THE MANY FACETS OF HISTONE TAILS IN
REGULATING TRANSCRIPTION

A Dissertation in
Biochemistry, Microbiology, and Molecular Biology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2011
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ABSTRACT

In eukaryotic cells, DNA is assembled into chromatin with histone proteins. The basic subunit of chromatin is the nucleosome, which consists of 147bp of DNA wrapped around a histone octamer. The N-terminal domains of histone proteins protrude from the nucleosome surface, maintain proper chromatin structure and serve as binding site of regulatory proteins. Genome wide gene expression profiling of histone tail mutants suggested that the tails are required for both transcription activation and repression in vivo. However, the mechanisms of how histone tails regulate these events remain largely unknown.

Here I explored the roles of different histone tails in gene regulation and my study provides evidence to show that different histone tails regulate distinct events at multiple stages of transcription. First, I demonstrate that deleting the H3 N terminal tails results in a shift of RNA polymerase density towards the 3’ end of gene, which indicates a defect in elongation. Further studies revealed that the H3 tail mutants severely impair H3 lysine 36 trimethylation. Recruitment of elongation factors and Set2 methyltransferase was examined and the H3 tails were dispensable for the association of these factors to genes. In vitro biochemistry assays carried out in the lab suggested that the H3 tail is critical for the catalytic activity of Set2. Thus, this novel “intra-tail” interaction added another level of regulation of Set2 activity, which in turn, controlled the dynamics of methylation during elongation.

Histone modifications not only regulate transcription directly, they also regulate one another, providing crosstalk. In the second part of this study, I describe a novel “trans-tail”
regulation involving the N-terminal tail of histone H2A. Deleting the N terminus of H2A reduces H2B ubiquitylation and H3K4 methylation, but does not affect the recruitment of the modifying enzymes to genes. I mapped the region primarily responsible for this regulation to the H2A Repression domain (HAR). Surprisingly, HAR locates in close proximity with H2BK123 in the nucleosome structure. Furthermore, the HAR is partially occluded by nucleosomal DNA, suggesting that the function of the H2A crosstalk pathway is to restrict histone modifications to nucleosomes altered by transcription.

Appropriate gene expression requires various factors, including histone chaperones and ATP dependent chromatin remodelers, to break through the chromatin barrier and facilitate RNAP II transcription. In the third section, I provided evidence to show that a conserved region in the histone H2B tail, the H2B Repression domain (HBR), regulates the activity of FACT, a histone chaperone. Deleting HBR, or the H2B tail, impairs the interaction between FACT and H2A/H2B dimer, and hampers FACT mediated chromatin remodeling, transcription activation and the redeposition of histone following the passage of polymerase. Therefore, in addition to its reported roles in repressing transcription, my study suggests a novel function of HBR in regulating histone chaperone activity and transcription activation.

The goal of this study is to provide a comprehensive analysis of how the different histone tails contribute to gene regulation. My initial hypothesis was that the tails mainly function to provide docking sites for transcription factors and regulatory proteins. However, our observations led me to investigate multiple aspects of chromatin biology and I conclude
that the histone tails are important regulators throughout the transcription cycle.
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ACKNOWLEDGEMENTS

As my life as a graduate student comes to an end, I am eager to start a new journey, but I also feel melancholy for what I am leaving behind. I am aware of how lucky I am to have worked in the Reese lab. This thesis could not be finished without the help and support of many people who are gratefully acknowledged here.

First, I would like to express my deepest gratitude to my advisor, Dr. Joseph Reese for starting me off on this journey, and for his constant “pushes” and supports throughout the years. Joe is a fantastic scientist with profound knowledge and rich research experience. Throughout my graduate study he has offered me valuable ideas and criticisms, and also “forced” me to shape my own ideas as an independent researcher. Besides the direct guidance in science, I would like to thank him for setting up the rule of “English only” in the lab, which greatly improved my ability to communicate in English. Moreover, I will always attribute the success I had, in graduate school and in future career as a scientist, to my outstanding mentor.

I next want to thank all the members of the Reese Lab, both past and present. I especially want to acknowledge Jimmy, Jenn, Arnob and Deb, who are the group of “senior” people in the lab that have accompanied me for the greater part of my graduate career. Jimmy, Jenn and Arnob taught me every detail of the experiments and shared with me whatever they know to make a better scientist. I also thank them for their patience and kindness in helping me with writing, presentations and countless other issues. Deb always brings sunshine to the
lab. She is the most energetic person I have ever seen and her dedication to work (as lab manager or technician) inspires me to perform my own role in the lab to the best of my ability. I also want to thank the "kids" in the lab who always bring in new techniques and fresh ideas. Thank Brooks for being my “replacement” and helping me with biochemical assays, and Jason for being my companion a lot of late nights in the lab. I cherish the days I have spent with them.

My thanks also go to the “Chinese Underground” whom I have built great relationship with during the last five years. They are incredibly helpful labmates and roommates who are always willing to lend me a hand in the lab or outside school. Our daily conversation, lunches and friendship helped me to persevere through graduate school and made my time at Penn State truly memorable.

I owe my parents too much for their unconditional support across the ocean. They have given me nothing but love and support throughout all aspects of my life. During the time in US, the trips back home are always my highest reward for working hard in the lab. Last but not least, I thank my husband, Jia. He accompanies me from China to the US, through every struggle and progress in pursuing my Ph.D. He influences me with his unwavering positive attitude toward life and I would not have come so far without his support and love.
Chapter 1

RNA POLYMERASE II TRANSCRIPTION THROUGH CHROMATIN
The regulation of gene expression is fundamental to the normal growth, development and survival of an organism. Gene regulation is executed mostly at the level of transcription of the DNA template to produce RNA. Numerous factors regulate transcription by influencing the ability of the RNA polymerase to access, bind and transcribe specific genes in response to environmental signals.

In eukaryotic cells, DNA is assembled into chromatin which consists of repeating subunits called nucleosomes. Each nucleosome is comprised of 147bp of DNA wrapped around a histone octamer containing two each of histones H2A, H2B, H3, and H4 (Luger et al, 1997; Richmond & Davey, 2003). Individual nucleosomes are linked together by short stretches of linker DNA to form oligonucleosomal fibers that are folded into a 30-nm diameter fiber of unknown configuration (Finch & Klug, 1976; Robinson et al, 2006; Tremethick, 2007; Woodcock et al, 1984).

The compaction of DNA into a nucleosome and higher order nucleoprotein structure forms a barrier to transcription (Bednar et al, 1999; Bondarenko et al, 2006). Eukaryotic ribosomal RNA genes usually lack nucleosomes on the transcribed repeats, which allow the passage of RNA polymerase I (Birch et al, 2009; Jones et al, 2007; Merz et al, 2008). In contrast, most genes transcribed by RNA polymerase II and III (RNAP II and RNAP III) have nucleosomes deposited along the genes. As a result, polymerases have to negotiate their way through nucleosomes. RNAP III possesses the intrinsic ability to transcribe through a mononucleosome by translocating the nucleosome to a position upstream of its original site during its passage without disrupting of the nucleosome (Studitsky et al, 1997); while RNAP II selectively remolds nucleosomes through the
orchestrated activity of ATP-dependent chromatin remodelers, histone chaperones, and histone posttranslational modifications, which provide access for the transcription apparatus throughout the transcription cycle (Armstrong, 2007; Saunders et al, 2006; Selth et al, 2010; Sims et al, 2004).
I. ATP-dependent chromatin remodelers

ATP-dependent chromatin remodelers use the energy from ATP hydrolysis to change the packaging state of chromatin by moving, ejecting, or restructuring the nucleosome. They form large, multi-component complexes which are highly conserved within eukaryotes. Based on distinct domain structures, the remodelers are classified into four well-characterized families: the SWI/SNF (switching defective/sucrose non-fermenting) family, the ISWI (imitation SWI) family, the NuRD (nucleosome remodeling and deacetylation)/CHD (chromodomain, helicase, DNA binding) family and the INO80 (inositol requiring 80) family (Becker & Horz, 2002; Clapier & Cairns, 2009; Gangaraju & Bartholomew, 2007; Wang et al, 2007). Members of the SWI/SNF family of ATPases contain a C-terminal bromodomain that binds to acetylated histone tails (Hassan et al, 2002). ISWI family members have a SANT and a SLIDE (SANT-like ISWI) domain that mediate interaction with unmodified histone tails and linker DNA (Boyer et al, 2004). NuRD/CHD family members have unique tandem chromodomains that specifically recognize methylated histone tails (Flanagan et al, 2005; Sims et al, 2005); while the INO80 family members are characterized by split ATPase domains (Bao & Shen, 2007). Although the ATPase domains are highly similar, the presence of distinct chromatin-interacting domains (bromo, chromo and SANT domains) in different ATPase remodelers suggest that they can be selectively targeted to chromatin regions with distinct modification patterns to carry out specialized roles (see below).

SWI/SNF is the first nucleosome-remodeling complex to be described in Saccharomyces cerevisiae. The genes encoding its various subunits were originally
identified in two independent screens for mutants affecting either mating type switching (SWI/switching defective, SWI) or growth on sucrose (Sucrose NonFermenting, SNF) (Stern et al, 1984; Wu & Winston, 1997). The complex plays direct roles in activator stimulated transcriptional initiation by RNAP II. \textit{In vitro}, Swi/Snf interacts with the acidic activation domains of activator proteins and drives nucleosome eviction. \textit{In vivo}, Swi/Snf is recruited to activator-binding sites, locally alters nucleosome positioning and density (Natarajan et al, 1999; Neely et al, 2002; Neely et al, 1999; Prochasson et al, 2003; Yudkovsky et al, 1999). It is required for the activation of a group of genes including the DNA damage inducible gene \textit{RNR3} and the galactose inducible gene \textit{GAL1} (Lemieux & Gaudreau, 2004; Sharma et al, 2003).

Recruitment of Swi/Snf to chromatin can also be mediated by specific modifications on the histone tails. A unique motif of the SWI/SNF family is the bromodomain which recognizes acetylated lysines. \textit{In vitro}, the stable anchoring of Swi/Snf to immobilized nucleosomal templates requires both acetylated histones and the Swi2/Snf2 bromodomain; while \textit{in vivo}, acetylation of the H3 and H4 tails by SAGA and NuA4 complexes greatly facilitates the targeting of Swi/Snf to active promoters (Awad & Hassan, 2008; Chandy et al, 2006; Hassan et al, 2001). Since most histone acetylation occur on the N terminal tails, people have studied the SWI/SNF remodeling on tailless nucleosomes and the result suggests that SWI/SNF can remodel nucleosome without tails. However, they cannot remodel multiple nucleosomal arrays in the absence of the histone termini, suggesting that the histone N terminal tails are important for the function of SWI/SNF \textit{in vivo} (Logie et al, 1999).
II. Chromatin Modifications

As the fundamental building blocks of chromatin, each histone protein consists of a globular core domain and an N terminal tail, which protrudes from the nucleosome surface. A striking feature of histone proteins is that they are decorated by multiple covalent modifications, including methylation, acetylation, ubiquitylation and phosphorylation. Most of these modifications occur on the tails and can be recognized by transcription regulatory proteins to modulate chromatin structure and gene expression. (Logie et al, 1999; Millar & Grunstein, 2006; Shahbazian & Grunstein, 2007; Shilatifard, 2006).

Acetylation

Acetylation neutralizes the positive charge on the ε-amino group of lysine, weakening the interaction between nucleosomal DNA and histone proteins (Hong et al, 1993). Thus, they are generally associated with decondensed chromatin regions, such as enhancers and promoters of active genes (Liu et al, 2005; Pokholok et al, 2005). The acetyltransferases are recruited to genes by sequence specific transcription activators. For instance, the DNA binding activator Gcn4 targets SAGA and NuA4 acetyltransferase complex to the promoter of different genes, where they acetylate histone H3/H2B and H4/H2A (Ikeda et al, 1999; Kuo et al, 2000; Utley et al, 1998). Acetylation of histones increases transient nucleosome unwrapping, which promotes the binding of general transcription factors to the promoter. In addition, acetylated lysine could be recognized by the bromodomain of multiple regulatory proteins. It is known that SWI/SNF
preferentially displaces histone acetylated by SAGA, and the RSC ATP-dependent chromatin remodeler binds to acetylated H3K14 through its tandem bromodomain and remodels chromatin nearby (Chandy et al, 2006; Kasten et al, 2004). Therefore, histone acetylation facilitates the transcription factor binding to nucleosomal sites to initiate transcription.

Low levels of acetylation are also found within the body of transcribing genes to loosen chromatin structure and facilitate the progression of polymerase. Targeting acetyltransferase to the coding region is mediated either through a direct interaction with RNA polymerase, or through binding to other histone modifications within the ORF. The elongator complex was purified as a multi-subunit complex associated with elongating RNAPII, and one of its subunits, Elp3, has been suggested to be a histone H3 acetyltransferase (Otero et al, 1999; Wittschieben et al, 1999); On the other hand, the NuA3 acetyltransferase is targeted to the ORF of genes through binding to trimethylated H3K4 and acetylates H3K14 at 5’ end regions (Taverna et al, 2006). Histone acetylations within the coding region are under the tight control of histone deacetylases to keep the chromatin in a condensed state, which prevent the use of cryptic initiation sites within the body of genes (Carrozza et al, 2005; Keogh et al, 2005; Li et al, 2007c).

**Methylation**

In yeast, lysine residues 4, 36 and 79 of histone H3 are subjected to methylation by Set1, Set2, and Dot1, respectively, and each modified lysine can exist in a mono-, di-, or tri-methylated form. Over the past few years remarkable progress has been made in
identifying the biological significance of these modifications and their roles in regulating

gene expression.

- **H3K4 methylation**

  The yeast Set1 protein was noted several years ago to be highly related to the
  MLL protein in human, which is involved in approximately 80% of the infant acute
  myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL). Since yeast is a great
  model organism for biochemical studies, characterization of the biochemical and
  biological properties of the yeast Set1 was initiated to learn more about MLL. Work from
different laboratories has shown that Set1 exists in a multi subunit complex known as
  COMPASS (COMplex Proteins ASSociated with Set1), and the complex is able to
  methylate histone H3K4 at the 5’ end of transcribing genes (Miller et al, 2001; Tenney &
  Shilatifard, 2005).

  COMPASS associates with the Serine-5 phosphorylated RNAPII via PAF complex
  (Polymerase II Associated Factors) and can mono-, di-, or trimethylate H3K4 (Figure 1-2)
  (Hampsey & Reinberg, 2003; Krogan et al, 2003a; Wood et al, 2003). The three states of
  H3K4 methylation represent different facets of transcription. Trimethylation of H3K4
  marks the early coding region and correlated with active transcription (Bernstein et al,
  2002; Santos-Rosa et al, 2002). It recruits the NuA3 and NuA4 acetyltransferase
  complexes which acetylates H3K14 and H4K8. Acetylated lysines further provide binding
  site for the chromatin remodeler RSC and SWI/SNF complex to associate and remodel
  chromatin (Ginsburg et al, 2009; Taverna et al, 2006). Dimethylation of H3K4 spread
  throughout the body of genes and may provide memory of recent transcriptional activity
(Ng et al, 2003c). H3K4 di, and trimethylation are subjected to “trans tail” regulation by H2BK123 monoubiquitylation (Nakanishi et al, 2009; Sun & Allis, 2002). It has been shown that nucleosomes at the 5’ coding region of genes are ubiquitylated at H2BK123 upon activation, and this modification serves as a prerequisite for the subsequent H3K4 methylation (see below) (Lee et al, 2007; Vitaliano-Prunier et al, 2008).

- H3K36 methylation

H3K36 methylation is another histone modification which shows tight correlation with transcription elongation. The methyltransferase, Set2, is directed to protein coding region through an interaction with the Serine 2-phosphorylated RNAP II CTD and methylates H3K36 within the body of genes (Krogan et al, 2003b; Strahl et al, 2002). H3K36 trimethylation is greatly enriched at the 3’ ends of genes and correlates strongly with transcription frequency; while H3K36 dimethylation spreads across the genes and correlates with whether a gene is expressed or not (Edmunds et al, 2008; Li et al, 2007c; Morillon et al, 2005; Rao et al, 2005). H3K36 di- and trimethylation negatively influences gene expression, including the repression of cryptic promoters within the coding region of genes. It recruits the Rpd3 histone deacetylase (HDAC) complex, leading to deacetylation and stabilization of newly transcribed chromatin and the repression of intragenic transcription (Carrozza et al, 2005; Joshi & Struhl, 2005; Li et al, 2007b).

Set2 has very low enzymatic activity in the histone H3 alone. The addition of H4 stimulated Set2 activity on H3 and from this observation it was determined that a residue within the core domain of H4, lysine 44, is required for Set2 activity on nucleosomes by allowing it to dock onto nucleosomes. This provides another example of “trans tail”
regulation of histone modifications and highlights the nucleosomal structure as a regulator of Set2 activity.

- **H3K79 methylation**

  The methyltransferase responsible for H3K79 methylation, Dot1, is the only lysine methyltransferase identified which lacks a SET domain. It is evolutionally conserved and can also produce different methylation states (Ng et al, 2003a; Ng et al, 2002; van Leeuwen et al, 2002). Methylation of lysine 79 inhibits the binding of Sir proteins which are responsible for maintaining heterochromatin at the telomere. Therefore, deletion of Dot1 leads to mislocalization of Sir proteins from heterochromatin and leads to a defect in silencing (Ng et al, 2003a). The roles of H3K79 methylation in transcription remain largely elusive. Recent study in budding yeast suggests that H3K79 di- and trimethylation are mutually exclusive, with M/G1 cell cycle-regulated genes significantly enriched for H3K79 dimethylation (Schulze et al, 2009).

**H2B ubiquitylation (H2Bub)**

Biochemical screens geared toward identifying the molecular machinery required for H3K4 methylation identified Rad6/Bre1, which ubiquitylates H2BK123 and regulates proper H3K4 methylation (Dover et al, 2002). Rad6 and Bre1 are initially recruited to promoters through interactions with activators and then associate with RNA polymerase II. The activation of Rad6 ubiquitin conjugase activity requires interactions with additional factors such as subunits of the PAF complex, Bur1/Bur2 cyclin dependent protein kinase and the Ser5-phosphorylation of RNAP II CTD (Laribee et al, 2005; Xiao et al, 2005).
H2Bub facilitate the RNAPII transcription through two distinct mechanisms. The first is to direct di- and trimethylation of H3K4, an important modification which regulates transcription elongation (Sun & Allis, 2002). Ubiquitylated H2B provides docking site for the Cps35 subunit of COMPASS complex, allowing it to associate with other subunits of the complex and fully activates Set1 (Lee et al, 2007); The second way is to recruit the FACT complex, a histone H2A/H2B chaperone which enhances the movement of RNAPII through removing H2A/2HB dimer from nucleosomes (Laribee et al, 2007; Pavri et al, 2006). Coordinated with FACT, H2Bub also appears to be required for restoration of chromatin structure in the wake of RNAPII transcription (Fleming et al, 2008).

