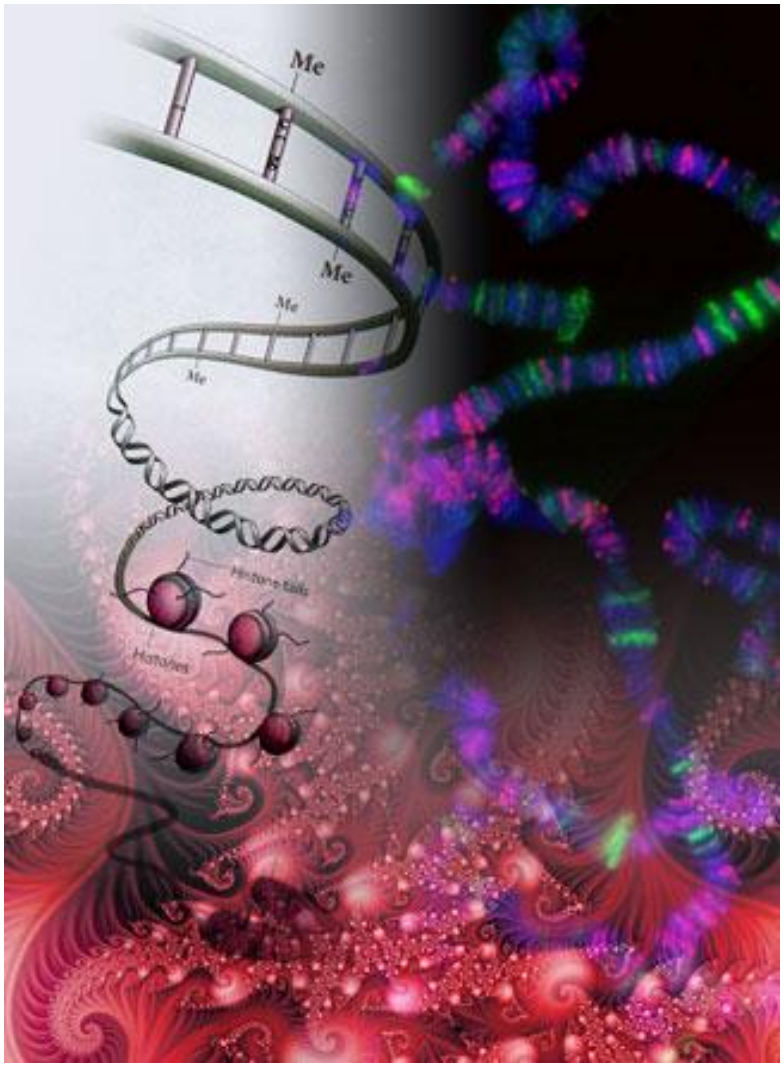
The role of phospho/methyl switch mechanisms in regulation of nuclear processes



Elke F. Roovers
July, 2011

Master thesis Cancer Genomics & Developmental Biology
Under supervision of Prof. Dr. H.Th.M. Timmers and A. Schram, MSc
Utrecht University

Contents

Introduction

The DNA of eukaryotes is packed into chromatin, a nucleoprotein complex that is subject to continuous adjustments, in order to regulate nuclear processes. Chromatin is build up out of nucleosomes, structures in which DNA is wrapped around a protein complex consisting of histones. Two copies of four different histones – H2A, H2B, H3 and H4 – form an octamer, around which 147 base pairs (bp) of DNA is wrapped. This results in the formation of a 'beads-on-a-string' construction (Fig. 1). Two nucleosomes are connected via a stretch of 'linker-DNA,' of 10-80bp in length. In metazoa, histone H1 binds the nucleosome at the incoming and exiting DNA and contributes to a higher order structure, in which the DNA-histone-complexes form a secondary helix, called a 'solenoid,' with a diameter of 30nm (Fig. 1). H1 contributes to the compactness of the chromatin, as in its absence, the chromatin is less condensed.

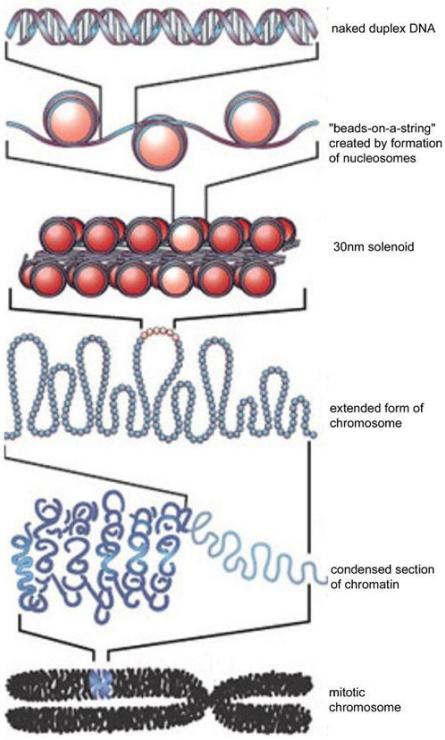


Figure 1: Illustration of chromatin. DNA is wrapped around nucleosomes, forming a DNA organization referred to as 'beads-on-a-string.' This can be further condensed to a 30nm solenoid. During mitosis, chromatin condensates and forms microscopically visible chromosomes .

Depending on the degree of condensation, chromatin is roughly divided into two states, heterochromatin and euchromatin. Heterochromatin is highly condensed and can be subdivided in constitutive and facultative heterochromatin. Both forms of heterochromatin are associated with transcriptionally inactive DNA. Constitutive heterochromatin mainly consists of repetitive sequences, such as centromeres and telomeres. The formation of facultative heterochromatin can differ between cell types and is reversible. Euchromatin is transcriptionally active chromatin. Its conformation is less condensed than heterochromatin and therefore accessible to transcription factors.

The organization of DNA is determined by covalent modifications, that can occur on nucleotides, such as cytosine methylation, or on N-terminal histone tails that protrude from the nucleosome. These tails are subject to a broad range of post-translational modifications (PTMs) that are important in chromatin modulation. Together, these modifications that alter genetic connotation without changing the DNA, are called epigenetic modifications.
They have consequences for the compaction and accessibility of the DNA. Different epigenetic modification mechanisms will be discussed below.

DNA methylation

In eukaryotes, DNA can be methylated on cytosine residues. In mammals, this usually occurs in a CpG dinucleotide context (in which 'p' denotes the phosphodiester bond between the cytosine and the guanine, indicating they are located on the same strand), that often reside in CpG-islands; regions of ~0.3-3 kb, with a high frequency of this di-nucleotide. This methylation of cytosines is a modification associated with transcriptionally silent regions, such as transposons, repeats and inactivated X-chromosomes. CpG-islands are also found in ~70% of the 5' ends of transcription start sites (Bajic et al., 2006; Deaton and Bird, 2011).

DNA methyltransferases (DNMTs), are the enzymes responsible for the catalyzation of the addition of methylgroup to the C5 of the pyrimidin ring of cytosine (Fig. 2). The methyl-donor for this reaction is S-adenolsyl-methionine (SAM), that is converted into S-adenosyl-homocysteine (SAH). DNA methylation can occur *de novo* by DNMT3A and B – in which new methylation marks are placed on unmethylated CpG sites – or can be maintained through mitosis by DNMT1, resulting in two daughter cells with the same methylation pattern (Zhao et al., 2009).

Recent findings have showed that cytosines are also found to be hydroxymethylated. The ten-eleven translocation (TET) enzyme family can be recruited to CpG islands and converts methylated cytosines into hydroxymethylcytosines (Tahiliani et al., 2009; Wu et al., 2011). Depletion of Tet1 in mice results in increased cytosine methylation within CpG islands and leads to downregulation of some Tet1 targets (Wu et al., 2011). Additionally, Tet1 also plays a role in the repression of several polycomb-group (PcG) genes, that play an important developmental role, and has therefore been proposed to play a regulatory role in DNA methylation and expression of PcG targets (Williams et al., 2011; Wu et al., 2011).

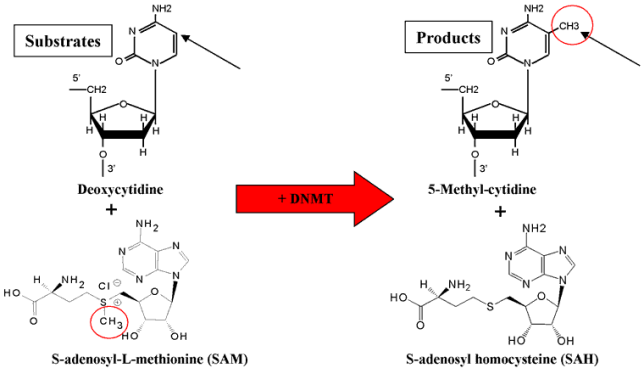


Figure 2: Cytosine methylation. A DNA methyltransferase catalyzes the transfer of a methylgroup from SAM to a cytosine in a CpG context. This methylation marks an inactive area on the DNA.

Histone modifications

The histone tails that are post-translationally modified can influence the overall chromatin structure, or they can adjust the accessibility of the DNA for DNA-binding factors at a specific region. Common types of PTMs are acetylation and ubiquitination, which occur on lysines, methylation of arginines and lysines, and phosphorylation of serines and threonines. To date, the existence and function of many different histone modifications have been described, some of which are associated with active DNA and some are typically found in inactive regions. Different types of modification mechanisms will be discussed below.

Acetylation

Lysines acetylation decreases the positive charge of the histone tail by one, since the acyl group (COCH3) has one free electron, that forms a chemical bond with the histone residue and therefore neutralize the histone. As a consequence, chromatin will become less condensed and accessible to transcription factors, and formation of the 30nm fiber is impeded (Shogren-Knaak et al., 2006). Additionally, the DNA, that is negatively charged will be less tightly bound and becomes more accessible. Acetylations are thus associated with transcriptionally active regions and can occur on all four histone tails (Table 1). Histone acetylation is a reversible process, which represents a dynamic mechanism for opening and closing of chromatin, and plays an important role transcriptional regulation.

Methylation

Histone methylations can occur as a single modification, but often di-or trimethylations are found (Reviewed in Zhang and Reinberg, 2001). Methylations do not change the histone charge, but rather serve as a docking site for downstream molecules (Berger, 2007). Therefore, these PTMs can be involved in both active and inactive chromatin formation, whereas acetylation is more unambiguous. Moreover, some methylation marks have been shown to be passed on to daughter cells, and can therefore play a role in bookmarking chromatin (Kouskouti and Talianidis, 2005; Sarraf and Stancheva, 2004). Methylation of a histone residue can be associated with inactive transcription, such as trimethylation of lysine 27 on histone 3 (H3K27me3) and H3K9me3. Furthermore, there are also examples of active methylation marks. For instance, H3K4me3 is an active mark, mainly associated with transcription start sites (Table 1) (Bernstein et al., 2005). In addition, distinction between symmetric or asymmetric dimethylation of arginines by downstream proteins can have a different outcome. For instance, symmetric H4R3me2 (H4R3me2s) correlates with gene repression, whereas H4R3me2as is linked to active genes (Table 1).

Table 1: Histone PTMs (Adapted from Berger, 2007)

Histone PTM	Residue	Repression/Activation
Acetylated Lysine	**H3**: 9, 14, 18, 56 **H4**: 5, 8, 13, 16 **H2A, H2B**	Activation
Phosphorylated Serine/Threonine	**H3**: 3, 10, 28 **H2A, H2B**	Activation
Methylated Arginine	**H3**: 17, 23 **H4**: 3as	Activation
	H4: 3s	Repression
Methylated Lysine	**H3**: 4, 36, 79	Activation
	H3: 9, 27 **H4**: 20	Repression
Ubiquitylated Lysine	**H2B**:123 (yeast), 120 (mammals)	Activation
	H2A: 119 (mammals)	Repression
Sumoylated Lysine	**H2B**: 6/7, **H2A**	Repression

Phosphorylation

Phosphorylation of proteins is an important PTM in various signaling pathways throughout the cytoplasm on serine, threonine or tyrosine residues. They often function as on/off switches, by increasing the energy of a protein, inducing a conformational change or provide a recognition domain for a downstream protein. Histone tails are subject to phosphorylation as well. Upon phosphorylation, the positively charged histone tail is being neutralized by two electrons, which can make the chromatin more relaxed, similar to acetylation (Figure 3), but it can also create a binding site for effector molecules. Histone residues that have been found to be phosphorylated are listed in Table 1. Interestingly, phosphorylation correlates with both transcriptional activation and chromosome condensation during mitosis, thus with opening and closing of the chromatin, respectively, which has been shown for phosphorylation of H3S10 (Reviewed in Johansen and Johansen, 2006). Therefore, similar to methylated residues, the function of phosphorylated residues is not clear-cut but depends on the genomic context.

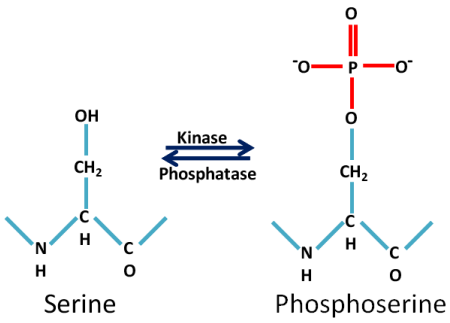


Figure 3: Serine phosphorylation. A kinase catalyzes the addition of a phosphate group to the OH of the serine, often using ATP as a donor. Conversely, serine phosphatases can remove the phosphate group.

