Critical Reviews in Biochemistry and Molecular Biology, 2010; 45(5): 440–452

informa
healthcare

REVIEW ARTICLE

Decoding the histone H4 lysine 20 methylation mark

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Abstract
The molecular biology of histone H4 lysine 20 (H4K20) methylation, like many other post-translational modifications of histones, has been the subject of intensive interest in recent years. While there is an emerging consensus linking H4K20me1, H4K20me2, and H4K20me3 to transcription, repair, and constitutive heterochromatin, respectively, the specific details of these associations and the biological mechanisms by which the methylated histones are introduced and function are now the subject of active investigation. Although a large number of methylases capable of methylating H4K20 have been identified and characterized; there is no known demethylase of H4K20, though the search is ongoing. Additionally, many recent studies have been directed at understanding the role of methylated H4K20 and other histone modifications associated with different biological processes in the context of a combinatorial histone code. It seems likely that continued study of the methylation of H4K20 will yield extremely valuable insights concerning the regulation of histone modifications before and during cell division and the impact of these modifications on subsequent gene expression.

Keywords: *Histone H4K20; chromatin; methylation; demethylation; epigenetics*

Introduction

Ever since Albrecht Kossel first showed that histones were bound to DNA, and also following the subsequent observation that some of these histones could be modified by acetylation (Allfrey *et al.*, 1964), there has been tremendous interest in understanding how modification of histones might affect the function of the associated DNA. Characterization of the nucleosome, the basic physical structure containing histones and associated DNA, through biochemical strategies (Kornberg, 1974) and x-ray crystallography (Richmond *et al.*, 1984) were critical first steps in understanding this regulation. Based upon the crystallography studies, the nucleosome was shown to consist of a core containing two copies of histones H2A, H2B, H3, and H4 with 146 bp of DNA wrapped around in 1.65 turns of a flat, left handed superhelix (Richmond *et al.*, 1984; Arents *et al.*, 1991; Wang *et al.*, 1994; Luger et al, 1997a; Wood *et al.*, 2005). Within the nucleosome, the bodies of histones H2A, H2B, H3, and H4 (two copies of each) interact very tightly with each other and the associated DNA (Luger *et al.*, 1997a) while the respective amino terminal tails of these histones are found in an unordered structure lying on the outside of the nucleosome. The tails of H2A and H4 project out from the nucleosome's core under the superhelical gyres of DNA, while the tails of H3 and H2B project out through the two superhelical gyres. *In vivo* it is thought that the tails are dynamic, with their specific positions determined by the type and extent of their modifications and bound factors. Consistent with the external positions of the amino terminal tails was the observation that the terminal domains were not necessary to maintain the stability of the histone octamer (Karantza *et al.*, 2001).

While there has been significant interest in understanding the function of histone modification for many years, recent technical advances have resulted in a virtual explosion of studies concerning histone modifications. The ability to prepare antibodies with high affinity and specificity, and that recognize peptides containing specific forms of histone modifications (Bannister and Kouzarides, 2004; Perez-Burgos *et al.*, 2004), combined with various

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(Received 28 March 2010; revised 23 May 2010; accepted 25 June 2010)

ISSN 1040-9238 print/ISSN 1549-7798 online © 2010 Informa Healthcare USA, Inc.
DOI: 10.3109/10409238.2010.504700

forms of chromatin immunoprecipitation (ChIP) analyses (Alberts *et al.*, 1998; Kuo and Allis, 1998; Luo *et al.*, 1998; Collas, 2009), has made it relatively easy to investigate the nature of histone modifications at specific sites in eukaryotic chromatin. At the same time mass spectrometric techniques have been developed for determining modification sites on histones (Burlingame *et al.*, 2005; Ueberheideand Mollah 2007; Phanstiel *et al.*, 2008; Young *et al.*, 2009). Using these techniques and their derivatives, a large and growing number of histone modifications have been identified and for many of these modifications their biological properties have been characterized. A number of different forms of histone modification have now been analyzed including acetylation, methylation, phosphorylation, ubiquitination, and polyADP ribosylation (reviewed in Girdwood *et al.*, 2004; Cheung and Lau, 2005; Iniguez-Lluhi, 2006; Shilatifard, 2006; An, 2007; Weake and Workman, 2008; Cerutti and Casas-Mollano, 2009; Choi and Howe, 2009; Delcuve *et al.*, 2009; Munshi *et al.*, 2009; Quenet *et al.*, 2009; Shukla, Chaurasia, and Bhaumik, 2009; Teyssier *et al.*, 2009; Thambirajah *et al.*, 2009; Wyrick and Parra, 2009).

Though evidence for regulation of gene expression by acetylation or methylation mediated histone modification was available as early as the 1960s (Allfrey *et al.*, 1964), acetylation took the early lead in the many discoveries that ensued in the study of epigenetic regulation, mainly due the early discoveries of acetyltransferases and deacetylases. Methylation of histones can occur on the ϵ-amino groups of lysine (K) or the guanidine group of arginine (R). Modifications of lysine residues assume different states, such as mono- (me1), di- (me2) and tri-methylation (me3), whereas arginines are mono-methylated and symmetrically or asymmetrically di-methylated (Shi and Whetstine, 2007). While histone H3 lysine tails are modified on K4, K9, K27 and K79, only K20 is modified on the tail of histone H4. However, all three states of modification, i.e. me1, me2 and me3, are reported on H4K20, marking the chromatin for distinct biological activities. This review will focus on the role of methylation of histone H4 lysine 20 on various cellular and viral biological processes. However, where appropriate we will relate the methylation of H4 to other forms of modification in order to place this H4 methylation in the overall context of epigenetic regulation.

Discovery and occurrence of methylated H4K20

The methylation of lysine 20 of histone H4 was first observed during the chemical sequencing of calf and pea H4 in 1969 (DeLange *et al.*, 1969). Since this original description of methylated H4K20 from calf and pea, there have been a number of subsequent reports indicating the universal nature of H4K 20 methylation. A representative listing of various eukaryotes that contain methylated H4K20 and the forms of methylated H4 is shown in Table 1.

