The Role of SAS-I in Mediating Crosstalk between Histone Modifications

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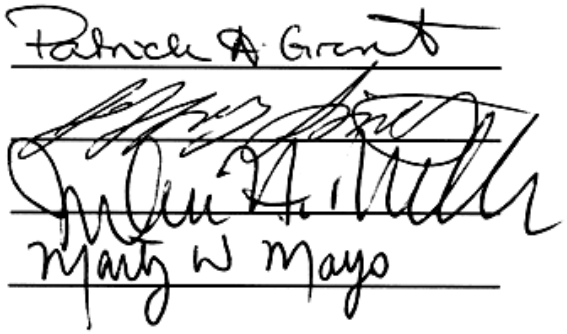

Abstract

Histone post translational modifications (HPTM) such as methylation, acetylation, phosphorylation, and ubiquitination play a role in regulating many cell processes including cell cycling, transcription, and DNA damage repair. Regulation of such processes is achieved through the ability of HPTMs to 1) recruit activating and repressive complexes via 'effector' proteins and 2) alter chromatin structure through changing DNA, inter-, and intranucleosomal interactions. Many marks, when studied alone, can correlate with an increase and/or a decrease in global gene expression. However, recent studies suggest that one HPTM rarely affects gene expression without 'crosstalk' with one or several other HPTMs.

The focus of this dissertation is to elucidate the mechanisms of targeting and regulation of Dot1 via histone crosstalk. Dot1 is a nonprocessive histone methyltransferase (HMT) responsible for H3K79 methylation. Dot1 requires H2BK123 ubiquitination in order to di- and trimethylate H3K79. However, how each H3K79 methylation state is regulated and what roles each state plays in specific cellular processes is unknown. Evidence provided in this dissertation illustrates that H3K79 trimethylation by Dot1 is dependent on H4K16 acetylation by the histone acetyltransferase (HAT) complex, SAS-I. *In vitro* HMT assays suggest that H4K16 acetylation-dependent trimethylation of H3K79 is achieved through changes in internuclesomal interactions, subsequent chromatin decondensation, and possible allosteric stimulation of Dot1 by H4K16ac. Upon loss of H4K16 acetylation, a significant loss in H3K79me3 is exhibited at genes bodies, while a loss of all three H3K79 methylation states is exhibited at subtelomeric regions. These results suggest that H3K79/H4K16 crosstalk may play a specific role in transcriptional regulation.

Data provided in this dissertation also shows that Dot1 is targeted to chromatin via various HPTMs that have been linked to conserved pathways involved in transcriptional regulation. Results shown here indicate that Dot1 binds to unmodified H4 tail and modified H3 peptides, including H3K4me, H3R2me, and H3K14/18ac. In addition, loss of H3K4 and H3R2 methylation is shown to affect Dot1 activity. Communication between H3K79 and the modifications discussed in this dissertation ultimately alters the degree to which H3K79 is methylated. Elucidating the mechanisms involved in regulating Dot1 will provide addition therapeutic targets for patients suffering from leukemia caused by the mistargeting of Dot1 and consequent dysregulatin of genes involved in hematopoietic development.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION TO CHROMATIN AND HISTONE MODIFICATIONS

Chromatin Organization

Chromatin is the organization of the eukaryotic genome into a condensed form due the function of many proteins and RNAs. In humans, approximately 2.0 meters of DNA is compacted into 24 chromosomes that fit into a nucleus that is 6 µm in diameter. The fundamental unit of the highly ordered chromatic fiber is the nucleosome, which consists of approximately146 base pairs of DNA wrapped around an octamer of core histones that contains two of each histone H2A, H2B, H3, and H4 (Figure 1-1) (Luger *et al.*, 1997). A linker histone, H1, binds to DNA as it enters and exits its 1.65 turns around the nucleosome. Nucleosomes serve as a repetitive array that facilitates the condensation of DNA. With the help of linker histone H1 to redirect the DNA path (Ramakrishnan 1997) and histone tails extending from the nucleosomal core (Luger and Ricmond 1998), a 30 nm fiber is formed (Figure 1-2) (Felsenfeld and McGhee 1986)

Naturally, the condensed structure forms a barrier to cell processes that require accessibility to DNA such as DNA replication, damage repair, and transcription (Workman *et al.*, 1998). Post-translational modifications of histones such as acetylation, methylation, and phosphorylation change the affinity between DNA and histones thus affecting the condensation of chromatin as to organize the genome into active and inactive regions, termed euchromatin and heterochromatin, respectively. Although the terms were defined by Heitz in 1929 based on cytological experiments, a better understanding of the composition of DNA and proteins in these distinct regions has provided a framework for much of the research included in this dissertation.

The field of genetics has grown by leaps and bounds within the last decade due to the completion and availability of the human genome sequence, but phenotypes still cannot be explained solely by an individual's DNA sequence. Complex coordination and communication between a plethora of well conserved chromatin modifying factors are

essential for all organisms. Regulation of cellular processes depends on HPTMs, DNA methylation, histone variants, remodeling enzymes, and effector proteins that influence the structure and function of chromatin, which affects a broad spectrum of activities such as DNA repair, DNA replication, growth, and proliferation. If mutated or deleted, many of these factors can result in human disease at the level of transcriptional regulation. The common goal of recent studies is to understand disease states at the stage of altered gene expression. Utilizing information gained from new high-throughput techniques and analyses will aid biomedical research in the development of treatments that work at one of the most basic levels of gene expression, chromatin.

Figure 1-1

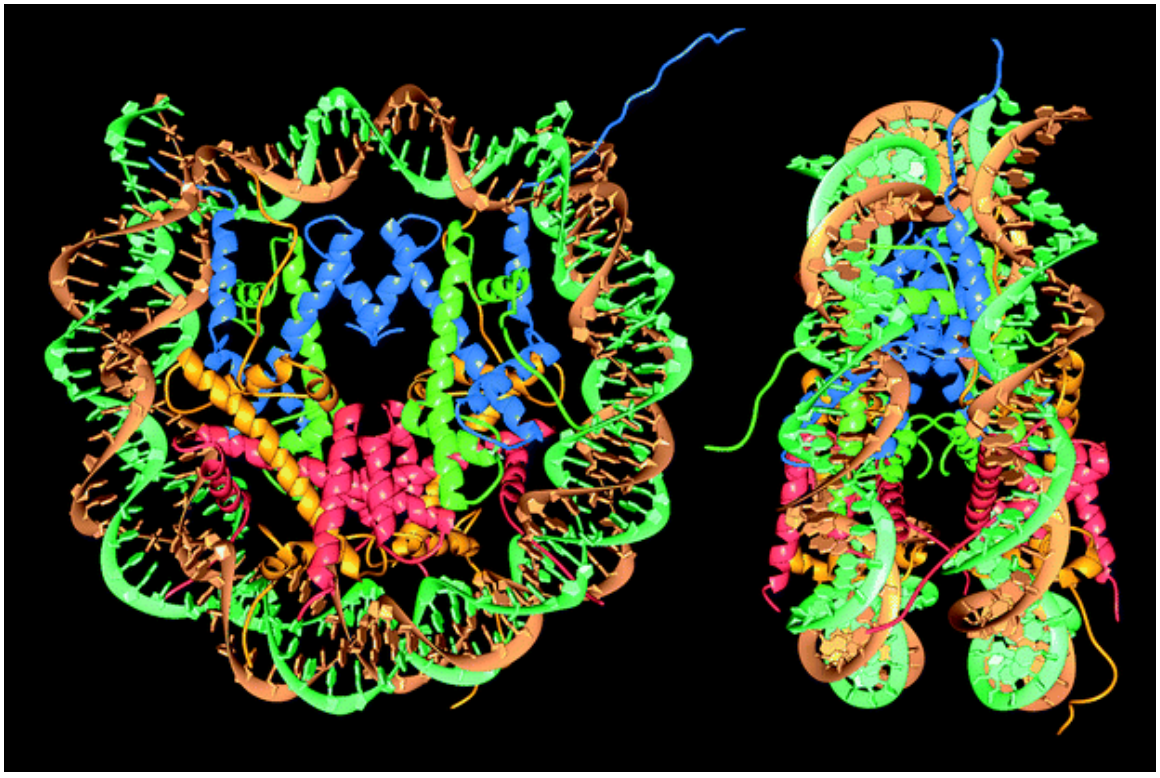


Figure 1-1: X-ray crystal structure of the nucleosomal core particle. Ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (H3: blue, H4: green, H2A: yellow, H2B: red). Interactions between the DNA and histones hold DNA in place. The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle with the center of the DNA helix shown at the top. Adapted from (Luger *et al.* 1997).

Figure 1-2

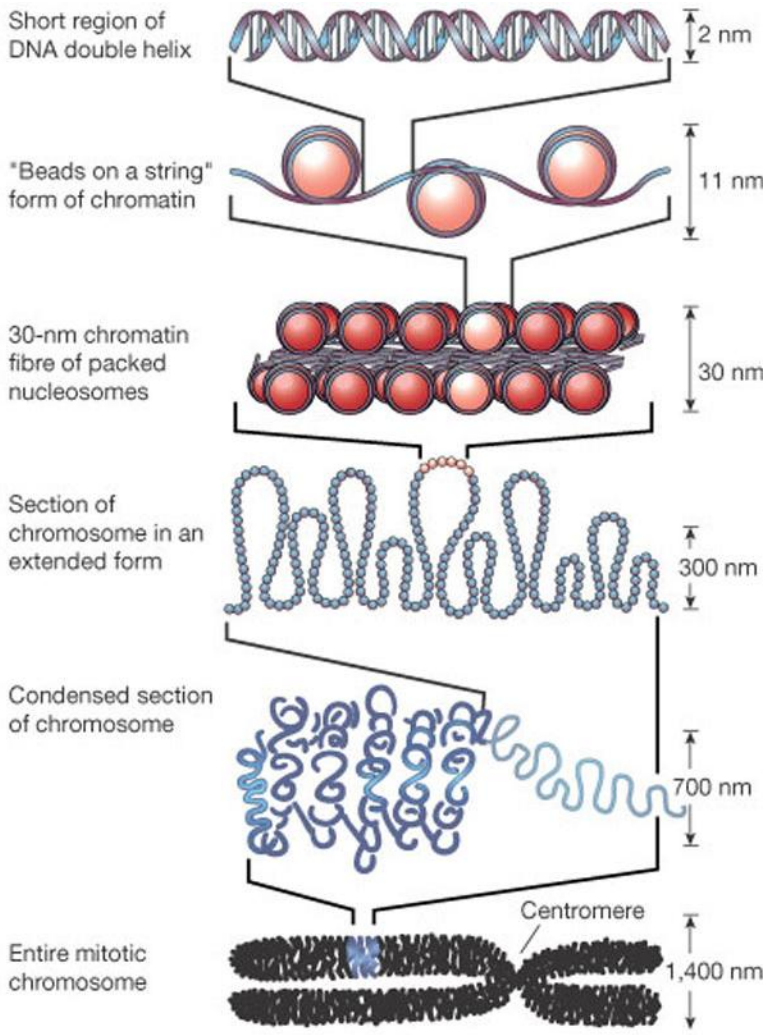


Figure 1-2: Condensation of DNA into chromosomes. DNA helices are wrapped around octamers of core histones to form nucleosomes and separated by 2 nm of linker DNA to form a first order structure in chromatin organization. In the next level of organization, nucleosomal core particles are compacted into a 30 nm fiber made possible by the inclusion of linker histone H1 and intramolecular nucleosome-nucleosome interactions. Lastly, the fiber is condensed into chromosomes by possible internucleosomal interactions that remain unclear. Adapted from (Felsenfeld and Groudine 2003).

Epigenetic Landscape

The human body is comprised of trillions of cells, each of which concurrently performs a specific function in order to form a functional human being. The function that one cell serves may be drastically different from another, yet each cell contains identical genetic information. Such phenotypic diversity is a result of a cell's distinctive gene expression profile. Gene expression is directly influenced by various factors including histone modifications, DNA methylation, histone variants, and availability of functional chromatin modifying complexes. Occasionally, DNA sequences targeted for modifications are expanded or contracted, or the enzymes that catalyze the addition or removal of modifications are lost or mutated. Respectively, these events cause a redistribution of DNA methylation and histone modification patterns. Alteration in the localization of these marks at sites such as promoters, repeat elements, and constitutive heterochromatin ultimately result in diseased states due to dysregulated gene expression (Kaufman and Rando 2010).

The idea that influences beyond the genetic code could determine phenotype is not by any means novel. In 1942, C.H. Waddington coined the phrase "epigenetic landscape" to denote changes in phenotype during development despite an identical genotype (Waddington *et al.* 1957). To date, the epigenetic landscape described by Waddington could be described by two important areas of chromatin research: the elaborate patterns of histone modifications and histone variant substitutions coined, "the histone code" (Jenuwein *et al.* 2001, Strahl and Allis 2000) and DNA methylation patterns (Bird and Wolffe 1999). Through its direct effects on transcriptional regulation, histone modifications and DNA methylation affect many essential cellular processes such as embryogenesis, genomic imprinting, DNA replication, microRNA expression, and X-chromosomal inactivation.

Evidence that some human diseases are caused by factors that influence gene expresstion beyond the genetic code is seen in cancer genetics (Estellar *et al.* 2007), autoimmune disorders (Javierre *et al*. 2010), and health related issues such as type 2 diabetes (Miao *et al.* 2008), coronary artery disease (Ordovas and Smith 2010), and obesity (Campion *et al.* 2009), to name a few. The role of epigenetics in the development of disease is further illustrated by the discordance of disease and trait development in monozyotic twins. Based on this study, environmental factors seem to play a significant role in disease susceptibility and dictating an individual's epigenetic landscape (Fraga *et al.* 2005). Ultimately, an increase in disease susceptibility can be attributed to environmentally influenced differences in DNA methylation and histone modification patterns that affect levels of gene expression.

With so many new advents in biomedical research, using human epigenetic profiling for understanding disease and even developing medical treatments has never seemed so tangible. Genome-wide association studies (GWAS) and high-throughput sequencing has allowed for high resolution comparison of modifications and gene expression in various organisms. With a future understanding of the basic functional roles these modifications play as transcriptional regulators in the cell, development of targeted treatments resulting in artificial epigenetic landscaping can potentially be established.

DNA Methylation

With respect to epigenetic research and a causal relationship to disorders resulting from transcriptional dysregulation, DNA methylation is the most characterized modification. The enzymatic addition of a methyl group to DNA is performed by DNA methyltransferase (DNMT) on the 5'-carbon of the pyrimidine ring in cytosine. Four

human DNMTs have been characterized: DNMT1 (Bestor *et al.* 1988), DNMT2 (Yoder and Bestor 1998), DNMT3a and DNMT3b (Okano *et al.* 1999). *De novo* DNA methylation patterns are established early in development by DNMT3a and DNMT3b and maintained by DNMT1, which prefers to methylate hemi-methylated templates during DNA replication through its recruitment by proliferating cell nuclear antigen (PCNA) (Clark *et al.* 1995). About 3% of cytosines are methylated in the human genome almost exclusively in the context of the dinucleotide, CpG. 5-methylcytosine (5-mC) is also found in very low abundance at the trinucleotide, CpNpG (Clark *et al.* 1995).

CpG dinucleotides are rarer than expected in the human genome (~1%) (Josse *et al.* 1961; Swartz *et al.* 1962) as a result of 5-mC deamination and subsequent mutation to thymine (Scarano *et al.* 1967). 70 to 80% of CpG dinucleotides are methylated and such dinucleotides tend to cluster in islands (Ehrlich *et al.* 1982). Regions containing the normal expected density of CpG dinucleotides are called CpG islands (CGI), which are regions no smaller than 200 bp that contain a GC content of more than 55% and an expected GC content to observed GC content ratio greater than 0.65 (Takai and Jones 2002).

Approximately 60% of human gene promoters and first exons are associated with CGIs. CGIs at promoters are frequently hypomethylated corresponding to a permissive chromatin structure in order to poise genes for transcriptional activation (Larsen *et al.* 1992; Antequera and Bird 1993), while some are hypermethylated during development, which stably silences the promoter (Figure 1-3a) (Straussman *et al.* 2009). Such programmed CGI methylation is important for genomic imprinting, which results in monoallelic expression through the silencing of a parental allele (Kacem and Feil 2009) and gene dosage compensation such as X-chromosome inactivation in females (Reik and Lewis 2005). Recently, Doi *et al.* has shown that limited gene expression in differing

tissue types is caused by differential methylation of CpG island shores (2009), which are located within 2.0 kb of CGIs (Figure 1-3b) (Saxonov *et al.* 2006). Still, a fraction of CGIs are prone to methylation in some tissues due to aging, in promoters of tumor suppressor genes in cancer cells (Issa *et al.* 2000), and committed cell lines (Jones *et al.* 1990). The remaining 40% of CGIs are located intra- and intergenically. Intragenically located CGIs within the coding region of genes are methylated at trinucleotides CpXpG (Lister *et al.* 2009) and are commonly found in highly expressed, constitutively active genes (Figure 1-3c) (Zhang *et al.* 2006) while intergenic CGIs may be used for transcription of non-coding RNAs (Illingworth *et al.* 2008).

More often than not, DNA methylation is usually associated with gene silencing due to 1) the occlusion of DNA binding proteins that act as or recruit transcriptional activators or 2) the recruitment of methyl-binding proteins (MBPs), which recruit transcriptional corepressor complexes (Figure 1-3) (Portela and Estellar 2010). Transcriptional activators and repressors recruit histone modifying and chromatin remodeling complexes that can remodel chromatin, which ultimately changes the transcriptional activity of a gene. Modifications made by such complexes and subsequent effects on transcription will be discussed later.

Even prior to DNA methylation, DNMTs can be recruited to DNA via DNA binding transcription factors, which results in specific promoter DNA methylation and regulatory gene repression. For example, studies showed that DNMTs interact with the oncogenic transcription factor formed by the fusion of promyelocytic leukemia protein and retinoic acid receptor (PML-RAR), found in acute promyelocytic leukemia (Di Croce *et al.* 2002). DNMT recruitment to the *RARβ2* gene promoter by PML-RAR results in promoter hypermethylation and subsequent gene silencing (Di Croce *et al.* 2002). A similar mechanism has been described for Myc, a DNA binding transcription factor. Myc

interacts with DNMT3a and is recruited to the p21 gene promoter resulting in subsequent DNA methylation and p21 gene repression (Brenner *et al.* 2005). In addition, p53 also interacts with DNMT3a and represses p53's transactivator function at the p21 gene promoter but in a DNA methylation independent manner (Wang *et al.* 2005). Both mechanisms elucidate cancer promoting pathways that intersect with DNA methylation and cause repression of expression of p21, a cyclin dependent kinase inhibitor. Moreover, one study showed that DNMT3a/b interacts with 79 different DNA binding transcription factors (Hervouet *et al.*2009). Some interactions were exclusive to each DNMT while some were shared between both (Hervouet *et al.* 2009). The diversity of interactions further illustrates the importance of DNA methylation on gene expression regulation through DNMT recruitment via DNA binding transcription factors.

Once DNA is methylated, DNA methyl-binding proteins (MBP) can bind to DNA and recruit transcriptional corepressors such as histone deacetylase (HDAC) complexes, polycomb proteins, and chromatin remodeling complexes. One family consists of MBPs, which possess a conserved methyl-CpG-binding-domain (MBD) and includes MBD1, MBD2, MBD3, MBD4, and MeCP2. MeCP2 is the founding member of the MBD family and contains a MBD in addition to an adjacent transcriptional repressor domain (TRD) (Klose and Bird 2006). The TRD of MeCP2 interacts with the Sin3 corepressor complex containing HDAC1 and 2 (Nan *et al.* 1998). MBD1 also contains three zinc-binding domains (CxxC), which have been shown to be responsible for its ability to bind unmethylated CpG sites (Jorgensen *et al.* 2004). MBD1 and 2 both contain a TRD that recruits different transcriptional corepressor complexes containing HDACs. MBD3 contains a MBD but does not bind methylated DNA due to two amino acid substitutions (Hendrich and Tweedie 2004) but is associated with the nucleosome remodelling and histone deacetylase (NuRD) corepressor complex, which contains HDACs necessary for

transcriptional silencing. MBD4 is a thymidine glycosylase DNA repair enzyme that excises mismatched thymines that have resulted from 5-methylcytosine deamination in the context of CpG dincleotides (Hendrich *et al.* 1999).

The second family of MBPs includes Kaiso, zinc finger and BTB (for BR-C, ttk, and bab) domain containing (ZBTB) 4 and ZBTB38 (Zollman *et al.* 1994). These are atypical MBPs, because they depend on a zinc-finger domain to recognize methylated DNA and a POZ (for Pox virus and Zinc finger) (Bardwell *et al.* 1994)/BTB domain to repress transcription through its interaction with nuclear receptor co-repressor-1 (N-CoR) (Prokhortchouk and Defossez 2008). Another study (Iioka *et al.*2009) showed that Kaiso can regulate transcription factor activity by modulating the interaction between β-catenin and HDAC1 activity. The third family of MBPs includes ubiquitin-like plant homeodomain and RING finger (UHRF)-domain containing protein 1 and 2. Both contain SET and RING associated (SRA) domains, which preferentially bind to DNMT1's substrate, hemi-methylated DNA (Bostick *et al.* 2007). Furthermore, UHRF1 has been shown to colocalize with DMNT1, which suggests that this family of MBPs may help target DMNT1 to DNA (Bostick *et al.* 2007).

DNA methylation is usually associated with transcriptional silencing, and one of the most well known cases where differential DNA methylation induces and suppresses expression is genomic imprinting at the *H19/IGF2* locus. Genomic imprinting is a form of gene regulation in which an allele is expressed from one of the two parental homologous chromosomes. *H19* and *IGF2* are reciprocally imprinted so that *H19* is expressed from the maternal allele and *IGF2* from the paternal allele (Bell and Felsenfeld 2000). Transcriptional regulation of these genes is dependent on a differentially methylated DNA domain (DMD) or imprinting control region (ICR) located upstream of *H19* and downstream of *IGF2.* The DMD/ICR is methylated on the paternal allele but not the

maternal allele (Bell and Felsenfeld 2000; Hark *et al.* 2000; Szabo *et al.* 2000; Kanduri *et al.* 2000). CCCTC-binding factor (CTCF) binds to the unmethylated ICR of the maternal allele, which blocks an enhancer region located downstream of *H19* from activating transcription of *IGF2* (Hark *et al.* 2000). CTCF binding also protects against *de novo* methylation and subsequent repression at the *H19* locus on the maternal allele (Rand *et al.* 2004). This is one of the most basic examples of how differentially methylated regions can determine levels of gene expression. Mutations or deletions in the *H19* promoter, ICR, or enhancer can lead to growth defects such as Beckwith-Wiedemann Syndrome or Silver-Russell dwarfism (Delaval *et al.* 2006).

With the advent of microarrays and high-throughput technologies, an explosion of gene expression profile comparisons in normal and diseased cells has occurred. Many studies have pursued genes of interest by comparing the DNA methylation status of a gene's 5' promoter region (Weber *et al.* 2005; Hatada *et al.* 2006), and presently, more comprehensive results are available as more direct solutions to discovering gene expression controlled by DNA methylation are established. Using *Arabidopsis thaliana* as a model system, Zhang *et al.* analyzed and compared whole genome methylome tiling arrays gathered from immunoprecipitating 5-mC or chromatin crosslinked MBPs in normal and mutant cells (Zhang *et al.* 2006). Another study (Javierre *et al.*2010) compared the DNA methylome of monozygotic twins who were differently affected by the disease, systemic lupus erythematosus (SLE) (Javierre *et al.* 2010). In comparison to the healthy twin, the twin affected by SLE had a decrease in promoter DNA methylation for many genes involved in immune system function including *IFNGR2, MMP14, LCN2, CSF3R, PECAM1, CD9, AIM2,* and *PDX1*. These genes had also previously been shown to participate in the development of SLE (Javierre *et al.* 2010).

Moreover, 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC) by an oxidation reaction carried out the ten-eleven-translocation (TET) family of proteins (Tahiliani *et al.* 2009). 5-hmC was first discovered in bacteriophage DNA in 1952 (Wyatt and Cohen 1952) and has since been found to be enriched in mouse brain (Kriaucionis and Heintz 2009), embryonic stem cells (Tahiliani *et al.* 2009), and human tissues (Li and Lui 2011).

Levels of 5-hmC are dynamically regulated by TET1-3 in stem cells and seem to be higher in pluripotent cells. Knockdown of *TET1* and *TET2* causes a decrease in 5-hmC levels and an increase in 5-mC at stem cell related gene promoters (Ficz *et al.* 2011). These genes are subsequently silenced. *TET3* is highly expressed in zygotes and oocytes (Wossidlo *et al.* 2011) and a recent study (Iqbal *et al.* 2011) has shown that after fertilization, 5-mC is converted to 5-hmC in the male but not the female pronucleus. This data (Iqbal *et al.* 2011) suggests an alternative to the global demethylation theory during cellular dedifferentiation where genome-wide 5-mC may be converted to 5-hmC by TET3 and differentiation is promoted by a decrease in TET3 and an increase in TET1 and 2 (Koh *et al.* 2011; Walter 2011). The mechanisms behind 5-hmC's role in cellular differentiation (Ito *et al.* 2010), carcinogenesis, (Li and Liu 2011) and association with actively transcribed genes is a mystery (Ficz *et al.* 2011). One clue provided is that 5-hmC prevents the binding of MBDs (Valinluck *et al.* 2004) and DNMTs (Valinluck and Sowers 2007).

Figure 1-3

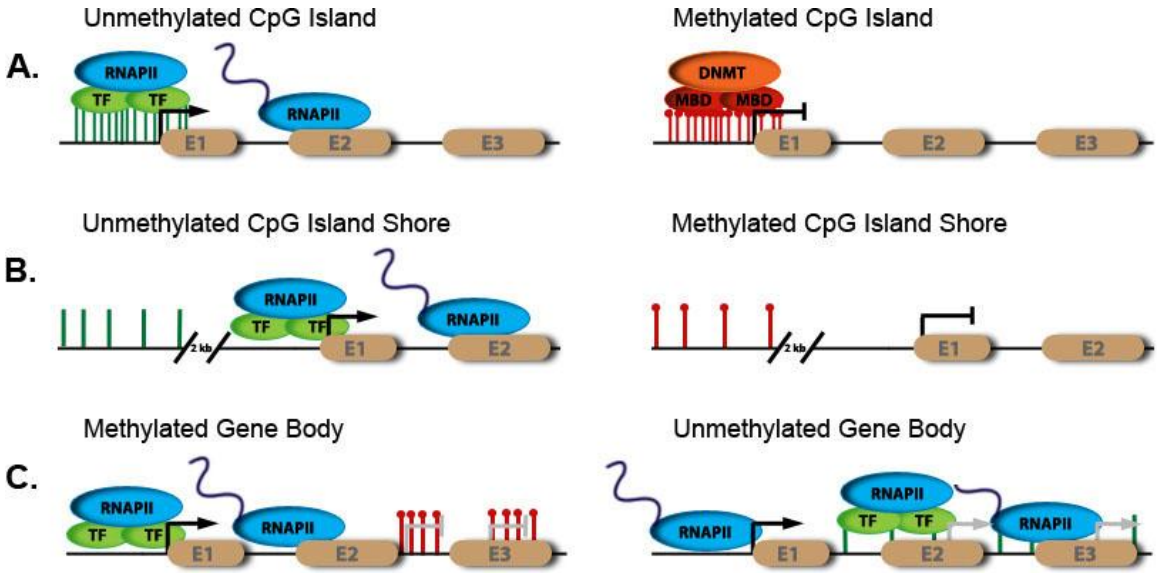


Figure 1-3: Various sites and effects of DNA methylation throughout the genome. DNA methylation is found at inter- and intragenic regions throughout the genome. DNA methylation dependent transcriptional activity is contingent on CpG dinucleotide genic location and density. Normal methylation events and subsequent effects are shown on the left. (a) CpG islands at promoters are normally unmethylated resulting in gene expression. However, aberrant hypermethylation at the same promoter results in corepressor complex recruitment and subsequent gene repression. (b) Intragenic regions characterized by scattered CpG dinucleodtides located 2kb upstream of the promoter called CpG island shores are regulated in the same manner as (a). (c) DNA methylation within the gene body prevents initiation of transcription from spurious sites in the gene. If unmethylated, these sites become transcriptional start sites resulting in an incorrect product. Adapted from (Portela and Estellar 2010).

Histone Modifications

As mentioned in the previous section, methylated DNA can recruit different transcriptional activator and repressor complexes. In most cases, these complexes contain histone modifying and chromatin remodeling enzymes that regulate chromatin structure, which ultimately changes the transcriptional activity of a gene. Such complexes are not just recruited by DNA methylation but also by various transcription factors and various other HPTMs. Covalent HPTMs such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deimination, and the non-covalent proline isomerization (Kouzarides *et al.* 2007) can affect the condensation of chromatin as to organize the genome into transcriptionally active and inactive regions termed euchromatin and heterochromatin (Heitz 1929). HPTMs can alter chromatin structure through perturbation of inter- and intranucleosomal interactions and recruitment of transcriptional complexes containing additional histone modifiers (Jenuwein 2001).