Ubiquitination and SUMOylation

Another type of modification of histones, is lysine ubiquitination or SUMOylation. Ubiquitination occurs in a similar way to ubiquitination of non-histone proteins, requiring activating, conjugating and ligating enzyme activity of E1, E2 and an E3 proteins respectively. Rather than small chemical groups as acetyl- and methyl molecules, one ubiquitin is 76 amino acids, which is about two-thirds of the histone it is being attached to. This will have consequences for the overall chromatin structure. For protein degradation in the proteasome, four or more ubiquitin units need to be ligated (Pickart, 2001). In histone ubiquitination, usually a single ubiquitin molecule is added, indicating this has rather a chromatin modifying or signaling function, rather than targeting for degradation. Ubiquitination can occur on lysines on histones H2A and H2B (Table 1). Similar to other PTMs, ubiquitination is reversible and can be removed by ubiquitin proteases (Wilkinson, 2000). Another ubiquitin-like molecule influencing chromatin is small ubiquitin-related modifier (SUMO), that is added to histones in a similar manner to ubiquitin, involving SUMO-specific E1, E2 and a E3 enzymes. H2A and H2B can be

SUMOylated, which has a repressing effect (Nathan et al., 2006). H2A can be ubiquitinated on lysine 119 in mammals, linked to transcriptional repression, whereas H2B ubiquitination – on K123 in yeast and K120 mammals – is associated with gene activation (Table 1) (Henry et al., 2003).

Writers and erasers

The enzymes responsible for decorating the histone tails are called 'writers.' Different histone modifications require different types of writers. For instance, histone methyltransferases (HMTs) can add one to three methylgroups to lysines or arginines. Lysines are commonly methylated by SET(Suvar, Enhancer of zeste, Trithorax)-domain-containing proteins, whereas arginines are modified by another class, the Protein arginine N-methyltransferases (PRMTs) (Ng et al., 2009). Lysines are methylated by a wide variety of enzymes, often with a high specificity for residue and non-, mono- or di-methylated modification state of the substrate, whereas PRMTs usually target arginines in a less defined manner (Bannister and Kouzarides, 2005; Ng et al., 2009). Asymmetric or symmetric methylation of arginines, depends on methylation by PRMT type I or II enzymes respectively (Fig. 4). The methyl-donor in HMT reactions is SAM, the same donor as in cytosine methylations (Fig. 4). Acetyl groups can be linked to lysine residues on histone tails by histone acetyltransferases (HATs) (Fig. 5).

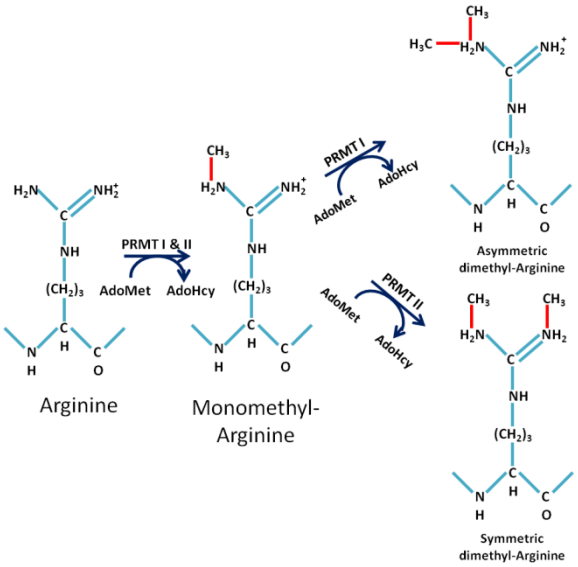


Figure 4: Arginine methylation. The first methylation step can be catalyzed by type I and II PRMTs. The second methylation will result in asymmetric or symmetric dimethylation, when catalyzed by PRMT I or II respectively (Adapted from Zhang and Reinberg, 2001).

The proteins responsible for removal of PTMs are called 'erasers.' This way, acetylations can be removed by histone deacetylases (HDACs) (Fig. 5) and methylations by demethylases. Writers and erasers can act in response to signalling pathways, activated by external or internal stimuli. It is the dynamic character of the addition and removal of the modifications that contributes to the important role that chromatin plays in nuclear processes.

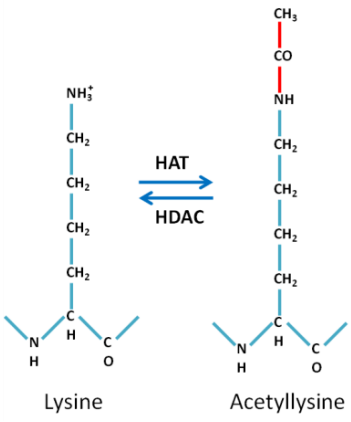


Figure 5: Lysine acetylation. A histone acetyltransferase (HAT) adds an acetylgroup to the NH_3^+ group of lysine. This modification is reversible and can be removed by a histone deacetylase (HDAC).

Readers

The modifications, or histone marks, are bound by proteins, called 'readers' or 'effectors.' This association determines their biological function. These proteins can bind to a specific modification with a wide variety of domains. They influence chromatin function, by for instance stimulating transcription, crosslinking different nucleosomes or promoting further chromatin modification (Reviewed in Ruthenburg et al., 2007). Methylated lysine residues can be recognized by different types of protein domains, including chromodomain proteins, plant homeodomain (PHD) finger proteins and tudor domain proteins (Yang et al., 2010). Methylated arginines are mainly recognized by tudor domains, however, just as unmodified H3K4, they can be recognized by ATRX-Dnmt3-Dnmt3L (ADD) domains as well, which is present on a DNA methylating enzyme Dnmt3 (Ooi et al., 2007; Zhao et al., 2009).

Acetylations and phosphorylations can function as chromatin remodelers by influencing the overall histone charge, but can function as an adaptor as well. Acetylated lysines form a binding site for bromodomain-containing proteins, motifs often found in transcription-enhancing complexes (Zeng and Zhou, 2002). So far,

not many readers of phosphorylated residues have been discovered. The BRCT domain is known for binding phosphorylated amino acids. The BRCT-containing protein Crb2 has been found to bind phosphorylated H2A in response to DNA double-stranded breaks (DSBs) (Sofueva et al., 2010). However, a more evident role for phosphorylated histone residues seems to be influencing other reader-PTM interactions, which will be discussed later.

Since methylated residues do not contribute to the histone tail charge to modulate the chromatin, the biological function is often determined by their readers and the complex they reside in. H3K4me3 is recognized by the PHD finger of TAF3, a subunit of the TFIID transcription factor complex, important for transcription initiation (Vermeulen et al., 2007). Furthermore, H3K4me3 is recognized by ING2, which is present in a HDAC and contributes to gene repression (Shi et al., 2006). The same reader can have different functions as well. The chromodomain of Eaf3 binds methylated forms of H3K4 and H3K36. However, Eaf3 is present in different complexes with opposite activities, a HAT (NuA4) and a HDAC (Rpd3S) (Eisen et al., 2001; Reid et al., 2004). Different connotations of the same mark or reader can be explained by the fact that PTMs usually do not occur as a single mark on a histone, but rather exist as specific patterns together with other PTMs. Readers therefore often recognize a specific PTM-signature, resulting in increased specificity and higher combined affinity.

Multiple marks can be recognized by the combination of different readers in an multivalent complex. For instance, the Rpd3S HDAC requires the PHD protein Rco1, which probably recognizes an additional (modified) lysine, in addition to the lysine recognized by Eaf3, to obtain a combinatorial affinity for K36me3 regions specifically (Li et al., 2007). However, it has become clear that many readers itself are sensitive to the modification state of multiple residues as well. For example, the TATA-binding protein associated factor 1 (TAF1) contains a double bromodomain to recognize diacetylated H4 (Jacobson et al., 2000). Another oligovalent protein, BPTF (bromodomain PHD transcription factor), comprises two domains to recognize H4K16Ac (bromodomain) and H3K4me3 (PHD finger) (Ruthenburg et al., 2011). This interaction not only combines two marks, but includes the modification state of both H3 and H4. Finally, a second modification can also have a negative influence on a reader-PTM interaction. The interaction between TAF3 and K4me3 is inhibited by asymmetric methylation of R2 (Vermeulen et al., 2007).

These examples illustrate that the epigenetic environment needs to be included to correctly interpret a histone modification. This context depends on other PTMs, DNA methylation, the writers/erasers in effector complexes and the continuous interplay between them. Next, examples of different histone communication mechanisms will be discussed , with a focus on histone switch mechanisms that regulate many nuclear functions at the chromatin level.

Histone crosstalk

Individual histone modifications and their writers, readers and erasers have been extensively studied. From this, it has become clear that many histone PTMs act in a combined or consecutive manner, to regulate regions more specifically. Therefore, the combination of PTMs will function as a code for the eventual chromatin output which has been described in the histone code hypothesis (Strahl and Allis, 2000). The presence of one PTM modulates the function of other modifications. Furthermore, one modification is often a signal for deposition or removal of another modification, emphasizing the intense intercommunication between writers, PTMs and effectors. This phenomenon, in which PTMs and their writers, erasers and readers/effectors function in downstream signaling mechanisms, is referred to as histone crosstalk (Fischle et al., 2003b). PTMs on the same histone tail can communicate, which is called *cis*-histone crosstalk (Fig 6). This can also occur via a *trans*-tail mechanism, in which different histone tails within the same nucleosome modulate each other(Fig. 6). Histone PTMs can influence modifications on other nucleosomes as well, which is referred to as *trans*-histone crosstalk. Additionally, histone PTMs and DNA methylation manipulate each other as well, linking these two epigenetic mechanisms (Fig. 6) (Reviewed in Fischle, 2008). As mentioned before, this can result in a combinatorial readout of PTMs by multiple proteins in the same complex or by proteins that have several binding domains. Below, different forms of crosstalk will be discussed using a number of examples.

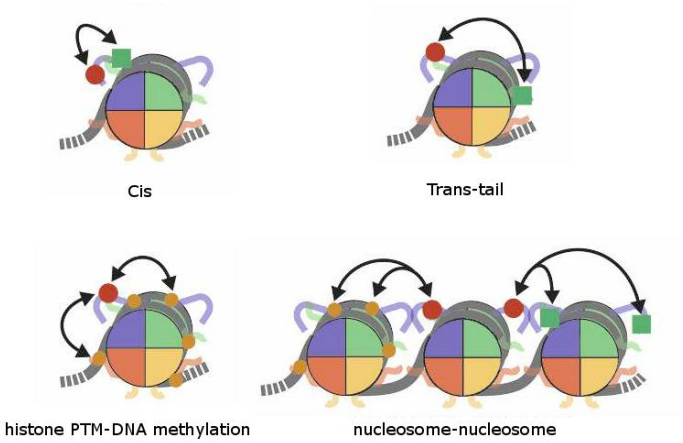


Figure 6: Different forms of crosstalk. Adapted from Fischle *et al.* 2008

Cis-histone crosstalk

Histone residues can communicate with each other by inducing or removing PTMs on other residues on the same tail. In this case, a writer or an eraser usually bind the first modification and act as a writer or eraser for the second. An example is the Spt-Ada-Gcn5 Acetyltransferase (SAGA) complex that plays an important role in transcription activation. The SAGA complex binds di-and trimethylated H3K4 on promoter regions via a tandem tudor domain Sgf29 (Bian et al., 2011; Vermeulen et al., 2010). Sgf29 recruits SAGA to these regions and is required for proper acetylation of H3 (Bian et al., 2011). Gcn5 is the catalytic acetyltransferase subunit of SAGA. Gcn5 acetylates H3 on K14, but can also acetylate H3K9, H3K18 and H3K23, according to both *in vivo* and *in vitro* studies (Grant et al., 1999; van Oevelen et al., 2006). Without Sgf29, acetylation of H3K9, H3K14 and H3K18 (and in humans also H3K23) is reduced *in vivo* and SAGA is not properly localized to promoters (Bian et al., 2011). This is also seen in Sgf29 that lack the tandem tudor domain and mutants that lack Spp1 or Swd1, to HMTs required for H3K4 methylation (Bian et al., 2011). These results illustrate that methylated H3K4 stimulates subsequent acetylation of lysines on H3 in *cis*.

One histone modification can also induce removal of another modification. Nucleosomes that are marked with the active mark H3K4me3 are also primed for removal of the H3K9me3 inactivating PTM via the demethylase JMJD2A. Via its tudor domain, JMJD2A can bind H3K4me3, and has a demethylating substrate preference for di- and trimethylated forms of H3K9 and H3K36 (Huang et al., 2006; Ng et al., 2007). Just as H3K4me3, methylated H3K36 is an active mark, but present in coding regions as opposed to H3K4me3-marked promoters. This way, both inactive marks (H3K9me3) and marks that interfere with proper transcription initiation (H3K36me3 in promoter regions) are being removed by the same demethylase in regions marked by H3K4me3.

Trans-histone crosstalk

Trans-tail modifications

Instead of influencing residues on the same tails, histone PTMs can influence PTMs on other tails within the same nucleosome as well. This *trans*-tail phenomenon can be illustrated by crosstalk between H3K4me3 and ubiquitination of H2B. The HMT Set1 is responsible for H3K4 methylation in promoter regions. It has been demonstrated that Set1-dependent H3K4 methylation requires H2B ubiquitination on lysine 123 (Sun and Allis, 2002). Set1 is part of the COMPASS (Complex Proteins Associated with Set1) complex in yeast and can di-and trimethylate H3K4 in the presence of Cps35 (Lee et al., 2007). Lee *et al*. 2007 showed that without ubiquitination of H2BK123, Cps35 does not associate with COMPASS, and Set1 can only mono-methylate H3K4. Another example is phosphorylation of H3S10 on enhancer regions of several *MYC* genes. This modification promotes acetylation of H4K16, which is required for activation of genes they regulate (Zippo et al., 2009). This example also demonstrates the influence of the epigenetic state of nucleosomes of the enhancer on transcription of more distal regions.