The presence of methylated H4K20 in a wide variety of higher eukaryotes suggests that the modification is evolutionarily conserved. However, there appear to be differences in the relative proportions of me1, me2, and me3 modified H4K20 progressing from simple to complex eukaryotes. While *Tetrahymena* had little methylated H4K20, the more complex yeast contained H4K20me1 and H4K20me2, and the most complex eukaryotes contained all three forms of methylated H4K20. Some higher eukaryotes have been shown to contain a higher proportion of H4K20me3 than of the less methylated forms (Garcia *et al.*, 2007) while in HeLa cells H4K20me2 was reported to be present in the greatest proportion (Pesavento *et al.*, 2008).

Table 1. Known H4K20 methylation states in model systems.

Source	Known methylated forms	Method of detection	Reference
Pea	me1, me2	Amino acid analysis	(DeLange *et al.*, 1969)
Yeast	me1, me2	Mass spectrometry	(Garcia *et al.*, 2007)
Arabidopsis	me1, me2, me3	Immunoblotting and immunostaining	(Naumann *et al.*, 2005)
Xenopus laevis	me1, me2, me3	Immunoblotting and mass immunostaining	(Shechter *et al.*, 2009)
Caenorhabditis elegans	me1	Amino acid sequencing spectrometry	(Vanfleteren *et al.*, 1987)
**Trypanosoma brucei*	me1, me2, me3	Mass spectrometry	(Janzen *et al.*, 2006)
Drosophila	me1, me2, me3	Immunostaining	(Karachentsev *et al.*, 2007)
SV40 virus	me1, me3	Chromatin immunoprecipitation	(Balakrishnan *et al.*, 2010)
Trout	me1, me2	Radiolabeling	(Honda *et al.*, 1975)
Mouse	me1, me2, me3	Immunostaining	(van der Heijden *et al.*, 2005); Kourmouli *et al.*, 2004
Rat	me1, me2, me3	Mass spectrometry	(Sarg *et al.*, 2002)
Chicken	not described	Radiolabeling	(Hendzel and Davie, 1989)
Calf	me1, me2	Amino acid analysis	(DeLange *et al.*, 1969)
Human (K562 cells)	me2	Mass spectrometry	(Galasinski *et al.*, 2002)
Human (HeLa cells)	me1, me2, me3	Chromatin immunoprecipitation	(Sims *et al.*, 2006)

*K17 and K18 are the equivalent positions for K20.

Similarly, there are also differences in the relative proportions of the three forms of methylated H4K20 in plants and animals (Fischer *et al.*, 2006). Finally, it appears that within a species there are major differences in the proportion of each form of methylated H4K20 depending upon the tissue/cell type. For example, terminally differentiated cells with low rates of transcription appear to have the highest levels of H4K20me3 (Shechter *et al.*, 2009). There also appear to be differences in the relative proportion of the various methylated forms of H4K20 during the cell cycle (Pesavento *et al.*, 2008). However, it is important to keep in mind that some of these studies were performed using techniques which may not be truly quantitative. For example, the apparent amount of signal from a particular methylated form of H4K20 in an antibody dependent assay will depend both upon the amount of the methylated form present in the target and the affinity of the antibody for that particular form.

H4K20 methyltransferases

Methylation of lysine residues on histones is catalyzed with remarkable specificity by a group of histone methyltransferases (HMTs), all belonging to the SET domain family of proteins, with the exception of the Dot1 methyltransferase (Qian and Zhou, 2006) (Figure 1). The SET methyltransferases were first discovered in Drosophila and took their name from the suppressor of variegation genes, Su(var)3-9, Enhancer of zeste and Trithorax (Qian and Zhou, 2006). The four main SET domain containing H4K20 methyltransferases are Pr-Set 7 (also referred to as Set 8 or KMT5A) (Fang *et al.*, 2002; Nishioka *et al.*, 2002), Suv4-20 (Suv4-20h1/KMT5B and Suv4-20h2/KMT5C) (Pannetier *et al.*, 2008), nuclear receptor-binding SET domain-containing protein (NSD1) (Huang *et al.*, 1998) and absent, small or homeotic-1 protein (Ash1) (Beisel *et al.*, 2002).

The bulk of the mono-methylation on H4K20 is catalyzed primarily by Pr-Set 7. It functions with remarkable specificity as a mono-methyltransferase, as it contains a tyrosine residue in its active site which hydrogen bonds with mono-methylated lysine residues, inhibiting addition of further methyl groups (Yang and Mizzen, 2009). Expression of Pr-Set7 is cell cycle regulated with an increased expression in late G2/M phase, with the enzyme being specifically localized to mitotic chromosomes (Rice *et al.*, 2002). Pr-Set7, independent of its