H2B ubiquitylation is highly dynamic. Ubp8, a subunit of the SAGA histone acetyltransferase, is recruited to genes through association with the SAGA complex and deubiquitylates H2BK123 (Ingvarsdottir et al, 2005; Lee et al, 2005). Constitutive H2Bub hampers the recruitment of Ctk1, which phosphorylates Ser2 on the RNAPII CTD (Wyce et al, 2007). Ubp10 also target H2Bub, but is more involved in deubiquitylation of telomeric-associated gene (Emre et al, 2005; Gardner et al, 2005). Therefore, the ubiquitin moiety on H2B is dynamically regulated during gene expression, which maintains the proper distribution of ubiquitylation and further regulates H3K4 methylation.

The PAF complex

Initially identified as a complex associated with RNAPII, the PAF complex coordinates different patterns of histone modifications and bridges them to active transcription. The complex is composed of five subunits: Paf1, Rtf1, Leo, Ctr9, and
Cdc73 (Jaehning, 2010; Krogan et al., 2002). At the 5’ ends of genes, PAF stimulates Rad6/Bre1 mediated H2BK123 ubiquitylation, which further regulates H3K4 methylation (Ng et al., 2003b; Wood et al., 2003). It also mediates the recruitment of COMPASS methyltransferase to genes (Krogan et al, 2003a). Thus, its roles in regulating H3K4 methylation are twofold. As polymerase moves towards the 3’ end of the gene, PAF is important for Set2 mediated H3K36 methylation. It promotes Ctk1 to phosphorylate Ser2 on the RNAPII CTD, which recruits Set2 methyltransferase. PAF may also regulate the activity of Set2 directly (Kizer et al., 2005; Krogan et al., 2003b; Xiao et al, 2003). Since H3K4 and K36 methylation peak separately at the 5’ and 3’ of genes, it is suggested that the PAF complex serves as a “switch” that controls the change from H3K4 to H3K36 methylation (Sims et al, 2004).

Cross talk between histone modifications

Covalent histone modifications play crucial roles in regulating gene expression. Moreover, these modifications also regulate one another, providing cross-talk. One modification can influence the occurrence of one or more subsequent modifications in cis, on the same histone molecule, or in trans, between histone molecules or across nucleosomes. The crosstalk between different modifications on one or more histones may provide a specific pattern of information for proper gene regulation.

In cis histone regulation, modification of one residue may create binding sites for the anchoring of another enzyme to modify the same histone. For example, the PHD finger of Yng1 recognizes methylated H3K4 and helps recruit the NuA3 histone
acetyltransferase complex to acetylate H3K14, which facilitates transcription (Martin et al, 2006; Taverna et al, 2006). The Yng1-related ING2 protein can also bind methylated H3K4; however, it is present in the mSin3a–HDAC1 histone deacetylase complex, and links H3K4 methylation to active gene repression (Shi et al, 2006). Therefore, one modification can serve as a docking platform for modifying enzymes with opposing activities. Modifications of nearby residues may also prevent the binding of another enzyme. For example, methylation of H3 Arginine 2 (H3R2) interferes with H3K4 methylation by Set1/COMPASS in yeast and COMPASS-like complexes in mammalian cells. Thus, H3K4me3 and H3R2me2 are enriched on active and inactive promoters respectively (Guccione et al, 2007)(Figure 1-3).

The first trans histone pathway was identified in yeast about 10 years ago. In this trans-histone pathway, H2BK123 monoubiquitylation is required for Set1-mediated H3 K4 di- and trimethylation by recruiting the Cps35 subunit of the COMPASS complex (Lee et al, 2007; Sun & Allis, 2002). H2Bub is also required for H3K79 di- and trimethylation, but the mechanism of this regulatory pathway remains elusive. A few more trans histone regulation pathways have been identified since then. For example, a lysine residue in the core domain of histone H4 (K44) is critical for the interaction of Set2 with nucleosome and proper H3 K36 di- and trimethylation (Du et al, 2008). H3 K36 methylation can in turn, mediate cis- and trans- histone deacetylation of histone H3 and H4 to repress transcription (Carrozza et al, 2005; Keogh et al, 2005) (Figure 1-3). By studying the chromatin structure and functional changes resulting from these cross talks, we will have a better understanding of how the specific combinations of histone
modifications result in distinct gene expression outcome.
III. Histone chaperones

Histone chaperones are histone-binding proteins involved in intracellular histone dynamics, histone storage and replication-associated chromatin assembly. Recent investigations have revealed their novel roles in regulating transcription coupled changes to chromatin. Histone chaperones help overcome the nucleosome barrier by partial or complete disassembly of the nucleosomes in front of polymerase. They also participate in the restoration of normal chromatin structure in the wake of transcription (Figure 1-4) (Eitoku et al, 2008; Selth et al, 2010). The absence of chaperone function may lead to severe defect in gene expression (Ivanovska et al, 2011; Jimeno-Gonzalez et al, 2006; Rufiange et al, 2007; Shimojima et al, 2003).

Spt6

*spt6* was first isolated as a mutation that suppresses the Ty element mediated repression of the *HIS4* gene (Clark-Adams & Winston, 1987). It was later demonstrated to have histone chaperone activities and binds histone H3/H4 preferentially (Bortvin & Winston, 1996). Several lines of evidences suggest that Spt6 play roles in regulating transcription elongation. It co-purifies with the CTD of RNAPII as well as with several elongation factors, including TFIIS, DRB-sensitivity-inducing factor (DSIF), FACT, and PAF (Hartzog et al, 1998; Kaplan et al, 2000; Krogan et al, 2002). Spt6, DSIF, and FACT inducibly co-localize to genes with Ser2 phosphorylated RNAPII, the elongation form of polymerase (Andrulis et al, 2000). *In vitro* biochemical study demonstrates that Spt6 is able to stimulate elongation both individually and in concert with DSIF (Endoh et al,
Inactivation of spt6 results in transcription initiation from cryptic sites within the body of genes (Kaplan et al, 2003). These results implicate Spt6 as a modulator of RNAP II activity during elongation, as well as a maintenance factor for chromatin structure.

**FAcilitates Chromatin Transcription (FACT)**

The FACT complex was initially identified as an activity which supports RNAP II transcription on chromatin template (Orphanides et al, 1998). The yeast FACT complex is composed of three subunits: Spt16, Pob3, and Nhp6. The small HMGB domain containing protein Nhp6 mediated the interaction between FACT and chromatin. (Brewster et al, 2001; Ruone et al, 2003).

As a histone chaperone, the major function of FACT involves the disassembly and reassembly of chromatin during transcription. During initiation, FACT interacts with TFIIE, and stimulates TBP and TFIIA binding to a nucleosomal TATA site (Biswas et al, 2005). It facilitates transcription elongation by RNA polymerase II on chromatin template *in vitro* and associates with RNAPII throughout the coding regions *in vivo* (Belotserkovskaya et al, 2003; Duroux et al, 2004; Mason & Struhl, 2003). In cooperation with H2BK123 ubiquitylation, FACT promotes the dissociation of H2A/H2B dimer from nucleosome, allowing RNAP II to transcribe through chromatin (Fleming et al, 2008; Hartzog & Quan, 2008; Pavri et al, 2006). Like Spt6, inactivation of FACT also leads to transcription initiation from internal cryptic initiation sites (Mason & Struhl, 2003). Therefore, FACT has clear roles in modulating productive elongation in a chromatin context *in vitro* and *in vivo*.
Asf1

The conserved histone H3/H4 chaperone Asf1 has multiple functions in chromatin metabolism. It modulates histone deposition at the promoter during repression of transcription, assembly of silent chromatin, and replication-independent deposition throughout the cell cycle (Green et al, 2005; Robinson & Schultz, 2003; Schermer et al, 2005; Sharp et al, 2001). Asf1 is also important for activator-dependent eviction of H3 and H4 and transcription activation of a subset of promoters, including PHO5, PHO8 and HO (Adkins et al, 2004; Gkikopoulos et al, 2009; Korber et al, 2006; Zabaronick & Tyler, 2005). In addition to maintaining the balance between chromatin assembly and disassembly, Asf1 plays role in regulating the post-translational modification of histones. It is required for Gcn5 and Rtt109 to acetylate H3K9 and K56 of newly synthesized H3 (Adkins et al, 2007; Driscoll et al, 2007; Kaplan et al, 2008), and it assists Set2-dependent trimethylation of H3K36 in chromatin (Lin et al, 2010). Deletion of Asf1 leads to a severe defect in gene expression that is seen genome wide, highlighting its role in chromatin assembly and remodeling during transcription activation.

In summary, the fact that nucleosomes are needed to be removed during transcription led to the evolution of many factors with overlapping and redundant functions. These factors regulate chromatin structure by covalently modifying histones, by temporarily moving or disassembling and reassembling nucleosomes. Understanding the roles that these factors play in regulating transcription is essential to our understanding of eukaryotic gene regulation.
IV. The role of histone tails in transcription

The histone N terminal tails, which constitute up to 30% by mass of histone proteins, protrude from the nucleosome surface and play a variety of roles in transcription. They modulate DNA accessibility within the nucleosome and are essential for stable folding of nucleosome arrays into condensed chromatin fibers (Allan et al, 1982; Fletcher & Hansen, 1995; Ling et al, 1996; Luger et al, 1997); a large number of residues within the tail are post-translationally modified, and these modifications functionally impact multiple stages of transcription. Histone tails also provide docking sites for chromatin remodeling/modification activities to regulate chromatin structure (Figure 1-5) (Fingerman et al, 2007; Georgel et al, 1997). Early studies about the histone tails suggested predominantly repressive roles in transcription. In vitro transcription assays revealed that RNAPII more readily transcribe “tailless” chromatin templates than wild type template (Chirinos et al, 1998; Hernandez et al, 1998; Protacio et al, 2000). Recently, genome wide expression studies have uncovered a more complex scenario. It seems that each histone tail has roles in both activation and repression and regulates specific groups of genes (Parra et al, 2006; Parra & Wyrick, 2007; Sabet et al, 2003; Sabet et al, 2004). However, the mechanism behind these phenotypes has not been established.

In S. cerevisiae, the histone N-termini are nonessential, which enables us to start a comprehensive analysis of how each histone tail contributes to transcription. Using Ribonucleotide-diphosphate reductase 3 (RNR3), a DNA damage inducible gene and Galactokinase (GALI), a galactose inducible gene to study transcription, data from our lab show that all four histone tails play roles in regulating transcription and their mechanisms
differ. We found that:

- Deletion of the H4 N-terminus results in the inability to release the Crt1-Ssn6-Tup1 repressor complex upon induction. This mutant also exhibits constitutive SWI/SNF recruitment and partial remodeling of promoter even in the absence of activation signal. This part of work was the major project of another graduate student in the lab.

- The H3 tail deletion (H3Δ1-28aa) and H3 Lysine 4,9,14,18,23,27 to glutamine (H3KtoQ, here and after) mutation causes “piling up” effect of RNAP towards the 3’ end of gene and impairs H3K36 trimethylation. In chapter II, I carried out experiments to examine the recruitment of elongation factors and Set2 methyltransferase to genes to dissect how the H3 tail regulates H3K36 methylation. Our data propose a model that H3K36 methylation is regulated by novel intra-tail interactions within H3.

- Deletion of the H2A tail causes a delay in the activation of inducible genes and a defect in H3K4 trimethylation. My analysis provides evidence to show that the H2A tails “*trans* tail” regulate H2BK123 monoubiquitylation, which further regulates H3K4 methylation. We propose that the significance of this regulatory pathway is to restrict histone modifications to nucleosomes altered by transcription. This part of work will be described in detail in chapter 3.

- Deletion of a large part of the H2B tail (H2BΔ1-32) did not lead to any transcription defect. However, deletion of a short basic patch including amino acid 30-37 of the H2B tail known as HBR (H2B Repression domain) strongly impairs transcription. In Chapter 4, we depict a critical role of HBR in mediating the interaction between histone chaperone FACT and H2A/H2B dimer. The biological consequence of this regulation will also be
discussed.

Collectively, this data suggests novel roles of histone tails in regulating different facets of transcription, which highlight their importance in the fast and accurate expression of eukaryotic genes.
Figure 1-1. ATP Dependent Chromatin Remodeler Families, defined by their ATPase
All remodeler families contain a SWI2/SNF2-family ATPase subunit characterized by an
ATPase domain that is split in two parts: DExx (red) and HELICc (orange). Remodelers
of the SWI/SNF, ISWI, and CHD families each have a distinctive short insertion (gray)
within the ATPase domain, whereas remodelers of INO80 family contain a long insertion
(yellow). Each family is further defined by domains adjacent to the ATPase domain and
other associating subunits.
Figure 1-2. Genome wide distribution pattern of histone modifications from a transcription perspective.
H3K4 trimethylation and H2BK123 ubiquitylation both peaks at promoter of gene. H3K4, H3K36 and H3K79 dimethylation all spread throughout genes; while H3K36 trimethylation peaks towards the 3’ end.
Figure 1-3. Examples of Histone Crosstalk

In cis histone regulation, one modification can either stimulate or repress another modification. For example, H3K4 methylation provides docking site for the NuA3 histone acetyltransferase to acetylate H3K14; while H3R2 methylation and H3K4 methylation are mutually exclusive.

The first identified trans histone regulation is that H2BK123ub trans regulate H3K4 and H3K79 di, and trimethylation. Other well studied examples includes K44 in the core domain of histone H4 anchorage Set2 methyltransferase on nucleosome to methylate H3K36, while H3K36 would in turn, recruit Rpd3S HDAC to deacetylate H3 and H4 and return the chromatin to a packed status.
Figure 1-4. Disassemble and reassemble of nucleosome during transcription
A) Transcription through a nucleosome can result in the complete release of histone proteins from DNA, or
B) The nucleosome can be only partially disassembled. FACT, and possibly other histone chaperones, can receive the dimer and load it in the wake of the progressing polymerase.
Figure 1-5 Known roles of histone tails during transcription.
The histone tails may (a) interact with adjacent nucleosomes to maintain the higher chromatin structure; (b) be post translationally modified and these modifications can be recognized by other factors to further modulate chromatin structure; (c) directly recruit factors to regulate transcription.
Chapter 2

ROLES FOR THE HISTONE H3 N-TERMINUS IN TRANSCRIPTION ELONGATION
Abstract

The packaging of chromosomal DNA by nucleosomes forms a barrier to RNAP II transcription. However, this constraint also allows nucleosomes and other numerous regulatory proteins to actively participate in the regulation of transcription. As the basic component of a nucleosome, the role of histone tails during transcription and the post-translational modifications to the tails have been studied extensively, but the requirement for the residues within the tails for different stages of transcription is less clear. Using RNR3 as a model, we find that the residues within the H3 N-terminus are predominantly required for transcription elongation. Specifically, deleting the first 28 amino acids of the tail, or substituting glutamines for lysines in the tail, greatly impaired H3K36 methylation by Set2. The mutations to the tail described here preserve the residues predicted to fill the active site of Set2. The H3 tail is dispensable for the recruitment of positive elongation factors, histone chaperones and Set2 methyltransferase to genes, but is required for the catalytic activity of Set2 in vitro. We propose that Set2 activity is controlled by novel intra-tail interactions, which can be influenced by modifications and changes to the structure of the H3 tail to control the dynamics and localization of methylation during elongation.
Introduction

Hundreds of proteins participate in transcription regulation in eukaryotes. Some factors act directly on RNAP II, whereas others manipulate the chromatin environment. Nucleosomes, the basic subunit of chromatin, form a barrier for transcription by RNAP II. The electrostatic interaction between histone and DNA need to be destabilized to allow the passage of polymerase. The potency of the nucleosomal barrier necessitates the involvement of various polymerase associated proteins to facilitate its progression through chromatin template.

The FACT complex (Facilitate chromatin transcription), consisting of Spt16 and Pob3, was first identified to have an activity which facilitates elongation on chromatin template. It destabilizes the nucleosome by selectively removing one H2A/H2B dimer from the octamer (Belotserkovskaya et al, 2003; Belotserkovskaya & Reinberg, 2004). SPT4, SPT5, and SPT6 were isolated in a genetic screen that identified multiple transcription factors which regulate chromatin and transcription, Suppressor of Ty insertion (Hartzog et al, 1998). Spt4 antagonizes the negative effects of RNAPII pausing imposed by the chromatin remodeling factor Isw1p (Morillon et al, 2003). It also facilitates loading of the PAF complex (RNA Polymerase II associated factor) onto the Ser5 phosphorylated RNAP II CTD (Qiu et al, 2006). Spt5 interacts with factors implicated in mRNA maturation and was shown to prevent premature termination and pausing during elongation (Lindstrom et al, 2003). Spt6 functions as a histone H3/H4 chaperone in the reassembly of nucleosomes displaced by activators and the elongating RNAPII complex (Bortvin & Winston, 1996). Spt6 and FACT both prevent cryptic
transcription within the coding region of genes, supporting the idea that they aid in maintaining chromatin structure during elongation (Kaplan et al, 2003).

Histones themselves possess information pertinent to transcription regulation in the form of posttranscriptional covalent modifications. One of the most important landmarks associated with elongation is H3K36 methylation, modified by the Set2 methyltransferase. Set2 associates with Ser2 phosphorylated RNAPII and methylate H3K36 in the wake of elongation (Krogan et al, 2003b). H3K36 trimethylation correlates strongly with transcription frequency and peaks at the 3’ end of genes. H3K36 dimethylation correlate with the “on” and “off” state of transcription and is more broadly distributed (Edmunds et al, 2008; Morillon et al, 2005; Rao et al, 2005). H3K36 methylation recruits the Rpd3 histone deacetylase (HDAC) complex to genes, which mediates chromatin reassembly once RNAPII has passed, and inhibits inappropriate initiation within coding regions (Carrozza et al, 2005; Joshi & Struhl, 2005; Li et al, 2007b).

The activity of Set2 is regulated by several events. Set2 is recruited to actively transcribed regions through an interaction between its SRI domain and Ser2 phosphorylated RNAPII CTD. Deletion of Ctk1, the kinase responsible for Ser2 phosphorylation, significantly lowers the Set2 protein level and H3K36 methylation (Kizer et al, 2005; Li et al, 2003). The PAF complex regulates Set2 activity in two folds: it is required for full levels of RNAP CTD Ser2 phosphorylation, and it may also regulate the activity of Set2 through an undefined mechanism (Hampsey & Reinberg, 2003; Krogan et al, 2003b; Xiao et al, 2003). In addition, the histone chaperone Spt6 regulates
the stability of Set2 protein. Inactivation of the conditional spt6-1004 mutant leads to
degradation of Set2 and loss of H3K36me3 (Yoh et al, 2008; Youdell et al, 2008). The last
but not least, a basic patch in histone H4 “trans” regulates H3K36 methylation by
providing docking site for Set2 on the nucleosomes (Du et al, 2008). Despite recent
advances, how the dynamics and localization of Set2-dependent methylation are
controlled in vivo remains largely unknown.