Histone PTMs and DNA methylation

Besides crosstalk between different histone modifications, they can also influence DNA methylation. This has been demonstrated for the Polycomb-group (PcG) proteins. EZH2 (Enhancer of Zeste Homolog 2) is a histone methyltransferase, present in the Polycomb repressor complex 2 (PRC2). It contains a SET-domain, with which it trimethylates H3K27. This recruits the PRC1 complex that binds K27me3 via the Polycomb chromodomain that contributes to a repressive chromatin state (Cao et al., 2002). EZH2, in the context of PRC2 proteins, was shown to interact with DNMTs. Moreover, common targets of EZH2 and DNMTs were dependent on EZH2 for DNA association of DNMTs and cytosine methylation. This was demonstrated by depletion of EZH2, which resulted in reduction of linked DNMTs and methylated CpG sites at that region (Vire et al., 2006).

Other residues associated with transcriptionally silent regions can be linked to DNA methylation. H4R3me2s functions as a binding site for the PHD of DNMT3A, which subsequently methylates DNA incorporated in that nucleosome (Zhao et al., 2009). Unmodified H3K4 can be bound by the PHD of DNMT3A that allosterically stimulates cytosine methylation enzyme activity. This is negatively regulated by methylation of H3K4, which ascertains prevention of cytosine methylation in promoter regions (Li et al., 2011). Conversely, there are examples of crosstalk from cytosine modification state to histones as well. Mouse *dnmt1* and *dnmt3b* cells, display aberrant H3K9 methylation patterns in heterochromatic regions (Espada et al., 2004; Gilbert et al., 2007).

Histone crosstalk between nucleosomes

Histone crosstalk is not limited to one nucleosome, but can also influence other nucleosomes. Trimethylated H3K9 is a PTM found in heterochromatic regions. H3K9me3 is required for gene silencing, the formation of centromeres and stabilization of telomeres (Grewal and Jia, 2007). During heterochromatin formation in *S. pombe*, methylated H3K9 is bound by heterochromatin protein 1 (HP1) homolog Swi6 via its chromodomain (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002). Swi6 can associate with itself, which is required for localization to heterochromatic regions (Haldar et al., 2011). It has been suggested that Swi6 exists as preformed dimers via a chromoshadow-chromoshadow interaction, and tetramerizes via their chromodomains (Canzio et al., 2011). Swi6 octamers have been detected as well (Haldar et al., 2011). This oligomerization could result in the bridging of adjacent nucleosomes, as depicted in Fig. 7, but also bridging different chromatin fibers, contributing to higher order heterochromatin (Canzio et al., 2011). This way, H3K9me3-marked nucleosomes communicate with other nucleosomes containing this mark, in order to form heterochromatin. H3K9me3 can also stimulate spreading of this mark to other nucleosomes, in cooperation with the RNA silencing machinery (Irvine et al., 2006). Moreover, in human cells, HP1 has been shown to interact with DNMT1 and DNMT3A, and associates with DNA methyltransferase activity, providing another link from readers of histone PTMs to cytosine methylation (Fuks et al., 2003). Communication can thus occur

between adjacent nucleosomes, such as nucleosome bridging and H3K9me3 spreading. However, nucleosomes can influence distal nucleosomes as well, such as the influence of the epigenetic state of the enhancer on the coding region of *MYC* genes (Zippo et al., 2009).

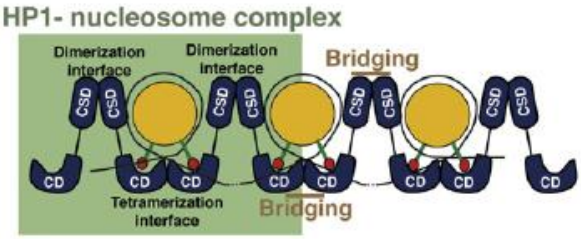


Figure 7: nucleosome bridging via HP1. HP1 dimerizes via its chromo-shadow domain (CSD) and binds H3K9me3 with its chromodomain (CD). HP1 is present as a pre-formed dimer, but on chromatin forms tetramers and possibly higher oligomers (Canzio et al., 2011).

Dynamics of histone PTMs

Several mechanism of histone crosstalk have been discussed so far. As the function of an increasing number of modifications becomes clear, examples of seemingly conflicting mechanisms are being revealed as well. The ubiquitination of H2BK123 is required for H3K4 di- and trimethylation via a trans-tail mechanism. The E2 conjugating enzyme that targets H2B is Rad6, together with the E3 ligase Bre1 (Robzyk et al., 2000; Wood et al., 2003). Methylated H3K4 marks promoters of active genes and can be bound by many activating complexes, such as SAGA. SAGA is an important co-activator complex in transcription and interacts with the TATA box and binds H3K4me3, after which it acetylates lysine residues on H3 and H2B in active genes.

Interestingly, the ubiquitin-specific protease responsible for ubiquitin removal of H2B, Ubp8, is a subunit of SAGA (Henry et al., 2003). Without Ubp8, H2B ubiquitination levels are upregulated, but transcription in SAGA-dependent genes was impaired (Henry et al., 2003). Apparently, the transcription stimulating mark H2BK123ub, needs removal by Ubp8 as well for transcription to be successful. It was therefore proposed that transcription requires both ubiquitination and deubiquitination, which was illustrated by following H2B ubiquitination levels during *GAL1* transcription.

Henry *et al*. 2003 show that induction of expression of *GAL1* with glucose, results in increased ubiquitination levels of H2BK123 after 30 to 60 minutes. This was followed by decrease in ubiquitination levels between 60-120 minutes after the glucose stimulant (Fig. 8, white bars). *GAL1* mRNA levels increased after 60 minutes, after decrease of H2B ubiquitination (Fig. 8, line graph).

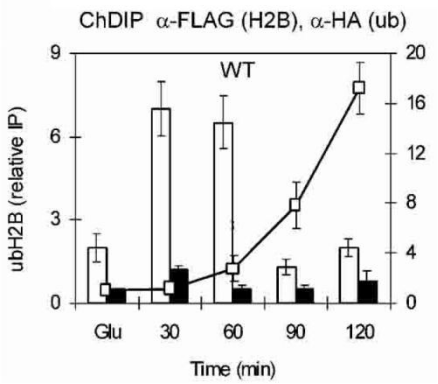


Figure 8: Transcription induction using glucose stimulates ubiquitination of H2B (white bars). The level of ubiquitination decreases again after 60 minutes, where after mRNA levels of *GAL1* become detectable (line). Control H2B molecules (black bars) show no change in ubiquitination levels (Henry et al., 2003).

This illustrates the dynamic character of crosstalk between PTMs. It seems that continuous changes are required for correct execution of chromatin functions during transcription. Another event that is coordinated by dynamic histone crosstalk is mitosis. Typically, mitosis is preceded by phosphorylations on H3 that influences adjacent methylated residues. Next, we will discuss these phospho/methyl switch mechanisms in more detail and explore their function in regulation of mitosis and possibly other nuclear functions, such as regulation of transcription.

Phospho/methyl switch mechanisms

We have seen that histone PTMs have important functions in regulation of various chromatin states in response to upstream signals. Direct on- or off-switching of genes or other regions can be required when cells instantaneously require change in gene expression, for instance after growth factor stimulation. Cell division is characterized by mitotic gene silencing and chromatin condensation to allow appropriate chromosome segregation. However, some methylmarks that interfere with these processes, such as H3K9me3 and H3K4me3, are stable marks that can be passed on to daughter cells. Mitosis coincides with phosphorylation of H3 on S10, T3 and S28. Negative crosstalk was observed between H3S10ph and HP1 binding to H3K9me3. Other phosphorylation sites on H3 – T3 and S28 – are in proximity of potentially methylated residues as well, such as K4 and K27. This lead to the suggestion by Fischle *et al.* 2003 that the addition of a phosphorylation mark next to a methylated lysine could function as on/off-switch, that allows instant mitotic changes at the chromatin level (Fischle et al., 2003a). Studies to the function of phosphorylations of these residues have revealed several 'phospho/methyl switch' mechanisms that contribute to chromatin changes during mitosis. Phosphorylation of H3 residues also occurs outside of mitosis, and it has become apparent that these switches play a role in other nuclear processes, such as transcription, as well. Below, known phospho/methyl switches of both types will be discussed.

K9/S10

HP1 dissociates from mitotic chromatin

Pericentromeric- and other constitutive heterochromatin, is typically bound by HP1 proteins. As discussed before, the heterochromatic mark H3K9me3 recruits HP1 and both are required for heterochromatin maintenance. They are both found concentrated in subnuclear dots, which is demonstrated for one of the HP1 homologs, HP1β, and H3K9me3 in Fig. 9A. However, in M-phase, HP1 is found throughout the nucleus, while H3K9 remains trimethylated (Fig. 9A). Mass-spectrometry of cells that were arrested in M-phase revealed the prevalence of phosphorylated H3S10 next to H3K9me3 marks (Fischle et al., 2005). The phosphorylation of H3S10 has been implicated in mitosis and occurs in early G2. This phospho-mark initially localizes to pericentromeric heterochromatin, similar to HP1/H3K9me3, but extends on chromatin until prophase, during which chromatin condensation takes place (Hendzel et al., 1997). In anaphase cells, dephosphorylation of H3S10 occurs, prior to telophase, in which chromosomes decondensate (Hendzel et al., 1997).

The relationship of this dual H3K9me3S10ph mark and its role in loss of HP1 binding to the chromatin during mitosis was therefore investigated. Fischle *et al.* 2003 demonstrated that the levels of both H3K9me3 and all HP1 isoforms do not change after entering mitosis (Fig. 9B). Phosphorylated H3S10 and the dual mark H3K9me3S10ph is only detectable in M-phase, but not when cells were treated with phosphatase (Fig. 9B). Moreover, in interphase cells, this dual mark is undetectable, but in G2, it is seen as pericentromeric dots, which co-occurs with spread of HP1β throughout the nucleus (Fig. 9C) (Fischle et al., 2005). *In vitro* experiments showed that the affinity of HP1 for the dual mark is 10^2 times lower than for the single K9me3 mark (Fischle et al., 2005). Aurora B is a mitotic kinase responsible for S10 phosphorylation and could therefore be causing HP1 dissociation. HeLa extracts from G2/M phase were analyzed on HP1 association on chromatin with or without hesperadin treatment, an Aurora B inhibitor. In hesperadin-treated cells, S10ph and the dual H3K9me3S10ph marks were significantly decreased compared to untreated cells (Fig. 9D). This co-occurred with prevention of HP1 release, indicating that phosphorylation of S10 by Aurora B is indeed responsible for HP1 dissociation. These results provide evidence for a mitotic switch mechanism, in order to release HP1 and stimulate chromosome condensation (Fischle et al., 2005).

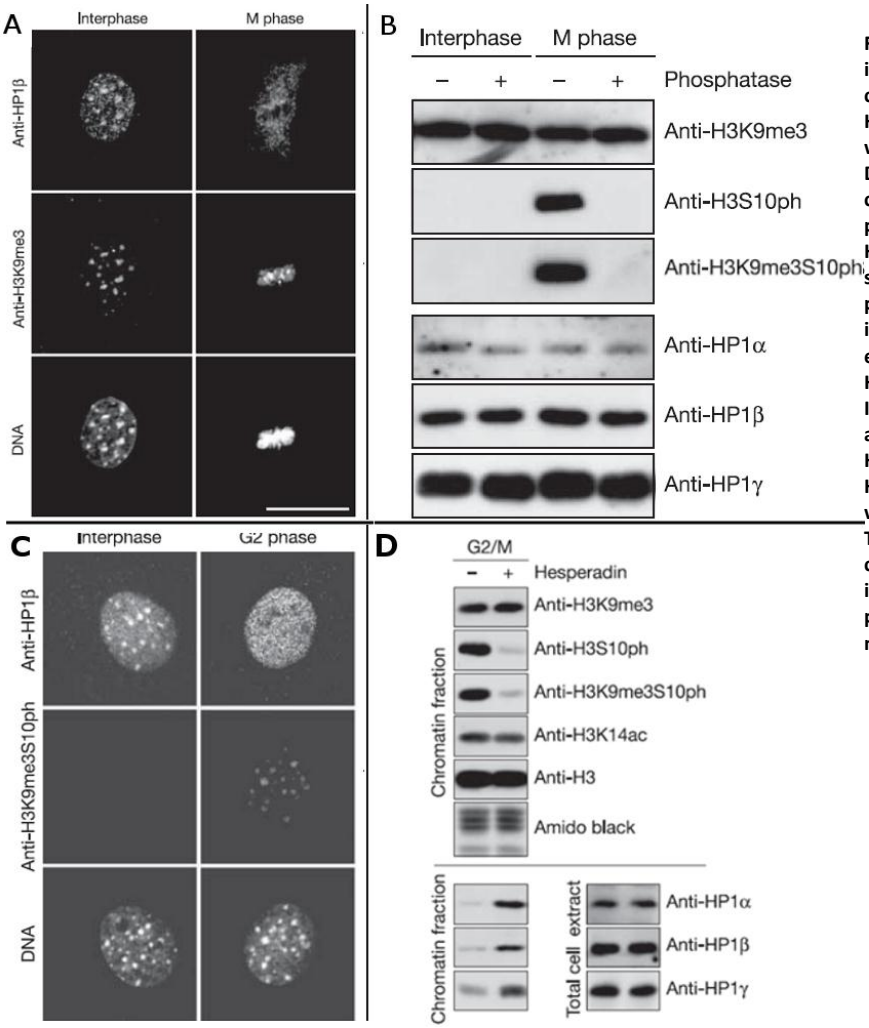


Figure 9: A. In Interphase cells, HP1β is found in dot-like subnuclear accumulations, colocalizing with H3K9me3. In M-phase, only H3K9me3 is still seen in these structures, while HP1β is found diffusely distributed. B. During Interphase, trimethylmarks on H3K9 can be measured but no H3S10ph. In M-phase, H3S10ph and the dual H3K9me3S10ph mark is detected. This is not seen when cells were treated with phosphatase. The amount of all HP1 variants in Interphase and M-phase in total cell extracts remains the same. C. The dual mark H3K9me3S10ph is not detected in Interphase, but during G2 this dual mark appears, which coincides with the dispersed HP1β localization. D. H3S10ph and the dual H3K9me3S10ph mark is severely reduced when Aurora B is inhibited by hesperadin. The chromatin fraction of hesperadin-treated cells still contains HP1 in the G2/M phase, indicating Aurora B is responsible for S10 phosphorylation and HP1 release during mitosis (Fischle et al., 2005).

