Beyond the double helix: writing and reading the histone code

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Abstract. Chromatin is the physiological carrier of not only genetic information, encoded in the DNA, but also of epigenetic information including DNA methylation and histone modifications. As such histone modifications are involved in many aspects of nuclear processes including gene regulation and chromosome segregation. Recently, a ‘histone code’ hypothesis was put forward to explain how patterns of histone modification may function in downstream processes. In support of the ‘histone code’ hypothesis, we found in vivo and in vitro evidence that effector proteins, HP1 (heterochromatin protein 1) and Pc (Polycomb) can discriminate and ‘read’ histone methylation marks on K9 and K27, respectively. Moreover, we propose a ‘binary switch’ model and suggest that binding and release of effector proteins to their cognate sites can be regulated by modifications on adjacent or nearby residues. Thus, combinations of adjacent histone modifications would function differently from singular modification, and static modifications (e.g. Lys methylation) may well be regulated by dynamic modifications (e.g. phosphorylation). Finally, we describe a novel histone phosphorylation event linking the function of Mst1 kinase and H2B Ser14 phosphorylation with apoptotic chromatin condensation in vertebrates. As this modification is not found during mitotic chromosome condensation, these findings suggest the intriguing possibility that a unique ‘death’ mark exists for chromatin condensation during apoptosis.


The human genome is estimated to contain 30 000–40 000 unique genes. The DNA sequence and the chromatic location of most of these genes has been determined and they are publicly available (Lander et al 2001, Venter et al 2001). The central challenge now facing the biomedical community is how to derive valuable medical knowledge about the function of these genes from DNA sequence data, and to

1This paper was presented at the symposium by David Allis, to whom correspondence should be addressed.
Me: DNA methylation inherited from mother cells
Me: Newly added DNA methylation after DNA replication
M: Old histone methylation from mother cells
M: New histone methylation after DNA replication
answer questions such as how the expression of these genes is orchestrated to carry out normal cellular functions and for responses to environmental and physiological changes. The genetic information encoded by the DNA sequence determines the sequence of RNAs and proteins. However, it is becoming clear that ‘epigenetic’ information plays a major role in determining when, where, and to what level the genetic information should be utilized. Epigenetic information refers to differential and inheritable changes of gene expression potentials that are not caused by mutations in DNA itself (Jaenisch & Bird 2003). Recent studies have focused on several molecular mechanisms of epigenetic gene regulation that include DNA methylation, histone modifications and small nuclear RNAs (SnRNAs) or RNA interference (RNAi). Whereas emerging evidence suggests that the three mechanisms are coordinated and affect each other, the discussion below will mainly focus on histone modifications as they relate to epigenetic-based forms of gene regulation.

**Histone proteins as ‘messengers’ of epigenetic information**

The human body contains multiple organs and diverse cell types, and every gene exists within every cell. However, only a small percentage of genes are activated in any given cell type, and each type of cell has its unique gene expression profile. These different expression profiles are formulated during early development in a multicellular organism, when cell division, cell differentiation, tissue and organ formation occur rapidly (Francis & Kingston 2001). Moreover, these gene expression potentials can be memorized and inherited after mitosis and even meiosis. To regulate this genetic information efficiently and in an epigenetic manner, nature has evolved a sophisticated system that controls access to specific genes. This system relies on packaging DNA into a DNA–histone complex called chromatin, which is the physiological substrate of all cellular processes involving the DNA (Felsenfeld & Groudine 2003). The dynamic change of the three dimensional architecture of chromatin makes certain genes more readily accessible to transcription factors and other machineries engaging the genetic template (Lomvardas & Thanos 2002). Because parental DNA and associated
histones are divided and incorporated into the newly duplicated chromatin during S phase of the cell cycle, it is possible that the epigenetic modifications carried by DNA and histones can be passed to the daughter cells after M phase and cell division, making DNA and histone proteins also the attractive messengers of epigenetic information (Fig. 1).

