INVESTIGATION OF THE ROLE AND REGULATION OF HISTONE H2B UBIQUITYLATION IN TRANSCRIPTION

Glenn Gerard Wozniak

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology.

Chapel Hill
2014

Approved by:

Brian Strahl

Scott Bultman

Jean Cook

Beverly Errede

Greg Matera

ABSTRACT

Glenn Gerard Wozniak: Investigation of the Role and Regulation of Histone H2B Ubiquitylation in Transcription
(Under the direction of Dr. Brian Strahl)

In eukaryotes, DNA is packaged by histone proteins to form nucleosomes – the fundamental unit of chromatin. Aside from their structural role in DNA compaction, histones are dynamic regulators of DNA accessibility and, hence, are important for DNA-templated processes including transcription and DNA repair. To regulate chromatin architecture, histones are covalently modified with numerous post-translational modifications (PTMs), e.g., methylation, acetylation, phosphorylation and monoubiquitylation. Histone PTMs function by either directly altering chromatin structure or serving as binding sites for effector proteins that mediate downstream functions. Because histone PTMs regulate many cellular processes, their specific deposition and removal throughout the genome are highly regulated. Accordingly, dysregulation of histone PTMs can result in human diseases such as cancer. One PTM that is carefully regulated and whose disruption results in disease is monoubiquitylation of lysine 123 on histone H2B (H2BK123ub1). The work in this dissertation focuses on understanding how H2BK123ub1 is regulated and functions with an emphasis on its role in transcription. These studies were performed using the highly tractable model organism *Saccharomyces cerevisiae*. Here, two novel forms of H2BK123ub1 regulation are identified. First, H2BK123ub1 was found to be regulated by a region of histone H2A

in a form of *trans*-histone regulation. This finding led to identification of a second form of regulation, which couples H2BK123ub1 catalysis to the stability of the responsible modifying enzyme Bre1. Lastly the functional role of H2BK123ub1 in transcription is expanded by connecting Bre1 stability to the regulation of gene silencing and by the finding that a histone PTM downstream of H2BK123ub1 facilitates interactions between histones and the transcriptional machinery. Altogether, the work in this dissertation expands our knowledge of the role and regulation of H2BK123ub1. These findings will help guide future studies focusing on H2BK123ub1 in chromatin regulation and disease.

ACKNOWLEDGEMENTS

I thank Dr. Brian Strahl for his support and guidance during the course of my graduate education. I also thank Brian and members of the Strahl lab (past and present) for creating a laboratory environment that challenged and encouraged me to think creatively. In addition, I thank my committee members Dr. Scott Bultman, Dr. Jean Cook, Dr. Beverly Errede and Dr. Greg Matera for their feedback and guidance.

I owe many thanks to my parents Barb and Greg, my brother Alex and my girlfriend Becca for their support and encouragement as well as for reminding me that there is more to life than just science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3HA	triple hemagglutinin
5-FOA	5-fluoroorotic acid
5mC	5-methylcytosine
6-AU	6-azauracil
ac	acetylation
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CENP-A	centromeric protein A
ChIP	chromatin immunoprecipitation
CHX	cyclohexamide
CTD	C-terminal domain
Da	dalton
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
GST	glutathione s-transferase
H2BK123	histone H2B lysine 123
H3K4	histone H3 lysine 4
H3K36	histone H3 lysine 36
H3K79	histone H3 lysine 79
H4K16	histone H4 lysine 16
HAR	histone H2A repression
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMD	histone modification domain

HRP	horseradish peroxidase
IP	immunoprecipitation
mA	milliampere
me	methylation
me1	monomethylation
me2	dimethylation
me3	trimethylation
ml	milliliter
mM	millimolar
NFR	nucleosome free region
NTP	nucleotide triphosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PHD	plant homeodomain
PTM	posttranslational modification
PVDF	polyvinylidene fluoride
RING	really interesting new gene
RNAPII	RNA Polymerase II
RT-PCR	reverse transcriptase polymerase chain reaction
SC	synthetic complete
SDS	sodium dodecyl sulfate
SET	Su(var)3-9, Enhancer of zeste and Trithorax
SeMet	selanomethionine
SIR	silent information regulator
SPOC	Spen paralogue and orthologue

TBS	Tris-buffered saline
TEL	telomere
TLD	TFIIS-like domain
TSS	transcriptional start site
ub1	monoubiquitylation
WCE	whole cell extract
WT	wild type
YPD	yeast extract, peptone, dextrose

CHAPTER 1 INTRODUCTION[1]

The human body displays an amazing diversity of structures and functions. Heart cells beat in unison to keep blood flowing through arteries, capillaries and veins. A layer of skin cells protects our insides from the surrounding environment. A vast network of neurons communicates via electrical pulses to allow us to process and respond to stimuli. The body is also highly resistant to change and has a stunning ability to adapt, which is evident in our immune system where a multitude specialized cells sense and fight off infection. Given the incredible specification and adaptability of cells within the human body described in these few examples, it is remarkable that every cell originates from just a single cell and, hence, shares the same set of instructions – the genome.

Over the past decades, efforts including and stemming from the Human Genome Project have helped clarify the basis of cellular diversity by demonstrating that the genome contains a massive amount of information in the form of genes. These genes not only encode the basic functions required for each cell such as metabolism and the ability to divide, but also include the information required for specialized functions. Given that every cell shares the same genes, several primary questions still remain. To begin, how does the any given cell select and utilize the necessary set of genes for a

[1] Portions of this chapter were adapted from Wozniak GG, Strahl BD. 2014. Hitting the 'Mark': Interpreting Lysine Methylation in the Context of Active Transcription. *Biochim Biophys Acta*. doi: 10.1016/j.bbagrm.2014.03.002.

given function? At the same time, how does a cell ensure that other genes, which may be detrimental in the given cellular context, are not inappropriately activated? Lastly, how is the pattern of gene activation and repression recapitulated after each cell division and even between parent and offspring? These questions form the basis of the field of epigenetics, which seeks to understand how the genome is utilized and regulated in order to uncover the mechanisms of establishment, alteration and maintenance of cellular diversity. The basic components of epigenetics and its advances in understanding gene regulation will be discussed here in further detail.

Chromatin Structure and Function

In the eukaryotic cell DNA does not exist on its own, but instead exists in a compacted structure called chromatin. The main, repeating, functional until of chromatin is called the nucleosome, which consists of 147 base pairs of DNA wrapped around a octameric protein complex made up of two copies of each of the four core histone proteins H2A, H2B, H3 and H4 (Luger et al. 1997; Kornberg and Lorch 1999). The histone proteins are small (~100-130 amino acid) and positively charged, which allows them to bind to the negatively charged DNA. Structurally, histones consist of unstructured N- and C-terminal tails and a globular central domain (Luger et al. 1997). As discussed in detail below, the histone tails play a prominent role in chromatin regulation. Once formed into nucleosomes, the histones facilitate the compaction of DNA into a structure referred to as the 30-nm fiber (Horn and Peterson 2002). Then, with the help of the non-core histone H1 and other proteins like cohesin and condensin, the DNA is further condensed into the poorly defined higher order structures observed in

the fully compacted mitotic chromosome (Kornberg and Lorch 1999; Horn and Peterson 2002; Hagstrom and Meyer 2003).

Functionally, chromatin serves multiple purposes. First, it acts to compact and protect the DNA from damage (e.g. breaks and recombination) and ensure the faithful passage from mother to daughter during cell division. Second, and in addition to its general compaction and protective function, chromatin acts as plastic barrier to the underlying DNA sequence (Li and Reinberg 2011; Voss and Hager 2014). It is this function that plays a primary role in specifying cellular identity, as the cell is able to manipulate chromatin structure to make different regions of the genome more or less accessible to the machinery of myriad DNA-template processes.

As a result of this manipulation, chromatin generally exists in two general states: euchromatin and heterochromatin (Li et al. 2007; Trojer and Reinberg 2007). The two states, originally distinguished by staining intensity as observed using cytological techniques, represent accessible and inaccessible regions of the genome, respectively. Euchromatin encompasses gene-rich and transcriptionally active loci. Depending on the activity at a given locus, euchromatin displays a range of compaction that also includes the complete removal of histones as observed at the most highly transcribed genes. Heterochromatin displays more variability and, hence, is further divided into two subtypes called constitutive and facultative (Trojer and Reinberg 2007). Constitutive heterochromatin is always repressed and mainly exists at specific chromosome features including telomeres, centromeres and at repetitive DNA elements such as transposons. Facultative heterochromatin consists of repressed genomic loci that may also exist as

euchromatin in other cellular contexts such as during development on in response to stimuli.

The ability of chromatin state to alter over the course of development and in response to stimuli is an important component of cellular specification and adaptation. As a result, the transition between states is heavily regulated and involves a multitude of components. Described below are the mechanisms by which the chromatin state is demarcated, altered and maintained.

DNA-Based Determinants

Aside from encoding genes and accompanied regulatory elements, DNA sequence itself can contribute to the structure and function of chromatin. One way this can occur is by promoting or preventing histone binding to modulate nucleosome formation. For instance, sequences rich in the bases adenine and thymine (AT-rich) will not form nucleosomes as readily as those with guanine and cytosine (Segal et al. 2006; Kaplan et al. 2009). Consistently, AT-rich DNA is often found at promoter regions of genes where DNA accessibility is a hallmark feature. Given that DNA sequence is static, unlike chromatin states, the extent to which it contributes to nucleosome formation, however, has not been fully determined.

DNA also contributes to chromatin through the binding of transcription factors (Figure 1.1). These proteins are capable of binding DNA with or without specificity and either harbor the activity to directly modify chromatin structure or recruit other proteins to carry out the same task (Rothbart and Strahl 2014). Transcription factors often display

specific expression patterns such that they are only found in specific cell types or under certain environmental conditions. The carefully regulated expression of these factors in addition to the specificity of binding across the genome plays an important role in dictating gene expression or repression in differing cellular contexts.

Adding another dimension to DNA is its ability to be modified. DNA can be methylated on the carbon 5 position of cytosine (5mC) by enzymes called DNA methyltransferases (Jones 2012). DNA methylation is also dynamic and can be removed, generating intermediate modifications including hydroxymethyl-, formyl- and carboxycytosine, which are ultimately repaired to cytosine. Canonically, 5mC is associated with gene repression and is partly thought to function by preventing the binding of transcription factors, which would otherwise alter chromatin accessibility (Jones 2012). Additionally, certain proteins contain domains that are capable of recognizing and binding 5mC and its derivatives (Bartke et al. 2010; Spruijt et al. 2013). Many of these proteins are found in complexes that function in gene repression such as histone deacetylases and chromatin remodelers, both of which alter chromatin structure and are discussed below. Lastly, the ability of DNA methylation to promote or prevent transcription factor binding helps explain, in part, why transcription factors are not found at every possible binding site across the genome. Taken together, both DNA sequence and its modifications can impinge on chromatin.

Histone Variants

As described above, histones constitute the main structural element of chromatin, however, every histone across the genome is not the same (Figure 1.1). Owing to the

fact that chromatin structure must be altered to govern DNA accessibility, histone proteins display considerable variability across the genome. Thus, by altering the histones associated with DNA, the cell can maintain the structure of chromatin and at the same time modulate the functional output of a given genomic region.

One way histones can be altered is through the use of histone variants. With the exception of histone H4, alternate versions exist for the core histones (Maze et al. 2014). Overall, the structures of the variants remain similar to that of the core histones, but can differ in both amino acid sequence and length. The inclusion of histone variants into nucleosomes can have varying affects on chromatin structure and can either act to stabilize or destabilize nucleosomes. One of the most conserved histone variants is the H2A variant H2A.Z, which is found in organisms ranging from yeast to human (Zlatanova and Thakar 2008). This variant, in particular, has been well studied with regard to its function. It is thought to destabilize nucleosomes and, interestingly, has been proposed to play a role in maintaining the memory of recent transcription at multiple genomic loci (Brickner et al. 2007; Zlatanova and Thakar 2008). Consistently, H2A.Z is typically found incorporated in the nucleosomes flanking the transcription start sites of genes (Zlatanova and Thakar 2008). Part of its destabilizing function may arise from its sequence differences from H2A, which include the alteration of a region important for inter-nucleosomal interactions. Another highly conserved variant called CENP-A replaces canonical H3 exclusively at centromeres and plays an important role in kinetochore formation (Black and Cleveland 2011; Maze et al. 2014). Therefore, the replacement of core-histones with variants can introduce variability in chromatin.

Histone Modifications

In addition to the use of histone variants in demarcating chromatin, histone proteins (canonical and variant) can be modified with a large number of post-translational modifications (PTMs, Figure 1.1). These include acetylation, methylation, phosphorylation, ubiquitylation and sumoylation as well as less characterized and defined modifications including ADP-ribosylation, citrullination, and glycosylation (Peterson and Laniel 2004; Kouzarides 2007; Bannister and Kouzarides 2011; Tan et al. 2011). Through the use of more sensitive mass spectrometry techniques, the number and types of modifications known continues to grow (Pesavento et al. 2004; Young et al. 2010). Histone PTMs primarily occur on the unstructured N- and C-terminal tails of the histone proteins where accessible and modifiable amino acid residues are enriched, however, a number of functionally significant modifications also occur within the globular domain (Luger et al. 1997; Kouzarides 2007; Bannister and Kouzarides 2011; Tan et al. 2011).

Functionally, histone PTMs can alter chromatin structure both directly and indirectly. The direct affect on chromatin structure mostly arises through alterations of amino acid charge. The histone tails are enriched in positively charged residues such as lysine and arginine, which may interact with the negatively charged DNA to create a repressed chromatin structure (Kornberg and Lorch 1999). Lysine acetylation, for example, can neutralize the positive charge, which is one reason it is considered a

modification associated with DNA accessibility (Kouzarides 2007). Histone phosphorylation (negative charge) may play a similar role.

It is thought, however, that the primary role by which histone PTMs affect chromatin structure is through their role in facilitating interactions with proteins and/or protein complexes that have the ability to directly alter chromatin (Kouzarides 2007; Bannister and Kouzarides 2011; Rothbart and Strahl 2014; Wozniak and Strahl 2014) (Figure 1.1 and 1.2). A number of protein domains (so-called "reader" domains) have been identified that recognize histone PTMs. Moreover, additional domains exist that are unable to bind histones when a given PTM is present (Figure 1.2A). Thus, like DNA methylation, histone modifications can help recruit proteins to chromatin, while at the same time oppose those that may otherwise play an aberrant role in chromatin.

Histone PTMs typically do not occur independently, but instead exist in distinct patterns that are thought to serve specific functions across the genome (Ruthenburg et al. 2007; Taverna et al. 2007; Young et al. 2010; Bannister and Kouzarides 2011; Musselman et al. 2012). This idea is the basis of the "histone code" hypothesis (Strahl and Allis 2000; Jenuwein and Allis 2001). Towards this idea, it has been observed that a number of proteins associated with chromatin contain more than one domain capable of recognizing histones (Jacobson et al. 2000; Vermeulen et al. 2007; Ruthenburg et al. 2011). It is thought that these proteins are able to "read" a particular chromatin state as dictated by the histone PTM pattern to faithfully perform its function only in specified genomic contexts. A notable example of this is a subunit of the NURF chromatin remodeling complex called BPTF, which is capable of simultaneously recognizing

trimethylation of lysine 4 on histone H3 (H3K4me3) and acetylation of lysine 16 on histone H4 (H4K16ac) through the PHD (plant homeodomain) finger and bromodomain, respectively (Ruthenburg et al. 2011). As described above, DNA methylation can also contribute to transcription factor binding specificity. It is now clear that the combination of histone modifications and DNA methylation can function together to dictate factor binding across the genome (Bartke et al. 2010; van Nuland et al. 2013). Consistently several proteins have been identified that can simultaneously recognize histone modifications and DNA methylation. One of these proteins is UHRF1, which is capable of binding methylation of lysine 9 on histone H3 and hemimethylated DNA (Arita et al. 2008; Rothbart et al. 2012a) (Figure 1.3A). Thus, multiple components exist to create the distinct chromatin patterns observed across cell types.

Mechanisms of Chromatin Alteration

As described above, chromatin states are demarcated by a number of structural features. However, when the cell accesses a previously repressed region of chromatin or represses an active region, a number of enzymatic processes must function together to alter chromatin structure. A general overview of these activities follows.

Histone Modifying Enzymes

Histone modifications act as landmarks of specific chromatin states and serve to recruit proteins capable of physically remodeling chromatin (Kouzarides 2007; Bannister and Kouzarides 2011; Rothbart and Strahl 2014; Wozniak and Strahl 2014) (Figure 1.2). Thus, altering histone modifications is an important step in the transition between

chromatin states. Histone modifications are governed by a wide array of enzymes and enzyme complexes, which typically display high specificity for a given histone residue. Histone modifying enzymes can also be separated into two types: “writers” and “erasers” (Gardner et al. 2011). These two enzyme classes catalyze either addition or removal of PTMs, respectively (Figure 1.2C). It is important to note that the recruitment of “writers” and “erasers” is not always mutually exclusive. Co-recruitment of both enzyme types is an important part in altering PTM patterns across the genome. Moreover, a competition between “writing” and “erasing” activities ensures a fine balance of histone PTMs and thus keeps chromatin accessibility properly regulated (Katan-Khaykovich and Struhl 2002; Black et al. 2012).

Recruitment of histone modifying enzymes occurs either alone or as part larger protein complexes. As with transcription factors, both DNA and histone recognition play an important role in enzyme recruitment (Rothbart and Strahl 2014). The enzymes utilize conserved domains that are able to bind specific histone modifications and/or DNA to catalyze modifications only in a defined chromatin context. As an interestingly extension of this concept, some histone modifiers are able to bind the modification that they catalyze, which may promote propagation of a modification across a specified genomic locus (Shi et al. 2007b; Milne et al. 2010; Eberl et al. 2013) (Figure 1.2D).

Chromatin Remodelers

Once a chromatin state is demarcated, it can be altered to change accessibility to the underlying DNA. The most basic mechanism by which chromatin structure is altered

is through the use of ATP-dependent chromatin remodelers (Clapier and Cairns 2009; Narlikar et al. 2013). Numerous proteins and protein complexes have been identified with chromatin remodeling activity, which includes the ability to deposit, slide and/or evict nucleosomes from chromatin (Figure 1.1). In addition, some of these complexes, for example the SWR complex in budding yeast, are able to facilitate the incorporation of variant histones into chromatin (Mizuguchi et al. 2004). Four general families of chromatin remodelers have been identified, which include SWI/SNF, ISWI, CHD and INO80 (Clapier and Cairns 2009; Narlikar et al. 2013). Each of these families contains multi-subunit complexes with a central ATPase catalytic subunit. While certain subunits and complex architectures are shared within a family, the activities of the specific complexes on chromatin can vary. For example, most complexes of the ISWI family function by maintaining the equal spacing of nucleosomes on DNA, however, the NURF complex can promote random spacing (Clapier and Cairns 2009).

Chromatin remodelers play a role in all DNA-templated processes including transcription, DNA replication and DNA repair. Although, the mechanisms by which chromatin remodeling activity is specified at different regions of the genome or how complexes are recruited to chromatin is, largely, still not understood. It is apparent that both histone and DNA modifications play an important role (Figure 1.2A). Interestingly, and as described above, chromatin-remodeling complexes often contain multiple chromatin and DNA binding domains that may increase the specificity of binding across the genome (Kasten et al. 2004; Ferreira et al. 2007; Ruthenburg et al. 2011).

Alternatively, the modifications may stimulate or attenuate the activity of the complexes that have already been recruited to chromatin.

Histone Chaperones

Working in concert with chromatin remodelers are proteins called histone chaperones (Avvakumov et al. 2011; Burgess and Zhang 2013). These proteins have the capability of binding free histones and facilitating their deposition and removal across the genome (Figure 1.1). Free histones can have a detrimental effect in the cell by binding non-specifically with the DNA template, thus histone chaperones help maintain the highly regulated process of histone incorporation and removal across the genome and throughout the cell cycle. One phase of the cell cycle where chaperones are especially important is during S-phase where a large pool of histones is generated for incorporation into the replicated strand of DNA. A cascade of histone chaperones including Asf1, CAF-1, and Rtt106 help facilitate this process. Histone chaperones also regulate replication-independent histone deposition such as during transcription where histones are removed and deposited during the passage of RNA polymerase (Li et al. 2007; Avvakumov et al. 2011).

While a number of histone chaperones have been described, each of them performs distinct functions. Many show specificity for either H3/H4 (Asf1) or H2A/H2B (Nap1), but some exist that can bind both (FACT) (Avvakumov et al. 2011; Burgess and Zhang 2013). Moreover, other chaperones are capable of binding histone variants such as H3.3 (Daxx and HIRA) or H2A.Z (Chz1) (Luk et al. 2007; Elsaesser and Allis 2010). Chaperones that bind the same histone can also display specificity for certain genomic

loci, as is the case for HIRA and Daxx, which associate with euchromatin and heterochromatin, respectively (Goldberg et al. 2010). This specificity can be contributed by a number of factors including recognition of specific histone modifications on the bound histone or through the association with distinct complexes such as chromatin remodelers (Clapier and Cairns 2009; Avvakumov et al. 2011).

General Mechanisms of Transcription

Chromatin ultimately impinges on all DNA-templated processes, but has been most well studied in the process of transcription (Berger 2007; Li et al. 2007; Rando and Winston 2012). This role is especially evident in the process of cellular specification where chromatin regulates large-scale, genome-wide changes in gene expression. Prior to exploring the role and regulation of chromatin in transcription, basic mechanisms and selected players of transcription will be discussed.

The Transcription Cycle

Transcription is defined by a number of highly regulated steps, which entail recruitment of RNA polymerase II (RNAPII) to genes and ultimately, the production of a fully processed and functional RNA molecule. The first phase of transcription is referred to as initiation and takes place at regions upstream of the transcription start site (TSS) called promoters (Figure 1.4). During initiation general and gene-specific transcription factors facilitate the recruitment and binding of RNAPII to the promoter to form the pre-initiation complex (Nikolov and Burley 1997; Shandilya and Roberts 2012). In addition to the promoter, sequences found both up- and downstream called enhancers interact

with other gene-specific transcription factors to promote initiation (Calo and Wysocka 2013). Once initiation is completed, RNAPII begins transcribing DNA into RNA and initial processing events occur such as the addition of the 7-methylguanosine cap on the 5' end of the RNA molecule. In higher organisms such as *Drosophila melanogaster* and humans, RNAPII may associate with the NELF (negative elongation factor) complex leading to transcriptional pausing just downstream of the TSS (Adelman and Lis 2012). This promoter proximal pausing, is thought to allow rapid induction of stress response genes in response to external stimuli by bypassing initiation. Either directly after initiation or after pause release by P-TEFb (positive transcription elongation factor), transcription progresses into the elongation phase where RNAPII continues along the length of the gene transcribing in a processive manner (Fuchs et al. 2009; Kwak and Lis 2013). During elongation RNA splicing occurs in a co-transcriptional fashion. Finally, elongation continues until a termination sequence is reached and the nascent RNA is processed with the potential 3' polyadenylation for its export from the nucleus.

C-Terminal Domain (CTD) Phosphorylation

The transcription cycle is highly coordinated to ensure faithful production and processing of the RNA molecule. An important player in coordinating the steps of transcription is RNAPII itself, and in particular the C-terminal domain (CTD) (Fuchs et al. 2009; Heidemann et al. 2013). The CTD is unique in that it contains multiple copies (26 in *S. cerevisiae*, 52 in *H. sapiens*) of a highly conserved heptapeptide repeat (YSPTSPS). The CTD is thought to function in transcription by serving as a binding

platform for a multitude of proteins capable of facilitating distinct transcription-associated events (Hsin and Manley 2012). Adding specificity to these binding events is the ability of the CTD repeats to be altered by modifications, which include phosphorylation of tyrosine 1, serine 2, threonine 4, serine 5 and serine 7 (Hsin and Manley 2012; Heidemann et al. 2013). Interestingly, each modification is only found on the CTD when RNAPII is at a specified region of a gene. For example, the CTD is phosphorylated at serine 5 when RNAPII is in the promoter and 5' end of the gene, while serine 2 is phosphorylated when RNAPII is in the gene body (Figure 1.4). Additionally several modifications can co-exist on the CTD as is the case for serine 5 and serine 2 phosphorylation in the middle of genes. The modifications are also dynamic and cyclical as evidenced by the fact that serine 5 of the CTD becomes dephosphorylated at the 3' end of genes. At the end of transcription all modifications are removed allowing RNAPII to restart the transcription cycle.

This cycle of CTD modifications plays an important role in co-transcriptional processing and does so by recruiting proteins (Fuchs et al. 2009; Hsin and Manley 2012). It is now evident that a number of proteins are capable of binding the CTD and akin to histone binding proteins, recognize specific modification states. Importantly, the CTD modification cycle must be carefully regulated to ensure proper control of gene expression. Changes in the modification states can lead to defects in transcription length or frequency as well as improper processing that can have adverse effects for the cell as a whole.

The Polymerase-Associated Factor (PAF) Complex

As described above, the CTD can serve as a binding site for numerous proteins. Some of the first proteins identified to associate with the CTD constitute a protein complex called the Polymerase-Associated Factor (PAF) complex (Shi et al. 1996; Wade et al. 1996; Jaehning 2010). The PAF complex is a five subunit complex consisting of the namesake protein Paf1 as well as Cdc73, Ctr9, Leo1 and Rtf1. Human PAF complex contains an additional subunit called Ski8 (Zhu et al. 2005). Functionally, the PAF complex plays diverse roles during transcription, but mainly functions in promoting transcription elongation and 3' end processing of select small RNAs (Jaehning 2010). Accordingly, the complex appears to associate with RNAPII during initiation and continue throughout the 3' ends of genes (Figure 1.4). The PAF complex mediates its functions by serving as a platform for protein recruitment. Interestingly, a number of these proteins are able to alter chromatin structure as described below. The complex also stimulates phosphorylation of serine 2 of the RNAPII CTD, which may explain its role in mediating the later phases of transcription (Fuchs et al. 2012). The role of the PAF complex in regulating chromatin is discussed further below.

Chromatin and Transcription

Chromatin plays a large role in transcription by regulating access to specific regions of the genome. As described above, numerous mechanisms exist in the regulation of chromatin structure. Discussed here are mechanisms of chromatin regulation during transcription with a particular emphasis on active transcription.

Chromatin Remodeling During Transcription

Transcription does not take place on a "naked" DNA template, but in the context of a compacted chromatin environment. Thus, chromatin acts as a significant physical barrier to the recruitment and binding of transcription factors as well as the passage of RNAPII. In order for transcription to occur, the barrier must be overcome by remodeling the chromatin. This process begins at promoter regions where DNA sequences required for transcription factor binding can be masked by the nucleosomes. Through interplay of transcription factors and chromatin remodelers, the promoter sequence can be unmasked leading to the creation of a nucleosome free regions (NFR) and formation of a transcriptionally competent initiation complex. One well-studied chromatin remodeler in this process, which may display gene-specific function, is the SWI/SNF complex (Yudkovsky et al. 1999; Clapier and Cairns 2009).

Once RNAPII transitions into the elongation phase, a distinct set of activities comes into play to allow passage of RNAPII across the gene. These activities include the coordinated removal and deposition of nucleosomes. While RNAPII is capable of dislodging histones from DNA on its own, chromatin remodelers assist in this function. Several chromatin remodelers function in this context especially those of the SWI/SNF, ISWI and CHD families like the RSC complex and CHD1 (Clapier and Cairns 2009; Narlikar et al. 2013). As described above the remodelers can play multiple roles including nucleosome sliding and eviction, although it is unclear how these activities are recruited to distinct loci to perform the specified function. Histone modifications may play a role, as is the case for CHD1 and RSC (Figure 1.2A). CHD1 in humans is able to

bind histone H3 methylated at lysine 4 and RSC is able to associate with H3 acetylated at lysine 14 (Kasten et al. 2004; Flanagan et al. 2005; Sims et al. 2005). Other complexes, namely the PAF complex can also facilitate recruitment since the subunit Rtf1 can interact with CHD1 (Simic et al. 2003).

While remodelers slide nucleosomes and regulate occupancy, mechanisms are also in place to ensure that histones are not lost from the gene bodies. This is the function of histone chaperones. Two important histone chaperones associated with transcription are the FACT complex and Spt6 (Hartzog et al. 1998; Orphanides et al. 1999). The FACT complex has been shown to bind all histones whereas Spt6 binds H3 and H4. As with CHD1, the PAF complex appears to play an important role in the recruitment of both chaperones (Squazzo et al. 2002; Dronamraju and Strahl 2014).

Loss of histone chaperones and chromatin remodelers lead to altered DNA accessibility in gene bodies, which can negatively affect transcription. Increased accessibility can increase the rate of DNA damage or recombination. Additionally many genes contain sequences that mimic promoters called “cryptic” promoters. The failure to restore nucleosome structure following the passage of RNAPII can expose these “cryptic” promoters and promote transcription (Cheung et al. 2008). Some of these “cryptic” transcripts can be translated into protein, but the process of “cryptic” transcription itself can decrease the transcriptional efficiency of the upstream promoter by titrating away the transcription machinery. Thus, regulation of chromatin architecture by remodelers and chaperones is important for maintaining the correct patterns of gene expression.

Histone Acetylation

The activity and localization of chromatin remodelers and chaperones is highly regulated during transcription. This regulation is mediated, in part, through histone PTMs. Three types of modifications that have been most well defined in transcription are acetylation, methylation, and monoubiquitylation. One of the first histone modifications linked to transcription is acetylation, which is found on a number of residues on each of the histones. Generally histone acetylation is associated with active transcription (Kouzarides 2007; Bannister and Kouzarides 2011). This is based on that fact that acetylation can neutralize the positive charge of lysine residues on histones to prevent DNA and internucleosomal interactions, thus promoting chromatin accessibility. Acetylation also functions by recruiting proteins such as the general transcription factor TFIID and the RSC chromatin-remodeling complex - both of which have domains called bromodomains that are capable of binding acetylated lysine (Jacobson et al. 2000; Kasten et al. 2004) (Figure 1.3B).

A number of histone acetyltransferases (HATs) function on chromatin. The HATs show specificity for each of the core histones and can function either at promoters or in the gene body. One of the most well studied HATs is Gcn5, which is capable of acetylating lysine residues on the H3 N-terminal tail (Kuo et al. 1996; Grant et al. 1997). Gcn5 exists as part of a complex called SAGA, which functions at promoters to facilitate chromatin remodeling and transcription initiation (Grant et al. 1997). Opposing the activity of the HATs are the histone deacetylases (HDACs). These enzymes remove

acetylation and play an important role in reducing chromatin accessibility. Paradoxically, HDACs can actually have a positive affect on transcription, by preventing the use of “cryptic” promoters, which as described above, can exist in gene bodies (Carrozza et al. 2005; Keogh et al. 2005; Li et al. 2009). HDACs reduce the accessibility of “cryptic” promoters to increase transcription efficiency. One such HDAC in budding yeast is Rpd3, which associates with RNAPII and histones during transcription elongation and suppress new transcription from “cryptic” promoters (Carrozza et al. 2005; Keogh et al. 2005; Li et al. 2009).

Histone Methylation

Another modification associated with transcription is histone methylation. Unlike acetylation, which is mainly associated with active transcription, methylation can play positive or negative roles based on the residue being modified (Bannister and Kouzarides 2011; Black et al. 2012). Active transcription is associated with methylation of lysine 4, 36 and 79 on histone H3, whereas repression involves methylation of lysine 9 and 27 on histone H3 and lysine 20 on histone H4. Arginine methylation is also associated with transcription, but interestingly, methylation of a single residue can play opposite roles in transcription depending on how it is methylated (symmetric or asymmetric) (Kouzarides 2007; Bannister and Kouzarides 2011). Methylation functions during transcription, as described above by either recruitment or repulsion of proteins involved in transcriptional activation or repression (Kouzarides 2007; Bannister and Kouzarides 2011; Wozniak and Strahl 2014) (Figure 1.2). While a number of

transcription-associated proteins have been identified to bind histone methylation, future work will be needed to uncover the full extent of reader proteins. One novel reader protein is further explored in Appendix A.

Histone H3 Lysine 4 Methylation

One modification that has been well studied with regards to its function in chromatin recruitment is methylation of lysine 4 on histone H3 (H3K4me). In budding yeast, where much work has been performed on elucidating the role of chromatin in transcription, a single SET domain-containing histone methyltransferase, Set1, mediates H3K4 methylation as part of the COMPASS complex. In humans SET1A, SET1B and the mixed lineage leukemia proteins MLL1-4 perform the same function, albeit in an apparent context-dependent manner (Shilatifard 2012).

Up to three methyl moieties can be added to a single lysine and it is thought that the number of moieties contributes to unique cellular function (Black et al. 2012). Trimethylation of H3K4 (H3K4me3) has been strongly linked to the process of transcription initiation and is most highly enriched at the nucleosome just downstream of the NFR centered at the TSS of actively transcribed genes (Strahl et al. 1999; Santos-Rosa et al. 2002; Schubeler et al. 2004; Bernstein et al. 2005; Pokholok et al. 2005) (Figure 1.5). Consistent with promoter localization, H3K4me3 requires S5ph of the CTD of RNAPII as well as the PAF complex (Ng et al. 2003b). Interestingly, and in contrast to H3K4me3, monomethylation (H3K4me1) is a modification associated with enhancer

elements (Calo and Wysocka 2013) (Figure 1.5). The role and regulation of this methylation state, however, has not been as fully investigated.

Of all the histone modifications H3K4me has been most well studied in its ability to recruit proteins to chromatin. For example, H3K4me3 can recruit proteins associated with RNAPII such as the general transcription factor TFIID (Vermeulen et al. 2007) (Figure 1.4B). Interestingly a direct connection to RNAPII has not yet been explored and is the focus of the work in Appendix A. It can also recruit accessory factors in transcription such as the chromatin remodeler CHD1 (Flanagan et al. 2005; Sims et al. 2005). Lastly, it can also recruit the complex that catalyzes H3K4me3, COMPASS, as a means to propagate the modification (Shi et al. 2007b; Milne et al. 2010; Eberl et al. 2013) (Figure 1.2D). At the same time H3K4 methylation can promoter active chromatin by preventing the binding of repressive proteins like BHC80 (a member of the LSD1 H3K4 demethylase complex) and the DNA methyltransferase DNMT3A/B (Lan et al. 2007; Ooi et al. 2007; Zhang et al. 2010) (Figure 1.2B).