S10 phosphorylation in gene activation

In addition to being phosphorylated during mitosis, S10 phosphorylation occurs during activation of immediate early genes (IEGs) (Thomson et al., 1999). The mitogen-and stress-activated kinase (MSK) family acts downstream of extracellular-signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) routes and can phosphorylate S10 during interphase. These pathways are involved in the response to various physiological and pathological stimuli (Soloaga et al., 2003; Vermeulen et al., 2009). Gene activation upon S10ph has also been reported for *HDAC1*, which requires more continuous phosphorylation by MAPK routes as opposed to the transient IEG-stimulation (Hauser et al., 2002). Both types have been reported to be coupled to acetylation (Clayton et al., 2000; Hauser et al., 2002; Lo et al., 2000). Furthermore, it has been demonstrated for HDAC1 that 'phosphoacetylation' of S10/K14 is required for 14-3-3 proteins and transcriptional activation (Winter et al., 2008).

A correlation was found between S10 phosphorylation and the *in vitro* activity of several HATs, particularly that of Gcn5 (Lo et al., 2000). S10ph increased deposition of the active mark K14Ac (Cheung et al., 2000; Lo et al., 2001). It has been shown that S10ph is required for SAGA and TBP recruitment at the *INO1* promoter, and that deletion of SAGA component Gcn5, mutation of S10 or K14 reduces *INO1* activation (Lo et al., 2001; Lo et al., 2005). The interaction of Gcn5 with S10ph, was required for increased activity of a subset of Gcn5 dependent genes. Substitution of the residue involved in this interaction, R164A, diminished this effect (Lo et al., 2000). Moreover, the expression of these genes was affected by both the Gcn5 R164A substitution and by mutated H3S10, demonstrating an *in vivo* role for the Gcn5-S10ph interaction in the activation of certain Gcn5-dependent genes (Lo et al., 2000). However, other studies report gene activation depending on S10ph without acetylation of K9/K14 (Nowak and Corces, 2000). It seems that S10ph during interphase can stimulate activation of a particular set of genes that can occur synergistically with acetylation of K14Ac, which might not be required for all genes.

The role of phosphoacetylation of H3 was demonstrated for activation of mammalian *HDAC1* and yeast *GAL1*. Phosphoacetylated H3 recruits 14-3-3 proteins to the *HDAC1* and *GAL1* promoter (Walter et al., 2008; Winter et al., 2008). These proteins are known to interact with chromatin modifiers and transcriptional regulators. The recruitment of 14-3-3 was shown to be required for full gene activation (Winter et al., 2008). In addition to K14Ac, K9 was found to be acetylated as well, but contributed less to 14-3-3 affinity (Walter et al., 2008; Winter et al., 2008) Interestingly, during *HDAC1* activation, phosphoacetylation and 14-3-3 recruitment correlates with dissociation of HP1γ at this locus to relieve silencing (Winter et al., 2008). Additionally, K9me2 remains present after S10ph, indicating the presence of another K9/S10 switch during interphase (Winter et al., 2008).

The fact that S10 can be phosphorylated by different types of kinases can be illustrative for the varied functions of S10ph in on one hand highly condensed mitotic chromatin and on the other hand the open and relaxed chromatin of transcribed genes. S10ph seems to play a role in stimulation of H3 acetylation of some genes, which has been shown to be required for 14-3-3 recruitment and activation of *HDAC1* and *GAL1,* and HP1γ displacement on *HDAC1*. It would be interesting to investigate the downstream effect of the other genes described to be subject to interphase phosphoacetylation, to see whether this also forms a docking site for effectors and/or displaces readers such as HP1γ.

Other K9me3 readers are subject to phospho/methyl switch

In 2010, Vermeulen *et al.* screened for the interactome of H3K9me3 using SILAC, in which one cell culture, containing amino acids labeled by a heavy isotope, is compared to another unlabeled group. Modified H3 peptides are exposed to cell extracts derived from the heavy labeled group and unmodified H3 peptides to cell extracts from the unlabeled group. This way, a shift is detected in MS-spectrum, so that it is possible to distinguish between binding preference for the unmodified or the modified peptide.

In addition to HP1, they found interactions with the chromodomain Y chromosome (CDY) family and PcG proteins, associated with gene repression as well, which confirmed previous findings (Fischle et al., 2008; Franz et al., 2009; Ringrose et al., 2004). Furthermore, subunits of the origin recognition complex (ORC) were found to interact with H3K9me3 (Vermeulen et al., 2010).

Next, they tried to identify preference of readers for single or double modification states of K9/S10, using a triple SILAC approach, in which two differently labeled cultures, exposed to single and double modified H3 peptides, were compared to an unlabeled culture, exposed to unmodified peptides. Surprisingly, HP1 affinity for the double modification H3K9me3S10ph seems unchanged and perhaps even slightly increased compared to the single H3K9me3 modification (Fig. 9). However, they did find a decreased affinity of CDY and ORC candidates for the double modification K9me3S10ph (Fig. 9) (Vermeulen et al., 2010). CDY proteins play a role in the formation of heterochromatin, via multimerization and K9me3 binding (Franz et al., 2009). Therefore, CDY removal could have a similar connotation as HP1 dissociation during mitosis; loss of heterochromatin in order to facilitate chromosome condensation. The ORC is required for the initiation of DNA replication and associates with DNA during G1 phase in a

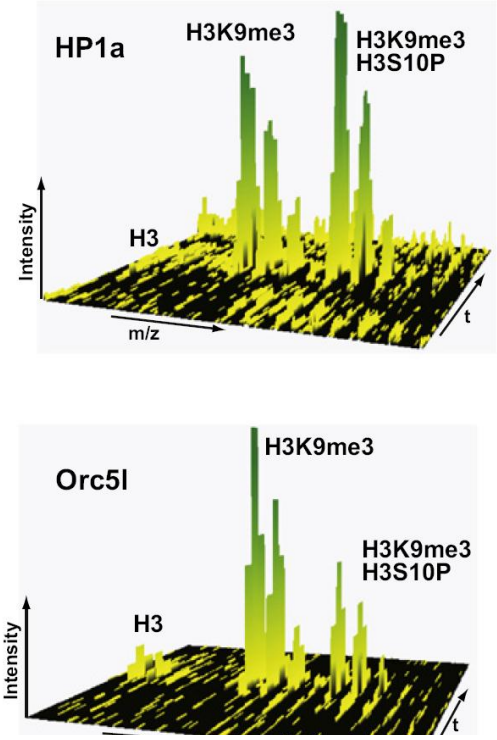


Figure 9: Triple SILAC. Unmodified, K9me3 or K9me3S10ph H3 peptides are exposed to lysates of unlabeled and two differently labeled cell cultures. The x axis represents the m/z scale, the MS signal is represented by the intensity on the y axis and the z axis is the chromatographic retention time. The three distinguishable groups are formed by the isotope-shift. The intensities correspond to binding preference to the specific modification state. Triple pull down of HP1, Orc5l and CDYL showed differential binding preference. HP1 showed similar affinity for both the single and double modification, while Orc5l and CDYL have decreased affinity for the double modification, indicating they could be subject to a phospho/methyl switch as well (Vermeulen et al., 2010).

sequence independent manner. Additionally, ORC plays a role in HP1 recruitment to heterochromatin (Prasanth et al., 2010). During mitosis, these functions and ORC localization might not be required anymore, explaining its sensitivity to S10ph. The results obtained by screening with SILAC indicate that S10ph might have an effect on multiple K9-readers. Further research should reveal if S10 phosphorylation can induce release of ORC and CDY components *in vivo* as well, similar to HP1. Additionally, it would be interesting to investigate the contribution of other (modified) histone residues present on nucleosomes that are not present on the peptides used for SILAC pull-down. Since HP1 affinity did not change in this assay, it is likely that the modification status of residues other than present on the peptides, are involved in its dissociation as well.

HP1 dissociation

Fischle *et al.* 2005 and Hirota *et al.*2005 demonstrated that S10ph was sufficient for HP1 dissociation from K9me3, using modified peptides. Moreover, they showed that this double modification is found *in vivo* in mitotic cells, concurrently with dispersed localization of HP1 (Fischle et al., 2005; Hirota et al., 2005). However, Mateescu *et al.* 2004 argue that S10ph is not sufficient and that an additional acetylation at K14 is required (Mateescu et al., 2004). In addition, they demonstrated that at the beginning of S10 phosphorylation, HP1 distribution even seemed more concentrated to the chromatin rather than diffuse (Fig. 12, panels B and F). When cells entered M-phase, HP1 delocalized (Fig. 12 D) (Mateescu et al., 2004). K14Ac upon S10ph occurs during MSK1-dependent gene activation as well. As mentioned earlier, this mechanism could be subject to the T3/K4 switch. Since T3 phosphorylation is only detectable from prophase on, K14Ac might occur before the onset of M-phase. Indeed, K14Ac acetylation has been detected during late G2/M phase (Mateescu et al., 2004). However, Mateescu *et al.* 2004 report increasing acetylation during M-phase as well, indicating that the involved HAT-complex is not removed by T3ph. Since mitotic chromatin acetylation plays an important role in successful mitosis, K14Ac could still contribute to HP1 dissociation (Ha et al., 2009). It would be interesting to follow the modification pattern of S10ph and K14Ac during mitosis together with HP1 localization, to see whether acetylation could indeed play a role in HP1 dissociation.

Recently, Vermeulen *et al.* 2010 used a triple SILAC approach to distinguish affinity of differently modified H3 peptides. They found that HP1 was insensitive to the double K9me3S10ph modification, whereas this seemed to be sufficient for CDY and ORC dissociation (Vermeulen et al., 2010).

All studies, including that of Vermeulen *et al.* 2010, used H3 peptides to investigate HP1 affinity for different modification states. Perhaps different peptide lengths caused their conflicting outcomes. HP1 might bind to residues further downstream or on other histones. Additionally, HP1 affinity is also increased by HP1 multimerization, which might be affected during mitosis as well. Investigation of HP1 affinity for complete nucleosomes would therefore provide a better insight in possible additional modifications that contribute to mitotic HP1 dissociation.

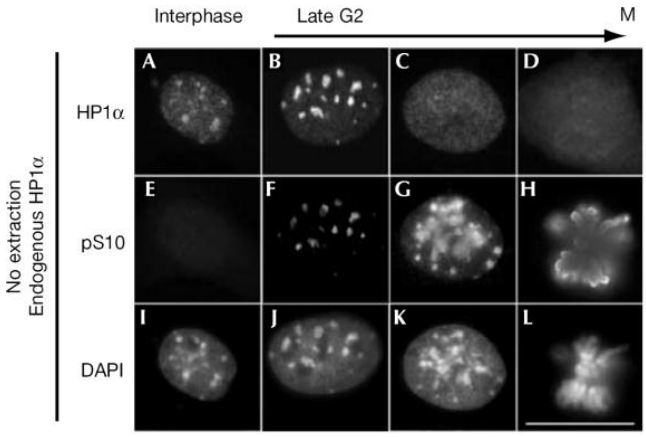


Figure 12: Mitotic phosphorylation of H3 co-exists with HP1 at pericentromeric regions (panels B and F). Towards M-phase, HP1 dissociates (Mateescu et al., 2004).

T3/K4

The core promoter is a region on the DNA that directs correct RNA-polII-driven transcription initiation. Promoter regions are characterized by sequences that can be recognized by transcription factors, such as the TATA box, the transcription start site Inr (initiator) and the motif ten element (MTE). They can be found in focused promoters, with one clear transcription start site, whereas dispersed promoters, containing multiple start sites, lack these sequences (Juven-Gershon and Kadonaga, 2009). These dispersed promoters are usually found in CpG islands, which have been discussed in the introduction. Generally, focused promoters are associated with regulated expressed genes, whereas dispersed promoters rather drive constitutive genes.