The core histones are highly conserved basic proteins composed of a globular domain and highly flexible N-terminal tails that protrude from the DNA wrapped nucleosome (Figure 1-1) (Luger *et al.* 1997). All histone N-termail tails and globular domains are subject to modification and more is known about the smaller covalent modifications methylation, acetylation, and phosphorylation. Lysine residues can be mono-,di-, and trimethylated while arginine residues can only be mono- or symmetrically or asymmetrically dimethylated (Bannister and Kouzarides 2011). The interactions between chromatin associated proteins that bind HPTMs can act synergistically or antagonistically with one another resulting in various gradients of transcriptional activation and repression across the genome. The term “histone code” was coined in order to convey that chromatin modifying proteins ultimately determine phenotype rather than simple possession of a certain genetic code (Strahl and Allis 2000; Jenuwein and

Allis 2001). HPTMs specific roles in gene expression and cellular activities are shown in Table 1-1 (adapted from Kouzarides and Berger 2007; Wang *et al.* 2008).

Euchromatin is characterized by high levels of acetylation and high levels of H3K4me1/2/3, H3K36me3 and H3K79me1/2/3. On the other hand, heterochromatin is characterized by low levels of acetylation and high levels of H3K9me2/3, H3K27me2/3 and H4K20me3 (Table 1.1) (Li *et al.* 2007). More recently, a group (Wang *et al.* 2008) performed chromatin immuneprecipitation sequencing (ChIP-seq) on 39 different core histone acetylations and methylations at 3,286 promoter regions. As shown in previous studies (Turner 1992), acetylated histones consistently correlate with increased gene transcription. However, certain modifications localized to specific gene regions rather than just at transcriptional start sites (TSS). H2AK9ac, H2BK5ac, H3K9ac, H3K18ac, H3K27ac, H3K36ac and H4K91ac were mainly located in the region surrounding the TSS, whereas H2BK12ac, H2BK20ac, H2BK120ac, H3K4ac, H4K5ac, H4K8ac, H4K12ac and H4K16ac were prominent in the promoter and transcribed regions of active genes (Wang *et al.* 2008).

Another group (Karlic *et al.* 2010) analyzed ChIP-seq data produced by the Zhao lab in order to create a model that could predict levels of gene expression based on HPTM levels present at promoters. They found that actively transcribed genes are characterized by high levels of H3K4me3, H3K27ac, H2BK5ac and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene body. Moreover, they found high levels of H4K20me1 and H3K27ac at promoters that contained high CpG content and H3K4me3 and H3K79me1 at promoters with low CpG content (Karlic *et al.* 2010).

Although there is no model that explains the HPTM difference at the two types of promoters, one can speculate that the difference is caused by different regulatory mechanisms and possibly, changes in DNA methylation. In agreement with this theory,

Table 1-1

Table 1-1: Transcriptional and cellular role of histone modifications

Modification	Histone Residues Modified	Role in Cell Activity and Transcription	Histone Modification Readers
Acetylated Lysine (Kac)	H3 (K4,9,14,18,23,27,36,56) H4 (K5,8,12,16,19) H2A (K5,9) H2B (K5,6,7,12,16,17,20,120)	Activation DNA Damage Repair	Bromodomain Tandem PHD
Phosphorylated Serine/ Threonine (S/Tph)	H3 (S10,28 and T3,11,45) H4 (S1,47) H2A (S1)	Apoptosis Activation Mitosis (Baker *et al.* 2010)	14-3-3 Domain
Methylated Lysine (Kme)	H3 (K4,23,36,79) H3 (K9, 27) and H4 (K20)	Activation Repression	MBT PHD Tudor Chromodomain WD40
Methylated Arginine (Rme)	H3 (R2,17,26) and H4 (R3) H3 (R8)	Activation Repression	Tudor (Yang *et al.* 2010; Chen *et al.* 2011) ADD (Zhao *et al.* 2009)
Ubiquitinated Lysine (Kub)	H2A (K119) H2B (K120/123 (yeast))	Repression Activation (Zhu *et al.* 2005)	Cps35 (Lee *et al.* 2007; Zheng *et al.* 2010)
Sumoylated Lysine (Ksu)	H4 (?)	Repression (Shiio and Eisenman 2003)	

previous ChIP-seq data was analyzed for HPTMs, CTCF, RNA Polymerase II (RNAPII), and the histone variant, H2A.Z, to describe 51 distinct chromatin states (Ernst and Kellis 2010). Each state is described by the enrichment of different HPTMs and chromatin associated proteins across the genome. Moreover, biological states of cells (cell cycle, developmental, T-cell activation, *etc.*) were predicted using the 51 states (Ernst and Kellis 2010). Another interesting study (Mikkelsen *et al.* 2007) showed that embryonic stem cells contained a bivalent pattern of HPTMs at promoters of genes that regulate development. Surprisingly, they found H3K4me3, an activation mark, and H3K27me3, a repressive mark, co-localizing at these promoters in stem cells (Mikkelsen *et al.* 2007). These bivalent domains can resolve into four different chromatin states: 1) marked with both H3K4me3 and H3K27me3; 2) not marked with either H3K4me3 nor H3K27me3; 3) marked with H3K4me3 alone; 4) marked with H3K27me3 alone. Maintenance or loss of both marks results in a poised transcriptional state while preservation of H3K27me3 alone or H3K4me3 alone results in inactive and active transcription respectively (Cui *et al.* 2009). This data (Mikkelsen *et al.* 2007) suggests that HPTM bivalency at promoters allows for plasticity during cellular differentiation and development (Bernstein *et al.* 2006).

Until recently, elucidating the mechanisms by which HPTMs interact with one another to control transcriptional activity has been complicated due to the layered complexity of combinatorial HPTMs and HPTM crosstalk. However, analysis of recently acquired ChIP-seq data and associated gene expression profiles has speedily facilitated decipherment of the histone code and its effect on transcriptional activity (Figure 1-4 and 1-5) (Barski *et al.* 2007; Wang *et al.* 2008; Heintzman *et al.* 2007; Mikkelsen *et al.* 2007). Three broad effects on transcription can be attributed to HPTMs: 1) HPTMs can prevent certain chromatin binding proteins from binding. For example, H3S10ph prevents

heterochromatin protein 1 (HP1) from binding H3K9me3 (Kouzarides and Berger 2007); 2) HPTMs can recruit certain chromatin binding proteins, which can enhance or inhibit gene activation. For example, H3K9me3, a marker for mammalian heterochromatin, is bound by the chromodomain of HP1 resulting in chromatin condensation and occlusion of DNA and nucleosomal binding sites utilized by coactivators, transcription factors, and RNAPII (Kouzarides and Berger 2007); 3) HPTMs can act *in cis* by affecting transcription through alteration of chromatin structure. For example, H4K16ac alone prevents the formation of a higher ordered compacted chromatin structure resulting in chromatin decondensation and increased transcriptional activity (Shogren-Knaak *et al.* 2006).

Much of the mechanistic research done on transcriptional regulation and HPTMs is pioneered through the use of yeast model systems because genetic manipulation and high-yield results have been easier to obtain as compared to humans. Importantly, many yeast proteins have correlative homologs that serve in the same manner as they do in mammals. However, there are some differences between the two eukaryotic organisms. For example, yeast do not possess the repressive mark H3K27me and in some cases, homolgous complexes may contain different chromatin targeting proteins.

Figure 1-4

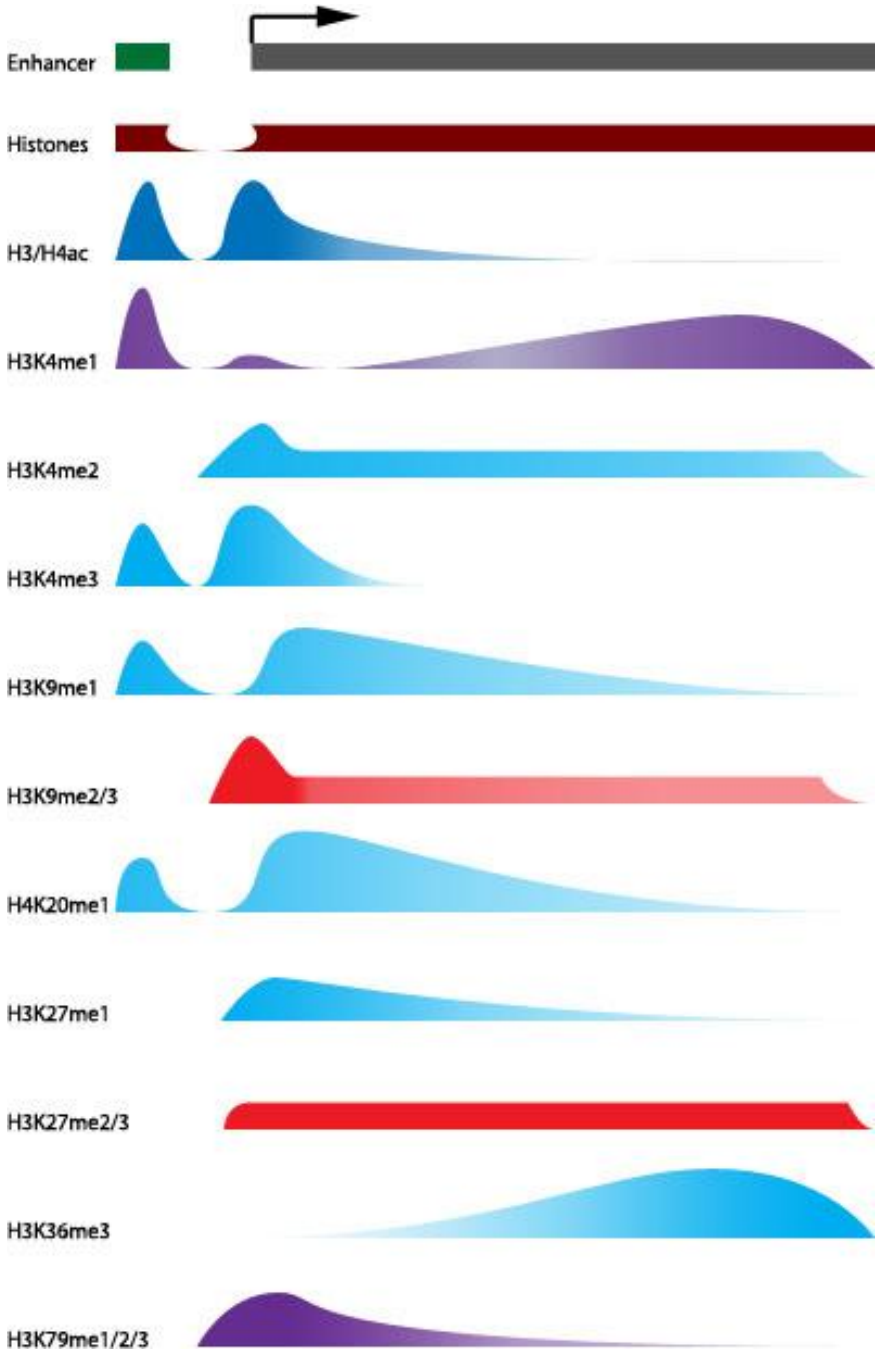

Enhancer
Histones
H3/H4ac
H3K4me1
H3K4me2
H3K4me3
H3K9me1
H3K9me2/3
H4K20me1
H3K27me1
H3K27me2/3
H3K36me3
H3K79me1/2/3

Figure 1-4: Localization of histone modifications across genes as it relates to transcriptional regulation. Patterns of histone modification enrichment are shown across an arbitrary enhancer and gene. The enhancer is shown as the smaller region succeeded by a gap denoting a nucleosome-free region and transcriptional start site as shown by the arrow. Data used to compile the profiles are from GWAS on histone modifications. The correlative effects of the modifications on gene expression are indicated by the labels: (+) expression, (-) repression, and (+/-) studies show enrichment in both expression and repressed genes. (Wang *et al.* 2008, Barski *et al.* 2007, Li *et al.* 2007)

Histone Acetylation

Overview

Histone acetylation at conserved lysine residues is the most intensely studied HPTM and was the first modification linked to transcriptional activity (Hebbes, Thorne, and Crane-Robinson 1988). It wasn't until 1996, that a link was made between acetylation and transcription factors. The first nuclear histone acetyltransferase (HAT) discovered, p55, was orthologous to a previously isolated transcriptional coactivator in *Saccharomyces cerevisiae*, Gcn5 (Brownell *et al.* 1996). HATs catalyze the addition of acetyl-coA to the ε-amino group on lysine side chains resulting in charge neutralization and affinity reduction between negatively charged DNA and basic histones. Acetylation ultimately creates an "open" chromatin structure (Shogren-Knaack *et al.* 2006) poised for active transcription through exposure of DNA-binding sites (Vettese-Dadey *et al.* 1996). There are two types of HATs: type-A (nuclear) and type-B (cytoplasmic). This discussion will only focus on type-A as they catalyze reactions related to active transcription (Bannister and Kouzarides 2011).

Type-A HATs are further divided into five families including the GCN5-related *N*-acetyltransferases (GNATs); the MOZ, Ybf2/Sas3, Sas2 and Tip60 (MYST)-related HATs; p300/CREB-binding protein (CBP) HATs; the general transcription factor HATs including the TFIID subunit TBP-associated factor-1 (TAF1); and the nuclear hormone-related HATs SRC1 and ACTR (SRC3) (Nagy and Tora 2007). They are often part of larger protein complexes and are recruited by DNA binding activators. For instance, in yeast, Gcn5 is part of the Spt-Ada-Gcn5-Acetyltransferase (SAGA) and Adaptor (ADA) complexes (Grant *et al.* 1997). In SAGA, Gcn5 is associated with three protein families known to be involved in gene expression: Spt, Ada, and a subset of TAFs (Grant *et al.* 1998). SAGA is recruited to active promoters via the SAGA subunit, Tra1's interaction

with acidic activator domains of transcriptional activators and subsequent recruitment of the TATA-binding protein (TBP) by the subunit, Spt3 (Grant *et al.* 1998; Larschan and Winston 2001; Brown *et al.* 2001; Reeves and Hahn 2005). Similar complex subunits have been found to be associated with Gcn5 human homologs, p300/CBP associated factor (P/CAF) and hGcn5 (Ogryzko *et al.* 1998; Martinez *et al.* 1998; Nagy and Tora 2007). Human Gcn5 is found in the SAGA complex homolog Spt3-Taf9-Gcn5-Acetyltransferase (STAGA) complex and is recruited to promoters by the Tra1 human homolog, Transactivation/transformation domain associated protein (TRRAP) via its interaction with the transactivation domain of c-Myc (McMahon *et al.* 2000; Lui *et al.* 2003).

Furthermore, Gcn5, P/CAF, and p300 contain a bromodomain that bind acetyl-lysine. Taf1 contains two bromodomains (Jacobson *et al.* 2000). The exact function of bromodomains in these proteins has yet to be elucidated. However, it is speculated that once HAT complexes are targeted to the promoter and perform acetylation, subsequent coactivators can stably bind to acetylated histone rich promoter regions via bromodomains, which would facilitate an acetylation cascade. Consistent with this hypothesis, SAGA requires the functional bromodomains of Gcn5 and the remodeling complex proteins Swi2/Snf2 for stable promoter occupancy, efficient HAT activity, and increase in gene expression resulting from an “open” chromatin conformation, and subsequent gene activation (Hassan *et al.* 2002).

HATs also acetylate non-histone proteins, including the tumor suppressor p53 and various transcription factors (Glozak *et al.* 2005), which ultimately regulates gene expression. The MYST (MOZ, Ybf2/ Sas3, Sas2 and Tip60) family of HATs requires active site lysine autoacetylation on a highly conserved residue for both efficient binding to its target substrate and subsequent histone acetylation (Yuan *et al.* 2012).

Like many bromodomains, DPF3b, a novel acetyl-lysine reader and BAF remodeling complex associated subunit also binds ambiguously to acetylated H3 and H4 (Lange *et al.* 2008) via its tandem plant homeodomain (PHD) fingers (Zeng *et al.* 2010). One PHD finger only has affinity for acetylated H3K14, which increases full-length DPF3b's affinity for acetylated H3 and H4 (Zeng *et al.* 2010). Loss of DPF3b affects both skeletal and heart muscle development through transcriptional deregulation of other transcriptional factors (Lange *et al.* 2008).

SAS-I Complex

The something about silencing (SAS) family of genes was discovered during a genetic screen for silencing restoration at mating type alleles in ***sir4Δ*** cells (Xu *et al.* 1999). However, the catalytic subunit Sas2 (39kDa) was found previous to the proteins Sas4 (55 kDa) and Sas5 (29kDa). *SAS2* was found in a complementation analysis for "enhancers of *sir1*Δ" mutants that caused haploid sterility in the silenced *sir1*Δ mating population (Reifsnyder *et al.* 1996). Mating assays performed with the *sir1*Δ*sas2*Δ strain indicated *HML* loci derepression while the *HMR* locus was unaffected (Ehrenhofer-Murray *et al.* 1997). Moreover, the *sas2*Δ strain showed complete loss of telomeric silencing as evident by expression of *URA3* markers strategically placed proximal to the telomere. After further investigation of the SAS2 gene, a BLASTP alignment showed that Sas2 also contained a conserved CysCysHisCys (C2HC) zinc-finger motif. The C2HC motif is common to human proteins MOZ and Tip60, which when translocated form chimeric oncogenes with HATs CBP and p300 (Reifsnyder *et al.*, 1997). Later research confirmed that Sas2 is a HAT that acetylates H4 at lysine 16. Specific localization of the HAT activity was substantiated by the comparable effects of H4 tail, HAT domain, and

zinc-finger domain mutants to that of *sas2*Δ strains in mating and telomeric silencing assays (Meijsing *et al.*, 2001).

SAS4 and *SAS5* were identified similarly to *SAS2*. As noted previously, *sas2*Δ has no effect on *HMR* silencing. However, if the Abf1 and Rap1 binding sites of the *HMR-E* silencer are mutated, loss of silencing occurs. Derepression of HMR silencing caused by the mutated silencers can be reversed in *sas2*Δ strains (Ehrenhofer-Murray *et al.*, 1997). A genetic screen was conducted with the *HMR-E* mutants for other genes that restore proper silencing at the *HMR* loci, which led to the discovery of *SAS4* and *SAS5*. Sas4 and Sas5 both are necessary for silencing at telomeres as well as at the *HML* locus in a *sir1*Δ strain (Xu *et al.*, 1999).

Sas2, 4, and 5 form a 230-450kDa trimeric complex termed SAS-I. However, other studies suggest a 125kDa complex based on a glycerol gradient sedimentation (Shia *et al.*, 2005). Sas4 is absolutely necessary for Sas2 HAT activity while Sas5 stimulates HAT activity *in vitro*. All subunits are needed for complex integrity and silencing function (Sutton *et al.*, 2003). Sas2 forms the catalytic core of the complex, which acetylates H4K16. Sas5 contains a conserved YEATS (Yaf9-ENL-AF9-Taf14-Sas5) domain, which is also found in human leukogenic proteins AF9, ENL, and Gas41. The YEATS domain is also found in two other yeast proteins, Taf14 and Yaf9. Yeast strains deficient for all three YEATS containing proteins are nonviable, which is suggestive of the domain's essential function in *S. cerevisiae* (Zhang *et al.*, 2004). Sas5 was also found in a higher molecular weight complex, which suggests its possible involvement in another complex (Meijsing *et al.*, 2001).

Sas4 and 2 have been shown to interact with both Cac1, a subunit of chromatin assembly factor complex (CAF-I), and Asf1 using both yeast two hybrid (Y2H) studies and co-IP (Osada *et al.*, 2001, Meisjing *et al.*, 2001). *Asf1* mutants have the same

HMsilencing phenotypes as *sas2, 4,* and *5*Δ but have no effect on telomeric silencing (Enomoto *et al.*, 1997, Kaufman *et al.*, 1997). *Cac1* mutants have similar but less pronounced silencing phenotypes as *sas2, 4,* and *5*Δ at both silent mating-type loci and telomeres (Singer *et al*., 1998). SAS-I does not acetylate nucleosomes efficiently but prefers acetylation of free histones (Shia *et al.* 2005, Meijsing *et al.* 2001). Moreover, SAS-I will not acetylate H4 in the presence of Asf1, a protein shown to bind H3 and H4 prior to histone deposition onto chromatin (Sutton *et al.*, 2003, Sharp *et al.*, 2001). Data showing SAS-I interaction with chromatin assembly factors and alteration of HAT activity in their presence suggests that SAS-I may acetylate histones previous to their deposition onto DNA.

As of recently, most of the research with SAS-I has focused primarily on its function at telomeres and as a factor in the prevention of heterochromatin spreading. The mutation of H4K16 to an alanine or the deletion of *SAS2* allows SIR (discussed below) to spread to the adjacent sub-telomeric region and can silence up to an extra 12kb beyond the telomere (Suka *et.al.* 2002). Moreover, an increase in SIR spreading, hypoacetylation beyond the telomeric boundary, and an increase in the repression of genes proximal to the telomere have been shown in *sas2*Δ strains (Kimura *et al.*, 2002). However, previous studies had placed *URA3* adjacent to the telomere of VII-L in a *sas2*Δ strain which produced a 50% chance of *URA3* expression and 5-fluoroorotic acid (5-FOA) sensitivity (Reifnyder *et al.*, 1997). One explanation has been deduced that although an increase in heterochromatin is formed due to the spread of the SIR complex beyond telomeres of *sas2*Δ cells, Sirtuins may be distributed in a more ubiquitous manner throughout the genome. A broader distribution of the SIR complex would leave genomic regions normally concentrated with Sir2 less inhabited by the SIR complex.

In support of the proposed redistribution hypothesis, data has shown a 30-60% decrease in the concentration of Sir2 0.5kb from the telomeric end and an increase in the spreading and localization of the SIR complex approximately 10kb further than normal (Suka *et.al.*, 2002). A redistribution of the SIR complex in the absence of SAS-I may explain why the *HMR* loci silencing is enhanced in ***sas2***Δ cells with a mutant *HMR-E* silencer. In the absence of Sas2, histones may be basally hypoacetylated, which favors recruitment of the SIR complex despite the inability of the silencer to recruit Abf1 or Rap1. An indication that histones are hypoacetylated initially at silencing boundaries has been shown in ***sas2***Δ cells, which show global reduction in all H3 and H4 lysine acetylation sites at sub-telomeric regions (Wang *et al.* 2013, Shia *et al.* 2006).

Sas2 has only recently become a HAT of interest when discussing transcriptional regulation. In yeast, approximately 80% of H4K16 is acetylated and marks euchromatin. The majority of H4K16 acetylation is performed by Sas2 (Kimura *et al.* 2002, Suka *et al.* 2002) while Esa1, a HAT contained in the NuA4 complex, can acetylate H4K16, but it mostly targets H4K5, 8, and12 (Chang and Pillus 2009, Suka *et al.* 2002, Suka *et al.* 2001, Grant *et al.* 1997). In fact, upon deletion of Sas2, H4K16ac is reduced significantly while Esa1 deletion mutants only show a slight decrease in H4K16ac. Double mutants exhibit ablation of H4K16ac in *Candida albicans*, suggesting that Sas2 and Esa1 are the only HATs responsible for H4K16ac (Wang *et al.* 2013). In higher eukaryotes, H4K16 is less ubiquitous and marks more specific regions of the genome. In male flies, histones on the X chromosome are highly acetylated at H4K16 (Turner *et al.* 1992), which is catalyzed by the HAT enzyme, MOF, and necessary for double the amount of transcription of male X chromosomal genes (Akhtar and Becker 2000, Hilkfiker *et al.* 1997). In humans, hMOF is also responsible for most H4K16ac (Smith *et al.* 2005) and is associated with the MLL complex, which is directed to gene promoters

where it catalyzes H3K4me, a mark commonly associated with active transcription (Dou *et al.* 2005).

As discussed previously, the modification of histone N-terminal tails can disrupt internucleosomal, intranucleosomal, and histone-DNA interactions, which changes the structure of chromatin. The H4 tail sits between an acidic H2A/H2B patch of the adjacent nucleosome (Figure 1-5) (Shogren-Knaak and Peterson 2006, Luger *et al.* 1997), and when modified, alters intra- and intermolecular compaction of chromatin (Dorigo *et al.* 2003). Acetylation of H4K16 would abolish the ionic interaction between the positive charge of K16 and the negative charged acidic patch. Indeed, acetylation of H4K16 disrupts chromatin compaction comparably to loss of the H4 tail (Shogren-Knaak *et al.* 2006).

Although H4K16ac has been linked to changes in chromatin structure, how H4K16ac functions in transcriptional regulation is not clearly understood. Generally, histone lysine acetylation promotes recruitment of activating complexes such as SAGA and the SWI/SNF ATP-dependent remodeling complex via subunits that contain bromodomains, which results in further acetylation, remodeling, and transcription (Hassan *et al.* 2002, Grant *et al.* 1998). In contrast, H4K16ac seems to destabilize the binding of chromatin associated proteins. Fruit fly SWI/SNF, ISWI, binds the H4 tail residues at and around H4K16 but once H4K16 is acetylated, the affinity of ISWI for H4 tail is disrupted (Clapier *et al.* 2001). Moreover, H4K16ac also reduces octamer mobilization by ISWI (Shogren-Knaak *et al.* 2006) suggesting that although chromatin is opened and made more accessible by H4K16ac, its rigidity may be increased.

Figure 1-5

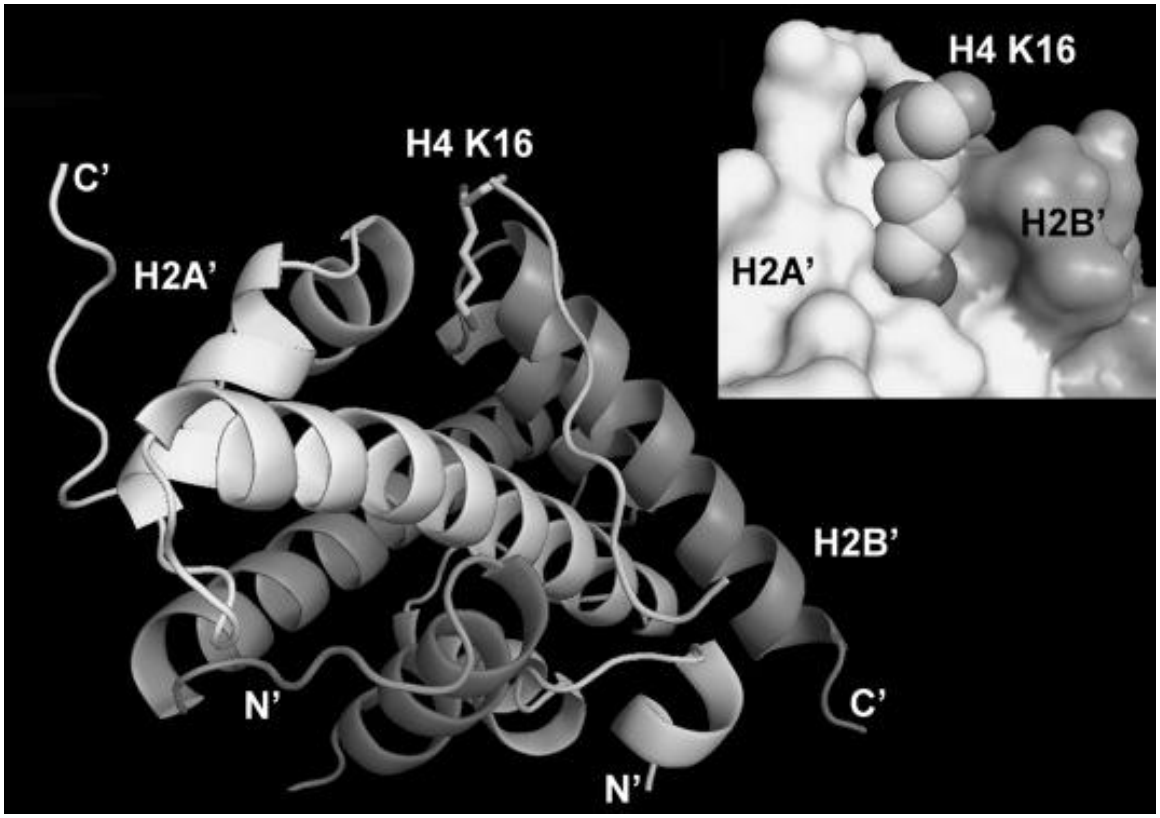


Figure 1-5: H4 tail interaction with H2A/H2B interface. Histones H2A (light grey) and H2B (dark grey) interact with residues 16-24 of H4 tail (grey) of an adjacent mononucleosome. The inset shows H4K16 sitting in an acidic pocket made by H2A/H2B. Adapted from (Shogren-Knaak and Peterson 2006).

Histone Deactylation

Overview

Histone deacetylases (HDACs) reverse the reaction carried out by HATs by removing acetyl marks on lysine to restore the positive charge. They fall into four classes: Class I (HDAC1, 2, 3, 8, Rpd3), II (HDAC4, 5, 6, 7, 9, 10, Hda1), III or NAD^+-dependent HDAC (Sir2 and other sirtuins), and class IV, which contains one member, HDAC11 (Yang and Set 2007).