Histone H3 Lysine 36 Methylation

As opposed to H3K4 methylation, methylation of lysine 36 of histone H3 (H3K36) is prevalent in gene bodies (Pokholok et al. 2005; Rao et al. 2005) (Figure 1.5). In budding yeast, a single histone methyltransferase, Set2, catalyzes all three methylation states (Strahl et al. 2002; Wagner and Carpenter 2012). In metazoans, several enzymes can methylate H3K36 including, but not limited to NSD1-3 and SETD2. As with H3K4 methylation, H3K36 methylation requires the PAF complex, but given its

presence in gene bodies, is dependent on serine 2 phosphorylation of the RNAPII CTD (Li et al. 2003; Kizer et al. 2005; Nordick et al. 2008).

The predominant function of H3K36 methylation is preventing "cryptic" transcription. This occurs mainly by recruiting the Rpd3 HDAC complex described above. At the same time H3K36 methylation can recruit an ISWI-family chromatin remodeling complex (Isw1b) as well as restrict histone turnover by preventing the binding of the histone chaperone Asf1 (Maltby et al. 2012; Smolle et al. 2012). These activities combined help maintain a relatively closed chromatin environment in gene bodies to increase overall transcriptional efficiency. It is important to note, however, that H3K36 methylation can also recruit the HATs NuA3 in yeast and MOF in complex organisms, suggesting that the role of H3K36 methylation in transcription is multifaceted (Shi et al. 2007b; Vezzoli et al. 2010).

Histone H3 Lysine 79 Methylation

Like H3K36 methylation, methylation of lysine 79 of histone H3 (H3K79) is also found in gene bodies, but its function is less well-defined (Figure 1.5). This modification is catalyzed by one enzyme called Dot1 in budding yeast and Dot1L in metazoans (Feng et al. 2002; Lacoste et al. 2002; Ng et al. 2002a; van Leeuwen et al. 2002). Displaying further similarity to H3K4 and H3K36 methylation, H3K79 methylation requires the PAF complex, but a direct connection with RNAPII has not been fully determined (Krogan et al. 2003a). Functionally, H3K79 methylation appears to play a primary role in preventing the binding of repressive proteins that would otherwise

negatively regulate transcription. The primary protein complex involved is the Silent Information Regulator (SIR) complex, which contains the HDAC Sir2 (Norris and Boeke 2010). Sir2 deacetylates lysine 16 of histone H4 (H4K16ac) and this is an important step in chromatin compaction and heterochromatin compaction. The Sir3 subunit of the SIR complex is not able to bind nucleosomes when H3K79 is methylated (Altaf et al. 2007; Onishi et al. 2007). Loss of H3K79 methylation in euchromatin regions leads to the spreading of silencing proteins into active gene regions leading to repression (Ng et al. 2002a; van Leeuwen et al. 2002). Conversely, increased H3K79 methylation reduces heterochromatin formation. To date, no protein has been identified to recognize and bind methylated H3K79.

Histone Monoubiquitylation

As opposed to small chemical modifications such as acetylation and methylation, histones can also be modified by the covalent addition of proteins (Komander and Rape 2012). The primary protein that can be attached to histones is ubiquitin, which is an 8.5 kDa protein that plays an important role in numerous cellular processes both in the nucleus and the cytosol. Ubiquitin is added to lysine residues via an isopeptide linkage, which links the C-terminal glycine residue to the epsilon amino group of the target lysine. This process is mediated by the concerted effort of three enzyme classes called ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Ubiquitin is activated for conjugation in an ATP-dependent manner by the E1 and then transferred to the active site of the E2. Then, in conjunction with the E3,

ubiquitin is transferred to the substrate. Interestingly the diversity of proteins involved increases with each step. Accordingly, less than ten E1 enzymes exist, whereas hundreds of E3 enzymes have been identified. This observation is likely due to the fact that the E3 provides substrate specificity. Lastly, multiple E3 enzymes can associate with each E2, thus increasing the utility of E2 enzymes.

Since ubiquitin itself also contains lysine residues, chains of ubiquitin can be formed (polyubiquitylation) (Komander and Rape 2012). Certain types of polyubiquitin chains can direct the modified proteins to degradation via the proteasome. In contrast to polyubiquitylation, histones are typically modified with one ubiquitin molecule (monoubiquitylation) and this is thought to serve as signaling molecule in chromatin (Weake and Workman 2008; Chandrasekharan et al. 2010b). Monoubiquitylation of histones is thought to function by two means. The first is that given its size relative to the histones, it may directly impinge on chromatin structure by altering the ability of chromatin to compact. The second function is similar to that of other histone modifications in that it can act to recruit proteins to chromatin. Two primary sites of histone monoubiquitylation exist on the C-terminal tails of histone H2A and histone H2B (Weake and Workman 2008). Interestingly, modification of these two sites have opposite effects on transcription with H2A monoubiquitylation generally playing a repressive role and H2B monoubiquitylation playing a role in activation.

Histone H2B Monoubiquitylation

Monoubiquitylation of histone H2B occurs on lysine 123 (H2BK123ub1) in budding yeast, and is a highly conserved modification (Robzyk et al. 2000). The modification is catalyzed in yeast by the concerted effort of the E2 ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin ligase Bre1 (Robzyk et al. 2000; Hwang et al. 2003; Wood et al. 2003a). H2BK123ub1 is also a very dynamic histone modification and this is due, in part, to the presence of deubiquitylating enzymes that can cleave the modification from histones. In yeast, these enzymes are Ubp8 and Ubp10 (Henry et al. 2003; Emre et al. 2005). As described above, monoubiquitylation of H2B is associated with active transcription and is predominantly found in gene bodies, but may exist transiently at promoters (Henry et al. 2003; Kao et al. 2004; Xiao et al. 2005; Pavri et al. 2006; Fleming et al. 2008; Chandrasekharan et al. 2009; Chandrasekharan et al. 2010b) (Figure 1.5). Accordingly, and consistent with active histone methylation, H2BK123ub1 is dependent on the PAF complex (specifically the Rtf1 subunit) and serine 5 phosphorylation of the RNAPII CTD (Ng et al. 2003a; Wood et al. 2003b; Xiao et al. 2005).

H2BK123ub1 is thought to function by multiple mechanisms in transcription. One mechanism ubiquitin may function in chromatin is by altering chromatin structure. H2BK123ub1 does appear to affect chromatin architecture, but the ultimate affect has not been fully resolved. First, *in vitro* studies have shown that H2BK123ub1 hinders chromatin compaction to maintain an open chromatin environment (Fierz et al. 2011). At the same time it has also been suggested to increase nucleosome stability (Fleming

et al. 2008; Chandrasekharan et al. 2009). The latter effect may be due to the interplay between H2BK123ub1 and the histone chaperone complex FACT.

H2BK123ub1 also facilitates the function of other proteins involved in transcription. For example, H2BK123ub1 has been shown to increase the recruitment of the chromatin remodeler SWI/SNF and, as stated above, the FACT complex (Fleming et al. 2008; Shema-Yaacoby et al. 2013). H2BK123ub1 has also been implicated in a unique phenomenon of 'cross-talk' called *trans*-histone regulation whereby one histone modification can stimulate the modification of another histone (Bannister and Kouzarides 2011). Ubiquitylation of histone H2B stimulates methylation of both lysine 4 and lysine 79 on histone H3 (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002). The mechanisms of this *trans*-histone regulation, however, have not been fully determined. Moreover the extent of *trans*-histone regulation in chromatin is not entirely known.

Similarly to H3K79 methylation, H2BK123ub1 is a carefully regulated modification and can impinge on the spreading of heterochromatic modifications into euchromatin regions. Therefore, too much or too little H2BK123ub1 can have adverse affects for the cell (Briggs et al. 2002; Emre et al. 2005). To avoid this, the cell carefully regulates the levels of H2BK123ub1, in part and as stated above, by the opposing activities of the ubiquitylating and deubiquitylating enzymes. In agreement, both activities are found to overlap across the genome (Schulze et al. 2011). In order for productive H2BK123ub1 to occur, however, the equilibrium needs to shift in a particular direction. Understanding how this occurs would provide important insight for the regulation of H2BK123ub1

genome-wide and how this balance is maintained or altered during changing cellular conditions or in disease.

Description of Work Included in Dissertation

Work over the past decades has demonstrated the existence and functional significance of histone modifications. Furthermore, it is clear that aberrant histone modification can trigger defects in chromatin structure and function and lead to disease such as cancer (Sharma et al. 2010; Bannister and Kouzarides 2011). Thus, histone modifications must be carefully regulated. The work in this dissertation focuses on one particular histone modification, monoubiquitylation of histone H2B (H2BK123ub1). This modification is of interest due its diverse functions in chromatin and the fact that it is both dynamically and carefully regulated. Moreover, it has been demonstrated that H2B ubiquitylation is altered in the context of disease (Shema et al. 2008; Blank et al. 2012; Chernikova et al. 2012; Wang et al. 2013). Interestingly, both the loss of gain of the modification genome-wide can contribute to disease. To better understand this modification, I initially focused on identifying novel regulators of this histone modification, which is discussed in Chapter 2. Then in an effort to understand the dynamics of histone H2B ubiquitylation, I identified a unique mechanism that keeps the modification in check. This work, which is presented in Chapter 3, also demonstrates a functional role for H2B ubiquitylation in transcription. As described above H2B ubiquitylation can partly function in transcription by regulating histone methylation. In Appendix A I present evidence connecting this downstream methylation directly to RNAPII and thus further make a connection between H2B ubiquitylation and

transcription. Finally, the work from these studies is put into the larger context of chromatin regulation in Chapter 4. Overall, the studies presented here provide new insight into the role and regulation of H2B ubiquitylation.

Figures

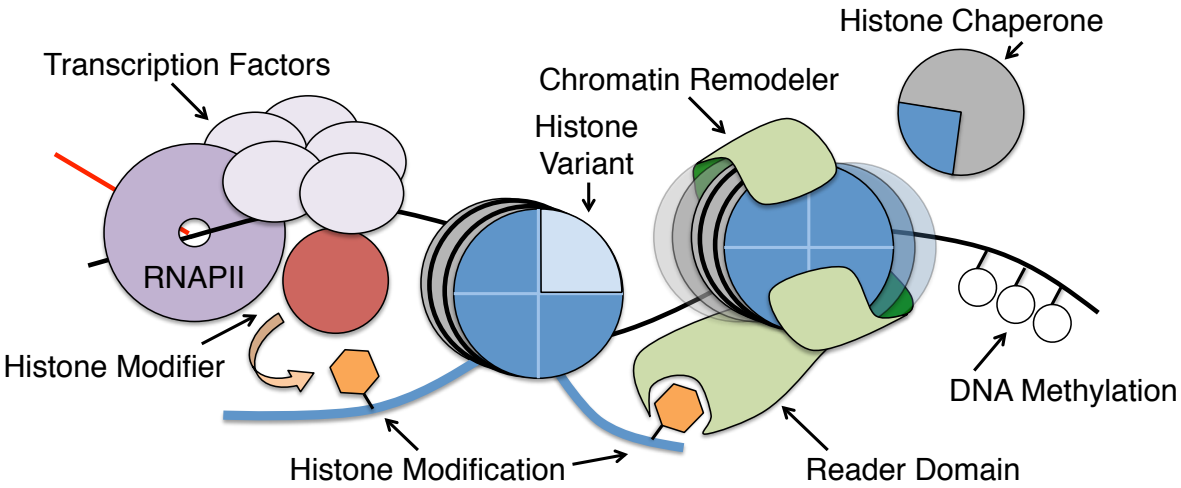

Figure 1.1 Components of Chromatin Regulation

Numerous proteins and complexes play a role in regulating chromatin structure and function. Transcription factors (light purple) associated with RNA Polymerase II (RNAPII) and DNA (black) can also recruit histone modifiers (red) such as methyltransferases and ubiquitin ligases to chemically modify histones (blue). Histone modifications (orange) can either directly alter chromatin structure or recruit proteins with so-called reader domains (green) that recognize the modification. Many types of chromatin-associated proteins contain reader domains including chromatin remodeler proteins (green) that can deposit, remove or slide nucleosomes. Working in concert with the chromatin remodelers are histone chaperones (gray) that bind free histones and/or facilitate the incorporation of histone variants (light blue). In addition to histone modifications, DNA can be methylated (white circles), which also impinges on chromatin binding proteins. See text for further details.

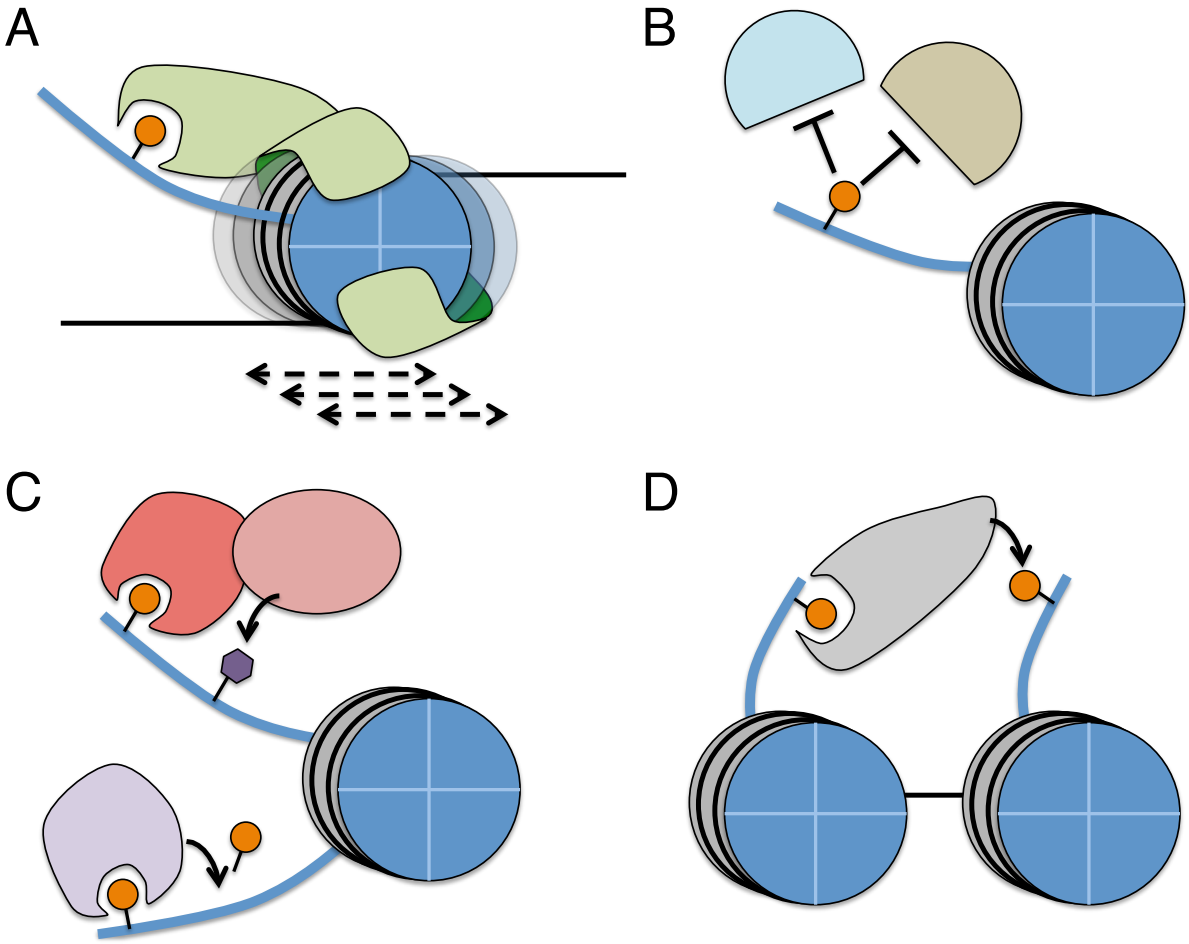

Figure 1.2 Types of Histone Modification Binding

Histone modifications regulate the association of proteins and protein complexes with chromatin. (A) Modifications can recruit proteins that can directly remodel chromatin or (B) repel the binding of proteins. (C) Histone modifying enzymes that add or remove histone modifications can also be recruited by other modifications. (D) Histone modifying enzymes can bind the modification that they catalyze as a potential mechanism for propagation. See text for further details.

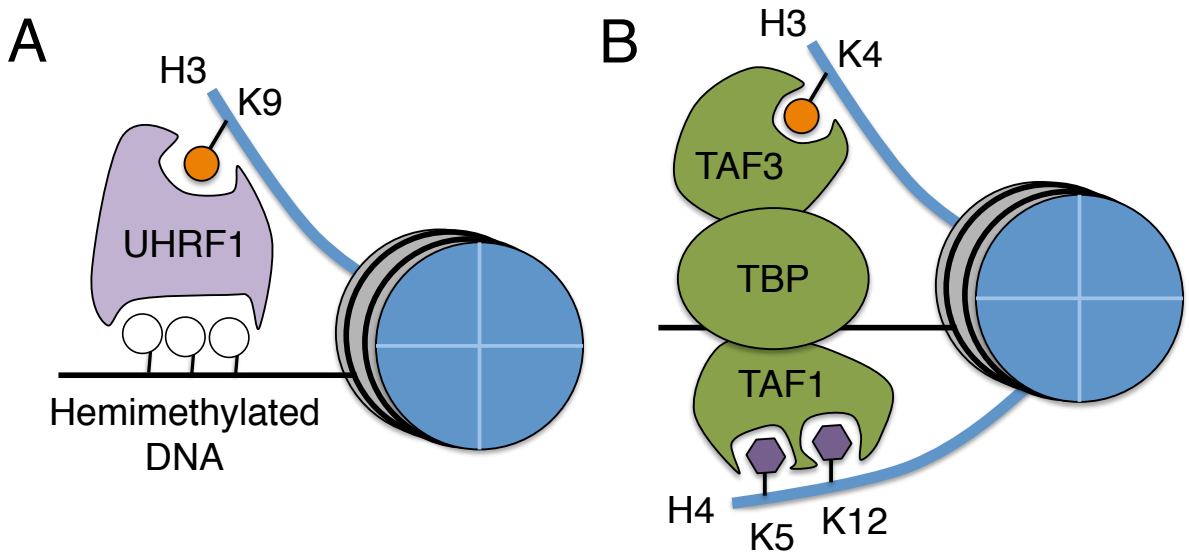

Figure 1.3 Multivalent Chromatin Binding

Shown are examples of an individual protein (A) or protein complex (B) that can simultaneously recognize histones and DNA. Both histones and DNA can be modified thus establishing a "code" that dictates protein binding across the genome.

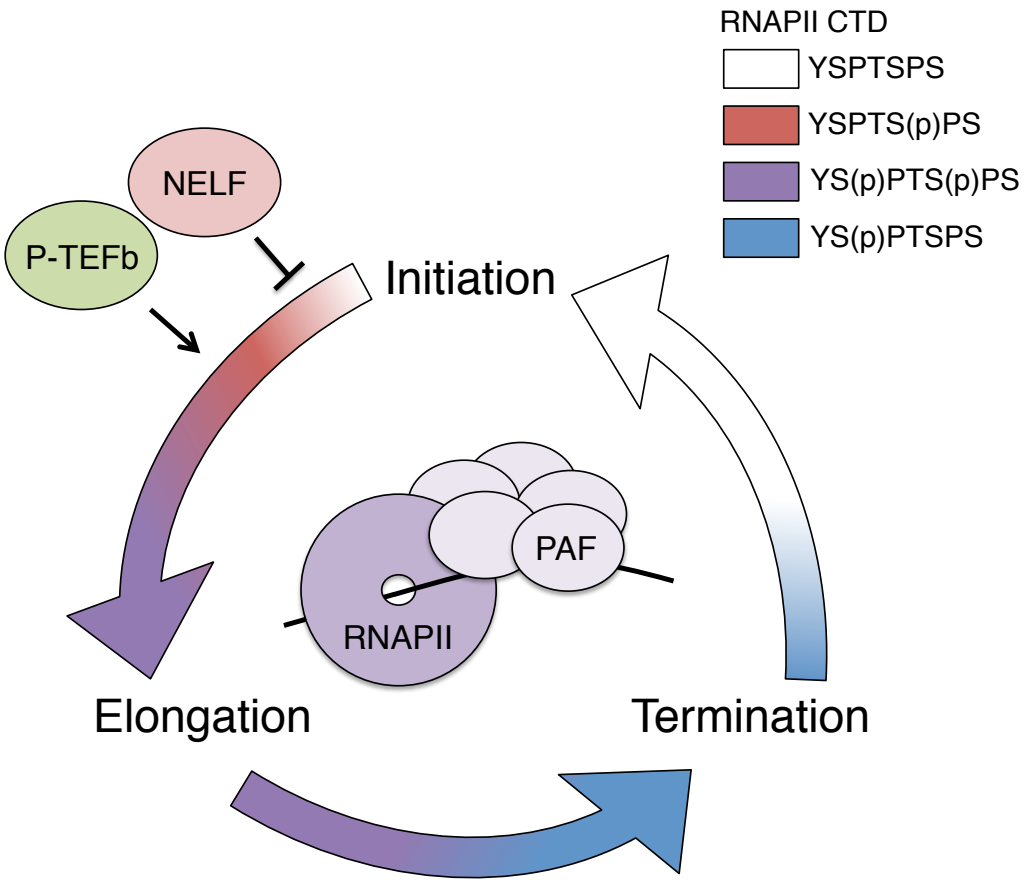

Figure 1.4 The Transcription Cycle

Transcription by RNA Polymerase II (RNAPII) proceeds in a cyclical fashion. RNAPII associates with factors such as the Polymerase Associated Factor (PAF) complex throughout the cycle to facilitate multiple steps. RNAPII is also modified on its repetitive C-terminal domain (CTD) with different modifications coinciding with distinct steps. Shown are the patterns of serine 2 and serine 5 phosphorylation on the CTD repeat. Pausing after initiation is an important step in metazoan transcription and is regulated by the opposing functions of the negative elongation factor (NELF) complex and the positive transcription elongation factor (P-TEFb). See text for details.

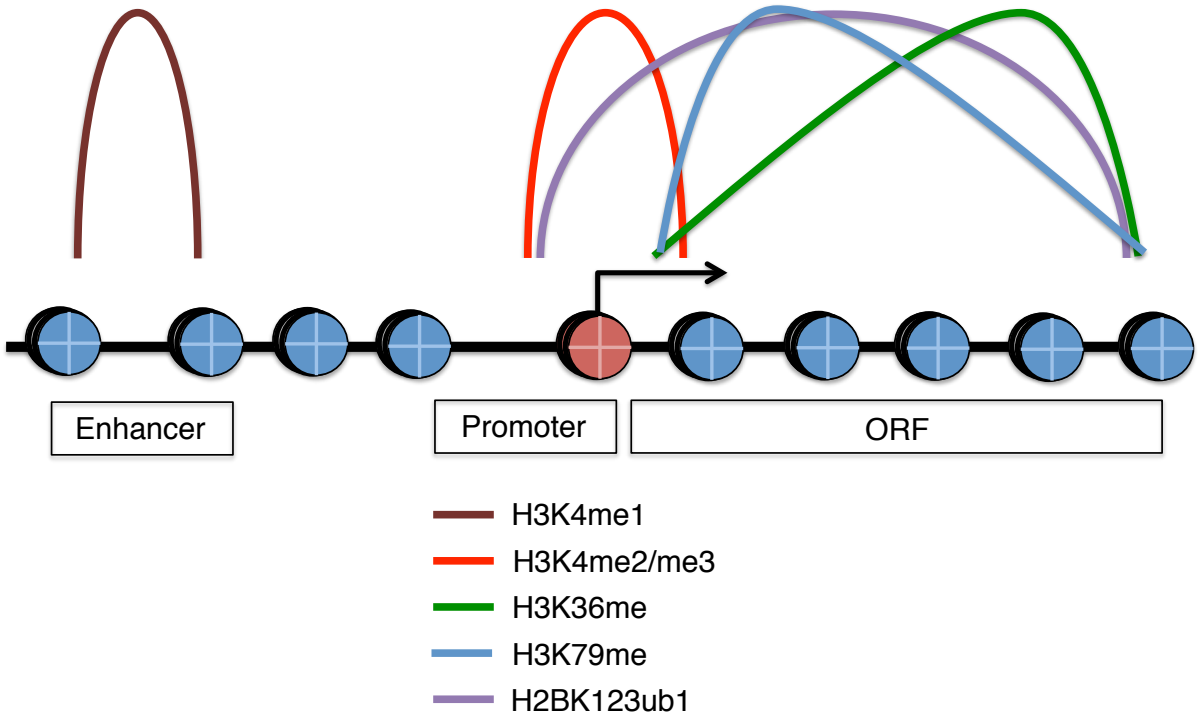

Figure 1.5 Patterns of Transcription-Associated Histone Modifications

Histone modifications exist in distinct patterns in and around actively transcribed genes. Shown is a representative model of a gene region with corresponding enrichment of selected modifications. Modification enrichment reflects patterns observed in mammals. The +1 nucleosome is displayed in red at the transcription start site (arrow), which is downstream of the nucleosome free regions (NFR).

CHAPTER 2 IDENTIFICATION OF THE HISTONE H2A REPRESSION DOMAIN AS A REGULATOR OF H2B UBIQUITYLATION AND TRANSCRIPTION ELONGATION IN YEAST[2]

Overview

Histone 'cross-talk' represents a fundamental mechanism by which histone post-translational modifications (PTMs) regulate the structure and function of chromatin. Here we show in the budding yeast *Saccharomyces cerevisiae* that a H2A N-terminal region referred to as the H2A repression (HAR) domain is important for trimethylation of H3K79 (H3K79me3). Consistent with a recently published report, we also find that the HAR domain regulates monoubiquitylation of H2BK123 (H2BK123ub1) which, as we show for H3K79me3, is a regulatory pathway observed across multiple genetic backgrounds. In contrast, we found that the regulation of H3K4 trimethylation by the HAR domain is context dependent regarding genetic background. We further show that the HAR domain promotes H3K79me3 by maintaining wild-type levels of H2BK123ub1, but this mechanism is independent of recruitment of the H2B ubiquitylation machinery to chromatin. Finally, we provide genetic evidence that the HAR domain contributes to telomeric silencing and the process of transcription elongation – consistent with the established role of H2BK123ub1 in these processes. In sum, these data highlight a

[2] Portions of this chapter were adapted from Wozniak GG, Strahl BD. 2014. Catalysis-dependent stabilization of Bre1 fine-tunes histone H2B ubiquitylation to regulate gene transcription. *Genes Dev.* 28:1647-1652

'cross-talk' pathway involving the H2A tail that governs H2B ubiquitylation and H3 methylation in the process of transcriptional regulation.

Introduction

Histone post-translational modifications (PTMs) play an important role in the regulation of chromatin structure and function, and occur primarily on the unstructured N- and C-terminal tails of the histones (Kouzarides 2007; Zentner and Henikoff 2013). A wide number of histone PTMs exists, and they include acetylation, methylation, phosphorylation and ubiquitylation. Histone PTMs can alter chromatin structure, at least in part, through the recruitment or occlusion of effector proteins that can carry out chromatin remodeling or further modification (Kouzarides 2007; Zentner and Henikoff 2013). On the histone tails, PTMs further exist in unique combinations, which add a fine level of control in the coordination of DNA-templated processes including transcription, replication and DNA repair (Strahl and Allis 2000; Kouzarides 2007).

Due to their importance for chromatin structure and function, histone PTMs are highly regulated. Regulation of histone modifications has been observed at both the level of the modifying enzymes and that of the histones themselves. The modifying enzymes can be regulated through their association with protein complexes that mediate recruitment to chromatin or by PTMs that can activate or inhibit enzymatic activity (Smith and Shilatifard 2010; Black et al. 2012). At the histone level, PTMs can regulate one another both in *cis* and *trans* (Kouzarides 2007). The first and well-characterized example of a *trans*-histone pathway involves the directed regulation of methylation at lysines 4 and 79 on histone H3 (H3K4 and H3K79) by monoubiquitylation

of histone H2B at lysine 123 (H2BK123ub1) (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002; Nakanishi et al. 2009). While the mechanism of how this occurs is still under investigation, it is known that this regulatory pathway is important for transcription (both at the levels of initiation and elongation) as well as DNA repair and replication (Henry et al. 2003; Kao et al. 2004; Xiao et al. 2005; Game et al. 2006; Pavri et al. 2006; Fleming et al. 2008; Chandrasekharan et al. 2010b; Schulze et al. 2011; Trujillo and Osley 2012).

Despite a wealth of studies investigating the prevalence and functional importance of histone PTMs, a thorough investigation to examine the extent to which the histone tails participate in *trans*-histone regulation of other histone PTMs remains to be performed. Moreover, it remains to be determined what the functional significance of *trans*-histone regulation may be. In the current study, we systematically examined the role of the individual histone N-terminal tails in *trans*-histone regulation of histone methylation and ubiquitylation. We found that the N-terminal tail of histone H2A, specifically a region in the H2A tail known as the HAR domain, acts as a *trans*-histone regulator of H3K79 methylation and H2BK123ub1. Our results with the regulation of H2BK123ub1 are consistent with a recently published report (Zheng et al. 2010); however, we find that the reported regulation of H3K4 methylation is yeast strain specific. We also find that the HAR domain regulates H3K79 methylation through H2BK123ub1, but does not regulate the recruitment of the ubiquitylation machinery to chromatin. Lastly, we show the functional importance of this regulation by establishing a role of the HAR domain in the process of transcription elongation.

Materials and Methods

Yeast Strains and Plasmids

The strains used in this study are listed in Table 2.1. Strains with wild-type (WT) or N-terminal tail truncations of histones (Figure 2.1) were obtained from C.D. Allis and have been described previously (Ahn et al. 2005). W303-derived WT and truncated H2A strains (Figure 2.2A) were obtained from M. Parra and have been described previously (Parra et al. 2006; Parra and Wyrick 2007). WT and mutant histone plasmids were introduced into FY406 and derivatives (Figure 2.2B, 2.4 and 2.5C, obtained from F. Winston (Hirschhorn et al. 1995)) using standard transformation and shuffling protocols. YZS276-derived strains (Figure 2.3, obtained from Z.W. Sun (Sun and Allis 2002; Chandrasekharan et al. 2009)) were generated by transforming YZS276 and YZS606 with pSAB6 (*CEN URA3 HTA1-HTB1*, obtained from F. Winston (Hirschhorn et al. 1995)) followed by two rounds of selection on synthetic media lacking uracil to promote loss of the *HIS3*-containing plasmid pZS145. The newly created histone shuffle strains (YGW063 and YGW066) were then transformed with pZS145 (obtained from Z.W. Sun (Sun and Allis 2002)) and mutant derivatives and shuffled as described above. Strains to assess telomeric silencing defects (Figure 2.5) were obtained from Z.W. Sun and have been described previously (Sun and Allis 2002).

Gene disruptions were performed using high efficiency transformation of a PCR product amplified from either genomic DNA of the gene of interest, which had already been replaced by *KanMx* (Open Biosystems) or from the *NatNT2* plasmid pFA6a-natNT2. Endogenous tagging of Rad6 with triple hemagglutinin (3HA) was performed

using plasmids and procedures previously described (Janke et al. 2004). Gene disruption and tagging were verified by both PCR and immunoblotting.

The plasmids pZS145 (*CEN HIS3 HTA1-Flag-HTB1*) and pZS146 (*CEN HIS3 HTA1-Flag-htb1-K123R*) were obtained from Z.W. Sun (Sun and Allis 2002). The plasmids pEG101 (*CEN HIS3 hta1 Δ1-20 Flag-HTB1*), pEG102 (*CEN HIS3 hta1 Δ1-16 Flag-HTB1*) and pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*) were derived from pZS145 using the QuikChange II Site-Directed Mutagenesis kit (Agilent). All mutant plasmids were verified by DNA sequencing.

Yeast Whole Cell Extracts and Western Blot Analysis

Yeast were grown in rich media (YPD) at 30˚C from a starting OD_{600} or 0.25 to mid-log phase (OD_{600} ~1.0). Five OD_{600} units were collected by centrifugation and used for preparation of whole cell extracts as previously described (Fuchs et al. 2012). For western blot analysis 5-10 μl of whole cell extract was separated by 15% SDS-PAGE. Gels were transferred to PVDF using a semi-dry apparatus (Hoefer) for 90 minutes at 45 mA and dried in methanol. Dried membranes were then rehydrated in methanol, washed briefly with TBS with 0.1% Tween-20 (TBST) and incubated for 30 minutes with gentle shaking in TBST containing 5% non-fat dry milk. Primary antibodies were diluted in TBST containing 2.5% non-fat dry milk and incubated overnight at 4˚C. Western blots were visualized using HRP-conjugated antibodies and ECL Plus chemiluminescence (GE Healthcare).

The following primary antibodies and dilutions were used: H3K79me3 (Abcam, ab2651) 1:2500, H3K79m2 (Active Motif, 39143) 1:2500, H3K79me1 (Active Motif, 39145) 1:2000, H3K4me3 (Active Motif, 39159) 1:2000, H3K4me2 (Millipore, 07-030) 1:2500, H3K4me1 (Millipore, 07-436) 1:1000, H3K36me3 (Abcam, ab9050) 1:1000, H3K36me2 (Active Motif, 39255) 1:1000, H3K36me1 (Abcam, ab9048) 1:1000, H2BK123ub1 (Cell Signaling Technology, 5546) 1:2000, H2A (Active Motif, 39235) 1:5000, H2B (Active Motif, 39237) 1:2500, H4 (Millipore, 05-858) 1:1000, H3 (EpiCypher, 13-0001; 1:1000) 1:2000, HA (Covance, MMS-101R) 1:1000, G6PDH (Sigma, A9521) 1:100,000.