Therefore, even though dispersed promoters are much more common, gene expression from focused promoters has been extensively studied (Juven-Gershon and Kadonaga, 2009).

RNA-polII cannot recognize the promoter by itself, but needs to be positioned by basal transcription factors, that form a pre-initiation complex (PIC) at the transcription start site. PIC formation starts when TFIID associates with the promoter region. TFIID contains a TATA box-binding protein (TBP) and several TBP-associated factors (TAFs). To recognize promoter DNA, TFIID binds the TATA box with TBP. TAFs mediate other interactions, such as the recognition of the Inr by TAF1 and TAF2 (Chalkley and Verrijzer, 1999). Furthermore, TAFs contain multiple histone modification recognition sites and can thus bind modified histones, which specify active promoters. For instance, TAF1 can bind acetylated lysines on histone tails and TAF3 recognizes H3K4me3 (Jacobson et al., 2000; Vermeulen et al., 2007). It has been demonstrated that H3K4me3 stimulates TFIID association and TFIID-mediated transcription via binding of the PHD of TAF3 (Vermeulen et al., 2007). This way, TFIID associates with active promoters and is more precisely targeted via sequence specific binding. TFIID subsequently recruits other transcription factors and RNA-polII, after which transcription can be initiated. Moreover, transcription factor-binding to PTMs might have a more important role in promoters without characteristic DNA sequences in the promoter.

T3ph interferes with TFIID-mediated transcription during mitosis

During mitosis, the transcription machinery receives inhibitory signals, to silence transcription. Several factors such as TFIID and RNA-polII are phosphorylated during early mitosis, which impedes their function (Segil et al., 1996). In the previous example, it became clear that histones undergo mitotis-specific phosphorylations as well, in which they interfere with reader-PTM interactions on the adjacent residue.

H3T3 has been identified as a mitotic phosphorylation site (Polioudaki et al., 2004). During early prophase, H3T3 becomes phosphorylated, which disappears again in anaphase (Dai and Higgins, 2005). The kinase responsible for H3T3ph, Haspin, associates with chromosomes on H3 throughout mitosis, starting at prophase (Dai and Higgins, 2005). Depletion of Haspin, results in decreased H3T3ph levels in mitotic cells and defects in chromosome alignment, whereas its overexpression results in H3T3ph throughout the cell cycle and prolonged mitosis (Dai and Higgins, 2005). This could indicate that T3 phosphorylation functions as a mitotic switch mechanism analogous to K9me3/S10ph.

In vitro and *in vivo* experiments confirmed that TFIID binding to H3K4me3 via TAF3 was reduced when H3T3 was phosporylated (Varier et al., 2010). Moreover, expression of Haspin could specifically inhibit TAF3-stimulated transcription. Haspin expression induced TFIID dissociation from the chromatin, whereas suppression of Haspin resulted in aberrant TFIID association during mitosis (Varier et al., 2010). This was unaffected by the S10 and S28 kinase Aurora B (Varier et al., 2010). These results support the idea that H3T3 phosphorylation by Haspin is responsible for the dissociation of TFIID during mitosis. Therefore this represents another mitotic binary switch mechanism, in this case regulating TFIID-driven gene expression during mitosis.

T3/K4 is a general switch for K4-readers

PHD fingers of other K4 readers were tested for reduced affinity in the presence of T3ph as well. GST-fusions of K4me3 readers BPTF, ING2 and ING4 were tested against several modified peptides. They showed affinity for K4me3, but not the double modification T3phK4me3 (Varier et al., 2010). The unmodified K4 reader BHC80 was sensitive to T3ph as well, and only bound to K4me0 and not to T3phK4me0 (Varier et al., 2010). This is supported by a screen in which the affinity of the PHD-fingers of several K4 readers – ING2, BHC80, AIRE and RAG2 – and the double tudor domain of JMJD2A, were tested against a peptide library containing combinatorial randomized PTMs on H3. This way, the influence of the modification state of surrounding residues on K4me0 readers (AIRE, BHC80) and K4me3 readers (ING2, RAG2, JMJD2A) could be explored (Garske et al., 2010). All candidates had a preference for the unmodified T3 residue, while no affinity for phosphorylated T3 was detected (Fig. 10) (Garske et al., 2010). The fact that several H3K4me3 binding modules, but also that of unmodified-H3K4 readers, are all affected by H3T3 phosphorylation, suggests a general disruption of H3K4 binding by this modification (Garske et al., 2010).

T6 phosphorylation modifies binding affinity

Besides T3, T6 was found to be an important binding regulator for these K4 readers (Garske et al., 2010). This residue is phosphorylated by protein kinase Cβ1 (PKCβ1) and correlates with gene expression in response to androgen receptor activation (Metzger et al., 2010). The PHD fingers of unmodified K4 readers BHC80 and AIRE, and K4me3 reader RAG2 were intolerant to phosphorylated T6 (Fig. 10) (Garske et al., 2010). The K4me3 reader ING2 showed less affinity as well, but to a lesser extent (Fig. 10). The JMJD2A DTD was not very sensitive to T6ph (Fig. 10) (Garske et al., 2010). T6 therefore functions as an on/off switch for some readers (BHC80, AIRE and RAG2), while having a negligible influence on K4 affinity of others (JMJD2A). Additionally, T6ph can have a negative influence, without completely abolish the interaction (ING2). This variable control on K4-readers could therefore indicate an important fine-tuning function of phosphorylated T6.

This way, binding modules of K4 could be displaced at target genes of T6 kinase, to induce or suppress transcription of specific genes. BHC80 is linked to gene repression via the demethylase LSD1, which depends on the interaction of the PHD finger of BHC80 to unmethylated K4 (Lan et al., 2007). T6ph by PKCβ1 has been demonstrated to prevent K4 demethylation by LSD1, in order to stimulate gene expression (Metzger et al., 2010). De-repression of LSD1 target genes by PKCβ1 could therefore be coupled to the release of BHC80 after T6ph.

Two other K4 readers that did not show affinity in the presence of T6ph were AIRE (*A*uto-*I*mmune *RE*gulator) and RAG2 (Recombination Activating Gene 2). AIRE binds unmethylated H3K4 and stimulates gene expression of tissue-specific antigens in the thymus for negative selection of self-reactive T-cells (Liston et al., 2003; Org et al., 2008). This protein has been demonstrated to bind CBP *in vitro*, a common co-activator with HAT-activity (Pitkanen et al., 2000). RAG2 plays a role in the V(D)J recombination that occurs during T- and B-cell development. Its function depends on the interaction between the RAG2 PHD finger and trimethylated H3K4 (Matthews et al., 2007). ING2 is present in a HDAC and is involved in gene repression following DNA damage. These results could indicate there is a regulatory function for a T6/K4 switch for readers involved in lymphocyte development, and has a less significant influence on readers involved in other processes.

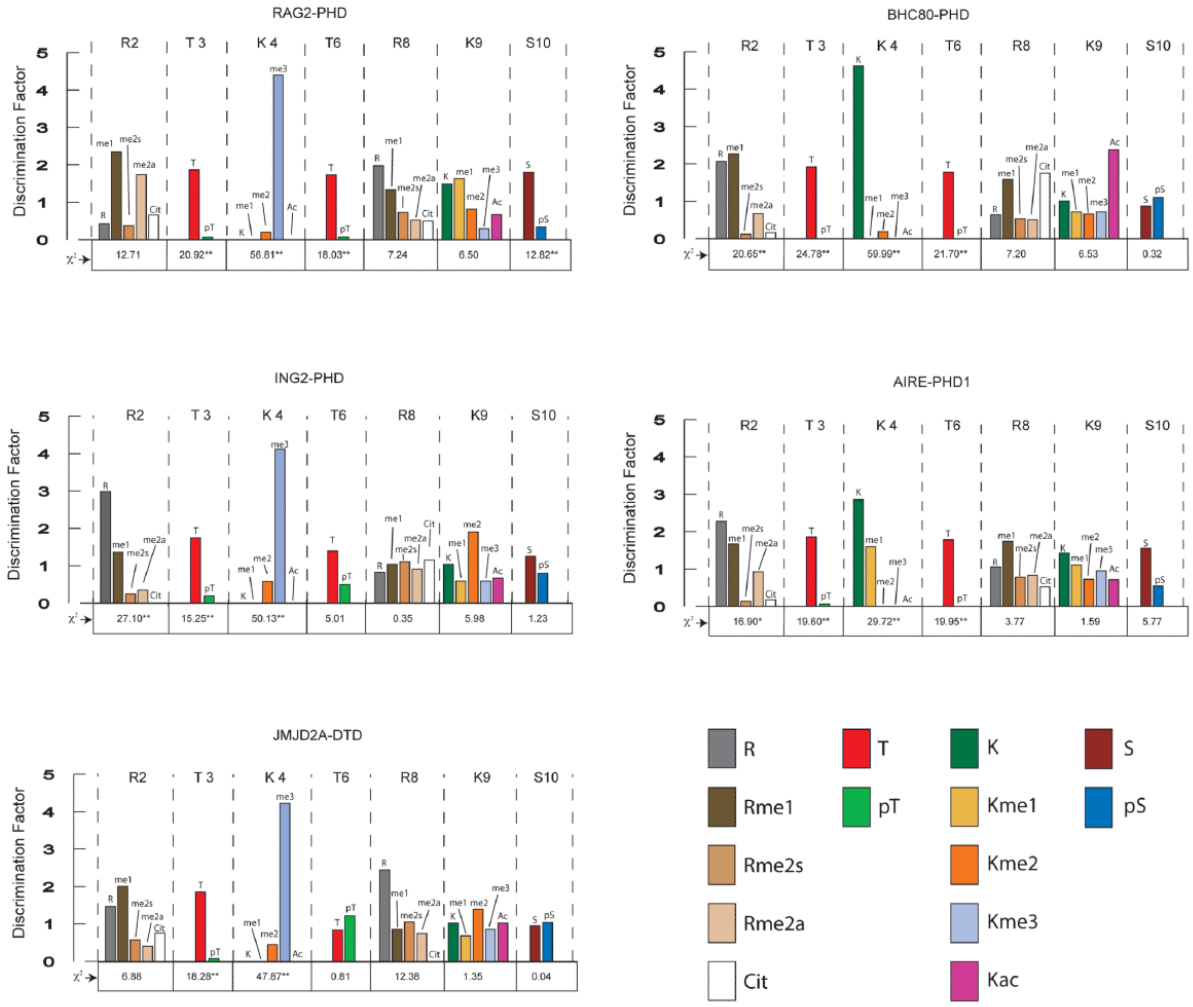


Figure 10: Relative affinity of the tested K4-binding domains for modification state of the histone residue. Some modifications exclude binding of all modules, such as T3ph compared to T3. Other modifications show differential modulation of binding affinity for different readers, such as T6/T6ph (Garske et al., 2010).

S28/K27

ORC and CDY proteins show decreased affinity to K27me3 in the presence of S28ph

Another lysine that can be methylated and bound by various readers is H3K27. Trimethylated H3K27 mediates docking of important silencing factors, as PcG proteins and members of the CDY family (Cao et al., 2002; Fischle et al., 2008). Next to S10 phosphorylation, Aurora B phosphorylates H3S28 (Goto et al., 2002). S10 phosphorylation is detectable from late G2 until metaphase, whereas S28 phosphorylation is found from prophase on (Goto et al., 2002). The fact that a serine that is known to be phosphorylated during mitosis, is localized adjacent to a lysine, indicates this could be another phospho/methyl switch.

The interactome of H3K27me3 was revealed using SILAC, in which binding of the CDY-family members and PcG proteins was confirmed (Vermeulen et al., 2010). Additionally, they found interactions with ORC subunits as well (Vermeulen et al., 2010). Furthermore, co-existence of K27me3 and S28ph on the same histone tail was detected via MS, in mitotic cells specifically (Vermeulen et al., 2010). This was followed by triple SILAC, to identify readers for the different modification states of K27 and S28.

All K27me3 readers showed the lowest affinity for unmodified H3 (Fig. 11). For ORC (Orc3I) and CDY (CDYL and CDYL2) proteins, they found a preference for the single modified K27me3 peptide over the double K27me3/S28ph mark (Fig. 11) (Vermeulen et al., 2010). This could mean that they are subject to a mitotic phospho/methylswitch, similar to ORC and CDY components that recognize H3K9me3. The PcG protein Ring1b, showed a similar affinity to the K27me3S28ph peptide compared to the K27me3 peptide (Fig. 11), which indicates that PcG proteins are less sensitive to this double modification state and that histone phosphorylations influence neighboring lysine readers in a selective manner, not affecting all binding proteins equally (Vermeulen et al., 2010).