In yeast, Rpd3, Sin3 and Ume1 form the core of the Sin3 complex, which is conserved from yeast to mammals (Yang and Seto 2008). Two functionally distinct complexes, Rpd3L and Rpd3S, have been characterized and both play a role in transcriptional regulation (Kremer and Gross 2009). Rpd3L is contained in the larger HDAC complex and is required for the heat stress response (Ruiz-Roig *et al.* 2010). It has also been implicated in Sir2-mediated silencing (Zhou *et al.* 2009) and replication origin firing (Knott *et al.* 2009). Rpd3S interacts with Set2-methylated histones and may contribute to transcription elongation and start site selection (Keough *et al.* 2005, Carrozza *et al.* 2005, Li *et al.* 2009). *Rpd3Δ* cells show an increase in telomeric repression of transcription (Rundlett *et al.* 1996) and exhibit an extended life span (Kim *et al.* 1999).

In humans, HDAC1 and 2 are found in the mammalian complexes Sin3A/B, NuRD, and corepressor for RE1 silencing transcription factor/neural-restrictive silencing factor (CoREST) while HDAC3 is found in nuclear receptor corepressor/silencing mediator for retinoid and thyroid hormone receptors (N-CoR/SMRT) (Yang and Seto 2008). Some of these corepressor complexes contain methyl-lysine binders that help target complexes to specific sites on chromatin. For instance, a subunit of the Sin3a complex, ING2, contains a PHD finger domain that binds H3K4me3 (Champagne and

Kutateladze 2009) in response to DNA damage. Once Sin3a is recruited, HDAC1 activity is stimulated, which stabilizes nucleosomes resulting in the repression of cell proliferation genes as a response to genotoxic events (Shi *et al.* 2006).

The class II HDAC, Hda1, shows sequence similarity to Rpd3 but is contained different complexes (Carmen *et al. 1996,* Rundlett *et al.* 1996). Both Rpd3 and Hda1 regulate transcription of two different set of genes with some overlapping sets (Suka *et al.* 1998, Rundlett *et al.* 1996). Yeast HDACs Hos1, 2, and 3 share sequence similarities to both Rpd3 and Hda1 but are less characterized.

SIR Complex

The silent information regulator (SIR) family of protein deacetylases named Sirtuins was discovered using a mating type assay in *S. cerevisiae*, wherein four *SIR* genes, *SIR1, 2, 3,* and *4* were deemed necessary for proper silencing of the mating type homothallic left (*HML)α* and homothallic right (*HMR)***a** loci (Rine and Herskowitz 1987). Under normal circumstances, haploid yeast cells express one locus and exhibit an α or **a**-mating phenotype. If both loci are expressed, the cells are considered diploid and sterile (Haber 1998). In the case of *sir2*Δ haploid cells, both loci were expressed and resulted in sterile haploids due to loss of silencing at flanking regions named silencers *E* or *I* (Rine and Herskowitz 1987). Moreover, this silencing defect was found at telomeres and was termed telomeric position effect (TPE). Using growth assays with strategically placed *ADE2* and *URA3* genes adjacent to the telomere, derepression of the genes was noticed in *sir2, 3,* and *4*Δ strains. Conclusions were drawn that, as in the previous mating assays, loss of silencing was occurring in both cases. Also, it was discovered that *SIR1* was not necessary to maintain silencing (Aparicio *et al.* 1991). Sir1 is necessary for establishment of silencing but not for maintenance. *Sir1*Δ cells contained

a mixed population of 20% silenced and 80% unsilenced cells and once cells were silenced, this phenotype was inherited stably through many generations of daughter cells (Pillus and Rine 1989).

The SIR complex is an HDAC complex that contains Sir2, Sir3, and Sir4, which catalyzes the deactylation of H4K16ac (Smith *et al.* 2002). SIR complex is recruited to silenced regions of chromatin using three regions of silencer sequences named *ARS, RAP1,* and *ORC*. Sir4 is recruited through its interactions with Rap1 and Sir1. Sir2 is recruited in a Sir4-Sir2 complex, which then recruits Sir3 (Rusche *et al.* 2002). Sir3 also binds Rap1 and possibly Abf1. This hierarchical formation of the SIR complex at the telomeres is the same except the major silencer region is composed of telomeric repeat, *RAP1*, which is bound by Rap1 and utilized though the binding of Sir4 with proteins Rap1 and Hdf1 (yKu80p) (Rusche *et al.* 2003).

H4K16ac has been shown to promote the binding of Sir2-Sir4 heterodimer but inhibits Sir3 binding (Oppikofer *et al.* 2011). Moreover, Sir4 is necessary for the stabilization of Sir2, which stimulates Sir2 HDAC activity (Martino *et al.* 2009). Once Sir2 deacetylates H4K16, Sir3 binds and forms a Sir2-3-4 holocomplex. Repeated rounds of deacetylation cause the release of *O*-AADPR, which facilitates Sir3 recruitment and SIR complex spreading (Oppikofer *et al*. 2011). In support of this mechanism, Sir2 was shown not to be necessary for its recruitment but necessary for spreading of the SIR complex (Buck *et al.* 2004). Also, combinatorial mutations in the tails of H3 and H4 have a profound derepressive effect on silencing. Specifically mutating H4K16 to alanine or glutamine abolishes silencing at telomeres and mating loci (Johnson *et al*., 1992).

Histone Methylation

Overview

Histone methylation is performed on the residues lysine and arginine by histone methyltranferase (HMT) enzymes. Lysines can be mono-, di-, and trimethylated while arginines can be mono- and symmetrically or asymmetrically dimethylated. There are over twenty sites of methylation that have been identified on the core histones. Given all the possible combinations of histone methylation, it is one of the most complex HPTMs to study in a static model. The modifications most relevant to transcriptional regulation have been listed in Table 1-1 and a few of the most studied histone methylations will be discussed in this section. Figure 1-4 summarizes the transcriptional effects and genomic enrichment of the HPTMs discussed below.

Lysine Methylation

H3K4

H3K4 methylation is usually enriched at the enhancers and promoters of actively transcribed genes (Wang *et al.* 2008; Santos-Rosa *et al.* 2002). H3K4me1 is highly enriched at enhancers (Wang *et al.* 2005). H3K4me2 is commonly found in the body of active genes while H3K4me3 is largely observed at the 5' ORF of genes (Pokholok *et al.* 2005). Methylation of H3K4 results from the recruitment of various H3K4 HMT enzymes by the transcriptional machinery, specifically RNAPII. Once RNAPII is poised for active transcription through phosphorylation of serine-5 of the carboxy-terminal domain (CTD) by TFIIH (Phatnani and Greenleaf 2006), the Set1 containing H3K4 HMT complex, COMPASS, is recruited by the PAF complex (Ng *et al.* 2003; Wood *et al.* 2003). RNAPII is released into an early elongating complex where H2BK120 (K123 in yeast) is ubiquitinated, which is required for further Set1 activity. Sometime during elongation,

RNAPII is phosphorylated at serine-2 resulting in the release of Set1 (reviewed in Martin and Zhang 2005).

Furthermore, PAF also interacts with chromodomain containing protein Chd1 (Simic *et al.* 2003). Proteins possessing methyl-binding domains, called chromodomains, are recruited to the H3K4me3 enriched promoter. SAGA also interacts with Chd1, which has two chromodomains, one which helps recruit SAGA to sites of H3K4me2/3 (Pray-Grant *et al.* 2005). As discussed earlier, SAGA recruitment results in an acetylation cascade that further promotes transcriptional activation. In humans, the HMT containing mixed-lineage-leukemia (MLL) complex is recruited by the H3K4me2 binding domain, WDR5. WDR5 interacts preferentially with H3K4me2 through its WD40-repeat domain (Wysocka *et al.* 2005). MLL can then convert H3K4me2 to H3K4me3.

H3K36

Unlike the 5' localization of H3K4 methylation, H3K36 methylation is highly enriched in the coding region and 3' ORF of genes. As mentioned in the previous section, once the CTD of RNAPII is phosphorylated at Serine-2 by Ctk1 and Bur1 kinases (Keogh *et al.* 2003; Qui *et al.* 2009), Set1 is released and chromatin is primed for transcriptional elongation through recruitment of Set2 (Xiao *et al.* 2003; Krogan *et al.* 2003). Set2 HMT catalyzes H3K36 methylation and specifically binds to phosphorylated Serine-2 of RNAPII's CTD (Hampsey and Reinberg 2003). This form of RNAPII is found in the transcribed regions of genes and the 3' end of genes, which correlates with H3K36me2/3 localization (Xiao *et al.* 2003; Krogan *et al.* 2003; Hampsey and Reinberg 2003; Li *et al.* 2003). The passage of RNAPII during transcriptional elongation results in histone displacement and positioning behind RNAPII. These histones are

hyperacetylated and subsequently methylated by Set2 (Hampsey and Reinberg 2003; Carrozza *et al.* 2005; Joshi and Struhl 2005; Keogh *et al.* 2005).

H3K36me2 is recognized by the chromodomain of Eaf3 and PHD finger of Rco1, which are subunits of the Rpd3S HDAC complex (Joshi and Struhl 2005; Govind *et al.* 2010). During transcriptional elongation, Rpd3S is recruited via the serine-2/serine-5-diphosphorylated CTD repeats followed by H3K36me2 binding by Eaf3 and Rco1 (Keogh *et al.* 2005, Govind *et al.* 2010). Once Eaf3 and Rci1 are recruited by H3K36me2, Rpd3 is transferred from the phosphorylated CTD to H3 where its HDAC activity creates a hypoacetylated environment within gene bodies and at the 3' end. (Li *et al.* 2007; Govind *et al.* 2010). Deletion of Rco1 or Eaf3 results in hyperacetylation of ORFs and the production of aberrant transcripts that are presumably initiated from cryptic promoters that are usually silenced by the Set2-Rpd3 pathway after RNAPII progression (Carrozza *et al.* 2005; Joshi and Struhl 2005; Keogh *et al.* 2005).

H3K79 and Dot1

Unlike the previously discussed HPTMs, H3K79 methylation occurs in the globular domain of H3 and within the core of the nucleosome. It is found within the coding regions of genes and is usually associated with active chromatin. H3K79 methylation is catalyzed by Dot1, a class I SAM dependent HMT that is conserved from yeast to humans (Sawada *et al.* 2004, Min *et al.* 2003).

DOT1 was found similarly to both *SIR* and *SAS* genes since overexpression and deletion of the gene leads to the loss of telomeric and *HM* silencing (Singer *et al.*, 1998). It was later discovered that Dot1 is responsible for all mono-, di-, and trimethylation of H3K79 in budding yeast and humans (van Leeuwen *et al.* 2002, Lacoste *et al.* 2002). Dot1 is the first lysine HMT that has been identified that's lacks an identifiable SET

domain (Feng *et al.* 2002). It is required to prevent encroachment of the SIR complex into active chromosomal regions (van Leeuwen *et al.* 2002) and recently, has also been shown to bind to actively transcribing RNAPII (Kim *et al.* 2012). H3K79me has also been shown to recruit mammalian protein 53BP1 tudor domain at sites of DNA damage (Huyen *et al.* 2004).

Human Dot1 (Dot1L) has been shown to be involved in both gene activation and gene repression, and its most notable role is its upregulation of *HOXA* cluster and *MEIS1* genes. *HOXA* overexpression results in defective hematopoiesis, Mixed Lineage Leukemia (MLL), and Acute Myelogenous Leukemia (AML) making regulation of H3K79 methylation a possible therapeutic target (Nguyen *et al.* 2011, Jo *et al.* 2011, Monroe *et al.* 2011). Dot1L is mistargeted to these genes due to *MLL* chromosomal translocations. MLL is a SET domain containing HMT contained in multicomponent complex that methylates H3K4, which is associated with transcriptionally active regions of chromatin (Tenney and Shilatifard 2005). In cases of acute myelogenous leukemia (AML), chromosomal translocations result in fusion proteins with the N-terminus of MLL fused most commonly with the C-terminus of AF6, 9, 10, or 17 (Aplan 2006). Dot1L has been shown to interact with AF10 and is mistargeted to *HOXA* genes via MLL-AF10 fusion proteins, causing aberrant expression of genes usually silenced during hematopoiesis (Okada *et al.* 2005). Inhibition of human Dot1L prevents leukemic transformation in cells containing MLL-AF10 and MLL-CALM rearrangements through the suppression of Dot1's ability to deregulate and overexpress genes targeted by MLL fusion proteins (Deshpande *et al.* 2013, Chen *et al.* 2012). Recently, two different groups (Deshpande *et al.* 2013, Chen *et al.* 2012) showed that leukemic transformation can be abrogated through Dot1 inhibition or transient deletion.

Dot1's methylation of H3K79 is also regulated by various other HPTMs. In yeast, Dot1 is required for proper telomeric silencing (Ng *et al.* 2003, van Leeuwen *et al.* 2002, Ng *et al.* 2002, van Leeuwen and Gottschling 2002, San Segundo and Roeder 2000, Xu *et al.* 1999, Singer *et al.* 1998) and competes with the bromo-associated homology (BAH) domain of Sir3 of the SIR complex for the same nucleosomal binding surface (Jean Armache *et al.* 2011, Altaf *et al.* 2007, Fingerman *et al.* 2007). Once H3K79 is methylated by Dot1, Sir3 cannot bind to nucleosomes which results in a failure to nucleate the SIR complex in order to form heterochromatin (Altaf *et al.* 2007). Dot1 also requires monoubiquitination of H2BK123 by the Rad6-Bre1 complex for efficient di- and trimethylation of H3K79 (Nakanishi *et al.* 2009, Briggs *et al.* 2002, Ng *et al.* 2002, Sun and Allis 2002).

Although the H4 tail is not required for Dot1 to bind to nucleosomes, Dot1 requires a basic patch of the H4 tail (residues 4-19) to efficiently di- and trimethylate H3K79 (Fingerman *et al.* 2007) and it methylates in a H4K16 acetylation-dependent fashion (Altaf *et al.* 2007). Sir3 outcompetes Dot1 for the same nucleosomal binding site as an increase in Sir3 depletes Dot1's ability to methylate (Altaf *et al.* 2007). Clearly, competition between Dot1 and Sir3 plays a role in the regulation of maintains proper heterochromatin-euchromatin boundaries. The role of Sas2 in this mechanism has not been studied but one might suspect that with the exclusion of Sir3 binding due to H4K16ac may result in the enhancement of Dot1 HMT activity (Oppikofer *et al.* 2011). Dot1 has not been shown to specifically interact with H4K16 nor has any study elucidated the effects that H4K16 acetylation may have on Dot1's activity.

H3K27

H3K27me does not exist in yeast. However, in mammals, H3K27 methylation serves as a repressive mark catalyzed by the Polycomb Repressor Complex 2 (PRC2), which contains the SET-domain containing lysine HMT, Enhancer of Zester 2 (EZH2). H3K27me3 serves as a repressive mark at homeotic genes, the inactive X-chromosome, and imprinted genes while H3K27me1 is enriched at pericentric heterochromatin (Martin and Zhang 2005). PRC2 is made up of four core components: EZH2, embryonic ectoderm development (EED), suppressor of zeste 12 homolog (SUZ12), and histone-binding protein retinoblastoma-binding protein p48/46 (RbAp48/46). Both EED and SUZ12 are necessary for EZH2 HMT activity (Simon and Kingston 2009). EED contains repeats of WD40 domains that bind H3K27me3 and promote PRC2 propagation (Margueron *et al.* 2009) and SUZ12 contains C_2-H_2 zinc finger and VEFS domain. RbAp48/46 contains six WD40 domains and is a core histone binding subunit.

PCR2 also interacts with AEBP2, PCLs and JARID2. AEBP2 contains three zinc-fingers that may play a role in DNA binding (Kim, Kang, and Kim 2009). PCL1, PCL2 and PCL3 (also known as PHF1, MTF2 and PHF19, respectively) contain a tudor domain and two PHD finger proteins, a PCL extended domain and a carboxy-terminal domain tail (Wang, Robertson, and Zhu 2004). PCL proteins interact with PRC2 through EZH2, and to some extent through SUZ12 and the histone chaperones RbAp46/48 (Nekrasov *et al.* 2007). JARID2 is the founding member of the Jumonji family of proteins that catalyses the demethylation of histone proteins. However, it lacks demethylase activity. JARID2 contains JmjC and JmjN domains and two potential DNA binding domains, ARID and a zinc finger (Margueron and Reinberg 2011). The core components of PRC2 and its associated proteins discussed above are all necessary for EZH2 optimal function.

The targeting of PRC2 in *D. Melanogaster* is a well understood mechanism compared to humans. In *D. Melanogaster*, transcription factors, such as Pho and PhoL, bind to the Polycomb responsive element and recruit EZ of PRC2. Only now is the mammalian mechanism coming to light with the recent discovery of long non-coding RNA (lncRNA) dependent PRC2 recruitment. The lncRNA, *HOTAIR*, is transcribed from the HOXC locus, binds PRC2, and targets the complex to the HOXD locus where several genes are repressed (Rinn *et al.*, 2007). Also, the lncRNA *Xist* and a short internal transcript *RepA* have been to shown target PRC2 to the inactivated female X-chromosome, which subsequently is repressed and enriched with H3K27me3. In contrast the lncRNA and antagonist to *Xist*, *Tsix*, also interacts with PRC2 suggesting an inhibitory mechanism to X-chromosome inactivation (Zhao *et al.* 2008).

H3K9

H3K9 methylation is one of the most intensely studied histone modifications to date. H3K9me1 is catalyzed by methyltransferases HMT1C/G9a or demethylases KDM3A/JMJD1A and KDM4D/JMJD2D (Shi and Whetstine 2007). The mark is enriched at the 5' UTR and found minimally in non-genic regions (Barski *et al.* 2007; Rosenfeld *et al.* 2009). Although no function has been ascribed to H3K9me1, its proposed mechanism of action may be to act as an intermediary between gene activation and repression through rapid methylation or demethylation (Black and Whetstine 2011). Most studies have focused on H3K9me2/3 as a heterochromatin mark catalyzed by the lysine HMT SUV39H1/2 and recognized by the chromodomain of heterochromatin protein-1 (HP1), which dictates the compaction of heterochromatin.

Futhermore, H3K9me2/3 is enriched in pericentromeric, subtelomeric, and gene desert regions. Gene deserts are megabase sized regions devoid of coding genes, and

unlike H3K9me3, H3K9me2 is rarely found in at individual active or silenced genes (Rosenfeld *et al* 2009). In support of H3K9me2's function as a repressive mark, it has been shown to associate with Lamin B1, a protein localized to the nuclear periphery and part of the nuclear lamina, which is commonly associated with inactive genes. Lamin B1 associated regions are also devoid of the activating mark, H3K4me3, and RNAPII further suggesting H3K9me2 is most likely a repressive mark that facilitates separation of active and inactive genes through chromosomal localization within the nuclear architecture (Guelen *et al.* 2008).

H3K9me3 is commonly found at heterochromatin and repressed promoters, and unlike H3K9me2, H3K9me3 is also localized to centromeres, subtelomeric regions, and in some cases, the coding region of genes (Vakoc *et al.* 2007; Mikkelsen *et al.* 2007). H3K9me3 is usually associated with H3K20me3 at heterochromatic locations such as pericentromeric chromatin, but this bivalent mark is absent at subtelomeric regions and gene deserts suggesting different silencing mechanisms at these different heterochromatic regions (Rosenfeld *et al.* 2009). In addition to its heterochromatin formation function, H3K9me2/3 is implicated in the silencing of euchromatic genes. RB and KAP1 corepressor complexes recruit lysine HMTs SUV39H1 and ESET/SETDB1 respectively to promoters of active genes. HP1 is recruited to sites of H3K9 methylation but is restricted to the promoter region of genes and does not spread (Kouzarides and Berger 2007). The role of H3K9me3 in the coding region of genes has not been elucidated, but enrichment of H3K9me3 at the 3' ORF increases and co-localizes with the elongating form of RNAPII during active transcription. Moreover, despite the accepted dogma that HP1 is thought to always be repressive, a γ-isoform of HP1 has been found to also be enriched in the coding regions of active genes (Vakoc *et al.* 2005). During transcriptional activation, promoter repression by HP1β is replaced by HP1γ,

which seems to facilitate RNAPII processivity through the coding region of the gene in addition to an increase in H3K9me3 (Matteescu *et al.* 2008).

H3K20

In addition to H3K9me2/3, H4K20me3 is also indicative of silenced chromatin. H4K20 methylation is catalyzed by two SET-domain containing lysine HMTs, SUV4-20H1 and SUV4-20H2. Interestingly, both of these HMTs have been shown to interact with the repressive HP1 isoforms, α and β, indicating a possible upstream function for H3K9 methylation and subsequent H4K20 methylation (Schotta *et al.* 2004). This idea is further illustrated by the dual enrichment of H3K9me3 and H4K20me3 at constitutively repressed regions such as transposons, satellite and long terminal repeats (LTRs), and pericentromeric chromatin, a region rich with repetitive satellite elements and interspersed with long and short interspersed nuclear elements (LINEs and SINEs). As discussed in the previous section, gene deserts are enriched with H3K9me2/3 but not H4K20me3. Interestingly, neither mark is found at telomeric and subtelomeric regions, which suggests a different mechanism of repression mediates constitutive heterochromatin at telomeres (Rosenfeld *et al.* 2009).

In contrast to H4K20me3, H4K20me1 is associated with highly expressed genes and is enriched at the 5' coding region along with H2BK5me1, H3K4me1/2/3, H3K9me1, H3K27me1, and H3K79me1/2/3 (Wang *et al.* 2008). As previously discussed, H3K36me3 is located at the 3' end of the coding region and marks transcriptionally active genes. Studies have shown that H4K20me1, H3K36me3, and H3K79me1/2/3 facilitate transcriptional elongation as all three marks fluctuate in a similar temporal manner during gene activation and subsequent transcription (Vakoc *et al.* 2006). H4K20me2 also seems to be required for checkpoint function and cell survival after DNA

damage through the recruitment of Tudor-domain containing protein Crb2 (Greeson *et al.* 2008).

Arginine Methylation

To date, nine protein arginine methyltransferases (PRMT) have been found in mammals and only four have been discovered in yeast (Hmt1/Rmt1, Rmt2 and Hsl7). Hmt1 and Hsl7 are homologous to human PRMT1 and 5 respectively. Arginine can be methylated in three different ways but these orientations from lysine methylation. They include monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginines (SDMA). PRMTs are classified as either type I, type II, or type III enzymes while type IV PRMT activity catalyzes monomethylation of the internal guanidino nitrogen and has only been described in yeast. Type I and type II enzymes catalyze the formation of an MMA intermediate. Type I PRMTs (PRMT1, 2, 3, 4, 6, 8, and Rmt1) further catalyze the production of ADMA, while type II PRMTs (PRMT5, 7, and Hsl7) catalyze the formation of SDMA. Type III enzymatic activity includes monomethylation by PRMT7. Type IV (Rmt2) activity catalyzes MMA of the internal guanidino nitrogen (Niewmierzycka and Clarke 1999). Rmt2 methylates ribosomal protein Rpl12 (Chern *et al.* 2002). Histone arginine methylation is a relatively new topic of interest in the realm of HPTM research, thus many methylarginine marks have been identified but the corresponding enzymes that catalyze the marks have not been discovered.

H3R2

H3R2 methylation is highly conserved from yeast to humans (Guccione *et al.* 2007, Kirmizis *et al.* 2007). In humans, H3R2me2a mark is catalyzed mainly by PRMT6

(Guccione *et al.* 2007) but no homolog or culprit has been found in yeast since deletion of Hmt1, Hsl7, and Rmt2 does not affect levels of H3R2me2a (Kirmizis *et al.* 2007). Based on ChIP-seq results, H3R2me2a mark is enriched at pericentromeric regions, and H3R2me1 mark is enriched at subtelomeric regions (Rosenfeld *et al.* 2009). H3R2me2a mark also results in transcriptional repression since it antagonizes trimethylation of H3K4 by Set1/MLL in the COMPASS/MLL complex. As discussed previously, H3K4me3 is a mark that is mostly associated with transcriptional activation through recruitment of activating complexes (Iberg *et al.* 2008, Kirmizis *et al.* 2007, Guccione *et al.* 2007).

H3R2me2s and H3R2me1, however, have the opposite effect as compared to H3R2me2a. In humans, PRMT5 and 7 catalyze the production of H3R2me2s at euchromatic regions and regions marked with H3K4me3 while Set1 catalyzes the same mark is yeast (Yuan *et al.* 2012). H3R2me2s inhibits binding of RBBP7, the targeting subunit for Sin3a co-repressor complex while promoting binding of WDR5, a subunit found in various coactivator complexes (Migliori *et al.* 2012). Like H3R2me2s, H3R2me1 does not inhibit H3K4me, is localized to the CDS of genes, and has been linked to increased transcription (Kirmizis *et al.* 2009). The PRMT responsible for MMA at this site has not been found in yeast or humans.

H4R3

Hmt1/Rmt1 is homologous to human PRMT1 (Gary 1996) and like Hsl7, is highly conserved from yeast to humans (Krause *et al.* 2007, Bachand 2007). Both PRMT1 and Rmt1 catalyze asymetrical dimethylation of H4R3 (H4R3me2a) (Kuo *et al.* 2009, Lacoste *et al.* 2002, Strahl *et al.* 2001 Wang *et al.* 2001). However, H4R3me2a serves two different functions in each organism. In mammals, PRMT1 serves as a transcriptional activator (Wang *et al.*2001 Strahl *et al.* 2001) whereas in yeast, Rmt1 acts a repressor

(Kuo *et al.* 2009). PRMT1 is recruited to promoters during the first cycle of transcription, which results in subsequent H3 and H4 acetylation (Li *et al.* 2010, Huang *et al.* 2005, Metivier *et al.* 2003). Also, mass spectrometric analysis of H4 modifications showed that less than 2% of H4R3 is asymmetrically/symmetrically methylated (Pesavento *et al.* 2008) and is usually found in combination with highly acetylated chromatin and H4K20me2 mark (Phanstiel *et al.* 2008).

Similar to the mammalian system, Rmt1 also preferentially binds and methylates H4R3 on acetylated histones (Kuo 2009). However, unlike PRMT1, Rmt1 facilitates the formation of silent chromatin through recruitment of Sir2 (Yu *et al.* 2006). When Rmt1 is overexpressed, histone acetylation decreases and vice versa (Yu *et al.* 2006). The SIR complex will be covered in a later section.

There is debate over whether H4R3me2s make is an activating or repressive mark. Keji Zhao's study of the human methylome suggested that H4R3me2s was not associated with transcriptional repression (Barski *et al.* 2007) whereas under a different method of analysis by Stefan Bekiranov's group showed that the mark is strongly associated with repression (Xu *et al.* 2010). Since H4R3me2a acts as an activating mark, H4R3me2s could act as an "off" switch for transcription, which would allow for transient regulation of gene expression (Di Lorenzo and Bedford 2011).

Histone Demethylation

Reversal of histone methylation was thought to be impossible due to the stable nature of the modification until the discovery of lysine-specific demethylase 1 (LSD1). LSD1 is a FAD dependent amine oxidase that catalyzes lysine demethylation and releases the product hydrogen peroxide (Shi *et al.* 2004). Protein arginine deiminase 4 (PADI4) converts methyl-arginine to citrulline rather than an unmodified arginine. PADI4

does not complete full demethylation and therefore requires processing by histone replacement or aminotransferases for complete arginine demethylation (Bannister et al. 2002). Lastly, the JumonjiC-domain containing histone demthylases (JHDMs) are Fe^{2+} and α-ketoglutarate dependent histone demethylases that release the product formaldehyde (Tsukada *et al.* 2006). Specifics about individual enzymes, mechanisms, specificity, and transcriptional activity can be found in Table 1-2.