Yeast Chromatin Fractionation

Chromatin fractionation was performed using a combination of previously described methods (Donovan et al. 1997; Keogh et al. 2006). Cells were grown in YPD from a starting OD_{600} of 0.25 to mid log phase (OD_{600} ~1.0). Forty OD_{600} units of cells were harvested by centrifugation, resuspended in 10 ml of sterile water. Following another round of centrifugation, cells were resuspended in 10 ml SB buffer (1 M Sorbitol, 20 mM Tris pH 7.4) then collected by centrifugation. The buffer was then aspirated and cell pellets were stored at -80˚C overnight. The cell pellets were then thawed on ice, resuspended in 1.5 ml PSB buffer (20 mM Tris pH 7.4, 2 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol) and transferred to a 2 ml microcentrifuge tube. Cells were allowed to mix for 10 minutes at room temperature on a rotating shaker. The cells were then pelleted by a flash spin in a microcentrifuge and the buffer was

aspirated. Cell pellets were then washed briefly in 1.5 ml SB buffer then quickly centrifuged as in the previous step. The cell pellet was then resuspended completely in 1 ml SB buffer followed by the addition of 125 µl of 10 mg/ml Zymolyase 20T (Seikagaku Biobusiness) prepared fresh in SB buffer. Tubes were then allowed to mix at room temperature for 30-60 minutes on a rotating shaker. Spheroplasting progress was assessed by addition of 10 µl of cells to 1 ml 1% SDS and vortexing followed by measuring the OD_{600} of the liquid. Once OD_{600} measurement decreased by more than 80% the starting value, spheroplasting was stopped by the addition of ice-cold SB buffer. Spheroplasts were pelleted at 2000 rpm for 5 minutes at 4˚C in a chilled microcentrifuge. The buffer was removed and the pellet was gently resuspended in 1 ml LB buffer (0.4 M Sorbitol, 150 mM potassium acetate, 2 mM magnesium acetate, 20 mM PIPES-KOH pH 6.8, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM PMSF) and pelleted as in the previous step. The LB buffer wash step was repeated once more. To lyse the cells, the pellet was then gently resuspended in 250 µl LB buffer with 1% Triton X-100, transferred to a 1.5 ml microcentrifuge tube and allowed to sit on ice for 10 minutes with occasional gentle mixing. Following lysis, 125 µl was removed for the whole cell extract (WCE) and the remainder was centrifuged at 5000 xg for 15 minutes at 4˚C. The supernatant was collected as the “soluble” fraction. The “chromatin” pellet was then washed once by resuspension in 125 µl of LB buffer with 1% Triton X-100 and spun as in the previous step. The supernatant was then discarded and the “chromatin” pellet was resuspended in 125 µl of LB buffer with 1% Triton X-100. All samples were normalized to total protein content of whole cell extract as determined

using Bradford reagent (Bio-Rad). Normalized whole cell extract and volume equivalents of “soluble” and “chromatin” fractions were boiled in 1x SDS loading buffer, separated by 15% SDS-PAGE and analyzed by standard immunoblot procedures.

Phenotypic Spotting Assays

Five-fold serial dilutions of saturated overnight yeast cultures were plated on YPD or synthetic complete dropout media with or without indicated drugs. Cells were plated at a starting OD_{600} of 0.5 on appropriate media and imaged after 2-4 days of growth at 30˚ C. For growth on 6-azauracil, drug was used at a final concentration of 200 μg/ml.

Results

The Histone H2A N-terminal Tail Regulates H3K79 Methylation and H2BK123 Ubiquitylation.

The N-terminal tail domains of histones play important roles in chromatin structure and function, which is regulated, in part, through *trans*-tail regulation of histone modifications (Kouzarides 2007). One well-characterized example is the regulation of H3K4me and H3K79me by H2BK123ub1. Additional examples include the regulation of H3K36 methylation (H3K36me) by surface core residues in H2A and H4, and the regulation of H3K79me by the basic patch found in the H4 N-terminal domain (Altaf et al. 2007; Fingerman et al. 2007; Du and Briggs 2010). Given these observations, we asked whether other *trans*-tail regulatory pathways might also exist, and whether they might have important roles in chromatin function. To explore this possibility, we

screened yeast strains containing or lacking the N-terminal tail domains from H3, H4, H2A and H2B for their histone methylation and H2B ubiquitylation status.

We first set out to recapitulate several recent *trans*-tail findings in regards to the loss of the H4, H3 and H2A N-terminal tails. In agreement with previous reports (Altaf et al. 2007; Fingerman et al. 2007), we found that loss of the H4 tail domain resulted in a complete abolishment of H3K79 methylation (Figure 2.1). In contrast, however, we were unable to observe the reported loss of H3K4me3 in the absence of the H2A tail, as was recently reported (Zheng et al. 2010). Interestingly, our studies have uncovered a basis for this discrepancy (see below). Furthermore, we were also unable to observe significant decreases in H3K36 methylation in the loss of the H3 tail domain, as was reported (Psathas et al. 2009).

In addition to confirming the role of the H4 tail in regulating H3K79 methylation, we found H3K79 methylation to be regulated further by other histone tails. Surprisingly, we found that loss of the H2A tail had decreased levels of H3K79me3 and increased H3K79me2 and H3K79me1 (Figure 2.1). Loss of the H3 tail had the opposite affect showing increased levels of H3K79me3 with a corresponding decrease in both H3K79me2 and H3K79me1. Given the role of H2BK123ub1 in regulating H3K79 methylation, we also screened the strains for this modification. We found that loss of the H2A tail resulted in decreased H2BK123ub1, consistent with a recently published report (Zheng et al. 2010). Taken together, our results reveal the H2A tail as a regulator of H3K79 methylation and H2BK123ub1.

The HAR Domain of H2A Regulates H3K79 Methylation and H2BK123 Ubiquitylation Independent of Genetic Background.

Given our finding that the H2A tail regulates H3K79 methylation and H2BK123ub1, we next wanted to determine which residues of the H2A tail are involved. We addressed this question using yeast strains harboring truncated versions of H2A. As shown in Figure 2.2A, loss of the entire N-terminal tail resulted in decreased H3K79me3 and H2BK123ub1 whereas loss of residues 4-16 had WT levels. In contrast, truncations lacking residues 16-20, a region of histone H2A known as the H2A repression (HAR) domain (Parra and Wyrick 2007), closely mimicked the methylation and ubiquitylation decrease observed in the absence of the entire tail, suggesting that the HAR domain is important for regulating both H3K79 methylation and H2BK123 ubiquitylation. We also validated these findings in another strain background and found similar results (Figure 2.2B). The results confirm that the HAR domain of histone H2A regulate H3K79 methylation and H2BK123 ubiquitylation in a manner that is independent of strain background.

The Regulation of H3K4me3 by Histone H2A is Dependent on Genetic Background.

The unique opportunity to examine multiple H2A truncation strains and backgrounds to identify the region responsible for affecting H3K79me also allowed us to further examine the discrepancy between our observations and those of others pertaining to the proposed role of the HAR domain in regulating H3K4me3. As mentioned, a recent report suggested that HAR domain regulates H3K4me3 (Zheng et

al. 2010), which is in contrast to the results from our screen (Figure 2.1). Using the originally published strains of that study, we were indeed able to recapitulate the previously reported findings (Figure 2.2A). We then independently addressed this questions in a third strain background and found that, as we observed in our screen, H3K4me3 was not affected by loss of the HAR domain (Figure 2.2B). Taken together, these results indicate that regulation of H3K4me3 by the HAR domain is not universal as is the regulation of H3K79me3, but instead dependent on the strain background.

The HAR Domain Promotes H3K79 Methylation Through Maintenance of H2BK123 Ubiquitylation.

Given the fact that H2BK123ub1 regulates H3K79 methylation in a *trans*-tail manner, we next asked whether the regulation of H3K79me3 by the HAR domain was dependent on the regulation of H2BK123ub1. In support of this possibility, analysis of the crystal structure of the nucleosome revealed that the HAR domain is located next to H2BK123 (Figure 2.3A). To address this question we hypothesized that increasing the amount of H2BK123ub1 in the HAR domain mutant strain would rescue the decrease in H3K79me3. We answered this question by introducing the HAR domain deletion into strains with or without the H2BK123-specific deubiquitylases Ubp8 and Ubp10. As reported previously, we found that the combined loss of Ubp8 and Ubp10 resulted in a dramatic increase in the amount of both H2BK123ub1 and H3K79me3 in cells (Figure 2.3B) (Gardner et al. 2005). In the absence of the HAR domain, we also observed a dramatic increase in both H2BK123ub1 and H3K79me3, albeit less than what is

observed in the strain with WT H2A. These data indicate that the ability of the HAR domain to regulate H3K79me3 likely occurs through H2BK123ub1. Moreover, the HAR domain is likely involved in the catalysis of H2BK123ub1.

Loss of the HAR Domain Does Not Alter Recruitment of the Histone Ubiquitylation Machinery to Chromatin.

The observation that loss of the H2BK123 deubiquitylating enzymes could not fully rescue the levels of H2BK123ub1 in the absence of the HAR domain (Figure 2.3B) suggested a problem with the ability to ubiquitylate histones. One question we next addressed is whether the histone ubiquitin ligase machinery consisting of Rad6 and Bre1 was still recruited to chromatin globally in the absence of the HAR domain. To address this question, we used a technique to fractionate cells into an insoluble chromatin-containing fraction and a soluble non-chromatin associated fraction, which has been used previously to assess association of factors with chromatin (Donovan et al. 1997; Keogh et al. 2006). Using an endogenously epitope tagged Rad6, we measured the chromatin association of the ubiquitylation machinery in either the presence or absence of the HAR domain. As opposed to loss of Bre1, which is required for Rad6 to associate with chromatin (Wood et al. 2003a; Kao et al. 2004), loss of the HAR domain did not affect the chromatin association of Rad6 (Figure 2.4). These results are consistent with previous results showing that the HAR domain was not required for Rad6 association with the *GAL* locus (Zheng et al. 2010). We conclude that the HAR domain is not required for the global association of the histone ubiquitylation

machinery with chromatin, but instead is likely important for catalysis of the ubiquitylation reaction.

The HAR Domain is Important for Telomeric Silencing and is linked with Transcription Elongation.

H2BK123ub1 has been implicated in numerous chromatin related processes including telomeric gene silencing and transcription elongation (Sun and Allis 2002; Xiao et al. 2005; Pavri et al. 2006; Fleming et al. 2008; Schulze et al. 2011). Since the HAR domain regulates H2BK123ub1, we hypothesized that it also plays a role in both telomeric silencing and transcription elongation. To determine if the HAR domain plays a role in telomeric silencing we made use of a common reporter strain containing the *URA3* gene integrated in the subtelomeric region of the left arm of chromosome VII (Sun and Allis 2002). We introduced different H2A tail truncations into this strain and plated cells on media containing 5-fluoroorotic acid (5-FOA) to measure phenotypes (Figure 2.5A). Serving as a control, loss of the silencing protein Sir2, which is vital for telomeric silencing results in a severe growth defect on 5-FOA. Compared to WT, we found that deletion of the entire H2A tail or just the HAR domain, but not residues 1-16 resulted in a growth defect on 5-FOA indicating an inability to silence the reporter gene (Figure 2.5A).

Loss of H2BK123ub1 also results in sensitivity to the drug 6-azauracil (Xiao et al. 2005), which has been used previously to study proteins involved in the transcription elongation process. Using the H2A tail truncation strains, we also find that loss of the

HAR domain, but not residues 1-16 results in sensitivity to 6-azauracil (Figure 2.5B). The observed sensitivity of the HAR domain deletion is not as strong as observed for loss of H2BK123ub1 consistent with the reduced levels, but not complete loss of H2BK123ub1 in HAR domain deletions. To further determine if the HAR domain plays a role in transcription elongation we assayed for genetic interactions between the HAR domain and several factors known to be involved in elongation. We focused on three proteins previously identified to genetically interact with H2BK123ub1: Rbp9, Elp3 and Spt4 (Xiao et al. 2005). As reported and recapitulated here, we found that loss of H2BK123ub1 using a lysine to arginine point mutation results in synthetic sickness with the genes encoding Rbp9, Elp3 and Spt4 (Figure 2.5C). Consistent with the ability of the HAR domain to regulate H2BK123ub1 we find that the loss of the HAR domain displays synthetic sickness when combined with either loss of Elp3 or Spt4. Surprisingly, and in contrast to the loss of H2BK123ub1, we found that loss of the HAR domain was able to dramatically rescue the growth defect observed for strains lacking Rpb9. The results from our genetic analyses suggest that similar to H2BK123ub1, the HAR domain plays a role in telomeric silencing and transcription elongation – a result that is likely manifested at the level of the HAR domain to regulate H2BK12ub1 and H3K79me3 in a *trans*-tail fashion.

Discussion

In the present study we uncover a role for the HAR domain of histone H2A in regulating both H3K79me3 and H2BK123ub1. We find that this regulation occurs across multiple strain backgrounds, and that there is strain background dependence for

the regulation of H3K4me3. We also show that the regulation of H3K79me3 by the HAR domain occurs through regulation of H2BK123ub1, but that the HAR domain is dispensable for recruitment of the ubiquitylation machinery to chromatin. Lastly we identify a novel role for the HAR domain in both telomeric silencing and transcription elongation.

Our results showing that the HAR domain regulates H2BK123ub1 are consistent with a recently published report (Zheng et al. 2010). This report also found that in the absence of the HAR domain, Rad6 recruitment to the *GAL* locus is not altered. Here we confirm and expand upon this finding and show that loss of the HAR domain does not affect global association of the ubiquitylation machinery with chromatin (Figure 2.4). These data provide further support that the HAR domain plays a role in regulating the activity of the ubiquitin ligase on chromatin. It is therefore interesting to speculate on how the HAR domain contributes to H2BK123ub1. Notably, the HAR domain is situated close to lysine 123 in H2B, forming a nucleosomal surface that likely contributes to either catalytic domain recognition and/or enzymatic function. Future work will be required to explore the role of the HAR domain in H2BK123ub1 function.

The previous report investigating the HAR domain also showed that it regulates H3K4me3. While we were able to recapitulate this result using the published strains we found that the regulation of H3K4me3 is strain dependent. In contrast, the HAR domain universally regulates H3K79me3 and H2BK123ub1. The mechanism by which H3K4me3 is differentially regulated in different strain backgrounds by the HAR domain remains unclear.

Our observation that the HAR domain affects H2BK123ub1 and H3K79me3 without H3K4me3 is especially interesting considering that the *trans*-histone regulatory pathway involving H2BK123ub1 is typically thought to involve both H3K4 and H3K79 methylation. Several recent observations, however, have begun to separate the regulation of each of these methylation states by H2BK123ub1. For example loss of Bur2, a component of a transcription-associated kinase complex in yeast, results in decreased H2BK123ub1 and H3K4me3, but not H3K79me3 (Laribee et al. 2005). Moreover during myogenic differentiation in mammals there is an observed decrease of H2BK123ub1 and H3K79 methylation, but not H3K4 methylation (Vethantham et al. 2012). Adding to this set of examples, the results presented here provide the first evidence for a specific mutation that in some instances affects H2BK123ub1 and H3K79 methylation, but not H3K4 methylation.

In addition to identifying a unique role in *trans*-histone regulation of histone PTMs, we discovered an additional functional role for the HAR domain in the process of transcription elongation. Consistent with the known role of H2BK123ub1 in transcription elongation and the observation that the HAR domain regulates H2BK123ub1 we found that the HAR domain exhibits genetic interactions with known elongation factors. Several of these interactions mimic those observed with H2BK123ub1, however this was not always the case. We were surprised by the genetic interaction between the HAR domain and Rpb9, where loss of the HAR domain was able to rescue the slow growth phenotype observed in the absence of Rpb9. This result is the opposite of that observed for the combined loss of H2BK123ub1 and Rpb9, which display synthetic

sickness. This result suggests that the role for the HAR domain in transcription elongation may be multifaceted. Given that the HAR domain is present throughout all of chromatin (unlike H2BK123ub1) it is possible that it may play multiple roles depending on the chromatin context and act as a binding platform for proteins with diverse functions. In line with this view is the observation in both fission yeast and humans that the condensin complex can bind the HAR domain (Tada et al. 2011). Future studies will need to address the full collection of proteins that interact with the HAR domain to further elucidate the unique functions of this domain.

Tables

Table 2.1 Yeast Strains and Genotypes

Strain	Genotype	Source
JHY311	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1Δ*::*KAN hhf2-hht2Δ*::*NAT hta1-htb1Δ*::*HPH hta2-htb2Δ*::*NAT* pQQ18 (*CEN LEU2 HTA1 HTB1 HHT2 HHF2*)	(Ahn et al. 2005)
JHY293	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1Δ*::*KAN hhf2-hht2Δ*::*NAT hta1-htb1Δ*::*HPH hta2-htb2Δ*::*NAT* pJH53 (*CEN LEU2 hta1 Δ1-20 HTB1 HHT2 HHF2*)	(Ahn et al. 2005)
JHY297	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1Δ*::*KAN hhf2-hht2Δ*::*NAT hta1-htb1Δ*::*HPH hta2-htb2Δ*::*NAT* pJH49 (*CEN LEU2 HTA1 htb1 Δ1-32 HHT2 HHF2*)	(Ahn et al. 2005)
JHY307	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1Δ*::*KAN hhf2-hht2Δ*::*NAT hta1-htb1Δ*::*HPH hta2-htb2Δ*::*NAT* pJH57 (*CEN LEU2 HTA1 HTB1 hht2 Δ1-30 HHF2*)	(Ahn et al. 2005)
JHY315	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1Δ*::*KAN hhf2-hht2Δ*::*NAT hta1-htb1Δ*::*HPH hta2-htb2Δ*::*NAT* pJH45 (*CEN LEU2 HTA1 HTB1 HHT2 hhf2 Δ1-27*)	(Ahn et al. 2005)
PY014	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP002 (*CEN6 TRP1 HTA1 HTB1*)	(Parra et al. 2006)
PY015	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP012 (*CEN6 TRP1 hta1 Δ4-20 HTB1*)	(Parra and Wyrick 2007)
PY050	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP072 (*CEN6 TRP1 hta1 Δ4-16 HTB1*)	(Parra and Wyrick 2007)
PY051	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP073 (*CEN6 TRP1 hta1 Δ4-12 HTB1*)	(Parra and Wyrick 2007)
PY052	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP074 (*CEN6 TRP1 hta1 Δ4-8 HTB1*)	(Parra and Wyrick 2007)

PY053	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP075 (*CEN6 TRP1 hta1 Δ8-20 HTB1*)	(Parra and Wyrick 2007)
PY054	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP076 (*CEN6 TRP1 hta1 Δ12-20 HTB1*)	(Parra and Wyrick 2007)
PY055	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 hta1-htb1Δ::HIS3 hta2-htb2Δ::LEU2* pMP077 (*CEN6 TRP1 hta1 Δ16-20 HTB1*)	(Parra and Wyrick 2007)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
YGW116	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dot1Δ::NAT*	This study
FY406	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pSAB6 (*CEN URA3 HTA1-HTB1*)	(Hirschhorn et al. 1995)
YGW062	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 dot1Δ::NAT* pSAB6 (*CEN URA3 HTA1-HTB1*)	This study
YGW067	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 dot1Δ::NAT* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YGW072	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YGW073	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pZS146 (*CEN HIS3 HTA1-Flag-htb1-K123R*)	This study
YGW074	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pEG101 (*CEN HIS3 hta1 Δ1-20 Flag-HTB1*)	This study
YGW075	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pEG102 (*CEN HIS3 hta1 Δ1-16 Flag-HTB1*)	This study
YGW076	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	This study
YZS276	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1*	(Sun and Allis 2002)

	pZS145 (*CEN HIS3 HTA1-Flag-HTB1)*	
YGW063	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pSAB6 (*CEN URA3 HTA1-HTB1*)	This study
YGW136	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pZS145 (*CEN HIS3 HTA1-Flag-HTB1)*	This study
YGW137	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	This study
YGW138	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pZS146 (*CEN HIS3 HTA1-Flag-htb1-K123R*)	This study
YZS606	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 ubp8Δ::KanMX6 ubp10Δ::NatMX4* pZS145 (*CEN HIS3 HTA1-Flag-HTB1)*	(Chandrasekharan et al. 2009)
YGW066	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 ubp8Δ::KanMX6 ubp10Δ::NatMX4* pSAB6 (*CEN URA3 HTA1-HTB1*)	This study
YGW139	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 ubp8Δ::KanMX6 ubp10Δ::NatMX4* pZS145 (*CEN HIS3 HTA1-Flag-HTB1)*	This study
YGW140	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 ubp8Δ::KanMX6 ubp10Δ::NatMX4* pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	This study
YGW141	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 ubp8Δ::KanMX6 ubp10Δ::NatMX4* pEG103 pZS146 (*CEN HIS3 HTA1-Flag-htb1-K123R*)	This study
YGW162	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 RAD6-3HA::KanMX* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YGW163	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 RAD6-*	This study

	3HA::KanMX pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	
YGW132	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 bre1Δ::NAT RAD6-3HA::KanMX* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YZS272	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL* pZS144 (*CEN TRP1 HTA1-Flag-HTB1)*	(Sun and Allis 2002)
YZS273	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL* pZS145 (*CEN HIS3 HTA1-Flag-HTB1)*	(Sun and Allis 2002)
YZS274	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL* pZS146 (*CEN HIS3 HTA1-Flag-htb1-K123R*)	(Sun and Allis 2002)
YZS275	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL sir2Δ::TRP1* pZS145 (*CEN HIS3 HTA1-Flag-HTB1)*	(Sun and Allis 2002)
YEG186	*MAT***a** □*leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL* pEG101 (*CEN HIS3 hta1 Δ1-20 Flag-HTB1*)	This study
YEG187	*MAT***a** □*leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL* pEG102 (*CEN HIS3 hta1 Δ1-16 Flag-HTB1*)	This study
YEG188	*MAT***a** □*leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 hta1-htb1Δ::LEU2 hta2-htb2Δ URA3-TEL* pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	This study
YGW131	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 elp3Δ::KanMX* pSAB6 (*CEN URA3 HTA1-HTB1*)	This study
YGW142	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 rbp9Δ::KanMX* pSAB6 (*CEN URA3 HTA1-HTB1*)	This study
YGW143	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 spt4Δ::KanMX* pSAB6 (*CEN URA3 HTA1-HTB1*)	This study

Figures

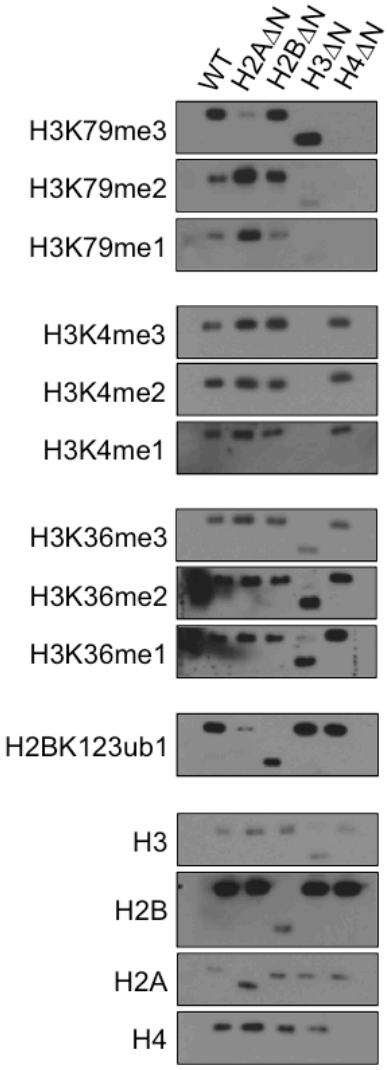

Figure 2.1 The H2A N-terminal Tail Regulates H3K79 Methylation and H2B Ubiquitylation

Shown is a screen of histone methylation and ubiquitylation states in wild-type (WT) and mutant strains lacking the N-terminal tails of each of the core histones (H2AΔN = Δ1-20, H2BΔN = Δ1-32, H3ΔN = Δ1-30 and H4ΔN = Δ1-27). Whole cell extracts (WCE) prepared from each strain were resolved by 15% SDS-PAGE and transferred to PVDF prior to immunoblotting with antibodies to the indicated histones or histone modifications. It should be noted that the H4 antibody recognizes an epitope in the N-terminal region, explaining the lack of signal in the H4 tail mutant strain.

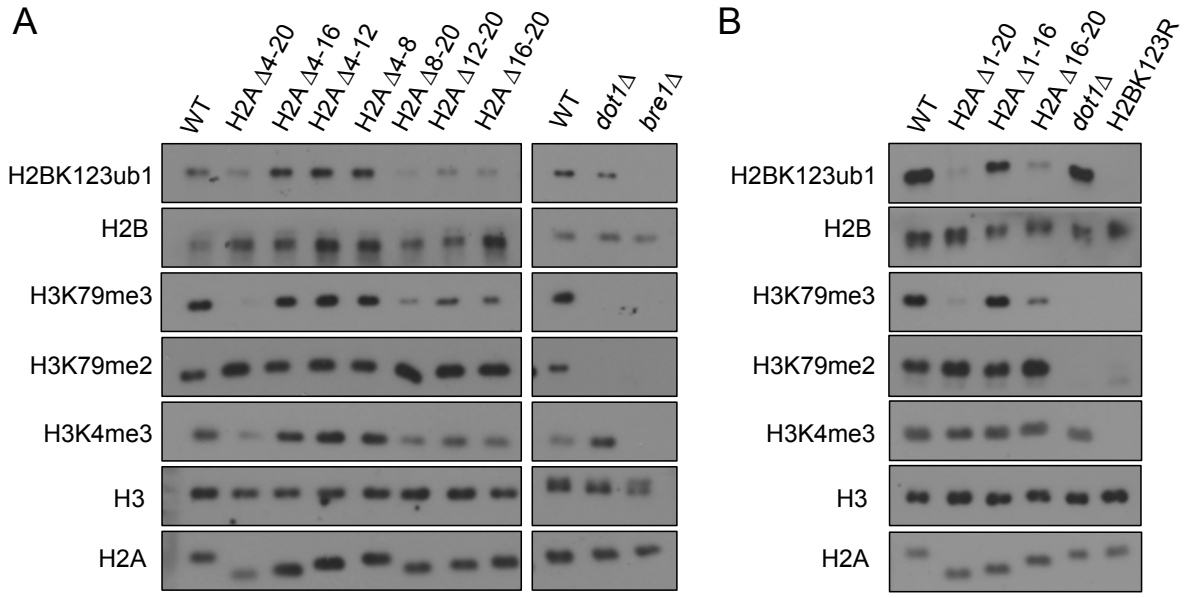

Figure 2.2 The H2A Repression (HAR) Domain is Important for the Regulation of H3K79me3 and H2BK123ub1

(A) WCE prepared from W303-derived WT and H2A N-terminal tail truncation mutants (left) were used for immunoblot analysis with antibodies to the indicated histones or histone modifications. Antibody specificity to the indicated histone modification was demonstrated in parallel using WCE prepared from BY4741-derived WT, *dot1Δ* and *bre1Δ* strains (right). (B) S288C-derived WT and mutant strains were subjected to Immunoblot analysis as described in (A). The H2BK123R strain contains an arginine instead of lysine at position 123 of histone H2B rendering the site non-ubiquitylated. The *dot1Δ* and H2BK123R strains are isogenic to WT.

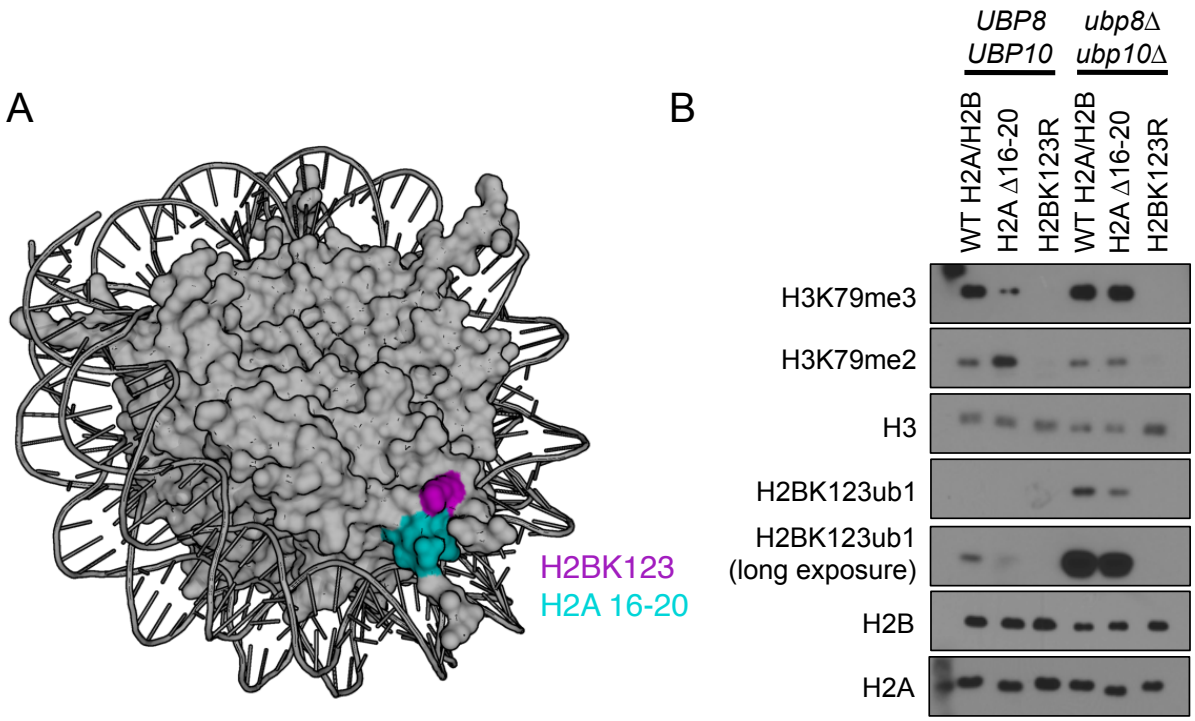

Figure 2.3 The HAR Domain Regulates the Catalysis of H2BK123ub1

(A) The HAR domain (cyan) is located next to H2BK123 (magenta) on the surface of the nucleosome (Protein Data Bank [PDB] ID: 1ID3). (B) WT and histone mutant plasmids were shuffled into *UBP8 UBP10* and *ubp8Δubp10Δ* strains and subjected to immunoblot analysis with antibodies to the indicated histones or histone modifications.

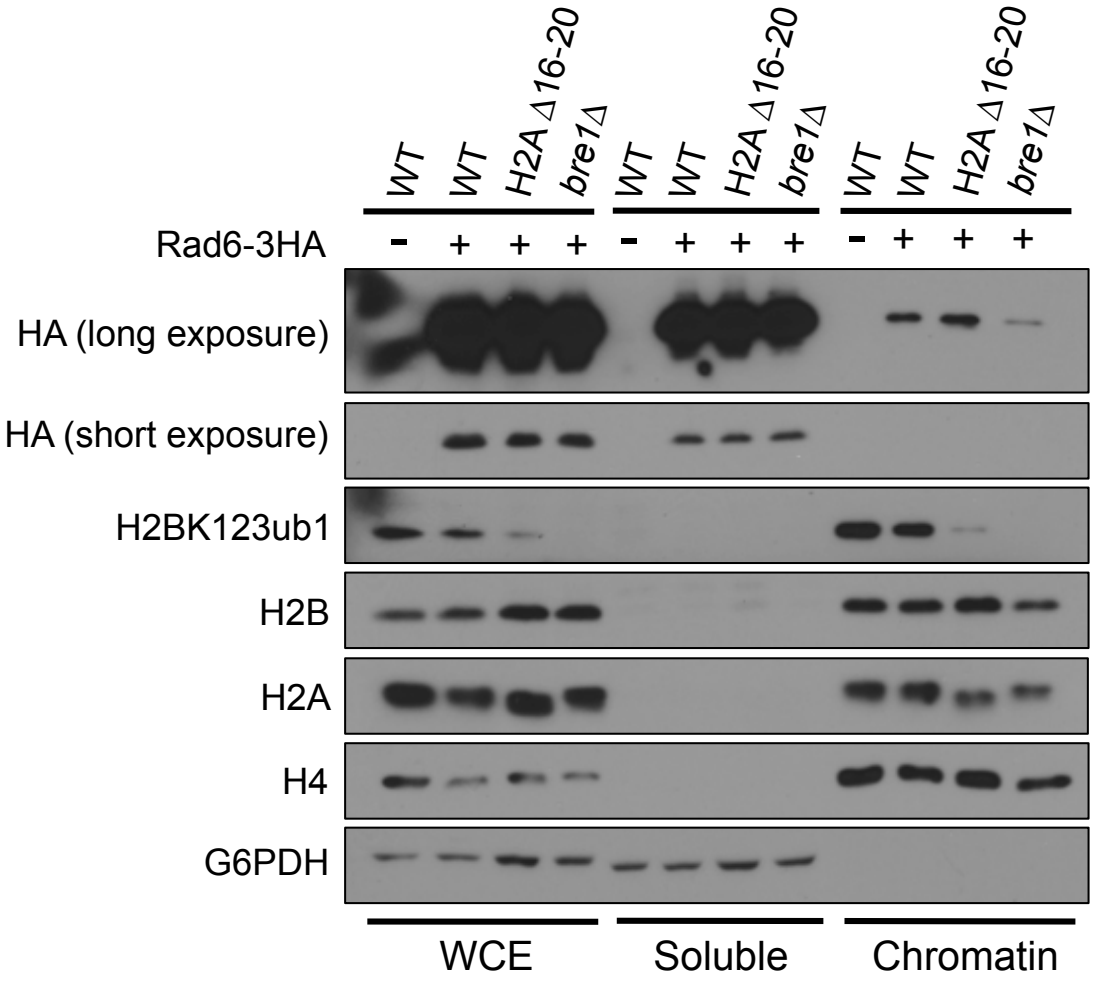

Figure 2.4 The HAR Domain is not required for the Global Recruitment of Rad6

Indicated WT and mutant strains, with or without endogenously triple HA epitope-tagged Rad6 (Rad6-3HA), were spheroplasted and subjected to detergent lysis to generate whole cell extract (WCE). The WCE was then lightly centrifuged to separate it into a soluble non-chromatin associated fraction (Soluble) and insoluble chromatin associated fraction (Chromatin). Samples of WCE along with both the soluble and chromatin fractions were subjected to SDS-PAGE and immunoblot analysis with antibodies to the indicated histones, histone modifications and G6PDH as a cytoplasmic control.