Here we characterized the role of histone H3 tail in transcription. We show that
deletion of the H3 tail, or mutating the lysine residues in the tail to glutamine, cause
“piling up” effect of RNAPII towards the 3’ end of RNR3 gene and impair Set2 mediated
H3K36 methylation. We show that the H3 tail is dispensable for the recruitment of
elongation factors and methyltransferase to genes. However, it does regulate the activity
of Set2. Biochemical analyses using recombinant components suggest that the tail directly
stimulates Set2 activity. We propose that Set2 methylation is regulated by novel intra-tail
interactions within H3.
Results and Discussion

Mutations to the H3 N terminus result in altered RNAPII density

Initial study in the role of histone H3 N terminal tail during transcription suggested that deletion of the tail was associated with derepression of transcription (Wan et al, 1995). However, recent gene expression profiling of H3 tail mutants revealed that it is required for both the activation and repression of different subset of genes. The mechanisms behind these observations remain elusive. We used the DNA damage-inducible \( RNR3 \) gene as a model to examine the role of the histone H3 tail in transcription activation. We found that deleting the first 28 amino acid of the H3 tail (H3 \( \Delta1-28 \)), or substituting lysines 4, 9, 14, 18, 23, and 27 in the tail with glutamines (H3 \( KtoQ \)), significantly reduced the levels of induced \( RNR3 \) transcription (Psathas et al, 2009).

To determine which steps in the activation of \( RNR3 \) were affected by the histone tail mutations, we examined the density of RNAPII across \( RNR3 \) by Chromatin Immunoprecipitation (ChIP) analysis. Upon activation, the increase in RNAPII recruitment is consistently ~5-7 folds above the uninduced level in the wild type strain, indicating its even distribution across the gene (Figure 2-1 and 2-2A). Deletion of the H3 N-terminus results in lower levels of RNAPII recruitment to the \( RNR3 \) promoter after induction. However, RNAPII levels within the ORF (at ORF 2 and ORF4) are equal to that observed in wild type cells (Figure 2-2A, comparing H3\( \Delta1-28 \) MMS+ to H3WT MMS+ at ORF2 and ORF4). To better illustrate the difference in the distribution of RNAPII between the wild type and the H3 \( \Delta1-28 \) mutant, we set the amount of
polymerase crosslinked to promoter to 1, and plotted its density across $RNR3$ in the wild type and mutant relative to that recruited to the promoter (Figure 2-2B). The wild type strain showed little variation in RNAPII density across $RNR3$, until it dropped off at the 3’UTR near the transcription termination site. While in the H3 Δ1-28 cells, RNAPII density increased progressively further downstream, up to 2.5-fold higher than that observed at the promoter. Assuming that RNAPII is loaded onto the promoter, the pattern is consistent with the “piling up” of RNAPII across $RNR3$, suggesting that RNAPII is having difficulties completing transcription across the gene.

The H3 N-terminus is not required for the recruitment of elongation factors.

The shift of polymerase density in the H3 tail deletion mutant suggested a defect in elongation. Although we did not observe a change in polymerase density at $RNR3$ promoter in the H3 KtoQ mutant, the mRNA level of $RNR3$ was greatly reduced (Psathas et al, 2009), indicating a defect after initiation.Mutations in the tail may alter chromatin structure, affect the recruitment of elongation factors, and therefore reduce transcription. To test this, we constructed strains with myc tagged elongation factors, including Pob3 (FACT), Spt4, Spt5 and Spt6, in the H3 tail deletion (H3 Δ1-28) and H3 KtoQ mutants. We then examined their recruitment to $RNR3$ by ChIP analysis.

The histone H2A/H2B chaperone FACT and H3/H4 chaperone Spt6 reorganize chromatin to facilitate the passage of polymerase during elongation. Recruitment of FACT complex (Pob3) and Spt6 across $RNR3$ was first examined in the wild type and H3 tail mutants. There was a 2-fold increase in FACT recruitment in the wild type cells at the
promoter of RNR3 upon treatment with MMS. This increase followed across RNR3 and reached about 3 fold in the ORFs, indicating a transcription dependent recruitment of histone chaperones (Figure 2-3A). In both the H3 tail deletion and KtoQ mutants, recruitment of FACT followed the same trends as the wild type. Noticeably, there was an increase of crosslinking in the mutants toward the 3’ end of gene in the repressed state (MMS-), which probably reflected a higher level of basal transcription in these mutants (Figure 2-3A). Recruitment of Spt6 mirrors that of the Pob3, and neither mutant reduced the crosslinking of Spt6 to RNR3 (Figure 2-3C). We also examined the association of FACT and Spt6 to a constitutively activated gene, PYK1. There was about the same amount, or a slight decrease of Pob3 crosslinked to PYK1 in the H3 tail mutants compared to the wild type. Recruitment of Spt6 to PYK1 was also examined and did not show any difference between the WT and mutant (Figure 2-3B and D), indicating that deleting the H3 tail, or substituting lysines with glutamine, does not affect the recruitment of histone chaperones to genes.

The Spt4/5 complex joins elongation complexes shortly after initiation and associates with RNAPII throughout the genes. Although the precise function of Spt4/5 in transcription has not been defined, a wealth of genetic data implicates their roles in regulating elongation and RNA processing in vivo. We next examined the recruitment of Spt4 and Spt5 to RNR3 in the wild type and H3 tail mutants. Spt4/5 associates with polymerase only in the ORF of genes. Recruitment of Spt4 increased ~5 to 10 fold across the ORF of RNR3 upon MMS treatment. Neither the H3 tail deletion nor the KtoQ mutations reduced Spt4 recruitment (Figure 2-3E). Recruitment of Spt5 to RNR3 gave the
same result (Figure 2-3G). Furthermore, Spt4/5 recruitment to PYK1 was equal in wild type and mutant cells (Figure 2-3F and H). Therefore, the transcription defect we have observed in the H3 tail mutants is not caused by an inability to recruit elongation factors.

**The H3 N-terminus is required for H3K36 methylation**

We next sought to identify other factors which may cause transcription defect and alter the distribution of polymerase. It has been reported that RNAPII level in the set2 deletion mutant was significantly increased in the middle to late coding region of genes (Kizer et al, 2005), the same phenomenon as we have observed in the H3Δ1-28 mutant. Since deletion of Set2 erases H3K36 methylation, we measured the H3K36me3 level in the H3 tail mutants to determine if there is a possible decrease. In the wild type cells, there was a transcription dependent increase in H3 K36me3 level towards the 3’ end of the coding region, and the level peaks over the ORF4 of RNR3 (Figure 2-4A). A similar pattern was also observed at the constitutively activated gene, PYK1. Furthermore, the increase in H3 K36me3 was dependent upon Set2 (Psathas et al, 2009)). Significantly, deleting the H3 tail (H3Δ1-28), or substituting lysines to glutamines (H3 KtoQ), caused more than 70% reduction in the level of H3K36me3 across RNR3 (Figure 2-4A). Deletion of the H3 tail also resulted in a significant decrease in H3K36me3 within the ORFs of PYK1 (Figure 2-4B). Thus, the same phenotype is observed at both constitutively transcribed and highly induced genes. It is worthwhile to point out that the mutated residues in the histone tail analyzed here are relatively distant from the region of H3 expected to fill the active site of Set2. Therefore, the H3 tails play an important role in
Set2 mediated H3K36methylation.

To determine if the defect in H3K36me3 is a global phenomenon, we examined its levels in chromatin from wild type, H3 Δ1-28, H3KtoQ, and set2Δ strains by Western blotting. The amount of total H3 was measured using an antibody recognizing the C-terminus of H3. Consistent with the ChIP analysis, H3K36me3 levels were significantly lower in the H3 tail mutants than wild type cells (Figure 2-4C), indicating that H3K36 methylation is reduced across the genome in the H3 mutants.

H3K36 methylation is required to recruit the Rpd3S HDAC complex, which in turn, deacetylates nucleosomes within the ORF of genes. Deleting SET2, or mutating the H3 tail which impairs H3K36 methylation, resulted in intragenic transcription within certain genes, and may lead to increased RNAP II density towards the 3’ end at these genes. The increased RNAP II density we have observed at 3’ end of RNR3 is not due to cryptic transcription because deleting components of the Rpd3S complex or mutating the H3 tail does not produce cryptic transcripts within RNR3 (data not shown). In addition, it has been reported that in a SET2 deletion mutant, RNAP II density increased significantly in the middle to late coding region of a variety of actively transcribing genes (Kizer et al, 2005), suggesting that H3K36 methylation regulates the precise localization of RNAPII on genes.

The H3 N-terminus is dispensable for Set2 and Paf1 recruitment

To determine the mechanism of how the H3 tail regulates H3K36 methylation, we first examined the recruitment of Set2 methyltransferase to genes. It is possible that
the H3 tails play a role, either directly or indirectly, in the recruitment of Set2 to genes. We constructed strains with myc-tagged Set2 in the wild type and H3 tail mutants and examined the recruitment of Set2 to \textit{RNR3} and \textit{PYK1} (Figures 2-5A). In the wild type strain, Set2-myc recruitment increased about 3 folds upon induction of transcription at ORF4 of \textit{RNR3}, the same location as the peak of H3K36me3 (Figure 2-5A). We also observed recruitment of Set2 to ORF 2 and the 3’ end of \textit{RNR3}, but to a lesser degree. Importantly, neither the H3 Δ1-28 nor the H3 KtoQ mutations reduced Set2 recruitment, and in fact, Set2 crosslinking was slightly higher in the H3 mutants than the wild type strain. Furthermore, Set2 recruitment to \textit{PYK1} was equal in wild type and mutant cells (Figure 2-5B). This data indicates that the H3 N-terminus is not required for Set2 recruitment, suggesting that the tail regulates Set2 activity at a post-recruitment step.

The PAF complex interacts with RNAPII and regulates H3K36 methylation. Deleting certain PAF subunits, such as Paf1 or Ctr9, eliminates H3K36 trimethylation and significantly reduces dimethylation at genes, presumably by reducing Set2 recruitment. While we do not observe a Set2 recruitment defect in the H3 mutants, it is possible that the PAF complex regulates Set2 by stimulating its activity. Thus, we examined the recruitment of the Paf1-myc to \textit{RNR3} (Figures 2-5C and 2-5D). In the wild type strain, Paf1-myc recruitment increased significantly in the ORF and 3’ end of \textit{RNR3} in the induced condition (+MMS). The peak of Paf1-myc recruitment occurs at ORF 2, a region where H3K4me3 and K36me3 methylation overlap considerably upon induction (data not shown). Neither H3 Δ1-28 nor the H3 KtoQ mutations affected the recruitment of Paf1-myc to most regions of \textit{RNR3} (Figures 2-6C). At ORF 2, Paf1-myc recruitment was
~50% less than the wild type in the H3 Δ1-28 mutant, and ~30% less in the H3KtoQ mutant. In addition, we also observed a significant decrease in the recruitment of Pafl to the constitutively activated gene *PYK1*. It seems that the H3 tails are required for the recruitment of PAF to at least some genes. At *RNR3*, since Pafl recruitment to ORF4, the location of the peak of Set2 recruitment and H3K36 methylation, was not affected in the H3 mutants, the defect in K36 methylation is not likely to be caused by an inability to recruit the PAF complex. However, the defect in PAF recruitment at *PYK1* gene in the H3 tail mutants may partially explain the decrease in H3K36 trimethylation in a subset of genes.

So far we have tested most known factors which may play a role in regulating Set2 activity *in vivo*, and we found these parameters to be intact. These data suggests that mutating the H3 N-terminus affects Set2 activity at a post-recruitment step, possibly by directly modulating its activity. Another graduate student in the lab (James Psathas) carried out biochemical analyses using recombinant Set2 protein and *in vitro* purified wild type and mutant nucleosomes. His results suggest that deleting the H3 tail, or mutating the lysine residues to glutamine, impairs the catalytic activity of Set2 which leads to the defect in H3K36 methylation. The H3 tail interacts with Set2 and probably keeps K36 in proper position inside the Set2 active site. The charges on the tails are also important for this stimulatory effect. Substituting glutamines for lysines neutralizes the positive charges on the tail, and greatly reduces H3 K36me3 *in vivo*; whereas substituting arginines for lysines, which maintains the positive charges, does not result in H3K36me3 defect (Psathas et al, 2009). With these results, we propose that Set2 activity is regulated by
novel intra-tail interactions within H3. Since residues within the tail of H3 are heavily modified during transcription, Set2 activity could be influenced by modifications and changes to the structure of the H3 tail, which in turn, regulates the dynamics and localization of H3K36 methylation during elongation.
Acknowledgements

We thank members of the Reese Lab and the Center for Eukaryotic Gene Regulation at Penn State for advice and comments on this work. We are grateful to James Psathas for initial characterization of the mutants, identifying the H3K36me3 defect, carrying out the \textit{in vitro} biochemical analysis of Set2 function, and providing Figures 2-2 and 2-4. Mitch Smith (University of Virginia) is acknowledged for providing the yeast H3 tail mutants. This research was supported by funds from National Institutes of Health (GM58672) to J.C.R.
Figure 2-1. Schematic of strains and primer pairs used in Chapter 2
(A) Schematic of histone H3 N-termini mutants examined.
(B) Schematic of primers used in ChIP experiments.
Figure 2-2. Mutations to the H3 N-termini result in altered RNAPII density*

(A) ChIP analysis of RNAPII crosslinking across RNR3. Wild type, H3 Δ1-28, and H3 KtoQ strains were grown and treated, or not, with MMS for 2.5 hrs prior to crosslinking. The data is represented as fold recruitment relative to the untreated (-MMS) wild type cells. Primer pairs directed to regions across RNR3 are indicated in Figure 2-1B.

(B) ChIP analysis of RNAPII crosslinking across RNR3 relative to the promoter. The induced (+MMS) data was expressed in each strain. RNAPII density at the promoter was set to 1.

* Figure provided by James N. Psathas
Figure 2-3. The H3 N-terminus is not required for the recruitment of elongation factors

(A) ChIP analysis of FACT recruitment across RNR3. Wild type, H3 Δ1-28, and H3 KtoQ strains with Spt16 myc-tagged were examined as described in the legend of Figure 2-2A.

(B) Analysis of FACT recruitment across PYK1. Same as in “A” except that cells were not induced with MMS.

(C and D) ChIP analysis of Spt6-myc recruitment across RNR3 and PYK1. Same as in “A” and "B"

(E and F) ChIP analysis of Spt4-myc recruitment across RNR3 and PYK1. Same as in “A" and "B"

(G and H) ChIP analysis of Spt5-myc recruitment across RNR3 and PYK1. Same as in “A” and "B"
Figure 2-4. The H3 N-terminus is required for H3 K36 methylation *

(A) Levels of H3K36me3 across \textit{RNR3}. Wild type, H3 Δ1-28, and H3 KtoQ strains were grown and treated as described in the legend of Figure 2-2A. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. Location of the PCR amplicons are shown in Figure 2-1B.

(B) Analysis of H3K36me3 across \textit{PYK1}. Same as in “A” except that cells were not induced with MMS.

(C) The N-terminus of H3 regulates K36me3 throughout the genome. Western blots were performed on whole cell extracts of wild type and mutant strains indicated in the panel. Blots were probed with H3K36me3 and H3 core antibodies. The gradient indicates a decreasing amount of extract loaded onto the gel.

* Figure provided by James N. Psathas
Figure 2-5. The H3 N-terminus is dispensable for Set2 and Paf1 recruitment.
(A) ChIP analysis of Set2 recruitment across RNR3. Wild type, H3 Δ1-28, and H3 KtoQ strains with Set2 myc-tagged were examined as described in the legend of Figure 2-2A.
(B) Analysis of Set2 recruitment across PYK1. Same as in “A” except that cells were not induced with MMS.
(C and D) ChIP analysis of Paf1-myc recruitment across RNR3 and PYK1. Same as in “A” and "B"
Chapter 3

NOVEL TRANS-TAIL REGULATION OF H2B UBIQUITYLATION AND H3 METHYLATION BY THE N-TERMINAL TAIL OF H2A
Abstract

Chromatin is regulated by crosstalk among different histone modifications, which can occur between residues within the same tail or different tails in the nucleosome. The later is referred to as trans-tail regulation, and the best-characterized example of this is the dependence of H3 methylation on H2B ubiquitylation. Here we describe a novel form of trans-tail regulation of histone modifications involving the N-terminal tail of histone H2A. Mutating or deleting residues in the N-terminal tail of H2A reduces H2B ubiquitylation and H3 K4 methylation, but does not affect the recruitment of the modifying enzymes, Rad6/Bre1 and COMPASS, to genes. The H2A tail is required for the incorporation of Cps35 into COMPASS and increasing the level of ubiquitylated H2B in H2A tail mutants suppresses the H3K4 methylation defect, suggesting that the H2A tail regulates H2B-H3 crosstalk. We mapped the region primarily responsible for this regulation to the H2A Repression domain, HAR. The HAR and K123 of H2B are in close proximity to each other on the nucleosome, suggesting that they form a docking site for the ubiquitylation machinery. Interestingly, the HAR is partially occluded by nucleosomal DNA, suggesting that the function of the H2A crosstalk pathway is to restrict histone modifications to nucleosomes altered by transcription.
Introduction

Posttranslational modification of histone proteins plays important roles in regulating chromatin dynamics and transcription (Kouzarides, 2007; Krebs, 2007; Strahl & Allis, 2000). Most of these modifications are located in the flexible N-terminal tails that protrude from the nucleosome, while some occur in the globular core domains of histones. In either case, nucleosome structure can be altered directly, or indirectly, by modifications and the activities that these modifications recruit.

Histone modifications are dynamic and highly regulated, and are under the tight control by enzymes that either add or remove them. For example, in yeast, histone H2B is monoubiquitylated by Rad6 at lysine 123 (H2BK123ub1) during transcription, and this mark is localized over the coding region of genes (Robzyk et al, 2000; Schulze et al, 2009; Xiao et al, 2005). Removal of H2BK123ub is mediated by the deubiquitylases Ubp8 and Ubp10, although Ubp10 functions more at heterochromatic regions (Daniel et al, 2004; Emre et al, 2005; Gardner et al, 2005; Geng & Tansey, 2008; Henry et al, 2003). In an ubp8Δ mutant, which has persistent H2BK123ub1, recruitment of Ctk1 kinase is hindered and the phosphorylation of Ser2 on the carboxyl terminal domain (CTD) of the large subunit of RNA polymerase II is altered (Wyce et al, 2007). Thus, both the timely addition and removal of ubiquitin on K123 are necessary for optimal transcription. In conjunction with the FACT complex (Spt16/Pob3), ubiquitylated H2B facilitates both the removal and reassembly of nucleosomes before and after the passage of RNA polymerase II (RNAP II) during elongation (Fleming et al, 2008; Pavri et al, 2006; Reinberg & Sims, 2006). H2BK123ub1 is clearly linked to chromatin dynamics during elongation, but it is
unclear how the ubiquitylation machinery recognizes nucleosomes and if disruption of the canonical nucleosome structure by RNAPII is required for modification of K123.