Recently, the chromatin field has witnessed an explosion of literature documenting the involvement of various histone modifications, such as methylation, phosphorylation, acetylation, ubiquitination and ADP ribosylation in essentially all DNA-templated processes. In the coming of a new epigenomic era, the regulation of the enzymes responsible for adding or subtracting these covalent marks are poised to take centre stage in the study of gene expression regulation, and understanding the molecular aetiology of human diseases such as cancer. Identification of altered DNA methylation and histone modification activities in a range of human cancers supports the involvement of epigenetic mechanisms during cancer development (Kondo et al 2003). Thus, it is important to investigate the role of epigenetic regulatory proteins and the way that epigenetic regulation works in order to get a more in-depth picture of pathways leading to oncogenesis and to assist the development of new therapeutic strategies.

New insights into the ‘histone code’ hypothesis

It is clear that the regulatory signals, either extracellular or intracellular, ultimately impinge on chromatin, which can be viewed as a gigantic signalling platform for integrating and recording these signalling events (Cheung et al 2000). The epigenetic information carried by the chromatin can in turn impact on most of the chromatin-templated processes with far-reaching consequences for cell fate decisions and for normal and pathological development (Jenuwein & Allis 2001, Fischle et al 2003). As mentioned above, epigenetic information is inheritable through the cell cycle and through meiosis from one sexual generation to the next. We and others have proposed that an epigenetic indexing system for our genome, a ‘histone’ or ‘epigenetic’ code, works as a fundamental regulatory mechanism in addition to the DNA and the genetic information itself (Strahl & Allis 2000, Turner 2000, Jenuwein & Allis 2001). The original histone code hypothesis proposed that ‘distinct covalent histone modifications, acting alone, sequentially, or in combination, form a “histone code” that is then read by effector proteins to bring about distinct downstream events’ (Strahl & Allis 2000). Although this hypothesis has received much attention and some strong experimental support (Agalioti et al 2002, Kanno et al 2004), it has been hard to derive definitive rules from our current knowledge of the ‘code’.

In this meeting, I will expand on this general concept by proposing the ‘methyl/phos’ (methylation/phosphorylation) switch hypothesis with ‘predictive rules’
that may govern the binding and release of effector proteins and complexes that engage the chromatin polymer. On the histone H3 tail, several clear examples of adjacent Lys residues and Ser/Thr residues exist, such as K9S10 and K27S28, that can be modified by methylation and phosphorylation, respectively (Fig. 2A). I will present recent work suggesting that methylation- and site-specific effector proteins exist, and their function is likely regulated by phosphorylation of adjacent residues (see below, Jacobs & Khorasanizadeh 2002, Fischle et al 2003a). Thus, these adjacent K/T/S sites may form ‘binary switches’ to regulate the binding of effector proteins (Fig. 2B and discussion below). Importantly, our ideas provide an explanation for several long-standing questions embedded in the existing literature, and are open to experimental tests. In addition, I will present a newly
discovered histone phosphorylation event and the responsible kinase, which link histone modification to apoptotic chromosome condensation.

Molecular basis for discrimination of repressive methyl-lysines in the histone H3 tail

On the histone H3 tail, lysines 9 and 27 are well-known methylation sites, and are often associated with epigenetic repression (Lys 27) and heterochromatin-mediated gene silencing (Lys 9) (Cao et al. 2002, Jacobs et al. 2001). Although these two sites are involved in different epigenetic events, it is remarkable that both ‘target’ lysines are embedded within a highly related sequence motif: TARK$^9$S versus AARK$^{27}$S (Fig. 2A). Moreover, as predicted by the histone code hypothesis, emerging evidence shows that Lys9 and Lys27 methylation sites are ‘read’ by distinct effector binding proteins: heterochromatin protein 1 (HP1) and Polycomb (Pc), respectively. Both HP1 and Pc are the prototype proteins in which the chromodomain was identified (Singh et al. 1991). Recent work suggests that chromodomains serve as methyl-lysine recognition and binding modules (Jacobs & Khorasanizadeh 2002, Nielsen et al. 2002).