Table 1-2: Histone demethylases

Enzymatic Family	Subfamily	Enzymes	Specific residue activity	Transcriptional Activity	References
PADI		PAD4	H3R2me1 H3R8me1 H3R17me1 H3R26me1 H4R3me1	Derepressors	Bannister *et al.* 2002; Wang *et al.* 2004; Cuthbert *et al.* 2004
Amine oxidase	LSD1		H3K4me1/2 H3K9me1/2	Repressors: CoREST, NuRD Activator: AR/ERα	Lee *et al.* 2005; Shi *et al.* 2005; Wang *et al.* 2009; Metzger *et al.* 2005; Garcia-Bassets *et al.* 2007
JmjC	JHDM1	JHDM1A JHDM1B	H3K36me1/2		Tsukada *et al.* 2006
	JHDM3/JMJD2	JMJD2/JHDM3A JMJD2B JMJD2C/GASC1 JMJD2D	H3K9me2/3 H3K36me2/3		Whetstine *et al.* 2006; Klose *et al.* 2006; Cloos *et al.* 2006; Fodor *et al.* 2006;
	JARID	JARID1A JARID1B JARID1C JARID1D	H3K4me2/3	Repressor of growth inhibitors	Iwase *et al.* 2007; Klose *et al*. 2007; Lee *et al*. 2007; Yamane *et al*. 2007
	UTX/UTY	JMJD3 UTX	H3K27me2/3	Activator: MLL	Agger *et al.* 2007; Issaeva *et al.* 2007
	JHDM2	JHDM2A JHDM2B JHDM2C	H3K9me1/2	Activator: AR	Yamane *et al.* 2006

Acronyms: Peptidyl arginine deiminase (PADI), Lysine specific demethylase (LSD), Jumonji C (JmjC), JmjC-domain-containing histone demethylase (JHDM), Androgen receptor (AR), Estrogen receptor (ER), Corepressor for RE1 silencing transcription factor/neural-restrictive silencing factor (CoREST), Nucleosome remodelling and histone deacetylase (NuRD)

Histone Proteolysis

In addition to demethylation and deactylation, previous reports of H3 N-terminal tail proteolytic cleavage have also been described as a mechanism that facilitates the removal of HPTMs (Allis *et al.* 1980). Recently, H3 tail cleavage by Cathepsin L has been linked to transcriptional activation and induction of differentiation in embryonic stem cells. N-terminal tail cleavage is also regulated by the HPTMs present on the tail (Duncan *et al.* 2008). Studies have shown (Santos-Rosa *et al.* 2009) that cleavage is inhibited by the activation mark H3K4me3 and facilitated by the repressive mark H3R2me2 suggesting that tail clipping is a rapid way to void promoters of repressive marks and complexes during the regulation of gene expression. Moreover, tail clipping directly precedes histone eviction at promoters, which provides strong evidence that H3 tail cleavage is a gene activating event (Santos-Rosa *et al.* 2009). A major challenge in the chromatin field remains in understanding how patterns of modifications are generated and interpreted by nuclear machinery.

Histone Crosstalk

Given all the histone modifications discussed in the previous sections, regulation of chromatin structure and transcriptional activity can be tightly controlled through the usage of combinatorial modifications. Histone modifications can affect the stimulation or inhibition of multiple cellular processes, which subsequently affects the capacity for the creation or erasure of other HPTMs (Figure 1-6). Some modifications can inhibit the target of other modifications as seen with H3K27, which can be exclusively methylated or acetylated. Modifications can also prevent the binding of certain effector proteins as is the case with the inhibition of HP1’s targeting to H3K9me2/3 by H3S10 phosphorylation (Fischle *et al.* 2005). Some marks can also facilitate the binding of

effector proteins that in turn perform other modifications. As mentioned above, ING2 contains a PHD finger domain that binds H3K4me3 (Champagne and Kutateladze 2009) in response to DNA damage. Once Sin3a is recruited, HDAC1 activity is stimulated to deacetylate histones and reduce transcriptional activity of genes that promote cell growth and division (Shi *et al.* 2006).

Various modifications are also dependent on one another. For example, H2B120/123 ubiquitination is necessary for H3K4 and H3K79 methylation in both yeast and humans (Lee *et al.* 2007, Kim *et al.* 2009). Both H3K79 and H3K4 methylation show similar patterns of enrichment on chromatin and correlate with transcriptional activation (Steger *et al.* 2008). Moreover, H3K79 methylation also inhibits the SIR complex from binding via nucleation factor, Sir3. Although H4K16 can still be deacetylated by the Sir2/Sir4 heterodimer, it cannot facilitate heterochromatin formation due to loss of Sir3 binding. As discussed previously, Sas2 and Dot1 catalyze H4K16ac and H3K79me, respectively. To date, the only link between these two enzymes in yeast is that both play a role in the inhibition of SIR complex nucleation and the formation of heterochromatin.

In this dissertation, a new link will be made between H4K16 and H3K79. Results indicate that H4K16 acetylation by the SAS-I HAT complex is necessary for H3K79 trimethylation. As opposed to previous research that has linked these two modifications to telomeric silencing, the effect of H4K16ac on H3K79me3 is most prominent in gene bodies, suggesting that SAS-I regulation of Dot1 activity may also be involved in transcriptional regulation. The role of Dot1 in transcriptional regulation is further described by its binding to histone peptides bearing marks involved in transcriptional activation at transcriptional start sites (TSS). In both yeast and humans, H4K16ac is localized to the TSS (Heise *et al.* 2012, Wang *et al.* 2008), and excluding its function in chromatin decondensation, the role of H4K16ac in transcriptional regulation has not

been elucidated. Data and discussions provided in this study show that H4K16 acetylation regulates H3K79 methylation by Dot1, which is achieved through changes in internuclesomal interactions, subsequent chromatin decondensation, and possible allosteric stimulation of Dot1 by H4K16ac.

Figure 1-6

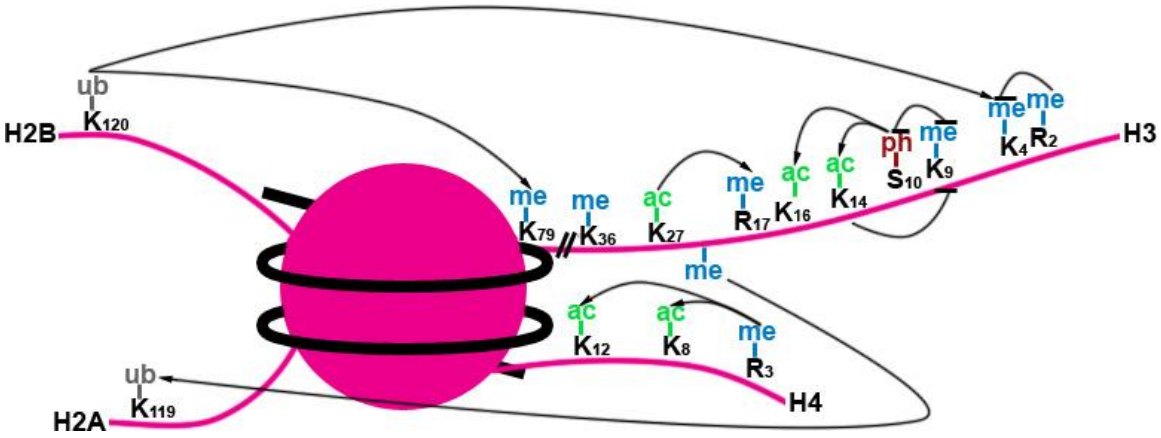


Figure 1-6: Histone modification crosstalk. Various post-translational histone modifications affect the binding of certain domains and catalysis of other HPTMs. Arrows indicate a positive effect and bars indicate inhibitory effects on other HPTMs.

CHAPTER 2: SAS-I ACETYLATION OF H4K16 IS NECESSARY FOR CATALYSIS OF H3K79 TRIMETHYLATION BY DOT1

Introduction

Dot1 and its regulation

Histones are subject to a plethora of modifications that have been linked to the regulation of various cell processes, including transcription. Most documented modifications are added to lysines (K) on the N-terminal tails of histone proteins but a few are situated in the nucleosome core. A highly conserved residue, lysine 79 on histone H3 (H3K79), is located in the core and at the surface of the nucleosome (Lu *et al.* 2008) and can be mono-, di-, and trimethylated (H3K79me1, 2, 3) by the histone methyltransferase (HMT) Dot1 (Lacoste *et al.* 2002, Ng *et al.* 2002, van Leeuwen *et al.* 2002). Dot1 is a non-SET domain containing, class I SAM-dependent methylase that is conserved from yeast to humans (Sawada *et al.* 2004, Min *et al.* 2003). It methylates H3K79 in a distributive manner (Fredericks *et al.* 2008) and preferentially methylates nucleosomes as opposed to core histones (Feng *et al.* 2002).

In yeast, Dot1 is required for proper telomeric silencing (Ng *et al.* 2003, van Leeuwen F *et al.* 2002, Ng *et al.* 2002, van Leeuwen and Gottschling 2002, San Segundo and Roeder 2000, Xu *et al.* 1999, Singer *et al.* 1998) and competes with the BAH domain of Sir3 of the SIR complex for the same nucleosomal binding surface (Jean Armache *et al.* 2011, Altaf *et al.* 2007, Fingerman *et al.* 2007). Once H3K79 is methylated by Dot1, Sir3 cannot bind to nucleosomes, which results in a failure to nucleate the SIR complex in order to form heterochromatin (Altaf *et al.* 2007). Dot1's methylation of H3K79 is also regulated by various other HPTMs. Dot1 requires monoubiquitination of H2BK123 by the Rad6-Bre1 complex for efficient di- and trimethylation of H3K79 (Nakanishi *et al.* 2009, Briggs *et al.* 2002, Ng *et al.* 2002, Sun and Allis 2002).

In addition to preventing chromatin silencing, Dot1 has also been linked to transcriptional regulation and elongation. In humans, Dot1L is contained in various activating complexes along with pTEF-B, a kinase responsible for Serine-2 phosphorylation on the CTD of RNAPII (Bitoun *et al.* 2007, Mueller *et al.* 2007). However, little is known about what role the three states of methylation play in transcriptional regulation and presently, conflicting results from completed studies have yet to be resolved.

Localization of H3K79 on the genome

A recent analysis of genome wide data (Wang *et al.* 2008) for combinatorial histone marks in humans did show that all three H3K79me states along with H2AK9ac, H4K16ac and H4K12ac recurred more frequently in combination with other marks in comparison to the marks' frequencies when analyzed alone (Linghu *et al.* 2013). In previous studies completed in humans, H3K79me1 was shown to be localized at the bodies of both activated and repressed genes with a slight preference towards activated genes (Barski *et al.*2007), and in later studies using a different ChIP approach (Wang *et al.* 2008, Steger *et al.* 2008), H3K79me1 was correlated with highly expressed genes. H3K79me2 has also been linked to transcriptional activation in humans (Okada *et al.* 2005, Steger *et al.* 2008), especially in the context of Dot1L's upregulation in leukemogenesis and dysregulation of *HOXA* genes (Nguyen *et al.* 2011, Jo *et al.* 2011, Monroe *et al.* 2011). A later study completed in yeast showed that both H3K79me2 and me3 were localized across the CDS of genes and decreased with genes' increasing transcriptional frequency (Schulze *et al.* 2011). The same study (Schulze *et al.* 2011) showed H3K79me2 and me3 marked shorter and longer genes, respectively.

Different functions for H3K79me2 and 3 have been discovered. Unlike H3K79me3, H3K79me2 has been linked to the cell cycle and peaks at G2/M phase. Both marks seem to be localized to mutually exclusive regions of the genome and associated with genes that tend to be less transcriptionally active. Di- but not trimethylation seems to be dependent on Swi4 and Swi6, subunits of the SBF complex. In contrast, tri- but not dimethylation colocalizes with H2BK123ub across the genome (Schulze *et al.* 2009). Ubp8 and Ubp10 deubiquitinate H2BK123. Upon Ubp10 deletion, H3K79me3 profiles similarly increase to that of H2BK123ub whereas H3K4me3 patterns increase similarly to H2Bub when Ubp8 is deleted (Schulze *et al.* 2011). The H2BK123ub link to H3K79me3 is further confirmed by the absence of H2BK123ub at genes that are cell cycle regulated (Schulze *et al.* 2011).

Ubiquitination of H2B promotes Dot1 function

H2BK123ub is localized at actively transcribed genes in concert with H3K4 methylation (Henry *et al.* 2003, Kao *et al.* 2004) and is also necessary for di- and trimethylation of H3K4 (Nakanishi *et al.* 2009). However, despite the loss of H3K79me2 and H3K79me3 upon Bre1 deletion or H2BK123 mutation, loss of Dot1 recruitment at activated genes is not observed (Shahbazian *et al.* 2005). Without the presence of other effector proteins, human Dot1L has been shown to perform methylation more efficiently on chemically engineered nucleosomes containing H2BK120ub in comparison to nucleosomes that are not ubiquitinated (Chatterjee *et al.* 2010, McGinty *et al.* 2008). Also, Dot1 can still dimethylate H3K79 in the absence of H2BK123ub, arguing in favor of a model where H2Bub causes a conformation change in the nucleosome, thus making H3K79 more accessible to Dot1 (Shahbazian *et al.* 2005, Fierz *et al.* 2011).

Histone H4 tail regulates Dot1 function

In addition to Dot1 activity being regulated by H2BK123ub, the H4 tail has also been implicated in the regulation of Dot1 activity (Fingerman *et al.* 2007, Altaf *et al.* 2007). Although the H4 tail is not required for Dot1 to bind to nucleosomes, Dot1 requires a basic patch of the H4 tail (residues 4-19) to efficiently di- and trimethylate H3 (Fingerman *et al.* 2007). Dot1 also methylates in an H4K16 acetylation-dependent fashion (Altaf *et al.* 2007). The HAT Sas2 is responsible for acetylating H4K16 in yeast but its role in regulating Dot1 HMT activity during transcription and anti-silencing has not been elucidated. An H4K16 HDAC complex subunit, Sir3, contains a BAH domain that has been shown to bind to the same nucleosomal binding surface as Dot1 (Onishi *et al.* 2007, Norris and Boeke 2010, Armache *et al.* 2011) with suggested greater affinity since an increase in Sir3 depletes Dot1's ability to methylate (Altaf *et al.* 2007). Dot1 has not been shown to specifically interact with H4K16 nor has any study elucidated the direct effects that H4K16 acetylation may have on Dot1's activity. Chapter 2 shows that H4K16ac is necessary for Dot1 to catalyze the transition from H3K79me2 to H3K79me3. We believe that this effect is due to structural changes in the nucleosome induced by the acetylation of H4K16 as *in vitro* results suggest that Dot1 HMT activity is not directly stimulated by H4K16ac.

Results

H2B Deubiquitinase, Ubp10, Regulates H3K79 Methylation and H4K16 Acetylation

Dot1 requires Bre1 and may require H2BK123ub in order to trimethylate H3K79 (Nakanishi *et al.* 2009, Briggs *et al.* 2002, Ng *et al.* 2002, Sun and Allis 2002). Dot1 HMT activity is also dependent on interaction with the H4 N-terminal tail and is enhanced upon H4K16 acetylation (Altaf *et al.* 2007). H3K79, H4K16, and H2BK123 are localized to the same surface of the nucleosome (Figure 2-8) (Armache *et al.* 2011, Turner 2008, Luger *et al.* 2007). This surface is bound by both Dot1 and Sir3-BAH domain (Armache *et al.* 2011). A link between all three marks in the regulation of one another has yet to be elucidated. Since Bre1, Ubp8, and Ubp10 have already been linked to the regulation of H3K79me (Schulze *et al.* 2011, Schulze *et al.* 2009, Wood *et al.* 2003), we decided to study the effects of each enzyme on H4K16ac and H3K79me.

In order to study the effects of Bre1-Rad6, Ubp8, and Ubp10 on the H3K79me and H4K16ac marks, histones from wild-type, *bre1Δ*, *ubp8*Δ, and *ubp10Δ* mutant cells were analyzed by immunoblotting for H3K79me2, H3K79me3, and H4K16ac (Figure 2-1). Based on previous studies (Schulze e*t al.* 2011), we expected H3K79me2/3 to increase in *ubp10Δ* cells but not *ubp8Δ* cells. As shown in figure 2-1, deletion of Ubp10 but not Ubp8 led to increased amounts of H3K79me2/3. In addition, H4K16ac is greatly increased in *ubp10Δ* but not *ubp8Δ* cells. As expected, *bre1Δ* cells lack both H3K79me2 and me3 marks, but H4K16ac is also minimally decreased suggesting that H2BK123ub may have an effect on acetylation of H4K16 at a diminutive amount of gene loci.

Figure 2-1

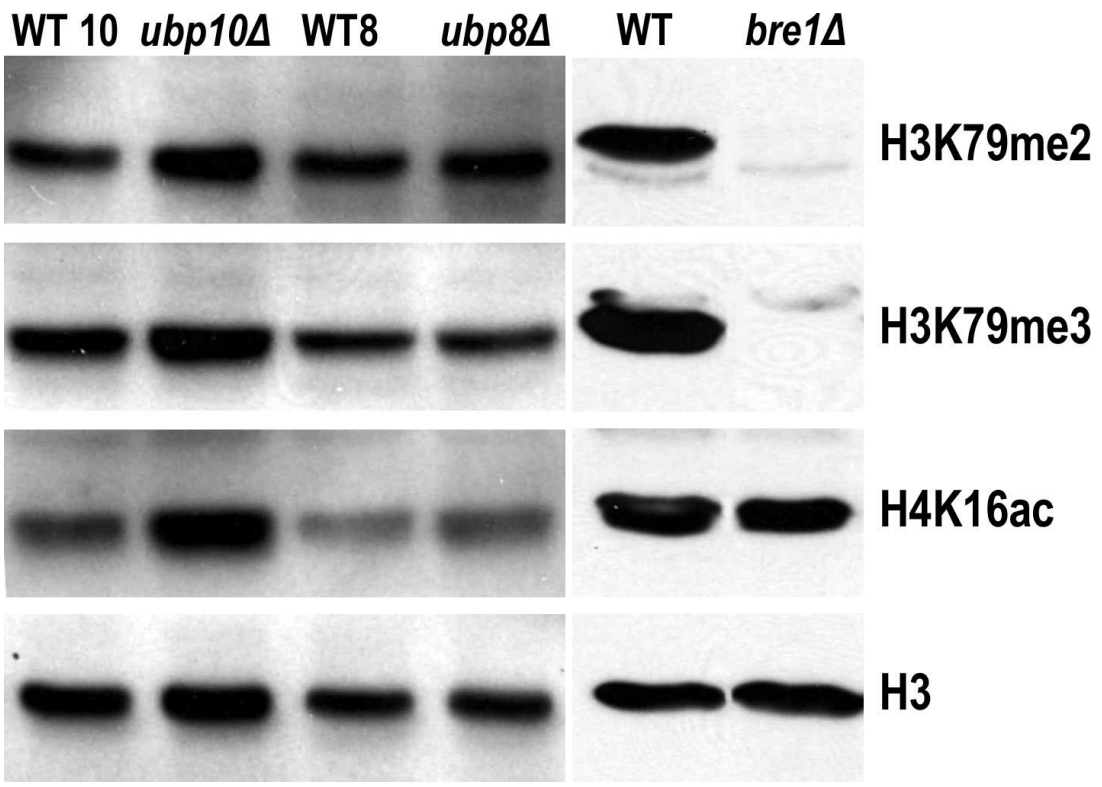


Figure 2-1: H3K79me and H4K16ac increase upon deletion of Ubp10 but not Ubp8. Acid extracted histones from whole cell lysate were run on an 18% SDS-PAGE gel, transferred to PVDF, and immunoblotted for H3K79me2, H3K79me3, and H4K16ac. H3 was used as a loading control. Each deletion mutant is compared to its wild-type strain: WT10 (IPY36T), WT8 and WT (BY4741). The four lanes on the left and the two lanes on the right are from different gels. *Ubp10Δ* and its wild-type strain were used in a previous study (Schulze *et al.* 2011) and kindly provided by the Kobor lab. All other strains are from the Mat**a** BY4741 Open Biosystems KO collection.

H4K16 Acetylation by SAS-I is Necessary for Dot1 to Optimally Trimethylate H3K79

In order to determine if acetylation of H4K16 has an effect on Dot1 activity *in vivo*, we performed an experiment similar to the one above (Figure 2-1) with three H4K16 mutants, H4K16Q, H4K16A, and H4K16R (Figure 2-2). H4K16ac is not detected in any H4K16 mutant when compared to wild-type cells. H3K79me2 increased in all three mutants while H3K79me3 decreased dramatically, which would suggest that H4K16ac is necessary for Dot1 to efficiently catalyze the transition from H3K79me2 to H3K79me3. Not surprisingly, the H3K79me3 mark was preserved best in H4K16ac mimic, H4K16Q, although still below wild-type (Figure 2-2). H4K16A exhibited the lowest levels of H3K79me3. The presence of lowered H3K79me3 in the unacetylatable H4K16R mutant suggests that although Dot1 has some ability to trimethylate H3K79 in the presence unacetylated H4K16, acetylation of H4K16 is necessary for full stimulation of Dot1 catalysis of the transition from H3K79me2 to H3K79me3.

To verify that Dot1 trimethylates H3K79 in a H4K16ac dependent manner *in vivo*, determining which HAT was responsible for the acetylation dependent transition from H3K79me2 to H3K79me3 was completed. In yeast, the majority of H4K16 acetylation is performed by Sas2, a MYST family HAT contained in the SAS-I complex with Sas4 and Sas5 (Kimura *et al.* 2002, Suka *et al.* 2002). While Esa1, an essential HAT contained in the NuA4 complex, can acetylate H4K16, it mostly targets H4K5 and H4K12 (Suka *et al.* 2001, 2002, Chang and Pillus 2009). To determine which HAT was responsible for the transition, the same immunoblotting experiment was performed on histones isolated from cells lacking SAS-I subunits or containing Esa1 mutants (Figure 2-3) (Decker *et al.* 2008, gift from Mitch Smith).

SAS-I is composed of three subunits, Sas2, 4, and 5, which are all necessary for its optimal activity on chromatin *in vivo* (Sutton *et al*. 2003). As shown in figure 2-3, both *sas2Δ* and *sas5Δ* cells show a decrease in both H4K16ac and H3K79me3. Lack of complete loss of H4K16ac may be accounted for by compensatory acetylation by other HATs including Esa1, an essential HAT in yeast (Smith *et al.* 1998, Clarke *et al.* 1999). In order to maintain viability, Esa1 catalytic activity can be ablated through mutations to residues in the catalytic core of the enzyme (Yan *et al.* 2000, 2002, Decker *et al.* 2008). Both *esa1-C304S* and *esa1-E338Q* lack H4 HAT activity *in vivo* (Decker *et al.* 2008). A recent study has also confirmed that a significant loss in H4K16ac is not observed in *esa1/esa1* cells (Wang *et al.* 2013). As shown in figure 2-3, a decrease in H4K16ac or H3K79me3 is not seen for Esa1 mutants suggesting that SAS-I and the catalytic activity of Sas2 are necessary for Dot1 to trimethylate H3K79 efficiently.

Figure 2-2

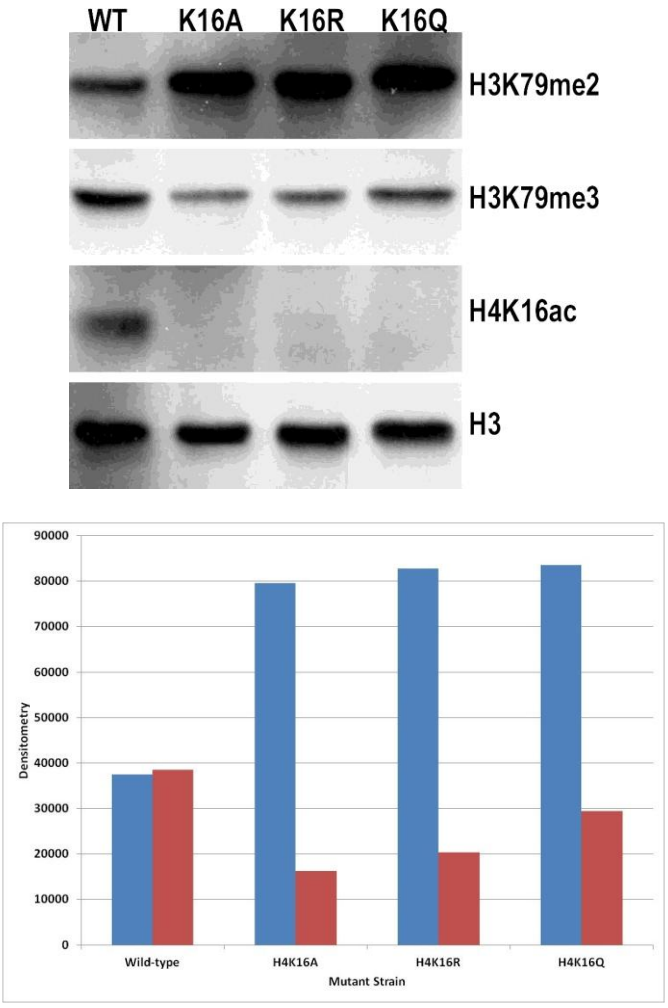


Figure 2-2: H4K16ac stimulates H3K79me2 to H3K79me3 transition by the HMT Dot1. Acid extracted histones from whole cell lysate were run on an 18% SDS-PAGE gel, transferred to PVDF, and immunoblotted for H3K79me2, H3K79me3, and H4K16ac. H3 was used as a loading control. H4K16A and R mutants were constructed using site mutagenesis on plasmid pQQ18. H4K16Q is a gift from Lucy Pemberton. Plasmid shuffle was performed in the wild-type strain JHY205 to make mutant strains. Densitometry measurements were taken for wild-type and mutant strains using ImageJ for H3K79me2 (blue) and H3K79me3 (red).

Figure 2-3

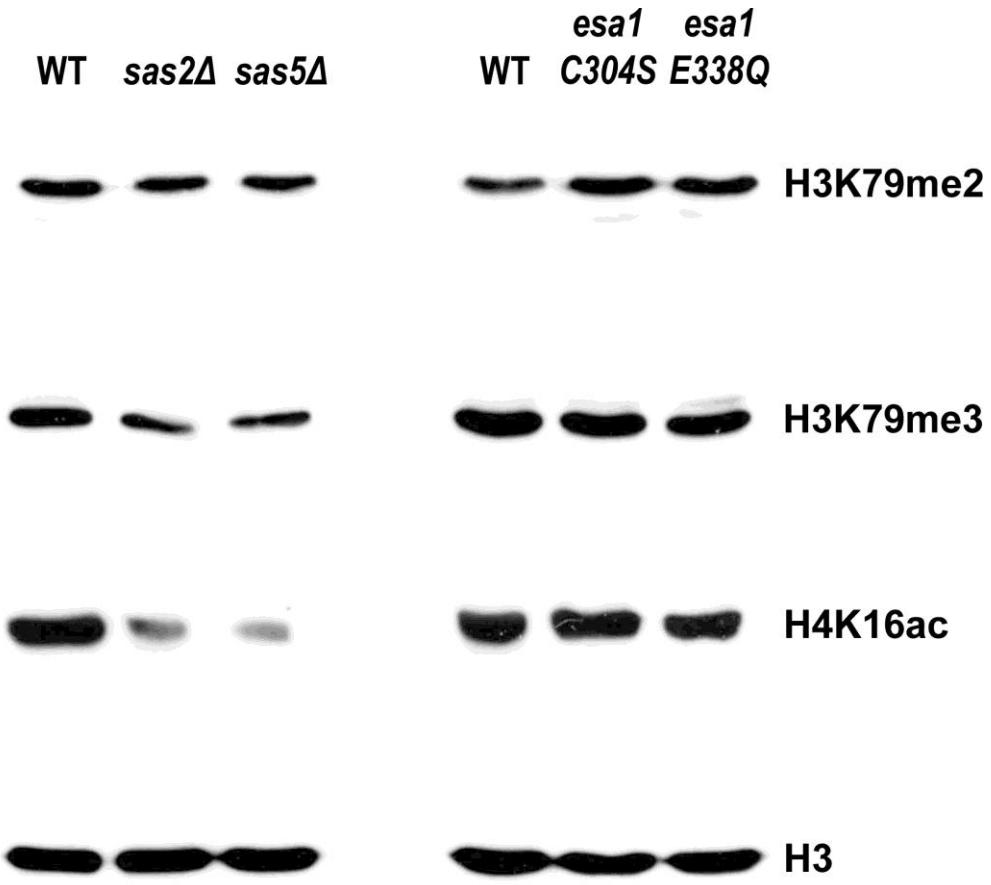


Figure 2-3: The HAT complex SAS-I, not Esa1, is responsible for efficient H3K79 trimethylation by Dot1. Acid extracted histones from whole cell lysates were run on an 18% SDS-PAGE gel, transferred to PVDF, and immunoblotted for H3K79me2, H3K79me3, and H4K16ac. H3 was used as a loading control. SAS-I mutants were obtained from the Open Biosystems Mat**a** deletion collection. Catalytically inactive Esa1 mutants and its wild-type strain were a gift from the Smith lab and utilized in a previous study (Decker *et al.* 2008).

Trimethylation of H3K79 by Dot1 is Dependent upon Structural Changes Elicited by H4K16 Acetylation

Evidence shows that an increase in ubiquitination of H2BK123 through the deletion of Ubp10 (Schulze *et al.* 2011) facilitates the acetylation of H4K16 and methylation of H3K79 whereas deletion of Bre1 reduces H4K16ac and abolishes H3K79me. I have also shown that acetylation of H4K16 by Sas2 facilitates a transition from di- to trimethylation of H3K79 by Dot1, which is further supported by evidence showing loss of H3K79me3 in H4K16 miutants. Two models can be formulated to explain H4K16's effect on Dot1's ability to trimethylate H3K79. H4K16ac may be acting: 1) in *cis* by altering additional intra- and internucleosomal interactions in order to make space for a third methyl group on H3K79 or 2) in *trans* through allosteric activation and subsequent alteration in the conformation of Dot1's active site to better catalyze trimethylation.