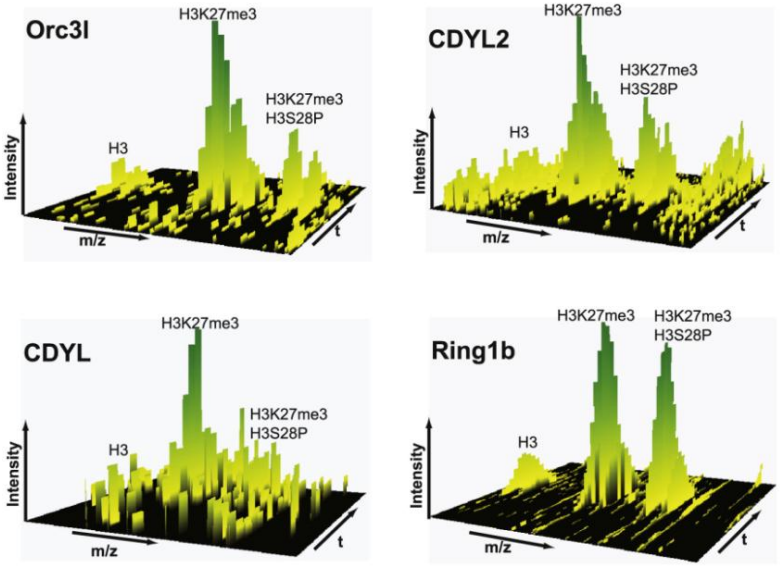


Figure 11: Triple SILAC. Unmodified, K27me3 or K27me3S28ph H3 peptides are exposed to lysates of unlabeled and two differently labeled cell cultures. The x axis represents the m/z scale, the MS signal is represented by the intensity on the y axis and the z axis is the chromatographic retention time. The three distinguishable groups are formed by the isotope-shift and the intensities correspond to binding preference to the specific modification state. Triple pull down of Orc3I, CDYL, CDYL2 and Ring1b showed differential binding preference. Orc3I, CDYL and CDYL2 preferred the single K27me3 modification, while the PcG protein Ring1b was found to interact well with both the single and the double modification (Vermeulen et al., 2010).

MSK1-dependent phosphorylation provides a switch for PcG-silenced genes

Vermeulen *et al.* 2010 detected the double K27me3S28ph modification mainly in mitotic cells. However, S28 is also phosphorylated during interphase by MSK1 and MSK2. Moreover, the double K27me3S28ph is found throughout the cell cycle (Dyson et al., 2005; Gehani et al., 2010; Soloaga et al., 2003). Similar to S10ph, S28ph is found on IEG-promoters and is often, not always, located on different H3 tails from S10ph (Dyson et al., 2005; Lau and Cheung, 2011). Targeted S28 phosphorylation by MSK1 was found to be correlated with transcription activation of a reporter gene, since the RSK2, that efficiently phosphorylates S10 but not S28, could not establish this effect (Lau and Cheung, 2011). Therefore, gene activation could be due to S28ph alone or in combination with S10ph. However, since S28ph and S10ph locations do not always show overlap *in vivo*, S28ph might be responsible for the activation of a specific set of genes. This was demonstrated for several genes that are repressed by PcG proteins, in which S28ph interferes with PcG-binding to K27me3.

The PRC2 complex trimethylates H3K27, providing a docking site for the PRC1 complex, that mediates gene silencing during development. The affinity of PRC2 for different forms of modified H3 was investigated by a peptide-binding experiment. PRC2 components showed affinity to peptides containing H3K27me3, whereas additional S28ph resulted in abolishment of PRC2 association (Gehani et al., 2010). When cells were treated with anisomycin, that induces MSK-dependent S10/S28 phosphorylation, several PcG-repressed genes were activated. ChIP analysis showed that this correlated with the presence of the double K27me3S28ph mark on promoters of activated PcG targets and displacement of PcG proteins at these regions (Gehani et al., 2010). WDR5, a component of the MLL H3K4 methyltransferase complex, showed to be recruited to these regions. Indeed, H3K4 showed to be trimethylated at these regions, and correlated with an increase of RNA-polII recruitment. These observations were not made in the presence of an MSK or when MSK was depleted, indicating that MSK activity was required for this switch.

Another example is the α-globin promoter that is principally phosphorylated at S28 by MSK1 (Lau and Cheung, 2011). The α-globin gene is specifically expressed in erythroid cells and suppressed in other tissues by H3K27me3-mediated silencing. Upon MSK1 expression, cells showed a decrease in both K27me3 and recruitment of PcG factors to the α-globin locus, concurrently with reactivation of the α-globin gene (Lau and Cheung, 2011). The S5-phosphorylated form of RNA-polII is recruited to these regions, which is found on promoters during early elongating transcription (Komarnitsky et al., 2000; Lau and Cheung, 2011). Additionally, ChIP-analysis showed that the dual K27Ac3S28ph mark was detectable on the activated α-globin and *c-fos* (another IEG) promoters (Lau and Cheung, 2011). This dual mark has been found before on promoters of activated PcG target genes in ES cells, and occurs in the absence of PRC2 activity at that region (Pasini et al., 2010). Moreover, the main HATs involved in H3K27 acetylation in p300 and CBP, two important coactivators of gene transcription that had been reported previously to co-immunoprecipitate with MSK1 (Janknecht, 2003; Pasini et al., 2010).

These examples demonstrate a link between the double K27me3S28ph mark, displacement of PRCs and transcriptional activation. S28ph seems to exclude PcG proteins from binding to adjacent K27me3, and induces gene activation this way (Gehani et al., 2010; Lau and Cheung, 2011). This provides another switch mechanism for PcG target regions during interphase. In mitotic cells, S28ph was found to reduce affinity for several K27me3 readers, which was not seen for the PcG protein Ring1b (Vermeulen et al., 2010). PRC2 components EZH2 and SUZ12 have been shown to stay attached to K27me3 during mitosis as well (Gehani et al., 2010; Hansen et al., 2008). This could indicate that mitotic S28ph does not play a role in relief of PcG mediated silencing and that other factors might be involved in establishing the different switches, such as K27 acetylation. Therefore, the K27/S28 switch occurs both during mitosis and interphase, but probably results in chromatin condensation – similar to the mitotic K9/S10 switch – and gene activation, respectively.

R2/K4

A 'methyl/methyl' switch regulates RNA-polII driven transcription

Besides T3 phosphorylation, readers of K4 are also influenced by methylation of R2 (Garske et al., 2010; Vermeulen et al., 2007). Negative crosstalk between R2 and K4 was demonstrated by the reduced affinity of the PHD-proteins TAF3 and Spp1p (part of the COMPASS complex) for H3K4me3 in the presence of asymmetric dimethylation of H3R2 (van Ingen et al., 2008; Vermeulen et al., 2007). The Type I arginine methylase PRMT6 was shown to be responsible for R2me2a and causes decreased gene expression (Guccione et al., 2007; Hyllus et al., 2007). R2me2a is found on inactive promoters and was found to be mutually exclusive K4me3 (Guccione et al., 2007; Kirmizis et al., 2007).

Van Ingen *et al.* 2008 showed that the PHD-finger of TAF3 forms hydrogen bonds with K4me3 and R2. Asymmetric dimethylation of R2 causes loss of these bonds and sterical hindrance within the binding pocket (van Ingen et al., 2008). Moreover, besides COMPASS, the K4 HMT complex MLL was prevented from binding to R2me2a regions as well (Guccione et al., 2007). However, not all K4me3 readers are affected by R2me2a. The K4me3 affinity of the PHD-fingers of ING2, BPTF and RAG2 – present in a HDAC, the NURF chromatin remodelling complex and the V(D)J-recombination complex respectively – are only mildly affected by R2me2a (Ramon-Maiques et al., 2007; Vermeulen et al., 2007).

Despite the fact that there are many methylated-K4-readers, R2me2a mainly affects binding RNA-polII promoting complexes. Therefore, the existence of a 'methyl/methyl'-switch was proposed by van Ingen *et al.*, 2008 which can instantly turn off RNA-polII-driven transcription by promoting dissociation of K4me3-bound transcription complexes, comparable to a phospho/methyl switch mechanism (van Ingen et al., 2008). The R2me2a mark is found in the cells that will give rise to the inner cell mass (ICM) in mouse embryos, just as R17me2a and R26me2a, and PRMT6-related PRMT4 has been shown to promote ICM formation (Torres-Padilla et al., 2007). Overexpression of PRMT6 results in repression of Hox-genes and Myc-dependent genes (Hyllus et al., 2007). The function of the R2/K4 switch could therefore be important in determining embryonic cell fate, by switching off specific genes in pluripotent cells.

This example demonstrates the existence of a switch without the involvement of a phosphorylated residue. Other switches have been reported that involve other PTMs as well. An acetylation-ubiquitination switch of H2B has been suggested to function in response to ER-stress at poised elements (Gatta et al., 2011). Another switch involving phosphorylation of T142 the H2A variant H2AX, has been proposed to function as a switch between promoting either apoptosis or DNA repair (Stucki, 2009). The usage of histone switch mechanisms might therefore be a more common strategy to execute a wide variety of nuclear processes, such as mitosis, gene expression, stress responses and DNA damage repair.

Discussion

When does it concern a switch and not merely crosstalk?

The involvement of epigenetic mechanisms in regulation of various nuclear processes has been a major focus of research. It has become clear that histone marks usually do not function by itself, but rather crosstalk to other modifications to provide an eventual read-out. In response to internal and external triggers, the cell needs to be able to instantly modulate chromatin. In order to establish this, the cell uses epigenetic switch mechanisms, in which the addition of one histone PTM influences binding of a reader to an adjacent residue. Phosphorylation of T6 contributes to a decrease in affinity for some K4 readers, representing a negative crosstalk mechanism without resulting in complete dissociation. The other switches that have been described provide an 'on/off' system, in which a residue is phosphorylated and the readers of the adjacent residue dissociate.

The switch mechanisms therefore seem to differ from merely negative crosstalk, that influences affinity but does not provide an unambiguous outcome. This is further supported by the fact that switches appear to be reversible, which can be illustrated by dephosphorylation of H3 at the end of mitosis. This will turn the mitotic H3 state back to an interphase state. Mitotic phosphatases play an important role in this process. The main histone phosphatase is PP1, that dephosphorylates T3ph, S10ph, and S28ph when cells exit mitosis (Qian et al., 2011). Dephosphorylation of T3ph and S28ph occurred before S10ph dephosphorylation, which can be explained by different PP1 interacting proteins (PIPs) that control PP1 activity and substrate selectivity (Qian et al., 2011). Removal of the blocking phosphate group has consequences for the readers that initially dissociated in its presence. When S10ph is dephosphorylated, HP1 reassociates again with K9me3 (Fischle et al., 2005; Qian et al., 2011). TFIID has been observed to reassociate with chromatin during late anaphase (Varier et al., 2010). This could result in rapid reactivation of pre-mitotic RNA-polII active genes, since they maintained H3K4me3 at promoters and, to a lesser extent, acetylated residues (Kouskouti and Talianidis, 2005; Valls et al., 2005). Therefore, dephosphorylation could stimulate reassociation of other K4me3 and possibly K27me3 readers as well, since their association will not be sterically hindered anymore.

This regulated histone dephosphorylation at the end of mitosis, provides additional evidence for the existence of a controlled switch mechanism that can be switched back during mitotic exit. However, phosphatases that control the interphase switches that have been described remain unknown. It would therefore be interesting to reveal other phosphatases that regulate phospho/methyl switches. This could be investigated by performing an RNAi screen of (predicted) phosphatases. This could reveal phosphatases that are involved in switches that are involved in gene activation, for instance interphase K9/S10 or K27/S28. Genes that are activated by one of these switches, such as PcG-taget genes and IEGs, often show transient rather than persistent expression. This could be established by a phosphatase that reverses gene activation. If a target gene of a switch remains active when a candidate phosphatase is knocked down, this phosphatase could play a role in switching this activated gene back into a suppressed state. This could underline the significant contribution of one switch PTM, that can instantly abolish reader affinity for the adjacent residue, which is reversed when this PTM not present anymore. This is different from the contribution of PTMs that provide negative or positive crosstalk, and modulate reader affinity in a less explicit manner.

Additional PTMs contribute to phospho/methyl switch selectivity

We have seen that several residues that are subject to phosphorylation – T3, S10 and S28 – can influence readers on adjacent residues. For instance, S28ph reduces the affinity of CDY and ORC proteins for H3K27me3. This was not seen for the PcG proteins (Vermeulen et al., 2010; Gehani et al., 2010; Hansen et al., 2008). However, PcG proteins showed to be sensitive to MSK1-dependent S28 phosphorylation (Lau and Cheung, 2011; Gehani et al., 2010). Therefore, the same modification seems to have a different outcome. Since it has become clear that many readers recognize multiple residues, by itself or indirectly via other readers in a complex, they often are sensitive to an extended modification state of the nucleosome. In phospho/methyl switch mechanisms, this could mean their dissociation probably not merely depends on the inducing phosphorylation. Readers might need to be 'sensitized' by the nucleosomal environment, in order to be prone

to a switch. If additional crosstalk stabilizes a complex to different regions on the nucleosome, the switch might not have an effect. Conversely, other crosstalking residues might reduce affinity, which is completed by the switch-modification. Therefore, phosphorylations could function as a switch for neighboring lysine-bound readers, that can be fine-tuned by other PTMs that are present on the nucleosome (Fischle et al., 2008; Franz et al., 2009; Vermeulen et al., 2010).

This can be illustrated by the differences in mitotic and non-mitotic S10ph-switch and the conflicting findings of HP1 affinity for the K9me3S10ph dual mark. S10 phosphorylation by Aurora B and MSK proteins result in HP1 delocalization and gene activation respectively. MSK-dependent phosphorylation stimulates H3 acetylation by Gcn5, that requires the interaction with S10ph, resulting in gene activation. During mitosis, this does not occur and gene activation is suppressed. How do Gcn5-containing HAT complexes distinguish between mitotic and non-mitotic S10 phosphorylation?