Our knowledge of the organization and function of heterochromatin has been greatly advanced by the study of HP1 and the histone H3 K9 methyltransferase, Su(var)3-9. The fact that the chromodomain of HP1 can recognize and bind the K9 methyl site generated by the Su(Var)3-9 offers a mechanistic insight on the epigenetic gene silencing phenomena associated with heterochromatin, such as position effect variegation (PEV) (Lachner et al. 2001, Bannister et al. 2001, Jacobs et al. 2001, Rea et al. 2000). On the epigenetic gene expression side, recent studies found that the E(z) (Enhancer of Zeste) complex can methylate histone H3 K27, and H3 K9 to a lesser extent, in *in vitro* enzymatic assays (Cao et al. 2002, Czermin et al. 2002, Kuzmichev et al. 2002, Muller et al. 2002). Likewise, the chromodomain of Pc was proposed to be able to ‘read’ both of these methylation sites. However, these promiscuous activities are paradoxical to the in *in vivo* observation that Pc and HP1 are involved in different pathways, and that the chromodomain of HP1 and Pc is responsible to target these protein to different destination in the nucleus (Messmer et al. 1992, Platero et al. 1995).

Here, we present new data to show that the chromodomain proteins Pc and HP1 are highly discriminatory for binding to these sites both *in vivo* and *in vitro*. Using newly developed methyl- and site-specific antibodies, we showed that trimethyl-Lys27 and Pc are colocalized and both excluded from heterochromatic areas that are enriched in di- and trimethyl-Lys9 and HP1 in *Drosophila* S2 cells and on polytene chromosomes. In addition, swapping of the chromodomain regions of Pc and HP1 is sufficient for switching the nuclear localization patterns of these
repressors, indicating a role for their chromodomains in both target site binding and discrimination (Fischle et al 2003b).

To better understand the molecular basis for the selection of methyl-Lys binding sites, we have recently solved the 1.8 Å structure of the Pc chromodomain in complex with a trimethyl-Lys27 H3 tail and compared it with our previously determined structure of the HP1 chromodomain complexed with a trimethyl-Lys9 H3 tail (Jacobs & Khorasanizadeh 2002). The structures show clear differences in how two chromodomains that are highly related in sequence and structure effectively distinguish methylation sites on the H3 tail (Fischle et al 2003). Whereas both the HP1 and the Pc chromodomains form aromatic ‘cages’ that bind the positively charged methylammonium ion, they distinguish the two binding sites by discriminating residues N-terminal to the common ARKS motif, which differ between the two target sites. The Pc chromodomain has evolved an extended ‘groove’ that provides more contact surfaces to engage five more residues to a modest degree in addition to the ARK27S. Together, these surfaces provide enough additional binding complimentarily to generate enhanced recognition and binding affinity (Fischle et al 2003b). On the HP1 side, it seems the residue T6 in front of ARK9S is critical for the binding specificity (Jacobs & Khorasanizadeh 2002, Fischle et al 2003).

HP1 and Pc proteins themselves have been implicated in fundamental nuclear processes including heterochromatin-mediated gene silencing, homeotic gene expression and chromosome dynamics (Simon & Tamkun 2002). The above studies of HP1 and Pc offer supportive evidence to a central tenet of the ‘histone code hypothesis’, that the covalent marks are docking sites for effector proteins that in turn bring about distinct downstream events (Strahl & Allis 2000). It is quite intriguing from an evolutionary aspect that the two effector proteins with the similar functional domain can recognize two binding sites embedded in the similar sequence context, and are evolved to participate in two different silencing pathways.

Binary switches as part of the histone code?

The density of modifiable residues on the histone tail, for example H3, is very striking (Fig. 2A). Recently, mass spectrometry analyses suggest that modification of two adjacent sites does coexist (C. D. Allis, D. F. Hunt, unpublished data). Many site- and modification-specific antibodies have been developed and have greatly benefited the field to tackle the histone modification problem. However, as many immunological tools were developed against specific histone modification sites, a recurring question to us and others is whether adjacent histone modifications might affect the epitope recognition by antibodies. Similarly, it is equally intriguing to know whether adjacent modifications, if they
exist, may affect the binding of effector proteins that normally recognize single modifications, such as HP1 and Pc.