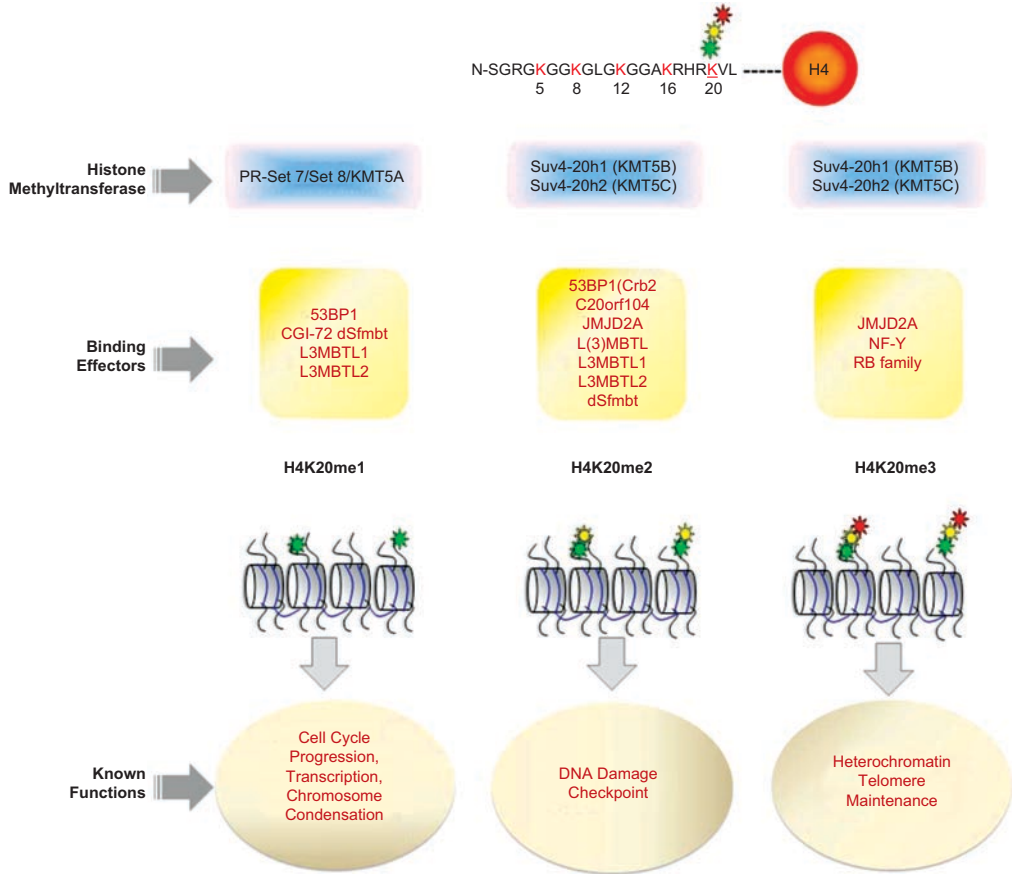

Figure 1. Histone H4K20 methylation: methylases, effectors and functions.

methyltransferase activity, has also been proposed to play a role in establishing a trans-tail histone code by recruiting a H3K4 methyltransferase to specific genes (Sims *et al.*, 2006).

The loss of Pr-Set7 *in vivo* causes a decrease in the formation not only of H4K20me1 but also of methylated subsequent states, i.e. H4K20me2 and H4K20me3 *in vivo* (Karachentsev *et al.*, 2005). This suggests that methyltransferases catalyzing di- and tri-methylation need a template in the form of mono-methylated H4K20 in order for enzymatic activity. The Suv4-20h1 (KMT5B) and Suv4-20h2 (KMT5C) enzymes are responsible for generating H4K20me2/me3. Using RNAi technology, it was shown in *Drosophila* S2 cells that knockdown of Suv4-20 led to a decrease in H4K20me2 levels and a corresponding increase in H4K20me1 (Yang *et al.*, 2008). Similarly, overexpression of FLAG-Suv4-20h2 caused a significant increase in the amount of H4K20me3 in HeLa cells (Yang *et al.*, 2008). Interestingly, *in vitro,* these enzymes are also capable of methylating the di- and tri- forms on unmodified histone H4K20 (Schotta *et al.*, 2004). The Suv4-20 enzymes interact with HP1 proteins and localize to constitutive heterochromatin sites (Schotta *et al.*, 2004). However, with contradictory evidence about the distribution of these proteins throughout chromatin, there is still a debate in the field of chromatin biology about the preferential localization of these enzymes to either active or inactive chromatin.

Histone methyltransferases NSD1 and the trithorax group activator, Ash1, mainly methylate other histone lysines and their ability to methylate H4K20 has only been characterized so far *in vitro* (Beisel *et al.*, 2002; Rayasam *et al.*, 2003). While certain studies have shown that H4K20 is not a natural substrate for Ash1 *in vivo* (Tanaka *et al.*, 2007), a recent study showed that inactivation of NSD1 in a transformed cell line reduced the amount of methylation of H4K20 (Berdasco *et al.*, 2009). Interestingly, in *Schizosaccharomyces pombe*, a single methyltransferase Set 9 is responsible for catalyzing the methylation of all three states of H4K20. The exact mechanism of how the enzyme regulates the formation of mono-, di- and tri- states of methylation is yet to be elucidated. Proteins containing various domains, e.g. the chromodomain (Eissenberg, 2001), Tudor domain (Sprangers *et al.*, 2003), PWWP (conserved proline and tryptophan) domain (Qiu *et al.*, 2002) and malignant brain tumor (MBT) domain (Wismar *et al.*, 1995), specifically recognize and bind the various states of methylation on H4K20 and recruit regulatory proteins to specify a biological function to the modified chromatin.

Demethylases

Until the discovery of a histone demethylase, methylation was considered to be a stable mark and a "true code" for epigenetic regulation. This hypothesis was supported in part by evidence from early studies that showed the rate of turnover of methylated histones and half-life of histones were well within the same range (Byvoet *et al.*, 1972). In 2004, Shi *et al.* reported the groundbreaking discovery of a histone demethylase termed LSD1 (lysine specific demethylase 1), belonging to the amine oxidase family. LSD1 was shown to specifically demethylate mono-methyl and di-methyl lysine 4 of histone H3. Subsequently another group of proteins containing the Jumonji C (JmjC) domain was discovered that were able to demethylate lysine residues of histones (Trewick *et al.*, 2005). The family of JmjC demethylases is relatively large with a number of enzymes known to specifically demethylate lysine residues of histone H3.

However, to date, there is no demethylase that is known to act on histone H4K20. It has also been proposed that along with H3K27me, H4K20me1 is a stable mark that is conserved during the various stages of development (Trojer and Reinberg, 2006). Additionally, mass spectrometry data of HeLa cells also show that the turnover of methyl groups on H4K20me2 is not detectable, suggesting the mark to be relatively stable *in vivo* (Pesavento *et al.*, 2008; Yang *et al.*, 2008). While technical challenges may be one reason why a specific H4K20 demethylase is yet to be discovered, the possibility of this mark being an irreversible one is also viable. Unlike histone deacetylases, discovery of demethylases have been relatively scarce and the exact mechanism involved in this reaction is still being elucidated. However, without doubt characterization of these enzymes will add to our understanding of the methylation mark as a key regulator of gene expression.