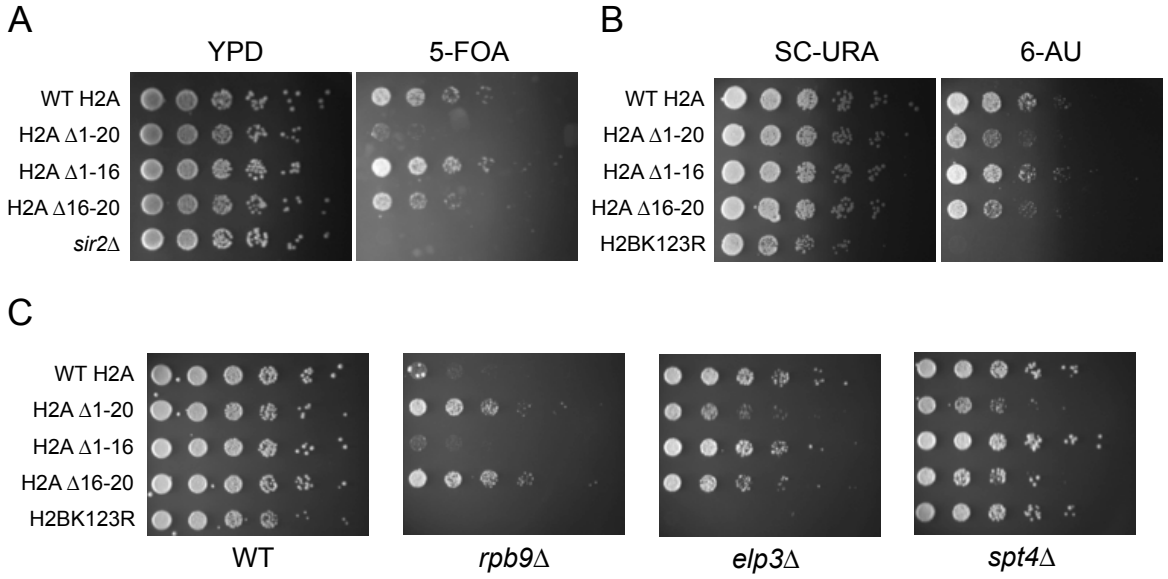

Figure 2.5 The HAR Domain Plays a Role in Telomeric Silencing and Transcription Elongation

(A) Loss of the HAR domain causes defects in telomeric silencing. Plasmids carrying WT and indicated H2A N-terminal tail mutations were introduced into a telomeric silencing reporter strain. The strains make use of the *URA3* gene inserted into the sub-telomeric region on the left arm of chromosome VII. Each strain was replica plated in five-fold serial dilutions from left to right on rich media (YPD) and media containing 5-fluoroorotic acid (5-FOA). (B) The HAR domain is important for cell growth under conditions of stressed transcription. WT and histone mutant strains carrying an empty *URA3*-containing vector (pRS316) were plated as five-fold serial dilutions on SC-URA plates with or without 200 μg/ml 6-azauracil (6-AU). (C) The HAR domain genetically interacts with transcription elongation factors. Indicated WT or histone mutant plasmids were shuffled into WT or elongation factor mutant (*rpb9Δ*, *elp3Δ* and *spt4Δ*) backgrounds and plated as five fold serial dilutions on SC-*HIS* plates.

CHAPTER 3 CATALYSIS-DEPENDENT STABILIZATION OF BRE1 FINE-TUNES HISTONE H2B UBIQUITYLATION TO REGULATE GENE TRANSCRIPTION[3]

Overview

Monoubiquitylation of histone H2B on lysine 123 (H2BK123ub1) plays a multifaceted role in diverse DNA-templated processes, yet the mechanistic details by which this modification is regulated are not fully elucidated. Here we show in yeast that H2BK123ub1 is regulated in part through the protein stability of the E3 ubiquitin ligase, Bre1. We find that Bre1 stability is controlled by the Rtf1 subunit of the polymerase associated factor (PAF) complex and through the ability of Bre1 to catalyze H2BK123ub1. Using a domain in Rtf1 that stabilizes Bre1, we show that inappropriate Bre1 levels lead to defects in gene regulation. Collectively, these data uncover a novel quality control mechanism used by the cell to maintain proper Bre1 and H2BK123ub1 levels, thereby ensuring proper control of gene expression.

Introduction

Histone post-translational modifications (PTMs) play essential roles in the regulation of chromatin structure and function (Kouzarides 2007; Zentner and Henikoff 2013). One such histone PTM that has been well studied as a regulator of multiple

[3] Portions of this chapter were adapted from Wozniak GG, Strahl BD. 2014. Catalysis-dependent stabilization of Bre1 fine-tunes histone H2B ubiquitylation to regulate gene transcription. *Genes Dev.* 28:1647-1652

DNA-templated processes is monoubiquitylation of histone H2B, which occurs at lysine 123 (H2BK123ub1) in the budding yeast *Saccharomyces cerevisiae* (Robzyk et al. 2000). This PTM functions in the context of transcriptional regulation (both initiation and elongation) (Henry et al. 2003; Kao et al. 2004; Xiao et al. 2005; Pavri et al. 2006; Fleming et al. 2008; Chandrasekharan et al. 2009; Chandrasekharan et al. 2010b), but has also been linked to other processes including DNA replication (Rizzardi et al. 2012; Trujillo and Osley 2012), repair (Game and Chernikova 2009) and kinetochore function (Latham et al. 2011).

H2BK123ub1 functions in chromatin by several means. First, this mark physically alters chromatin compaction and nucleosome stability (Fleming et al. 2008; Chandrasekharan et al. 2009; Fierz et al. 2011). Another function of H2BK123ub1 is to promote histone H3 methylation at lysines 4 (H3K4me) and 79 (H3K79me) in a mechanism of histone 'cross-talk' referred to as *trans*-histone regulation (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002). H3K4me and H3K79me, in conjunction with H2BK123ub1, serve as markers of euchromatin and act to facilitate transcription factors recruitment and prevent the binding of silencing factors (Wozniak and Strahl 2014). Accordingly, loss of these PTMs leads to aberrant gene regulation.

In yeast, H2BK123ub1 is catalyzed by the concerted efforts of the ubiquitin-conjugating enzyme (E2) Rad6 and the RING finger domain-containing ubiquitin ligase (E3) Bre1 (Robzyk et al. 2000; Hwang et al. 2003; Wood et al. 2003a). Similar to other E3 ligases, Bre1 serves as the substrate recognition module for the complex and is important for the recruitment of Rad6 to chromatin (Wood et al. 2003a). Studies have

also found that the polymerase associated factor (PAF) complex associates with Rad6 and facilitates its recruitment to gene bodies (Ng et al. 2003a; Wood et al. 2003b; Xiao et al. 2005). Although the mechanistic underpinnings of this recruitment are not entirely clear, it is known that the Rtf1 subunit of the PAF complex plays a major role (Wood et al. 2003b; Xiao et al. 2005). In addition to Bre1 recruitment and catalysis, H2BK123ub1 levels are also controlled by the deubiquitylases Ubp8 and Ubp10 (Henry et al. 2003; Emre et al. 2005). Loss of Ubp8 or Ubp10 leads to similar phenotypes as the loss of H2BK123ub1, indicating that the levels of this PTM are carefully regulated in the cell.

In this report, we find that H2BK123ub1 is regulated through the control of Bre1 protein stability. Surprisingly, Bre1 stability is primarily controlled through its catalytic activity, in addition to its association with the PAF complex that is likely responsible for its recruitment to chromatin. By taking advantage of a region in Rtf1 of the PAF complex that can stabilize Bre1, we find that inappropriate stabilization of Bre1 under normal conditions leads to defects in gene regulation. Our results suggest a 'rheostat' control mechanism for H2BK123ub1 that contributes to proper transcriptional control.

Methods

Yeast Strains and Plasmids

Strains and plasmids used in this study are listed in Tables 3.1 and 3.2. Gene disruptions and endogenous overexpression were performed as previously described (Janke et al. 2004) and verified by both PCR and immunoblotting.

Yeast Whole Cell Extracts and Western Blot Analysis

Yeast were grown in YPD or synthetic complete dropout (SC) media at 30˚C to mid-log phase and extracts were prepared as previously described (Mehta et al. 2010). Western blots were performed as described previously (Fuchs et al. 2012). The following primary antibodies and dilutions were used: H3K79me3 (Abcam, ab2651) 1:2500, H2BK123ub1 (Cell Signaling Technology, 5546) 1:2000, H2A (Active Motif, 39235) 1:5000, H2B (Active Motif, 39237) 1:2500, H3 (EpiCypher, 13-0001; 1:1000) 1:2000, FLAG (Sigma) 1:5000, Myc Tag (Millipore, 05-724) 1:5000, G6PDH (Sigma, A9521) 1:100,000.

Phenotypic Spotting Assays

Five-fold serial dilutions of saturated overnight yeast cultures were plated on YPD or synthetic complete dropout media with or without indicated drugs. Cells were plated at a starting OD_{600} of 0.5 on appropriate media and imaged after 2-4 days of growth at 30˚ C.

RNA Isolation and RT-PCR

RNA was prepared from 10 OD_{600} units of mid-log phase cells using hot acid phenol-chloroform extraction followed by ethanol precipitation. Crude RNA was DNaseI treated (Promega) then purified using an RNeasy Mini Kit (QIAGEN). cDNA was synthesized using SuperScript II First Strand Synthesis System (Life Technologies) and diluted 1/10 prior to amplification by PCR. Primers are listed in Table 3.3. Reactions were run on

2% agarose gels and visualized by UV with SYBR Safe DNA gel stain (Life Technologies). Bands were quantified using ImageJ software.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described previously (Jha and Strahl 2014) with some exceptions. Sonication for each sample was performed for 20 minutes with alternating on/off cycles of 30 sec using a Bioruptor Standard (Diagenode). Immunoprecipitation was performed overnight with 1 mg clarified, sonicated extract and 20 μl equilibrated FLAG M2 agarose (Sigma). For PCR analysis 1 μl ChIP DNA and 0.25 μl of 1:4 diluted input DNA was used per reaction. Reactions were run on 2% agarose gels and visualized by UV with SYBR Safe DNA gel stain (Life Technologies). Bands were quantified using ImageJ software. Relative IP was calculated as follows: the intensity of the IP band for each sample was divided by the intensity of the corresponding input (IP/input). IP/input for all samples were then normalized to the untagged sample, which was set at 1. Statistical analysis was performed using an unpaired t-test. $P < 0.05$ was considered significant. Primer sequences are listed in Table 3.3**.**

Results and Discussion

The HAR domain regulates H2BK123ub1 by stabilizing Bre1

As presented in Chapter 2, we identified a region of the histone H2A N-terminal tail comprising residues 16-20 (HAR domain), which is important for the regulation of H2BK123ub1. We next sought to uncover the mechanism by which the HAR domain regulates H2BK123ub1. Given the close physical proximity of the HAR domain to

H2BK123 on the nucleosomal surface (Figure 2.3A), we hypothesized that the HAR domain may play a role in the ubiquitylation reaction itself. Thus, we investigated whether loss of the HAR domain had any effect on the E2 or E3 ubiquitin ligases Rad6 and Bre1, respectively. We previously found that loss of the HAR domain did not alter either total or bulk chromatin bound levels of Rad6 (Figure 2.4), but Bre1 was not previously investigated. To assess Bre1 levels, we transformed *bre1Δ* strains either containing or lacking the HAR domain with a low copy plasmid expressing *ADH1*-driven, N-terminally FLAG-tagged Bre1. Importantly, this expression construct restores H2BK123ub1 to WT levels in the *bre1Δ* strain and behaves similarly to a version containing the native *BRE1* promoter (Figure 3.1A and Figure 3.2). Surprisingly, the levels of Bre1 in the HAR deletion strain were reduced, matching the decrease in H2BK123ub1 (Figure 3.1A). Moreover, this was not the result of decreased *BRE1* transcription as measured by RT-PCR (Figure 3.3), indicating that the HAR domain regulates Bre1 levels through a mechanism that is post-transcriptional.

Bre1 stability is dependent on its ability to ubiquitylate H2BK123

Given the possibility that the HAR domain might regulate Bre1 stability through its contribution to a nucleosomal surface required by Bre1 to catalyze H2BK123ub1, we next asked if the loss of H2BK123ub1 itself might also regulate Bre1 stability. Strikingly, we found Bre1 proteins levels were nearly abolished in strains harboring a point mutation at H2BK123 (H2BK123R) (Figure 3.1B). As with the loss of the HAR domain, the H2BK123R mutation did not affect *BRE1* expression, suggesting that the regulation

occurs at the level of the protein stability (Figure 3.3). Consistent with this, a cyclohexamide (CHX) pulse-chase analysis revealed that Bre1 is more rapidly turned over in the H2BK123R strain (Figure 3.1C, compare WT to H2BK123R at 30 minutes post CHX treatment). Taken together, these data provide strong support that Bre1 in the HARΔ and H2BK123R strains is subject to post-transcriptional control. We note that Bre1 regulation may not involve the proteasome since MG132 treatment failed to stabilize Bre1 (Figure 3.4). This result is in agreement with another report showing MG132 decreases H2BK123ub1 levels (Mimnaugh et al. 1997).

We next ascertained if mutations in the ubiquitylation machinery would also affect Bre1 stability. We found that loss of Rad6, like the H2BK123R mutant, also decreased Bre1 levels (Figure 3.1B). Moreover, both deletion of the catalytic RING finger domain of Bre1 (1-650) and a point mutation that disrupts its enzymatic function (H665A) (Wood et al. 2003a) destabilize Bre1 (Figure 3.1D). Additionally, RING finger mutants of Bre1 also had a destabilizing effect on the protein when expressed in the context of WT endogenous Bre1 indicating that destabilization is not merely the consequence of a global loss of histone ubiquitylation (Figure 3.1D). Thus, the ability of Bre1 to ubiquitylate chromatin is important for its stability.

The PAF complex contributes to Bre1 stability via a conserved domain in Rtf1

Given that Bre1 stability is dependent on catalysis, we next sought to determine if other proteins that promote H2BK123ub1 also regulate Bre1 stability. We focused on the PAF complex, which has been well studied as a regulator of H2BK123ub1 (Jaehning

2010). As shown in Figure 3.5A, deletions of individual members of the complex have varying effects on H2BK123ub1 with the *paf1Δ* and *rtf1Δ* strains having the strongest effect. Significantly, we found that the loss of H2BK123ub1 correlates with the loss of Bre1 levels in these mutant strains, thereby linking the PAF complex to Bre1 stability and H2BK123ub1.

Rtf1 is the only subunit of the PAF complex that is absolutely required for H2BK123ub1 (Ng et al. 2003a; Wood et al. 2003b; Xiao et al. 2005). This appears to be mediated by a small conserved domain of Rtf1 called the histone modification domain (HMD), which is capable of facilitating H2BK123ub1 independently of the PAF complex (Piro et al. 2012). Based on this finding, we hypothesized that the HMD promotes H2BK123ub1 by stabilizing Bre1. To test this idea, we co-expressed Myc-tagged HMD fused to a nuclear localization sequence (NLS-Myc-HMD) and FLAG-Bre1 in the *rtf1Δ* strain. In agreement with published data (Piro et al. 2012), we found that the HMD could restore H2BK123ub1 in the *rtf1Δ* strain (Figure 3.5B). Moreover, we found that expression of the HMD could also rescue Bre1 levels, indicating a critical role for the HMD in stabilizing Bre1.

To examine the functional relevance of HMD-mediated Bre1 stabilization, we investigated its role in telomeric silencing – a function linked to both Bre1 and Rtf1. We made use of a telomeric silencing reporter strain, which has the *URA3* gene inserted near the telomere of chromosome VII. Loss of Rtf1 in this strain shows a severe growth defect when grown on media containing 5-FOA, indicating a loss of silencing (Figure 3.5C). In line with the finding that the HMD could rescue Bre1 levels and H2BK123ub1,

expression of the HMD was able to restore the silencing defect of the *rtf1Δ* strain (Figure 3.5C). These data demonstrate that the HMD plays an important role in gene silencing by stabilizing Bre1.

Altering the balance of Bre1 leads to defects in gene regulation

The ability of the HMD to stabilize Bre1 allowed us to use it as a tool to ask why Bre1 is under such careful regulation. To address this question, we again utilized the telomeric silencing reporter strain used above. In this strain we overexpressed Bre1 from the highly expressed *GPD* promoter either alone or in combination with the HMD and measured growth on 5-FOA. Overexpression of Bre1 alone did not result in any growth defect on 5-FOA (Figure 3.6A), consistent with inability of Bre1 overexpression to increase the levels of H2BK123ub1 (Figure 3.1D). In contrast, we found that overexpression of the HMD resulted in reduced growth on 5-FOA and this effect was exacerbated when Bre1 was also overexpressed indicating loss of silencing of the *URA3* reporter (Figure 3.6A). In validation of the reporter strain, we also observed increased transcription of two naturally silenced subtelomeric genes (*YFR057W* (chromosome VI) and *COS12* (chromosome VII)) with Bre1 stabilization indicating that aberrant levels of Bre1 impact transcription of normally silenced telomere-proximal genes (Figure 3.6B).

Lastly, we sought to determine if the observed changes in gene expression were the result of HMD-mediated binding of Bre1 at telomeres. To determine this we performed chromatin immunoprecipitation (ChIP) to measure Bre1 binding to a

subtelomeric region of chromosome VI proximal to *YFR057W* where the HMD has been previously shown to bind (Piro et al. 2012). In agreement with the upregulation of *YFR057W*, we found increased Bre1 binding in this region in the presence of the HMD (Figure 3.6C). Taken together, these observations demonstrate that aberrant stabilization of Bre1 at telomeres leads to defects in gene silencing. Given loss of Ubp8 and Ubp10 also result in increased H2BK123ub1 levels at euchromatic and telomeric regions (Henry et al. 2003; Emre et al. 2005), the collective data support a model wherein the ubiquitylation machinery is present across the genome, but is kept in check by the opposing functions of RNAPII-dependent PAF recruitment and the deubiquitylating enzymes that reduce H2BK123ub1 – both of which would control Bre1 stability and hence H2BK123ub1 levels genome-wide.

Concluding Remarks

In this study, we uncover a novel pathway of H2BK123ub1 regulation that involves the precise control of Bre1 protein stability. Using mutants that disrupt i) the nucleosomal surface targeted by Bre1, ii) Bre1 catalytic activity, or iii) proteins that aid in Bre1 catalysis (i.e., Rad6 and the PAF complex), we show that the ability to ubiquitylate H2B is critical for the stabilization of this E3 ligase. By expressing a domain in Rtf1 that couples the PAF complex with Bre1 and leads to its stabilization, we show that aberrant Bre1 levels results in adverse consequences for gene silencing. Taken together, these findings reveal a novel control mechanism for Bre1 that we suggest functions to fine-tune the appropriate levels of H2BK123ub1 genome-wide.

In addition to the regulation of Bre1, another mechanism that acts to fine-tune the levels of H2BK123ub1 across the genome is the deubiquitylating enzymes Ubp8 and Ubp10. A question remains as to why the cell would utilize two distinct mechanisms to control H2BK123ub1 levels. Perhaps similar to histone acetylases and deacetylases, where the equilibrium of the "on" and "off" enzymes define the precise levels of histone acetylation at any given point across the genome, it may be that the level of H2BK123ub1 across the genome is similarly governed by the equilibrium of Rad6/Bre1 and Ubp8/Ubp10. Consistent with this idea, deletion of the heterochromatin-associated Ubp10 deubiquitylase results in increased levels of H2BK123ub1 in silenced regions of the genome (Emre et al. 2005). This finding implies Bre1/Rad6 can localize to these regions but is prevented from functioning by the removal of H2BK123ub1. Notably, we were unable to detect Bre1 at a subtelomeric region of chromosome VI under normal conditions (Figure 3.6C), suggesting that it may interact transiently with these regions. In contrast, within transcribed regions where Bre1 is stabilized by the PAF complex, the equilibrium shifts toward productive H2BK123ub1 (Figure 3.7A). Thus, a possible surveillance mechanism comprising the deubiquitylating enzymes ensures loss of Bre1 and erasure of H2BK123ub1 where it would otherwise drive inappropriate functions (Figure 3.7B).

Our observations also provide insight into the regulation of H2BK123ub1 by the PAF complex. Previous work has shown that Bre1 directly interacts with the PAF complex *in vitro* using purified recombinant proteins (Kim and Roeder 2009). In addition, we have demonstrated that Rad6/Bre1 is associated with the PAF complex in

yeast (Xiao et al. 2005). Given these observations, we propose that the PAF complex, through the HMD, stabilizes Bre1 in transcribed regions, which in turn promotes Rad6 recruitment (Wood et al. 2003a) and H2BK123ub1 (Figure 3.7A). It is not entirely clear how a potential interaction with Rtf1 could stabilize Bre1, but the interaction may either mask specific degradation sequences within Bre1 or aid in the recruitment of Bre1 to its nucleosomal substrate, which may be the actual stabilizing interaction.

One of the important mechanistic functions of H2BK123ub1, in addition to promoting nucleosomal disruption and stability during transcription elongation, is the regulation of histone methylation at H3K4 and H3K79 (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002; Chandrasekharan et al. 2010b). This form of histone 'cross-talk' has been the focus of numerous studies over the past decade, but the mechanism remains to be fully elucidated. Two primary models exist, which suggest that H2BK123ub1 either acts as a wedge in chromatin to facilitate enzyme access (Fierz et al. 2011) or as a bridge to the histone methyltransferases (either directly (McGinty et al. 2008; Kim et al. 2013) or indirectly (Lee et al. 2007; Vitaliano-Prunier et al. 2008)). The indirect recruitment mechanism has been proposed to involve Cps35/Swd2, which is a subunit of the H3K4 methylating COMPASS complex and has been suggested to interact with the H3K79 methyltransferase Dot1 (Lee et al. 2007). Both these models share the common theme, however, that the ubiquitin moiety itself at H2BK123 mediates the 'cross-talk'. Intriguingly, our data demonstrate that the same mutations used to characterize H2BK123ub1-mediated 'cross-talk' also disrupt the stability of Bre1. Thus, it will be intriguing to determine if any aspect of the *trans*-histone

pathway of H3K4 and H3K79 methylation might involve Bre1 itself independent of H2BK123ub1. In support of this idea, Bre1 has been shown to interact with Cps35/Swd2 *in vivo* (Vitaliano-Prunier et al. 2008) and, intriguingly, mutations that disrupt H2BK123ub1 (and hence Bre1 stability) also disrupt the ability of Cps35/Swd2 to facilitate COMPASS-mediated H3K4 methylation (Lee et al. 2007; Vitaliano-Prunier et al. 2008). Thus, Cps35/Swd2 may be a link between Bre1 and H3K4 methylation. Future studies will be required to revisit some of the basic assumptions of H2BK123ub1-mediated histone 'cross-talk' and the details that underlie Bre1 regulation.

Tables

Table 3.1 Yeast Strains and Genotypes

Strain	Genotype	Source
FY406	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pSAB6 (*CEN URA3 HTA1-HTB1*)	(Hirschhorn et al. 1995)
YGW067	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 dot1Δ::NAT* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YGW072	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YGW073	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pZS146 (*CEN HIS3 HTA1-Flag-htb1-K123R*)	This study
YGW076	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	This study
YGW161	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 bre1Δ::HPH* pZS145 (*CEN HIS3 HTA1-Flag-HTB1*)	This study
YGW169	*MAT***a** *leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63 hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 bre1Δ::HPH* pEG103 (*CEN HIS3 hta1 Δ16-20 Flag-HTB1*)	This study
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
YJJ662	*MAT***a** *leu2Δ1 his3Δ200 ura3-52*	(Shi et al. 1997)
YJJ577	*MAT***a** *leu2Δ1 his3Δ200 ura3-52 paf1Δ::HIS3*	(Shi et al. 1997)
YJJ665	*MAT***a** *leu2Δ1 his3Δ200 ura3-52 cdc73Δ::HIS3*	(Shi et al. 1997)
YJJ1197	*MAT***a** *leu2Δ1 his3Δ200 ura3-52 ctr9Δ::KanMx*	(Nordick et al. 2008)
YJJ1336	*MAT***a** *leu2Δ1 his3Δ200 ura3-52 leo1Δ::KanMx*	(Nordick et al. 2008)
YJJ1303	*MAT***a** *leu2Δ1 his3Δ200 ura3-52 rtf1Δ::KanMx*	(Nordick et al.

		2008)
YCB647	*MATa ura3-52 his3Δ200 leu2Δ1 trp1 Δ63 lys2Δ202*	(Brachmann et al. 1995)
YNL012	*MATa ura3-52 his3Δ200 leu2Δ1 trp1 Δ63 lys2Δ202 rtf1Δ::KanMx*	This study
YGW203	*MATa ura3-52 his3Δ200 leu2Δ1 trp1 Δ63 lys2Δ202 GPD-3HA-BRE1::NatNT2*	This study

Table 3.2 List of Plasmids

Plasmid	Features	Source
pZS145	*CEN HIS3 HTA1-FLAG-HTB1*	(Sun and Allis 2002)
pZS146	*CEN HIS3 HTA1-FLAG-htb1-K123R*	(Sun and Allis 2002)
pEG101	*CEN HIS3 hta1 Δ1-20 FLAG-HTB1*	This study
pEG102	*CEN HIS3 hta1 Δ1-16 FLAG-HTB1*	This study
pEG103	*CEN HIS3 hta1 Δ16-20 FLAG-HTB1*	This study
FLAG-Bre1 p416 ADH	*CEN URA3 pADH1-FLAG-BRE1*	This study
FLAG-Bre1 H665A p416 ADH	*CEN URA3 pADH1-FLAG-bre1-H665A*	This study
Myc-NLS-HMD p416 ADH	*CEN URA3 pADH1-Myc-NLS-HMD*	This study
Myc-NLS-HMD p415 ADH	*CEN LEU2 pADH1-Myc-NLS-HMD*	This study
Myc-NLS-HMD pAD4M	*2μ LEU2 pADH1-Myc-NLS-HMD*	This study
pRS315-9XMyc-Bre1	*CEN LEU2 pBRE1-9xMyc-BRE1*	(Wood et al. 2003a)
pRS315-9XMyc-Bre1 C663A/H665A	*CEN LEU2 pBRE1-9xMyc-bre1-C663A/H665A*	(Wood et al. 2003a)

Table 3.3 List of Primers

Primer	Sequence	Application
ACT1 F	TCACCAACTGGGACGATATGG	RT-PCR
ACT1 R	CAAGGACAAAACGGCTTGGA	RT-PCR
BRE1 F	CAAGCAGAAGGCATCTCATCTA	RT-PCR
BRE1 R	CATCGCTCGAGCCCTTATTT	RT-PCR
COS12 F	TGGAATTCGCCAATACTGTTC	RT-PCR
COS12 R	ACAAAGACGCTTGCGAAGAT	RT-PCR
YRF057W F	CTAGTGTCTATAGTAAGTGCTCGG	RT-PCR
YFR057W R	CTCTAACATAACTTTGATCCTTACTCG	RT-PCR
TEL-VI F	GCGTAACAAAGCCATAATGCCTCC	ChIP
TEL-VI R	CTCGTTAGGATCACGTTCGAATCC	ChIP

Figures

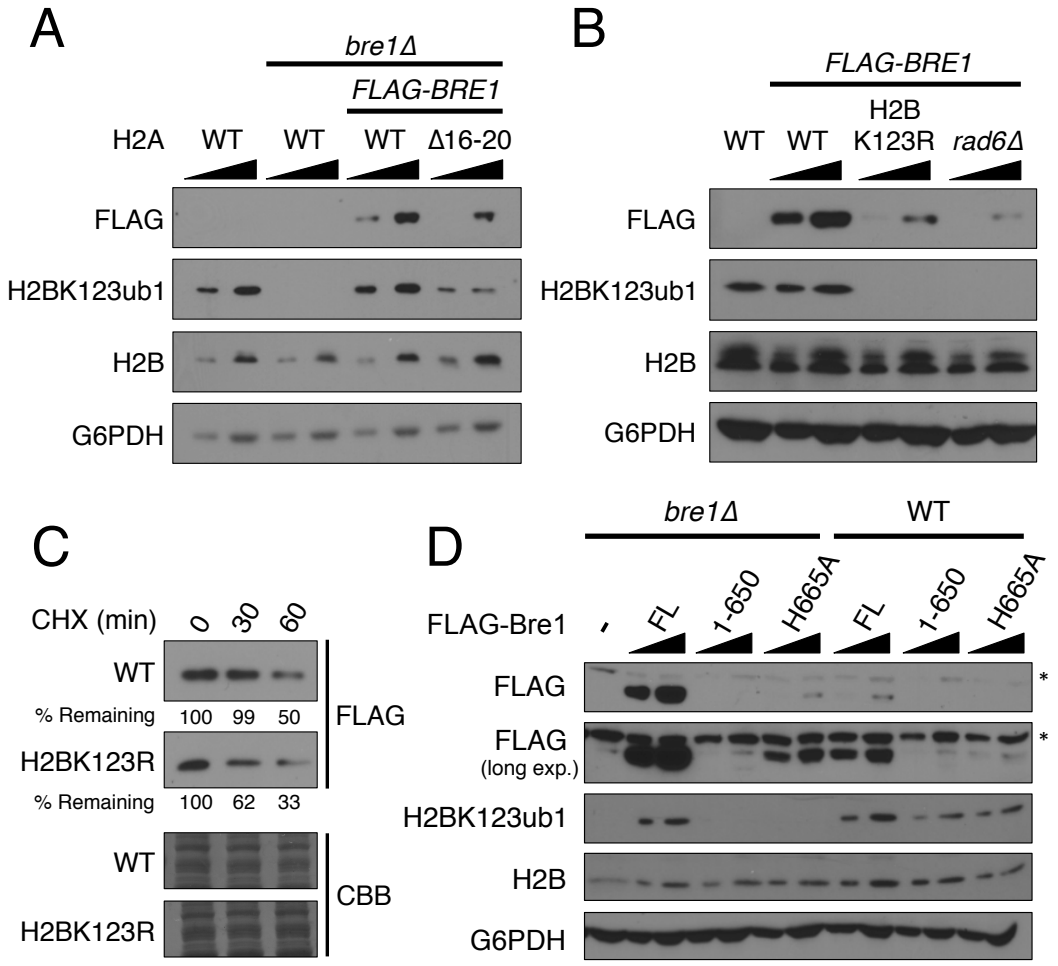

Figure 3.1 Bre1 Stability is Dependent on the Catalysis of H2BK123ub1

(A) The HAR domain is important for the stability of Bre1. Indicated mutant strains were transformed with empty vector or *ADH1*-driven *FLAG-BRE1* and subjected to immunoblot analysis with indicated antibodies. G6PDH serves as a loading control. Increasing amounts of extract were loaded for each sample as indicated by solid black triangles. (B) Catalysis of H2BK123ub1 is required for Bre1 stability. Indicated strains were analyzed as in (A). (C) Loss of H2BK123ub1 destabilizes Bre1. WT and H2BK123R strains were treated with cyclohexamide (CHX) for the indicated amount of time. Samples taken at each time point were analyzed by immunoblot analysis and Coomassie Brilliant Blue (CBB) staining. Percentage of signal compared to 0 minute time point for each sample is indicated (% remaining). (D) The RING finger domain of Bre1 is required for stability. WT or *bre1Δ* strains expressing empty vector (-), full length (FL) FLAG-Bre1 or mutant derivatives lacking the RING finger domain (1-650) or harboring an inactivating point mutation (H665A) were analyzed by immunoblot analysis. Asterisk indicates a non-specific band.

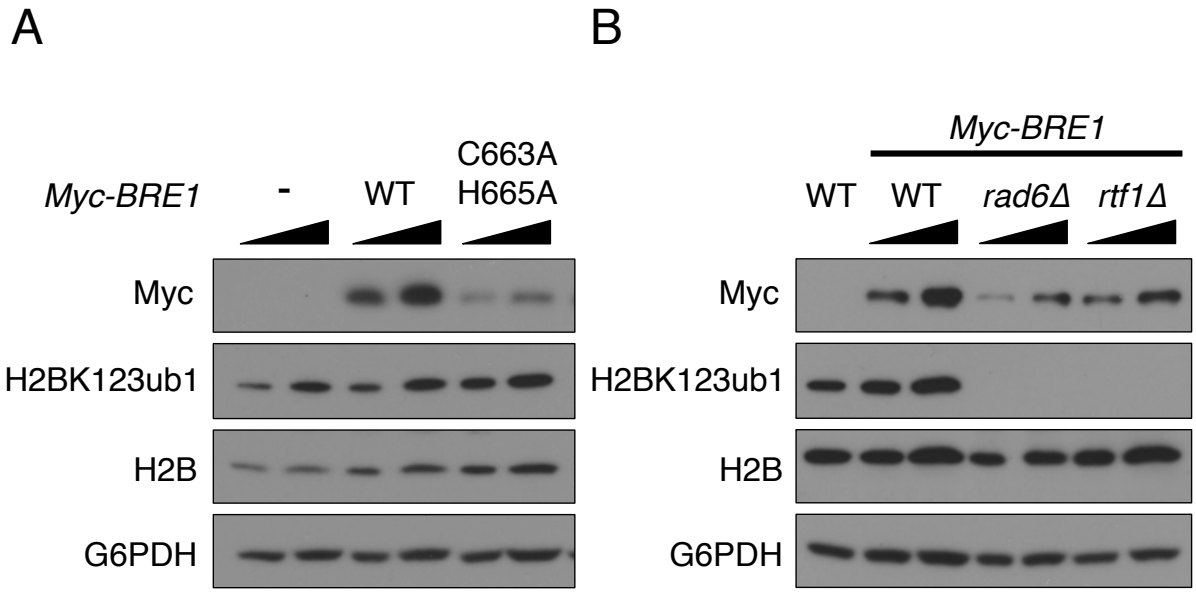

Figure 3.2 Stability of Bre1 Expressed from its Native Promoter

(A) Catalysis is important for the stability of natively expressed Bre1. WT strains were transformed with empty vector or a constructs expressing WT or mutant 9xMyc-tagged Bre1 (*Myc-BRE1*) from its native promoter and subjected to immunoblot analysis with indicated antibodies. G6PDH serves as a loading control. Increasing amounts of extract were loaded for each sample as indicated by solid black triangles. (B) Rad6 and Rtf1 are important for natively expressed Bre1. Indicated WT and mutant strains expressing WT *Myc-BRE1* were analyzed as in (A).