On the basis of the presence of close dual modification sites on the histone tails, we wish to extend the histone code hypothesis and to propose the concept of ‘binary switches’ (Fig. 2B). We hypothesize that binary switches in the histone tails regulate the ‘ON/OFF’ state for the binding of effector proteins, such as HP1 and Pc. Specifically, a ‘phos/methyl’ or ‘methyl/phos’ switch likely operates on the histone tails to regulate effector binding at the correct time in the cell cycle or appropriate stage of development.

The central tenet of this hypothesis is as follows: ‘on–off’ binding of effectors is firstly regulated by adding chemical moieties, such as the methyl groups, to their cognate site; secondly, the addition and subtraction of modification at the nearby or adjacent site can release and recruit binding effectors, respectively, without changing the primary modification site. For example, it is conceivable that the K9S10 sites might form such a binary switch. Structural analyses of the chromodomain of HP1 bound to the H3 tail methylated on Lys9 (Jacobs & Khorasanizadeh 2002), argue whether mitosis-driven phosphorylation of Ser10 will significantly diminish the binding affinity of this module.

Mitotic phosphorylation of H3 at Ser10 and/or Ser28, catalysed by aurora B-type kinases, is well documented in organisms ranging from yeast to humans (Hsu et al 2000). If the general concept is correct, mitosis (or meiosis) may drive the phosphorylation side of the phos/methyl switch allowing for the release, and potential clearing, of chromatin effectors (e.g. HP1) that dock on stable methylation marks during interphase. In support, a large portion of HP1 and Pc protein is released from the mitotic chromosome in early embryos and in the Drosophila S2 cells (Dietzel et al 1999, Kellum et al 1995 and Y. Wang, C. D. Allis, unpublished results). For the proposed binary phos/methyl switches to work, we predict that methyl-specific chromodomains would change their binding affinities to methyl target sites with adjacent phosphorylation marks. New binding data with the chromodomain of HP1 show that this is indeed correct; the binding affinity is dramatically decreased when the chromodomain of HP1 was tested with a H3 peptide that is both tri-methylated at Lys9 and phosphorylated at Ser10 (S. A. Jacobs, W. Fischle, unpublished results).

It is intriguing to consider to what range this concept might be applied. To our knowledge, a binding partner has yet to be identified that ‘reads’ the H3 (Lys4) methyl mark, a mark often associated with an ‘on’ or ‘competent’ transcriptional state (Santos-Rosa et al 2002). If such a protein exists, we would predict that its binding to the H3 Lys4 methylation site will be regulated by phosphorylation at Thr3, a mark which has recently been found to be a strong mitotic phosphorylation site in mammalian cells (C. Barber, F. Turner, and D. Allis, unpublished data). Similar arguments can be made for T22K23, although to our knowledge, it is not
yet known whether Thr22 and Lys23 in the H3 tail are phosphorylation and methylation sites, respectively. On the other side, it is intriguing to postulate that the acetyl-Lys binding protein of the K23 site may be regulated by an ‘acetyl-phos’ switch. Nevertheless, we suggest that there may be at least four ‘binary switches’ operating on the H3 tail alone (Fig. 2A).

In turn, we predict that the interaction of phos-binding effectors (yet to be found) and their cognate sites may be regulated by nearby or adjacent ‘off’ methyl switches. Histone methylation on the Lys residues is relatively stable epigenetic mark, and no histone (Lys) demethylases have been identified so far. However, phosphorylation can be reversibly regulated by kinase/phosphatase. We note that the Ipl1/aurora kinase and type 1 protein phosphatase (PP1ase) have been identified as the mitotic kinase/phosphatase responsible for regulating H3 Ser10 phosphorylation as cells enter/exit mitosis (Hsu et al 2000). Interestingly, PP1ase was also identified in the same genetic screens in *Drosophila* as Su(Var)3-6 (Baksa et al 1993), suggesting that its activity facilitates silencing by unknown mechanisms. However, it is tempting to think about the suppressor function of PP1 in the context of the ‘methyl/phos’ binary switch. The switch model makes a clear and testable prediction regarding the role of Su(Var)3-6 in the above silencing pathway: one role, if not the major role, of PP1ase, Su(Var)3-6, is to remove phosphates at Ser10 on H3 at the end of mitosis. Thus, the released and dispersed HP1 (also identified in the same genetic screen as Su[Var]2-5) in the M phase cells can be recruited back to H3 Lys9 methylation sites, which are themselves added by a H3 (Lys9) methyltransferase, Su(Var)3-9.