Histone modifications also regulate one another, providing cross-talk among the histones (Latham & Dent, 2007; Shukla et al, 2009; Suganuma & Workman, 2008). In “trans histone” regulation, the modification on one histone modulates the modification on another histone protein. One of the most well known examples of “trans histone” regulation is the requirement of H2BK123ub1 for H3K4 di-, and trimethylation during transcription (Dover et al, 2002; Lee et al, 2007; Nakanishi et al, 2009; Sun & Allis, 2002; Vitaliano-Prunier et al, 2008). In S. cerevisiae, methylation of H3K4 is catalyzed by COMPASS, which contains the Set1 methyltransferase (Dehe & Geli, 2006; Miller et al, 2001; Roguev et al, 2001). H3K4 can be mono-, di- and trimethylated and the different methylation states represent different facets of active chromatin. H3K4 trimethylation peaks at the promoter of actively transcribed genes and has been suggested to recruit Isw1 chromatin remodelers and the NuA3 histone acetylation complex to genes, both of which remodel chromatin and facilitate transcription (Ginsburg et al, 2009; Santos-Rosa et al, 2003; Taverna et al, 2006). H3K4 dimethylation presents in the middle of genes and may serve as a mark of recent transcription while H3K4 monomethylation rises toward the 3’ end (Kim & Buratowski, 2009; Ng et al, 2003c). H2BK123ub1 is required for H3K4 di-, and trimethylation by controlling the incorporation of Cps35 into the COMPASS complex (Lee et al, 2007). The presence of Cps35 within COMPASS is required to form a highly active complex capable of di-, and tri-methylating H3K4.

Most histone modifications occur on the N-terminal domain of histone H3 and
H4. For this reason, the roles of H3 and H4 tails in regulating nuclear functions have been studied extensively. In contrast, very little is known about the function of the H2A and H2B tails in transcriptional regulation. Interestingly, the limited amount of data suggests that the H2A and H2B tails play a more prominent role in repression of transcription (Lenfant et al, 1996; Parra et al, 2006; Parra & Wyrick, 2007; Recht et al, 1996; Wyrick & Parra, 2009). To explore the function of the histone H2A N terminal domain in transcription, we analyzed modification levels for various histone modifications in the histone H2A tail mutants. Our results revealed that the H2A tail is required for the activation of highly induced genes and that a region previously identified as a H2A Repression domain, HAR, controls the level of H2BK123 monoubiquitylation and subsequently H3K4 methylation. The close proximity of the HAR to H2BK123, and its partial occlusion by nucleosomal DNA, suggest a novel histone cross talk between H2A and H2BK123ub1 that may depend on the exposure of the HAR during transcription-linked nucleosome disruption. The implications of this pathway on the coordination of histone modifications during transcription are discussed.
Results

The N-terminal tail of histone H2A is required for H3K4 methylation

Recent genome wide expression analysis has revealed the importance of histone H2A and H2B N-terminal tails in transcriptional regulation (Parra et al, 2006; Parra & Wyrick, 2007; Wyrick & Parra, 2009). However, little is known about how the tails regulate transcription or if they influence the modification of other histone tails. Since lysine K4 methylation is tightly linked to transcription activation, this modification was examined in H2A tail mutants. Interestingly, we found that deleting the majority of the H2A tail, residues 4-20, significantly reduced the level of trimethylated lysine 4 (K4me3) on histone H3 (Figure 3-1A); the level was 25% of that in wild type cells. Examination of strains with smaller deletions within the tail revealed that the more C-terminal portion of the tail is primarily responsible for this phenotype. The Δ12-20 mutant showed a significant reduction in K4me3, albeit not as great as deleting the entire tail (Figure 3-1A, compare lanes 4 vs 5). Correspondingly, deleting residues 4-12, or mutating the two lysine residues known to be acetylated (K4,7), had little to no effect on H3K4me3. To test the specificity of this phenotype we examined a mutant containing a deletion of the first 32 residues in the N-terminal tail of H2B, and found that in this experiment, a small reduction in H3K4me3 was observed (Figure 3-1A, lane 6). However, this was not observed in all samples tested (not shown).

Next, we examined the levels of di- (me2) and monomethylated (me1) K4 in the histone H2A mutants by western blotting. Interestingly, the levels of H3K4me2 and H3K4me1 were not significantly reduced, suggesting that the H2A tail is more important
for trimethylation across the genome (Figure 3-1A). We examined two other histone lysine methylation marks associated with gene activity, H3K36me3 and H3K79me2 (Li et al, 2003; Li et al, 2002a; Miao & Natarajan, 2005; Pokholok et al, 2005; Schubeler et al, 2004). Relatively little, if any, change in these two modifications was detected (Figure 3-1B), suggesting that the H2A tail is specifically required for H3K4me3. The commercial antiserum raised to H3 K79me3 peptides also recognizes the dimethylated form of K79, so we could not determine if deletion of the H2A tail affects the level of trimethylated K79.

The requirement for the H2A tail in regulating H3K4me3 was examined in greater detail at highly expressed genes in vivo. GAL1 is commonly used to study transcription-linked histone modifications. The level of H3K4me3 at GAL1 was examined in wild type and H2A tail mutant cells grown in either dextrose (repressed state) or galactose (activated state). Wild type cells displayed a ~ 20 fold higher level of H3K4me3 at GAL1 in cells grown in galactose versus dextrose (Figure 3-2A). Further, there was a 70% and 85% loss in trimethylation in the H2A Δ12-20 and H2A Δ4-20 mutants, respectively (Figure 3-2A). The reduction in H3K4me3 at GAL1 was somewhat greater than that observed in bulk chromatin by western blotting, but the trends among all four mutants are very similar. Interestingly, the ChIP assay revealed that the levels of K4me2 and K4me1 were significantly reduced in H2A Δ12-20 and H2A Δ4-20 mutants (Figure 3-2B and 3-2C). This is very different from what was observed in bulk chromatin. It appears as though H3K4me1 is not increased in the Δ4-20 mutant when the gene was activated, yet a small increase in both H3K4me2 and H3K4me3 was observed under the
same condition (compare panel C to panels A and B). This can be explained if the small increase in H3K4me1 in the Δ4-20 mutant is not detectable because of the overall lower level of this mark compared to the others or differences in the quality of the antibodies to each form of methylated K4 used in ChIP. Examination of H3K4me3 levels at GAL1 in H2A mutants with deletions in residues 4-12 and in the double K4,7G substitution, confirmed that the more C-terminal portion of the tail, residues 12-20, is primarily responsible for regulating H3K4me (Figure 3-2D).

Deleting the H2A tail did not affect the level of H3K36me in chromatin (Figure 3-1B), so we examined this mark at the 3’ end of the ORF of GAL1. The results show that deleting the tail had a very small affect of H3K36me3 levels; however, the reduction is barely statistically significant in the H2A Δ4-20 mutant and not significant in the H2A Δ12-20 mutant (Figure 3-2E).

To determine if the H3K4me defects are unique to GAL1, we analyzed H3K4me3 at another highly induced gene, RNR3. RNR3 is induced by the DNA damaging agent methyl methanesulfonate (MMS), and a strong increase in the levels of H3 K4me3 was detected at the promoter (Figure 3-2F). As observed at GAL1, deleting the H2A N-terminus greatly reduced the level of H3K4me3 at RNR3 (Figure 3-2F).

The levels of all three forms of K4me were significantly lower at GAL1 compared to that observed in bulk chromatin by western blotting. This is especially true of H3K4me2 and H3K4me1. This suggests that the H2A tail may be particularly important for methylating histones at the promoter of induced genes, versus constitutive genes or untranscribed regions of the genome. To test this, we analyzed H3K4me levels at PMA1
and PYK1, two well characterized, constitutively expressed genes. Deletion of the H2A tail resulted in a significant decrease in H3K4me3, and a modest decrease in K4me2 and K4me1 at both genes (Figure 3-2G, H, I). Among all three forms of H3K4me, trimethylation was reduced the most. Interestingly, the reductions in all three forms of K4me at PMA1 and PYK1 were not as dramatic as those observed at the activated GAL1 gene, and were closer in magnitude to that observed in bulk chromatin by western blotting.

RNAPII is required for COMPASS recruitment (Ng et al, 2003c; Pokholok et al, 2005), and it is possible that the H3K4me defect is caused by reduced amounts of RNAPII at the genes we examined. Others have shown that mutating the N-terminus of H2A leads to a slight depression of GAL1 under the repressed condition, but no detectable defect in activation when cells are grown in galactose for a long period of time (Durrin et al, 1991; Lenfant et al, 1996). Deleting the H2A tail led to a 6-7 fold derepression of GAL1 in raffinose, consistent with the results of others (Figure 3-3A, data not shown). Interestingly, while the steady state level of GAL1 mRNA was similar in the wild type cells and the mutants after prolonged growth in galactose, deleting the H2A tail impaired the activation kinetics of GAL1 (Figure 3-3A).

The ChIP assay was used to measure RNAPII levels at GAL1 in the mutants when the cells were grown overnight in galactose, which were the same conditions used to measure histone modification levels. RNAPII levels were indistinguishable among the wild type and mutant cells under these conditions, indicating the loss of H3K4me is not caused by reduced transcription (Figure 3-3B). Likewise, RNAPII levels were unaffected
at *PYK1* in the H2A mutants, but K4me3 levels were significantly reduced (Figure 3-2G and data not shown). The recruitment of COMPASS is dependent upon phosphorylation of serine 5 (Ser5p) in the heptad repeats in carboxyl-terminus of the large subunit of RNAPII (Ng et al, 2003c). Thus, we examined this modification in whole cells extracts and at *GAL1*. Figure 3-3C shows that the level of Ser5p is no less in the H2A tail mutants than in wild type cells. Furthermore, when we examined the level of Ser5p at *GAL1* by ChIP, there was no detectable difference between mutant and wild type cells (Figure 3-3D). Therefore, the loss of H3K4me is not caused by the lack of Ser5p or reduced RNAPII levels.

**Mutation of H2A tail impairs H2B ubiquitylation and disrupts H3K4me-H2BK123Ub crosstalk through changes in COMPASS composition**

The PAF complex (Paf1c) recruits COMPASS to chromatin and deleting *PAFI* or *CTR9* abolishes H3K4me3 and significantly reduces H3K4me2 (Krogan et al, 2003a; Ng et al, 2003c; Tenney et al, 2006; Warner et al, 2007). Diminished H3K4me levels could be caused by defective COMPASS or Paf1c recruitment; thus, the recruitment of these factors to active genes was examined. Both Cps60 and Paf1 are recruited robustly to the ORF of the *GAL1* gene in wild type cells grown in galactose medium (Figure 3-3E and F). Importantly, both were recruited to *GAL1* in the H2A tail deletion mutants (Figure 3-3E and F). There was a slight, but statistically insignificant, reduction in Cps60 recruitment to *GAL1* in both H2A mutants. Even if this small reduction is real, it cannot account for the very strong loss of H3K4me3 at *GAL1*. Thus, the reduced H3K4me levels cannot be
explained by a lack of Paf1c or COMPASS recruitment to genes in vivo.

Monoubiquitylation of lysine 123 on histone H2B (H2BK123ub1) by the Rad6/Bre1 complex is a prerequisite for histone H3K4 methylation (Briggs et al, 2002; Dover et al, 2002; Sun & Allis, 2002). This raises the possibility that the H2A tail may affect H2BK123ub1. The levels of H2BK123ub1 in the mutants were measured in bulk chromatin by western blotting using antiserum against histone H2B. The slower migrating band in Figure 3-4A represents the monoubiquitylated form of H2B (H2B-Ub), as its presence was dependent upon RAD6. Interestingly, the H2A Δ12-20 and Δ4-20 mutants have significantly less H2BK123ub1 compared to that observed in the wild type strain (compare lanes 1 versus 4 and 5), 60- and 75%, respectively. The reduction in H2BK123ub1 levels in the tail mutants is similar in magnitude to the decrease in H3K4me3 (compare Figure 1A to 4A). Only a weak reduction in H2BK123ub1 was detected in the H2A Δ4-12 mutant, which correlates also with the slight reduction in H3K4me3 in this strain. This, and the observation that COMPASS recruitment to genes is minimally affected in the H2A tail mutants, suggests that the loss of H3K4 methylation is due largely to a decrease in H2BK123ub1. It was surprising that we failed to see a significant reduction in H3K79me2 in the H2A mutants by western blotting (Figure 3-1B). It is possible that the residual H2BK123ub1 may be sufficient to maintain H3K79me2 levels.

The loss in H2B ubiquitylation in the H2A tail mutant may be caused by reduced recruitment of Rad6, the ubiquitin-conjugating enzyme for H2B. To test if the H2A tails are important for the recruitment of Rad6 to chromatin, we used ChIP analysis to study
the crosslinking of Rad6 to \textit{GAL1}. The recruitment of Rad6 was unaffected in both the H2A tail mutants compared to the wild type (Figure 3-4B). This result indicates that the N-terminal of H2A is not required for Rad6 recruitment, and suggests that it may be important for the activity of the Rad6/Bre1 complex.

Spt16, a subunit of the FACT complex, has been tied to histone H2B ubiquitylation during transcription elongation \textit{in vivo} and \textit{in vitro} (Belotserkovskaya et al, 2003; Fleming et al, 2008; Reinberg & Sims, 2006). FACT acts as an H2A/H2B chaperone \textit{in vitro} and associates with the dimer (Belotserkovskaya et al, 2003). It is possible that mutations to the H2A tail could impair FACT recruitment; therefore, this was examined at \textit{GAL1} during gene activation. Spt16 crosslinked robustly to the activated \textit{GAL1} gene in wild type cells, and importantly, its recruitment is not affected by deleting the H2A tail (Figure 3-4C).

It has been proposed that H2BK123ub1 regulates H3K4me3 by recruiting Cps35/Swd2 into COMPASS on chromatin because the amount of Cps35 in COMPASS is significantly reduced in \textit{rad6}\textDelta and H2B K123R mutants (Lee et al, 2007). However, an alternative model has been proposed that attributes the cross-talk to the Rad6-dependent ubiquitylation of Cps35/Swd2 and not Cps35 incorporation into COMPASS (Vitaliano-Prunier et al, 2008). We next examined if the reduced H3K4me3 in the H2A mutants is linked to the disruption of the pathway controlling the association of Cps35 with COMPASS. COMPASS was purified from wild type cells and the H2A \textDelta4-20 mutant by tandem affinity purification (TAP) using a tagged version Cps60. The composition of the complex and stoichiometry of each subunit was analyzed by
SYPRORuby staining. When normalized to the amount of Cps60-TAP in the preparations, the amount of Cps35 in COMPASS isolated from the H2A mutant strain was more than three-fold lower than that isolated from wild type cells (Figure 3-4D). The approximately 70% reduction in Cps35 incorporation correlates well with the 75% loss of H2BK123ub1 observed in the H2A Δ4-20 strain. To determine if there is a difference in Cps35 total protein level between the wild type and mutant, western blotting was performed using Cps35-Myc tagged strains. Deletion of the H2A tail leads to about 60% reduction of Cps35 in the H2A tail deletion mutant (Figure 3-4E). This may reflect a defect in Cps35 protein synthesis or stability when it is unable to be incorporated into the COMPASS complex. Together, these data provide a separate line of evidence indicating that deletion of the N-terminal tail of H2A reduces H2BK123ub1, and disrupts H3K4-H2BK123ub1 crosstalk by affecting the incorporation of Cps35 into COMPASS. It also confirms previous results that H2BK123ub1 regulates the incorporation of Cps35 into COMPASS.

**Increasing H2BK123ub1 suppresses the H3K4 methylation defect and impaired GAL1 induction in the H2A tail mutants**

The data obtained so far suggest that the H3K4me defect results from reduced H2BK123ub1 levels, but it is also possible that the H2A tail is required for both modifications independently. To provide further evidence that the H2A tail regulates H3K4me through the H2BK123ub pathway, we tested if increasing the level of H2BK123ub1 genetically could suppress the H3K4me defect. Ubp8 and Ubp10 are two deubiquitylases that remove ubiquitin from H2B (Daniel et al, 2004; Gardner et al, 2005;
Geng & Tansey, 2008; Henry et al, 2003). It has been shown that deleting \textit{UBP8} or \textit{UBP10} individually partially increased H2BK123ub1 due to redundancy, but deleting both strongly increased H2BK123ub1 levels \textit{in vivo} (Geng & Tansey, 2008). The genes encoding the Ubp8 and Ubp10 proteins were deleted in the wild type and the H2A tail mutant background, and the levels of H2BK123ub1 and H3K4me3 were analyzed by western blotting. As expected, the \textit{ubp8/10\Delta} double mutants displayed increased H2BK123ub1 levels in both the wild type and H2A mutant strains (Figure 3-5A, lanes 4-6). Furthermore, deleting \textit{UBP8} and \textit{UBP10} significantly increased H3K4me3 levels in the H2A tail mutants, almost fully restoring the levels of this modification to that observed in wild type cells (Figure 3-5A compare lanes 2 and 3 to lanes 5 and 6 for H3 K4me3).

Next, we tested if restoring H3K4me and H2BK123ub1 levels in the H2A \textit{\Delta}4-20 mutant has consequences on gene expression by analyzing the timing of \textit{GAL1} induction. As shown in Figure 2A, the induction of \textit{GAL1} was delayed in the H2A tail deletion mutant (Figure 3-5B). Deleting both \textit{UBP8} and \textit{UBP10} accelerated \textit{GAL1} induction in the wild type background, but the maximum level of \textit{GAL1} mRNA was similar to that of a wild type strain by 90 minutes (Figure 3-5B). Importantly, deleting \textit{UBP8} and \textit{UBP10} suppressed the slow \textit{GAL1} induction phenotype observed in the H2A \textit{\Delta}4-20 mutant, and \textit{GAL1} mRNA accumulated to wild type levels within 90 min (Figure 3-5B). Thus, the data indicates that the H2A tail regulates H3K4me through the Rad6-H2BK123ub pathway and suggests that the \textit{GAL1} activation defect in the H2A tail mutant is caused, at least in part, by reduced H2BK123ub1 and H3 K4me.
The HAR domain of H2A tail regulates H2BK123ub1

To narrow down the region within H2A required for H2BK123ub1 and H3K4me, we screened additional mutants with smaller deletions or point mutations within the N-terminal tail. Since residues between 12-20 are especially important for controlling H3K4me levels, we focused on this region. A subdomain in the H2A tail between residues 16-20, referred to as the H2A repression domain (HAR), has been identified (Parra & Wyrick, 2007). Interestingly, mapping the HAR on the x-ray crystal structure of the nucleosome revealed that it is located next to K123 of H2B (Figure 3-6A). The levels of H3K4me3 and H2BK123ub1 were examined in strains containing a more precise deletion, Δ16-20, or the HAR domain. Residues S17 and R18 were identified as being particularly important in HAR function, so the levels of histone modifications were examined in a double H2A S17R18A mutant as well. The results in Figure 3-6B clearly show that the Δ16-20 mutation reduces both H2BK123ub1 and H3K4me3 in bulk chromatin similar to that caused by deleting the majority of the H2A tail, Δ4-20. The amount of both H2BK123ub1 and H3K4me3 was reduced in the double point mutant (S17R18A) also, albeit not to the same level of that observed in the HAR deletion mutant (Δ16-20).