Biological function

Replication and repair

Faithful replication of DNA is essential for genome viability. Many histone marks have been implicated in ensuring efficient cell cycle regulated DNA replication. Along with the DNA, the chromatin is also duplicated during the S phase of the cell cycle. While studies have reported acetylation of lysine tails of *de novo* synthesized H3 and H4, the transmission and maintenance of methylated marks on histones are less well understood.

Histone H4K20 methylation is a mark that is specifically associated with DNA replication. In HeLa cells H4K20 methylation occurs progressively during the G2, M, and G1 phases of the cell cycle (Pesavento *et al.*, 2008). Interestingly, expression of Set8, which is responsible for H4K2me1, is increased during S phase, thereby linking the methyltransferase to the replication process (Rice *et al.*, 2002). It was further shown that Pr-Set7 associates

with S phase chromatin and regulates the cell cycle progression (Jorgensen *et al.*, 2007; Huen *et al.*, 2008). Proliferating cell nuclear antigen (PCNA) is a key component of the replisome. It is a toroidal homodimer and functions as the processivity factor for the main replicative DNA polymerases on both the leading and lagging strand of replication. Set8 interacts with PCNA via the PIP interacting box sequence and physically tethers the PCNA to replication foci. Furthermore, inhibition of Set8 expression via siRNA mediated knockdown increases DNA double strand breaks, consequently slowing down replication fork movement (Jorgensen *et al.*, 2007; Huen *et al.*, 2008). Depletion of Rad51, a protein that assists in repairing double strand breaks, and Set8 also slowed down replication fork progression. It has also been suggested that Set8 acts downstream of Rad51 in resolving damaged DNA and maintaining genomic integrity (Jorgensen *et al.*, 2007). While this evidence suggests a role for Pr-Set7 in replication there is no conclusive proof linking H4K20me1 to these processes. It is difficult to pinpoint the role of H4K20me1 mainly because the mono-methyl form serves as a substrate for action by the Suv4-20 enzymes responsible for H4K20me2/3. Depletion or knockdown of Set8 will reduce the H4K20me1 mark, subsequently decreasing the higher states of modification. We recently studied SV40 minichromosomes undergoing replication late in infection and also found that they exclusively contain H4K20me1 (Balakrishnan *et al.*, 2010).

In fission yeast, sites of damaged DNA marked with H4K20me2 by Set9/Kmt5 histone methyltransferase act as the focal point for interaction between damaged DNA and the checkpoint protein Crb2 (or 53BP1, human homolog) (Greeson *et al.*, 2008). Any change made to the histone methyltransferase (deletion, inhibition or loss of catalytic activity) uncouples the repair process. Crb2 via its Tudor domain specifically interacts only with the di-methylated form of H4K20. While H4K20 methylation is present constitutively on the chromatin even in the absence of DNA damage, phosphorylated H2AX is present only at sites of damage. Crb2, via its Tudor domain (recognizing H4K20me2) and BRCT domain (recognizing phosphorylated H2AX), can specifically interact and respond to damaged DNA sites (Greeson *et al.*, 2008).

H3K9me3 recruits heterochromatin protein 1 (HP1) and subsequently Suv4-20h1/2 to form H4K20me3 to mark heterochromatin. H4K20me3 serves as a mark for interaction with members of the retinoblastoma family (RB) that help maintain genomic integrity in constitutive heterochromatin (Gonzalo *et al.*, 2005). Finally, H4K20me3, along with H3K9me3, has also been implicated in the maintenance of telomeres. Loss of activity of Suv4-20h1/2 and consequent loss of the H4K20me3 mark results in telomere lengthening. This is possibly caused by the loss of the repressive marks at the ends of the genomes, which open up the telomere for lengthening by telomerase. Abnormal lengthening of telomeres can drive the cells into aberrant cell growth and finally cancer.

While H4K20me1 marks newly replicating chromatin, histone methyltransferases Suv4-20h1/2 methylate H4K20 to higher states of modification in a cell cycle dependent manner and in the context of the biological need of the cell.

Transcription

Because the regulation of gene expression is critical to all biological processes, the relationship between various histone modifications and transcription has been the topic of a number of studies. Although most of these studies concern the role of acetylation of H3 and H4 and the methylation of histone H3K4 and H3K9, a number of studies have been reported concerning the methylation of H4K20. In evaluating the results of these studies with respect to the function of methylation during transcription it is important to keep in mind that the function is the result of presumably at least two independent events. First, the methylated H4 must be targeted in a regulated fashion to mark a specific region of chromatin, and second the methylated H4 must exert some influence on the function of the chromatin through a change in structure of the chromatin or in the binding capability of the chromatin for a regulatory protein. Thus, if two cell types differ in the function of a regulatory protein which interacts with chromatin carrying a particular form of methylation, it is possible that the same form of methylation could give very different readouts with respect to transcription. An additional consideration is that some factors which interact with methylated H4 appear to interact with more than one form with similar affinities. For example, lethal 3 malignant brain tumor 1 (L3MBTL1) interacts with both K20me1 and me2, while JMJD2A interacts with K20me2 and me3 *in vitro* (Kim *et al.*, 2006; Kalakonda *et al.*, 2008) and all three modified forms of K20 *in vivo* (Spektor and Rice, 2009). Nevertheless, certain themes are developing with respect to the role of the methylated forms of H4K20 (Figure 1).