In summary, this model and its predictions provide new insights into a potential role for protein phosphatases, kinases, methyltransferases, and potentially histone demethylases in regulating the binding and release of critical effector proteins. More importantly, since histone demethylation activity has not been discovered to date, to regulate histone methylation and the associated methyl binding protein by reversible phosphorylation is a means to dynamically regulate histone methylation.

**A ‘life’ versus ‘death’ histone code?**

Histone phosphorylation is one of the best-characterized histone modifications. The function of histone phosphorylation has been linked with many aspects of chromatin biology, including mitotic chromosome condensation, gene expression, dosage compensation in *Drosophila*, DNA double-strand breakage and repair (Fig. 3A). The linker histone H1 and histone H3 phosphorylation are well documented and are linked to both transcription regulation and mitotic chromosome condensation (Dou et al 2002, Hsu et al 2000). Much less well
characterized are molecular events that influence the remarkable changes in chromatin condensation that characterize dying cells. During apoptosis chromatin is digested into oligonucleosomal fragments and is condensed to form pycnotic chromatin bodies, two hallmark properties of this process in most cells (Wyllie 1980). Because of the intimate association between histones and DNA, histone phosphorylation was suggested to be involved in the change of chromatin integrity and compaction (Ajiro 2000). However, how histone phosphorylation is induced and involved in apoptosis remains poorly understood (Cheung et al 2000). Phosphorylation at the C-terminal tail of a relatively minor histone variant, H2A.X (at serine 139 in human), increases during early stages of DNA fragmentation in apoptosis (Redon et al 2002). However, H2A.X phosphorylation correlates with all known double-stranded DNA breaks suggesting that it acts more as a ‘DNA-damage sensor’ than a specific chromatin mark linked to the apoptotic process.

To further investigate the relationship between histone phosphorylation and mitotic and/or apoptotic chromatin condensation, we have generated a
novel phos-specific antibody against Ser14 residue on histone H2B (hereafter \(\alpha\)-Phos\[Ser14\]H2B) (commercially available from Upstate Biotech. Inc.; Lake Placid, NY). Using this antibody in several vertebrate and mammalian models, we found that H2B Ser14 phosphorylation specifically correlates with the onset of apoptotic chromatin condensation and DNA fragmentation in human cells (Cheung et al 2003). This correlation was also found in cells undergoing programmed cell death during *Xenopus* tail resorption. Using in-gel kinase assays, we detect and have identified an apoptotic-induced H2B (Ser14) kinase with a molecular weight of 34 kDa as the caspase cleaved form of Mst1 (Mammalian Sterile Twenty), which is a well studied kinase activated by multiple apoptotic stimuli (Feig & Buchsbaum 2002, Graves et al 2001). Interestingly, the Ser14 phosphorylation site is only conserved among vertebrates, ranging from frog to human (Fig. 3B), leaving open the intriguing possibility that additional apoptotic phosphorylation sites might exist on other sites of the histone tails of invertebrates. Nevertheless, these studies define what may be an apoptotic ‘histone code’ conserved among vertebrates, and cast new light on physiological substrates of Mst1 kinase.

Mst1 is a member of sterile 20-like superfamily of which approximately 30 related kinases exist in humans (Graves et al 2001). Kinases contained in this superfamily are most often regarded as an upstream regulator of MAPK pathways with roles in cellular morphogenesis and cytoskeletal rearrangements, as well as apoptotic cell death (Feig & Buchsbaum 2002). Our finding that the cleaved-form of Mst1 is likely a nuclear-bound kinase directly responsible for H2B (Ser14) phosphorylation, at least in higher eukaryotic cells under some inducing conditions, might shed new light on it as a potential drug target. Chromatin condensation and DNA fragmentation have been viewed as the last committed step of apoptosis. Considerable evidence exists suggesting that many cells die under stress by undergoing apoptosis (Wyllie 1980). However, using caspase inhibitors has not been very effective to decrease cell death after the initial stress, such as ischaemia, has occurred (Natori et al 2003). Perhaps, after effector caspases initiate the death pathway leading to defined chromatin changes, caspases are no longer needed. It is possible that effective prevention of cell death may be best brought about by combining caspase inhibitors with drugs that target downstream activities such as are caused by Mst1 to prevent chromatin changes during apoptosis.