Finally, we further confirmed that the HAR is required for H3K4me3 by using the ChIP assay to monitor this modification at GAL1. As expected, the Δ16-20 and S17R18A mutations reduced H3K4me3 at GAL1, which did not result from lower levels of RNAPII at the promoter when the cells were grown overnight in galactose (Figure 3-6C and 6D).

Taken together, our study has uncovered a novel role for the HAR domain of H2A in mediating a trans-tail regulation of H2BK123ub1 and H3K4me.
Discussion


A few trans tail histone modification pathways have been described in eukaryotes. In *S. cerevisiae*, the Bre1/Rad6 ubiquitin ligase complex is required for methylation of histone H3K4 and H3K79 (Dover et al, 2002; Jeltsch & Rathert, 2008; Nakanishi et al, 2009; Shahbazian et al, 2005). Additionally, Dot1, which methylates histone H3 at lysine79, requires three basic residues (R17H18R19) on the tail of histone H4 for its binding and activity (Altarf et al, 2007; Fingerman et al, 2007). Here, we are the first to report that the N-terminal tail of histone H2A, the HAR domain specifically, regulates monoubiquitylation of K123 on H2B, and subsequently, H3K4me (Figure 3-7). In addition to the molecular analysis presented here, the H2A-H2B-H3 axis is also supported by common phenotypes of the H2A tail deletion mutants and strains defective for either H2BK123ub1 or H3K4me. HAR mutants are sensitive to DNA damaging agents, similar to *rad6Δ* and *set1Δ* strains (Giannattasio et al, 2005; Huang et al, 1997; Parra & Wyrick, 2007; Sun & Allis, 2002; Venkatasubrahmanyam et al, 2007). The overlap in the genes whose expression changes in H2A tail mutants and a *set1Δ* mutant and a H2B K123R is clear, and correlate best with genes that are derepressed in the mutants (data not shown). This reinforces other studies showing that the HAR is important in gene repression (Lenfant et al, 1996; Parra & Wyrick, 2007). Even though genome-wide expression studies identified the H2A tail as primarily playing a role in repression, proper timing of induction of *GAL1* is dependent upon the H2A tail,
H2BK123ub1 and H3K4me (this study and (Carvin & Kladde, 2004; Shukla & Bhaumik, 2007)). This suggests that the activation of some genes is also dependent upon the H2A-H2Bub-H3K4me pathway. The role of the HAR in activation may have been obscured because previous global gene expression studies were conducted under conditions that measured constitutively expressed genes. The results we obtained indicate that the H2A tail is particularly important for the induction phase of genes and that the defects in H3K4me are strongest at highly induced genes such as GAL1 and RNR3 compared to the rest of the genome (Figure 3-2).

It is striking that the HAR domain is adjacent to K123 of H2B in the nucleosome (Figure 3-6A). The proximity of the HAR domain and H2BK123 on the nucleosome structure raises the possibility that these residues form a docking site for the Bre1/Rad6 ubiquitin ligase and/or the Cps35 subunit of COMPASS. While the data presented here shows that the H2A tail is not required for Rad6 or COMPASS recruitment, it is possible that the ubiquitylation machinery is recruited to the gene through Paf1c-RNAPII or other factors in the absence of the H2A tail. The HAR and H2B C-terminal tail may be the site where Bre1/Rad6 directly interacts with nucleosome. Alternatively, the HAR domain may stimulate the ubiquitin ligase activity of the Bre1/Rad6 complex, in a post-recruitment manner. Similarly, Paf1c is believed to stimulate Rad6 activity because Paf1c deletion mutants display reduced H2BK123ub1, yet Rad6 is recruited in these mutants (Xiao et al, 2005). We have tried different methods to detect an interaction between Rad6/Bre1 and nucleosomes in an attempt to test this mechanism, but we were unable to observe a stable interaction (not shown). Indeed, although the binding of Rad6 or Bre1 to histone
substrates has been demonstrated, a stable interaction between Bre1 or Rad6 and nucleosomes has not been reported (Kim & Roeder, 2009; Robzyk et al, 2000).

Histone H2A mutants were identified that reduced H3K4me in a screen of a comprehensive library of histone mutants (Nakanishi et al, 2008). Alanine substitutions in E65, L66, N69, or D79 reduce the di- and trimethylated forms of H3K4. Of these, only the L66A mutant reduced H2BK123ub1. These residues form a patch on the surface of H2A, but are relatively distant to the HAR domain, and unlike the HAR domain, are exposed on the surface of the nucleosome. These mutants were only screened for histone modification levels in bulk chromatin and it is unknown if they affect the recruitment of the remodeling enzymes. Thus, the relationship and functional similarities between the H2A acidic patch identified in this screen and the HAR is unclear. Single alanine substitutions within the HAR domain were not identified in the screen as affecting H3K4me. This is not surprising as the S17AR18A double point mutant analyzed here reduced H3K4me3 by 50% (Figure 3-6B). A single mutation in the HAR may not lead to a significant enough reduction in H3K4me to be identified in the GPS screen (Global Proteomic Screen, (Nakanishi et al, 2008).

H2B ubiquitylation is known to be required for both histone H3K4 and H3K79 di-, and trimethylation (Lee et al, 2007; Sun & Allis, 2002). Deletion of HAR impairs H2B ubiquitylation and hence H3K4 di-, and trimethylation. However, we did not observe a defect in H3K79 methylation in the HAR mutant in our analysis. It is possible that the low level of ubiquitylation present in the mutant is enough to support the normal level of K79 methylation. Mechanistic studies on how H2Bub1 stimulates H3K79 methylation
suggest that the ubiquitylation mainly affects the *in vivo* chromatin substrate such that H3K79 on the nucleosome core becomes more accessible for interaction with Dot1 (Frederiks et al, 2008). Unlike the H2BK123R or *rad6Δ* mutants which completely erased ubiquitylation, the diminished H2Bub1 in the H2A tail mutants might still be able to keep the chromatin in an “opened” status to allow the di-, and trimethylation of H3K79.

**Does the HAR play a role in restricting H2B ubiquitylation and H3K4me to nucleosomes modified by transcription?**

The N-terminal tail of histones H2A and H2B are the least conserved among the four core histones. As noted previously (Parra & Wyrick, 2007), the residues within the HAR are the exception. The HAR is very well conserved, suggesting that this regulatory pathway is utilized across the eukaryotic kingdom. It is tempting to speculate that modifications to the H2A tail regulate H2BK123ub1 and H3K4me, but there are no known modifications to the HAR domain and the only two residues known to be modified in H2A, K4 and K7, apparently play a minor role at best according to our data (Figure 3-1A). While some residues in the HAR are exposed on the surface of the nucleosome, others lie underneath the DNA and arginine 18 makes contact with the minor groove of nucleosomal DNA ((Parra & Wyrick, 2007) and Figure 3-6). Since R18 is critical for HAR repression activity (Parra & Wyrick, 2007) and H2BK123ub1 (this study), residues required for the H2A-H2B crosstalk may be inaccessible in the intact nucleosome. Thus, the recognition of the HAR-H2B interface by histone modifying enzymes may require transcription-dependent nucleosome remodeling, thereby linking histone ubiquitylation
and methylation to transcription (Figure 3-7). Human FACT (FAcilitates Chromatin Transcription) is required for H2Bub1 \textit{in vitro}, and recently yeast FACT was shown to be required for H2Bub1 \textit{in vivo} (Belotserkovskaya et al, 2003; Fleming et al, 2008; Pavri et al, 2006). The regulation of H2BK123ub1 by FACT, a histone chaperone/remodeling complex, suggests that Bre1/Rad6 requires the disruption of the nucleosome during transcription to modify H2B. Why ubiquitylation of K123 requires nucleosome disruption was not clear because the H2B C-terminal tail is exposed and, in principle, should be accessible to the ubiquitylation machinery. Our data argue that the exposure of the HAR may be required for the ubiquitylation enzymes to recognize the nucleosome. This would restrict H2BK123ub1 to nucleosomes with an HAR made accessible by transcription and/or chromatin remodeling activities. Conversely, reassembly of nucleosomes by chaperonees such as FACT after the passage of RNAP II, would block accessibility of the HAR to the ubiquitylation machinery and allow for the de-ubiquitylases to return the chromatin to the original state until another round of transcription starts the process over again. Therefore, the highly conserved HAR domain is important for maintaining the proper timing and localization of H2Bub1 and other histone modifications.
Acknowledgements

We thank members of the Reese Lab and the Center for Eukaryotic Gene Regulation at Penn State for advice and comments on this work. We are grateful to James Psathas for comments and initial observations suggesting a link between the H2A tail and H3 K4 methylation. We thank Dr. John Wyrick for providing the histone H2A mutant strains and performing genome wide gene expression analysis in the H2A tail deletion mutant. This research was supported by funds from National Institutes of Health (GM58672) to J.C.R.
Figure 3-1. The N-terminal tail of histone H2A is required for global H3K4 methylation

(A) Western blotting of whole cell extracts prepared from the wild type and mutant strains using the antibodies described in the panel. The asterisk in the H3K4me2 blot designates a non-specific band also present in set1Δ control. Histone modification levels were normalized to the amount of histone H3 on each blot (H3 core). Numbers below each panel are the levels of modification in the mutants relative to the wild type cells, which was set at 100.

(B) Same as in “A” except blots were probed for H3K36me3 and H3K79me2. The asterisk designates a non specific band.
Figure 3-2. ChIP analysis of histone methylation in H2A mutants.

(A-C) ChIP analysis of H3K4 methylation at the 5’ end of GAL1 gene. Wild type and mutant cells were grown to log phase in media containing 2% dextrose (Dex, white bars) or 2% galactose (Gal, black bars), and chromatin was precipitated with antibodies to the modified histone. The Student’s t-test was used to determine significance between the modification levels in mutant and wild type cells under the induced condition. The level of significance is marked by asterisks in each panel (* , p<0.05; **, p<0.01, ***, p<0.005). (A) H3K4me3, (B) H3K4me2 and (C) H3K4me1. The methylation signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. Cells were grown in galactose for 16 hours.

(D) ChIP analysis of H3K4me3 at the 5’ end of GAL1 gene in the wild type, H2A K4,7G and H2A Δ4-12 mutants.

(E) Levels of H3 K36me3 at the 3’ ORF of GAL1.

(F) H3 K4me3 at the promoter of the DNA damage inducible RNR3 gene. The gene was induced by treating cells with 0.03% MMS for 2.5 hours prior to crosslinking (black bars).

(G-I) ChIP analysis of H3K4 methylation at the 5’ end of PMA1 and PYK1 genes. Same as in (A-C) except that the cells were grown in media containing 2% dextrose.
A

B

C

D
Figure 3-3. Characterization of transcription factor recruitment to *GAL1* in H2A mutants.

(A) Northern blot of *GAL1* mRNA. Wild type (solid line, triangles), H2A Δ12-20 (dashed line, diamonds) or H2A Δ4-20 (dotted line, squares) strains were grown to log phase in media containing 2% raffinose and induced with 2% galactose for the times indicated in figure. For the overnight induction (O/N), cells were grown to log phase in medium containing 2% galactose. The levels of RNA were normalized to the signal of *scR1*, a loading control.

(B) RNAPII crosslinking to the 5' ORF of *GAL1*. 8WG16 was used to IP chromatin in cells grown in dextrose (Dex, white bars) or galactose (gal, black bars). ChIP was performed as described in the legend of figure 3-2. Cells were grown in galactose for 16 hours.

(C) Western blot analysis of RNAPII Ser5 phosphorylation levels in whole cell extracts. As a control for the selectivity of the antibody, extract from the wild type cells was treated with lambda phosphatase (WT pptase) in lane 5. The extracts were also probed with antibody against Rpb3 to control for the amount of RNAPII.

(D) Same as (B) except measuring the crosslinking of Ser5 phosphorylated RNAP II (H14 antibody) over *GAL1*.

(E-F) ChIP analysis of Cps60-myc (E) and Paf1-myc (F) recruitment to the 5’ ORF of *GAL1*. 

![Graphs showing data](image-url)
A

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B

![Graph showing Rad6-Myc % IP with Dextrose and Galactose]

C

![Graph showing Spt6-Myc % IP with Dextrose and Galactose]

D

![Marker gel with Set1, Cps60, Cps50, Cps40, Cps35, Cps30, Cps25]

Cps35/Cps60 = 0.36 0.11
Figure 3-4. The N terminus of H2A regulates H2B K123 monoubiquitylation (H2BK123ub1) and Cps35 association with COMPASS.

(A) Western blot analysis of H2B in chromatin. The experiment was performed and quantified as described in the material and methods section and in the legend of figure 3-2. Monoubiquitylated form of H2B is marked (H2B-Ub).

(B) and (C) Rad6 (B) and Spt16 (C) crosslinking to the 5’ end of GAL1.

(D) COMPASS complex was purified from wild type and H2AΔ4-20 mutant cells thorough the Cps60 subunit. Preparations were analyzed by SDS-PAGE followed by SYPRORuby stain. The locations of identifiable COMPASS subunits are indicated on the right. The relative amount of Cps35 subunit in each complex was calculated by dividing the intensity of Cps35 band by Cps60 band and is indicated below the panel.

(E) Western blot analysis of Cps35 protein level in wild type and H2AΔ4-20 mutant. Whole cell extracts were probed with 9E10 antibody to measure the Cps35-Myc protein level. The gradient indicates a decreasing amount of extract loaded onto the gel. The same blot was also probed with antibody against TBP to control for the amount of total protein level.
Figure 3-5. Increasing H2B ubiquitylation in the H2A tail mutants restores H3K4 methylation and rescues the slowed induction of \textit{GAL1}.

(A) Western blot analysis of H2B monoubiquitylation and H3K4me3 in chromatin. The experiment was performed as described in the legend of figure 3-1. The lighter exposure of the H2B and the H3 blots were used as a loading control.

(B) Northern blot of \textit{GAL1} mRNA levels in wild type, \textit{ubp8/ubp10}\(\Delta\) and triple H2A \(\Delta4\)-20/\textit{ubp8/ubp10}\(\Delta\) mutants.
Figure 3-6. The histone H2A repression (HAR) domain accounts for the major function of the H2A tail in regulating H2BK123ub1 and H3K4me3.

(A) Location of the histone H2A HAR domain (red) and H2B K123 (blue) in the yeast nucleosome structure. The figure was drawn using POLYVIEW.

(B) Western blot analysis of H2B monoubiquitylation and H3K4 methylation in whole cell extracts of wild type and mutant strains indicated in the panel.

(C) ChIP analysis of H3K4 methylation at the 5’ end of GAL1 gene in wild type and HAR mutants. Cells were grown in galactose for 16 hours. The Student’s t-test was used to determine significance between the modification levels in mutant and wild type cells under the induced condition. The level of significance is marked by asterisks in each panel (*, p<0.05; **, p<0.01).

(D) ChIP analysis of RNAPII at the 5’ end of GAL1 gene.
Figure 3-7. H2A trans-tail regulation of H2BK123ub1-H3K4me
Model for the regulation of histone modifications by the HAR. Transcription by RNAPII through the nucleosome exposes the HAR to allow for Rad6/Bre1 ubiquitylation of K123 of H2B.
Chapter 4

A CONSERVED REGION ON THE HISTONE H2B TAIL IS CENTRAL FOR HISTONE CHAPERONE ACTIVITY
Abstract

Transcription through a chromatin template necessitates the involvement of multiple factors facilitating the progression of polymerase. The histone chaperones are essential component of the transcription machinery used to overcome the nucleosomal barrier. Among them, FACT (FAcilitates Chromatin Transcription) was the first histone chaperones to be identified which possesses an activity to partially disassemble chromatin and facilitate the progression of polymerase. However, little is known about how FACT interacts with nucleosomes and how these interactions contribute to the function of chaperones. Here we identified a conserved region on the histone H2B tail, known as the H2B Repression Domain (HBR), which regulates the activity of histone chaperone FACT. HBR is not required for FACT binding to chromatin, but it is essential for FACT to interact with the H2A/H2B dimer. Deleting HBR, or the H2B tail, impairs FACT mediated chromatin remodeling, transcription activation and the redeposition of histone following the passage of polymerase. Therefore, in contrast to its known role in repressing gene expression, our study suggests a novel function of HBR in regulating histone chaperone activity and transcription activation.
Introduction

Eukaryotic gene expression can be regulated at multiple steps during the transcription cycle, including chromatin remodeling, recruitment of general transcription factors, elongation and termination (Buratowski, 2009; Lee & Young, 2000; Svejstrup, 2004). The packaging of eukaryotic DNA into nucleosomes severely hampers the progression of RNAP II. As a result, numerous chromatin remodeling factors associate with polymerase and facilitate its progression through chromatin template (Cairns, 2005; Kulaeva et al, 2007; Li et al, 2007a; Workman, 2006). Histone chaperones, such as FACT (facilitates chromatin transcription), Spt6, and Asf1, possess activity to disassemble and reassemble chromatin (Eitoku et al, 2008; Formosa, 2008; Park & Luger, 2008). The roles of histone chaperones in regulating transcription have been of great interest in recent years.

The FACT complex, consisting of Spt16/Pob3, was first identified as an activity which facilitates transcription on a chromatin template in a highly purified system (Orphanides et al, 1998). It facilitates transcription initiation by promoting TBP binding to TATA box in a TFIIA-dependent manner (Biswas et al, 2005; Mason & Struhl, 2003). Depletion of Spt16 strongly affects the transcription of a group of genes which exhibit well positioned nucleosomes at their 5’end; while the genes bearing random nucleosome structures are only mildly influenced, highlighting its role in chromatin remodeling and transcription activation (Jimeno-Gonzalez et al, 2006).

During elongation, FACT facilitates the passage of polymerase by destabilizing nucleosomes. Initial studies proposed that FACT displaces one H2A/H2B dimer from the
nucleosome to form a hexasome, therefore partially disassembling chromatin
(Belotserkovskaya & Reinberg, 2004; Orphanides et al, 1998; Orphanides et al, 1999; Reinberg & Sims, 2006). A recent study suggests that in addition to H2A/H2B displacement, FACT may also reorganize nucleosomes into their original composition but looser and more dynamic in structure (Xin et al, 2009). A reciprocal regulation between FACT and H2B ubiquitylation has been described; both stimulate elongation on a chromatin template and facilitate the generation of longer transcripts (Fleming et al, 2008; Hartzog & Quan, 2008; Pavri et al, 2006). In the wake of transcribing polymerase, FACT positions histones back onto chromatin and to restore normal nucleosome structure.