In order to test which mechanism best fits a model for Dot1 to catalyze trimethylation, *in vitro* HMT assays were performed with full length recombinant Dot1, unlabeled adenosyl-methionine, and oligonucleosomes (LaCoste *et al.* 2002, Fingerman *et al.* 2007) that exhibited partial or complete loss of H4K16ac (Figure 2-4 and 2-5). The assays were performed with oligonucleosomes that were purified from *dot1Δ*, *sas2Δ* and H4K16R yeast. Figure 2-4 shows that *dot1Δ* cells are also minimally deficient in H4K16ac to the same degree as *bre1Δ* cells. In order to test the stimulatory properties of H4K16ac, oligonucleosomes were incubated alone with Dot1 and in the presence of $H4_{1-20}$ and H4K16ac peptides (Figure 2-5). After incubation, reactions were analyzed by immunoblotting for H3K79me2 and me3 (radioactivity HMT assays are not sensitive to different states of histone methylation).

In reactions with *dot1Δ* nucleosomes, H4K16ac peptide was not stimulatory for either di- or trimethylation in comparison to reactions containing no peptide. Reactions supplemented with $H4_{1-20}$ peptide displayed even less di- and trimethylation by Dot1. In both *sas2Δ* and H4K16R HMT reactions, histones are modestly di- and trimethylated without adding Dot1. Neither peptide had an effect on H3K79me2 or H3K79me3 in *sas2Δ* or H4K16R reactions in comparison to the no peptide control (Figure 2-5).

Surprisingly, $H4_{1-20}$ peptide did not stimulate the reactions in comparison to the no peptide control despite previous reports that Dot1 binds to the H4 N-terminal tail, which is necessary for *in vivo* Dot1 activity (Altaf *et al.* 2007, Fingerman *et al.* 2007). One explanation for this discrepancy is that Dot1 normally binds to the H4 tail within the context of the nucleosome and its' activity is being sequestered by binding to the free $H4_{1-20}$ peptide. In order to test this hypothesis, increasing amounts of $H4_{1-20}$ and H4K16ac peptide were added to Dot1 HMT reactions containing *dot1Δ* nucleosomes (Figure 2-6, top). Reactions were processed and analyzed as discussed in figure 2-6. Based on densitometry measurements, (Figure 2-6, bottom), increasing amounts of H4 tail does, in fact, reduce Dot1 trimethylation activity whereas H4K16ac peptide does not have the same effect.

Figure 2-4

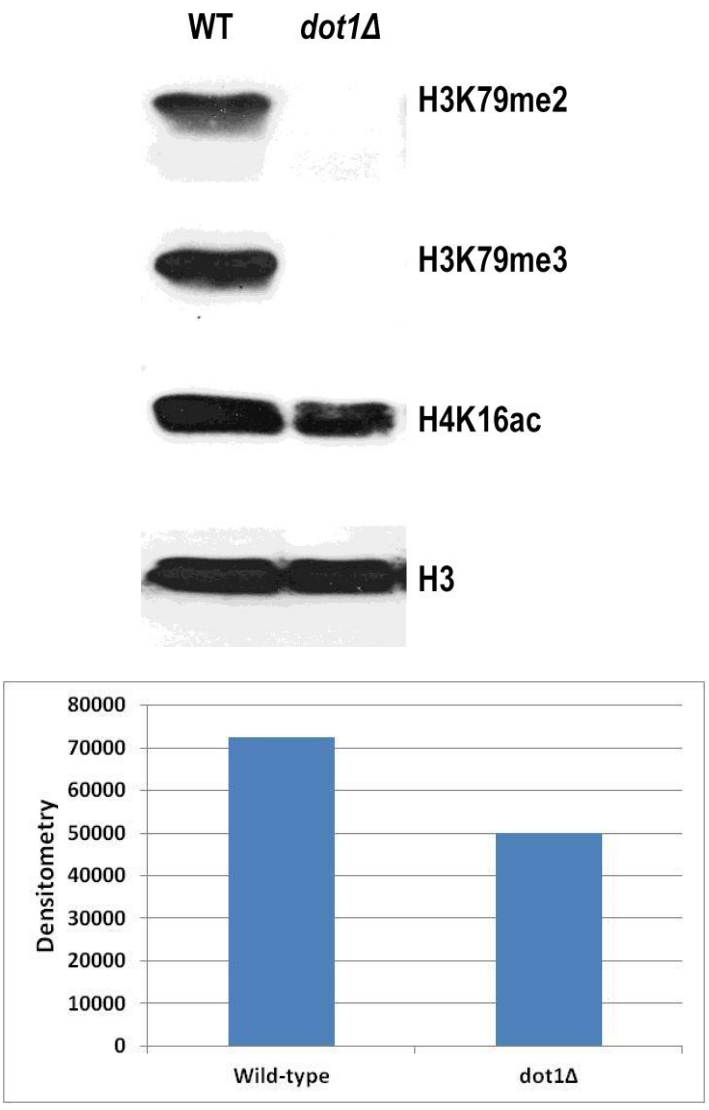


Figure 2-4: H4K16ac is reduced in *dot1Δ* strain background. Immunoblotting was repeated as in the previous experiments. Dot1 deletion strain was obtained from Open Biosystems mat**a** KO collection. Densitometry measurements were taken for H4K16ac using ImageJ.

Figure 2-5

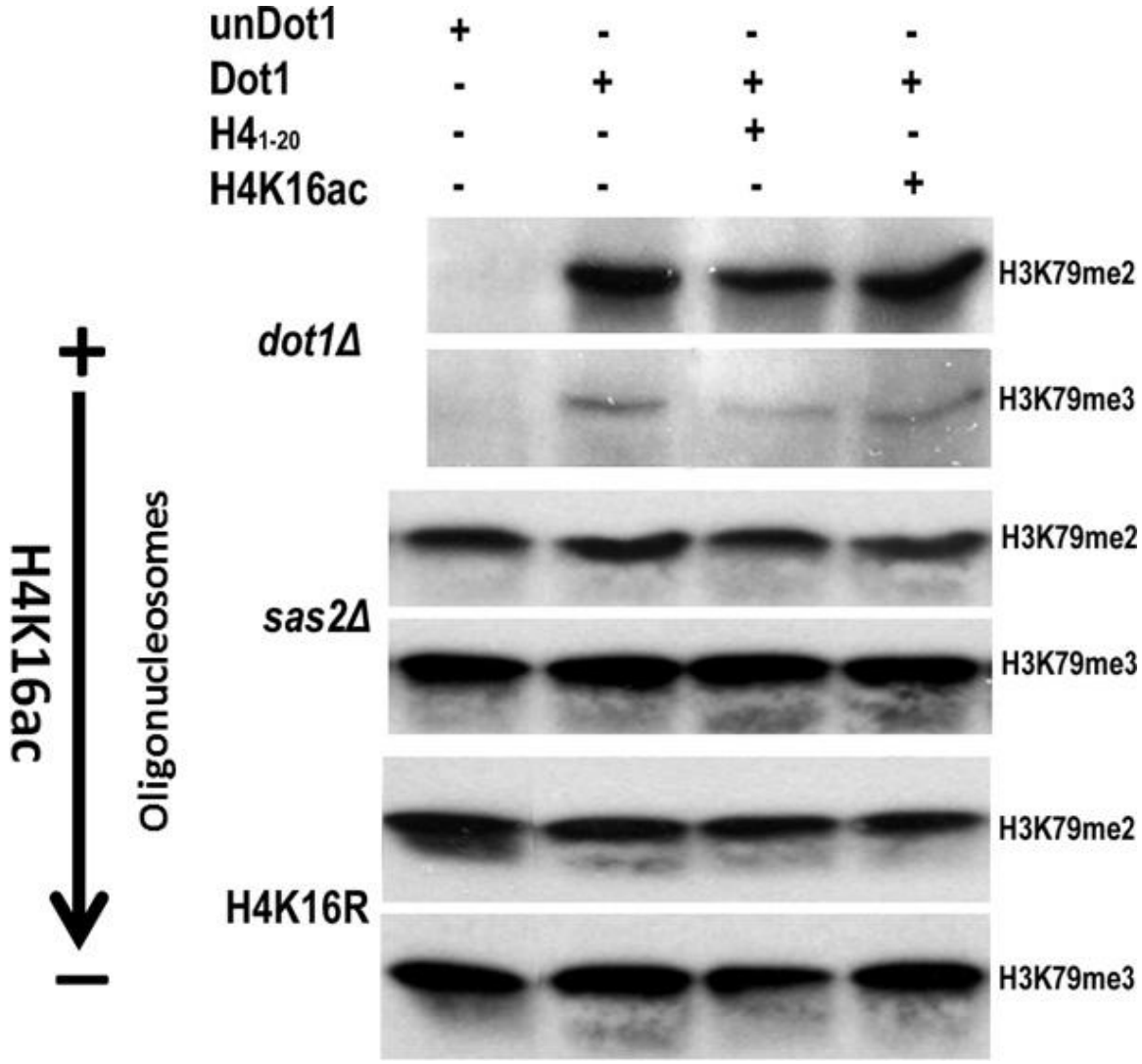
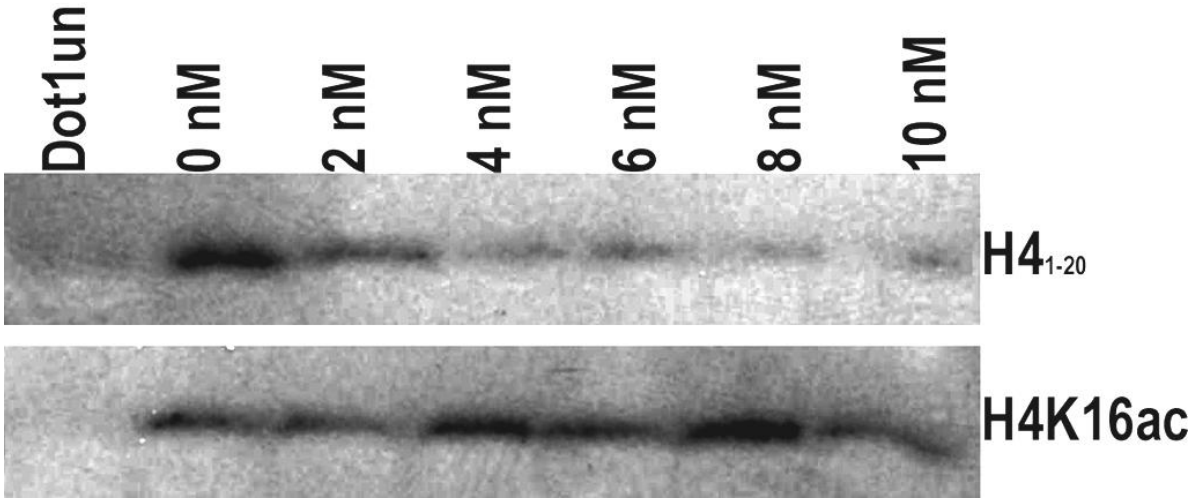


Figure 2-5: Dot1 is not allosterically stimulated in *trans* by H4K16ac. *In vitro* HMT assays were performed with native yeast oligonucleosomes (labeled to the left of immunoblots) supplemented with lysate from uninduced (unDot1) and induced bacterial cells harboring an expression plasmid with recombinant Dot1. Reactions were performed in the presence of $H4_{1\text{-}20}$, H4K16ac, or no peptide. Samples were run on an 18% SDS-PAGE gel and processed as in the previous immunoblots.

Figure 2-6


Dot1un
0 nM
2 nM
4 nM
6 nM
8 nM
10 nM
H4 1-20
H4K16ac

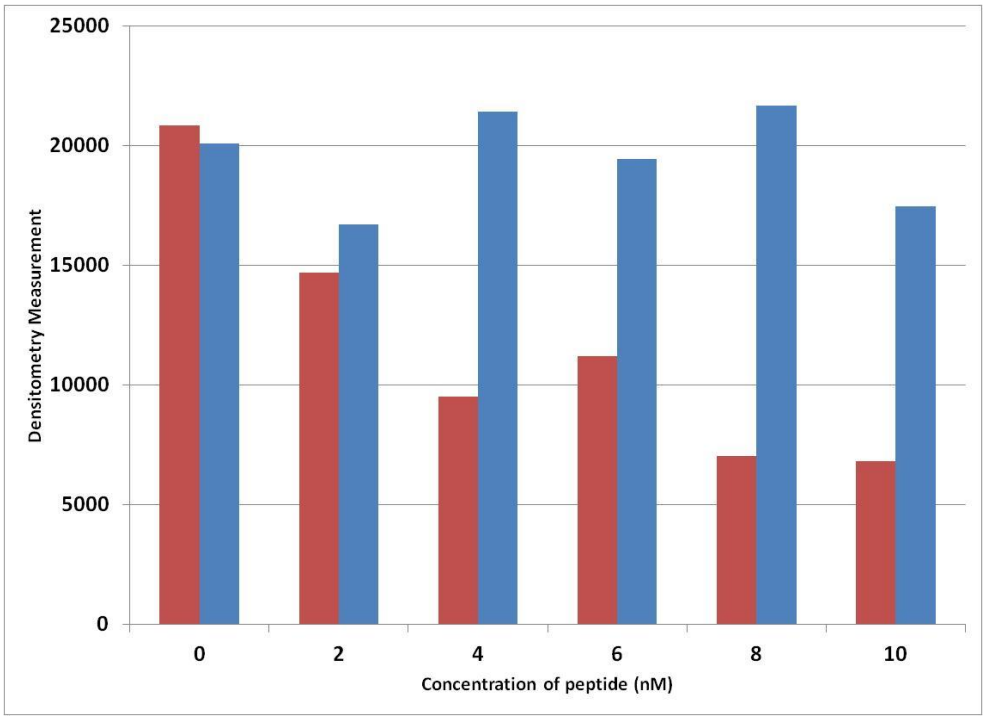

25000
20000
15000
10000
5000
0
Densitometry Measurement
0
2
4
6
8
10
Concentration of peptide (nM)

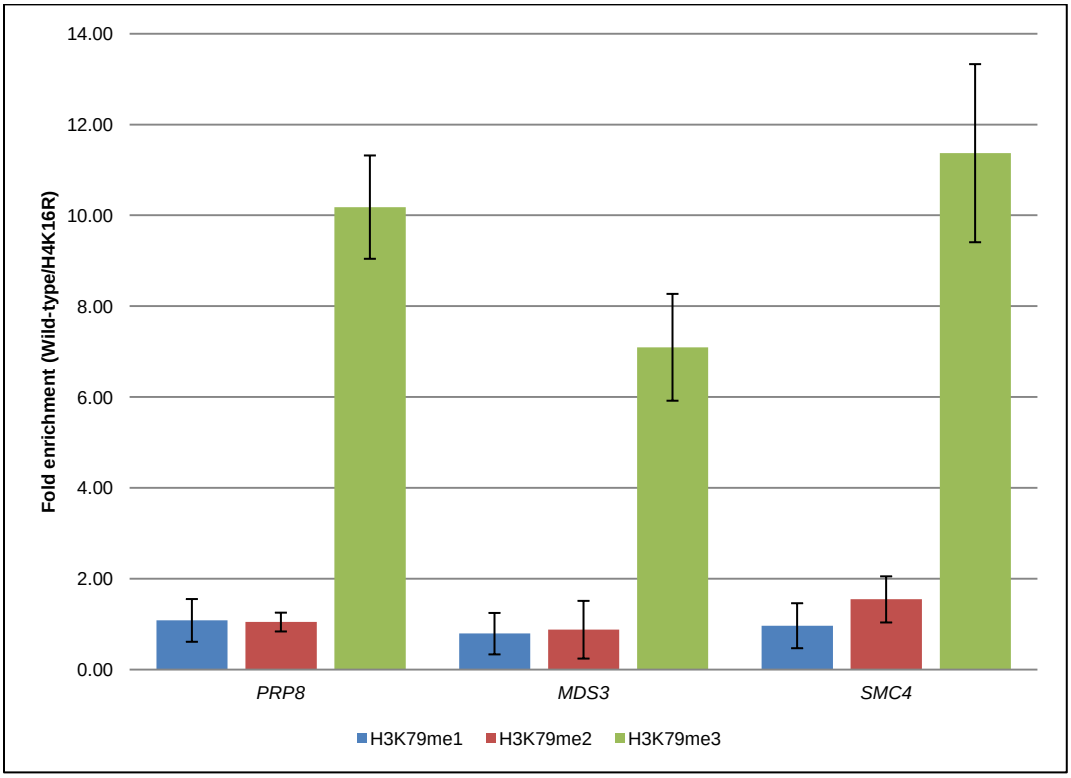
Figure 2-6: *In vitro* Dot1 activity is sequestered by $H4_{1-20}$ peptide. Dot1 HMT assays were carried out with *dot1Δ* nucleosomes as previously described and analyzed by immunoblotting for H3K79me3. (Top panel) Reactions were supplemented with increasing amounts of unacetylated peptide and H4K16ac peptide. Peptide concentrations are indicated. Reactions containing lysate from uninduced bacteria harboring pET28a-6xHIS-Dot1 are shown in the first lane (Dot1un). (Bottom panel) Band intensities were measured using ImageJ software to determine if H4 (red) or H4K16ac (blue) peptide inhibited or stimulated Dot1 as performed in the assay in the top panel.

Gene Bodies Acetylated by Sas2 are specifically Affected by H4K16ac Dependent H3K79 Trimethylation

Both Dot1 and SAS-I were discovered during genetic screens searching for proteins that caused silencing defects in yeast (Xu *et al.* 1999, Singer *et al.* 1998). Dot1 was specifically found during a screen that tested for disruptors of telomeric silencing (Singer *et al.* 1998). Although a role of Dot1 in silencing, DNA damage repair, and cell cycle control has been clearly established, how Dot1 and different H3K79 methylation states function at the transcriptional level has yet to be clearly elucidated. Newer studies have shown that Dot1 plays a clearer role in transcription by binding directly to RNAPII (Kim *et al.* 2012) and promoting leukemic transformation by hypermethylating *HOX* genes targeted by MLL fusion proteins (Deshpande *et al.* 2013, Zhang *et al.* 2012). In light of discovering that Dot1 requires H4K16ac in order to optimally trimethylate H3K79, I set out to determine if this effect is localized to telomeres and/or genes that possess the H4K16ac mark.

Chromatin immunoprecipitation (ChIP) of H3K79me1, 2, and 3 for wild-type and H4K16R mutant strains was completed at the subtelomeric regions on chromosome VIR and gene bodies that were previously shown to decrease in the H4K16ac mark upon Sas2 deletion (Shia and Workman 2006, Heise *et al.* 2012). Figure 2-7 shows fold enrichment by ChIP of the three H3K79 methylation states in wild-type cells compared to H4K16R mutants. On average, H3K79me3 decreases 10-fold at both telomeres and gene bodies in the H4K16R mutant while a decrease in H3K79me1 and 2 is only seen at telomeres, which suggests that H3K79me1, 2, and 3 may contribute to proper silencing while the Sas2 dependent trimethylation of H3K79 by Dot1 may be localized to gene regions. Specific loss of just H3K79me3 at gene bodies marked by H4K16ac suggests a role for H4K16ac dependent H3K79 trimethylation in the regulation of transcription.

Figure 2-7


14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Fold enrichment (Wild-type/H4K16R)
PRP8
MDS3
SMC4
H3K79me1
H3K79me2
H3K79me3

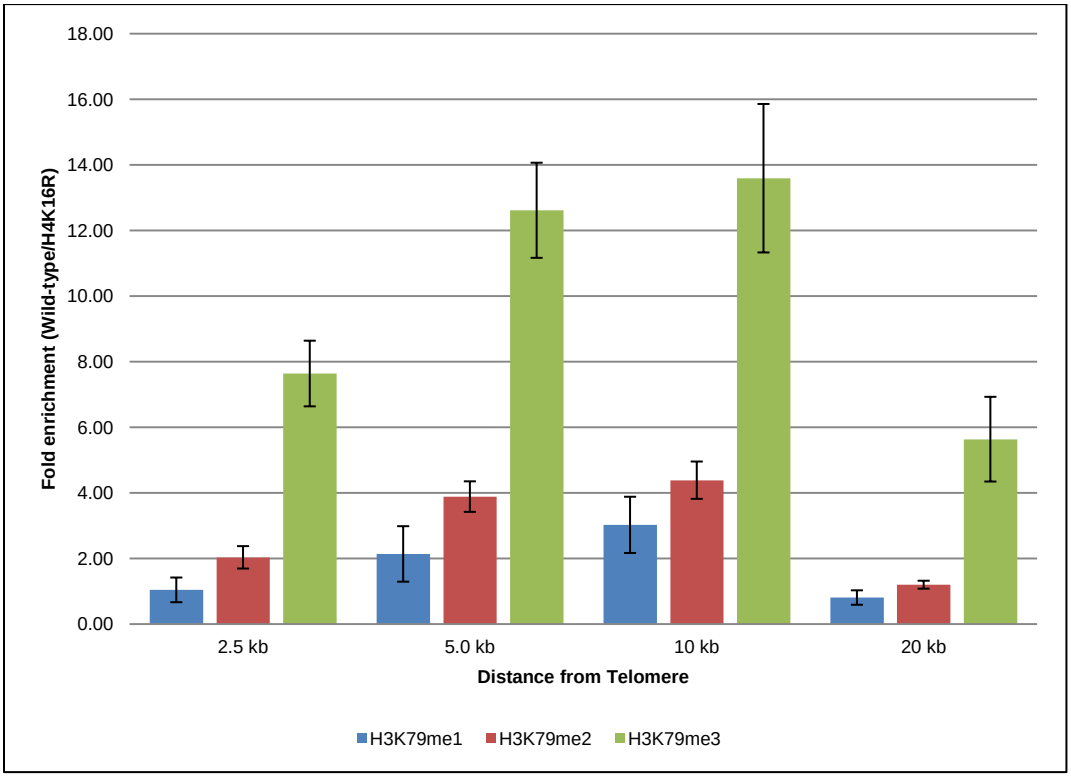

18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Fold enrichment (Wild-type/H4K16R)
2.5 kb
5.0 kb
10 kb
20 kb
Distance from Telomere
H3K79me1
H3K79me2
H3K79me3

Figure 2-7: Sas2 H4K16 acetylation-dependent H3K79me3 is observed at both telomeres and gene bodies. ChIPs for H3K79me1 (blue), 2 (red), and 3 (green) were performed in wild-type and H4K16R mutants. (Top panel) ChIPs performed at gene bodies acetylated by Sas2 (Heise *et al.* 2012). Genes are labeled below corresponding bars. (Bottom panel) ChIPs performed at the subtelomeric region of VIR. Distances indicated start from chromosomal base pair position 269660. Data is represented as a fold enrichment of H3K79me at loci in wild-type cells compared to mutant cells and is normalized to H3. Triplicate real time measurements were collected for biological replicates. Error bars are a measurement of standard deviation.

Discussion

H3K79 methylation states are functionally redundant in telomeric silencing

Although Dot1 has been linked to the regulation of silencing, DNA repair, cell cycling, and transcription, what role H3K79 mono-, di-, and trimethylation play in these processes requires further investigation. Moreover, a mechanism for the reversal of H3K79 methylation has yet to be elucidated, suggesting that H3K79me is a static mark that acts to demarcate euchromatin from heterochromatin. However, both H3K79me2 and H3K79me3 exhibit a slight decrease upon transcriptional activation (Schulze *et al.* 2011, Shahbazian *et al.* 2005) suggesting that histone exchange during transcription may act as a mechanism for H3K79me removal.

Dot1 was first described as a distributive enzyme, implying the three methylation states of H3K79 could not be generated independently and they are functionally redundant (Fredericks *et al.* 2008). Dot1's role in the regulation of silencing supports this notion. At telomeres, acetylation of H4K16 by Sas2 is opposed by the HDAC activity of Sir2. Opposition between these two enzymes forms the heterochromatic-euchromatic boundary (Suka *et al.* 2002, Kimura *et al.* 2002). Loss of H4K16ac at the subtelomeric region of chromosome VIR conferred a noticeable decrease in all H3K79 methylation states in comparison to wild-type (Figure 2-7 (bottom)). Loss of all three methylation states would suggest that H3K79 methylation states serve redundant roles in silencing.

Even more, Sas2 and Dot1 substrates, H4K16 and H3K79, are separated by a mere distance of 18 Å and located on the surface of the nucleosome (Armache *et al.* 2011), suggesting that crosstalk between these residues is possible. Moreover, the same nucleosomal binding surface is shared with the SIR HDAC complex subunit, Sir3 (Figure 2-8). Structural analysis of the interaction between Sir3-BAH and nucleosomes showed that the positively charged H4 tail is stabilized by the negatively charged surface

of Sir3-BAH (Armache *et al.* 2011). Not surprisingly, mutating H4K16 or H3K79 disrupts silencing in yeast, suggesting that maintaining residues at both H4K16 and H3K79 are important for silencing.

H3K79me3 is affected at gene bodies upon H4K16ac loss suggesting a role for Dot1 in transcriptional regulation

A possible role in the regulation of transcription by Sas2 and Dot1 is indicated by the regulation of H3K79 trimethylation by H4K16 acetylation on gene bodies. Figures 2-2 and 2-3 indicate that the deposition of H3K79me3 by Dot1 requires H4K16 acetylation by Sas2 and not Esa1. Furthermore, a role for H4K16ac-H3K79me3 crosstalk in transcription can be postulated based on specific loss of H3K79me3 at genic regions upon mutating H4K16 (Figure 2-7, top). H3K79me2 and H3K79me3 have been shown to mark the bodies of genes. Genome wide analysis of H3K79me2 and me3 showed that genes can be enriched in either one or both marks, suggesting that the degree of H3K79 methylation may indicates different modes of regulation in gene expression (Takahashi *et al.* 2011, Schulze *et al.* 2011, Wang *et al.* 2008, Barski *et al.* 2007, Shahbazian *et al.* 2005). Furthermore, both H3K79 di- and trimethylation are dependent on H2BK123ub (Nakanishi *et al.* 2009, Briggs *et al.* 2002, Ng *et al.* 2002, Sun and Allis 2002), which is also in close proximity to H4K16 and H3K79 on the nucleosome (Figure 2-8).

Figure 2-8

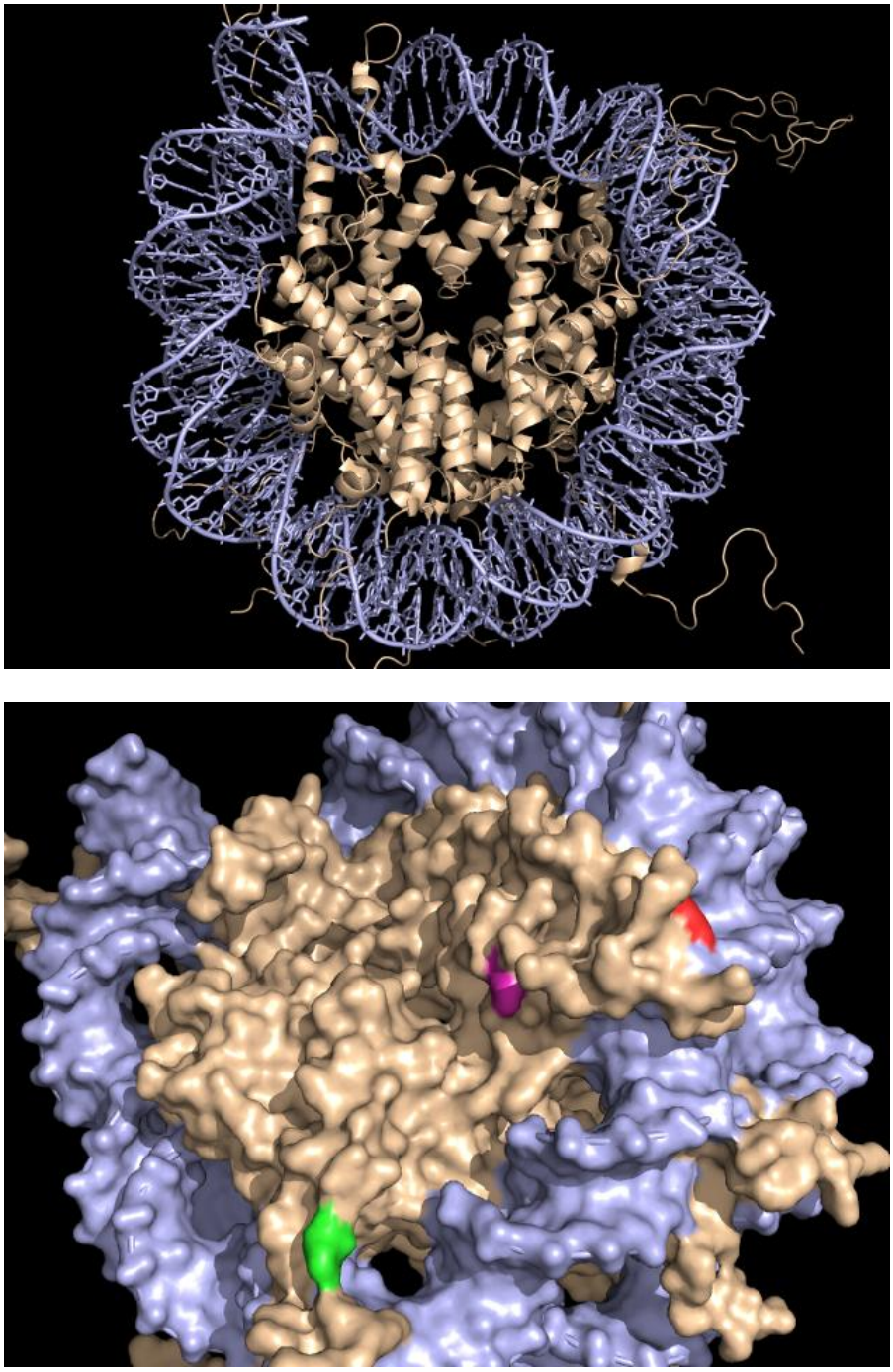


Figure 2-8: Nucleosomal surface bound by both Dot1 and Sir3-BAH domain. Looking from the bottom of the nucleosome as shown in the top panel, H2BK123 (green), H3K79 (purple), and H4K16 (red) are within close proximity of one another. The span of this surface is bound by both Dot1 and Sir3-BAH. Residues in this model have not been modified. DNA (blue) and histones (white) were modeled using PyMol.