Unlike the H3 Ser10 and Ser28 phosphorylation, the Phos (Ser14) H2B mark is not detected in mitotic chromosomes, at least in the cell types that we have examined. Thus, we are intrigued with the possibility that there may be a non-overlapping set of phosphorylation marks that discriminate ‘mitotic’ from ‘apoptotic’ chromatin. It is becoming well established that bromodomains ‘read’ acetyl-lysine mark on histones (and likely non-histone proteins), and
chromodomains ‘read’ methyl-lysine marks in a sequence context-dependent fashion (see above, Jenuwein & Allis 2001). However, it is not clear whether there are effector proteins docking on phosphorylated histone tails peptides, which is currently pursued in my laboratory (C. Barber and C. D. Allis, work in progress).

**Epigenomics and human diseases**

It has been widely accepted that DNA methylation and histone modifications serve as two major mechanisms for the function and inheritance of epigenetic information (Jaenisch & Bird 2003). Recent advance has suggested that DNA methylation and histone methylation are correlated in *Neurospora crassa*, plant and mammals (Tamaru et al 2003, Soppe et al 2002, Fuks et al 2003). Importantly, histone deacetylation, histone methylation, and DNA methylation are involved in the aberrant silencing of certain tumour suppressor genes in tumour cells (Bachman et al 2003, Kondo et al 2003). In the case of the p16INK4a tumour suppressor gene, Bachman et al (2003) have found that histone Lys9 methylation precedes DNA methylation. The promising result of applying HDAC inhibitors in the treatment of leukaemia is a harbinger for cancer treatment by interfering with epigenetic histone modifications. Collectively, these exciting developments make a compelling argument for investments in developing new therapies centered in attacking epigenetic forms of gene regulation. We predict that chromatin modifications will revolutionize our view of cancer as new mechanisms of ‘epigenetic’ carcinogenesis are discovered.

Given the emerging link between histone modifications and DNA methylation, it is conceivable that histone modifications might be involved in the multiple diseases caused by epigenetic disorder, including deregulation of imprinted genes. In addition to the direct control of tumour suppressor gene expression, members of the Pc and HP1 family have been suggested to play a role in the cell proliferative capacity (Lessard & Sauvageau 2003, Varambally et al 2002, Kirschmann et al 2000). Furthermore, it remains to be discerned whether histone modifications are involved in various genomic imprinting disorders, such as Beckwith-Wiedemann, Angelman, and Prader-Willi Syndromes (Wolfe & Matzke 1999).

**Conclusions and perspective**

Chromatin is the physiological template of our genetic information. Well known is the understanding that this polymer is subject to a diverse array of post-translational modifications that largely impinge on histone N-termini, thereby
regulating access to the underlying DNA. The combinatorial nature of histone N-terminal modifications thus reveals a ‘histone code’ that significantly extends the information potential of our genetic code. As is well documented in the literature and in the meeting itself, this covalent modification-based histone code may well exist in non-histone proteins, suggesting a more universally applied protein code. Current evidence suggests that it is a fundamental regulatory mechanism that impacts on most, if not all, chromatin-templated processes with far-reaching consequences for cell fate decisions, and normal and pathological development.

References


DISCUSSION

Khochbin: Some of the acetyltransferases from the MYST family have a chromodomain, like Tip60 or Mof. Do you have any evidence for the targeting of methylated lysine by these chromodomains?