Inactivating histone chaperones, such as FACT and Spt6, lead to the activation of cryptic initiation sites within coding region of genes and the generation of intragenic transcripts (Kaplan et al, 2003; Mason & Struhl, 2003). Therefore, both the chromatin disassembly and reassembly functions of FACT are crucial for the efficiency and accuracy of transcription.

The many roles that FACT plays during transcription all stem from its histone chaperone activity. Biochemical and structural studies suggest that FACT binds to nucleosomes and destabilizes the interactions between H2A/H2B dimers and H3/H4 tetramers (Belotserkovskaya et al, 2003; Reinberg & Sims, 2006). The acidic C terminal domain of Spt16 binds the histone H2A/H2B dimer, while its N terminus interacts with H3/H4 tetramer (Belotserkovskaya et al, 2003; Stuwe et al, 2008; VanDemark et al, 2006; VanDemark et al, 2008). Pob3 also contacts with the H3/H4 tetramer (Belotserkovskaya et al, 2003). Within the nucleosome, FACT has binding sites on the H3 and H4 N-terminal
tails and a “docking domain” on the C-terminus of H2A (VanDemark et al, 2008). Despite these advances, little is known about how FACT recognizes the H2A/H2B dimer and how these interactions contribute to its chromatin remodeling activity.

The histone N terminal tails protrude from the nucleosome surface and play multiple roles in regulating gene expression. Previously, it has been suggested that the H2B N-terminal domain is principally required for the transcription repression of a large subset of the yeast genome. The H2B repression (HBR) domain, comprised of residues 30 to 37, is necessary and sufficient for this function (Parra et al, 2006). We set out to study the mechanism of how HBR, or the H2B tails, represses transcription. To our surprise, our data indicates that the HBR, or the H2B tail, is also required for the transcription activation of multiple genes examined. These observations led us to investigate known events associated with active transcription and we found that the HBR is essential for the activity of FACT. Here we provide evidence to show that HBR, or the H2B tail, critically mediates the interaction between FACT and the H2A/H2B dimer. Deleting HBR, which impairs the binding of FACT to H2A/H2B dimer, leads to defects in chromatin remodeling at the promoter, reduces transcription, and hampers the restoration of chromatin structure after the passage of polymerase. The partial occlusion of HBR by nucleosomal DNA suggests that the changes in nucleosome structure in the absence of HBR might be the major cause of chromatin remodeling and assemble defect. Taken together, we propose that in addition to its known roles in repression, the HBR may play more important roles in activating transcription through regulating the function of histone chaperones.
Results

The H2B Repression domain (HBR) is required for the transcription activation of subsets of genes

Previous gene expression profiling of the H2B tail mutants identified a functional region within the H2B tail known as H2B repression domain (HBR) (Lenfant et al, 1996; Parra et al, 2006). Consisting of amino acids 30 to 37, HBR has been shown to repress the transcription of groups of gene in the yeast genome. However, the mechanism by which HBR represses transcription, and whether it has other roles in gene regulation remains elusive. The GAL1 gene was used as a model to study the role of HBR in transcription since changing the carbon source can easily control the activation and repression of this gene. The basal level of GAL1 transcription was examined in strains harboring deletion of the majority of the H2B tail (H2B Δ1-32), deletion of HBR (H2B Δ30-37), or the entire tail (H2B Δ3-37). Deleting different regions of the tail, or the entire tail led to ~2-3 fold derepression of GAL1 in raffinose (Figure 4-1A), consistent with the role of H2B N-terminus in repressing basal, uninduced transcription. We next examined the activation of GAL1 by adding galactose to the medium and measuring GAL1 transcription level at different time points following induction. In the wild type cells, there was a progressive accumulation of transcripts within 90min of induction, and the same increase was also observed in the H2B Δ1-32 mutant. Surprisingly, deleting either HBR or the entire H2B tail diminished GAL1 transcription to only 60% or 40% of the wild type level within 90min of induction, suggesting that the HBR is required for the normal level of induced GAL1 expression (Figure 4-1B).
To determine if the HBR may be specifically required for the fast induction of genes, we examined the mRNA level of *PMA1* and *PYK1*, two constitutively expressed genes that have been used extensively as models to study transcription. Deleting HBR resulted in 50% reduction in *PMA1* transcription comparing to the wild type. There was a small yet reproducible decrease in *PYK1* mRNA level (Figure 4-1C). Therefore, it seems that the H2B “Repression Domain” may also regulate the transcription activation of a subset of genes.

Posttranslational modifications of histone proteins are tightly linked to transcription, and different modifications “landmark” distinct stages of the transcription cycle. Since we have observed a decrease in transcription at several genes, we wonder if HBR could potentially regulate any of the histone modifications. Multiple histone modifications were examined in the H2B tail deletion mutants. Deleting different regions of the tail (H2BΔ1-32 and H2BΔ30-37), or the entire H2B tail, did not affect histone H3 or H4 acetylation (Figure 4-1D). However, deleting HBR, or the entire tail, significantly reduced the histone methylation modifications we examined, including H3K4me2, H3K4me3, H3K36me3 and H3K79me2, while deleting the region before HBR (H2BΔ1-32) had no effect (Figure 4-1D). These results are consistent with the transcription defect we have observed in the HBR and tail deletion mutant, and suggest that the HBR is of particular importance in H2B tail mediated function; In addition, the decrease in transcription and histone modifications in the HBR deletion mutant strongly point to a role of HBR in transcription activation.

To explore which steps in the activation of *GAL1* were affected by the HBR or
H2B tail deletion mutations, we first examined the preinitiation complex (PIC) formation by measuring the recruitment of TBP to GAL1 promoter. We focused on the two mutants, H2BΔHBR and H2BΔ3-37, which showed reduced transcription and histone modifications. Upon addition of galactose, there was a gradual increase in TBP occupancy at GAL1 promoter in the wild type strain, reaching ~7 folds greater than the basal level within 90 min of induction. Deleting HBR, or the H2B tail, resulted in a large decrease in TBP recruitment to GAL1 promoter. The crosslinking reached only 60% and 25% that of the wild type cells and correlated well with the decrease in mRNA level (Figure 4-1E), suggesting that the transcription defects in the HBR deletion mutant occur during transcription initiation.

**HBR domain promotes chromatin disassembly during initiation**

An early event in transcription initiation is the remodeling of chromatin. Promoter nucleosomes are evicted during activation to allow access to DNA binding proteins, coactivators, and the general transcription machinery to the DNA. We next examined chromatin remodeling in the wild type and H2B tail mutants upon activation. Nucleosome density was examined by ChIP analysis using antibodies to core histones. There was a comparable level of histone H2A and H3 crosslinked to GAL1 promoter in the wild type and H2B tail mutants in the non-inducing condition (Raffinose). An activation dependent decrease in histone H3 and H2A density was detected over the promoter in wild type cells, and more than 80% of the nucleosomes are removed at the end of 90min (Figure 4-2A and 4-2B). Significantly, deleting HBR, or the H2B tail, resulted in the dwelling of
nucleosome over GAL1 promoter, and the final nucleosome density is 2-3 folds higher than that in the wild type cells, suggesting that HBR deletion impairs the removal of promoter nucleosomes during activation.

Removal of promoter nucleosomes is carried out by chromatin remodelers. The reduced nucleosome eviction in the ΔHBR may be caused by a delay in the recruitment or slowed activation of remodelers to “open up” the promoter; while prolonged growth in galactose may eventually allow the removal of most nucleosomes from the mutant promoter and the final nucleosome level may end up comparable to that in the wild type cells. To determine if the nucleosomes persisting over the GAL1 promoter could be removed after prolonged activation, we grew the cultures in dextrose and galactose overnight and the nucleosome density in the repressed and activated conditions were analyzed. Growing cultures in galactose led to a dramatic decrease in H2A and H3 crosslinking comparing to the dextrose condition in the wild type cells; However, in the HBR deletion mutants, the H2A density was approximately 2 folds higher, and the H3 density was 4-6 folds higher than that in the wild-type cells after prolonged activation (Figure 4-2 C and D), implying that the remodeling defect we have observed in the mutant is more likely due to an impaired enzymatic activity, rather than a delay in the recruitment or activation of the chromatin remodelers. Likewise, nucleosome occupancy at the two constitutively activated genes, PMA1 and PYK1, were also higher in the mutants. These results support the conclusion that the HBR is important for the disassembly of nucleosomes at promoter, which is essential for the transcription activation of a large number of genes.
The HBR domain is dispensable for the recruitment of histone chaperones and chromatin remodelers

The ATP dependent chromatin remodelers use energy derived from ATP hydrolysis to enhance the accessibility of nucleosomal DNA or change the histone composition of nucleosomes. Among them, the SWI/SNF complex has been shown to participate in chromatin remodeling at GAL1 and is required for its transcription activation and memory. It is possible that the HBR plays a role in the recruitment of SWI/SNF to genes. The cultures were grown in dextrose (repressed) and galactose (activated) overnight, and the steady level of Swi/Snf recruitment was examined. In the wild type cells, Swi2p crosslinking increased ~10 fold upon induction of transcription at GAL1 promoter, consistent with its role during activation. Neither the HBR deletion nor deleting the entire tail led to any defect in Swi2 recruitment (Figure 4-3A), indicating that the HBR is dispensable for the recruitment of chromatin remodelers, but it may be important for the activity of the remodeling complex.

Histone chaperones enhance the accessibility of DNA sequence in chromatin through partially disassembling the nucleosome. The FACT complex, consisting of Spt16/Pob3, shows strong genetic interaction with basal transcription factors, and promotes TBP binding to TATA box within a chromatin template both in vitro and in vivo (Biswas et al, 2005). Since FACT functions as an H2A/H2B chaperone, it is possible that the N-terminal tail of H2B provides a docking site for FACT to associate with chromatin. The crosslinking of Spt16-myc to GAL1 promoter was examined in cultures grown in either dextrose or galactose. There were no defects in the recruitment of FACT in the HBR
deletion mutants (Figure 4-3B). As a comparison, we analyzed the crosslinking of TBP to the same region as indications of PIC formation. In contrast to SWI/SNF and FACT, TBP recruitment was reduced to about 50% of the wild type level in the HBR deletion mutant, again indicating a defect in PIC formation (Figure 4-3C). This observation is consistent with the view that general transcription factors bind to promoter after chromatin remodeling. Since both chromatin remodelers and histone chaperones can to be recruited to genes in the HBR deletion mutant, these results suggest that the HBR may regulate their activity at a post-recruitment step.

The HBR is essential for the reassembly of nucleosome after the passage of polymerase

Besides their roles in disassembly of nucleosomes, histone chaperones are also implicated in restoration of normal chromatin structure following the passage of polymerase, preventing intragenic transcription. Inactivating FACT (histone H2A/H2B chaperone), or Spt6 (histone H3/H4 chaperone), results in the generation of shorter transcripts within certain genes, such as \textit{FLO8} and \textit{FMP27} (Kaplan et al, 2003; Mason & Struhl, 2003). Although the binding of histone chaperones to genes has been demonstrated in the mutants, it is possible that HBR deletion affects their activity at a post-recruitment step. To test if FACT reassembles nucleosome properly in a HBR deletion mutant, we examined the RNAs produced from \textit{FLO8} in the HBR deletion and wild type. Inactivation of the conditional \textit{spt16-197} mutant resulted in the appearance of short \textit{FLO8} transcripts, consistent with its known role in maintaining proper chromatin
structure in the wake of transcription. Significantly, this phenotype was also observed in
the HBR or H2B tail deletion mutants, providing evidence that the HBR is required for
the activity of histone chaperones (Figure 4-3D). In addition, it is known that SET2 (the
methyltransferase of H3K36) or CTK1 (the RNAPII CTD Ser2 kinase required for Set2
activity) mutants also generate intragenic transcription independent of the histone
chaperone pathway. Set2 mediated H3K36 methylation recruits the Rpd3S histone
deadacetylate complex to deacetylates nucleosomes within ORFs of genes, resetting
chromatin to an organized structure. We examined the FLO8 transcripts in set2Δ, and
ctk1Δ mutants as well and both showed cryptic transcription (Figure 4-3D). Importantly,
the patterns of the short transcripts generated from the set2Δ or ctk1Δ mutants were
distinct from the ones generated in the H2B tail or FACT mutants. This is consistent with
previous reports (Cheung et al, 2008) showing that histone chaperones and H3K36
methylation work in parallel pathways to prevent cryptic transcription, and strongly
suggests that HBR modulates chromatin structure through the histone chaperone pathway.

To determine if the generation of the cryptic transcripts at FLO8 was due to an
inability to recruit FACT to this locus, we analyzed the crosslinking of Spt16 to FLO8
promoter, as well as a functionally optimal TATA element lying just upstream of the
cryptic transcripts (Kaplan et al, 2003). Deletion of HBR did not affect the recruitment of
FACT and there was the same amount of the chaperon crosslinked to the promoter and
ORF. Interestingly, there seems to be more FACT recruited to both loci in the H2B tail
deletion mutant (Figure 4-3E). It is possible that in the wild type cells, FACT remodels
nucleosome and leave; whereas in the tail deletion mutant, FACT binds to nucleosome but
is unable to remodel it, therefore causing it to interact with nucleosome for a longer time.

We have also measured the TBP and polymerase recruitment at both sites. In the wild type cell, RNAP density was equal at promoter and the internal cryptic initiation site, while TBP crosslinking was reduced to half in the coding region. In the HBR deletion mutants, the RNAPII and TBP level were comparable to that of the wild type at FLO8 promoter. However, TBP and polymerase occupancy over the cryptic TATA sequence (TATAAA +1625 - +1631) were about 2 fold, and 1.5 fold higher than the wild type strain (Figure 4-3F and G), consistent with the internal initiation within coding region.

We confirmed the HBR deletion induced cryptic transcription in another gene, FMP27. A previous investigation reported that inactivating Spt16 led to intragenic transcription from this 8kb long gene (Mason & Struhl, 2003). FMP27 transcripts were probed in the FACT and HBR deletion mutants as well as their respective wild type strain. The expression level of FMP27 in the H2B mutants and their wild type was lower than that in the FACT mutant and its wild type, probably due to a difference in strain background. Nonetheless, the Northern blot clearly demonstrated that deleting HBR, or the H2B tail, resulted in the production of short transcripts as observed in the Spt16 mutants (Figure 4-3H), confirming that the HBR domain has roles in regulating the activity of histone chaperones and chromatin structure.

The HBR domain mediates the interaction between FACT and the H2A/H2B dimer

Our data obtained so far suggest that the H2B tail mutants display all the hallmark phenotypes of FACT mutants, including chromatin remodeling defects and production of
cryptic transcripts from the ORFs of genes. However, deleting HBR, or the H2B tail, does not affect the recruitment of FACT to genes. Previous reports suggested that FACT could be recruited to chromatin through multiple interactions, including binding to histone modifications (H2BK123 ubiquitylation) and associating with other components of the transcription machinery, such as the PAF complex (Fleming et al, 2008; Krogan et al, 2002; Pavri et al, 2006; Squazzo et al, 2002). It is possible that these factors mediated the crosslinking of FACT to chromatin in the absence of a functional HBR. Therefore, we used a pull down assay to examine the direct interaction between FACT and nucleosome. Nucleosomes purified from wild type or HBR deletion mutant were incubated with immobilized Spt16/Pob3 and the bound fraction was detected by Western blotting. Both the wild type and HBR deletion nucleosomes interacted with Spt16-IgG, but not the IgG resin alone. Furthermore, Spt16-IgG binds the wild type and ΔHBR nucleosomes equally well (Figure 4-4A), indicating that the HBR is not essential for FACT to bind nucleosome.

FACT has multiple binding sites on the nucleosome. The histone H3 and H4 N terminal tails are required for high affinity interaction between FACT and chromatin, and a stirrup-like “docking domain”, located on the C-terminal domain of histone H2A play important roles in FACT function (VanDemark et al, 2008). Although the HBR is dispensable for FACT binding to nucleosome, it might be required for the interaction between FACT and H2A/H2B dimer, and trigger its activity on nucleosome. To determine the specific binding of Spt16 to H2A/H2B, we reconstituted the yeast H2A/H2B dimer in vitro. The quality of the dimers was determined by SDS-PAGE and native gel
electrophoresis. Correctly folded dimer could enter the native gel, whereas free histones, which tend to aggregate, will be trapped in the well (Figure 4-4B). When equal amount of wild type and mutant dimer without HBR were loaded onto the native gel, almost all the samples could enter the gel, indicating that the individual histones were folded into dimer. In addition, the wild type and mutant dimer migrated at about the same positions, suggesting that deletion of HBR did not change the configuration of dimer significantly (Figure 4-4B). Pull down assay was then performed using immobilized Spt16/Pob3 and reconstituted dimer. The fractions bound to resin were revealed by Coomassie Blue staining. Surprisingly, Spt16/Pob3 only interacted with the wild type dimer, but not the dimer bearing HBR deletion (Figure 4-4C), indicating that the HBR is critical for FACT to bind H2A/H2B dimer. Deleting HBR hampers the interaction between FACT and H2A/H2B dimer, and impairs FACT mediated chromatin remodeling and reassembly. This result strongly suggests that HBR is a major regulator of FACT activity.

**Deletion of the HBR domain causes the accumulation of free histones within cells**

It has been reported that FACT dysfunction triggers the accumulation of free histones within cell because the histones evicted from disassembled nucleosome are not reassembled into chromatin (Morillo-Huesca et al, 2010). Since our result suggested that the HBR deletion impaired FACT mediated chromatin reassembly, we asked if there was also an up-regulation of non-chromatin associated histones in the HBR mutants. Free histone level was quantified by measuring the amount of histones associated with chaperones in the soluble fraction of cell extracts (Gunjan & Verreault, 2003;
Morillo-Huesca et al., 2010; Singh et al., 2009). Pull down assay was performed using Myc-tagged Asf1, which binds H3/H4 tetramer, and Spt16-Myc, which interact with H2A/H2B dimer. Deleting HBR did not affect the level of Asf1-Myc or Spt16-Myc within the cells (Figure 4-5A and B). Significantly, there was a substantial increase in the amount of histone H2A associated with Spt16 in the HBR deletion mutant. Since H2A and H2B form dimers which are bound by histone chaperones, this result indicates an accumulation of free H2A/H2B dimer within the cells (Figure 4-5A). Similarly, an increase in the amount of histone H3 associated with Asf1 was also observed in the HBR and tail deletion mutants, indicating an increase in the non-chromatin associated histone H3/H4 tetramer, which are captured by the chaperone Asf1 (Figure 4-5B).