Dot1 activity is stimulated intranucleosomally by H2Bub and H4K16ac

Based on structural analysis performed on nucleosomes (Peterson 2012, Feitz *et al.* 2011) and data shown in this chapter, a hypothesis was formed that Dot1 dimethylates H3K79 upon H2BK123 ubiquitination by Rad6/Bre1 and trimethylates H3K79 upon H4K16 acetylation by Sas2 (Figure 2-9). Figure 2-4 supports the unidirectionality of this pathway as *dot1Δ* nucleosomes exhibit complete loss of H3K79me and minimal loss of H4K16ac. An increase in H3K79me2 and me3 is also observed when recombinant Dot1 is added *in vitro* to nucleosomes isolated from *dot1Δ* cells (Figure 2-5), in comparison to the control reaction supplemented with uninduced bacterial lysate. In contrast, both *sas2Δ* and H4K16R nucleosomes exhibit a loss of H4K16ac (Figure 2-2 and 2-3) and no increase in H3K79me3 in comparison to reactions without added peptide (Figure 2-5). This suggests that H4K16ac stimulates H3K79 trimethylation when the two marks are located intranucleosomally.

H4K16ac does not seem to recruit Dot1 to chromatin, as a non-acetylated peptide served as an effective competitor of methylation reactions (Figure 2-6). Instead, acetylation by SAS-I may promote H3K79 trimethylation by Dot1 in both a structural and direct stimulatory manner. This is further supported by evidence in previous studies indicating that although H4 tail is necessary for Dot1 activity, it is not necessary for nucleosomal binding. The same studies also showed that Dot1 methylates in a H4K16ac dependent manner (Fingerman *et al.* 2007, Altaf *et al.* 2007). Therefore, Dot1 may not have been directly stimulated by the H4K16ac peptide because it needs both H3K79 and H4K16 within the same vicinity to stabilize Dot1 binding and the reaction (Figure 2-9 and Figure 2-10).

Structural changes in the nucleosome upon H2B ubiquitination and H4K16 acetylation affect Dot1 methyltransferase activity

Acetylation of H4K16 has been shown to decondense chromatin (Shogren-Knaak *et al.* 2006) and may play a role in creating enough space for Dot1 to add a third methyl group, which would otherwise possibly be limited by spatial constrictions between nucleosomes. In support of this notion and the model proposed in figure 2-9A, chromatin fibers exhibiting both H2BK123ub and H4K16ac marks together synergistically inhibit chromatin compaction suggesting different modes of action in the alteration of chromatin structure by each modification (Fierz *et al.* 2011).

Upon quantification of H3K4me and H3K79me in comparing H2BK123R mutants to normal cells, a near loss of H3K4me2 and me3 was observed whereas H3K79me2 was reduced by half and H3K79me3 exhibited an extreme decrease (Shahbazian *et al.* 2005). Dot1 was shown to bind to actively transcribed genes in both normal and mutant cells suggesting that loss of H3K79me2 and me3 is not due to loss of Dot1 recruitment. While Dot1 can perform monomethylation on H3K79 in the absence of H2BK123ub, results suggest that other factors are necessary for Dot1 to efficiently di- and trimethylate H3K79 even in the presence of H2BK123ub (Shahbazian *et al.* 2005). Moreover, H3K79me2 and me3 have been found to be enriched at specific genes independently of the gene's transcriptional frequency, suggesting that other chromatin associated factors that are not related to the basic transcriptional machinery (such as SAGA, COMPASS, Paf1, SLIK, etc.) may also have an effect on Dot1's ability to mono-, di-, or trimethylate H3K79 at specific locations on the genome (Shahbazian *et al.* 2005). One such factor may be Sas2 as shown in the data provided in this chapter. The simplified model shown in figure 2-9 describes how both H2BK123ub and H4K16ac affect Dot1 activity based on the results discussed in this chapter.

Figure 2-9

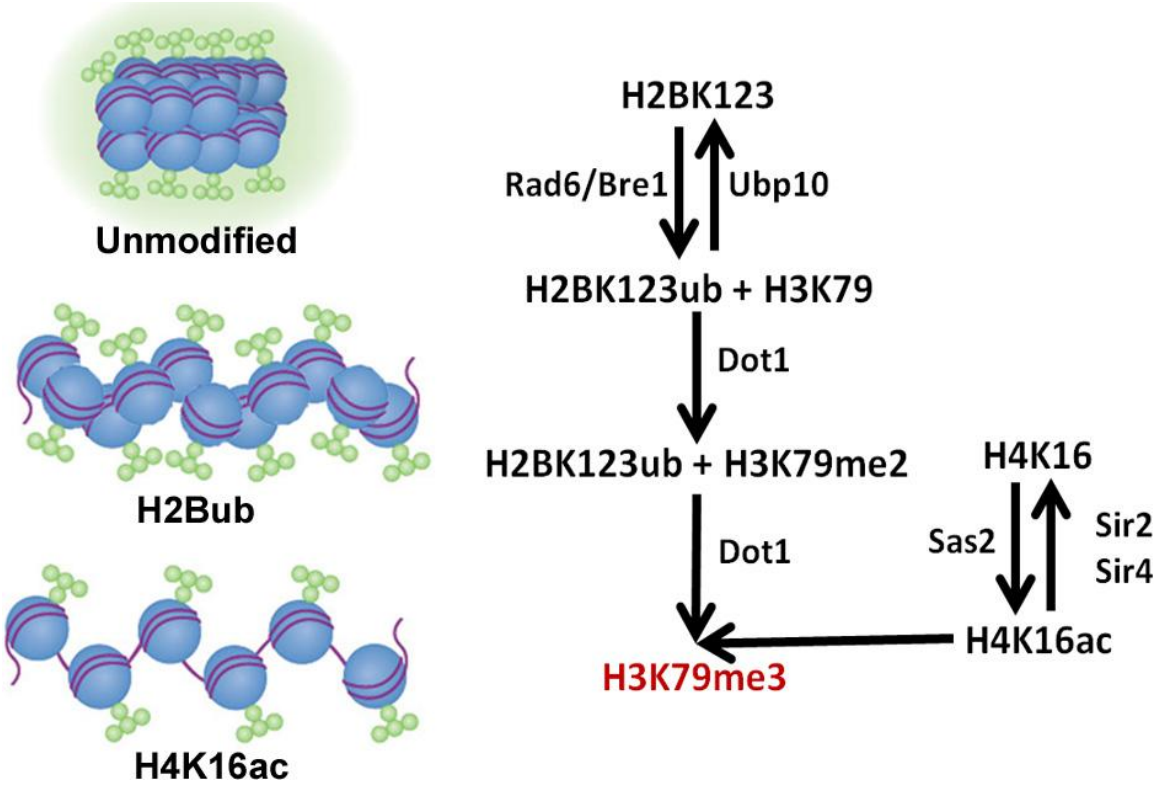


Figure 2-9: Regulation of nonprocessive H3K79 methylation by Dot1 through changes in internucleosomal interactions. A summary of findings presented thus far are shown in the flow chart to the right. Ubiquitinatin of H2BK123 by Rad6/Bre1 results in the decondensation of chromatin and allows Dot1 to dimethylate H3K79. Release of the H4 tail and further chromatin decondensation upon H4K16 acetylation by Sas2 regulates the transition from di- to trimethylation of H3K79. Structural changes in chromatin as a consequence of H2BK123ub and H4K16ac are shown to the left of the flow chart (Peterson 2011).

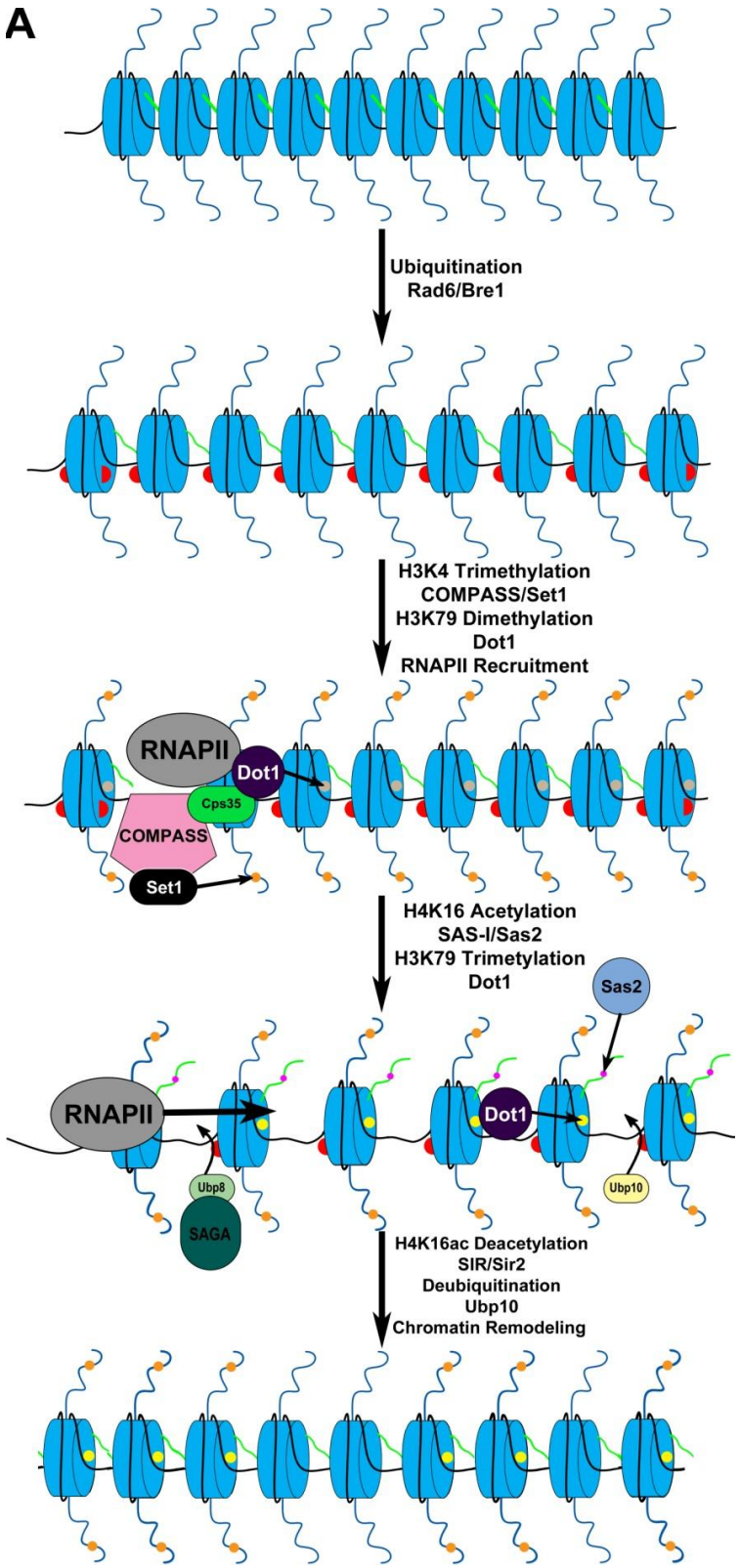
Genes marked by H3K79me3 and H4K16ac may be dynamically regulated by histone modifiers

H4K16 acetylation by Sas2 is necessary for efficient trimethylation of H3K79 by Dot1 (Figure 2-2 and 2-3). H4K16ac is localized to the bodies of actively transcribed genes and has also been observed to pause RNAPII during transcriptional initiation in addition to regulating transcription at the 3' end of longer genes (Heise *et al.* 2012). Based on previous studies and the evidence presented, the pathway by which H4K16ac controls catalysis of H3K79me3 may have more of an effect on the 3' end of longer genes (Schulze *et al.* 2011), which would be consistent with data showing that H3K79me3 is enriched on the bodies and 3' end of genes (Wang *et al*. 2008). Moreover, Ubp10 rather than Ubp8 seems to also have more of an effect on the deubiquitination of H2BK123ub enriched on longer genes. In fact, *ubp10Δ* exhibits an increase of H3K79me3 in gene bodies whereas *ubp8Δ* exhibits an increase in H3K4me3 at promoter regions (Schulze *et al.* 2011).

Gene promoters that are poised for transcription are marked by H3K4me2 and H3K4me3 and seem to be dynamically regulated by both HATs and HDACs. Upon HDAC inhibition, H4K16ac increases dramatically at silent genes marked with H3K4me (primed) and within 12 hours, 60% of these gene promoters are bound by RNAPII compared to 0.7% not marked with H3K4me (Wang *et al.* 2009). Despite RNAPII recruitment, these genes were not transcribed suggesting the possibility that H4K16ac/H3K4me acts as a bivalent mark for poised genes (Figure 2-10B). Interestingly, a recent finding showed that BPTF binds to histones marked with both H3K4me3 and H4K16ac via its PHD and bromodomain, respectively. The PHD module is known to recruit the NURD remodeling complex but no recruitment mechanism has been established for the bromodomain (Ruthenburg *et al.* 2011). The possibility of the

bromodomain recruiting Dot1 or RNAPII has not been tested. Moreover, our data may imply that an equivalent bivalent reader of H3K79me3 and H4K16ac may also exist.

Figure 2-10


A
Ubiquitination
Rad6/Bre1
H3K4 Trimethylation
COMPASS/Set1
H3K79 Dimethylation
Dot1
RNAPII Recruitment
RNAPII
Dot1
Cps35
COMPASS
Set1
H4K16 Acetylation
SAS-I/Sas2
H3K79 Trimetylation
Dot1
Sas2
RNAPII
Dot1
Ubp8
SAGA
Ubp10
H4K16ac Deacetylation
SIR/Sir2
Deubiquitination
Ubp10
Chromatin Remodeling

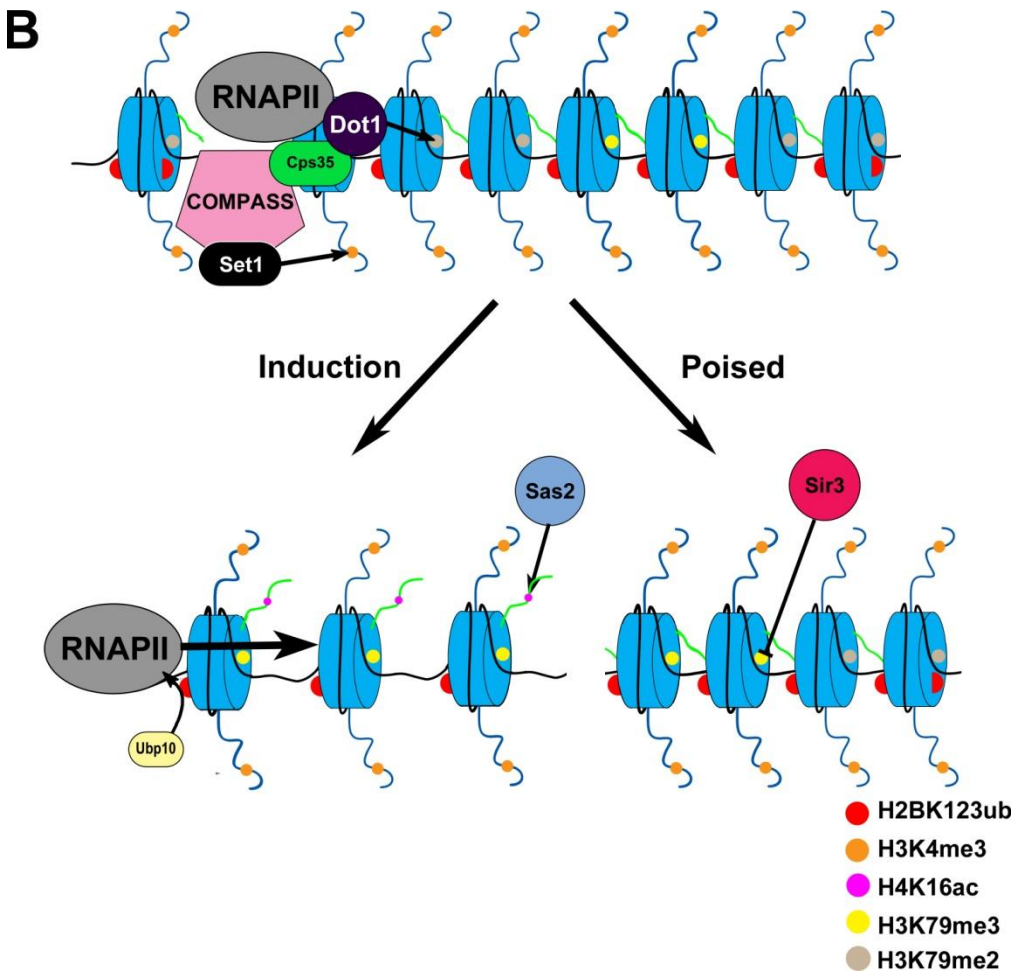

B
RNAPII
Dot1
Cps35
COMPASS
Set1
Induction
Poised
Sas2
Sir3
RNAPII
Ubp10
H2BK123ub
H3K4me3
H4K16ac
H3K79me3
H3K79me2

Figure 2-10: Model for the cycle of chromatin marks during gene transcription: (A) Unmarked chromatin is ubiquitinated by Rad6/Bre1 at H2BK123. Dot1 is recruited to active genes through mechanisms not yet elucidated, and dimethylates H3K79. Cps35 is recruited to H2Bub and recruits COMPASS/Set1, which methylates H3K4. RNAPII is recruited to gene promoters by transcription factors (not shown) but is paused (initiation). In addition to H2Bub, H4 acetylation by SAGA and SAS-I/Sas2 results in a more open conformation of chromatin structure. This facilitates Dot1 methyltransferase activity. Dot1 may also be stimulated by H4K16ac intranucleosomally by H4K16ac. H3K79me3 permits RNAPII to actively transcribe the gene (elongation) upon Ubp8 to deubiquitination of H2BK123. Ubp10 deubiquitinates H2B on longer genes. Sir3 binding is inhibited by H3K79me (Oppikofer *et al.* 2011). Chromatin is reverted back to its semi-condensed state. (B) In order to reestablish a poised state for genes that must be induced in a short amount of time after a given external stimulus, newly deposited histones are ubiquitinated by Bre1/Rad6, dimethylated by Dot1, and trimethylated at H3K4 by COMPASS HMT, Set1. Genes can either stay in the poised state and are marked by newly catalyzed H3K79me2, H3K4me3, H2BK123ub, and previously catalyzed H3K79me3. H3K79me prevents SIR complex from binding and eliciting gene repression. Upon induction, acetylation of H4K16 by Sas2 and H2B deubiquitination stimulates Dot1 to trimethylate H3K79 on longer genes, which releases RNAPII for efficient elongation. Despite Dot1 detection at genes only during activation, presence of H3K79me at inactive genes has been previously report (Shahbazian *et al.* 2005). H3K79me present at a gene during an inactive state may serve to poise the gene for temporal induction by SAS-I while preventing SIR complex from binding.

CHAPTER 3: DOT1 RECRUITMENT TO CHROMATIN AND METHYLTRANSFERASE ACTIVITY DURING TRANSCRIPTION IS DEPENDENT ON HISTONE CROSSTALK

Introduction

Dot1 is regulated by various transcription factors and histone modifiers in humans

A model of Dot1 recruitment to actively transcribed genes has not been well established. In humans, it has been shown to bind to various histone modifications directly or through effector proteins employed in larger chromatin modifying complexes (Table 3-1). In yeast, however Dot1 is not contained in any complexes. In this chapter, I will attempt to decipher the mechanism by which Dot1 is targeted to chromatin and how this affects the specificity to which genes it targets.

In flies, P-TEFb is a cyclin-dependent kinase complex that phosphorylates serine-2 (S2) on the RNAPII C-terminal domain (CTD), which is required for the transition of RNAPII from a transcriptional initiation to a transcriptional elongation phase. Dot1L has been shown to bind to RNAPII directly when phosphorylated on its CTD. This interaction is necessary for transcription of *NANOG*, *OCT4*, and genes that promote the maintenance of pluripotency in embryonic stem cells (Kim *et al.* 2012). Dot1 also co-purifies with various complexes and subunits that are involved in transcriptional regulation, including P-TEFb (Table 3-1). Although SEC does not contain Dot1, the common denominator between these complexes is ENL, which serves to recruit Dot1 to complexes involved in transcriptional elongation (Nguyen and Zhang 2011, Bitoun *et al.* 2009). P-TEFb has also been linked to H2B deubiquitination, H3S10 phosphorylation (H3S10ph), and double bromodomain-containing protein, Brd4 (Brès *et al.* 2008).

Table 3-1: Transcriptional Elongation Complexes Linked to hDot1L

Complex	Proteins							
	Dot1L	AF4	AF9	AF10	ENL	P-TEFb	Other	Reference
EAP core	+	+			+	+		Mueller *et al.* 2009
Unnamed	+	+	+	+	+	+		Bitoun *et al.* 2009
DotCom	+		+	+	+		AF17, TRRAP, Skp1, β-catenin	Mohan *et al.* 2010
SEC		+	+		+		ELL, AFF4	Lin *et al.* 2010

ENL-associated proteins (EAP), Super elongation complex (SEC), plus signed indicates subunits co-purified in complex

Figure 3-1

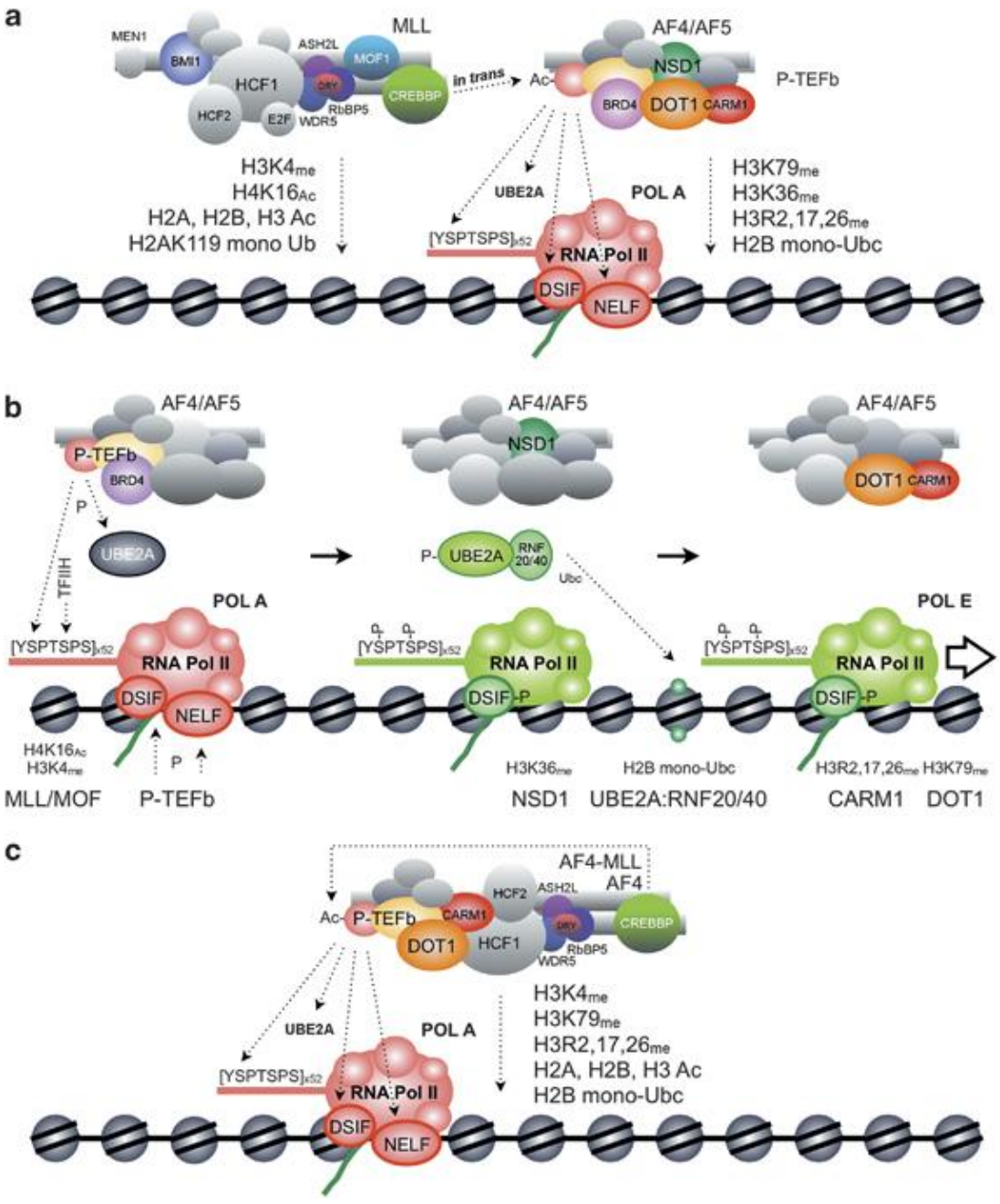

a
MEN1
BMI1
HCF1
HCF2
E2F
ASH2L
MOF1
DPY
RbBP5
WDR5
CREBBP
MLL
in trans
Ac-
AF4/AF5
NSD1
BRD4
DOT1
CARM1
P-TEFb
H3K4me
H4K16Ac
H2A, H2B, H3 Ac
H2AK119 mono Ub
UBE2A
POL A
[YSPTSPS]x52
RNA Pol II
DSIF
NELF
H3K79me
H3K36me
H3R2,17,26me
H2B mono-Ubc
b
AF4/AF5
P-TEFb
BRD4
P
UBE2A
TFIIH
NSD1
DOT1
CARM1
P-
RNF 20/40
Ubc
POL A
POL E
[YSPTSPS]x52
RNA Pol II
DSIF
NELF
DSIF-P
H4K16Ac
H3K4me
MLL/MOF
P-TEFb
H3K36me
H2B mono-Ubc
NSD1
UBE2A:RNF20/40
H3R2,17,26me
H3K79me
CARM1
DOT1
c
AF4-MLL
AF4
HCF2
ASH2L
Ac-
P-TEFb
CARM1
DOT1
HCF1
DPY
RbBP5
WDR5
CREBBP
UBE2A
POL A
[YSPTSPS]x52
RNA Pol II
DSIF
NELF
H3K4me
H3K79me
H3R2,17,26me
H2A, H2B, H3 Ac
H2B mono-Ubc

Figure 3-1: AF4-MLL combines crucial functions of MLL and AF4 protein complexes. (A) Molecular functions performed by the MLL and AF4 complex are depicted. Chromatin modifications mediated by the MLL complex lead to the activation of promoter regions. MLL complex catalyzes H3K4me (SET–domain complex), H4K16Ac (MOF), general histone acetyltransferase activity (CREBBP) and mono-ubiquitinylation of H2AK119 (BMI1). The AF4 complex activates RNAPII by CTD S2 phosphorylation carried out by P-TEFb. DOT1L, NSD1 and CARM1 modify the chromatin during the elongation state of RNAPII. (B) The AF4 complex exerts three independent chromatin-modifying functions. BRD4 recruits P-TEFb via H3K9/14ac binding domain. Active P-TEFb phosphorylates RNAPII and UBE2A/DSIF, whereas NELF gets destroyed after phosphorylation. Phosphorylated UBE2A associates with RNF20/40 and monoubiquitinates histone H2B lysine 120 residues in histone core particles. The associated HMT NSD1 methylates H3K36. The HMTs DOT1 and CARM1 are able to methylate H3K79 and H3R2, R17 and R26, respectively. All these chromatin signatures enhance transcriptional processes. (C) The AF4–MLL fusion protein concurrently exerts functions of AF4 and MLL, thereby changing chromatin properties. CREBBP activates P-TEFb by K44-Acetylation (in the absence of BRD4), which results in ectopic activation of promoter-arrested RNA Pol II. The functions of the SET–domain complex, DOT1L and CARM1 result in chromatin signatures that confer active chromatin to enhance transcriptional processes. Adapted from (Benedikt *et al.* 2011).