Allis: That’s a great question. Just in case anyone is not following, the comment was that in the histone acetyltransferase (HAT) field, there is a group of HATs that are known collectively as the ‘MYST’ family. Some members of this family have chromodomains themselves, so it is tempting to think that there might be a methyl mark that might be read. One of the members of this family that you might be thinking about is Mof. This was originally discovered by John Lucchesi and his colleagues at Emory University in Atlanta (Hilfiker et al 1997). That Mof is a famous HAT because it is well known to be the up-regulator for the fly male X chromosome. It puts on the Lys16 acetyl mark. I didn’t say anything about H4, but one of the conserved lysines in the H4 tail is Lys16. The H4 tail has lysines at K5, K8, K12 and K16. Most of these are separated by runs of glycine, until you get to this K16, where it then goes KRHRK. The K20 is a well known methyl site in histone H4. It might be attractive to have a chromodomain that could dock on K20 methyl near where a HAT would have to go to put on this acetyl site (Nishioka et al 2002). In fact, if you ask me why the H4 tail suddenly goes from lysines separated by Gly to KRHRK, I think this is another hot area for something to be binding. I suspect we will have bromodomains that will be docking on the acetylated form of this lysine. It would be very clever if nature is exploiting this tail a little more, by putting on HATs that are required through binding via chromodomains. If you search for the KRHRK in the available databases, it goes well past histones. These might be small protein modules that have been exploited in histones with their abundant post-translational modifications.
Verdin: How big is the chromodomain family? How many chromodomains are there in the whole genome?

Allis: There are lots of chromodomains in the human genome. At least 40. I don’t think they will necessarily all be histone-docking motifs. The chromodomain of Mof that might be reading methyl K20, was originally described to be an RNA-binding motif. With the employment of small RNAs and guide RNAs in the nucleus, we should be open to other roles for chromodomains beside histone methylation.

Moazed: I have a couple of questions concerning your methyl-phospho switch. First, is it known whether HP1 or homologues such as polycomb dissociate from chromatin during mitosis?

Allis: This has been published by others in Drosophila and Schizosaccharomyces pombe.

Moazed: How do you propose that the kinase can gain access to the serine, if HP1 is stably bound to the histone tail?

Allis: It is hard for me to tell this from crystal structural information. The question would be can the kinase get at its target in the presence of what might be docking here? I think the cell can do it. These are all in giant complexes. New studies suggest that HP1 is ‘on and off’ chromatin in a very dynamic way (Festenstein et al 2003, Cheutin et al 2003). I am sure that it will prove to be more than just a recombinant kinase getting in here and doing the job.

Moazed: Is it possible that there is a second step: that something else dissociates the complex?

Allis: It is possible. This sort of two-step model is possible. All I can tell is that our mass spectrometry studies have no problem in picking up that di-modified state. How it is achieved, I am not sure.

Marmorstein: If it binds to the HP1 chromodomain with some given dissociation constant, there are times when it is off, so the kinase could gain access then.

Jenuwein: There is another explanation. If you look at the phosphorylation status of the enzyme, the Suv39h HMTase (histone methyltransferase) becomes phosphorylated during mitosis, and along with it it dissociates from mitotic chromatin (Aagaard et al 2000). One could therefore argue that there may be another model. The phosphorylation event of the Suv39h enzyme could attenuate the activity of the HMTase itself.

Allis: That is another possibility: the methyltransferase is phosphorylated as part of a reaction that then may do many things we have yet to figure out.

Turner: The ideas you have presented are tremendously exciting, but they present us with enormous problems in terms of using the antibody. If we have an antibody to methyl K4, as many of us do, is that still going to bind when P3 is phosphorylated, and vice versa? This means we are in danger of getting a whole bunch of false negative results.
**Allis:** We could spend some quality time discussing this. If you have a methyl site, get a great looking antibody, employ it and suggest that there is some very interesting cell cycle or developmental regulation, you could be totally fooled by what you don’t know is going on next door. This density of modifications may almost make the antibodies—as beautiful as they have been—potentially problematic as tools. Louis Mahadevan has all kinds of evidence that Ser10 phosphorylation in the immediate early mitogen response can work with Lys9 acetylation next door, so antibody recognition here with a Ser10-selective antibody is a concern. We are comforted a bit that we can do mass spec, which gets us away from the use of antibodies alone.

**Turner:** It doesn’t have the flexibility of the antibodies. Louis particularly has attempted to raise antibodies that recognize two motifs. Is this what we are looking at: raising a batch of antibodies for every site?