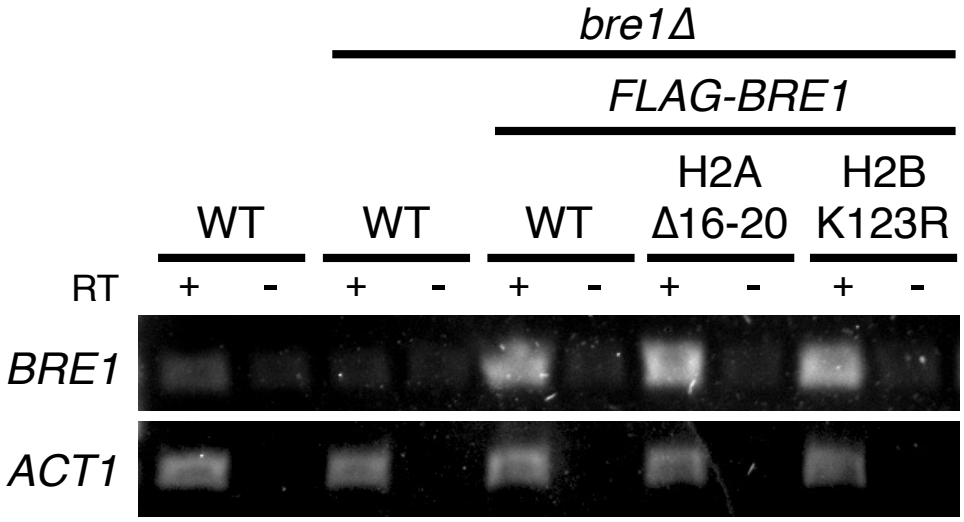

Figure 3.3 Bre1 Transcript Levels are not affected by the Level of H2BK123ub1

Reverse transcriptase (RT) PCR was performed using indicated strains with primers directed towards *BRE1* or the housekeeping gene *ACT1*. Reactions performed with or without RT were run on a 2% agarose gel and visualized by UV.

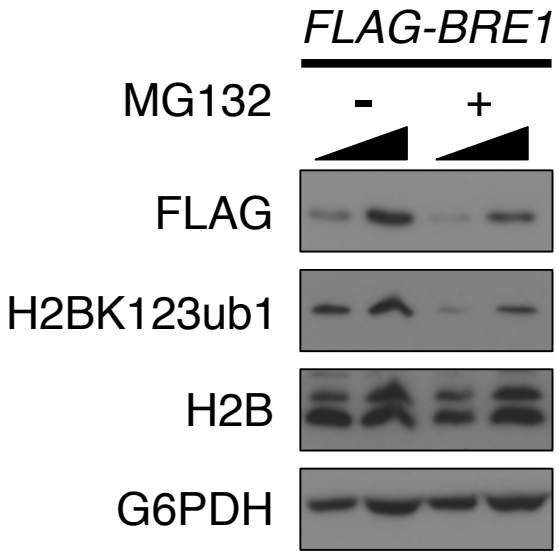

Figure 3.4 The Stability of Bre1 is not regulated by the Proteasome

WT strains expressing *FLAG-BRE1* were grown in presence of MG132 (75μM) or DMSO vehicle control for 4 hours prior to immunoblot analysis with indicated antibodies. Cells were grown in 0.004% SDS as previously described to increase drug permeability (Liu et al. 2007). Increasing amounts of extract were loaded for each sample as indicated by solid black triangles. Decreased levels of H2BK123ub1 were used as a positive control for treatment (Mimnaugh et al. 1997).

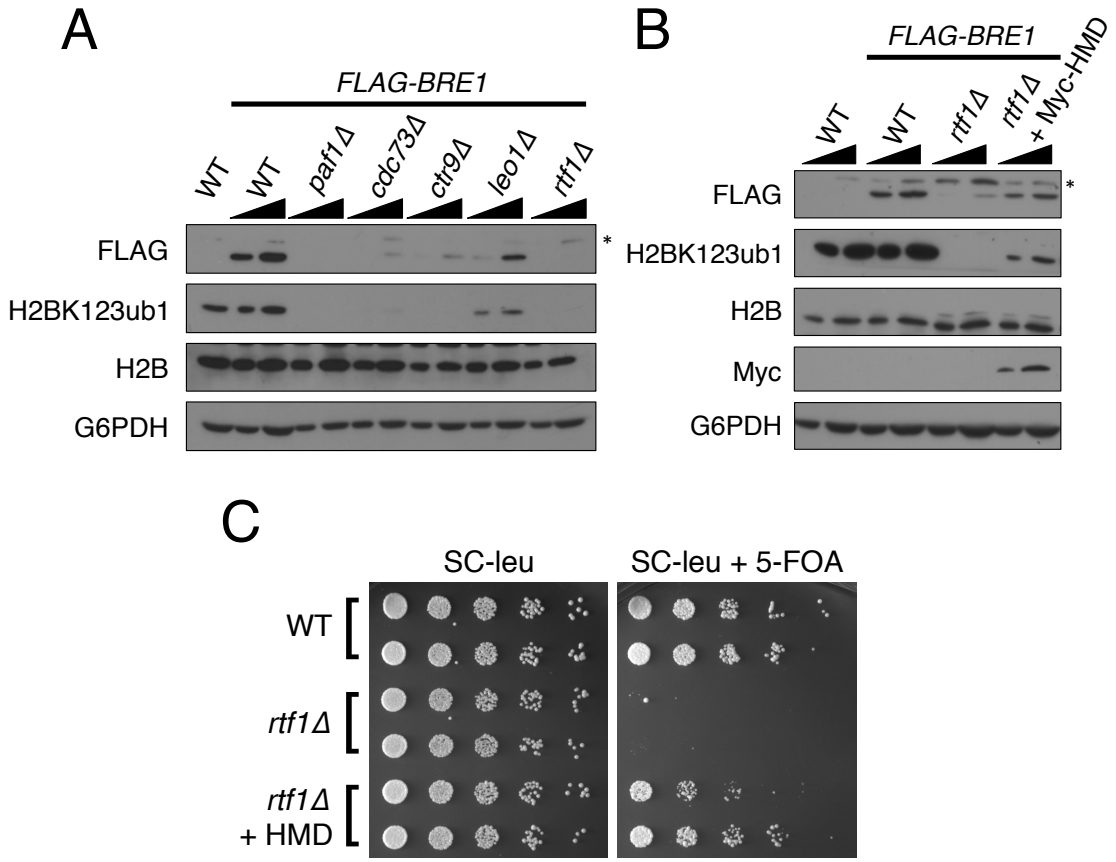

Figure 3.5 The Histone Modification Domain (HMD) of Rtf1 Stabilizes Bre1

(A) The PAF complex regulates Bre1 stability. Indicated strains transformed with empty vector or *FLAG-BRE1* were subjected to immunoblot analysis. Increasing amounts of extract were loaded for each sample as indicated by solid black triangles. Asterisk indicates a non-specific band. (B) The HMD of Rtf1 stabilizes Bre1. Indicated strains were transformed with *FLAG-BRE1* and/or NLS-Myc-HMD (Myc-HMD, CEN) and subjected to immunoblot analysis as in (A). (C) The HMD is sufficient for mediating the telomeric silencing function of Rtf1. Empty vector or a plasmid expressing NLS-Myc-HMD (HMD, 2*μ*) were transformed into WT or *rtf1Δ* telomeric silencing reporter strains harboring the *URA3* gene inserted within a subtelomeric region of chromosome VII. Strains were plated on SC-leu media with or without 5-FOA.

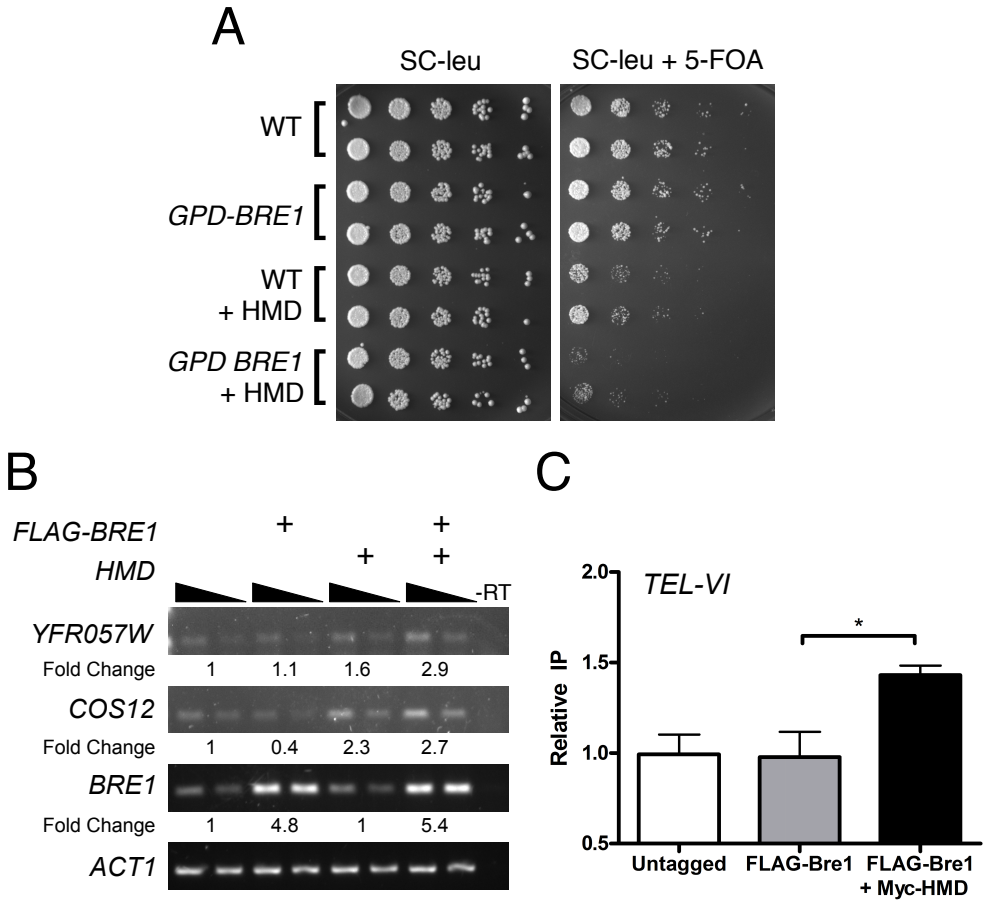

Figure 3.6 Aberrant Bre1 Stabilization Disrupts Gene Silencing

(A) Stabilization of Bre1 causes defective silencing at telomeres. Telomeric silencing strains that overexpressed Bre1 from the *GPD* promoter (*GPD-BRE*) and/or the HMD (2*μ*) were used and analyzed as in Fig. 3c. (B) Stabilized Bre1 alters the expression of naturally silenced telomeric genes. Reverse transcriptase (RT) PCR was performed with RNA isolated from strains expressing *FLAG-BRE1* (CEN) and/or the HMD (2*μ*) with primers directed towards the subtelomeric genes *YFR057W* (chromosome VI), *COS12* (chromosome VII), *BRE1* or the housekeeping gene *ACT1*. Decreasing amounts of cDNA were used for each PCR as indicated by solid black triangles. The expression of each target was normalized to *ACT1* and the fold change versus WT was calculated and shown below each strain. (C) The HMD recruits Bre1 to telomeres. ChIP was performed with M2 FLAG agarose under each of the indicated conditions. ChIP and input DNA were used as template for PCR reactions containing primers specific to a subtelomeric region of chromosome VI (*TEL-VI*). Relative IP represents fold change enrichment versus untagged. See Supplemental Methods for further details. Data represent mean +/- SEM (n = 3; $*P < 0.04$).

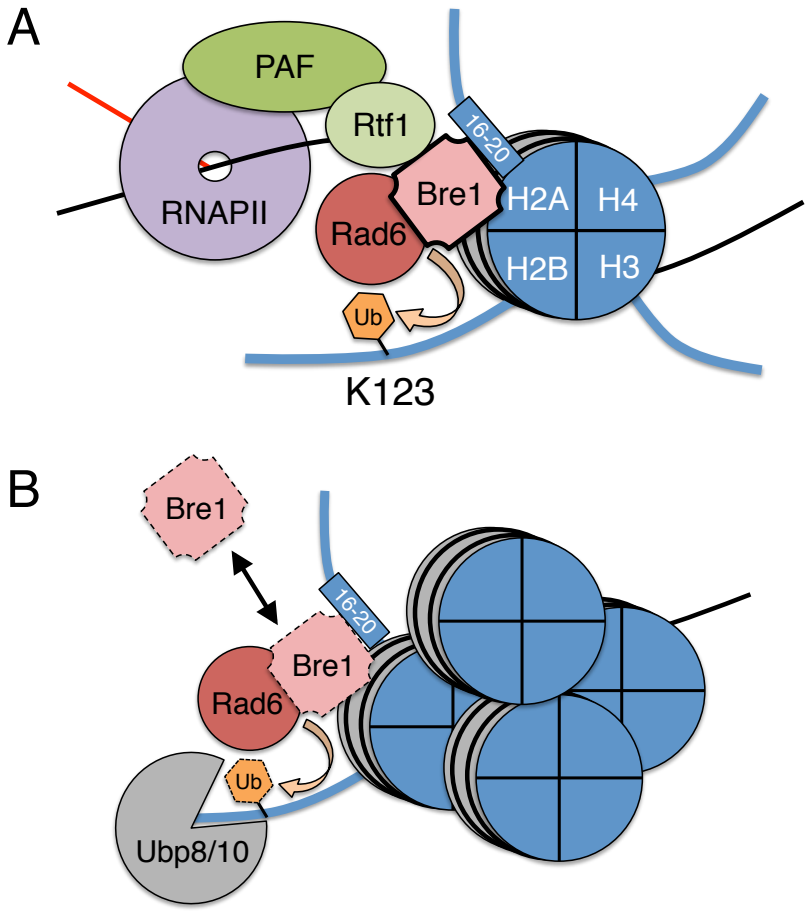

Figure 3.7 Transcription-Coupled Stabilization of Bre1 Fine-Tunes H2B Ubiquitylation

(A) We find that the Rtf1 subunit of the PAF complex is important for stabilizing Bre1 and promoting H2BK123ub1. Given the close association of the PAF complex with transcribing RNA Polymerase II (RNAPII) we propose that Rtf1, residues 16-20 of histone H2A and perhaps other proteins associated with the transcriptional apparatus interact with and stabilize Bre1 (indicated by solid black outline) to promote H2BK123ub1 in active regions of the genome. (B) Once transcription is complete or in repressed regions, the absence of the transcriptional machinery leads to Bre1 instability (indicated by dashed outline). Transient interactions of Bre1/Rad6 with chromatin in repressed regions catalyze short-lived H2BK123ub1 (dashed outline) that is rapidly removed by the deubiquitylating enzymes Ubp8/10.

CHAPTER 4 CONCLUSIONS

Ubiquitylation of histone H2B (H2BK123ub1) is a dynamic histone modification that plays important roles in many DNA-templated processes. Consistent with its diverse functionality, alterations of this modification have been implicated in disease, underscoring the importance of understanding its regulation and function. Over the past decades, much has been learned about H2BK123ub1 such as the proteins involved and downstream functions, but there are still important open questions as evidenced in the previous chapters. It is clear that H2BK123ub1 is associated with euchromatin, but it remains to be determined how the ubiquitylation machinery recognizes its substrate to catalyze H2BK123ub1. Additionally, despite the observation that H2BK123ub1 is dynamically regulated, it is unclear how this occurs. How H2BK123ub1 mediates its affect in chromatin and fits into the larger picture of chromatin and cellular regulation also warrants further investigation. The work presented in this dissertation does not provide all the answers, but adds important insights into each of these questions. These insights will be important for guiding future research on the role and regulation of H2BK123ub1.

A Role for the HAR Domain in Catalysis of H2BK123ub1

As presented in Chapter 1, a region of the histone H2A N-terminal tail called the HAR domain was identified to regulate and function analogously to H2BK123ub1. It is

still not clear, however, how exactly the HAR domain participates in this regulation. We and others have shown that it does not play a role in the recruitment of Rad6 to chromatin (Zheng et al. 2010). Instead, loss of the HAR domain reduces the stability of Bre1. As revealed in Chapter 2, Bre1 becomes unstable when it is unable to catalyze H2BK123ub1, indicating that the HAR domain regulates the catalysis of the modification. This is idea is further bolstered by the observation that the HAR domain sits in close physical proximity to H2BK123 on the surface of the nucleosome.

One possible mechanism for how this occurs is that Bre1 physically interacts with the HAR domain. To test this question, *in vitro* binding experiments using purified recombinant Bre1 and nucleosomes will be required due to the issue of Bre1 stability *in vivo*. An alternative possibility is that the HAR domain is important for the structural integrity of the nucleosomal surface surrounding H2BK123 and, thus, is important for accessibility of the lysine residue. In support of this idea, replacement of the amino acid residues within the HAR domain with alanine does not dramatically affect H2BK123ub1 (data not shown). Alanine may be able to maintain the nucleosomal surface and this also indicates that the residues themselves do not necessarily matter. Structural analysis of nucleosomes lacking the HAR domain may be required to fully test this possibility.

Either of these possibilities will shed light on the interaction surface utilized by the histone ubiquitylation machinery. Knowledge of such interactions could be useful to guide the design of small molecule chemical probes to disrupt H2BK123ub1 catalysis. Small molecules that disrupt the contribution of the HAR domain to H2BK123ub1 could

prove a useful in cancers, which are dependent on H2BK123ub1 (Wang et al. 2013). Loss of the HAR domain does not abrogate H2BK123ub1, which is also detrimental to cells, and thus, may restore H2BK123ub1 to normal levels in these cells.

Functionality of the HAR Domain

While it is unclear how the HAR facilitates H2BK123ub1, it is clear that it shares similar biological functions with H2BK123ub1. With the two functions tested (transcription elongation and telomeric silencing) the HAR domain showed similar phenotypes with the notable exception of genetic interaction with Rpb9. As suggested in Chapter 2, this may be the consequence of multiple functions for the HAR domain in chromatin. Alternatively, reduction of H2BK123ub1 may lead to different phenotypes than complete loss. This possibility could be tested using other mutations that reduce H2BK123ub1.

It remains to be seen, however, whether the HAR domain plays a similar biological function in more complex organisms especially given the conservation of H2B ubiquitylation. A comparison of histone H2A between organisms also reveals that the HAR domain is highly conserved (Parra and Wyrick 2007). Until recently, this question could not be addressed due to the inability to completely mutate histones genes in a complex organism. This has recently changed with histone replacement methods in the fruit fly *Drosophila melanogaster* (Gunesdogan et al. 2010; Pengelly et al. 2013). With this technology it is now possible to test the effect of HAR domain mutation and/or the loss of H2B ubiquitylation on an organismal level.

Mechanisms of *Trans*-Histone Regulation

The regulation of H2BK123ub1 by the HAR domain represents a type of *trans*-histone regulation whereby one histone influences the modifications on another. One of the most well-defined forms of *trans*-histone regulation involves the regulation of H3K4 and H3K79 methylation by H2BK123ub1 (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002; Chandrasekharan et al. 2010b; Pengelly et al. 2013). Consistent with this pathway, the HAR domain also regulates H3K4 and H3K79 methylation in addition to H2BK123ub1. Surprisingly, however, the regulation of H3K4 appears to be dependent on genetic background. Part of this observation may be explained by how H3K4 methylation is catalyzed. It has been reported that the H3K4 methyltransferase complex COMPASS can bind the H2B C-terminus independently of H2BK123ub1 (Chandrasekharan et al. 2010a). As suggested above, loss of the HAR domain could create structural aberrations in or around the H2B C-terminus that would preclude binding of COMPASS and hence H3K4 methylation. Why this would only be affected in one genetic background but not the other is still an open question. An in depth DNA sequence and RNA expression comparison of the two backgrounds will likely be required to understand this phenomenon.

Despite the differences in H3K4 methylation with mutation of the HAR domain, the mechanism of *trans*-histone regulation of histone methylation by H2BK123ub1 is still poorly defined. Two general hypotheses have been put forth, which state that either H2BK123ub1 promotes chromatin accessibility or acts as a binding site for the methylation enzymes (Chandrasekharan et al. 2010b). Focusing on H3K79 methylation, the enzyme responsible for this modification (Dot1) has been shown to bind

ubiquitin, but does not show altered binding to nucleosomes in the presence or absence of H2BK123ub1 (McGinty et al. 2008; Oh et al. 2010). Remarkably, H2BK123ub1 stimulates H3K79 methylation within the context of the same nucleosome *in vitro*, which suggests that ubiquitin may be functioning allosterically (McGinty et al. 2008). It also suggests that other accessory factors may not be required for the *trans*-histone pathway as previously suggested (Lee et al. 2007).

Not investigated in this context, however, was the effect of H2BK123ub1 on internucleosomal interactions. Increased internucleosomal interaction in the absence of H2BK123ub1 could disrupt histone binding sites required for the activity of Dot1. In agreement with this idea, unmodified mononucleosomes serve as better substrates for Dot1 than nucleosomal arrays (Fierz et al. 2011). Moreover, addition of H2BK123ub1 to the arrays eliminates this difference. One of these potential internucleosomal interaction involves the histone H4 N-terminal tail, which has been shown to stimulate Dot1 activity, but not contribute to overall nucleosome binding (Fingerman et al. 2007). Interestingly the H4 tail has also been proposed to mediate internucleosomal interactions with a region of histone H2A on a neighboring nucleosome (Luger et al. 1997). Thus a competition between Dot1 and the neighboring nucleosome for binding the H4 tail may govern H3K79 methylation. Furthermore, H2BK123ub1 may block the internucleosomal interaction between H4 and H2A to help resolve the competition in favor of Dot1. Future studies will be required to integrate the contribution of internucleosomal interactions into the *trans*-histone regulatory pathway.

Regulation of H2BK123ub1 via Bre1 Stability

Initial work on the HAR domain led to the surprising finding that Bre1 stability is coupled to the catalysis of H2BK123ub1. Moreover stability is linked to transcription via the PAF complex, thereby ensuring that H2BK123ub1 is only found at actively transcribed genes. While this mechanism provides a framework for how H2BK123ub1 can by dynamically regulated during transcription, some questions still remain. It is still unknown how Bre1 is degraded and, moreover, what upstream signals trigger the degradation process. Initial experiments indicated that, perhaps, Bre1 is not degraded by the proteasome owing to the fact that treatment with the proteasome inhibitor MG132 decreased protein levels. Upon further investigation it appears that this result may be the result of cellular stress induced by MG132, since other forms of cellular stress such as heat shock also reduced Bre1 levels – a point further explored below. Interestingly, a recent study on the human homolog of Bre1 called RNF20 showed that it is degraded in a proteasome-dependent manner (Blank et al. 2012). Therefore, the mechanism of Bre1 degradation in yeast will need to be revisited using other techniques that reduce the stress response such as genetic inactivation of the proteasome over short time courses. Understanding the mode of degradation is important because it will help focus work to identify the players involved in degradation.

Despite this gap in knowledge, a few candidate regulators of Bre1 have been identified. One comes from the aforementioned study on RNF20, which found that a E3 ubiquitin ligase called Smurf2 can polyubiquitylate RNF20 to target it for degradation (Blank et al. 2012). Whether this is linked to transcription has not yet been explored. Intriguingly, however, is that a putative yeast homolog of Smurf2, Rsp5, has been

extensively linked to transcription and has been demonstrated to participate in the degradation of RNAPII (Huibregtse et al. 1997; Somesh et al. 2005). This evidence, while circumstantial, puts Rsp5 at the right place and time to serve a regulator of Bre1 stability and warrants further study. Moreover, it will be interesting to see if the coupling of Bre1 stability to transcription is conserved in more complex organisms.

Another unknown with regards to Bre1 stability is how degradation is signaled. Interestingly, as described above, Bre1 levels decrease in response to cellular stress. It is not clear, however, which proteins or if a common set of stress response proteins are involved. Moreover, several possibilities exist for the initiation of Bre1 degradation. Since Bre1 stability is coupled with transcription, it could be a more passive process whereby when a stalled or terminated RNAPII leaves the gene, Bre1 becomes more vulnerable to degradation. Alternatively, transcriptional repressors recruited to genes during inactivation may also recruit the proteins that degrade Bre1. Degradation of Bre1 would lead to the loss of H2BK123ub1 and stalled transcription leading to complete gene inactivation. In this scenario, Bre1 degradation would be an early event in gene repression.

Purpose of Bre1 Degradation

As mentioned above, one reason Bre1 may be degraded is that it may be an early event in the process of gene inactivation to reduce H2BK123ub1. Having Bre1 as a control point for transcriptional regulation could prove useful in the event of large, genome-wide changes in transcription. A prime example of this is during the yeast stress response where many genes change their expression and, intriguingly, Bre1

levels are also altered. Another surprising result is that Bre1 is still degraded in mutants where H2BK123 cannot be ubiquitylated (H2BK123R). The degradation of Bre1 in this mutant may be the result of reduced global transcription, although this possibility is not likely since loss of the PAF complex, which plays a much larger role in transcription (Jaehning 2010), reduces Bre1 to a similar level at the H2BK123R mutant. Bre1 degradation in the H2BK123R mutant may, instead, suggest that Bre1 has additional ubiquitylation substrates or binding partners during transcription. Thus Bre1 has to be degraded to prevent interaction with and, perhaps, ubiquitylation of the non-histone substrates.

Currently, no other substrates of Bre1 have been identified, but intriguingly Bre1 has been linked to the ubiquitylation status of other proteins. One of these proteins is Swd2/Cps35, which is involved in both histone methylation and RNA 3' processing. Swd2/Cps35 has been shown to be ubiquitylated and this is dependent on the presence of H2BK123ub1 in cells (Vitaliano-Prunier et al. 2008). Because Bre1 levels are also dependent on H2BK123ub1, Swd2/Cps35 ubiquitylation may actually be dependent on Bre1. Bre1 has also been shown to physically interact with the RNA processing factor Npl3 (Moehle et al. 2012). Thus Bre1 may have additional roles outside of H2BK123ub1, which could explain its careful regulation. Future studies including identification of binding partners using unbiased mass-spectrometry as well as identification of novel substrates using substrate labeling techniques (Zhuang et al. 2013) will help identify the full spectrum of Bre1 function.

A Role for Bre1 and H2BK123ub1 in Ubiquitin Homeostasis

H2B is just one of many ubiquitylation substrates in the cell, but given the large number of histones required to cover the genome, it may reflect one of the most substantial substrates. In fact, about 25% of total cellular ubiquitin in human cells is associated with histones (Kaiser et al. 2011). Important to note, the contribution of H2A and H2B ubiquitylation to this quantity has not been determined. In budding yeast, however, only H2B is ubiquitylated. Therefore, alterations in histone H2B ubiquitylation alone in yeast could affect the total cellular level of ubiquitin. Moreover, histones could be a key player in the regulation of ubiquitin homeostasis by serving as an excess supply of ubiquitin.

Two studies support this idea. The first found that treatment of cells with the proteasome inhibitor MG132 leads to an increase in polyubiquitylated proteins, consistent with the inability to degrade these proteins (Mimnaugh et al. 1997). Interestingly, however, they also found that the levels of histone ubiquitylation decreased. The second study used microscopy techniques to more carefully assess ubiquitin levels in response to proteasome inhibition (Dantuma et al. 2006). They found that the nuclear pool of ubiquitin decreases rapidly after treatment, while the cytosolic pool increases along with the amount of polyubiquitylated proteins. They also provide evidence that the decrease in nuclear ubiquitin is not due to deubiquitylation of histones. Instead, the decrease is due to changes in the utilization of free ubiquitin. Thus, there appears to be a competition between the histone ubiquitylation enzymes and the cytosolic ubiquitylation enzymes for the pool of free ubiquitin. Under stress conditions,

where ubiquitin is required for protein degradation, the cytosolic pathway takes over and decreases the pool of ubiquitin available for histone ubiquitylation.

How could the competition for free ubiquitin be resolved? As illustrated in Chapter 3, treatment of cells with MG132 decreases the levels of Bre1. Moreover, Bre1 stability depends on its ability to ubiquitylate histones. Therefore, Bre1 may act as a sensor of both cellular stress and free ubiquitin to modulate the cellular distribution of ubiquitin. Coupling Bre1 stability to the stress response would not only regulate ubiquitin homeostasis, but also impinge on transcription and perhaps mediate the gene expression changes observed during this time. Lastly, Bre1 serves as an ideal component of the histone ubiquitylation machinery to regulate owing to its exclusive nuclear function as opposed to its partner Rad6, which is involved in ubiquitylation of non-histone and potentially cytosolic proteins. Future studies will be required to determine if H2BK123ub1 itself playes into ubiquitin homeostasis and identify how the stress response regulates Bre1 stability.

Final Thoughts

Bre1 and H2BK123ub1 play diverse roles in the cell and as evidenced here and elsewhere it is clear why this pathway is under such strict regulation. Since Bre1 sits at a potential intersection between protein homeostasis and chromatin regulation, it represents a key player in the cell. The dynamic regulation of Bre1 and H2BK123ub1 allow for a rapid cellular response to stress that is stably maintained through other histone modifications. Bre1 and H2BK123ub1 are likely also important players during cellular specification. As discussed in Chapter 1, changes in genome utilization account

for the diversity of cellular states. The transient nature of H2BK123ub1 may place it as an important regulator of cellular differentiation, by facilitating genomic plasticity. It is, therefore, no surprise that disrupting the careful balance of H2BK123ub1 leads to diseases like cancer. Given the diverse roles of H2BK123ub1 and Bre1, the human homologues of Bre1 (RNF20/40) could prove useful drug targets for the treatment of disease. It is likely, however, that in order for such drugs to succeed the levels of H2BK123ub1 will need to be carefully monitored to restore and maintain the fine-tuned balance of the modification.

In addition to the regulation of H2BK123ub1, much work is still needed to understand how it functions in chromatin. Recent work identifying factors that associate with H2BK123ub1 has begun to shed light on the mechanism, but this is only half of the story. Since H2BK123ub1 is an important regulator of histone methylation, future work will also need to identify how H3K4 and H3K79 methylation function in transcription. This will require, like for H2BK123ub1, the identification of effector proteins for each modification. Our work on Bye1 in Appendix A adds another piece to the puzzle by linking H3K4 methylation directly to RNAPII. These studies as well as others will help unveil the mechanisms of H2BK123ub1 function.

APPENDIX A STRUCTURES OF RNA POLYMERASE II COMPLEXES WITH THE CHROMATIN-BINDING PHF3/DIDO1 HOMOLOGUE BYE1[4]

Overview

Bye1 is a nuclear protein with a domain resembling the central domain in the transcription elongation factor TFIIS. Here we show that Bye1 binds with its TFIIS-like domain (TLD) to RNA polymerase II (RNAPII), and report crystal structures of the Bye1 TLD bound to RNAPII and three RNAPII-nucleic acid complexes. Like TFIIS, Bye1 binds to the RNAPII jaw and funnel, but in contrast to TFIIS it neither alters the conformation nor the *in vitro* functions of RNAPII. *In vivo*, Bye1 is recruited to chromatin via its TLD and occupies the 5'-region of active genes. A PHD domain in Bye1 binds histone H3 tails with trimethylated K4, and this interaction is enhanced by the presence of additional marks for active transcription, but impaired by repressive marks. These data indicate that Bye1 is a novel type of chromatin transcription factor that tethers histones with active marks to transcribing RNAPII. Finally, we detect putative human homologues of Bye1, the proteins PHF3 and DIDO1, which were implicated in cancer.

[4] This chapter is based on Kinkelin K, Wozniak GG, Rothbart SB, Lidschreiber M, Strahl BD, Cramer P. 2013. Structures of RNA polymerase II complexes with Bye1, a chromatin-binding PHF3/DIDO homologue. *Proceedings of the National Academy of Sciences of the United States of America* 110: 15277-15282.

Introduction

For transcription of eukaryotic protein-coding genes, RNA polymerase II (RNAPII) associates transiently with dozens of transcription factors. Different RNAPII-associated factors are required for transcription initiation, for RNA chain elongation through chromatin, for pre-mRNA processing, including 5'-capping, splicing, and 3'-processing of the nascent transcript, and for transcription termination (Perales and Bentley 2009; Hahn and Young 2011; Mischo and Proudfoot 2013). In order to understand how these factors cooperate with RNAPII and achieve their functions, structural information on RNAPII in complex with transcription factors is required. Thus far, X-ray crystallographic structural information on such complexes is limited to two transcription factors, the initiation factor TFIIB (Bushnell et al. 2004; Kostrewa et al. 2009; Liu et al. 2010; Sainsbury et al. 2013), and the elongation factor TFIIS (Kettenberger et al. 2003; Kettenberger et al. 2004; Wang et al. 2009; Cheung and Cramer 2011). TFIIS contains three domains, a mobile N-terminal domain, a central domain that binds directly to the RNAPII jaw and funnel domains, and a C-terminal zinc ribbon domain that inserts into the polymerase pore (or secondary channel) and reaches the RNAPII active site (Kettenberger et al. 2003), to stimulate cleavage of backtracked RNA during transcriptional proofreading and arrest (Wind and Reines 2000).