H3K4 functions as an important docking site. Many modified-and unmodified-K4 readers show downstream *cis*-crosstalk functions. For instance, the JMJD2A demethylase binds H3K4me3 and removes methylations from H3K9 or H3K36. The Gcn5-containing SAGA complex binds H3K4me3-marked promoter regions via Sgf29 and subsequently acetylates residues such as K14, stimulating gene expression. During mitosis, additional phosphorylations are present, such as T4ph, which functions as a general switch for dissociation of multiple H3K4 readers. This might interfere with docking of SAGA as well, preventing stimulation of transcription by Gcn5-dependent lysine acetylation this way. Another explanation for the prevention of gene activation is that the 14-3-3 proteins, important interactors with the transcription machinery, are excluded from mitotic chromosomes (Macdonald et al., 2005). It would be interesting to study SAGA affinity to histone tails containing multiple modifications, for instance H3K4me3S10ph and H3T3phK4me3S10ph, using the triple SILAC method. Differences between SAGA affinity for H3 of interphase and mitotic cells, could be investigated by exposure of SAGA to IPs of FLAG-tagged H3 from nuclei in both cell cycle phases. MS can reveal the epigenetic states that cause the degree of affinity.

Revealing new switch mechanisms

Since the first indication of the existence of histone PTM switch mechanisms, by Fischle *et al.* in 2003, several phospho/methyl switches have been described. New modification sites on histones are revealed, that might be candidates for new switches. For instance, H3S57 and H3T80 have been found to be phosphorylated *in vivo* and are both found next to a lysine residue (Vermeulen et al., 2010). As mentioned briefly, novel switches are being discovered, that not only comprise methylations and phosphorylations, but other types of PTMs as well, playing diverse nuclear roles. Asymmetric dimethylations of H4R3, H3R17 and H3R26 correlate with active genes and could be candidates to negatively influence suppressing readers.

Furthermore, to obtain full understanding of known switch mechanisms, it seems important to uncover the complete epigenetic state of the nucleosome, since readers often appear to be sensitive to the modification state of multiple residues at once. Furthermore, increased specification can be obtained by the presence of additional readers in the same complex. These multivalent interactions of single readers or a combination of readers with histones seem to be more important in specifying the interaction of chromatin-bound complexes. These interactions can therefore be misinterpreted when modified peptides are used, that only represent part of the histone tail. Also, more than one histone tail can be recognized. To investigate the combination of PTMs required for the specific interaction with readers, it seems important to investigate this with whole nucleosomes, rather than peptides or single histones.

This can be established by exposing nucleosomes containing a specific combination of PTMs to cell extracts, followed by IP of a reader of interest and investigate affinity. Nucleosomes can be isolated by performing sequential IPs, to obtain nucleosomes with specific PTM combinations. It is also possible to synthesize chromatin, from semi-synthetic histones, that contain incorporated PTMs. These histones can be assembled into nucleosomes with other recombinant histones and DNA, and contain multiple modifications of interest (Shogren-Knaak and Peterson 2004). This way, optimal PTM combinations required for interaction with readers can be investigated. This could reveal the differences in epigenetic environments that underlie the dissimilar outcomes of known switches. Moreover, novel switches could be revealed if a reader shows affinity that is abolished by an additional PTM.

References

Bajic, V. B., Tan, S. L., Christoffels, A., Schonbach, C., Lipovich, L., Yang, L., Hofmann, O., Kruger, A., Hide, W., Kai, C. et al. (2006). Mice and men: their promoter properties. *PLoS Genet* **2**, e54.

Bannister, A. J. and Kouzarides, T. (2005). Reversing histone methylation. *Nature* **436**, 1103-6.

Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C. and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**, 120-4.

Berger, S. L. (2007). The complex language of chromatin regulation during transcription. *Nature* **447**, 407-12.

Bernstein, B. E., Kamal, M., Lindblad-Toh, K., Bekiranov, S., Bailey, D. K., Huebert, D. J., McMahon, S., Karlsson, E. K., Kulbokas, E. J., 3rd, Gingeras, T. R. et al. (2005). Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell* **120**, 169-81.

Bian, C., Xu, C., Ruan, J., Lee, K. K., Burke, T. L., Tempel, W., Barsyte, D., Li, J., Wu, M., Zhou, B. O. et al. (2011). Sgf29 binds histone H3K4me2/3 and is required for SAGA complex recruitment and histone H3 acetylation. *EMBO J* **30**, 2829-42.

Canzio, D., Chang, E. Y., Shankar, S., Kuchenbecker, K. M., Simon, M. D., Madhani, H. D., Narlikar, G. J. and Al-Sady, B. (2011). Chromodomain-mediated oligomerization of HP1 suggests a nucleosome-bridging mechanism for heterochromatin assembly. *Mol Cell* **41**, 67-81.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S. and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**, 1039-43.

Chalkley, G. E. and Verrijzer, C. P. (1999). DNA binding site selection by RNA polymerase II TAFs: a TAF(II)250-TAF(II)150 complex recognizes the initiator. *EMBO J* **18**, 4835-45.

Cheung, P., Tanner, K. G., Cheung, W. L., Sassone-Corsi, P., Denu, J. M. and Allis, C. D. (2000). Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell* **5**, 905-15.

Clayton, A. L., Rose, S., Barratt, M. J. and Mahadevan, L. C. (2000). Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. *EMBO J* **19**, 3714-26.

Dai, J. and Higgins, J. M. (2005). Haspin: a mitotic histone kinase required for metaphase chromosome alignment. *Cell Cycle* **4**, 665-8.

Deaton, A. M. and Bird, A. (2011). CpG islands and the regulation of transcription. *Genes Dev* **25**, 1010-22.

Dyson, M. H., Thomson, S., Inagaki, M., Goto, H., Arthur, S. J., Nightingale, K., Iborra, F. J. and Mahadevan, L. C. (2005). MAP kinase-mediated phosphorylation of distinct pools of histone H3 at S10 or S28 via mitogen- and stress-activated kinase 1/2. *J Cell Sci* **118**, 2247-59.

Eisen, A., Utley, R. T., Nourani, A., Allard, S., Schmidt, P., Lane, W. S., Lucchesi, J. C. and Cote, J. (2001). The yeast NuA4 and Drosophila MSL complexes contain homologous subunits important for transcription regulation. *J Biol Chem* **276**, 3484-91.

Espada, J., Ballestar, E., Fraga, M. F., Villar-Garea, A., Juarranz, A., Stockert, J. C., Robertson, K. D., Fuks, F. and Esteller, M. (2004). Human DNA methyltransferase 1 is required for maintenance of the histone H3 modification pattern. *J Biol Chem* **279**, 37175-84.

Fischle, W. (2008). Talk is cheap--cross-talk in establishment, maintenance, and readout of chromatin modifications. *Genes Dev* **22**, 3375-82.

Fischle, W., Franz, H., Jacobs, S. A., Allis, C. D. and Khorasanizadeh, S. (2008). Specificity of the chromodomain Y chromosome family of chromodomains for lysine-methylated ARK(S/T) motifs. *J Biol Chem* **283**, 19626-35.

Fischle, W., Tseng, B. S., Dormann, H. L., Ueberheide, B. M., Garcia, B. A., Shabanowitz, J., Hunt, D. F., Funabiki, H. and Allis, C. D. (2005). Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature* **438**, 1116-22.

Fischle, W., Wang, Y. and Allis, C. D. (2003a). Binary switches and modification cassettes in histone biology and beyond. *Nature* **425**, 475-9.

Fischle, W., Wang, Y. and Allis, C. D. (2003b). Histone and chromatin cross-talk. *Curr Opin Cell Biol* **15**, 172-83.

Franz, H., Mosch, K., Soeroes, S., Urlaub, H. and Fischle, W. (2009). Multimerization and H3K9me3 binding are required for CDYL1b heterochromatin association. *J Biol Chem* **284**, 35049-59.

Fuks, F., Hurd, P. J., Deplus, R. and Kouzarides, T. (2003). The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res* **31**, 2305-12.

Garske, A. L., Oliver, S. S., Wagner, E. K., Musselman, C. A., LeRoy, G., Garcia, B. A., Kutateladze, T. G. and Denu, J. M. (2010). Combinatorial profiling of chromatin binding modules reveals multisite discrimination. *Nat Chem Biol* **6**, 283-90.
Gatta, R., Dolfini, D., Zambelli, F., Imbriano, C., Pavesi, G. and Mantovani, R. (2011). An acetylation-mono-ubiquitination switch on lysine 120 of H2B. *Epigenetics* **6**, 630-7.
Gehani, S. S., Agrawal-Singh, S., Dietrich, N., Christophersen, N. S., Helin, K. and Hansen, K. (2010). Polycomb group protein displacement and gene activation through MSK-dependent H3K27me3S28 phosphorylation. *Mol Cell* **39**, 886-900.
Gilbert, N., Thomson, I., Boyle, S., Allan, J., Ramsahoye, B. and Bickmore, W. A. (2007). DNA methylation affects nuclear organization, histone modifications, and linker histone binding but not chromatin compaction. *J Cell Biol* **177**, 401-11.
Goto, H., Yasui, Y., Nigg, E. A. and Inagaki, M. (2002). Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation. *Genes Cells* **7**, 11-7.
Grant, P. A., Eberharter, A., John, S., Cook, R. G., Turner, B. M. and Workman, J. L. (1999). Expanded lysine acetylation specificity of Gcn5 in native complexes. *J Biol Chem* **274**, 5895-900.
Grewal, S. I. and Jia, S. (2007). Heterochromatin revisited. *Nat Rev Genet* **8**, 35-46.
Guccione, E., Bassi, C., Casadio, F., Martinato, F., Cesaroni, M., Schuchlautz, H., Luscher, B. and Amati, B. (2007). Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. *Nature* **449**, 933-7.
Ha, G. H., Kim, H. S., Lee, C. G., Park, H. Y., Kim, E. J., Shin, H. J., Lee, J. C., Lee, K. W. and Lee, C. W. (2009). Mitotic catastrophe is the predominant response to histone acetyltransferase depletion. *Cell Death Differ* **16**, 483-97.
Haldar, S., Saini, A., Nanda, J. S., Saini, S. and Singh, J. (2011). Role of Swi6/HP1 self-association-mediated recruitment of Clr4/Suv39 in establishment and maintenance of heterochromatin in fission yeast. *J Biol Chem* **286**, 9308-20.
Hansen, K.H., Bracken, A.P., Pasini, D., Dietrich, N., Gehani, S.S., Monrad, A., Rappsilber, J., Lerdrup, M. and Helin, K. (2008). A model for transmission of the H3K27me3 epigenetic mark, *Nat. Cell Biol.* **10** 1291–1300.
Hauser, C., Schuettengruber, B., Bartl, S., Lagger, G. and Seiser, C. (2002). Activation of the mouse histone deacetylase 1 gene by cooperative histone phosphorylation and acetylation. *Mol Cell Biol* **22**, 7820-30.
Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-60.
Henry, K. W., Wyce, A., Lo, W. S., Duggan, L. J., Emre, N. C., Kao, C. F., Pillus, L., Shilatifard, A., Osley, M. A. and Berger, S. L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev* **17**, 2648-63.
Hirota, T., Lipp, J. J., Toh, B. H. and Peters, J. M. (2005). Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature* **438**, 1176-80.
Huang, Y., Fang, J., Bedford, M. T., Zhang, Y. and Xu, R. M. (2006). Recognition of histone H3 lysine-4 methylation by the double tudor domain of JMJD2A. *Science* **312**, 748-51.
Hyllus, D., Stein, C., Schnabel, K., Schiltz, E., Imhof, A., Dou, Y., Hsieh, J. and Bauer, U. M. (2007). PRMT6-mediated methylation of R2 in histone H3 antagonizes H3 K4 trimethylation. *Genes Dev* **21**, 3369-80.
Irvine, D. V., Zaratiegui, M., Tolia, N. H., Goto, D. B., Chitwood, D. H., Vaughn, M. W., Joshua-Tor, L. and Martienssen, R. A. (2006). Argonaute slicing is required for heterochromatic silencing and spreading. *Science* **313**, 1134-7.
Jacobs, S. A. and Khorasanizadeh, S. (2002). Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science* **295**, 2080-3.
Jacobson, R. H., Ladurner, A. G., King, D. S. and Tjian, R. (2000). Structure and function of a human TAFII250 double bromodomain module. *Science* **288**, 1422-5.
Janknecht, R. (2003). Regulation of the ER81 transcription factor and its coactivators by mitogen- and stress-activated protein kinase 1 (MSK1). *Oncogene* **22** 746–755.
Johansen, K. M. and Johansen, J. (2006). Regulation of chromatin structure by histone H3S10 phosphorylation. *Chromosome Res* **14**, 393-404.
Juven-Gershon, T. and Kadonaga, J. T. (2009). Regulation of gene expression via the core promoter and the basal transcriptional machinery. *Dev Biol* **339**, 225-9.