The accumulation of non-nucleosomal histones is toxic to the cell and may lead to DNA replication defects (Gunjan et al., 2005; Gunjan & Verreault, 2003). We examined the sensitivity of the HBR mutants to hydroxyurea (HU), a drug which blocks DNA replication and both mutants showed clear sensitivity to HU, indicating a defect in replication stress response (Figure 4-5C). This result provides a separate line of evidence supporting the role of HBR in regulating FACT activity, and indicates that the altered chromatin structure in these mutants has biological consequences.
Discussion

The role of HBR in regulating histone chaperone activity

FACT was one of the first histone chaperones to be identified as having a role in transcription elongation through chromatin template. It binds to chromatin and facilitates elongation by partially destabilizing the nucleosome structure (Orphanides et al, 1998; Orphanides et al, 1999). The specific structural changes that occur during FACT mediated nucleosome reorganization are not known, but it has been suggested that H2A/H2B dimers might be partially or fully displaced. Therefore, mechanistic studies on how FACT interacts with H2A/2HB dimer and how these interactions affect its function would greatly increase our understanding of FACT mediated chromatin remodeling activity.

Here we report that a basic region located on the N terminus of histone H2B, the HBR, is essential for the binding of FACT to H2A/H2B dimer, which in turn regulates multiple FACT related activities. The HBR domain locates in a minor groove channel between the DNA strands of the superhelix (Figure 4-6A). Deletion of HBR may abolish the local DNA-histone interaction, which alters the nucleosomal structure and may hamper the docking of FACT onto chromatin. HBR also provides a critical binding site for FACT on the H2A/H2B dimer, and allows the dimer to be removed by the chaperone during transcription. Deletion of HBR slows down the nucleosome disassembly upon activation, reduces the transcription level of multiple genes. HBR is also essential for FACT mediated reassembly of chromatin structure in the wake of polymerase, represses cryptic transcription and maintains normal level of free histones within the cells (Figure 4-6).
FACT has multiple binding sites on the nucleosome. Biochemical and structural studies have identified several domains within the complex that interact with different histones redundantly (Belotserkovskaya et al, 2003; Stuwe et al, 2008; VanDemark et al, 2008). Therefore, it is not surprising to see that FACT had no bias binding to the wild type and HBR deletion nucleosome. The HBR domain, consisting of amino acids KKRSKARK, is highly basic. It is tempting to speculate that the HBR provides a binding site for the acidic C terminus of Spt16 to grasp H2A/H2B through electronic interaction. However, studies in nucleosome structure indicated that HBR protrude into the minor groove of nucleosomal DNA (Luger & Richmond, 1998). Thus, one explanation is that the HBR keeps local DNA-histone interaction and maintains a proper chromatin structure. Deletion of HBR may prevent the docking of FACT onto this region, although the chaperone is still able to associate with other parts of the nucleosome. A second possibility is that activation dependent chromatin remodeling allows the exposure of HBR. ATP dependent chromatin remodelers, or other histone chaperones may partially disassemble chromatin, enable the recognition of HBR by FACT, and allow it to further remodel chromatin. In either scenario, it is certain that the HBR directly or indirectly mediates the interaction between chaperone and the H2A/H2B dimer.

Besides its role in chromatin disassembly upon activation, FACT is also essential for the reassembly of the nucleosome in the wake of polymerase, maintaining an organized chromatin structure. Deletion of HBR leads to intragenic transcripts from multiple genes bearing cryptic initiation site within ORF, indicating that the nucleosomes are not positioned properly over cryptic promoters, most likely due to the impaired FACT
activity. In addition, the chaperone pull down assay suggested an increase in free histone level in the HBR deletion mutants, again indicating a partially unpacked chromatin environment. We have also tried to monitor the reassemble of nucleosome following repression to see if deleting HBR hampers the deposition of core histones onto chromatin. However, ChIP analysis measuring the histone H2A and H3 density at *GAL1* following its repression did not suggest a defect in nucleosome reassembly in the mutants. It is possible that other histone chaperones, including Spt6 and Asf1, redundantly restore chromatin structure at *GAL1*. In addition, histones might be placed back onto DNA but not in the proper positions; whereas histone chaperones, such as FACT, rearrange them into highly organized structure. Since ChIP analysis were unable to distinguish the random placed versus highly organized nucleosome structure, the cryptic transcription we have observed in the HBR deletion mutants might be a better indication of partially organized chromatin structure. Thus, we conclude that the HBR is critical for FACT mediated chromatin remodeling as well as reassembly of proper chromatin structure during transcription.

**The HBR modulates chromatin structure**

Multiple cues suggest that the chromatin structure is altered in the HBR mutants. First, HBR deletion alters the DNA superhelical turns in plasmid topology assay and the Micrococcal Nuclesase (MNase) accessibility in the MNase digestion assay (Lenfant et al, 1996; Nag et al, 2010), suggesting a change to the organization of nucleosomes. Second, structural study indicates that the HBR sits in a minor groove between the DNA superhelix, with its highly basic residues directly contact the nucleosomal DNA. Thus,
deletion of HBR results in a partial loss of association of the nucleosomal DNA along the histone octamer. Third, as has been shown in our study, the H2A/H2B dimer without HBR cannot be bound by the histone chaperone FACT. The changes in dimer configuration could possibly induce an alternation in local chromatin structure. Whereas deleting HBR made the H2A/H2B dimer unrecognizable by FACT, it is also possible that the changes in nucleosome structure are the major inhibitor of chromatin remodeling activities. Preliminary data from our lab suggest that reconstituted yeast nucleosomes without HBR are unable to be remodeled by the SWI/SNF chromatin remodeling complex. Therefore, the HBR, through stabilizing the association and position of genomic DNA, keeps the nucleosome structure in a proper configuration which can be remodeled by the chromatin remodeler and chaperones.

The role of HBR in regulating histone modification and genome wide transcription

Genome wide expression analysis has identified the H2B tail as primarily playing a role in the repression of transcription. However, our data presented here argues that the HBR may play more important roles in the activation of transcription. Deleting HBR reduces multiple histone modifications associated with active transcription, implying a down regulation of genome wide transcription. It is of our interest to determine how HBR regulates these modifications. Data from Wyrick’s lab suggest that H2BK123 ubiquitylation is decreased in the HBR deletion mutants (personal communication). Since FACT and H2Bub reciprocally regulate each other, the impaired activity of FACT in the HBR mutants may probably cause the ubiquitylation defect. The
decrease in H2Bub could in turn, lead to reduction in H3K4 and K79 methylation as we have observed in the western blot analysis. Indeed, when we artificially increased the H2Bub by deleting the deubiquitylases Upb8 and Upb10, an increase in H3K4 methylation was detected in the HBR deletion mutants, providing evidence for the HBR - H2Bub - H3K4 methylation regulatory pathway. The decrease in FACT activity affect the transcription of a large group of genes, which may account for the reduction of other histone modifications associated with transcription, such as H3K36 methylation. It is also possible that the HBR plays direct role in regulating these histone modifications to maintaining proper chromatin structure and function.

In summary, the HBR may regulate genome wide transcription through multiple mechanisms. It maintains a proper chromatin structure and post translational modifications; it is essential for the activity of histone chaperones and maybe ATP dependent chromatin remodelers. Therefore, our study prompts a re-evaluation of the importance of HBR during transcription.
Acknowledgement

We thank members of the Reese Lab and the Center for Eukaryotic Gene Regulation at Penn State for advice and comments on this work. We thank John Crickard for helping with the purification of H2A/H2B dimer. We are grateful to Dr. John Wyrick for histone H2B mutant strains. This research was supported by funds from National Institutes of Health (GM58672) to J.C.R.
A

![Graph showing GAL1 mRNA levels with Raffinose treatment](image)

B

![Graph showing GAL1 mRNA levels over time with different H2B variants](image)

C

![Bar graph showing mRNA/WT ratio for PMA1 and PYK1](image)

D

![Western blot analysis of H3K4me2 and H3K4me3](image)
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**E**

![Graph showing GAL1 Pro TBP % IP over time](image)

- Red: Raffinose
- Green: add Galactose 15min
- Blue: add Galactose 30min
- Orange: add Galactose 60min
- Light Blue: add Galactose 90min
Figure 4-1. The H2B Repression (HBR) domain is required for the transcription of subsets of genes.

(A) Basal level of GAL1 expression in wild type, H2BΔ30-37, H2BΔ3-37 measured by RNA isolation and RT-PCR. Cultures were grown to log phase in media containing 2% raffinose. The levels of RNA were normalized to the signal of scR1, a loading control.

(B) GAL1 induction in wild type, H2BΔ30-37, H2BΔ3-37 measured by RNA isolation and RT-PCR. Cultures were grown to log phase in media containing 2% raffinose and induced with 2% galactose for the times indicated in figure.

(C) PMA1 and PYK1 expression in wild type, H2BΔ30-37, H2BΔ3-37.

(D) Western blotting of whole cell extracts prepared from the wild type and mutant strains using the antibodies described in the panel. Histone modification levels were normalized to the amount of histone H3 on each blot (H3 core). Numbers below each panel are the levels of modification in the mutants relative to the wild type cells, which was set at 100.

(E) ChIP analysis of TBP recruitment at the promoter of GAL1 gene upon activation. Wild type and mutants were grown to log phase in media containing 2% raffinose and induced with 2% galactose for the times indicated in figure. Chromatin was precipitated with TBP antibody.
Figure 4-2. HBR domain promotes chromatin disassembly during transcriptional activation

(A and B) ChIP analysis to measure the eviction of nucleosome at GAL1 promoter upon activation. Experiment was performed as described in the legend of figure 4-1C except that H2A(A) and H3(B) antibodies were used in the IP.

(C and D) Histone H2A and H3 level at the promoter of GAL1 gene after overnight induction. Wild type and mutant cells were grown to log phase in media containing 2% dextrose (Dex, white bars) or 2% galactose (Gal, black bars), and chromatin was precipitated with antibodies to histone H2A (C) and H3 (D).

(E) ChIP analysis of histone H3 level at PMA1 and PYK1 promoter. Cells were grown in media containing 2% dextrose.
Figure 4-3. The HBR domain is dispensable for the recruitment of histone chaperone, but is required for its activity

(A-C) ChIP analysis of SWI/SNF, Spt16-Myc, TBP crosslinking to the promoter of GAL1. Cultures were grown in media containing dextrose or galactose overnight. Antibodies raised against the N terminal of Swi2 (A), 9E10(B), and TBP(C) was used to IP chromatin.

(D) Northern blot analysis of FLO8 RNA. Total RNA prepared from the indicated strains were run on formaldehyde-containing agarose gels and transferred to HyBond membrane. Blots were probed with sequences complementary to the 3' region of FLO8. The full-length and short cryptic transcript signals for FLO8 are indicated.

(E-G) Recruitment of Spt16 (E), RNAP (F), and TBP (G) to the promoter and the internal cryptic TATA box of FLO8. Cultures were grown in dextrose medium.

(E) Northern blot analysis of FMP27 RNA. Same as in D except that the FMP27 3' region probe was used.
Figure 4-4. The HBR domain mediated the interaction between FACT and H2A/H2B dimer.

(A) FACT nucleosome pull down. Spt16-TAP or IgG resin was incubated with wild type or H2BΔ30-37 nucleosomes. Interactions were examined by silver stain and western blotting: the upper part of the gel was silver stained to reveal Spt16/Pob3; the lower part of the blot was probed for H3 core.

(B) Native and SDS-PAGE gel analysis of the reconstituted dimers.

(C) FACT H2A/H2B dimer pull down. Spt16-TAP or Calmodulin resin was incubated with wild type H2A/H2B dimer or H2A/H2BΔ30-37 dimer. Interactions were examined by silver stain.
Figure 4-5. HBR domain deletion cause the accumulation of free histones within cells

(A) The histone H2A/H2B levels associated with Spt16/Pob3 in the WT, H2BΔ30-37, H2BΔ3-37 strains. The indicated strains carrying myc-tagged Spt16 were grown to log phase and harvested for WCE preparation. The amount of H2A/H2B associated with Spt16/Pob3 was shown by Western blot using H2A antibody. Total histone H2A and Spt16-myc levels in the WCE were shown to demonstrate that roughly equal amounts of WCE were used in the IP.

(B) The histone H3/H4 levels associated with Asf1. The indicated strains carrying myc-tagged Asf1 were harvested for WCE preparation. The amount of H3/H4 associated with Asf1 was shown by Western blot using H3 antibody.

(C) Spot tests for growth on HU. Serial dilutions of wild type and mutant strains were spotted onto YPAD or YPAD+50ug/ml HU media and incubated at 30°C for 48hours.
Figure 4-6. Model for regulation of nucleosome assembly and disassembly by HBR
(A) Position of HBR in the nucleosome structure.
(B) HBR regulates FACT mediated nucleosome disassembly and reassembly. In the wild type cells, nucleosome disassembly and assembly can be quickly achieved in the presence of histone chaperone. HBR deletion impairs the activity of FACT, and greatly slows down the conversion. This leads to more stabilized nucleosome during activation and an excess of free histones during repression when chromatin needs to be reassembled.
Chapter 5

TRANSCRIPTION REGULATION BY HISTONE TAILS: CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS
The histone N terminal tails regulate multiple facets of transcription

It has long been suggested that the histone N-terminal tails play roles in gene regulation, but the exact mechanisms through which they act still remains elusive. Early on, in vitro transcription assays with chromatin templates showed that the deletion of histone tails gave rise to higher transcription efficiency, suggesting that the presence of histone tails repress transcription (Chirinos et al, 1998; Hernandez et al, 1998; Protacio et al, 2000; Ujvari et al, 2008); In vivo analysis, however, indicate more complicated and gene specific roles of histone tails during transcription. It has been reported that all four histones contain short regions which suppress the basal, uninduced transcription (HAR within the H2A tail and HBR in the HBR tail in my study). These sequences locate at the junction of the hydrophilic N-terminal tails and the highly $\alpha$-helical structured core domain of each histone. Deletion of these residues causes derepression of the GAL1 promoter (Lenfant et al, 1996). The very N-terminal sequences of each histone have more versatile roles in gene expression. It has been shown that the H4 tail is required for GAL1 activation and deletion of H4 tails (H4 $\Delta$4-23) led to about 20-fold decrease in GAL1 mRNA level (Durrin et al, 1991); In contrast, deletion of the H3 tails (H3 $\Delta$4-15) seemed to “hyperactivate” GAL1 at a rate about 3-fold greater than the wild type level (Mann & Grunstein, 1992). Besides studies from model genes, recent genome wide expression profiling suggest that different histone tails play roles in both activation and repression of specific sets of genes (Parra et al, 2006; Parra & Wyrick, 2007; Sabet et al, 2003; Sabet et al, 2004). Since the mechanisms behind these observations are not quite established, we set out to explore how each tail regulates transcription specifically.
Our initial hypothesis was that residues on the tails mainly serve as docking sites for the direct recruitment of transcription factors, chromatin remodelers, or chaperones, to chromatin, thereby promoting transcription (Fingerman et al, 2007; Georgel et al, 1997; Vitolo et al, 2000). In addition, multiple studies suggest that modifications on the tail could be recognized by chromatin regulatory proteins, and target these proteins to genes (Carrozza et al, 2005; Ginsburg et al, 2009; Joshi & Struhl, 2005; Lee et al, 2007; Taverna et al, 2006). Indeed, data from our lab shows that the histone H4 tails are required for the recruitment of several elongation factors, including FACT, Spt4/5, Spt6, and the PAF complex. The inability to recruit chaperones or elongation factors to genes led to strong transcription defect in the H4 Δ2-26 mutant (James Psathas, unpublished data). Albeit true in the case of the H4 tail, my analysis with the other histone tails indicate that the H2A, H2B, and H3 tails are mainly dispensable for the recruitment of regulatory factors. It is highly possible that multiple histone tails play redundant roles in the recruitment of these factors. For example, it is known that the tails are important for the interaction between FACT and nucleosomes, and nucleosomes treated with trypsin to remove histone tails were no longer able to bind to FACT (VanDemark et al, 2008). While in my analysis, deleting a single H2B tail has no effect in FACT recruitment to genes or binding to nucleosomes in vitro. Therefore, in order to change the local nucleosome structure, elongation factors, or remodeling enzymes may have multiple binding sites within nucleosome to achieve a stable interaction. The deletion or mutation of single histone tails may not impair the interaction of these factors with nucleosome.

Although not essential for the recruitment of regulatory enzymes, my analysis
suggests that individual histone tails play more specific roles in regulating their activity.

Here I provide evidence to show that the H2B tail mediates the interaction between histone chaperone FACT and the H2A/H2B dimer, and regulate the function of FACT in chromatin remodeling and reassembly; the H2A tails may provide binding site for the Rad6/Bre1 ubiquitylation machinery or trigger its activity to ubiquitylate H2BK123; and the H3 tail positions K36 within the active site of Set2 for proper H3K36 methylation. These results suggest that instead of acting as a “docking site” passively, individual histone tails may actively regulate the function of specific chromatin modifying or remodeling enzymes that use the tails as substrates. Therefore, we propose that the tails of multiple histones may provide stable binding sites for regulatory factors to dock onto the chromatin, while individual tails specifically modulate the activity of each enzyme, allowing them to remodel or modify nucleosome.

A common feature with the H2A, H2B and H3 tails is that they all have roles in regulating histone modifications, and the mechanisms by which they regulate modifications are quite distinct from the known cis or trans regulatory pathways. For the H2A and H2B tails, the regions involved in regulation are occluded by nucleosomal DNA, therefore, both pathways require a local nucleosome environment modified by transcription. The HAR stays in close proximity to the H2Bub site, which provides a good chance for it to modulate the activity of the ubiquitylation machinery; while the HBR regulates multiple modifications in cis or trans, most likely through affecting the activity of histone chaperones. The intra-tail regulation of H3K36 methylation by the H3 tail is also of novelty because most intra-tail regulations described before are through affecting
the recruitment of another enzyme which docks in close proximity (Guccione et al, 2007; Martin et al, 2006; Nelson et al, 2006). In contrast, we demonstrate that the H3 tail is not required for the recruitment of Set2; however, it helps to align K36 within the catalytic site of the methyltransferase and stimulates its activity. These results suggest that an important pathway by which the tails modulate transcription is to regulate histone modifications. Since histone modifications are essential regulators of transcription, regulating the enzymatic activity of histone modifiers is a major aspect of histone tails mediated gene regulation mechanism.