In the yeast *Saccharomyces cerevisiae*, there is only a single protein that contains a domain that is distantly homologous to the central, RNAPII-associated domain of TFIIS. This protein, Bye1, has been identified as a multi-copy suppressor of Ess1 (Wu et al. 2000), a peptidyl-prolyl cis-trans isomerase that is involved in proline isomerization of the C-terminal domain (CTD) of RNAPII (Hani et al. 1995; Morris et al.

1999). In Bye1, the central TFIIS-like domain (TLD, residues 232-365), is flanked by an N-terminal PHD domain (residues 74-134) and a C-terminal SPOC domain (residues 447-547, Figure A.1A). PHD domains are mostly found in proteins involved in chromatin-mediated gene regulation (Aasland et al. 1995). Consistent with this, the Bye1 PHD domain binds to a histone H3 tail peptide containing trimethylated lysine 4 (H3K4me3) (Shi et al. 2007a). The function of SPOC domains in yeast is unclear, but in higher eukaryotes SPOC domains are implicated in developmental signaling (Ariyoshi and Schwabe 2003). Bye1 localizes to the nucleus (Kumar et al. 2000), consistent with harboring putative nuclear localization signals in the N-terminal protein region. Based on yeast genetics, it was suggested that Bye1 plays an inhibitory role during transcription elongation (Wu et al. 2003). It is unknown whether Bye1 binds to RNAPII directly, and what the consequences of such binding are for polymerase structure and function.

Here we show that Bye1 binds directly to the core of RNAPII and report four crystal structures of different RNAPII functional complexes bound by Bye1. The structures reveal similarities and differences to the RNAPII-TFIIS complex. Together with functional data our results indicate that Bye1 binds to early RNAPII elongation complexes at the beginning of transcribed regions of active genes without changing polymerase structure or function. The polymerase interaction recruits Bye1 to chromatin, where it tethers promoter-proximal RNAPII at early stages of elongation to actively transcribed chromatin via a second, direct contact to histone H3 tails with modification marks for active transcription.

Materials and Methods

Protein Preparation

Saccharomyces cerevisiae 12-subunit RNAPII was prepared as described (Sydow et al. 2009). Full-length Bye1 was cloned into pOPINF with an N-terminal hexahistidine tag and expressed in *E. coli* BL21 (DE3) (Novagen). The culture was grown in LB medium at 37 °C until an OD_{600} of 0.9 was reached, induced with 0.25 mM IPTG, and grown for 18 h at 20 °C. Cells were collected by centrifugation and flash-frozen. Protein was purified by nickel affinity, anion exchange and size-exclusion chromatography. Cells were lysed by sonication in buffer A (20 mM Tris pH 7.5, 100 mM NaCl, 10 µM $ZnCl_2$, 10% (v/v) glycerol, 5 mM dithiothreitol (DTT), supplemented with 20 mM imidazole, 1 u/µl DNase (Fermentas) and 1× protease inhibitors (100× stock: 1.42 mg leupeptin, 6.85 mg pepstatin A, 850 mg PMSF, 1,650 mg benzamidine in 50 ml ethanol)). After centrifugation at 16,000g for 20 min, the cleared lysate was applied to a pre-equilibrated (buffer A) Ni-NTA agarose column (Qiagen). The column was washed with 10 column volumes of buffer A containing 20 mM imidazole before step-wise elution of the protein with buffer A containing 50/100/200 mM imidazole. Fractions containing Bye1 were pooled and applied to a MonoQ 10/100 GL column (GE healthcare) equilibrated in buffer A. The protein was eluted with a linear gradient from 100 mM to 1 M NaCl (buffer B. 20 mM Tris pH 7.5, 1 M NaCl, 10 µM $ZnCl_2$, 10% (v/v) glycerol, 5 mM DTT). To remove any minor contaminants a final size exclusion step using a Superdex 200 10/300 GL column (GE Healthcare) in 20 mM Tris pH 7.5, 100 mM NaCl, 10 µM $ZnCl_2$, 10% (v/v) glycerol, 5 mM DTT was carried out. SeMet-substituted Bye1 was grown in 2 L SelenoMet Base, 100 ml nutrient mix (Molecular Dimensions), 80 mg Selenomethionine

(Acros Organics) at 37 °C until absorbance at 600 nm of 0.6. 0.5 mM IPTG, 50 mg Selenomethionine, 100 mg Lysine, Threonine, Phenylalanine (Sigma-Aldrich), 50 mg Leucin, Isoleucin, Valin (Sigma-Aldrich) was added per 2 liter culture and the culture was grown for a further 18 h at 20 °C. Protein was purified as above. Bye1 TLD (residues 225-370) was expressed as a larger variant (residues 69-370) containing a protease cleavage site at the N-terminal border of the TLD, cloned into pOPINI with an N-terminal hexahistidine tag. The protein was expressed and purified as above except that buffers did not contain glycerol and the protein was eluted from the Ni-NTA column with 200 mM imidazole. After ion exchange purification, 300 μg precision protease was added and cleavage was carried out overnight at 4°C. To separate the cleavage products, the protein was applied to a pre-equilibrated (buffer A) Ni-NTA column. Bye1 TLD could be collected in the flow-through fraction and was then applied to size-exclusion chromatography using a Superdex 75 10/300 GL column.

Surface Plasmon Resonance

Approximately 2500 resonance units of yeast RNAPII were immobilized in immobilization buffer (Na-Acetate, pH 5) on the surface of a biosensor CM5 chip (Biacore) using the amine coupling kit (Biacore) (Löfås 1990; Johnsson et al. 1991). Recombinant Bye1 full-length was injected for 60 sec at 10 μl/min in running buffer (5 mM HEPES (pH 7.25 at 20 °C), 40 mM $(NH_4)_2SO_4$, 10 μM $ZnCl_2$, 5 mM DTT, 0.005% P20) at different concentrations (19 nM to 20 μM). The complex was allowed to dissociate for 5 min between injections. Affinity was measured for three independent

dilution series. Raw data were corrected for the bulk signal from buffer and by identical injection through a flow cell in which no RNAPII was immobilized. Data were analyzed with BIAevaluation software (Biacore).

Crystallization and X-ray Structure Determination

Complexes of RNAPII and Bye1 were formed by incubating RNAPII with a ten-fold molar amount of Bye1 at 4°C overnight. For the elongation complex (EC) and the arrested complex (AC), purified RNAPII (3.5 mg ml^{-1}) was mixed with a two-fold molar excess of template (EC template see (Cheung et al. 2011), AC template see (Cheung and Cramer 2011)) prepared as described (Kettenberger et al. 2004), 8 mM magnesium chloride and 2 mM CTP (AC), and incubated for 1 h (EC) or 2 h (AC) at 20 °C before crystallization by vapor diffusion with 5-7% PEG 6000, 200 mM ammonium acetate, 300 mM sodium acetate, 50 mM HEPES pH 7.0 and 5 mM TCEP as reservoir solution. Crystals were grown for 5–10 days, cryo-protected in mother solution supplemented with 22% glycerol and containing 4 µM tailed template and 2 mM CTP, 8 mM magnesium chloride (AC), followed by overnight incubation at 8 °C before harvesting and freezing in liquid nitrogen. Bye1 TLD or SeMet substituted Bye1 was added to the cryo-protectant at 1 mg ml^{-1} and crystals were incubated overnight at 8 °C. For complexes containing AMPCPP, RNAPII was co-crystallized with nucleic acids in the presence of 8 mM magnesium chloride and was soaked with 2 mM AMPCPP in all cryo protectant solutions. For co-crystallization of RNAPII and Bye1 full-length, purified RNAPII (3.5 mg ml $^{-1}$) was mixed with a tenfold molar excess of recombinant Bye1 and incubated over

night at 4°C before crystallization by vapor diffusion with 750 mM tri-Na-citrate and 100 mM HEPES pH 7.5 as reservoir solution. Crystals were grown for 13 days, cryo-protected in 22% glycerol, followed by one hour incubation before harvesting and flash-freezing in liquid nitrogen. Diffraction data were collected at 100 K at beamline X06SA of the Swiss Light Source. Data were collected at 0.91887 Å, the K-absorption peak of bromine and 0.9797 Å, the K-absorption peak of selenium. Structures were solved with molecular replacement using BUSTER (Bricogne et al. 2012) and the structure of 12-subunit RNAPII (1WCM) as search model. Refinement was performed using iterative cycles of model building in COOT (Emsley et al. 2010) and restrained refinement in BUSTER.

Chromatin Fractionation

Strains used in yeast chromatin fractionation were derived from W303. Plasmids containing HA-tagged, full-length Bye1, Bye1 ΔPHD (Δ1-177) and Bye1 ΔTLD (Δ177-354) (obtained from S.D. Hanes, (Wu et al. 2003)) were transformed into wild-type yeast. Chromatin fractionation was performed as described in Chapter 2.

Histone Peptide Microarrays

Full-length Bye1 (residues 1-594) and Bye1 PHD (residues 47-134) were expressed as GST-fusions from exponentially growing (OD_{600} ~0.6) BL21 RIL cells by overnight induction with 0.4 mM IPTG at 16°C. Cells were lysed by sonication in cold 1x PBS pH 7.6 containing 1 mM (PHD) or 5 mM (full-length) DTT, 1 mM PMSF, 1 mM $ZnSO_4$, and

10% glycerol (full-length only). Proteins were captured on GST-Bind Resin (Novagen) and eluted in buffer containing 50 mM Tris-HCl pH 8.0 and 10 mM glutathione. Proteins were dialyzed into buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1 mM DTT prior to microarray hybridization. Peptide synthesis and validation, microarray fabrication, effector protein hybridization and detection, and data analysis were performed essentially as described (Rothbart et al. 2012b) with the following modification. Each peptide was spotted in triplicate eight times per array. Triplicate spots were averaged and treated as a single value for subsequent statistical analysis.

Synthetic Lethality Screen

Strains used to validate candidates from the synthetic lethality screens were derived from BY4741. Synthetic genetic array analysis was performed as described previously (Tong et al. 2001; Tong et al. 2004). Briefly, strain BY5563 *bye1*Δ was crossed to the complete knockout library of nonessential genes (Giaever et al. 2002). After sporulation and selection for the respective double knockout, the latter was screened for viability. The screen was performed on a Beckman-Coulter Biomek FX.

In Vitro Transcription Assay

Nuclear extracts of *BY4741* and *bye1Δ* were prepared from 3L of yeast culture as described (24,25). Activator-dependent *in vitro* transcription assays were carried out using 150 ng of recombinant full-length Gcn4 (26) and addition of recombinant Bye1. The transcript was detected by primer extension using the 5′-Cy5-labelled

oligonucleotide 5′-TTCACCAGTGAGACGGGCAAC-3’ (24). The resulting gel was scanned on a typhoon scanner FLA9400 and data was analyzed with ImageQuant Software (GE Healthcare).

RNA Extension Assay

RNA extension assays were carried out as described (27). All samples were incubated ON at 4°C prior to addition of NTPs to allow complex formation of RNAPII and Bye1.

Results

Bye1 Interacts with RNAPII

To test whether RNAPII binds directly to Bye1 *in vitro*, we incubated pure yeast RNAPII with recombinant Bye1 and subjected the sample to size-exclusion chromatography (Materials and Methods). A stable and apparently stoichiometric RNAPII-Bye1 complex was obtained (Figure A.1B). To characterize the RNAPII-Bye1 interaction, we used surface plasmon resonance. We immobilized RNAPII on a Biacore sensor chip by amine coupling (Löfås 1990; Johnsson et al. 1991) and determined Bye1 association and dissociation rates k_a and k_d, respectively. The ratio of these rates provided a dissociation constant of K_D=3.8 ± 2.2 μM.

Structure of Bye1-bound RNAPII Elongation Complex

Co-crystallization of RNAPII with full-length Bye1 yielded crystals diffracting to 4.8 Å resolution. Structure solution by molecular replacement with free RNAPII (Armache et al. 2005) revealed strong positive difference density for the Bye1 TLD on

the Rpb1 surface, but no density for the two other Bye1 domains. To obtain better diffraction, the Bye1 TLD was expressed in isolation and soaked into preformed RNAPII elongation complex crystals containing a DNA-RNA scaffold. Diffraction data to 3.15 Å resolution were obtained (Table A.1). Phasing with the RNAPII structure (Armache et al. 2005) revealed positive difference density at the same location observed with full-length Bye1 (Figure A.1C, D). The Bye1 TLD structure was built with the aid of sequence markers obtained with selenomethionine-labeled protein, and the complex structure was refined to a free R-factor of 20.7% (Table A.1).

Bye1 Binds the Polymerase Jaw

The Bye1 TLD fold comprises an N-terminal three-helix bundle (helices α1-α3) followed by two short helices (α4, α5) that link to an extended C-terminal helix α6 (Figure A.1E). This fold resembles that of TFIIS domain II (helices α1-α6) (Kettenberger et al. 2003), and helix α6 corresponding to the TFIIS linker between domains II and III (Figure A.2A). The Bye1 TLD binds the Rpb1 jaw domain at the location where TFIIS domain II binds the polymerase (Figure A.3). Despite an overall similarity in the interactions of Bye1 and TFIIS with RNAPII, the detailed contacts differ. The Bye1 helix α3 binds the loop ß30-ß31 and helix α40 of the Rpb1 jaw domain and induces ordering of loop α40-ß29. Helix α6 extends from the jaw into the RNAPII funnel, contacting the Rpb1 loops α20-α21 and ß29-α41, and strand ß32 of the Rpb1 funnel domain. The Bye1 loop α2-α3 contacts the N-terminus of Rpb5 (Figure A.2B-D).

Bye1 Does Not Change RNAPII Conformation

TFIIS binding to RNAPII induces three major conformational changes. It repositions the large jaw-lobe module, traps the trigger loop in a locked conformation (Kettenberger et al. 2003), and realigns the RNA in the active site (Kettenberger et al. 2004). Although Bye1 resembles part of TFIIS and binds to a similar position on RNAPII, it does not induce conformational changes (Figure A.2E). These observations predicted that Bye1 does not impair nucleoside triphosphate (NTP) binding to RNAPII, which requires closure of the trigger loop. Indeed, an additional structure of Bye1 bound to the RNAPII elongation complex with an NTP substrate revealed a closed trigger loop (Figure A.1F, A.4A). Bye1 binding also did not prevent backtracking of RNA into the RNAPII pore, as revealed by another structure of Bye1 bound to arrested RNAPII with backtracked RNA (Figure A.4B).

Bye1 Does Not Influence Basic RNAPII Functions

These observations suggested that Bye1 had no functional influence on basal transcription. Indeed nuclear extracts prepared from yeast cells lacking the gene encoding Bye1 were active in promoter-dependent *in vitro* transcription assays, and addition of purified Bye1 to WT nuclear extracts did not alter their activity (Figure A.5, (Ranish et al. 1999; Seizl et al. 2011a; Seizl et al. 2011b)). In contrast to TFIIS, Bye1 did not induce RNAPII backtracking and RNA cleavage on DNA-RNA scaffolds, but allowed for unperturbed elongation activity in RNA extension assays (Figure A.6, (Damsma et al. 2007)). In addition, overall mRNA levels did not change upon Bye1 depletion, as shown

by comparative dynamic transcriptome analysis (Sun et al. 2012) (not shown). All these data indicate that Bye1 does neither induce structural changes in RNAPII functional complexes nor influence their function *in vitro*.

Bye1 Associates with Chromatin via its TLD Domain

The above results suggested that Bye1 functions in a chromatin context. To investigate whether Bye1 associates with chromatin *in vivo* and whether its RNAPII-binding TLD is required for this, we fractionated cell extracts into an insoluble, chromatin-containing fraction and a soluble, non-chromatin associated fraction as described (Donovan et al. 1997; Keogh et al. 2006). We used strains harboring plasmids containing HA-tagged, full-length Bye1 (WT) or variants lacking either the PHD domain (ΔPHD) or the TLD domain (ΔTLD) (Wu et al. 2003). All variants of Bye1 were present at the same level in unfractionated whole cell extract (Figure A.7, lanes 1-4). WT Bye1 was present in the chromatin fraction. The ΔPHD variant was also present in the chromatin fraction, but the ΔTLD variant associated with chromatin only weakly (Figure A.7, lanes 9-12). These results demonstrate that the TLD of Bye1 is important for the association of Bye1 with chromatin, but regions outside the TLD contributes to chromatin association.

Bye1 Binds Active Histone Marks via its PHD Domain

These results suggested that the PHD domain of Bye1 contributes to chromatin association, consistent with a report that this domain can bind trimethylated H3K4 peptides (Shi et al. 2007a). We therefore investigated binding of the Bye1 PHD domain

to about 200 different histone peptides on a microarray (Rothbart et al. 2012b). Microarrays were spotted at high density (~4,000 individual features) with unique histone peptides that encompass known single and combinatorial post-translational modifications on the core and tail domains of the four histone proteins H3, H4, H2A, and H2B, and their variants. The Bye1 PHD domain as well as full-length Bye1 bound specifically to H3K4me3 peptides (Figure A.8A). The high correlation between arrays probed with full length Bye1 and the isolated PHD domain indicated that the histone binding potential of Bye1 is harbored solely within its PHD domain (Figure A.8B). The interaction of Bye1 with H3K4me3 was strongly influenced by neighboring modifications (Figure A.8C). In particular, marks of active transcription (H3K9ac, H3K14ac, H3K18ac, and H3S10p) enhanced Bye1 affinity to H3K4me3, whereas marks of transcription repression (H3R2 and H3R8 methylation, Cit2, T3 and T6 phosphorylation, and H3K9me3) impaired the interaction (Figure A.8D).

Bye1 Occupies the 5'-Region of Active Genes

These results suggested that Bye1 is recruited to actively transcribed genes *in vivo*. To test this, we carried out genomic occupancy profiling with the use of chromatin immunoprecipitation (ChIP) as described (Mayer et al. 2012). Metagene analysis by averaging occupancy profiles of genes of similar length revealed a Bye1 occupancy peak 110 nucleotides downstream of the transcription start site (TSS). No significant signals were observed in promoter regions or downstream of the polyadenylation (pA) site (Figure A.9A). Bye1 was found on all active genes and its occupancy level

correlated with those for *bona fide* RNAPII elongation factors such as Spt5 (Figure A.10A). Published ChIP data for active histone H3 marks show a peak at a similar location downstream of the TSS (Figure A.10B, (Schulze et al. 2011)). These results indicated that Bye1 is recruited to the 5'-region of active genes *in vivo*, and that active histone marks contribute to Bye1 recruitment.

In order to interpret the ChIP data, we generated a three-dimensional topological model of the Bye1-bound RNAPII elongation complex approaching the +2 nucleosome of an active yeast gene (Figure A.9C). For the modeling we assumed that Bye1 crosslinks to DNA via RNAPII in ChIP experiments, and set the RNAPII active center to nucleotide position +110 downstream of the TSS. We positioned the +2 nucleosome based on its experimentally defined average position. We also included models of the flexible Bye1 SPOC and PHD domains, with the latter bound to the H3 tail emerging from the core nucleosome particle (Figure A.9C). The resulting model explained the position of the ChIP peak with high H3K4me3 occupancy, and shows that it is structurally possible that Bye1 interacts simultaneously with the RNAPII core and the trimethylated H3 tail in the 5' region of active genes.

Bye1 Genetically Interacts with Paf1 and Tho2

In order to identify genes that interact functionally with the gene encoding Bye1 and thereby further elucidate Bye1 function, we screened a yeast deletion strain collection (Tong et al. 2001; Tong et al. 2004) for synthetic growth defects with *bye1Δ*, which does not show any obvious phenotype (Wu et al. 2003). This screen revealed two

candidate genes, *paf1* and *tho2*. Generating *bye1Δpaf1Δ* and *bye1Δtho2Δ* double mutants in a different genetic background confirmed the synthetic interaction between these genes (Figure A.9D). The genes *paf1* and *tho2* encode for subunits of two *bona fide* elongation factor complexes. Paf1 belongs to the 5-subunit Paf1 complex that recruits the histone methyltransferase Set1 to transcribed genes (Krogan et al. 2003b; Wood et al. 2003b; Dehe and Geli 2006; Shilatifard 2008; Jaehning 2010). Set1 in turn is responsible for H3K4 trimethylation during transcription (Roguev et al. 2001). The interaction of Bye1 with H3K4me3 hence closes the link between Paf1 and Bye1. Tho2 resides in the 4-subunit THO complex that is required for efficient transcription elongation (Rondon et al. 2003). These results strongly support an involvement of Bye1 in transcription elongation through chromatin.

PHF3 and DIDO1 are Human Homologues of Bye1

No homologues in higher eukaryotes have been reported for Bye1. We performed a bioinformatics search based on the Pfam database (Punta et al. 2012) to identify potential homologues based on domain organization. We found two human proteins, PHD finger protein 3 (PHF3) and Death-inducer obliterator 1 (DIDO1), which show the same domain organization as Bye1. Both proteins contain an N-terminal PHD domain, a central TLD domain, and a C-terminal SPOC domain, with linkers of varying lengths in between these domains. PHF3 has been associated with glioma development as its expression is significantly reduced or lost in glioblastomas (Fischer et al. 2001).

DIDO1 is a potential tumor suppressor showing abnormal expression patterns in patients with myelodysplastic and myeloproliferative diseases (Futterer et al. 2005).

To corroborate the homology of PHF3 and DIDO1 with Bye1, we analyzed the conservation of the RNAPII-TLD interface. Both yeast RNAPII and Bye1 surfaces forming the interface are well conserved in human RNAPII and PHF3/DIDO1, respectively (Figure A.11, A.12). In particular, a salt bridge between Bye1 residue K314 and E1168 in the largest RNAPII subunit Rpb1 is conserved in the predicted human PHF3/DIDO1-RNAPII complexes. Similarly, many hydrogen bonds observed between the Bye1 TLD and Rpb1 (Bye1 residues N292, S311, D315, R355, N362, F363) are predicted to be conserved in the homologous human complexes. These results predict that PHF3 and DIDO1 contain RNAPII-binding TLD domains and are human homologues of yeast Bye1, and indicate that our structures and results are relevant for understanding the human proteins.

Discussion

Here we show that the nuclear protein Bye1 binds to RNAPII, and report crystal structures of the central TLD domain of Bye1 bound to free RNAPII, a RNAPII elongation complex with DNA template and RNA transcript, an elongation complex with an NTP analogue, and an arrested elongation complex with backtracked RNA. These studies represent only the third high-resolution structural analysis of a transcription factor complex with the polymerase core. Whereas the previously studied factors TFIIB and TFIIS alter RNAPII function by directly affecting catalytic events, Bye1 does not alter basic RNAPII functions *in vitro*. Consistent with this, Bye1 binding to RNAPII does

not alter RNAPII conformation in the structures. Additional functional data *in vitro* and *in vivo* indicate that Bye1 occupies active genes in their 5'-region and can bind to histone H3 tails with active marks using its PHD domain.

What could be the function of Bye1 in chromatin transcription? We show that the TLD of Bye1 is required for chromatin association of Bye1. It is thus unlikely that Bye1 would recognize active chromatin marks, and then recruit RNAPII to active chromatin regions. In contrast, our data indicate that Bye1 binds RNAPII during early elongation and tethers surrounding H3 histones containing active marks to RNAPII, maybe to cooperate with other chromatin elongation factors such as Spt6 and FACT and prevent loss of histones during polymerase passage through chromatin. This model is consistent with the observation that PHF3 and DIDO1, the putative homologues of Bye1 in human cells that we detected here, both may prevent deregulated transcription that may arise from histone loss, thus suppressing cancer development.

Author Contributions

K.K. carried out structural analysis, surface plasmon resonance experiments, genetic screens and transcription and RNA extension assays. G.G.W. carried out chromatin assays. S.B.W. carried out peptide array analysis. M.L. carried out ChIP-chip experiments. P.C. and B.D.S. designed and supervised research. K.K. and P.C. prepared the manuscript, with help from all authors.

Tables

Table A.1 Diffraction Data and Refinement Statistics

	Pol2-Bye1 FL	Pol2-Bye1 TLD	Pol2-Bye1 TLD + AMPCPP	Arrested Pol2+Bye1 TLD
Data collection				
Space group	$C222_1$	$C222_1$	$C222_1$	$C222_1$
Unit cell axes (Å)	220.55 392.09 279.80	222.8 391.0 282.2	222.4 391.7 281.1	222.7 392.2 280.7
Unit cell angle (°)	α=ß=γ=90	α=ß=γ=90	α=ß=γ=90	α=ß=γ=90
Resolution range (Å)	49.63-4.80 (4.92-4.80)	48.84-3.15 (3.23-3.15)	48.95-3.60 (3.69-3.60)	49.08-3.28 (3.37-3.28)
Unique reflections	59394 (4352)	210346 (15471)	141065 (10391)	187168 (13766)
Completeness (%)	99.97 (100)	99.98 (100)	99.98 (100)	99.98 (99.98)
Redundancy	7.50 (7.82)	7.66 (7.74)	7.62 (7.61)	7.66 (7.49)
Rsym (%)	40.9 (173.0)	11.6 (165.2)	21.2 (193.4)	12.9 (185.4)
I/σ(I)	6.05 (1.24)	15.97 (1.60)	9.95 (1.57)	14.66 (1.52)
CC(1/2)	98.5 (60.8)	99.8 (63.2)	99.6 (56.3)	99.8 (67.1)
Refinement				
Non-H atoms	31510	33234	33026	32753
B-factor (mean)	199.00	109.12	125.34	120.59
Rmsd bonds	0.010	0.010	0.010	0.010
Rmsd angles	1.33	1.27	1.29	1.29
Rcryst (%)	19.06	18.92	17.49	18.03
Rfree (%)	25.27	20.70	20.62	20.68

Values in parenthesis are for the highest resolution shell. All data were collected with a radiation wavelength of 0.9188 Å.

Figures

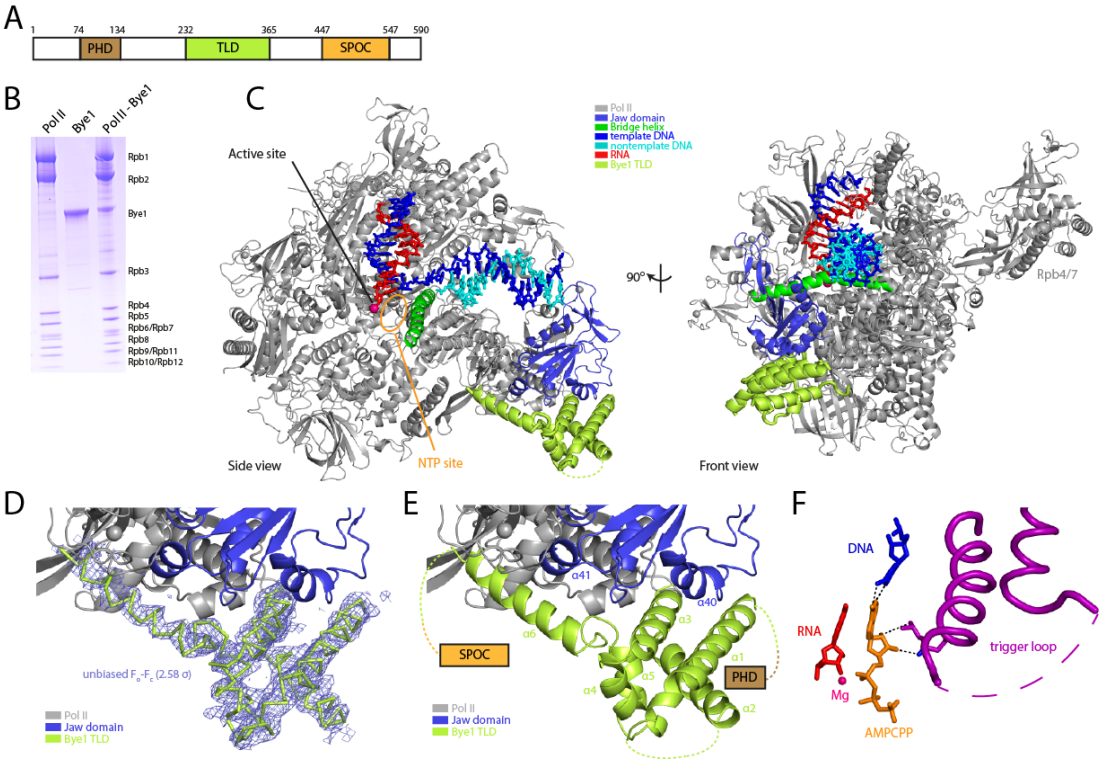

Figure A.1 Structure of the RNAPII-Bye1 Elongation Complex

(A) Bye1 domain organization. PHD: Plant Homeodomain, TLD: TFIIS-like domain, SPOC: Spen paralogue and orthologue C-terminal domain. Bordering residue numbers are indicated. (B) SDS–PAGE analysis (Coomassie staining) of endogenous yeast Pol II (left), recombinant Bye1 (center), and the Pol II-Bye1 complex after size exclusion chromatography (right). (C) Ribbon model of the Pol II-Bye1 elongation complex crystal structure. The views correspond to the side and front views of Pol II used before (Cramer et al. 2001) and are related by a 90° rotation around a vertical axis. (D) Unbiased difference electron density (blue mesh, contoured at 2.6 σ) for Bye1 TLD after phasing with the Pol II structure. (E) Close-up view of the Pol II-Bye1 interaction. Mobile loops are indicated by dashed lines. (F) Contacts of AMPCPP with the closed trigger loop in the AMPCPP-containing Pol II-Bye1 elongation complex structure. Residues involved in hydrogen bond formation are shown as sticks, hydrogen bonds are indicated by dashed lines.

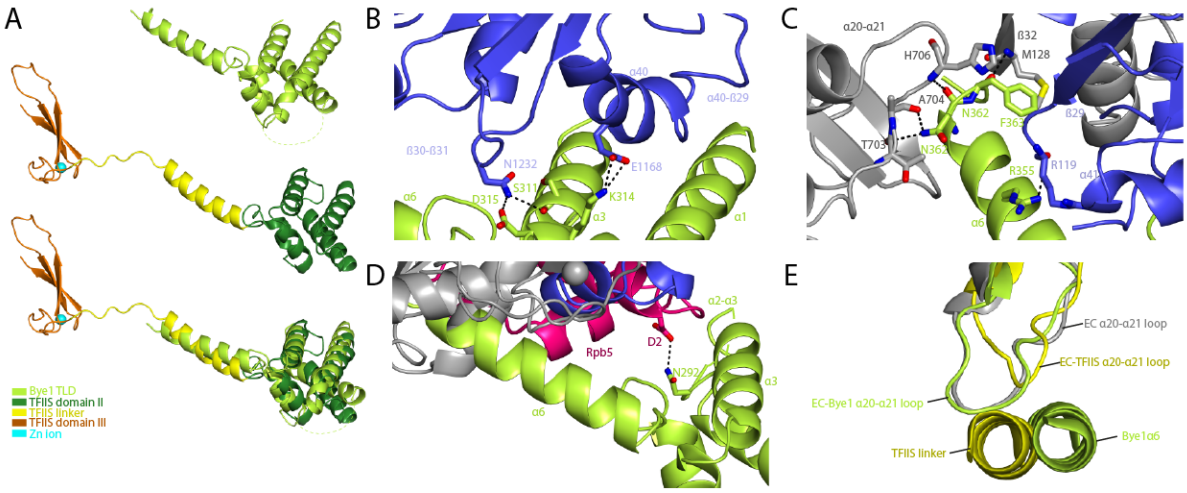

Figure A.2 RNAPII-Bye1 Interaction and Comparison with TFIIS

(A) Side view of the Bye1 TLD bound to Pol II (top), TFIIS (middle) in its Pol II-bound state (Cheung and Cramer 2011) with its central domain II (green), linker helix (yellow), and the C-terminal zinc ribbon domain III (orange), and superposition of the two structures (bottom). (B) Details of the interaction of the Bye1 TLD with the Pol II Rpb1 jaw domain (blue). The view is from the side. (C) Side view of the Pol II Rpb1 funnel and jaw domain-Bye1 TLD interaction. Residues involved in hydrogen bond formation or salt bridges (dashed lines) are shown as sticks. (D) Details of the interaction of the Bye1 TLD with the Pol II Rpb5 jaw domain (magenta). (E) In the Pol II-TFIIS complex structure, conformational changes in Pol II are induced by movements of the Pol II Rpb1 loop α20-α21, which results in opening of a crevice in the polymerase funnel. Loop movements are observed for TFIIS-bound Pol II (yellow) (Cheung and Cramer 2011), but not for Bye1-bound (this study, green) or unbound (Kettenberger et al. 2004) Pol II (silver).

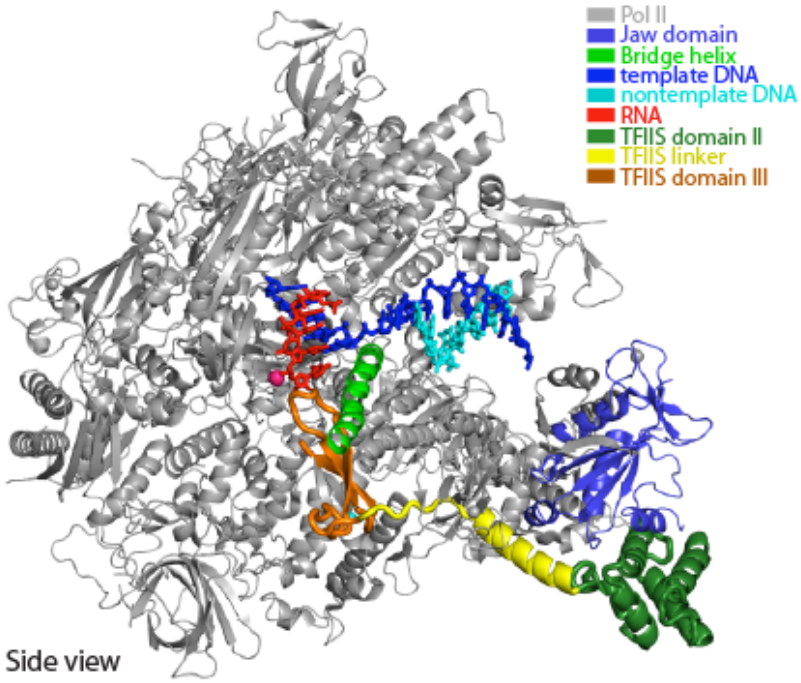

Figure A.3 Structure of RNAPII-TFIIS Complex

Adapted from (Kettenberger et al. 2004).