**Allis:** That is a reasonable way to go. Louis knows better than anyone that if we have a K9 acetyl in conjunction with a neighbouring Ser10 phospho, the dimodified antibody is a gorgeous reagent for the immediate early mitogen response. It doesn’t seem impossible to then reach for K9 methyl Ser10 phospho, and then play this out over and over again.

**Mahadevan:** I think eventually we will have to raise highly specific antibodies against all these combinations. These problems are accessible to much more specific well characterized antibodies. We should also consider quantitative issues. When we talk about serine 10 phosphorylation, for example in mitotic cells, would you have any idea about the stoichiometry of phosphorylation? When we read about it we hear that it is very highly phosphorylated. Do you think it is completely phosphorylated?

**Allis:** I wouldn’t want to say it is all phosphorylated. When we run acid-urea gels that permit us to separate unmodified from a mono-modified protein, the mitotic sample jumps up to a significant amount of the total. If you got a pretty respectable enrichment for mitotic samples, I’d say that over half is jumping up. Unless you want to say that is mono-acetyl (and I don’t think it is in mitotic samples), I would suggest over 50% of the molecules are phosphorylated. A healthy dose of the H3 picks up the phospho mark in a mitotic culture.

**Verdin:** Given the fact that methylation apparently is not reversible, wouldn’t this be one of the best candidates in terms of maintaining a memory?

**Allis:** I think that is right. Acetylation has beautiful reversibility. Could it be that the decision to methylate was very purposeful because of its permanent chemical nature, whether on DNA or histone.

**Berger:** At one point you mentioned that you think you have effectors that bind to phosphorylated Ser10 on histone H3. But you said that they wouldn’t bind to the histone when it is methylated. For a unified model, this presents a bit of a problem. If the methyl marks are permanent, then how does the binding come about?
Allis: If we have just a Ser10 phospho effector (which I called ‘Y’), and we eject this by methylation on the adjacent Lys9—or, to be fair, acetylation—what happens? All I can say is that we tested our candidate Y with the methyl-phos, and it didn’t bind at all. But you are right, if you put on that methyl mark to be the ejector, how do you remove that? It is a problem. Some people will say that it is possible to dilute histones out of chromatin and replace them.

Berger: Is there a difference between mono-, di- and trimethylation in terms of that binding?

Allis: It is modest. Whether the HP1 on K9 is mono-, di- or trimethylated only has a modest effect. If you generate antibodies of Lys9 that are mono- versus di-versus trimethylated, these are like different beasts. The regulation opportunities that are available to the cell from just two residues are awesome. If you look at the H3 tail I have been telling you about, in my early Tetrahymena postdoc days I found out that the Tetrahymena in one of its nuclei (the silent nucleus) quantitatively cleaves the H3 between residues 6 and 7. The only thing we knew at the time was that the H3 extreme N-terminus was AR. After you cleave off this six residue piece, there is a new AR. For Tetrahymena this would remove the Lys4. This is the positive up-regulator that ties in to activation, especially if it is trimethylated. I think this may be something that Tetrahymena does purposefully in its silent nucleus. In yeast, their chromatin is clipped in the H3 region. We recently sequenced this when we knew that GCN5 liked to hit on H3, and found that there is a new AR utilization. We have now a little glimmer that mammalian cells are doing this too, in constitutive heterochromatin. The trimethylated K9 mark is very much the constitutive heterochromatin. If this is something that Tetrahymena and yeast do, and it might extend to mammalian cells, then all bets are off on how unwanted methyl marks are removed. Do you demethylate them, clip them or replace them?

Atadja: Have people really looked for demethylases?

Allis: We have looked very hard and come up with nothing. But there is a breath of fresh air: we have made some progress on arginine demethylation. Arginine looks reversible.

Li: Does the K9 methylation re-establish every cell cycle if you just focus on one particular gene locus?

Allis: I don’t know whether I can answer that.

Li: What about Ser10 phosphorylation? Is it directly involved in chromatin condensation?

Allis: If we make that mutation in Tetrahymena it causes severe mitotic dysfunction. I’m not sure why this doesn’t happen in yeast, but redundancy could be the explanation. There is a published report that the Ser10Ala mutation in Tetrahymena had a severe mitotic failure of condensation with some chromosome segregation defects (Wei et al 1999).
References


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