Regulation of transcription by H3S10 phosphorylation

Although most of what is known about H3S10ph is in the context of mitosis and cell cycle regulation, newer studies have shown that it also plays a role in the activation of transcription. H3S10ph was discovered as a marker of transcription when, in *Drosophila,* it was found to be necessary for recruitment of P-TEFb at heat shock genes (Ivaldi *et al.* 2007). Two human H3S10 kinases, MSK1 and 2, mediate H3S10ph at c-Jun and c-Fos genes in response to various external stimuli. In mammals and yeast, H3S10ph in the presence of H3K9ac or H3K14ac induces the recruitment of 14-3-3 domain and ejectiontion of HP1γ from H3K9me3 sites (Macdonald *et al.* 2005, Waiter *et al.* 2008, Winter *et al.* 2008), which facilitates the release of RNAPII. It was also found that in 20% of c-Myc targeted genes in humans, c-Myc recruits the H3S10 kinase, Pim1, to enhancers of the *c-FosL1* and *ID2* genes. Depletion of Pim1 blocks transcription due to loss of S2 phosphorylation on the CTD of RNAPII suggesting that H3S10ph is necessary for P-TEFb recruitment and transcriptional elongation (Zippo *et al.* 2007).

In yeast, Gcn5 is recruited to acetylated H3K14 by Snf1-mediated phosphorylation of H3S10 (Fuchs *et al.* 2009). During transcription, RSC subunit, Rcs4, recruits RSC chromatin remodeling activities and binds to H3K14ac via its tandem bromodomains. Rsc4 recruits RNAPII via interactions with Rpb5. Rsc4 can also be directly acetylated by Gcn5, leading to inactivation of the complex (Fuchs *et al.* 2009). In flies, upon binding H3S10ph, 14-3-3 recruits Sas2 related HAT, MOF (males absent on first), which acetylates H4K16. H4K16ac results in the tandem acetylation of H3K9 and H3K14, which is bound by Brd4. As mentioned, Brd4 recruits P-TEFb and results in the release of promoter proximal RNAPII (Zippo *et al.* 2009). Moreover, H3K14 can also be acetylated by a MYST family HAT, MOZ (monocytic leukemia zinc finger protein), which is functionally related to Sas3 in yeast. It is commonly mutated in leukemia and is

essential in the maintenance of hematopoietic stem cells and normal development (Sapountzi and Côté 2011). Recently, it was found that MOZ's PHD12 finger binds to both H3K14ac and unmodified H3R2, which results in the increased expression of *HOXA9* (Qiu *et al.* 2012), a gene also overexpressed when hypermethylated on H3K79.

H3R2 Methylation

H3R2 methylation is highly conserved from yeast to humans (Guccione *et al.* 2007, Kirmizis *et al.* 2007) and can be monomethylated, symmetrically dimethylated (Rme2s) and asymmetrically dimethylated (Rme2a). H3R2me2s is catalyzed by PRMT5 and PRMT7 in humans (Migliori *et al.* 2012) and recently was discovered in yeast. The mark is deposited by Set1 and is tightly correlated with H3Kme4me3 at active promoters throughout the genome (Yuan *et al.* 2012). In humans, H3R2me2a is catalyzed mainly by PRMT6 (Guccione *et al.* 2007) but no homolog or culprit has been found in yeast since deletion of Hmt1, Hsl7, and Rmt2 does not affect levels of H3R2me2a (Kirmizis *et al.* 2007). H3R2me2a is mutually exclusive with H3K4me3 and like H3K79me3, marks the mid- to 3'- regions of genes in addition to inactive promoters (Guccione *et al.* 2007, Kirmizis *et al.* 2007). In yeast, H3R2me2a inhibits the recruitment of COMPASS subunit, Cps40, to H3K4 through its PHD domain due to steric hindrance imposed by H3R2me2a (Kirmizis *et al.* 2007), which abolishes H3K4 methylation (Schneider *et al.* 2005). Similarly, in humans MLL subunit, WDR5, is inhibited from binding to H3K4 via its WD40 domain (Guccione *et al.* 2007, Hyllus *et al.* 2007). Vice versa, H3K4me3 inhibits methylation of H3R2, which is important for transcriptional activation through recruitment of activating complexes (Iberg *et al.* 2008, Kirmizis *et al.* 2007, Guccione *et al.* 2007).

H3R2me2s, however, has the opposite effect as compared to H3R2me2a and has only been found in humans. PRMT5 and 7 catalyze the production of H3R2me2s at

euchromatic regions and regions marked with H3K4me3 (Yuan *et al.* 2012). H3R2me2s inhibits binding of RBBP7, the targeting subunit for Sin3a co-repressor complex while promoting binding of WDR5, a subunit found in various coactivator complexes (Migliori *et al.* 2012). Like H3R2me2s, H3R2me1 does not inhibit H3K4me, is localized to the CDS of genes, and has been linked to increased transcription (Kirmizis *et al.* 2009).

As described in chapter 2, H3K79 methylation by Dot1 is stimulated by Sas2 acetylation of H4K16 (Figure 2-9), which may facilitate the release of RNAPII into the elongation phase of transcription (Figure 2-10). The exact mechanism of how Dot1 is targeted to charomatin is still unkown. Many marks have been shown to correlate with actively transcribed genes, which may facilitate in the targeting of Dot1 to chromatin. To determine if Dot1 is targeted to chromatin by any of the marks discussed, a peptide binding study using Epicypher histone peptide arrays spotted with histone peptides from H3, H4, H2A, and H2B that bear various HPTMs was performed with recombinant Dot1. Results from this experiment were further analyzed by studying the effects of mutating various histone residues and deleting histone modifiers thought to act in the recruitment of Dot1. Special attention was paid to changes in H3K79 methylation and H4K16 acetylation.

A figure that summarizes the modifications discussed in this introduction is shown in figure 3-1, which is from a human study that purified AF4 and its fusion protein AF4-MLL for specific binding partners (Benedikt *et al.* 2011). As mention in the overall introduction, chromosomal translocations occurring with *MLL* result in various fusion proteins that mistarget Dot1 to genes that play a role in hematopoiesis. Due to the recruitment of Dot1, these gene bodies exhibit H3K79 hypermethylation and experience aberrant upregulation, resulting in excessive proliferation of white blood cells and acute myelogenous leukemia. Therefore, discovering novel ways in which Dot1 is regulated

by various transcriptional complexes may provide distinct modes of individual therapy in hope of conserved pathways from yeast to humans.

Results

Dot1 Binds to Unmodified H4 N-terminal Tail and Modified H3 Tail

The mechanism by which Dot1 is targeted to chromatin is still not clear based on the studies completed to date. Although it has been speculated that Dot1 is targeted by H2Bub either directly or indirectly, Dot1 targeting to chromatin does not change in H2BK123R mutants, thus invalidating this idea as the primary mechanism (Shabazian *et al.* 2005). Dot1L has been shown to bind to the phosphorylated CTD of RNAPII of actively transcribing RNAPII (Kim *et al.* 2012) but this cannot account for sites of H3K79 di- and trimethylation on inactive genes (Shahbazian *et al.* 2005). Therefore, in order to determine if Dot1 is targeted by histones, recombinant Dot1 was used to probe tiling arrays from Epicypher spotted with various H3 and H4 tail peptides bearing combinations of different HPTMs. As shown in figure 3-2, Dot1 seems to have the greatest affinity for unmodified H4 (aa 1-23). Previous studies have also confirmed that Dot1 does bind unmodified H4 tail with greater affinity than H4K16ac modified tail (Fingerman *et al.* 2007, Altaf *et al.* 2007). Dot1 also bound H3R2me2s/H3K4me2 and H3K4me3/K14ac/K18ac peptides, all of which are found at active promoters (Figure 3-2).

Figure 3-2

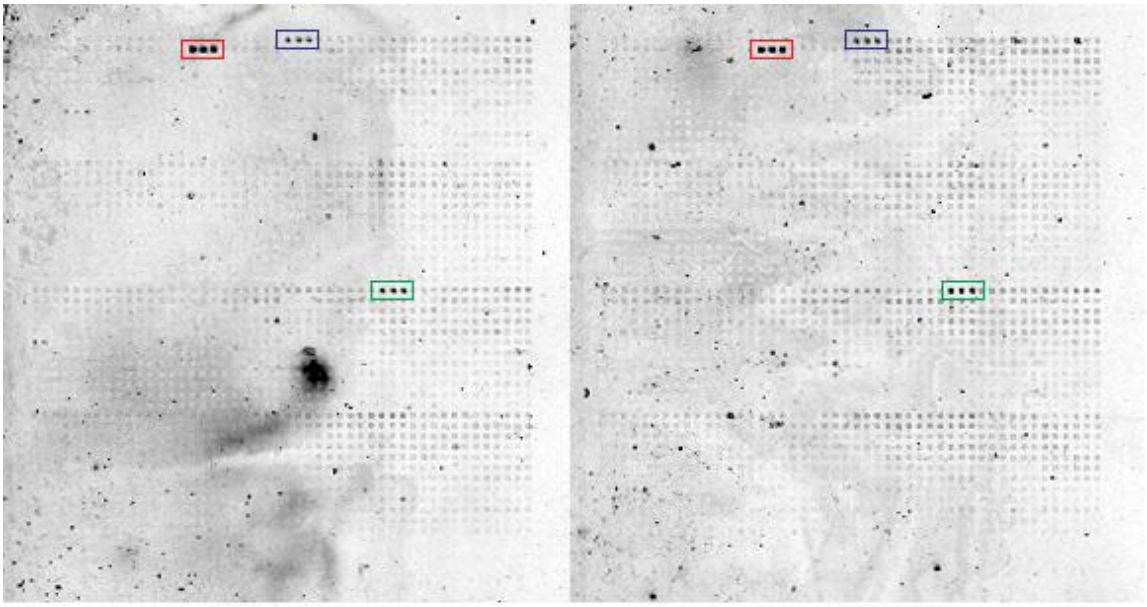


Figure 3-2: Dot1 binds to unmodified H4 tail and modified H3 tail. Purified recombinant Dot1 was incubated on Epicypher histone peptide arrays as instructed. Penta-his monoclonal antibody conjugated with Alexafluor 532 (Qiagen) was used to detect Dot1 binding on the array. The array was scanned using a Typhoon Trio at 50 µm resolution. Dot1 was shown to bind to triplicate peptides of H4 (1-23) (red box), H3R2me2s/H3K4me2 (blue box) and H3K4me3/K14ac/K18ac (green box).

Arginine and H3K4 methylation affect H3K79 methylation by Dot1

Previous studies have found that H3R2me2s highly correlates with H3K4me3 at transcriptionally active promoters in both yeast (Yuan *et al.* 2012) and humans (Migliori *et al.* 2012, Kirmizis *et al.* 2007). The effects of H3R2 and H3K4 methylation on H3K79 methylation were analyzed to gain a further understanding into the discovery that Dot1 bound to the dual modified H3 tail peptide, H3K4me3/H3R2me2s (Figure 3-3). H3K79me2, H3K79me3, and H4K16ac levels were analyzed in H3R2A, H3R2Q, and H3K4A mutants (Figure 3-3). Increases in H3K79me3 and H4K16ac were observed for the H3R2A strain. Although H3K79me2 levels remained unchanged in the H3R2A mutant, the bands for each of these modifications ran faster than wild-type suggesting a possible global loss of a charged modification such as phosphorylation or acetylation on histones marked with H3K79me2 or H4K16ac. H3R2Q exhibited slightly reduced H4K16ac while H3K79me levels remained normal. H3K4A showed a slight increase in H3K79me3 while H3K79me2 and H4K16ac levels remained normal.

Only three known histone arginine methylases have been identified in yeast, Hmt1, Hmt2, and Hsl7. Hmt1 has been shown to methylate H4R3 but, a H3R2 demethylase has not been elucidated (Di Lorenzo and Bedford 2011). Levels for H3K79me2, H3K79me3, and H4K16ac were analyzed in the three deletion mutants for Hm1, Hmt2, and Hsl7 (Figure 3-3). All three mutants exhibited an increase in H4K16ac and *hmt2Δ* and *hsl7Δ* showed a mild increase in both H3K79me2 and H3K79me3. Based on these results, it is likely that these putative arginine methylases do not play a significant role in transcriptional regulation through methylation by Dot1. Moreover, deletion of the H4R3 methylase, Hmt1, had the least effect on H3K79me suggesting that H4R3me does not influence the transcription of genes that are regulated by Dot1 methylation.

Figure 3-3

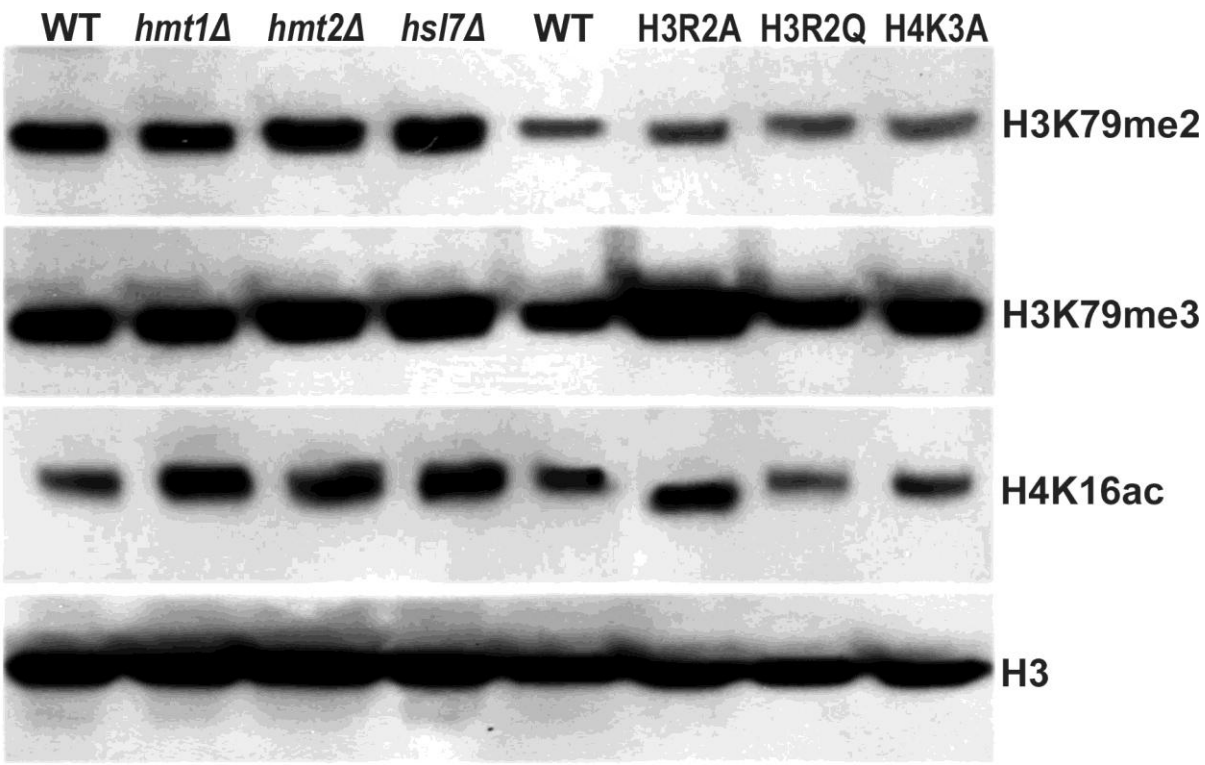


Figure 3-3: Arginine and H3K4 Methylation Affect Dot1 Activity. Acid extracted histones from whole cell lysates were run on an 18% SDS-PAGE gel, transferred to PVDF, and immunoblotted for H3K79me2, H3K79me3, and H4K16ac. H3 was used as a loading control. Hmt1, Hmt2, and Hsl7 mutants were obtained from the Open Biosystems Mat**a** deletion collection. H3R2 and H3K4 mutants and its wild-type strain were a gift from the Kirmizis lab and utilized in a previous study. (Kirmizis *et al.* 2007).

H3S10 Phosphorylation by Snf1 Inhibits H3K79 Trimethylation by Dot1

H3S10 phosphorylation has been shown to be very important for transcriptional activation and has also been linked to H4K16ac (Zippo *et al.* 2009). In yeast, H3S10ph is catalyzed by Ipl1 and Snf1. Phosphorylation of H3S10 by Ipl1 is related to cell cycle regulation whereas phosphorylation by Snf1 has been linked transcriptional regulation. Snf1 is a nutrient sensing kinase that promotes the activation of genes involved in metabolism (Lo *et al.* 2001). Analysis of the H3K79me and H4K16ac on *snf1Δ* histones was completed (Figure 3-4), since H3S10ph has already been linked to both transcriptional regulation and H4K16ac. Changes in the levels for these modifications upon deletion of Snf1 could also help elucidate if the size change seen in H3K79me2 marked histones is related to H3S10ph. As shown in figure 3-4, *snf1Δ* exhibit a large increase in H3K79me3 although H3K79me2 levels do not change. In agreement with previous studies (Zippo *et al.* 2009, Walter *et al.* 2008, Winter *et al.* 2008), H4K16ac decreases upon Snf1 deletion. Although the level of increase in H3K79me3 for *snf1Δ* cells is comparable to that of H3R2A, there is no size change in histones bearing H3K79me2 or H4K16ac. This suggests that the mechanism by which H3R2 methylation controls H3K79 methylation by Dot1 and acetylation of H4K16 by Sas2 may act in concert with Snf1, albeit via different regulatory pathways. Unlike Snf1 phosphorylation of H3S10, methylation of H3R2 may also serve as a regulatory pathway for histone tail proteolysis.

Figure 3-4

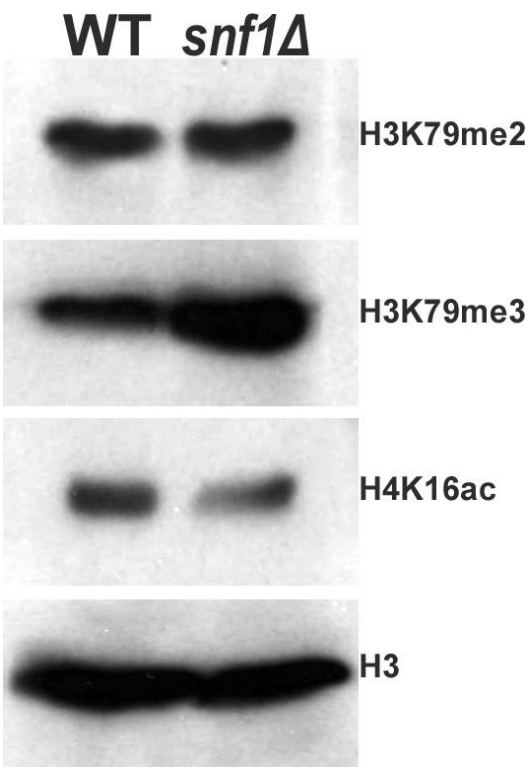


Figure 3-4: Snf1 negatively regulates H4K16ac-H3K79me3 crosstalk. Acid extracted histones from whole cell lysates were run on an 18% SDS-PAGE gel, transferred to PVDF, and immunoblotted for H3K79me2, H3K79me3, and H4K16ac. H3 was used as a loading control. Snf1 mutant was obtained from the Open Biosystems Mat**a** deletion collection.

Discussion

Dot1 binds but is unlikely targeted by histones H4 Tail

The data presented in this chapter shows that Dot1 HMT regulation depends on various modifications that have already been implicated in transcriptional control. Figure 3-2 shows that Dot1 has affinity for the H4 N-terminal tail and peptides marked with modifications linked to transcriptional activation. As proposed in chapter 2, an unacetylated H4 tail may facilitate targeting Dot1 to chromatin since previous studies have shown that Dot1 binds to both H4 and an H4K16Q mutant. It also binds in a charge-dependent manner as there is no loss in binding when lysines are mutated to arginines (Altaf *et al.* 2007). Therefore, it is not surprising that Dot1 would bind to the H4 tail with an appreciable affinity. Studies also revealed that although the H4 tail was necessary for Dot1 activity, Dot1 did not require it for nucleosomal binding (Fingerman *et al.* 2007, Altaf *et al.* 2007). Dot1 may act to protect the H4 tail in anticipation of Sas2 or MOF (humans), which acetylate H4K16. Previous studies illustrated that H3K79 methylation prevented nucleation of the SIR complex and gene silencing through inhibition of Sir3 binding (reviewed in Turner 2008). Later, it was found that Sir2-Sir4 heterodimer can still deacetylate H4K16ac despite the presence of H3K79 methylation. The same study also showed that Sir3 is not necessary for Sir2 HDAC activity (Oppikofer *et al.* 2011). Therefore, although the presence of H3K79me prevents Sir3 from binding, it still cannot prevent H4K16 deactylation by Sir2. Protection of the H4 tail by Dot1 on newly deposited histones may preserve the ability for genes to be temporally activated upon H4K16 acetylation. Figure 3-2 shows that Dot1 also binds to histone modifications that have been linked to transcriptional activation. These marks may act as a code for the targeting of Dot1 to specific genes that are ready to transition from transcriptional initiation to elongation.

Dot1 binds to histone acetyl-lysines catalyzed by SAGA

Histone acetylation is a very important HPTM that is associated with transcriptional activation. Dot1 was shown to bind to H3R2me2s/H3K4me2 and H3K4me3/K14ac/K18ac peptides in addition to the unmodified H4 tail (Figure 3-2). One study sought to distinguish a regulatory model for transcription between 181 transcription factors and levels of acetylation for 11 lysines at 3221 promoters in yeast. H4K16ac was shown to be regulated by only 2 transcription factors whereas the H3K18ac mark was the most widely regulated with association to 15 TFs (Pham *et al.* 2007). This suggests that H4K16ac regulation is involved in the regulation of a specific subset of genes and H3K18ac is a general marker of transcriptional activation. Moreover, Sum1 was linked to the regulation of both H3K14 and H3K18 acetylation (Pham *et al.* 2007).

In yeast, H3K14ac and H3K18ac are acetylated by Gcn5-containing HAT complexes in yeast, including SAGA (Spt–Ada–Gcn5 acetyltransferase) (Grant *et al.* 1997), ADA (Grant *et al.* 1999) and SLIK (Pray-Grant *et al.* 2002). Acetylation of these residues has been shown to confer transcriptional activation (Grant *et al.* 1998). Figure 3-5 shows the different subunits in contained in each complex, which are color coordinated according to function and spaced apart according to subunit interactions discovered using immunoprecipitation upon deletion of subunits, yeast two hybrid assays, and genetic screens (Lee *et al.* 2011). More recently, Sgf29 was shown to bind H3K4me2/3 via its tandem Tudor domains, which was shown to be important for acetylation of H3K9, H3K18, and recruitment of Gcn5 HAT activity to activated gene bodies and promoters (Bian *et al.* 2011). The chromodomain of Chd1 was previously implicated to have a similar function (Pray-Grant *et al.* 2005). Moreover, H4K16ac stimulates the catalysis of H3K14ac by SAGA and is dependent on the bromodomain of

Gcn5 (Li and Shogren-Knaak 2009). Therefore, Dot1 recruitment or stabilization at promoter regions may be dependent on SAGA/Gcn5's acetylation of H3K14 and H3K18 and COMPASS/Set1's ability to trimethylate H3K4.

Figure 3-6 is a model showing how these co-activator complexes are sequentially recruited to chromatin by activators at inducible genes such as *GAL1* (Weake and Workman 2010). Figure 3-6 shows a more accurate picture of the events surrounding gene activation as shown in figure 2-9B. Based on the peptide array data (Figure 3-2) and previous studies, Dot1 may be recruited to chromatin after COMPASS methylates H3K4, and SAGA acetylates H3K14/18. Therefore, loss of H3K79me upon Bre1 deletion may be due to loss of COMPASS recruitment and subsequent H3K4me. H3K14ac, H3K18ac, H3K4me3 may serve as a combinatorial code most favorable for Dot1 recruitment to specific genes or regulation of Dot1 activity. Acetylation of H4K16 may confer an additional level of regulation and complexity to the model. SAGA HAT activity's stimulation by H4K16ac (Li and Shogren-Knaak 2009) may promote a more rapid recruitment of Dot1 to promoters or favor its' retention there, and induction of gene activation.

H3R2 and H3K4 methylation affects Dot1 activity

H3R2A and H3K4A mutants exhibit reciprocal loss of H3K4me3 and H3R2me2s (Kirmizis *et al.* 2007, Yuan *et al.* 2012). H3R2A has been shown to abolish H3K4me3 while still exhibiting H3K4me1/2 (Kirmizis *et al.* 2007). Table 3-2 summarizes the results shown in figure 3-3 based on modifications present in the cells, change in the charge of H3R2/H3K4, and changes observed in H3K79me3 and H4K16ac. Based on these results, SAS-I and Dot1 seem to be dynamically regulated by both marks. In contrary to HPTM effects exerted in the H4K16A mutant (Figure 2-3), a large increase in both

H4K16ac and H3K79me3 was observed in H3R2A mutants. This may be attributed to the loss of Sas2 or Dot1 negative regulators that usually bind to H3K4me3, H3R2me2s, or unmodified H3R2. For example, in flies, MOZ acetylates H3K9 and K14, and has been shown to bind to H3K9ac, H3K14ac, and unmodified H3R2 via its PHD12 domain. H3K4me3 has been shown to lessen its binding. Similarly to Dot1L, MOZ has been shown to regulate *HOX* genes during development and the cell cycle (Qui *et al.* 2012, Ali *et al.* 2012). In humans, BPTF (bromodomain PHD finger transcription factor) binds to H3K4me2/3 via its PHD finger and H4K16ac with its bromodomain. Upon purification of H4K16ac and H3K4me3 marked histones, H3K4me2, and H3K79me2 were also enriched (Ruthenburg *et al.* 2011) suggesting that both Dot1 and SAS-I may be regulated by H3K4me. Figure 3-3 also suggests that H3K14ac may play an important role in SAS-I/Dot1 crosstalk as SAGA enrichment via Sgf29 is compromised upon loss of H3K4me2/3 while H3K14ac remains largely unaffected (Bian *et al.* 2011). Acetylation of H3K14 and K18 by the ADA complex (Grant *et al.* 1999) at sites of Dot1 recruitment is also possible, since the SAGA and SLIK subunit, Ubp8, has not been linked to H3K79me3 (Schulze *et al.* 2011). Although, a Ubp8-indepedent function for SAGA or SLIK may occur.

When all putative arginine methylases were deleted in a screen for changes in H3K79me and H4K16ac, an increase in H4K16ac was observed for all samples when compared to wild-type (Figure 3-3). Hmt1 is known to be an H4R3me2a methylase while the roles of Hsl7 and Hmt2 in histone arginine methylation have yet to be discovered. Deletion of Hsl7 and Hmt2 showed a subtle increase for all three marks suggesting that it may play a role in silencing.

Dot1 is regulated by Snf1 indirectly

Lastly, results from figure 3-4 will be discussed. Upon deletion of Snf1, H4K16ac decreases and H3K79me3 increases with no change in H3K79me2. Snf1 phosphorylates H3S10 in yeast and has been shown to unidirectionally facilitate acetylation of H3K14 by Gcn5 to activate gene transcription (Lo *et al.* 2001). Based on the peptide array data, and evidence supporting that Dot1 is recruited to active promoters by H3K14ac and H3K18ac (Figure 3-2) catalyzed by SAGA-related complexes, it is not a necessity that H3S10ph has an effect on SAS-I/Dot1 crosstalk. Instead, Snf1-mediated H3S10 phosphorylation could stimulate SAGA activity, thus bypassing any requirement for SAS and H4K16ac. The possibility that Dot1 or any subunit in SAS-I could be regulated by Snf1 by serving as a substrate is also possible. Similar to Snf1, SAS-I has been implicated in the regulation of genes related to nutrient utilization (Raisner and Madhani 2008).

Figure 3-5

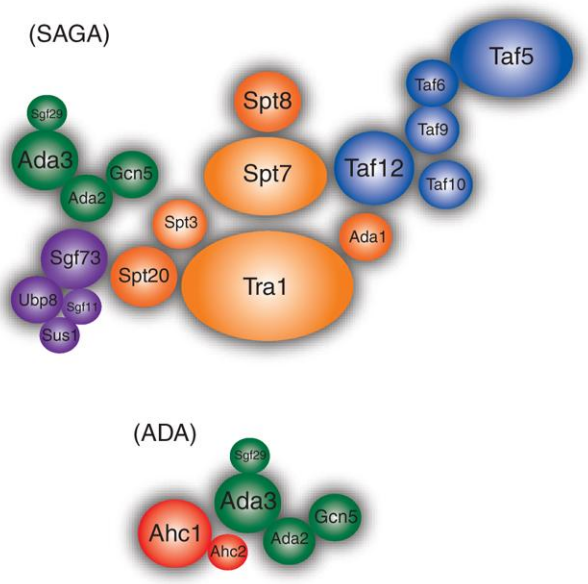


Figure 3-5: Subunit composition of SAGA and ADA HAT complexes in yeast. Subunits are color coordinated according to function including: recruitment module (orange), the acetylation module (green), the TBP interaction unit (blue), and the deubiquitinase module (purple). ADA specific subunits are labeled in red. The SLIK complex (not shown) is highly related to SAGA and contains Rtg2, but lacks Spt8. Space between subunits represents a measure of proximity between subunits. Subunits that are touching symbolize direct interactions founded by yeast two-hybrid data. Other subunits have been analyzed using deletion purifications, yeast two-hybrid and gene deletion experiments (Lee *et al.* 2011).