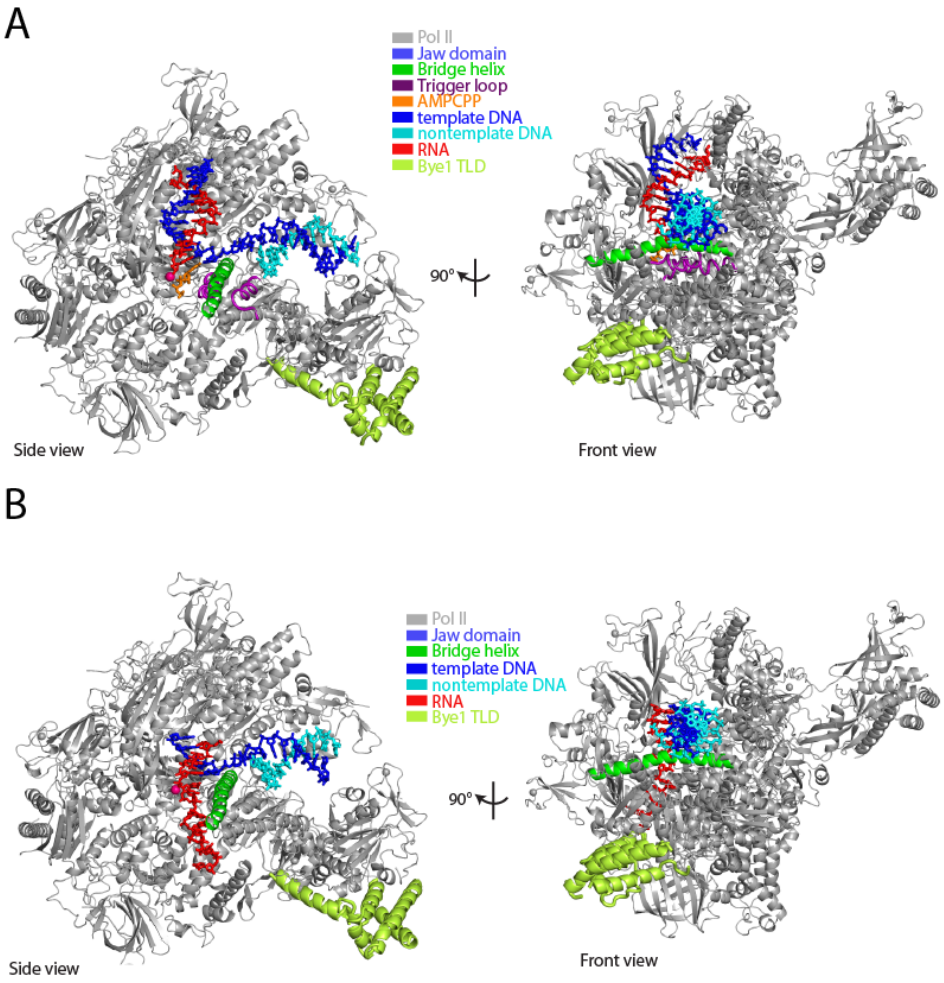

Figure A.4 Structures of Additional RNAPII-Bye1 Complexes

(A) Ribbon model of the Pol2-Bye1 complex containing an additional nucleotide. (B) Ribbon model of the arrested Pol2-Bye1 complex.

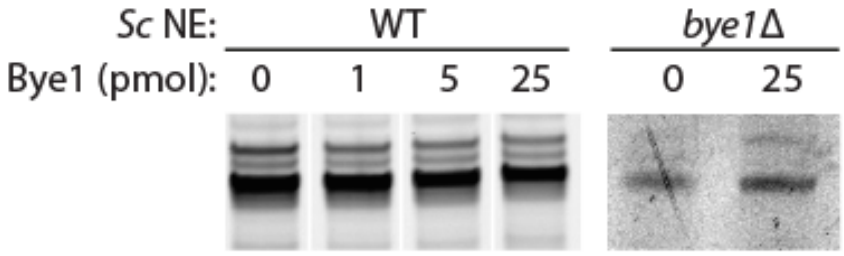

Figure A.5 Transcriptional Activity of Bye1-Depleted Nuclear Extracts

Transcriptional activities of wild type (WT) and Bye1-depleted (*bye1*Δ) nuclear extracts (NE) in an *in vitro* transcription assay using a nucleosome-free DNA template.

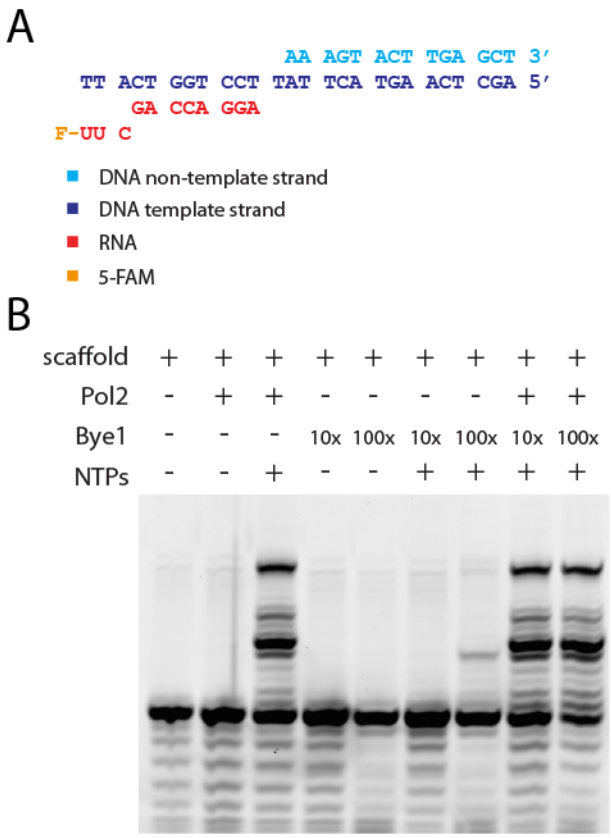

Figure A.6 Effect of Bye1 on RNAPII Elongation *in Vitro*

(A) Nucleic acid scaffold for reconstitution of Pol II-EC. (B) Gel electrophoresis separation of RNA products obtained in RNA extension assay.

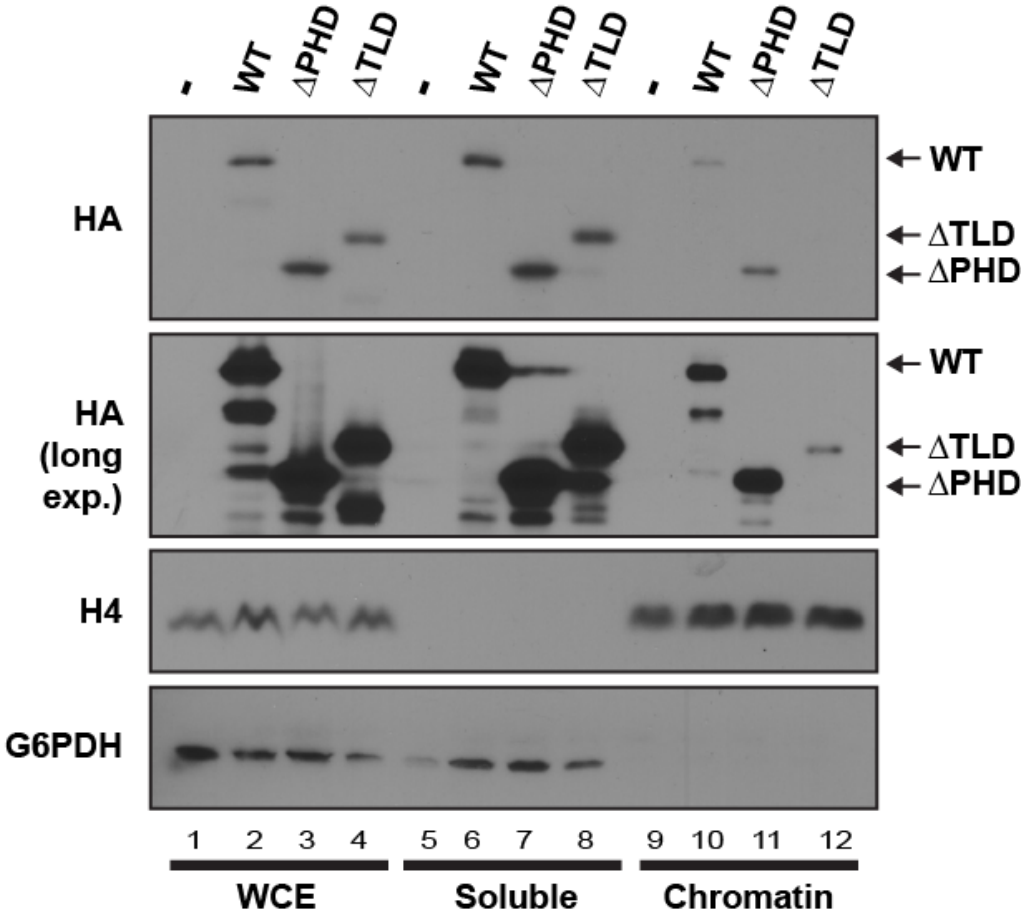

Figure A.7 Bye1 Associates with Chromatin via its TLD Domain

Immunoblot of whole-cell extract (WCE), chromatin-bound and soluble cell fraction to hemagglutinin tag (HA), histone H4 (H4) and glucose-6-phosphate-1-dehydrogenase (G6PDH). For details compare text.

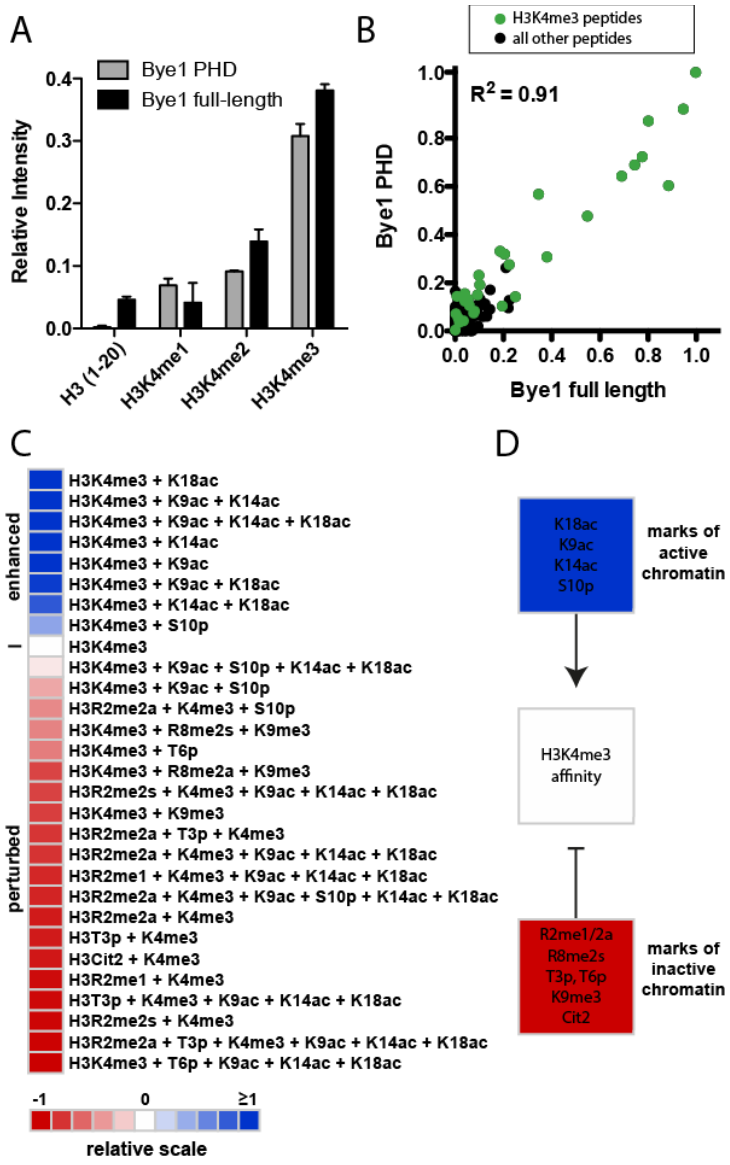

Figure A.8 Bye1 Preferentially Binds Histone Peptides Carrying Active Modifications

(A) Peptide array binding analysis reveals that Bye1 preferentially binds a H3K4me3 peptide and its PHD domain is sufficient for this interaction. (B) Scatter plot showing the correlation of two arrays probed with full-length Bye1 and the Bye1 PHD domain for H3K4me3 binding (green dots). All other peptides are shown as black dots. (C) Heat map depicting the effects of combinatorial modifications on the binding of Bye1 to H3K4me3-containing peptides. Binding intensities are represented relative to H3K4me3 (0, white). Enhanced (1, red) and occluded (– 1, blue) interactions are depicted. (D) Summary of modifications enhancing (blue) and impairing (red) Bye1 affinity to H3K4me3.

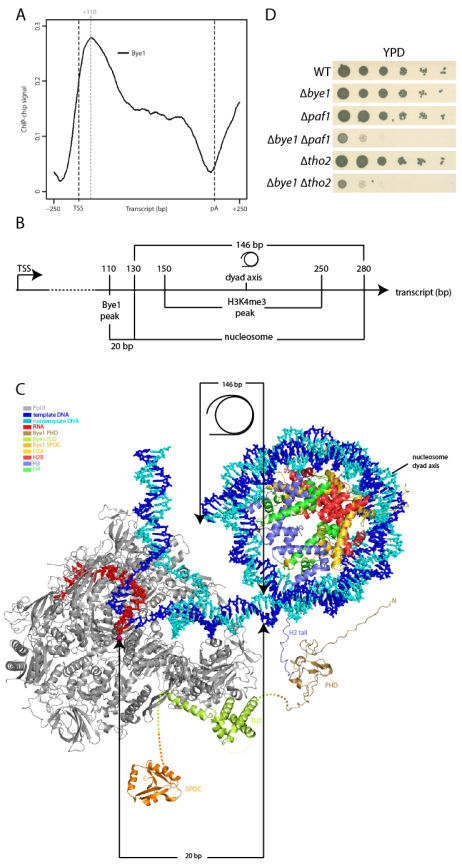

Figure A.9 Bye1 Associates with Active Genes in Front of the +2 Nucleosome

(A) Gene-averaged Bye1 ChIP occupancy profile for the median gene length class (1,238 ± 300 nt, 339 genes). TSS, transcription start site; pA, polyadenylation site. (B) Scheme showing occupancies of Bye1 and H3K4me4 derived from ChIP data and nucleosome position derived from the model in C. (C) Model of a Pol II-nucleosome-Bye1 complex based on crystal structures and ChIP occupancy peak positions. Distances in base pairs (bp) are indicated between the Pol II active center and the nucleosome as well as for the nucleosomal DNA. The model is based on the structure of the nucleosome core particle by Luger et al. (1997). Modeling was performed with Coot (Emsley et al. 2010). Bye1 PHD and SPOC domain were modeled using Modeller (Sanchez and Sali 1997). The PHD domain model is based on structures 3kqi, 1wem, 1wew, 2lv9 and 1wep, which were identified by HHpred (Soding et al. 2005) to be most similar to Bye1 PHD. Binding of the PHD domain to H3K4me3 was modeled based on structure 2jmj. The SPOC domain model is based on structure 1ow1. (D) Bye1 genetically interacts with Paf1 and Tho2. Serial dilutions of strains *bye1*Δ, *paf1*Δ, *bye1*Δ*paf1*Δ, *tho2*Δ, *bye1*Δ*tho2*Δ, and an isogenic wild-type (WT) control strain were placed on YPD plates and incubated at 30°C for 3 days.

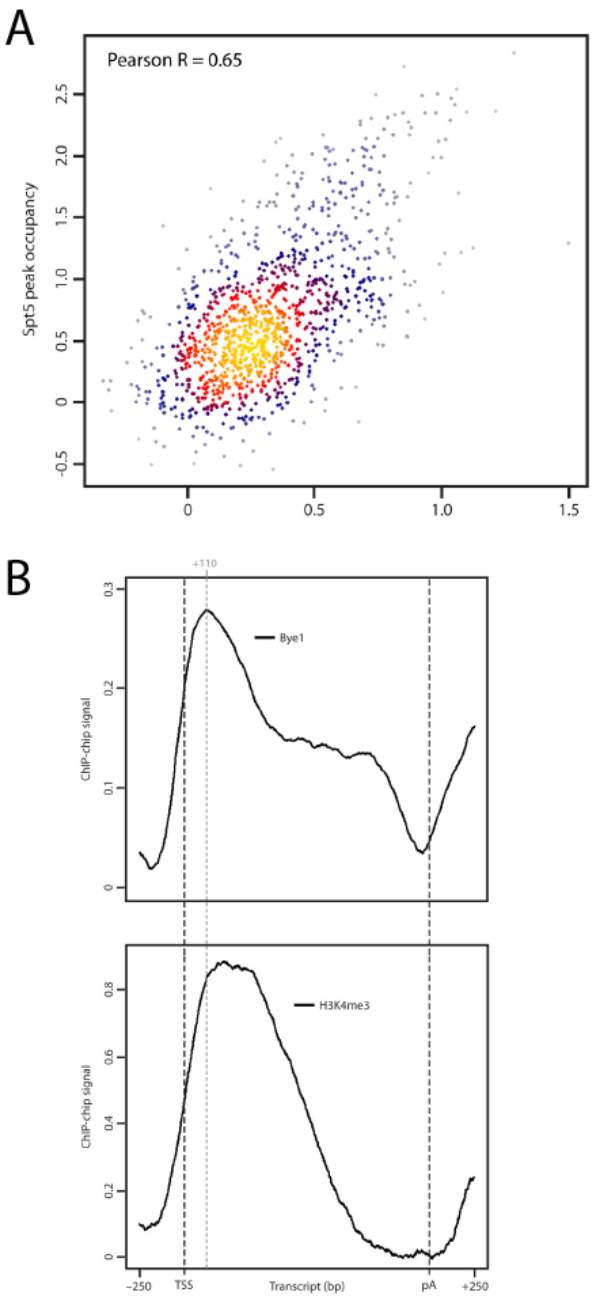

Figure A.10 ChIP-chip Analysis of Bye1

(A) Correlation of Bye1 and Spt5 occupancies. (B) Comparison of Bye1 and H3K4me3 occupancy profiles.

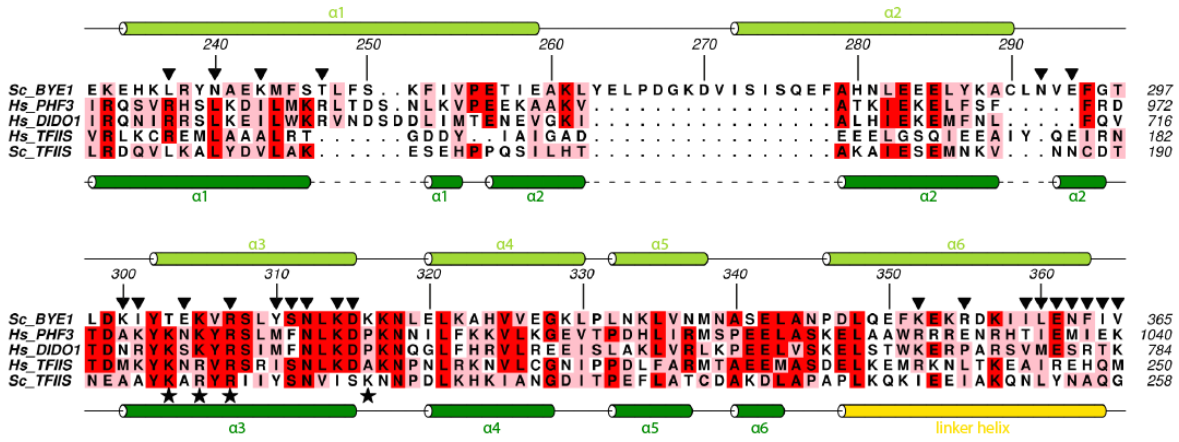

Figure A.11 Conservation of RNAPII-Binding Residues in Bye1 Human Homologues

Amino-acid sequence alignments of *S. cerevisiae* Bye1, *H. sapiens* PHF3, *H. sapiens* DIDO1, *H. sapiens* TFIIS and *S. cerevisiae* TFIIS. Secondary structure elements are indicated as arrows (ß-strands) or rods (α-helices). Loops are indicated with solid lines. Residues that are part of the Pol II-Bye1 interface are marked with black triangles. Residues essential for the Pol II-TFIIS interaction (Awrey et al. 1998) are marked with black asterisks.

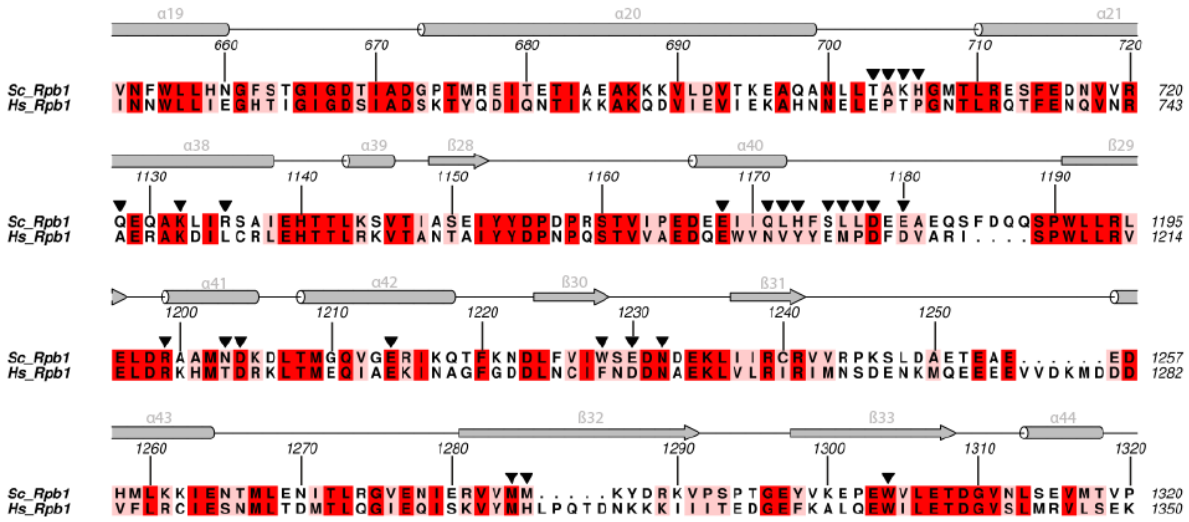

Figure A.12 Conservation of RNAPII-Bye1 Interface in Human Homologues

Amino-acid sequence alignments of *S. cerevisiae* Rpb1 and *H. sapiens* Rpb1.Secondary structure elements are indicated as arrows (ß-strands) or rods (α-helices). Loops are indicated with solid lines. Residues that are part of the Pol II-Bye1 interface are marked with black triangles.

REFERENCES

Aasland R, Gibson TJ, Stewart AF. 1995. The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci* **20**: 56-59.

Adelman K, Lis JT. 2012. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. *Nat Rev Genet* **13**: 720-731.

Ahn SH, Cheung WL, Hsu JY, Diaz RL, Smith MM, Allis CD. 2005. Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in S. cerevisiae. *Cell* **120**: 25-36.

Altaf M, Utley RT, Lacoste N, Tan S, Briggs SD, Cote J. 2007. Interplay of chromatin modifiers on a short basic patch of histone H4 tail defines the boundary of telomeric heterochromatin. *Molecular cell* **28**: 1002-1014.

Arita K, Ariyoshi M, Tochio H, Nakamura Y, Shirakawa M. 2008. Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism. *Nature* **455**: 818-821.

Ariyoshi M, Schwabe JW. 2003. A conserved structural motif reveals the essential transcriptional repression function of Spen proteins and their role in developmental signaling. *Genes Dev* **17**: 1909-1920.

Armache KJ, Mitterweger S, Meinhart A, Cramer P. 2005. Structures of complete RNA polymerase II and its subcomplex, Rpb4/7. *J Biol Chem* **280**: 7131-7134.

Avvakumov N, Nourani A, Cote J. 2011. Histone chaperones: modulators of chromatin marks. *Molecular cell* **41**: 502-514.

Awrey DE, Shimasaki N, Koth C, Weilbaecher R, Olmsted V, Kazanis S, Shan X, Arellano J, Arrowsmith CH, Kane CM et al. 1998. Yeast transcript elongation factor (TFIIS), structure and function. II: RNA polymerase binding, transcript cleavage, and read-through. *J Biol Chem* **273**: 22595-22605.

Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. *Cell research* **21**: 381-395.

Bartke T, Vermeulen M, Xhemalce B, Robson SC, Mann M, Kouzarides T. 2010. Nucleosome-interacting proteins regulated by DNA and histone methylation. *Cell* **143**: 470-484.

Berger SL. 2007. The complex language of chromatin regulation during transcription. *Nature* **447**: 407-412.

Bernstein BE, Kamal M, Lindblad-Toh K, Bekiranov S, Bailey DK, Huebert DJ, McMahon S, Karlsson EK, Kulbokas EJ, 3rd, Gingeras TR et al. 2005. Genomic

maps and comparative analysis of histone modifications in human and mouse. *Cell* **120**: 169-181.

Black BE, Cleveland DW. 2011. Epigenetic centromere propagation and the nature of CENP-a nucleosomes. *Cell* **144**: 471-479.

Black JC, Van Rechem C, Whetstine JR. 2012. Histone lysine methylation dynamics: establishment, regulation, and biological impact. *Molecular cell* **48**: 491-507.

Blank M, Tang Y, Yamashita M, Burkett SS, Cheng SY, Zhang YE. 2012. A tumor suppressor function of Smurf2 associated with controlling chromatin landscape and genome stability through RNF20. *Nat Med* **18**: 227-234.

Brachmann CB, Sherman JM, Devine SE, Cameron EE, Pillus L, Boeke JD. 1995. The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes Dev* **9**: 2888-2902.

Brickner DG, Cajigas I, Fondufe-Mittendorf Y, Ahmed S, Lee PC, Widom J, Brickner JH. 2007. H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. *PLoS biology* **5**: e81.

Bricogne G, Blanc E, Brandl M, Flensburg C, Keller P, Paciorek P, Roversi P, Sharff A, Smart O, Vonrhein C et al. 2012. BUSTER Version 2.11.5.

Briggs SD, Xiao T, Sun ZW, Caldwell JA, Shabanowitz J, Hunt DF, Allis CD, Strahl BD. 2002. Gene silencing: trans-histone regulatory pathway in chromatin. *Nature* **418**: 498.

Burgess RJ, Zhang Z. 2013. Histone chaperones in nucleosome assembly and human disease. *Nature structural & molecular biology* **20**: 14-22.

Bushnell DA, Westover KD, Davis RE, Kornberg RD. 2004. Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms. *Science* **303**: 983-988.

Calo E, Wysocka J. 2013. Modification of enhancer chromatin: what, how, and why? *Molecular cell* **49**: 825-837.

Carrozza MJ, Li B, Florens L, Suganuma T, Swanson SK, Lee KK, Shia WJ, Anderson S, Yates J, Washburn MP et al. 2005. Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. *Cell* **123**: 581-592.

Chandrasekharan MB, Huang F, Chen YC, Sun ZW. 2010a. Histone H2B C-terminal helix mediates trans-histone H3K4 methylation independent of H2B ubiquitination. *Molecular and cellular biology* **30**: 3216-3232.

Chandrasekharan MB, Huang F, Sun ZW. 2009. Ubiquitination of histone H2B regulates chromatin dynamics by enhancing nucleosome stability. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 16686-16691.

Chandrasekharan MB, Huang F, Sun ZW. 2010b. Histone H2B ubiquitination and beyond: Regulation of nucleosome stability, chromatin dynamics and the trans-histone H3 methylation. *Epigenetics : official journal of the DNA Methylation Society* **5**: 460-468.

Chernikova SB, Razorenova OV, Higgins JP, Sishc BJ, Nicolau M, Dorth JA, Chernikova DA, Kwok S, Brooks JD, Bailey SM et al. 2012. Deficiency in mammalian histone H2B ubiquitin ligase Bre1 (Rnf20/Rnf40) leads to replication stress and chromosomal instability. *Cancer Res* **72**: 2111-2119.

Cheung AC, Cramer P. 2011. Structural basis of RNA polymerase II backtracking, arrest and reactivation. *Nature* **471**: 249-253.

Cheung AC, Sainsbury S, Cramer P. 2011. Structural basis of initial RNA polymerase II transcription. *The EMBO journal* **30**: 4755-4763.

Cheung V, Chua G, Batada NN, Landry CR, Michnick SW, Hughes TR, Winston F. 2008. Chromatin- and transcription-related factors repress transcription from within coding regions throughout the Saccharomyces cerevisiae genome. *PLoS biology* **6**: e277.

Clapier CR, Cairns BR. 2009. The biology of chromatin remodeling complexes. *Annual review of biochemistry* **78**: 273-304.

Cramer P, Bushnell DA, Kornberg RD. 2001. Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science* **292**: 1863-1876.

Damsma GE, Alt A, Brueckner F, Carell T, Cramer P. 2007. Mechanism of transcriptional stalling at cisplatin-damaged DNA. *Nature structural & molecular biology* **14**: 1127-1133.

Dantuma NP, Groothuis TA, Salomons FA, Neefjes J. 2006. A dynamic ubiquitin equilibrium couples proteasomal activity to chromatin remodeling. *J Cell Biol* **173**: 19-26.

Dehe PM, Geli V. 2006. The multiple faces of Set1. *Biochem Cell Biol* **84**: 536-548.

Donovan S, Harwood J, Drury LS, Diffley JF. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 5611-5616.

Dover J, Schneider J, Tawiah-Boateng MA, Wood A, Dean K, Johnston M, Shilatifard A. 2002. Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. *J Biol Chem* **277**: 28368-28371.

Dronamraju R, Strahl BD. 2014. A feed forward circuit comprising Spt6, Ctk1 and PAF regulates Pol II CTD phosphorylation and transcription elongation. *Nucleic acids research* **42**: 870-881.

Du HN, Briggs SD. 2010. A nucleosome surface formed by histone H4, H2A, and H3 residues is needed for proper histone H3 Lys36 methylation, histone acetylation, and repression of cryptic transcription. *J Biol Chem* **285**: 11704-11713.

Eberl HC, Spruijt CG, Kelstrup CD, Vermeulen M, Mann M. 2013. A map of general and specialized chromatin readers in mouse tissues generated by label-free interaction proteomics. *Molecular cell* **49**: 368-378.

Elsaesser SJ, Allis CD. 2010. HIRA and Daxx constitute two independent histone H3.3-containing predeposition complexes. *Cold Spring Harb Symp Quant Biol* **75**: 27-34.

Emre NC, Ingvarsdottir K, Wyce A, Wood A, Krogan NJ, Henry KW, Li K, Marmorstein R, Greenblatt JF, Shilatifard A et al. 2005. Maintenance of low histone ubiquitylation by Ubp10 correlates with telomere-proximal Sir2 association and gene silencing. *Molecular cell* **17**: 585-594.

Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**: 486-501.

Feng Q, Wang H, Ng HH, Erdjument-Bromage H, Tempst P, Struhl K, Zhang Y. 2002. Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. *Current biology : CB* **12**: 1052-1058.

Ferreira H, Flaus A, Owen-Hughes T. 2007. Histone modifications influence the action of Snf2 family remodelling enzymes by different mechanisms. *J Mol Biol* **374**: 563-579.

Fierz B, Chatterjee C, McGinty RK, Bar-Dagan M, Raleigh DP, Muir TW. 2011. Histone H2B ubiquitylation disrupts local and higher-order chromatin compaction. *Nature chemical biology* **7**: 113-119.

Fingerman IM, Li HC, Briggs SD. 2007. A charge-based interaction between histone H4 and Dot1 is required for H3K79 methylation and telomere silencing: identification of a new trans-histone pathway. *Genes Dev* **21**: 2018-2029.

Fischer U, Struss AK, Hemmer D, Michel A, Henn W, Steudel WI, Meese E. 2001. PHF3 expression is frequently reduced in glioma. *Cytogenet Cell Genet* **94**: 131-136.

Flanagan JF, Mi LZ, Chruszcz M, Cymborowski M, Clines KL, Kim Y, Minor W, Rastinejad F, Khorasanizadeh S. 2005. Double chromodomains cooperate to recognize the methylated histone H3 tail. *Nature* **438**: 1181-1185.

Fleming AB, Kao CF, Hillyer C, Pikaart M, Osley MA. 2008. H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. *Molecular cell* **31**: 57-66.

Fuchs SM, Kizer KO, Braberg H, Krogan NJ, Strahl BD. 2012. RNA polymerase II carboxyl-terminal domain phosphorylation regulates protein stability of the Set2 methyltransferase and histone H3 di- and trimethylation at lysine 36. *J Biol Chem* **287**: 3249-3256.

Fuchs SM, Laribee RN, Strahl BD. 2009. Protein modifications in transcription elongation. *Biochim Biophys Acta* **1789**: 26-36.