Kirmizis, A., Santos-Rosa, H., Penkett, C. J., Singer, M. A., Vermeulen, M., Mann, M., Bahler, J., Green, R. D. and Kouzarides, T. (2007). Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. *Nature* **449**, 928-32.
Komarnitsky, P., Cho, E. J. and Buratowski, S. (2000). Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. *Genes Dev* **14**, 2452-60.
Kouskouti, A. and Talianidis, I. (2005). Histone modifications defining active genes persist after transcriptional and mitotic inactivation. *EMBO J* **24**, 347-57.
Lan, F., Collins, R. E., De Cegli, R., Alpatov, R., Horton, J. R., Shi, X., Gozani, O., Cheng, X. and Shi, Y. (2007). Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression. *Nature* **448**, 718-22.
Lau, P. N. and Cheung, P. (2011). Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. *Proc Natl Acad Sci U S A* **108**, 2801-6.
Lee, J. S., Shukla, A., Schneider, J., Swanson, S. K., Washburn, M. P., Florens, L., Bhaumik, S. R. and Shilatifard, A. (2007). Histone crosstalk between H2B monoubiquitination and H3 methylation mediated by COMPASS. *Cell* **131**, 1084-96.
Li, B., Gogol, M., Carey, M., Lee, D., Seidel, C. and Workman, J. L. (2007). Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. *Science* **316**, 1050-4.
Li, B. Z., Huang, Z., Cui, Q. Y., Song, X. H., Du, L., Jeltsch, A., Chen, P., Li, G., Li, E. and Xu, G. L. (2011). Histone tails regulate DNA methylation by allosterically activating de novo methyltransferase. *Cell Res*.
Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C. (2003). Aire regulates negative selection of organ-specific T cells. *Nat Immunol* **4**, 350-4.
Lo, W. S., Duggan, L., Emre, N. C., Belotserkovskya, R., Lane, W. S., Shiekhattar, R. and Berger, S. L. (2001). Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science* **293**, 1142-6.
Lo, W. S., Gamache, E. R., Henry, K. W., Yang, D., Pillus, L. and Berger, S. L. (2005). Histone H3 phosphorylation can promote TBP recruitment through distinct promoter-specific mechanisms. *EMBO J* **24**, 997-1008.
Lo, W. S., Trievel, R. C., Rojas, J. R., Duggan, L., Hsu, J. Y., Allis, C. D., Marmorstein, R. and Berger, S. L. (2000). Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. *Mol Cell* **5**, 917-26.
Macdonald, N., Welburn, J. P., Noble, M. E., Nguyen, A., Yaffe, M. B., Clynes, D., Moggs, J. G., Orphanides, G., Thomson, S., Edmunds, J. W. et al. (2005). Molecular basis for the recognition of phosphorylated and phosphoacetylated histone h3 by 14-3-3. *Mol Cell* **20**, 199-211.
Mateescu, B., England, P., Halgand, F., Yaniv, M. and Muchardt, C. (2004). Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. *EMBO Rep* **5**, 490-6.
Matthews, A. G., Kuo, A. J., Ramon-Maiques, S., Han, S., Champagne, K. S., Ivanov, D., Gallardo, M., Carney, D., Cheung, P., Ciccone, D. N. et al. (2007). RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination. *Nature* **450**, 1106-10.
Metzger, E., Imhof, A., Patel, D., Kahl, P., Hoffmeyer, K., Friedrichs, N., Muller, J. M., Greschik, H., Kirfel, J., Ji, S. et al. (2010). Phosphorylation of histone H3T6 by PKCbeta(I) controls demethylation at histone H3K4. *Nature* **464**, 792-6.
Nathan, D., Ingvarsdottir, K., Sterner, D. E., Bylebyl, G. R., Dokmanovic, M., Dorsey, J. A., Whelan, K. A., Krsmanovic, M., Lane, W. S., Meluh, P. B. et al. (2006). Histone sumoylation is a negative regulator in Saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications. *Genes Dev* **20**, 966-76.
Ng, S. S., Kavanagh, K. L., McDonough, M. A., Butler, D., Pilka, E. S., Lienard, B. M., Bray, J. E., Savitsky, P., Gileadi, O., von Delft, F. et al. (2007). Crystal structures of histone demethylase JMJD2A reveal basis for substrate specificity. *Nature* **448**, 87-91.
Ng, S. S., Yue, W. W., Oppermann, U. and Klose, R. J. (2009). Dynamic protein methylation in chromatin biology. *Cell Mol Life Sci* **66**, 407-22.
Nowak, S. J. and Corces, V. G. (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci. *Genes Dev* **14**, 3003-13.
Ooi, S. K., Qiu, C., Bernstein, E., Li, K., Jia, D., Yang, Z., Erdjument-Bromage, H., Tempst, P., Lin, S. P., Allis, C. D. et al. (2007). DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* **448**, 714-7.

Org, T., Chignola, F., Hetenyi, C., Gaetani, M., Rebane, A., Liiv, I., Maran, U., Mollica, L., Bottomley, M. J., Musco, G. et al. (2008). The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression. *EMBO Rep* **9**, 370-6.
Pasini, D., Malatesta, M., Jung, H. R., Walfridsson, J., Willer, A., Olsson, L., Skotte, J., Wutz, A., Porse, B., Jensen, O. N. et al. (2010). Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. *Nucleic Acids Res* **38**, 4958-69.
Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**, 503-33.
Pitkanen, J., Doucas, V., Sternsdorf, T., Nakajima, T., Aratani, S., Jensen, K., Will, H., Vahamurto, P., Ollila, J., Vihinen, M. et al. (2000). The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein. *J Biol Chem* **275**, 16802-9.
Polioudaki, H., Markaki, Y., Kourmouli, N., Dialynas, G., Theodoropoulos, P. A., Singh, P. B. and Georgatos, S. D. (2004). Mitotic phosphorylation of histone H3 at threonine 3. *FEBS Lett* **560**, 39-44.
Prasanth, S. G., Shen, Z., Prasanth, K. V. and Stillman, B. (2010). Human origin recognition complex is essential for HP1 binding to chromatin and heterochromatin organization. *Proc Natl Acad Sci U S A* **107**, 15093-8.
Qian, J., Lesage, B., Beullens, M., Van Eynde, A. and Bollen, M. (2011). PP1/Repo-man dephosphorylates mitotic histone H3 at T3 and regulates chromosomal aurora B targeting. *Curr Biol* **21**, 766-73.
Ramon-Maiques, S., Kuo, A. J., Carney, D., Matthews, A. G., Oettinger, M. A., Gozani, O. and Yang, W. (2007). The plant homeodomain finger of RAG2 recognizes histone H3 methylated at both lysine-4 and arginine-2. *Proc Natl Acad Sci U S A* **104**, 18993-8.
Reid, J. L., Moqtaderi, Z. and Struhl, K. (2004). Eaf3 regulates the global pattern of histone acetylation in Saccharomyces cerevisiae. *Mol Cell Biol* **24**, 757-64.
Ringrose, L., Ehret, H. and Paro, R. (2004). Distinct contributions of histone H3 lysine 9 and 27 methylation to locus-specific stability of polycomb complexes. *Mol Cell* **16**, 641-53.
Robzyk, K., Recht, J. and Osley, M. A. (2000). Rad6-dependent ubiquitination of histone H2B in yeast. *Science* **287**, 501-4.
Ruthenburg, A. J., Li, H., Milne, T. A., Dewell, S., McGinty, R. K., Yuen, M., Ueberheide, B., Dou, Y., Muir, T. W., Patel, D. J. et al. (2011). Recognition of a mononucleosomal histone modification pattern by BPTF via multivalent interactions. *Cell* **145**, 692-706.
Ruthenburg, A. J., Li, H., Patel, D. J. and Allis, C. D. (2007). Multivalent engagement of chromatin modifications by linked binding modules. *Nat Rev Mol Cell Biol* **8**, 983-94.
Sarraf, S. A. and Stancheva, I. (2004). Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Mol Cell* **15**, 595-605.
Segil, N., Guermah, M., Hoffmann, A., Roeder, R. G. and Heintz, N. (1996). Mitotic regulation of TFIID: inhibition of activator-dependent transcription and changes in subcellular localization. *Genes Dev* **10**, 2389-400.
Shi, X., Hong, T., Walter, K. L., Ewalt, M., Michishita, E., Hung, T., Carney, D., Pena, P., Lan, F., Kaadige, M. R. et al. (2006). ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**, 96-9.
Shogren-Knaak, M. and Peterson, C. L. (2004). Creating designer histones by native chemical ligation. *Methods Enzymol.* **375**, 62-76
Shogren-Knaak, M., Ishii, H., Sun, J. M., Pazin, M. J., Davie, J. R. and Peterson, C. L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science* **311**, 844-7.
Sofueva, S., Du, L. L., Limbo, O., Williams, J. S. and Russell, P. (2010). BRCT domain interactions with phospho-histone H2A target Crb2 to chromatin at double-strand breaks and maintain the DNA damage checkpoint. *Mol Cell Biol* **30**, 4732-43.
Soloaga, A., Thomson, S., Wiggin, G. R., Rampersaud, N., Dyson, M. H., Hazzalin, C. A., Mahadevan, L. C. and Arthur, J. S. (2003). MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J* **22**, 2788-97.
Strahl, B. D. and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* **403**, 41-5.
Stucki, M. (2009). Histone H2A.X Tyr142 phosphorylation: a novel sWItCH for apoptosis? *DNA Repair (Amst)* **8**, 873-6.
Sun, Z. W. and Allis, C. D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* **418**, 104-8.
Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L. M., Liu, D. R., Aravind, L. et al. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324**, 930-5.

Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J. and Mahadevan, L. C. (1999). The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO J* **18**, 4779-93.
Torres-Padilla, M. E., Parfitt, D. E., Kouzarides, T. and Zernicka-Goetz, M. (2007). Histone arginine methylation regulates pluripotency in the early mouse embryo. *Nature* **445**, 214-8.
Valls, E., Sanchez-Molina, S. and Martinez-Balbas, M. A. (2005). Role of histone modifications in marking and activating genes through mitosis. *J Biol Chem* **280**, 42592-600.
van Ingen, H., van Schaik, F. M., Wienk, H., Ballering, J., Rehmann, H., Dechesne, A. C., Kruijzer, J. A., Liskamp, R. M., Timmers, H. T. and Boelens, R. (2008). Structural insight into the recognition of the H3K4me3 mark by the TFIID subunit TAF3. *Structure* **16**, 1245-56.
van Oevelen, C. J., van Teeffelen, H. A., van Werven, F. J. and Timmers, H. T. (2006). Snf1p-dependent Spt-Ada-Gcn5-acetyltransferase (SAGA) recruitment and chromatin remodeling activities on the HXT2 and HXT4 promoters. *J Biol Chem* **281**, 4523-31.
Varier, R. A., Outchkourov, N. S., de Graaf, P., van Schaik, F. M., Ensing, H. J., Wang, F., Higgins, J. M., Kops, G. J. and Timmers, H. T. (2010). A phospho/methyl switch at histone H3 regulates TFIID association with mitotic chromosomes. *EMBO J* **29**, 3967-78.
Vermeulen, L., Vanden Berghe, W., Beck, I. M., De Bosscher, K. and Haegeman, G. (2009). The versatile role of MSKs in transcriptional regulation. *Trends Biochem Sci* **34**, 311-8.
Vermeulen, M., Eberl, H. C., Matarese, F., Marks, H., Denissov, S., Butter, F., Lee, K. K., Olsen, J. V., Hyman, A. A., Stunnenberg, H. G. et al. (2010). Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. *Cell* **142**, 967-80.
Vermeulen, M., Mulder, K. W., Denissov, S., Pijnappel, W. W., van Schaik, F. M., Varier, R. A., Baltissen, M. P., Stunnenberg, H. G., Mann, M. and Timmers, H. T. (2007). Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. *Cell* **131**, 58-69.
Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J. M. et al. (2006). The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**, 871-4.
Walter, W., Clynes, D., Tang, Y., Marmorstein, R., Mellor, J. and Berger, S. L. (2008). 14-3-3 interaction with histone H3 involves a dual modification pattern of phosphoacetylation. *Mol Cell Biol* **28**, 2840-9.
Wilkinson, K. D. (2000). Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol* **11**, 141-8.
Williams, K., Christensen, J., Pedersen, M. T., Johansen, J. V., Cloos, P. A., Rappsilber, J. and Helin, K. (2011). TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* **473**, 343-8.
Winter, S., Simboeck, E., Fischle, W., Zupkovitz, G., Dohnal, I., Mechtler, K., Ammerer, G. and Seiser, C. (2008). 14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation. *EMBO J* **27**, 88-99.
Wood, A., Krogan, N. J., Dover, J., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Golshani, A., Zhang, Y., Greenblatt, J. F. et al. (2003). Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. *Mol Cell* **11**, 267-74.
Wu, H., D'Alessio, A. C., Ito, S., Xia, K., Wang, Z., Cui, K., Zhao, K., Sun, Y. E. and Zhang, Y. (2011). Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. *Nature* **473**, 389-93.
Yang, Y., Lu, Y., Espejo, A., Wu, J., Xu, W., Liang, S. and Bedford, M. T. (2010). TDRD3 is an effector molecule for arginine-methylated histone marks. *Mol Cell* **40**, 1016-23.
Zeng, L. and Zhou, M. M. (2002). Bromodomain: an acetyl-lysine binding domain. *FEBS Lett* **513**, 124-8.
Zhang, Y. and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev* **15**, 2343-60.
Zhao, Q., Rank, G., Tan, Y. T., Li, H., Moritz, R. L., Simpson, R. J., Cerruti, L., Curtis, D. J., Patel, D. J., Allis, C. D. et al. (2009). PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat Struct Mol Biol* **16**, 304-11.
Zhao, Z., Tavoosidana, G., Sjolinder, M., Gondor, A., Mariano, P., Wang, S., Kanduri, C., Lezcano, M., Sandhu, K. S., Singh, U. et al. (2006). Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat Genet* **38**, 1341-7.
Zippo, A., Serafini, R., Rocchigiani, M., Pennacchini, S., Krepelova, A. and Oliviero, S. (2009). Histone crosstalk between H3S10ph and H4K16ac generates a histone code that mediates transcription elongation. *Cell* **138**, 1122-36.