H4K20me1

H4K20me1 has been associated with active transcription in a number of different cell models (Talasz *et al.*, 2005; Vakoc *et al.*, 2006; Barski *et al.*, 2007; Wang *et al.*, 2008; Wakabayashi *et al.*, 2009; Karlic *et al.*, 2010). Interestingly, the association between H4K20me1 and transcription was greatest in those genes whose promoters contained a high CpG content (Karlic *et al.*,) suggesting that different histone modifications might reflect differences in initiation or elongation occurring during the transcription

of a particular gene. However, H4K20me1 has also been associated with repressed regions of chromatin (Karachentsev *et al.*, 2005) including regions repressed during X inactivation (Kohlmaier *et al.*, 2004). Similarly, H4K20me1 was not lost as expected as a mark of active transcription following repression by c-myc (Talasz *et al.*, 2005). Taken together these results suggest that the role of H4K20me1 in transcription is complex and may be a marker more for potential transcription than active transcription. One way that H4K20me1 is thought to play a role in repression of expression is through interactions with the (L3MBT1) protein, a polycomb homolog (Kalakonda *et al.*, 2008). It is not clear why H4K20me1 appears to be associated extensively with expression and repression. However, one possibility is that H4K20me1 is required as a substrate for the generation of H4K20me3, which has been shown to be associated with repression in a number of model systems (see below). Consistent with this possibility was the observation that the absence of PR-Set 7 in mouse embryos containing a knockout of the gene for PR-Set7 failed to produce all three methylated forms of H4K20 (Oda *et al.*, 2009).

H4K20me2

The relationship between H4K20me2 and transcription has not been extensively studied to date. One report in human cells indicated that the presence of H4K20me2 essentially paralleled that of H4K20me3, which is thought to be a repressive mark (see below) in the poly (A) binding protein C1 gene, and was very different from what was found for H4K20me1 (Vakoc et al., 2006). However, further characterization in this model system was not undertaken.

Using immunocytology, in *Arabidopsis* H4K20me2 has been associated with euchromatin in regions undergoing transcription (Fischer *et al.*, 2006; Naumann *et al.*, 2005). As described above there appears to be a significant difference in the function of H4K20 methylation in plants compared to animals. Most likely this represents differences in the protein factors which ultimately recognize the different forms of methylated H4K20.

H4K20me3

The results from a number of studies using independent approaches have led to the conclusion that H4K20me3 is associated with repressed chromatin in most higher eukaryotes. First, H4K20me3 is found in constitutive heterochromatin regions (Kourmouli *et al.*, 2004; Schotta *et al.*, 2004; Gonzalo *et al.*, 2005; Terranova *et al.*, 2005). H4K20me3 has been reported specifically in centromeres where there is a change in its presence in paternal chromatin following fertilization (Probst *et al.*, 2007), and in telomeres (Benetti *et al.*, 2007; Schoeftner and Blasco, 2008). H4K20me3 is also enriched in regions of chromatin which contain genes when the genes are silenced (Frye *et al.*, 2007; Regha *et al.*, 2007; Henckel *et al.*, 2009; Pauler *et al.*, 2009). Moreover, the presence of H4K20me3 has been correlated with the silencing of genes during the development of cancers (Kwon *et al.*,; Pogribny *et al.*, 2007). Inhibition of tri-methylation of H4K20 either with a chemical inhibitor (Miranda *et al.*, 2009) or using knockouts of Suv4-20H in the mouse (Schotta *et al.*, 2008) has confirmed that H4K20me3 plays a role in repression of expression in higher eukaryotes. Following inhibition of tri-methylation in both systems, de-repression of previously repressed genes was shown to occur. However, in *Arabidopsis* H4K20me3 has been associated with actively transcribed chromatin, suggesting that there may be significant differences between the function of H4K20me3 in plants and animals (de la Paz Sanchez and Gutierrez, 2009).

The effects of H4K20 methylation on nucleosome structure and the binding of transcriptional factors

It is reasonable to speculate that methylation of H4K20 most likely would have its effects on biological processes by either directly affecting the structure and properties of nucleosomes containing the modified H4 or by influencing the binding to these nucleosomes of proteins which are capable of affecting the function indirectly. These two possibilities may be linked if a change in conformation also results in the exposure of an otherwise cryptic binding site. While both of these effects have been observed under certain circumstances described below, the extent to which each of these possibilities contributes to the biological function of a particular form of methylation in a given cell still needs to be determined.

The effect of tri-methylation of H4K20 on nucleosome structure has been investigated through a combination of crystal structure and biochemical analysis (Lu *et al.*, 2008). Compared to its unmodified counterpart the amino terminal tail of H4 showed significant conformational changes in the vicinity of K20 and also around K16. The tail of H4K20me3 was observed to point at the DNA backbone of the nucleosome, while His 18 and Arg 19 each showed distinct conformational changes affecting their ability to interact with DNA on adjacent nucleosomes. The net result of these changes was an observed increased ability to condense into more compact structures. This result was not unexpected, since H4K20me3 is thought to play a role in repression and is found in condensed regions of chromatin. However, as noted below, H4K20me3 also interacts with specific proteins. There have been no similar reports to date on the effects of H4K20me1 or H4K20me2 on the structure of the nucleosome. It will be interesting to learn whether mono- or di-methylation of H4K20 will also result in conformational changes to the amino terminal tail.

Each of the methylated forms of H4K20 has been shown to be bound by one or more regulatory proteins (Figure 1). H4K20me1 has been shown to be bound by 53BP1, CGI-72, dSfmbt, L3MBTL1, and L3MBTL2 (Kim *et al.*, 2006; Min *et al.*, 2007; Grimm *et al.*, 2009; Guo *et al.*, 2009), H4K20me2 by 53BP1, C20orf104, JMJD2A, L(3) MBTL, L3MBTL1, L3MBTL2, dSfmbt, and Crb2 (Botuyan *et al.*, 2006; Kim *et al.*, 2006; Min *et al.*, 2007; Grimm *et al.*, 2009; Guo *et al.*, 2009), and H4K20me3 by JMJD2A and NF-Y (Kim *et al.*, 2006; Ceribelli *et al.*, 2008; Lee *et al.*, 2008). This list includes both Tudor and MBT domain proteins. Interestingly, chromodomain proteins such as HP-1 were not observed to bind to methylated H4K20, even though HP-1 is typically found in regions of chromatin along with H4K20me3 (Kim *et al.*, 2006). Functionally, the list includes the histone demethylase JMJD2A (Kim *et al.*, 2006), known repressors such as the polycomb member dSfmbt (Grimm *et al.*, 2009) and L3MBTL1 (Min *et al.*, 2007), bifunctional transcription regulators such as NF-Y (Ceribelli *et al.*, 2008) and repair signaling proteins such as 53BP1 and Crb2 (Botuyan *et al.*, 2006).