Table 3-2: Effects of H3R2 and H3K4 on H4K16ac and H3K79me3

Mutants	HPTM Present on Histones			Charge of Residues		Changes Compared to WT	
	H3K4me2	H3K4me3	H3R2me2s	H3K4	H3R2	H4K16ac	H3K79me3
H3R2A	+	-	-	+	+/-	+	+
H3R2Q	+	-	-	+	-	-	normal
H3K4A	-	-	-	+/-	+	normal	+

+ mutant contains a HPTM/positive charge/increase

- mutant lacks a HPTM/negative charge/decrease

+/- neutral/no change

Figure 3-6

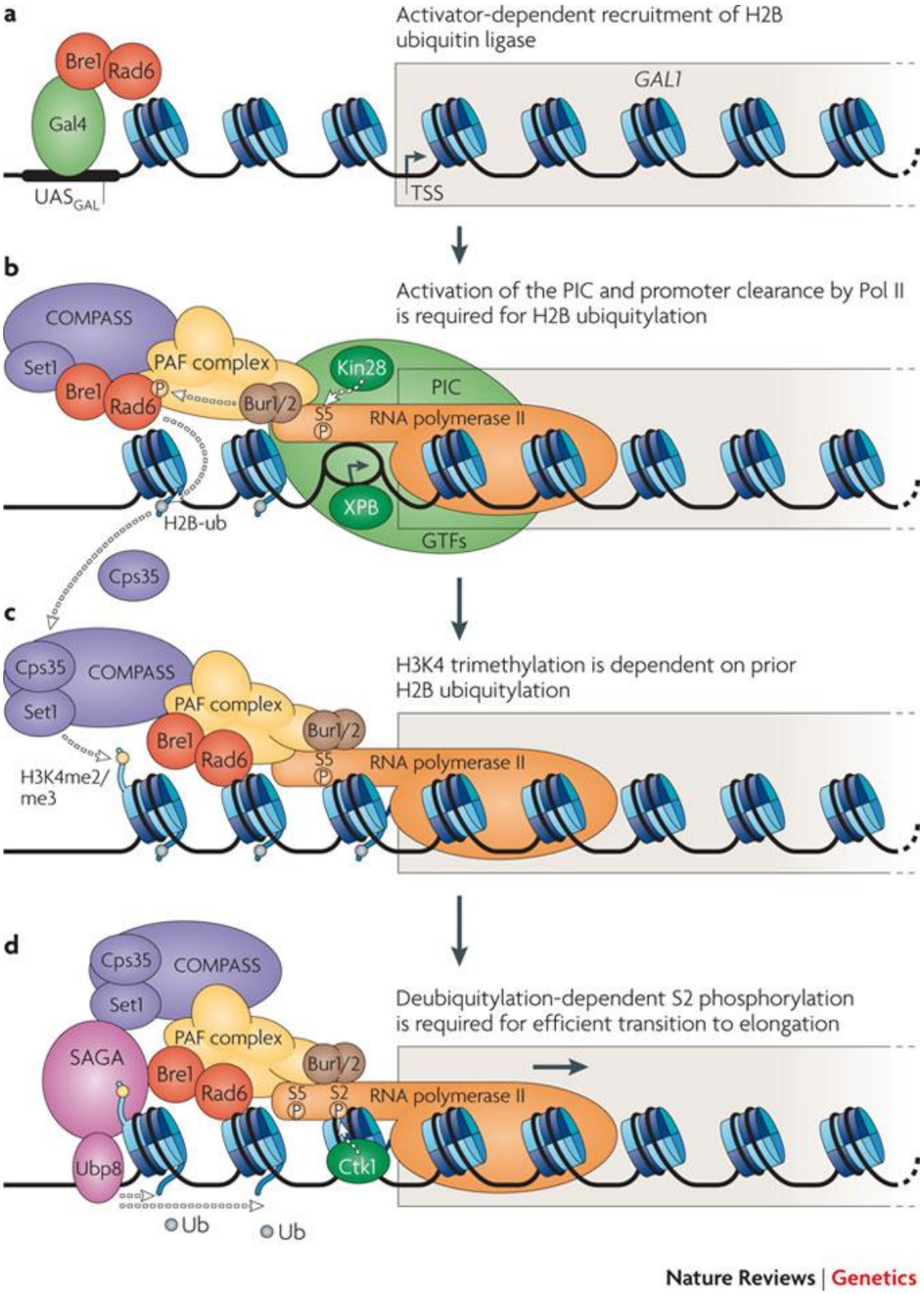

a
Activator-dependent recruitment of H2B ubiquitin ligase
Bre1
Rad6
Gal4
UAS$_{GAL}$
GAL1
TSS
b
Activation of the PIC and promoter clearance by Pol II is required for H2B ubiquitylation
COMPASS
Set1
Bre1
Rad6
PAF complex
Bur1/2
Kin28
PIC
S5
RNA polymerase II
XPB
GTFs
H2B-ub
Cps35
c
H3K4 trimethylation is dependent on prior H2B ubiquitylation
Cps35
COMPASS
Set1
PAF complex
Bre1
Rad6
Bur1/2
S5
RNA polymerase II
H3K4me2/ me3
d
Deubiquitylation-dependent S2 phosphorylation is required for efficient transition to elongation
Cps35
COMPASS
Set1
SAGA
PAF complex
Bre1
Rad6
Bur1/2
S5
S2
RNA polymerase II
Ctk1
Ubp8
Ub
Ub
Nature Reviews | Genetics

Figure 3-6: Sequential chromatin modifications during *GAL* gene induction. (a) Upon induction, Gal4 activator is recruited to the upstream activation sequence. H2BK123 (H2Bub) ubiquitinase complex, Rad6/Bre1 is recruited. (b) Pre-initiation complex and transcription factors recruit RNAPII to promoter. PAF associated Rad6/Bre1 ubiquitinates H2Bub, which results in a cascade of events that release RNAPII from initiation. DNA helicase begins unwinding DNA and RNAPII CTD S5 is phosphorylated. (c) Cps35 is recruited via H2Bub and COMPASS methylates H3K4. (d) SAGA subunit, Ubp8, or Ubp10 deubiquitinates H2Bub. SAGA or ADA complex acetylates H3 and H3ac or Ctk1 activated RNAPII recruits Dot1, which dimethylates H3K79. SAS-I complex acetylates H4K16ac allowing Dot1 to trimethylate H3K79. RNAPII is released into elongation phase (Weake and Workman 2010).

CHAPTER 4: MATERIALS AND METHODS

Yeast Strains and Plasmids

Strains used in this study are listed in Table 4-1. Recombinant Dot1 was expressed from pET28a-His_6-Dot1 and was constructed by amplification of yeast genomic *DOT1* using primers:

Dot1BamHI CGGGATCCATGGGCGGTCAAGAAAGTATATC

Dot1NotI TTTTCCTTTTGCGGCCGCTCATCTGGTATACTTCAC

Primers and *DOT1* were flanked with BamHI/NotI restriction sites that were used to ligate *DOT1* into pET28a using T4 DNA Ligase. Plasmids were verified by sequencing. Enzymes used during cloning are from New England Biolabs and were used per instructions included with the enzymes.

Histone H4 mutants were generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). Plasmid pQQ18, which contains all four wild-type histone genes (*HHT2, HHF2, HTA1, HTB1)* was used as a template for the mutagenesis reaction. Each mutant plasmid was verified by DNA sequencing. Mutant plasmids were transformed into JHY205 and grown on complete synthetic media plates lacking leucine (CSM-LEU) in order to select for successful transformation of pQQ18. Colonies were patch plated on to CSM plates supplemented with 5'-fluroorotic acid in order to counterselect for pHJ33 plasmid. Loss of pHJ33 was verified by lack of growth on CSM-URA plates.

Immunoblotting

Histone samples used in immunoblotting experiments were isolated by TCA extraction. 50 mL of yeast cells were grown in YPD to an OD_{600} of ~1.0, harvested by centrifugation, and washed 2x with sterile water. Cells were resuspended in 250 µl of concentrated HCl and left on ice for 1 hour. 125 µl of HCl was added to the cells, which

were then broken with the addition of glass beads and vortexed for a total of 5 minutes. Supernatant was cleared by centrifugation at 20,000 g for 10 minutes to which 18% TCA was added. Histones were precipitated overnight on ice. Histone pellets were washed 1x with acetone supplemented with 0.1% HCl, 1x with acetone, and dried. Pellets were resuspended in 200 µl of 2x SDS sample buffer, boiled for 10 minutes, and electrophoresed on a 18% SDS-Page gel. Histones were transferred to PVDF membrane in Towbin buffer supplemented with 0.0375% SDS at 100V for 120 minutes.

Immunoblotting was performed in TBST with 3% BSA. Membranes were blocked with 3% BSA solution for 1 hour at room temperature. Antibodies used in this study were H3K79me1 (Abcam ab2886), H3K79me2 (Abcam ab3594), H3K79me3 (Abcam ab2621), H4K16ac (Millipore 07-329) and H3 C-terminal (Active Motif 39164). H3K79 antibodies were used at a 1:1000 dilution in TBST+3% BSA and incubated O/N at 4°C. H3 C-terminal and H4K16ac antibodies were used at 1:2000. Secondary antibodies were used at a 1:10000 and incubated with the membranes for 1 hour at room temperature. After thorough washing of the membrane, ECL was added to the membrane, exposed to film, and developed. Densitometry measurements were taken using ImageJ software.

In vitro histone methyltransferase assays

In vitro HMT assays were completed as previously described (Fingerman *et al.* 2008) with some modifications. Oligonucleosomes from mutant strains were isolated from yeast using a protocol that has been previously described (Zhang and Reese 2006). Nuclei were resuspended in 1.0 mL of digestion buffer (50 Tris, pH 7.5, 1 mM $MgCl_2$, 2 mM $CaCl_2$) per 100 mL of cell culture grown to an OD_{600} of ~ 2.0. Protein concentrations were measured using a BCA assay (BioRad). Samples were diluted

accordingly based on concentrations to ensure uniform oligonucleosome sizes. Nuclei were treated with micrococcal nuclease (Worthington) at 10 Units/mL for 10 minutes at 37° C and quenched with 10 mM EDTA. Reactions were centrifuged at ~10,000 g for 10 minutes and supernatant was dialyzed O/N at 4°C in dialysis buffer (50mM Tris, pH 8.0, 10% glycerol, 100 mM NaCl). Samples were equalized according to protein concentration and used in HMT assays.

BL21 DE3 pLysS cells harboring pET28a-His_6-Dot1 plasmid were grown in LB supplemented with 50 µg/mL Kanamycin and 30 µg/mL Chloramphenicol. Bacteria were induced with 0.4 mM IPTG at an OD_{600} of ~0.8 for 4 hours at room temperature. An uninduced control was also grown for the same amount of time. Cells were centrifuged, resuspended in 100 µL/mL of culture of lysis buffer (20 mM Tris pH 8.0, 300 mM NaCl, 2 µg/mL leupeptin, aprotinin, pepstatin, 1 mM PMSF), and lysed using lysozyme at 1.0 mg/mL for 30 minutes on ice. Lysate was centrifuged at 20,000 g for 20 minutes, supernatant was decanted and glycerol was added to 10%. 10 µg of oligonucleosomes were used in 20 µl reactions with 2 µl of bacterial extract and 1x HMT buffer (20 mM Tris pH 8.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10 mM sodium butyrate, 100 µM S-adenosyl-methionine, 5 mM nicotinamide, 1mM PMSF). 1.0 µM of peptide was supplemented to the peptide stimulated reactions. Peptides were purchased from Millipore (12-346, 12-347). Reactions were incubated at 30° C O/N, electrophoresed on an 18% SDS-Page gel, transferred to PVDF membrane, and immunoblotted for H3K79me2 and H3K79me3.

Chromatin Immunoprecipitation

Yeast cells were grown in YPD to an OD_{600} of ~1.0 and fixed in 1% formaldehyde for 15 minutes at room temperature. Fixation was quenched with 125 mM glycine for 5

minutes. Samples were processed as previously described (Kuo and Allis 1999) with some modifications. Cells were lysed in a mini bead beater using 8 cycles of 40 seconds on and 1 minute off. Chromatin shearing was done using a Diagenode water bath sonicator set on high for 20 cycles of 30 seconds on/off. Real time PCR data was collected and analyzed using a BioRad iCycler. Telomeric primers used were previously characterized in a study that analyzed similar modifications (Shia *et al.* 2006). ORF primers used in this study were:

SMC4: GAACAGAGCGAACAACTGAAGGA, ATCGTCTAACATTCCCAGGGTGT

MSD3: CGTAGATCGTCGCATATCGGTAG, GGAAAGAGTTCATCCTTGGCTGT

Data was analyzed using percent input and normalized to H3 signal. Antibodies used are noted in the immunoblotting section.

Dot1 Purification

2.0 L of bacteria harboring pET28a-His_6-Dot1 were grown, induced, and harvested as in the HMT assay section. Cells were resuspended in 5.0 mL of lysis buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 1% TWEEN-20, 10 mM imidazole, 2 µg/mL leupeptin, aprotinin, pepstatin, 1 mM PMSF) per gram of cell pellet. Following incubation with lysozyme, cells were frozen using liquid nitrogen and thawed at 37°C for 3x cycles (Johnson and Hecht 1994). Cell pellets were spun down at 20,000 g for 30 minutes. The supernatant was loaded on to Ni^{2+}-NTA agarose resin (Qiagen) and bound O/N at 4°C. The resin was washed with lysis buffer plus 20 mM imidazole and eluted from the resin with lysis buffer supplemented with 300 mM imidazole. The eluate was dialyzed O/N into low salt buffer containing 100 mM NaCl and no imidazole. Dialyzed eluate was loaded onto a Mono Q HR 5/5 column (Amersham Pharmacia). Bound proteins were eluted with a 25-ml linear gradient from 100 to 600 mM NaCl.

Fractions were analyzed on 10% SDS-Page gels by both coomassie staining and immunoblotting (Invitrogen P-21315 antibody at 1:2000) for purity. Proteins were transferred to nitrocellulose membrane and immunoblotting was performed as described above. Fractions exhibiting no contamination were pooled and concentration using Centriprep YM-30 centrifugal filtering devices (Millipore).

Histone Peptide Array

Epicypher histone peptide arrays were probed with recombinant His_6-Dot1 purified in the previous section. All solutions were 0.2 micron filtered. Arrays were incubated with 1xTBST (pH 7.5) + 5% BSA at 4°C for 30 minutes. 200 µL of 1 µM recombinant Dot1 in 1xTBST + 3% BSA was placed on the slide and covered with a large coverslip. Arrays were incubated in a humidified chamber O/N at 4°C. The array was washed 3 times for 10 minutes with cold 1xTBST followed by incubation with Penta·His Alexa Fluor 532 Conjugate (Qiagen 35330) antibody for 1 hour at 4°C. The array was washed 3 times with cold TBST and 2 times with room temperature TBS. The array was centrifuged at 800 g for 2 minutes and scanned using a Typhoon Trio (Excitation: Green, PMT: 650, Sensitivity: Normal, Resolution: 50 µm).

Table 4-1: Yeast Strains

Background	Genotype	Gifted By
BY4741[h]	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	
BY4741	h sas5Δ::KanMX	
BY4741	h dot1Δ::KanMX	
BY4741	h sas2Δ::KanMX	
BY4741	h ubp8Δ::KanMX	
BY4741	h bre1Δ::KanMX	
BY4741	h hmt1Δ::KanMX	
BY4741	h rmt2Δ::KanMX	
BY4741	h hsl7Δ::KanMX	Mitch Smith
JHY205	h hhf2-hht2::NAT hta1-htb1::HPH hhf2-hht2::KAN hta2-htb2::NAT + pJH33-HHT2-HHF2-HTA1-HTB1-URA3	
JHY205	h hhf2-hht2::NAT hta1-htb1::HPH hhf2-hht2::KAN hta2-htb2::NAT + pQQ18[HHT2-hhf2-K16A-HTA1-HTB1-LEU2]	
JHY205	h hhf2-hht2::NAT hta1-htb1::HPH hhf2-hht2::KAN hta2-htb2::NAT + pQQ18[HHT2-hhf2-K16Q-HTA1-HTB1-LEU2]	Lucy Pemberton
JHY205	h hhf2-hht2::NAT hta1-htb1::HPH hhf2-hht2::KAN hta2-htb2::NAT + pQQ18[HHT2-hhf2-K16R-HTA1-HTB1-LEU2]	
W303-1b[j]	MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1	Mitch Smith
W303-1b	j esa1-C304S:URA3	Mitch Smith
W303-1b	j esa1-E338Q:URA3	Mitch Smith
JHY6	MAT**a** ura3-52 lys2-801 ade2-101 trp1-289 his3Δ1 leu2-3,112 Δhhf2-hht2 Δhhf1-hht1 pMR206[TRP1-HHT2-HHF2]	Antonis Kirmizis
JHY6	MAT**a** ura3-52 lys2-801 ade2-101 trp1-289 his3Δ1 leu2-3,112 Δhhf2-hht2 Δhhf1-hht1 pMR206[TRP1-hht2-R2A-HHF2]	Antonis Kirmizis
JHY6	MAT**a** ura3-52 lys2-801 ade2-101 trp1-289 his3Δ1 leu2-3,112 Δhhf2-hht2 Δhhf1-hht1 pMR206[TRP1-hht2-R2Q-HHF2]	Antonis Kirmizis
JHY6	MAT**a** ura3-52 lys2-801 ade2-101 trp1-289 his3Δ1 leu2-3,112 Δhhf2-hht2 Δhhf1-hht1 pMR206[TRP1-hht2-K4A-HHF2]	Antonis Kirmizis
IPY36T[c]	MAT**a** his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG	Michael Kobor
IPY36T	c ubp10Δ::HIS5+	Michael Kobor

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

The Ubiquitin Dilemma

Based on previous studies, Dot1 was long thought to be recruited to chromatin via its putative ubiquitin binding domain. *In vitro*, Dot1 was shown to bind both human and yeast H2Bub directly and indirectly. Dot1 was shown to bind ubiquitin directly through a lysine rich region (amino acids 101-140). Moreover, deletion of this region resulted in loss of nucleosomal binding and catalysis of H3K79me2/me3 (Oh *et al.* 2010). This data was supported by the confirmed loss of H3K79me2/me3 in *bre1Δ* cells. Dot1 also binds to Cps35, a subunit in the COMPASS complex, which interacts with H2BK123ub and is required for H3K79me3 (Ezhkova and Tansey 2004, Lee *et al.* 2007). Nonetheless, studies have showed that H2Bub results in a structural change in the nucleosome and acts concomitantly with H4K16ac to further open chromatin (Feitz *et al.* 2011).

Data presented in chapter 2 also supports the theory that Dot1 activity is not regulated by ubquituin. Although, the question remains as to what purpose the lysine rich region contained in Dot1 serves. It could encompass a putative bromodomain as data from chapter 3 shows that Dot1 binds to acetyl-lysine *in vivo.* Moreover, ubiquitin is a sizeable molecule (over half the size of a histone), with varying charges located on its surface. H2BK123 is also close to the acidic patch located between H2A and H2B as shown in Figure 1-5. This has been shown to be important for chromatin condensation as the unmodified H4 tail seems to be sequestered in this region. It would seem likely that addition of an 8kDa molecule that is charged would result in intranucleosomal changes at the least. A model showing how exactly ubiquitin and H4K16ac would act to open chromatin is shown in figure 5-1 (Peterson 2011).

Dot1 is also a relatively enzyme (~60kDa) and its substrate, H3K79, is located in the core of the nucleosome. Access to H3K79 may be hindered by nucleosomal

condensation and from an evolutionary stand-point, it seems more energetically favorable to regulate the deposition of HPTMs through structural alterations as opposed to creating a cascade for every new methyl mark added to the core of a nucleosome. Set1 is over 100 kDa and has not been shown to methylate any residues at the core of the nucleosome. An interesting study would be too analyze the difference is sizes of possible histone modifiers in relation to their substrate location on the nucleosome. This could potentially rule out possible enzymes during screens for novel modifications.

Figure 5-1

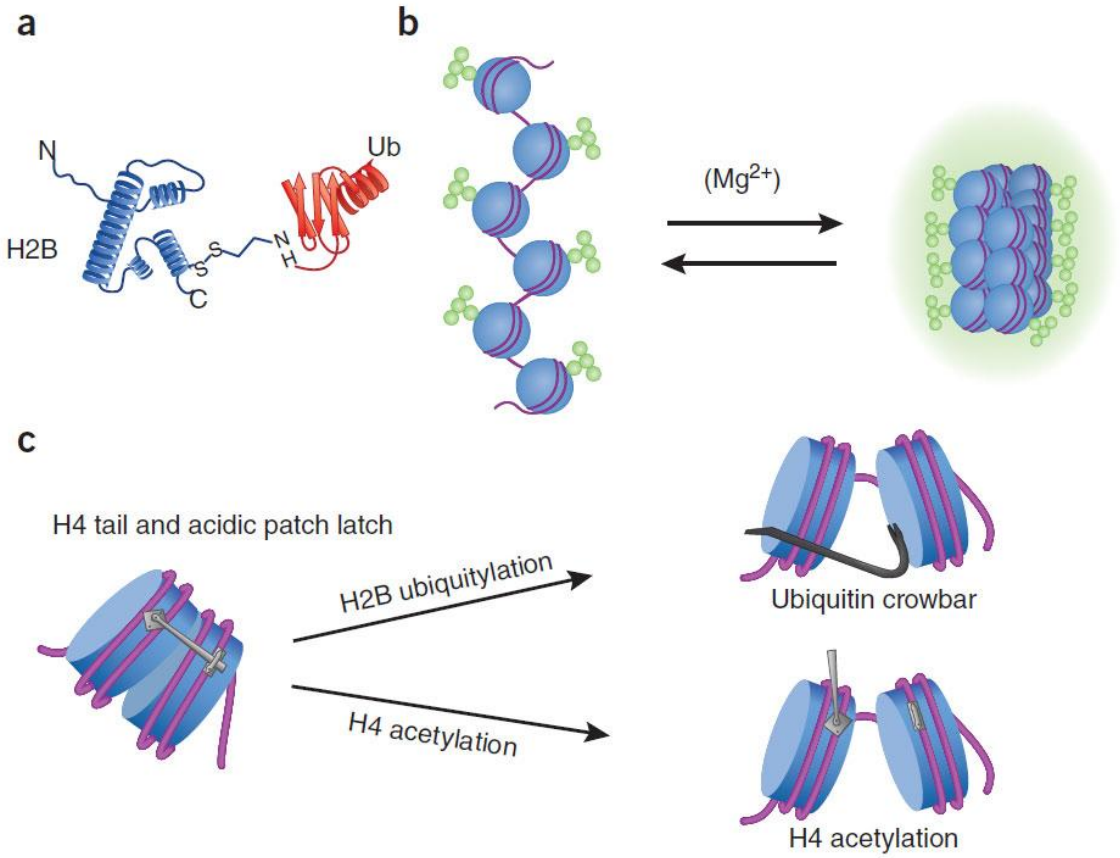


Figure 5-1: H2B ubiquitination and H4K16 acetylation act to open chromatin via different mechanisms. (**A**) H2B is shown in blue and ubiquitin in red (**B**) Studies using HOMO-Fret analyzed chromatin compaction upon the addition of magnesium. (**C**) Disruption of chromatin folding by H4 acetylation or H2B ubiquitylation. The H4 N-terminal tail interacts with an acidic patch on the H2A/H2B dimer surface to form a nucleosome-nucleosome contact that is essential for array condensation. This chromatin 'latch' is disrupted by H4K16ac. The C-terminal tail of H2B lies at the interface of each pair of nucleosome disks within a folded fiber. H2BK123ub may disrupt fiber folding by disrupting nucleosome-nucleosome stacking interactions. (Peterson 2011)

Dot1 Recruitment via COMPASS

Dot1 is involved in a plethora of processes. It has been linked to transcriptional regulation, DNA damage response, silencing, cell cycle regulation, and development. It is a ubiquitous enzyme that methylates ~90% of histones in yeast (van Leeuwen *et al.* 2002). Surprisingly, no histone demethylase has been identified for H3K79me, suggesting the possibility that it may have the capacity to store epigenetic memory in order to preserve active and inactive after DNA replication (Schulze *et al.* 2009).

According to the data presented, Dot1 bound to two separate peptides containing H3K4me2 and H3K4me3 modifications. Both are catalyzed by COMPASS subunit, Set1 and sites of Paf1C recruitment. Paf1C subunits, Rtf1 and Paf1 are necessary for H2BK123 ubiquitination and subsequent H3K4 and H3K79 trimethylation, which is accomplished by the subunits' facilitation in associating Rad6 with active RNAPII (Krogan *et al.* 2003, Wood *et al.* 2003, Ng *et al.* 2003). Moreover, a direct interaction between Paf1C and Bre1 was shown *in vitro* suggesting that Paf1C targets Rad6/Bre1 to RNAPII during transcriptional elongation (Kim and Roeder 2009). Once H2BK123 is ubiquitinated, COMPASS via Cps35 is recruited, which is required for H3K4 and H3K79 di- and trimethylation. Cps35 is recruited in an H2Bub-dependent and Set1-independent manner and is necessary for H3K79 methylation (Lee *et al.* 2007). Dot1 could be recruited to sites of transcriptional elongation via Cps35. However, Dot1 and Cps35 have not been shown to physically interact. Therefore, based on the peptide array data, Dot1 may be recruited independently of Cps35 and targeted after H3K4 methylation by COMPASS.

SAS-I and RNAPII Pausing

Along with H3K4me and H3K79me, H2BK123ub has been shown to mark the bodies of active genes that contain inducible promoters (Schule *et al.* 2011). Microarray analysis of transcript levels H2BK123R mutants revealed that H2BK123ub may repress genes rather than activate them throughout the yeast genome due to an increase in expression for many genes in the mutant (Mutiu *et al.* 2007). A similar study was completed with *sas2Δ* mutants and exhibited a similar phenomenom. Although histone acetylation is usually correlated with an increase in gene expression, Sas2 has also been linked to transcriptional repression, specifically at the 3' end of longer genes (Heise *et al.* 2012). Consistent with repressive functions, removal of ubiquitin by SAGA subunit, Ubp8, is required for induction of genes, including *GAL1*, *GAL10*, and *SUC2* (Daniel *et al.* 2004, Kao *et al.* 2004, Henry *et al.*2003). Furthermore, Paf1C was also shown to repress a subset of genes, including the *ARG1* gene, through the facilitation of H2BK123 ubiquitination (Crisucci and Ardnt 2011). As shown in chapter 2, H4K16ac does decreases similarly in *Δdot1* and *Δbre1* strains, suggesting that H2BK123ub may also play a role in targeting SAS-I to inducible promoters. A similar amount of H4K16ac loss for both strains may suggest that H4K16ac loss is correlated to sites regulated by both Dot1 and Bre1. The following section discusses putative binding partners that may target Dot1 and SAS-I concomitantly.

Dot1 and SAS-I are linked through mutual binding subunits

I decided to research what other binding partners were linked to the subunits of the SAS-I complex or Dot1 that may implicate a mechanism for the regulation of their catalytic. A database of yeast protein interactions has already been established for this purpose (Figure 5-2) (Collins *et al.* 2007). For brevity, functions and definitions for subunits were taken from *Saccharomyces* genome database. Two subunits that were not included in figure 5-2: 1) TIM13 is involved in delivering hydrophobic molecules to inner membrane of mitochondria and 2) Subunit has an undefined function.

Dot1 was shown to correlate with two subunits that validate a link between SAS-I, Dot1, and COMPASS. It was shown to correlate with Cps25, a subunit of the COMPASS complex that is conserved from yeast to humans (Takahashi *et al.* 2011). It also correlated with Arp6, a component in the Swr1 complex that exchanges H2A for H2A.Z. Previous studies have shown that H2A.Z incorporation is closely linked to H4K16ac deposition by the SAS-I complex (Shia *et al.* 2006). H2A.Z has also been linked to dynamically regulated genes involved in hematopoiesis (Abraham *et al.* 2013).

SAS-I subunits were also linked to subunits found to be linked to Dot1 and H3K4me effectors. The core components of SAS-I bound to one another with an approximate correlation of 0.5 and its associated subunits bound with an average coefficient of 0.3 (Collins *et al*. 2011). Based on interaction studies, added subunits for SAS-I may include a unbiquitination component (Ubc5/Ubp5), an H2A.Z incorporation subunit (Swc7), and a WD40 containing subunit of Set3c HDAC complex (Sif2). Moreover, Ubc5 may solve the dilemma of an undiscovered demethylase for H3K79 as it has been shown to mark H3 for degradation. In addition, most these subunits also have human paralogs. Moreover, that additive molecular weight of this complex is near 400kDA which would explain the discrepancies in SAS complex sizes upon purification.

Figure 5-2

Figure 5-2: SAS-I complex and binding partners form a larger complex. Binding partners for each subunit contained in SAS-I were founded using a yeast protein interaction grid that was obtained from genetic screens in deletion mutants. Subunits that are touching showed a correlation of binding of ~0.3. Future experiments need to be completed to verify these subunits as actual binding partners.

CHAPTER 6: REFERENCES

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