Futterer A, Campanero MR, Leonardo E, Criado LM, Flores JM, Hernandez JM, San Miguel JF, Martinez AC. 2005. Dido gene expression alterations are implicated in the induction of hematological myeloid neoplasms. *J Clin Invest* **115**: 2351-2362.

Game JC, Chernikova SB. 2009. The role of RAD6 in recombinational repair, checkpoints and meiosis via histone modification. *DNA Repair (Amst)* **8**: 470-482.

Game JC, Williamson MS, Spicakova T, Brown JM. 2006. The RAD6/BRE1 histone modification pathway in Saccharomyces confers radiation resistance through a RAD51-dependent process that is independent of RAD18. *Genetics* **173**: 1951-1968.

Gardner KE, Allis CD, Strahl BD. 2011. Operating on chromatin, a colorful language where context matters. *J Mol Biol* **409**: 36-46.

Gardner RG, Nelson ZW, Gottschling DE. 2005. Ubp10/Dot4p regulates the persistence of ubiquitinated histone H2B: distinct roles in telomeric silencing and general chromatin. *Molecular and cellular biology* **25**: 6123-6139.

Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A, Anderson K, Andre B et al. 2002. Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418**: 387-391.

Goldberg AD, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ, Stadler S, Dewell S, Law M, Guo X, Li X et al. 2010. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* **140**: 678-691.

Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, Candau R, Ohba R, Owen-Hughes T, Allis CD, Winston F et al. 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev* **11**: 1640-1650.

Gunesdogan U, Jackle H, Herzig A. 2010. A genetic system to assess in vivo the functions of histones and histone modifications in higher eukaryotes. *EMBO reports* **11**: 772-776.

Hagstrom KA, Meyer BJ. 2003. Condensin and cohesin: more than chromosome compactor and glue. *Nat Rev Genet* **4**: 520-534.

Hahn S, Young ET. 2011. Transcriptional regulation in Saccharomyces cerevisiae: transcription factor regulation and function, mechanisms of initiation, and roles of activators and coactivators. *Genetics* **189**: 705-736.

Hani J, Stumpf G, Domdey H. 1995. PTF1 encodes an essential protein in Saccharomyces cerevisiae, which shows strong homology with a new putative family of PPIases. *FEBS Lett* **365**: 198-202.

Hartzog GA, Wada T, Handa H, Winston F. 1998. Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae. *Genes Dev* **12**: 357-369.

Heidemann M, Hintermair C, Voss K, Eick D. 2013. Dynamic phosphorylation patterns of RNA polymerase II CTD during transcription. *Biochim Biophys Acta* **1829**: 55-62.

Henry KW, Wyce A, Lo WS, Duggan LJ, Emre NC, Kao CF, Pillus L, Shilatifard A, Osley MA, Berger SL. 2003. Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev* **17**: 2648-2663.

Hirschhorn JN, Bortvin AL, Ricupero-Hovasse SL, Winston F. 1995. A new class of histone H2A mutations in Saccharomyces cerevisiae causes specific transcriptional defects in vivo. *Molecular and cellular biology* **15**: 1999-2009.

Horn PJ, Peterson CL. 2002. Molecular biology. Chromatin higher order folding--wrapping up transcription. *Science* **297**: 1824-1827.

Hsin JP, Manley JL. 2012. The RNA polymerase II CTD coordinates transcription and RNA processing. *Genes Dev* **26**: 2119-2137.

Huibregtse JM, Yang JC, Beaudenon SL. 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 3656-3661.

Hwang WW, Venkatasubrahmanyam S, Ianculescu AG, Tong A, Boone C, Madhani HD. 2003. A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. *Molecular cell* **11**: 261-266.

Jacobson RH, Ladurner AG, King DS, Tjian R. 2000. Structure and function of a human TAFII250 double bromodomain module. *Science* **288**: 1422-1425.

Jaehning JA. 2010. The Paf1 complex: platform or player in RNA polymerase II transcription? *Biochim Biophys Acta* **1799**: 379-388.

Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, Moreno-Borchart A, Doenges G, Schwob E, Schiebel E et al. 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**: 947-962.

Jenuwein T, Allis CD. 2001. Translating the histone code. *Science* **293**: 1074-1080.

Jha DK, Strahl BD. 2014. An RNA polymerase II-coupled function for histone H3K36 methylation in checkpoint activation and DSB repair. *Nat Commun* **5**: 3965.

Johnsson B, Lofas S, Lindquist G. 1991. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal Biochem* **198**: 268-277.

Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* **13**: 484-492.

Kaiser SE, Riley BE, Shaler TA, Trevino RS, Becker CH, Schulman H, Kopito RR. 2011. Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. *Nat Methods* **8**: 691-696.

Kao CF, Hillyer C, Tsukuda T, Henry K, Berger S, Osley MA. 2004. Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. *Genes Dev* **18**: 184-195.

Kaplan N, Moore IK, Fondufe-Mittendorf Y, Gossett AJ, Tillo D, Field Y, LeProust EM, Hughes TR, Lieb JD, Widom J et al. 2009. The DNA-encoded nucleosome organization of a eukaryotic genome. *Nature* **458**: 362-366.

Kasten M, Szerlong H, Erdjument-Bromage H, Tempst P, Werner M, Cairns BR. 2004. Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. *The EMBO journal* **23**: 1348-1359.

Katan-Khaykovich Y, Struhl K. 2002. Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev* **16**: 743-752.

Keogh MC, Kim JA, Downey M, Fillingham J, Chowdhury D, Harrison JC, Onishi M, Datta N, Galicia S, Emili A et al. 2006. A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. *Nature* **439**: 497-501.

Keogh MC, Kurdistani SK, Morris SA, Ahn SH, Podolny V, Collins SR, Schuldiner M, Chin K, Punna T, Thompson NJ et al. 2005. Cotranscriptional set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. *Cell* **123**: 593-605.

Kettenberger H, Armache KJ, Cramer P. 2003. Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage. *Cell* **114**: 347-357.

Kettenberger H, Armache KJ, Cramer P. 2004. Complete RNA polymerase II elongation complex structure and its interactions with NTP and TFIIS. *Molecular cell* **16**: 955-965.

Kim J, Kim JA, McGinty RK, Nguyen UT, Muir TW, Allis CD, Roeder RG. 2013. The n-SET domain of Set1 regulates H2B ubiquitylation-dependent H3K4 methylation. *Molecular cell* **49**: 1121-1133.

Kim J, Roeder RG. 2009. Direct Bre1-Paf1 complex interactions and RING finger-independent Bre1-Rad6 interactions mediate histone H2B ubiquitylation in yeast. *J Biol Chem* **284**: 20582-20592.

Kizer KO, Phatnani HP, Shibata Y, Hall H, Greenleaf AL, Strahl BD. 2005. A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. *Molecular and cellular biology* **25**: 3305-3316.

Komander D, Rape M. 2012. The ubiquitin code. *Annual review of biochemistry* **81**: 203-229.

Kornberg RD, Lorch Y. 1999. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**: 285-294.

Kostrewa D, Zeller ME, Armache KJ, Seizl M, Leike K, Thomm M, Cramer P. 2009. RNA polymerase II-TFIIB structure and mechanism of transcription initiation. *Nature* **462**: 323-330.

Kouzarides T. 2007. Chromatin modifications and their function. *Cell* **128**: 693-705.

Krogan NJ, Dover J, Wood A, Schneider J, Heidt J, Boateng MA, Dean K, Ryan OW, Golshani A, Johnston M et al. 2003a. The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Molecular cell* **11**: 721-729.

Krogan NJ, Dover J, Wood A, Schneider J, Heidt J, Boateng MA, Dean K, Ryan OW, Golshani A, Johnston M et al. 2003b. The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Molecular cell* **11**: 721-729.

Kumar A, Cheung KH, Ross-Macdonald P, Coelho PS, Miller P, Snyder M. 2000. TRIPLES: a database of gene function in Saccharomyces cerevisiae. *Nucleic acids research* **28**: 81-84.

Kuo MH, Brownell JE, Sobel RE, Ranalli TA, Cook RG, Edmondson DG, Roth SY, Allis CD. 1996. Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. *Nature* **383**: 269-272.

Kwak H, Lis JT. 2013. Control of transcriptional elongation. *Annu Rev Genet* **47**: 483-508.

Lacoste N, Utley RT, Hunter JM, Poirier GG, Cote J. 2002. Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. *The Journal of biological chemistry* **277**: 30421-30424.

Lan F, Collins RE, De Cegli R, Alpatov R, Horton JR, Shi X, Gozani O, Cheng X, Shi Y. 2007. Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression. *Nature* **448**: 718-722.

Laribee RN, Krogan NJ, Xiao T, Shibata Y, Hughes TR, Greenblatt JF, Strahl BD. 2005. BUR kinase selectively regulates H3 K4 trimethylation and H2B ubiquitylation through recruitment of the PAF elongation complex. *Current biology : CB* **15**: 1487-1493.

Latham JA, Chosed RJ, Wang S, Dent SY. 2011. Chromatin signaling to kinetochores: transregulation of Dam1 methylation by histone H2B ubiquitination. *Cell* **146**: 709-719.

Lee JS, Shukla A, Schneider J, Swanson SK, Washburn MP, Florens L, Bhaumik SR, Shilatifard A. 2007. Histone crosstalk between H2B monoubiquitination and H3 methylation mediated by COMPASS. *Cell* **131**: 1084-1096.

Li B, Carey M, Workman JL. 2007. The role of chromatin during transcription. *Cell* **128**: 707-719.

Li B, Howe L, Anderson S, Yates JR, 3rd, Workman JL. 2003. The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. *The Journal of biological chemistry* **278**: 8897-8903.

Li B, Jackson J, Simon MD, Fleharty B, Gogol M, Seidel C, Workman JL, Shilatifard A. 2009. Histone H3 lysine 36 dimethylation (H3K36me2) is sufficient to recruit the Rpd3s histone deacetylase complex and to repress spurious transcription. *The Journal of biological chemistry* **284**: 7970-7976.

Li G, Reinberg D. 2011. Chromatin higher-order structures and gene regulation. *Current opinion in genetics & development* **21**: 175-186.

Liu C, Apodaca J, Davis LE, Rao H. 2007. Proteasome inhibition in wild-type yeast Saccharomyces cerevisiae cells. *Biotechniques* **42**: 158, 160, 162.

Liu X, Bushnell DA, Wang D, Calero G, Kornberg RD. 2010. Structure of an RNA polymerase II-TFIIB complex and the transcription initiation mechanism. *Science* **327**: 206-209.

Löfås SaJ, Bo. 1990. A Novel Hydrogel Matrix on Gold Surfaces in Surface Plasmon Resonance Sensors for Fast and Efficient Covalent Immobilization of Ligands *Journal of The Chemical Society, Chemical Communications*.

Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**: 251-260.

Luk E, Vu ND, Patteson K, Mizuguchi G, Wu WH, Ranjan A, Backus J, Sen S, Lewis M, Bai Y et al. 2007. Chz1, a nuclear chaperone for histone H2AZ. *Molecular cell* **25**: 357-368.

Maltby VE, Martin BJ, Schulze JM, Johnson I, Hentrich T, Sharma A, Kobor MS, Howe L. 2012. Histone H3 lysine 36 methylation targets the Isw1b remodeling complex to chromatin. *Molecular and cellular biology* **32**: 3479-3485.

Mayer A, Heidemann M, Lidschreiber M, Schreieck A, Sun M, Hintermair C, Kremmer E, Eick D, Cramer P. 2012. CTD tyrosine phosphorylation impairs termination factor recruitment to RNA polymerase II. *Science* **336**: 1723-1725.

Maze I, Noh KM, Soshnev AA, Allis CD. 2014. Every amino acid matters: essential contributions of histone variants to mammalian development and disease. *Nat Rev Genet* **15**: 259-271.

McGinty RK, Kim J, Chatterjee C, Roeder RG, Muir TW. 2008. Chemically ubiquitylated histone H2B stimulates hDot1L-mediated intranucleosomal methylation. *Nature* **453**: 812-816.

Mehta M, Braberg H, Wang S, Lozsa A, Shales M, Solache A, Krogan NJ, Keogh MC. 2010. Individual lysine acetylations on the N terminus of Saccharomyces cerevisiae H2A.Z are highly but not differentially regulated. *J Biol Chem* **285**: 39855-39865.

Milne TA, Kim J, Wang GG, Stadler SC, Basrur V, Whitcomb SJ, Wang Z, Ruthenburg AJ, Elenitoba-Johnson KS, Roeder RG et al. 2010. Multiple interactions recruit MLL1 and MLL1 fusion proteins to the HOXA9 locus in leukemogenesis. *Molecular cell* **38**: 853-863.

Mimnaugh EG, Chen HY, Davie JR, Celis JE, Neckers L. 1997. Rapid deubiquitination of nucleosomal histones in human tumor cells caused by proteasome inhibitors and stress response inducers: effects on replication, transcription, translation, and the cellular stress response. *Biochemistry* **36**: 14418-14429.

Mischo HE, Proudfoot NJ. 2013. Disengaging polymerase: terminating RNA polymerase II transcription in budding yeast. *Biochim Biophys Acta* **1829**: 174-185.

Mizuguchi G, Shen X, Landry J, Wu WH, Sen S, Wu C. 2004. ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* **303**: 343-348.

Moehle EA, Ryan CJ, Krogan NJ, Kress TL, Guthrie C. 2012. The yeast SR-like protein Npl3 links chromatin modification to mRNA processing. *PLoS genetics* **8**: e1003101.

Morris DP, Phatnani HP, Greenleaf AL. 1999. Phospho-carboxyl-terminal domain binding and the role of a prolyl isomerase in pre-mRNA 3'-End formation. *J Biol Chem* **274**: 31583-31587.

Musselman CA, Lalonde ME, Cote J, Kutateladze TG. 2012. Perceiving the epigenetic landscape through histone readers. *Nature structural & molecular biology* **19**: 1218-1227.

Nakanishi S, Lee JS, Gardner KE, Gardner JM, Takahashi YH, Chandrasekharan MB, Sun ZW, Osley MA, Strahl BD, Jaspersen SL et al. 2009. Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1. *J Cell Biol* **186**: 371-377.

Narlikar GJ, Sundaramoorthy R, Owen-Hughes T. 2013. Mechanisms and functions of ATP-dependent chromatin-remodeling enzymes. *Cell* **154**: 490-503.

Ng HH, Dole S, Struhl K. 2003a. The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. *J Biol Chem* **278**: 33625-33628.

Ng HH, Feng Q, Wang H, Erdjument-Bromage H, Tempst P, Zhang Y, Struhl K. 2002a. Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. *Genes & development* **16**: 1518-1527.

Ng HH, Robert F, Young RA, Struhl K. 2003b. Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Molecular cell* **11**: 709-719.

Ng HH, Xu RM, Zhang Y, Struhl K. 2002b. Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. *J Biol Chem* **277**: 34655-34657.

Nikolov DB, Burley SK. 1997. RNA polymerase II transcription initiation: a structural view. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 15-22.

Nordick K, Hoffman MG, Betz JL, Jaehning JA. 2008. Direct interactions between the Paf1 complex and a cleavage and polyadenylation factor are revealed by dissociation of Paf1 from RNA polymerase II. *Eukaryot Cell* **7**: 1158-1167.

Norris A, Boeke JD. 2010. Silent information regulator 3: the Goldilocks of the silencing complex. *Genes & development* **24**: 115-122.

Oh S, Jeong K, Kim H, Kwon CS, Lee D. 2010. A lysine-rich region in Dot1p is crucial for direct interaction with H2B ubiquitylation and high level methylation of H3K79. *Biochem Biophys Res Commun* **399**: 512-517.

Onishi M, Liou GG, Buchberger JR, Walz T, Moazed D. 2007. Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. *Molecular cell* **28**: 1015-1028.

Ooi SK, Qiu C, Bernstein E, Li K, Jia D, Yang Z, Erdjument-Bromage H, Tempst P, Lin SP, Allis CD et al. 2007. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* **448**: 714-717.

Orphanides G, Wu WH, Lane WS, Hampsey M, Reinberg D. 1999. The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. *Nature* **400**: 284-288.

Parra MA, Kerr D, Fahy D, Pouchnik DJ, Wyrick JJ. 2006. Deciphering the roles of the histone H2B N-terminal domain in genome-wide transcription. *Molecular and cellular biology* **26**: 3842-3852.

Parra MA, Wyrick JJ. 2007. Regulation of gene transcription by the histone H2A N-terminal domain. *Molecular and cellular biology* **27**: 7641-7648.

Pavri R, Zhu B, Li G, Trojer P, Mandal S, Shilatifard A, Reinberg D. 2006. Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. *Cell* **125**: 703-717.

Pengelly AR, Copur O, Jackle H, Herzig A, Muller J. 2013. A histone mutant reproduces the phenotype caused by loss of histone-modifying factor Polycomb. *Science* **339**: 698-699.

Perales R, Bentley D. 2009. "Cotranscriptionality": the transcription elongation complex as a nexus for nuclear transactions. *Molecular cell* **36**: 178-191.

Pesavento JJ, Kim YB, Taylor GK, Kelleher NL. 2004. Shotgun annotation of histone modifications: a new approach for streamlined characterization of proteins by top down mass spectrometry. *Journal of the American Chemical Society* **126**: 3386-3387.

Peterson CL, Laniel MA. 2004. Histones and histone modifications. *Current biology : CB* **14**: R546-551.

Piro AS, Mayekar MK, Warner MH, Davis CP, Arndt KM. 2012. Small region of Rtf1 protein can substitute for complete Paf1 complex in facilitating global histone H2B ubiquitylation in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 10837-10842.

Pokholok DK, Harbison CT, Levine S, Cole M, Hannett NM, Lee TI, Bell GW, Walker K, Rolfe PA, Herbolsheimer E et al. 2005. Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* **122**: 517-527.

Psathas JN, Zheng S, Tan S, Reese JC. 2009. Set2-dependent K36 methylation is regulated by novel intratail interactions within H3. *Molecular and cellular biology* **29**: 6413-6426.

Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J et al. 2012. The Pfam protein families database. *Nucleic acids research* **40**: D290-301.

Rando OJ, Winston F. 2012. Chromatin and transcription in yeast. *Genetics* **190**: 351-387.

Ranish JA, Yudkovsky N, Hahn S. 1999. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. *Genes Dev* **13**: 49-63.

Rao B, Shibata Y, Strahl BD, Lieb JD. 2005. Dimethylation of histone H3 at lysine 36 demarcates regulatory and nonregulatory chromatin genome-wide. *Molecular and cellular biology* **25**: 9447-9459.

Rizzardi LF, Dorn ES, Strahl BD, Cook JG. 2012. DNA replication origin function is promoted by H3K4 di-methylation in Saccharomyces cerevisiae. *Genetics* **192**: 371-384.

Robzyk K, Recht J, Osley MA. 2000. Rad6-dependent ubiquitination of histone H2B in yeast. *Science* **287**: 501-504.

Roguev A, Schaft D, Shevchenko A, Pijnappel WW, Wilm M, Aasland R, Stewart AF. 2001. The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. *The EMBO journal* **20**: 7137-7148.

Rondon AG, Jimeno S, Garcia-Rubio M, Aguilera A. 2003. Molecular evidence that the eukaryotic THO/TREX complex is required for efficient transcription elongation. *J Biol Chem* **278**: 39037-39043.

Rothbart SB, Krajewski K, Nady N, Tempel W, Xue S, Badeaux AI, Barsyte-Lovejoy D, Martinez JY, Bedford MT, Fuchs SM et al. 2012a. Association of UHRF1 with methylated H3K9 directs the maintenance of DNA methylation. *Nature structural & molecular biology* **19**: 1155-1160.

Rothbart SB, Krajewski K, Strahl BD, Fuchs SM. 2012b. Peptide microarrays to interrogate the "histone code". *Methods Enzymol* **512**: 107-135.

Rothbart SB, Strahl BD. 2014. Interpreting the language of histone and DNA modifications. *Biochim Biophys Acta* **1839**: 627-643.

Ruthenburg AJ, Li H, Milne TA, Dewell S, McGinty RK, Yuen M, Ueberheide B, Dou Y, Muir TW, Patel DJ et al. 2011. Recognition of a mononucleosomal histone modification pattern by BPTF via multivalent interactions. *Cell* **145**: 692-706.

Ruthenburg AJ, Li H, Patel DJ, Allis CD. 2007. Multivalent engagement of chromatin modifications by linked binding modules. *Nature reviews Molecular cell biology* **8**: 983-994.

Sainsbury S, Niesser J, Cramer P. 2013. Structure and function of the initially transcribing RNA polymerase II-TFIIB complex. *Nature* **493**: 437-440.

Sanchez R, Sali A. 1997. Evaluation of comparative protein structure modeling by MODELLER-3. *Proteins* **Suppl 1**: 50-58.

Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, Emre NC, Schreiber SL, Mellor J, Kouzarides T. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature* **419**: 407-411.

Schubeler D, MacAlpine DM, Scalzo D, Wirbelauer C, Kooperberg C, van Leeuwen F, Gottschling DE, O'Neill LP, Turner BM, Delrow J et al. 2004. The histone

modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes & development* **18**: 1263-1271.

Schulze JM, Hentrich T, Nakanishi S, Gupta A, Emberly E, Shilatifard A, Kobor MS. 2011. Splitting the task: Ubp8 and Ubp10 deubiquitinate different cellular pools of H2BK123. *Genes Dev* **25**: 2242-2247.

Segal E, Fondufe-Mittendorf Y, Chen L, Thastrom A, Field Y, Moore IK, Wang JP, Widom J. 2006. A genomic code for nucleosome positioning. *Nature* **442**: 772-778.

Seizl M, Hartmann H, Hoeg F, Kurth F, Martin DE, Soding J, Cramer P. 2011a. A conserved GA element in TATA-less RNA polymerase II promoters. *PloS one* **6**: e27595.

Seizl M, Lariviere L, Pfaffeneder T, Wenzeck L, Cramer P. 2011b. Mediator head subcomplex Med11/22 contains a common helix bundle building block with a specific function in transcription initiation complex stabilization. *Nucleic acids research* **39**: 6291-6304.

Shandilya J, Roberts SG. 2012. The transcription cycle in eukaryotes: from productive initiation to RNA polymerase II recycling. *Biochim Biophys Acta* **1819**: 391-400.

Sharma S, Kelly TK, Jones PA. 2010. Epigenetics in cancer. *Carcinogenesis* **31**: 27-36.

Shema E, Tirosh I, Aylon Y, Huang J, Ye C, Moskovits N, Raver-Shapira N, Minsky N, Pirngruber J, Tarcic G et al. 2008. The histone H2B-specific ubiquitin ligase RNF20/hBRE1 acts as a putative tumor suppressor through selective regulation of gene expression. *Genes Dev* **22**: 2664-2676.

Shema-Yaacoby E, Nikolov M, Haj-Yahya M, Siman P, Allemand E, Yamaguchi Y, Muchardt C, Urlaub H, Brik A, Oren M et al. 2013. Systematic identification of proteins binding to chromatin-embedded ubiquitylated H2B reveals recruitment of SWI/SNF to regulate transcription. *Cell reports* **4**: 601-608.

Shi X, Chang M, Wolf AJ, Chang CH, Frazer-Abel AA, Wade PA, Burton ZF, Jaehning JA. 1997. Cdc73p and Paf1p are found in a novel RNA polymerase II-containing complex distinct from the Srbp-containing holoenzyme. *Molecular and cellular biology* **17**: 1160-1169.

Shi X, Finkelstein A, Wolf AJ, Wade PA, Burton ZF, Jaehning JA. 1996. Paf1p, an RNA polymerase II-associated factor in Saccharomyces cerevisiae, may have both positive and negative roles in transcription. *Molecular and cellular biology* **16**: 669-676.

Shi X, Kachirskaia I, Walter KL, Kuo JH, Lake A, Davrazou F, Chan SM, Martin DG, Fingerman IM, Briggs SD et al. 2007a. Proteome-wide analysis in Saccharomyces cerevisiae identifies several PHD fingers as novel direct and selective binding modules of histone H3 methylated at either lysine 4 or lysine 36. *J Biol Chem* **282**: 2450-2455.

Shi X, Kachirskaia I, Walter KL, Kuo JH, Lake A, Davrazou F, Chan SM, Martin DG, Fingerman IM, Briggs SD et al. 2007b. Proteome-wide analysis in Saccharomyces cerevisiae identifies several PHD fingers as novel direct and selective binding modules of histone H3 methylated at either lysine 4 or lysine 36. *The Journal of biological chemistry* **282**: 2450-2455.

Shilatifard A. 2008. Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. *Curr Opin Cell Biol* **20**: 341-348.

Shilatifard A. 2012. The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. *Annual review of biochemistry* **81**: 65-95.

Simic R, Lindstrom DL, Tran HG, Roinick KL, Costa PJ, Johnson AD, Hartzog GA, Arndt KM. 2003. Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. *The EMBO journal* **22**: 1846-1856.

Sims RJ, 3rd, Chen CF, Santos-Rosa H, Kouzarides T, Patel SS, Reinberg D. 2005. Human but not yeast CHD1 binds directly and selectively to histone H3 methylated at lysine 4 via its tandem chromodomains. *The Journal of biological chemistry* **280**: 41789-41792.

Smith E, Shilatifard A. 2010. The chromatin signaling pathway: diverse mechanisms of recruitment of histone-modifying enzymes and varied biological outcomes. *Molecular cell* **40**: 689-701.

Smolle M, Venkatesh S, Gogol MM, Li H, Zhang Y, Florens L, Washburn MP, Workman JL. 2012. Chromatin remodelers Isw1 and Chd1 maintain chromatin structure during transcription by preventing histone exchange. *Nature structural & molecular biology* **19**: 884-892.

Soding J, Biegert A, Lupas AN. 2005. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic acids research* **33**: W244-248.

Somesh BP, Reid J, Liu WF, Sogaard TM, Erdjument-Bromage H, Tempst P, Svejstrup JQ. 2005. Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest. *Cell* **121**: 913-923.

Spruijt CG, Gnerlich F, Smits AH, Pfaffeneder T, Jansen PW, Bauer C, Munzel M, Wagner M, Muller M, Khan F et al. 2013. Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives. *Cell* **152**: 1146-1159.

Squazzo SL, Costa PJ, Lindstrom DL, Kumer KE, Simic R, Jennings JL, Link AJ, Arndt KM, Hartzog GA. 2002. The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. *The EMBO journal* **21**: 1764-1774.

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. *Nature* **403**: 41-45.

Strahl BD, Grant PA, Briggs SD, Sun ZW, Bone JR, Caldwell JA, Mollah S, Cook RG, Shabanowitz J, Hunt DF et al. 2002. Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. *Molecular and cellular biology* **22**: 1298-1306.

Strahl BD, Ohba R, Cook RG, Allis CD. 1999. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 14967-14972.

Sun M, Schwalb B, Schulz D, Pirkl N, Etzold S, Lariviere L, Maier KC, Seizl M, Tresch A, Cramer P. 2012. Comparative dynamic transcriptome analysis (cDTA) reveals mutual feedback between mRNA synthesis and degradation. *Genome Res* **22**: 1350-1359.

Sun ZW, Allis CD. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* **418**: 104-108.

Sydow JF, Brueckner F, Cheung AC, Damsma GE, Dengl S, Lehmann E, Vassylyev D, Cramer P. 2009. Structural basis of transcription: mismatch-specific fidelity mechanisms and paused RNA polymerase II with frayed RNA. *Molecular cell* **34**: 710-721.

Tada K, Susumu H, Sakuno T, Watanabe Y. 2011. Condensin association with histone H2A shapes mitotic chromosomes. *Nature* **474**: 477-483.

Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N et al. 2011. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* **146**: 1016-1028.

Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ. 2007. How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nature structural & molecular biology* **14**: 1025-1040.

Tong AH, Evangelista M, Parsons AB, Xu H, Bader GD, Page N, Robinson M, Raghibizadeh S, Hogue CW, Bussey H et al. 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* **294**: 2364-2368.

Tong AH, Lesage G, Bader GD, Ding H, Xu H, Xin X, Young J, Berriz GF, Brost RL, Chang M et al. 2004. Global mapping of the yeast genetic interaction network. *Science* **303**: 808-813.

Trojer P, Reinberg D. 2007. Facultative heterochromatin: is there a distinctive molecular signature? *Molecular cell* **28**: 1-13.

Trujillo KM, Osley MA. 2012. A role for H2B ubiquitylation in DNA replication. *Molecular cell* **48**: 734-746.

van Leeuwen F, Gafken PR, Gottschling DE. 2002. Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* **109**: 745-756.

van Nuland R, Schram AW, van Schaik FM, Jansen PW, Vermeulen M, Marc Timmers HT. 2013. Multivalent Engagement of TFIID to Nucleosomes. *PloS one* **8**: e73495.

Vermeulen M, Mulder KW, Denissov S, Pijnappel WW, van Schaik FM, Varier RA, Baltissen MP, Stunnenberg HG, Mann M, Timmers HT. 2007. Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. *Cell* **131**: 58-69.

Vethantham V, Yang Y, Bowman C, Asp P, Lee JH, Skalnik DG, Dynlacht BD. 2012. Dynamic loss of H2B ubiquitylation without corresponding changes in H3K4 trimethylation during myogenic differentiation. *Molecular and cellular biology* **32**: 1044-1055.

Vezzoli A, Bonadies N, Allen MD, Freund SM, Santiveri CM, Kvinlaug BT, Huntly BJ, Gottgens B, Bycroft M. 2010. Molecular basis of histone H3K36me3 recognition by the PWWP domain of Brpf1. *Nature structural & molecular biology* **17**: 617-619.

Vitaliano-Prunier A, Menant A, Hobeika M, Geli V, Gwizdek C, Dargemont C. 2008. Ubiquitylation of the COMPASS component Swd2 links H2B ubiquitylation to H3K4 trimethylation. *Nature cell biology* **10**: 1365-1371.

Voss TC, Hager GL. 2014. Dynamic regulation of transcriptional states by chromatin and transcription factors. *Nat Rev Genet* **15**: 69-81.

Wade PA, Werel W, Fentzke RC, Thompson NE, Leykam JF, Burgess RR, Jaehning JA, Burton ZF. 1996. A novel collection of accessory factors associated with yeast RNA polymerase II. *Protein Expr Purif* **8**: 85-90.

Wagner EJ, Carpenter PB. 2012. Understanding the language of Lys36 methylation at histone H3. *Nature reviews Molecular cell biology* **13**: 115-126.

Wang D, Bushnell DA, Huang X, Westover KD, Levitt M, Kornberg RD. 2009. Structural basis of transcription: backtracked RNA polymerase II at 3.4 angstrom resolution. *Science* **324**: 1203-1206.

Wang E, Kawaoka S, Yu M, Shi J, Ni T, Yang W, Zhu J, Roeder RG, Vakoc CR. 2013. Histone H2B ubiquitin ligase RNF20 is required for MLL-rearranged leukemia. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 3901-3906.

Weake VM, Workman JL. 2008. Histone ubiquitination: triggering gene activity. *Molecular cell* **29**: 653-663.

Wind M, Reines D. 2000. Transcription elongation factor SII. *Bioessays* **22**: 327-336.

Wood A, Krogan NJ, Dover J, Schneider J, Heidt J, Boateng MA, Dean K, Golshani A, Zhang Y, Greenblatt JF et al. 2003a. Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. *Molecular cell* **11**: 267-274.

Wood A, Schneider J, Dover J, Johnston M, Shilatifard A. 2003b. The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. *J Biol Chem* **278**: 34739-34742.

Wozniak GG, Strahl BD. 2014. Hitting the 'Mark': Interpreting Lysine Methylation in the Context of Active Transcription. *Biochim Biophys Acta*.

Wu X, Rossettini A, Hanes SD. 2003. The ESS1 prolyl isomerase and its suppressor BYE1 interact with RNA pol II to inhibit transcription elongation in Saccharomyces cerevisiae. *Genetics* **165**: 1687-1702.

Wu X, Wilcox CB, Devasahayam G, Hackett RL, Arevalo-Rodriguez M, Cardenas ME, Heitman J, Hanes SD. 2000. The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery. *The EMBO journal* **19**: 3727-3738.

Xiao T, Kao CF, Krogan NJ, Sun ZW, Greenblatt JF, Osley MA, Strahl BD. 2005. Histone H2B ubiquitylation is associated with elongating RNA polymerase II. *Molecular and cellular biology* **25**: 637-651.

Young NL, Plazas-Mayorca MD, Garcia BA. 2010. Systems-wide proteomic characterization of combinatorial post-translational modification patterns. *Expert review of proteomics* **7**: 79-92.

Yudkovsky N, Logie C, Hahn S, Peterson CL. 1999. Recruitment of the SWI/SNF chromatin remodeling complex by transcriptional activators. *Genes Dev* **13**: 2369-2374.

Zentner GE, Henikoff S. 2013. Regulation of nucleosome dynamics by histone modifications. *Nature structural & molecular biology* **20**: 259-266.

Zhang Y, Jurkowska R, Soeroes S, Rajavelu A, Dhayalan A, Bock I, Rathert P, Brandt O, Reinhardt R, Fischle W et al. 2010. Chromatin methylation activity of Dnmt3a and Dnmt3a/3L is guided by interaction of the ADD domain with the histone H3 tail. *Nucleic acids research* **38**: 4246-4253.

Zheng S, Wyrick JJ, Reese JC. 2010. Novel trans-tail regulation of H2B ubiquitylation and H3K4 methylation by the N terminus of histone H2A. *Molecular and cellular biology* **30**: 3635-3645.

Zhu B, Mandal SS, Pham AD, Zheng Y, Erdjument-Bromage H, Batra SK, Tempst P, Reinberg D. 2005. The human PAF complex coordinates transcription with events downstream of RNA synthesis. *Genes Dev* **19**: 1668-1673.

Zhuang M, Guan S, Wang H, Burlingame AL, Wells JA. 2013. Substrates of IAP ubiquitin ligases identified with a designed orthogonal E3 ligase, the NEDDylator. *Molecular cell* **49**: 273-282.

Zlatanova J, Thakar A. 2008. H2A.Z: view from the top. *Structure* **16**: 166-179.