Because of the relative structural similarity between H4K20me1, me2, and me3 there has been much interest in determining how the binding proteins are able to discriminate between the different methylation states of the histone. The crystal structures of binding sites from the regulatory factors with methylated peptides corresponding to the appropriate region of H4K20 have been determined for JMJD2A (Lee *et al.*, 2008), L3MBTL2 (Guo *et al.*, 2009), dSfmbt (Grimm *et al.*, 2009), and L3MBTL1 (Min *et al.*, 2007). Binding in the three MBT domain proteins, L3MBTL1, L3MBTL3, and dSfmbt, was relatively similar. In each case the MBT domains formed a binding pocket which, through a combination of hydrophobic, electrostatic, and hydrogen bonding interactions, define the specificity of the binding to the methylated H4. The hydrophobic pocket was generated from three aromatic amino acids in each case, while other amino acids located around the pocket contributed to the electrostatic interactions and hydrogen bonding. For example, in L3MBTL1, H4K20me2 makes maximal contact with the binding pocket and auxiliary proteins while H4K20me3 would be too bulky to fit into the hydrophobic binding site without significant conformational changes to the site and would not be able to interact electrostatically with Asp355 (Min *et al.*, 2007). Similar observations were described in the other studies. It is interesting to note that despite the similarity between the MBT domain proteins and their mode of discrimination, the actual MBT domain which interacted directly with the methylated H4K20 was not necessarily the same. Binding of H4K20me3 in the Tudor domain protein JMJD2A was also similar. Again three aromatic amino acids formed a hydrophobic pocket while other amino acids were optimally placed in the protein for electrostatic interactions and hydrogen bonding of the trimethylated histone (Lee *et al.*, 2008).

Because of the potential variation in structure and amount of protein binding factors in cells under different conditions, the interaction between a methylated form of H4K20 and the factors could easily explain some of the variations noted above. For example, a variation in the splicing pattern generating the factor or the presence or absence of a particular factor could result in activation of transcription in one case and repression in another situation. Changes in splicing patterns for protein binding factors might also be important in reading a combinatorial histone code. A bi-functional protein binding factor could recognize a methylated form of H4K20 at one of its binding sites and a second form of modification at another binding site. Although a small number of binding factors have been identified to date, as described above, it remains unclear how the binding of the factor to chromatin containing methylated H4K20 influences its biological activity. *Does the bound factor form a physical barrier preventing the binding of other factors such as transcription activators/repressors, or does the factor serve as a scaffold to bring in other important cofactors?* Further in-depth studies to address these specific questions will be needed.

Viral modification

Although most studies concerning the role of histone modifications in biological processes have been carried out with cells and tissues, there are now an increasing number of reports in which histone modifications have been reported during viral infections. To a large extent studies on the epigenetics of viruses has paralleled the development of antibodies to specific histone modifications and small molecule inhibitors. Thus, most of the earlier reports deal with histone acetylation and the effects of deacetylase inhibitors. Many of these earlier studies have been reviewed (Lieberman, 2008). Since many nuclear viruses can exist as either episomes or as integrants in host chromatin, many of the studies concerning the epigenetics of viruses have focused on the regulation of the two forms of viral chromatin and the mechanism of latency (Bloom *et al.*,; Takacs *et al.*, 2009). It is only relatively recently that studies have been undertaken concerning the role of histone methylation.

Methylated histone H3 has been reported in viral chromatin following infection with HSV-1 (Cliffe *et al.*, 2009), cytomegalovirus (Cuevas-Bennett and Shenk, 2008), HIV (Pearson *et al.*, 2008), and HPV (Wooldridge and Laimins, 2008). We are the first to report the presence of methylated histone H4 in a virus, SV40 (Balakrishnan *et al.*, 2010). Using ChIP validated antibodies, we analyzed SV40 minichromosomes present in virions and

in infected cells at various time post-infection for the presence of H4K20me1, H4K20me2, and H4K20me3. Unexpectedly, we found H4K20me1 to be present in all forms of SV40 chromatin tested, H4K20me2 to be absent from SV40 chromatin, and H4K20me3 to be present only in SV40 minichromosomes obtained at the earliest times in infection (Balakrishnan *et al.*, 2010).

Because H4K20me1 was found throughout the SV40 genome, was not present in transcribing minichromosomes, but was present in actively replicating minichromosomes and virion chromatin, we proposed that this modification was associated with targeting newly replicated minichromosomes for encapsidation at late times. However, the specific function of H4K20me1 in the encapsidation process has not yet been elucidated. Two possible roles are obvious. The mono-methylation of H4 at K20 could be part of a signal which determines which of the newly replicated minichromosomes begins the encapsidation process, or the mono-methylation could be necessary to allow the minichromosomes to fit within the spatial confines of the virion. The latter hypothesis is particularly appealing because in order for an SV40 minichromosome to become encapsidated at least two conditions need to be fulfilled. First, the chromatin structure of the minichromosomes must be modified to allow for the binding of the virion structural proteins VP-1, VP2, and VP3. Second, the nucleosomes in the minichromosomes need to be able to come relatively close together to allow for the compaction necessary for the minichromosomes to fit within the virion. The location of the tail of histone H4 on the face of the nucleosome suggests that it might play a critical role in determining the ability of two nucleosomes to be close together. However, it is not clear how methylation of lysine 20 could accomplish this other than by simply disrupting normal protein–protein or protein–DNA interactions in open chromatin.

Since H4K20me3 was only present in SV40 minichromosomes at the earliest times in infection, was present throughout the genome, and disappeared rapidly from SV40 chromatin along with a general loss of SV40 minichromosomes, we proposed that the H4K20me3 was functioning as part of a cellular mechanism to remove incoming infecting SV40 minichromosomes. We hypothesized that cells might be able to recognize foreign chromatin as a result of the combination of histone marks present on the incoming foreign chromatin and use additional histone modifications to label the foreign chromatin for subsequent degradation. While this process has not been observed yet with other viruses, there is an increasing literature indicating that infecting viruses can be recognized by cells and activate cellular signaling pathways related to DNA repair (Butin-Israeli *et al.*,; Lilley *et al.*, 2010). We would like to suggest that this activation of DNA repair may be related to our observed degradation of incoming SV40 chromatin and is also likely to occur through a mechanism in which the cell recognizes foreign chromatin because the histone modifications present in the exogenous chromatin are not correct for the context of the chromatin location within the nucleus.

We do not know why H4K20me2 was not found in SV40 chromatin given the evidence for large amounts of H4K20me2 in cells (Pesavento *et al.*, 2008). Since we used ChIP validated commercial antibodies for these studies, it is seems unlikely that it is simply an issue with the antibody. Most likely it is a consequence of either the mechanisms by which H4 is methylated following replication of the cellular and viral chromatin or the different functions of both kinds of chromatin. H4K20me2 in HeLa cells appears to require 2–3 cell cycles before reaching the observed high levels (Pesavento *et al.*, 2008). SV40 chromatin most likely does not undergo the same kind of maturation pathway. Newly replicated minichromosomes are most likely directed to become virions late in the infection, which removes these SV40 minichromosomes from the pool of available minichromosomes before they undergo further modifications, like cellular chromatin. The other forms of minichromosomes, e.g. transcribing minichromosomes, appear to be relatively stable in their function and complement of histone modifications.

We believe that the use of relatively simple viral models like the SV40 minichromosome will be extremely useful in determining the function and regulation of H4K20 methylation and other forms of histone modifications. Because of the limited coding capacity of these viruses, they extensively utilize host cell enzymes and processes to regulate their intracellular life cycle. Moreover, they are an excellent model system to investigate how multiple epigenotypes can coexist and how an epigenotype can be modified into a new form. For example, late in an SV40 infection there are biologically distinct minichromosomes present responsible for transcription, replication, and encapsidation. Presumably these minichromosomes represent distinct epigenotypes that can be maintained despite the competing biological processes. As described in our publication, replicating minichromosomes contain H4K20me1 and many of the daughter minichromosomes resulting from replication continue to carry this form of modification as they undergo encapsidation. It will be interesting to learn whether the H4K20me1 is being introduced into actively replicating minichromosomes by a process in which existing histone modifications are conserved in the daughter minichromosomes, or whether the H4K20me1 is introduced post-replicatively in a process resulting in the generation of daughter minichromosomes carrying different forms of modifications then their parents. Viral models, such as SV40, should be very useful in addressing this and similar questions concerning the regulation of histone modifications.

Relationship of H4K20 methylation to other forms of histone modification

The large number of histone modifications along with the complexity of most biological processes such as transcription has led to the hypothesis that there is a combinatorial "histone code" which is associated with the biological function of chromatin (Jenuwein and Allis, 2001). This combinatorial code is thought to consist of multiple histone modifications on either the same histone or combinations of histones which either define the biological function of the associated chromatin or is a consequence of the biological function. Various types of study have been undertaken to test this hypothesis by determining whether certain forms of histone modifications are typically found together.

Using a genome wide mapping strategy with a resolution of a single nucleosomes, a number of different forms of histone modification were analyzed for colocalization (Wang *et al.*, 2008). Using this approach H4K20me1 was found to be likely collocated in nucleosomes from transcribed regions that also contain H2BK5me1, H3K9me1, H3K4me1/2, and H3K79me1/2/3. In an earlier analysis by the same group using a different approach (Barski *et al.*, 2007) H4K20me1 was found associated with H2BK5me1, H3K27me1, and H3K9me1 in actively transcribed regions. In a separate approach analyzing across the poly (A) binding protein C1 gene (Vakoc *et al.*, 2006) H4K20me1 was found to be associated with H3K9me3, H3K36me3, and H3K79me3. However, it should be noted that antibodies to H2BK5me1, HeK9me1, H3K79me1, and H3K79me2 were not used in this latter analysis. Because of the relatively small number of studies to date and the lack of consistent associations, it is not clear whether a specific set of marks including H4K20me1 is invariably associated with a particular biological function. Adding to the complexity of interpreting these analyses was the observation that differences in association were also noted between the enhancer, promoter, and transcribed regions in all the studies, indicating that the association of various forms of histone modification in transcribed chromatin is very complex. Most likely this complexity reflects differences in the targeting of the specific protein factors which interact with the different forms of histone modification. Interestingly, H4K20me1 and H4K16ac are antagonistic marks with respect to one another (Rice *et al.*, 2002).

In a similar analysis H4K20me3 was found in association with H3K9me3 in large repeat-related chromatin regions such as those located near centromeres (Barski *et al.*, 2007). Both of these marks have been associated with centromeres in other studies. In our studies with the virus SV40, H4K20me3 was also found in the same minichromosomes with H4K20me1 early in infection (Balakrishnan *et al.*, 2010). Again, there are simply not enough studies to determine whether H4K20me3 is invariably associated with certain other modifications.

H4K20me2 has been shown to be associated with unacetylated, mono, di, tri and tertra-acetylated isoforms of H4 in HeLa cells (Zhang *et al.*, 2002). H4K20me2 in human embroyonic stem cells has also been shown to be associated with H4R3 methylation (Phanstiel *et al.*, 2008). The H4R3 methylation was only present when H4K20me2 was also present.

In an alternative type of analysis, H4 has been analyzed by mass spectrometry to determine which combination of modifications can exist on the same molecule of H4 (Pesavento *et al.*, 2008; Phanstiel *et al.*, 2008). Total H4 from differentiating cells was analyzed for the presence of methylated, acetylated, and phosphorylated modifications in the amino terminal tail. H4K20me1 was diacetylated on approximately 4% of the molecules, while H4K20me2 was diacetylated on as much as 30% of the molecules. H4K20me1 and me2 were also present on a significant number of molecules carrying three and four acetyl groups, however at much lower values. H4K20me3 on the other hand was generally present on less than 1% of the molecules which were also acetylated. In this system methylation and phosphorylation appeared to be mutually exclusive (Phanstiel *et al.*, 2008). Similar results were obtained in HeLa cells (Pesavento *et al.*, 2008). It would appear that it is quite likely to find H4K20me1 and H4K20me2 together with acetylated lysines 5, 8, 12, and/or 16 in molecules of the histone.

The combinatorial nature of histone modifications has also been investigated by looking directly at the nature of the histone modifications that allow for optimal interaction with a bound protein (Garske *et al.*, 2008). In this analysis a combinatorial library of peptides containing all of the possible modifications known for the amino terminal tail of H4 was selected by binding to JMJD2A, and the bound peptides were identified and characterized by mass spectrometry. As expected JMJD2A was found to bind best to peptides containing either H4K20me2 or me3 (Garske *et al.*, 2008). Moreover, binding was observed in these peptides when R3 was methylated and K5, K8, K12, and K16 were acetylated. In order to characterize further the extent of binding, isothermal titration calorimetry was used to determine dissociation constants. Based on this analysis it appeared that JMJD2A bound best to a peptide containing K20me3 when K5 was acetylated and to peptides containing K20me2 when R3 was methylated, K8 and K12 were both acetylated, or K5 and K16 were both acetylated. Peptides containing K20me2 and R3me2 did not bind as well when all four lysines at 5, 8, 12, and 16 were acetylated (Garske *et al.*, 2008).

The known combinatorial codes along with H4K20 methylation are shown in Figure 2.

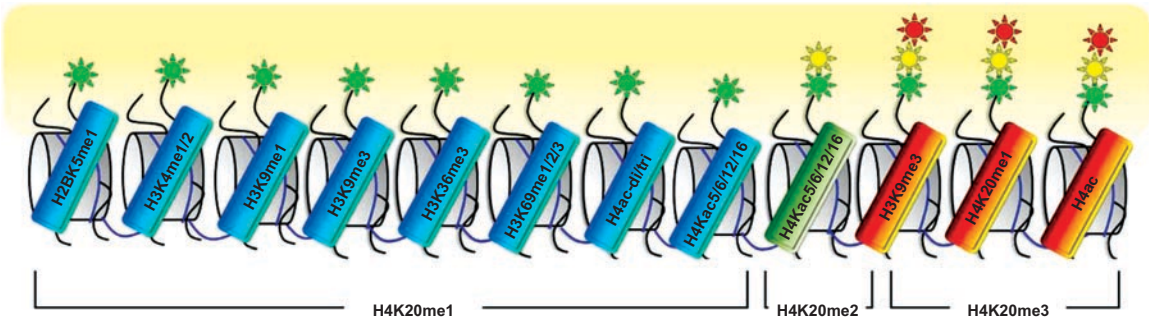

Figure 2. Histone H4K20 methylation and other known combinatorial codes.

Concluding remarks

H4K20 methylation has been shown to be associated with a number of diverse biological processes in various eukaryotic systems. However, a number of significant questions remain concerning the biological role of H4K20 methylation in cells. Perhaps the most important question is "how does a particular form of histone modification influence the function of the associated chromatin?" The answer to this question should help to explain the apparent contradictory observations that H4K20me1 like some other forms of histone modification may be associated with transcriptional activation in some cells and repression in others. Similarly, the answer should also help to explain the variations reported for the association of methylated forms of H4K20 with other modified histones.

A second important regulatory question concerns the targeting of the methylase enzymes to their appropriate substrates. Are the methylases targeted to a replication or transcription complex, and do they function as the complex moves through chromatin, as we proposed for histone acetylation (Balakrishnan and Milavetz, 2007a; 2007b), or are the methylases targeted by sequences or other proteins to specific portions of chromatin? The former seems unlikely for cellular chromatin (Pesavento *et al.*, 2008) based upon a mass spectrometry study in HeLa cells (Pesavento *et al.*, 2008), but may be the case for SV40 replication since we observed that H4K20me1 was present in replicating minichromosomes (Balakrishnan *et al.*, 2010).

A third question concerns the relationship between the different forms of H4 methylation to each other and to the modification of other histones. Methylated H4 has been associated with various other forms of modification using genome wide studies. However, as indicated above there is no consensus on which combinations of histone modifications are typically associated with certain biological functions. The characterization of the relationship between the different methylation states of each histone to other states of that histone and other modified histones should be very useful for a better understanding of the combinatorial aspects of the histone modifications.

The last question that we would like to consider is whether histone modifications in general and methylation of H4K20 in particular may play an important role in defining self and non-self in cellular chromatin. In our studies with SV40 we noted that incoming viral chromatin was marked with H4K20me3 and then subjected to degradation during the earliest stages of infection. We proposed that this process would be similar to restriction in bacteria and would have the same effect. In the first step the foreign chromatin would be recognized as foreign because its chromatin modifications are incorrect for its cellular context. In the second step this foreign chromatin would be marked with one or more histone modifications which would result in the chromatin serving as a substrate for degradative enzymes. We would like to suggest that this process may share some similarities to DNA repair, where chromatin appearing foreign as a consequence of damage is recognized and subjected to the repair process.

Acknowledgements

The authors acknowledge the work of all the investigators in the field of histone methylation and regret the omission of several references due to space limitations.

Declaration of interest

The authors declare they have no conflict of interest. This work was supported by grants from the National Institutes of Health GM074811 and AI070193 to BM.

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Editor: Michael M. Cox