**Structural-functional analysis of the HAR and HBR domain**

The histone residues that comprise the HAR and HBR subdomains are well conserved across eukaryotic species, while the other parts of the H2A and H2B N-terminal tails show considerable sequence divergence (Figure 5-1A). The high sequence conservation of the HAR and HBR suggests that these sequences are under selective pressure to keep their function. Indeed, one striking features of HAR and HBR is that both domains interact with nucleosomal DNA. The HBR domain sits in a minor groove channel between the DNA strands of the superhelix, while the HAR domain interact with the minor groove of the DNA on the outside of the superhelix in close proximity to HBR (Fig. 5-1B). This juxtaposition of the H2A and H2B subdomains was noted by Luger and Richmond, and they postulated that these subdomains might tether the H2A/H2B dimer to the nucleosome (Luger & Richmond, 1998). The intriguing position of HAR and HBR within the nucleosome leads us to ask how the sequences normally hidden under the
nucleosomal DNA exert their stimulatory effect on chromatin remodeler and modifying enzymes. We propose that in the presence of transcription machinery, the highly packed chromatin structure is reorganized, allowing the exposure of the HAR and HBR. Following their exposure, the HAR modulate the activity of Rad6 to ubiquitylate H2BK123; while the HBR allows the histone chaperone FACT to bind the H2A/H2B dimer and disassemble the nucleosome. Therefore, the unique position of HAR and HBR prevent the unlimited accessibility of regulatory factors to remodel chromatin, and restrain these activities to transcription dependent nucleosome structure changes.

**Future directions**

**The HAR regulates H2BK123 ubiquitylation**

Regulation of COMPASS and Dot1 mediated H3K4, K79 methylation by H2BK123 ubiquitylation is an evolutionarily conserved “trans histone” crosstalk pathway (Briggs et al, 2002; Lee et al, 2007; Nakanishi et al, 2009). However, the factors which regulate H2B ubiquitylation remain largely unidentified. The histone H2A and H2B dimerize in a head to tail manner, placing the N- terminus of H2A in close proximity to the C- terminal of H2B. Here we show that the HAR, a conserved region in the N-terminal of H2A, regulates H2BK123 ubiquitylation through a novel “trans tail” mechanism. Deleting HAR impairs H2B ubiquitylation and the subsequent H3K4methylation, while increasing H2B ubiquitylation rescues the H3K4 defect in the mutant.

Having identified the regulatory pathway, the next question we want to ask is how
HAR regulates H2B ubiquitylation? Our data suggests that HAR may regulate the activity of Rad6/Bre1 ubiquitylation machinery at a post recruitment step. We have tried different methods to detect an interaction between Rad6/Bre1 and nucleosomes in an attempt to test this mechanism, but we were unable to observe a stable interaction. It is possible that Rad6/Bre1 ubiquitylates H2B and release its substrate shortly after the reaction. A more direct experiment to examine the stimulatory effect of HAR is to establish in vitro ubiquitylation assay using purified Rad6/Bre1 and wild type or mutant nucleosomes (Kim & Roeder, 2009). Since Rad6/Bre1 ubiquitylates multiple residues in all the four histones (Geng & Tansey, 2008; Singh et al, 1998; Sung et al, 1988), an H2BK123ub specific antibody will be used to detect this modification (Nakanishi et al, 2009). Result from this assay will provide a better mechanistic view of the HAR-H2B regulatory pathway.

Another interesting direction of study is to identify novel post-translational modifications and their functions within the histone H2A tails, particularly modifications located in functional domains such as the HAR. Several residues of the HAR domain locate on the outside of the DNA superhelix, and thus are readily accessible to histone modifying enzymes. Advances in mass spectrometry based studies will facilitate the identification of novel histone modifications, and characterizing the roles of these modifications will add to our understanding of the role of H2A tail in regulating transcription.

The HBR and chromatin remodeling

The HBR domain sits in a minor groove channel between the DNA strands of the
superhelix, with its basic residues contacting directly with the nucleosomal DNA.

Deletion of HBR abolishes local DNA-histone interaction and may lead to changes in chromatin structure. Our study suggests that the HBR is critical for histone chaperon mediated chromatin remodeling. However, the altered chromatin structure in the HBR mutant may also hinder the activity of other chromatin remodelers. Using *in vitro* reconstituted nucleosome with yeast histones, our preliminary data suggests that the ATP dependent chromatin remodeler SWI/SNF remodels the HBR deletion nucleosomes less efficiently than the wild type, providing another piece of evidence to show the importance of HBR in maintaining a proper chromatin structure and transcription activation.

Since we have observed a defect in chromatin remodeling by SWI/SNF in the HBR deletion mutant, several other assays can be employed to further dissect the nucleosome structure changes without HBR. First, gel mobility shift and ATPase assays can be performed to determine if HBR deletion affects the binding of SWI/SNF to nucleosome or impairs its ATP hydrolysis activity. Second, DNase 1 footprinting and endonuclease accessibility assays can be applied to reconstituted nucleosome before and after SWI/SNF remodeling, and the patterns of digestion may reveal the differences in nucleosome structure between the wild type and mutant in detail. Third, whether HBR deletion affects the activities of other chromatin remodelers, such as the ATP dependent chromatin remodeler RSC or ISW1 complexes, may also be explored (Carey et al, 2006; Clapier et al, 2001). These analyses will illuminate how HBR coordinates with chromatin remodeling activities to maintain a transcription-competent chromatin structure.

The HBR is named after its ability to repress transcription of subsets of genes,
mainly involved in amino acid and carbohydrate metabolism (Parra et al, 2006). Genome
wide expression analysis suggests that transcriptions of these genes are up-regulated upon
HBR deletion. The mechanism of how HBR represses their transcription remains unclear;
whereas our analysis point to more prominent roles of HBR in transcription activation. It
would be interesting to determine the differences between HBR repressed and activated
genes. Probing for the local chromatin structure and nucleosome density at these genes
will shed light on how changing local nucleosome structure may lead to distinct
transcription outcome.
**Figure 5-1. Sequence alignment of the HAR and HBR subdomains and their location in the nucleosome structure.**

A. Sequence alignment of the HAR and HBR among species.
B. Locations of the HAR and HBR domains in the nucleosome structure.

The HAR domain is colored red, and the HBR domain is colored blue.
Chapter 6

MATERIALS AND METHODS
**Strains and media**

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Cells were grown in YP media (1% yeast extract, 2% peptone) with appropriate carbon source: 2% dextrose, 3% raffinose, or 2% galactose. Media was supplemented with 0.05mg/ml adenine sulphate. For the *GAL1* induction studies, cells were grown to an optical density at 600 nm (OD600) of 0.6 in YP+ 3% raffinose media supplemented with 0.05mg/ml adenine sulphate, then galactose was added to a final concentration of 2% for the times indicated in figure. For hydroxyurea sensitivity, cultures were grown overnight and diluted to an OD600=1.0. Three-fold dilutions of cultures were spotted on YPAD or YPAD+50ug/ml HU media and incubated at 30°C for 48 hours. Gene deletion and epitope tagging were carried out by homologous recombination using PCR-generated cassettes (Brachmann et al, 1998; Longtine et al, 1998).

**Western Blotting**

For analysis of histone modifications, 10ml of yeast culture (OD600= 0.6-0.8) was collected and washed with 10% Tri-Chloro Acetic Acid (TCA). Cell pellet was disrupted by vortexing with glass beads. The lysate was clarified by centrifugation and washed once with 0.5M TrisHCl pH7.4. The pellet was suspended in 0.5M TrisHCl pH7.4 and SDS loading buffer. Whole cell extract was separated on SDS-PAGE gel and subjected to Western blot analysis. The following antibodies were used: H3core (Abcam), H3K4me1(Active Motif), H3K4me2(Upstate), H3K4me3(Active Motif), H3K36me3(Abcam), H3K79me2(Upstate), H2B(Active Motif), Acetyl H3 (Millipore),
Acetyl H4 (Millipore), H14(Covance). Immunoblots were quantified using ImageJ software (NIH).

**Northern blotting.**

RNA isolation and northern blotting were carried out as previously described (Reese & Green, 2003). Cells from 10 ml of yeast culture (OD600 = 0.7) were harvested for total RNA extraction. 15ug of total RNA was separated on 1.2% formaldehyde-containing agarose gels and transferred to a Hybond-XL membrane (GE Biosciences, Piscataway NJ) by capillary blotting. After UV crosslinking and prehybridization at 65°C for 4hours, radioactively labeled gene-specific probes were added. Probes for *GAL1*, *FLO8*, *FMP27*, and *ScR1* were prepared using PCR. Signal was detected using Phosphor Screen (Molecular Dynamics), scanned with the Typhoon system (Molecular Dynamics), and quantified using ImageQuant. The *ScR1* signal of each sample was used to correct for loading of RNA.

**mRNA Quantification via RT-qPCR**

RNA preparation was performed as described above. 20μg of RNA was digested with DNase I (Worthington, Lakewood, NJ) for 30min at 37C. RNA was phenol/chloroform extracted, ethanol precipitated, resuspended in DEPC-treated ddH2O and diluted to a concentration of 200ng/μL. 2μl RNA was used in RT-PCR with random primers to generate cDNA. Dilutions of 1:50 and 1:100 of cDNA was used in qPCR. RNA template, following DNase I digestion but prior to RT, was used a negative control for
qPCR. Signal of ScR1 was used as loading control.

**Chromatin Immunoprecipitation (ChIP)**

The chromatin immunoprecipitation (ChIP) assay was performed as described in previous publications (Sharma et al, 2007). 100ml of yeast culture (OD600 = 0.7) was crosslinked with formaldehyde (1% v/v) for 15 min at room temperature and quenched by adding glycine to 125 mM. Whole cell extract was prepared by glass beads disruption and sheared into fragments averaging 200 to 600 bp in size by using a Bioruptor (Diagenode, Philadelphia PA). 100ul of whole cell extract was incubated with 1–2ul of antibody overnight. The immunoprecipitated DNA and input DNA were analyzed by real time PCR. The percent IP represents the (IP signal/ input signal) x100. For histone modifications ChIPs, the data is represented as the (%IP modified / %IP total H3) in order to account for variations in histone levels. Data are presented as the means and standard deviations of results from at least three independent experiments.

**TAP tag purification**

Purification of a Tandem Affinity Purification (TAP) - tagged protein was performed essentially as previously described (Takahashi et al, 2009). For TAP purification of COMPASS complex, 6L of yeast culture (Cps60-TAP, OD=1.0) was collected, washed and lysed with glass beads in E-buffer (40 mM HEPES pH 7.5, 0.1% Tween20, 200 mM NaCl, 10% Glycerol, 1mM PMSF, 2ug/ml leupeptin, 2ug/ml pepstatin A). After clarification of the lysate with centrifugation, TAP-tagged proteins were bound
to immunoglobulin G-Sepharose beads (IgG-sepharose fast flow, GE healthcare) overnight at 4°C. The IgG beads were washed with TEV cleavage buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 0.1% NP-40, 0.5 mM EDTA and 1 mM DTT), followed by cleavage of the TAP tag with tobacco etch virus protease at 4°C overnight. The TEV elute was incubated with calmodulin-Sepharose 4B (GE healthcare) in calmodulin binding buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2.0 mM CaCl$_2$, 1.0 mM MgAc$_2$, 1.0 mM Imidazole, 0.1% Tween20, 10% glycerol, 5 mM β-mercaptoethanol) for 2 hours at 4°C. The beads were washed with calmodulin binding buffer 3 times and the bound proteins were recovered in elution buffer (50 mM Tris-Cl pH 8.0, 2.0 mM EGTA, 150 mM NaCl, 10% Glycerol, 0.1% Tween 20). The purity of each fraction was then analyzed by SDS-PAGE and SYPRO Ruby staining (Invitrogen) of the protein complexes. For TAP purification of the FACT complex, 2L of yeast culture (Spt16-TAP OD=1.5) were used in purification.

**Purification of yeast histone**

Purification of yeast histones were performed as previously described, with minor changes (Saha et al, 2002). *S. cerevisiae* histones H2A, H2B, or H2BΔ were PCR amplified and cloned into the BamHI - BsrG1 sites of pST50TRC3 using restriction enzyme sites that were engineered into the PCR primers. The integrity of the ORFs were confirmed by DNA sequencing. Expression constructs were transformed into Rosetta (DE3) LysS (Novagen) which supplies tRNA for 7 rare codons. These were maintained in the BL21 cells by selection in chloramphenicol (30 μg/ml) and ampicillin (100 μg/ml).
Once transformed, the expression constructs were kept under selection in both chloramphenicol and ampicillin.

Fresh transformants were used to inoculate a 50 ml culture of LB containing ampicillin and chloramphenicol and grown for 3 to 4 hours at 37°C. This culture was used to inoculate 2 liters of culture, which was grown to an OD600 of 0.6–0.8 and induced with 0.2 mM IPTG for 4 h at 37°C. Cells were harvested at 5000 g for 10 min. Each liter of cells was suspended in 16.7 ml wash buffer (50 mM TrisHCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.2 mM PMSF). Suspensions were frozen down in liquid nitrogen and stored at −80°C.

Cell suspensions were thawed in 30°C water bath with constant gentle shaking. The volume was adjusted to 25 ml/Liter cells and sonicated until viscosity was reduced. Inclusion bodies were spun down at 14K for 20 min at 4°C. The pellets were rinsed with 25 ml/liter culture of TW buffer (wash buffer + 0.1% triton X-100) by pipeting up and down while digging at the pellets with a 10 ml pipet (Pellets never completely resuspend, but should be broken up as much as possible). The pellets were washed four times in total, twice with TW buffer and twice with wash buffer followed by spun down at 14K at 4°C after each wash. The pellets were drained and stored at −80°C.

For all of the subsequent steps, buffers containing guanidinium hydrochloride, urea, or reductants should be made fresh and used within a day. Freshly made urea stocks were deionized with 25 g/liter of IRN-150L monobed mixed resin (Amberlite) while stirring for 30 min at RT, then filtered. Protease inhibitors were not needed during the subsequent purification steps.
Inclusion body pellets were soaked in 350ul DMSO for 30min at RT and combined into one tube. The pellets were minced with spatula, denatured in 13.3 ml unfolding buffer (20 mM TrisHCl, pH 7.4; 7M guanidinium hydrochloride, 10 mM DTT) and rotated for 1 h at RT. Cell debris were removed by centrifuging at 14K for 20 min. The supernatant was transferred to a new tube, and the pellet was reextract with 3.3 ml unfolding buffer. The solubilized histones were dialyzed against 2 liters of urea dialysis buffer (100 mM NaCl, 10mM TrisHCl, pH 8, 7M urea, 1mM EDTA, 5mM 2-mercaptoethanol, 0.2mM PMSF) using 6000–8000 MWCO tubing, for 3 hours with one buffer changes at RT.

While the sample was dialyzing, a Q- and a SP-Sepharose column (5 ml HiTrap ion exchange columns, GE healthcare) was equilibrated arranged in tandem, with the Q-column first in line. Column buffers contain 10 mM TrisHCl pH 8, 7M urea, 1 mM EDTA, 1 mM DTT (buffer A), or with the addition of 1 M NaCl (buffer B). The columns were finally equilibrated in 10% buffer B.

The dialyzed histones were filtered through 0.45 µmcellulose acetate membrane and loaded onto Q column, followed by washing the column with one column volumes of starting buffer (10% buffer B). The Q-column was removed from the system before eluting the protein. SP-bound proteins were eluted with 35% B over 8 column volumes, followed by 100%B over 2 column volumes. 3ml fractions were collected and flow through was saved. The peak protein fractions were analyzed by 15% SDS-PAGE and coomassie blue staining, then pooled and dialyzed against three changes of ddH2O (with 5 mM 2-mercaptoethanol and 0.2mM PMSF) at 4°C. The second dialysis step should be
done overnight.

Histone fractions were lyophilized and dissolved in a small volume of water. Measure the OD276 and determine the approximate concentration of the yeast histone using the extinction efficient (mg/ml) as follow: H2A=0.320, H2B=0.562, H2BΔ30-37=0.523. The proteins were lyophilized in aliquots appropriate for dimer reconstitution.

**Reconstitution of H2A/H2B dimer**

Five microgram of each histone were suspended in unfolding buffer and allowed to sit at RT for 30min. H2A/H2B or H2A/H2B Δ30-37 were mixed in equal molar ratios and the volume was adjusted to 2 mg/ml with unfolding buffer. The histone mixtures were dialyzed against 2 liters of refolding buffer (10 mM HEPES KOH pH7.4, 50mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol) using 6000-8000 MWCO tubing at 4°C for a total of 4 buffer changes. The second step had to be overnight.

Equilibrate a S1 column (1 ml HiTrap ion exchange columns, GE healthcare) with HS50 buffer (10 mM HEPES pH7.4, 50mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol), or HS1500 buffer (10 mM HEPES pH7.4, 1500mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol). The column was finally equilibrated in HS50 buffer.

The dialysate was spun down at 14K at 4°C before loaded onto column. The column was washed with 3 column volume of HS50, and the dimers were eluted with a gradient of 0–100% HS1500 over 20 column volume at 4ml/min followed by 3 column volume of HS1500. Collect 0.5ml fractions. Peak fractions (A280) were analyzed by 15%
SDS-PAGE and coomassie blue staining, then pooled and dialyzed against one liter of storage buffer (10 mM TrisHCl pH 7.4; 100 mM NaCl; 1mM EDTA; 10 mM 2-mercaptoethanol) for 4 hours with one buffer changes at 4°C.

Reconstituted dimers were concentrated to approximately 5mg/ml and stored in 20% glycerol at -80°C.

**Purification of yeast nucleosome**

Two liters of yeast culture were grown to a density of OD600 = 1 and harvested at 5000g for 10 min at 4°C. The cell pellet was washed twice with 30ml cold SB buffer (1.4M Sorbitol, 40mM HEPES pH7.5, 0.5 mM MgCl₂, 0.5mM EGTA, complete protease inhibitor) supplemented with 10mM 2-mercaptoethanol. The pellet was weighted and suspended in 4X weight in volume pre-warmed SB buffer supplemented with 2mM 2-mercaptoethanol. Zymolyase [seikagaku 100T] was added to 0.5mg/ml and incubated at 30°C for 25-30 min with gentle shaking. When spheroblast was complete, volume of cell suspension was adjusted to 30ml/liter of culture with cold SB buffer and cells were spun down. Cell pellets were suspended in 40 ml of cold NIB (nuclei isolation buffer; 0.25 M sucrose, 60 mM KCl, 14 mM NaCl, 5 mM MgCl₂, 15mM PIPES pH 6.6, 0.8% Triton X-100, 0.5mM EGTA, 0.2 mM PMSF), held on ice water for 15 min, and spun at 5K for 5 min at 4°C. The NIB wash was repeated two more times. The pellets were then washed two times in "A" wash (10 mM Tris at pH 8.0, 0.5% NP-40, 75 mM NaCl, 0.5mM EGTA, 0.2 mM PMSF), once in "B" wash (10 mM Tris at pH 8.0, 0.4 M NaCl, 0.5mM EGTA, 0.2 mM PMSF) and once in “C” wash (10 mM Tris at pH 8.0, 0.4 M NaCl, 0.5mM...
EGTA, 0.2 mM PMSF). Pellet suspensions were held on ice water for 15 min in-between each wash. To obtain mononucleosomes, the nucleus-containing pellets were washed with 10ml of digestion buffer buffer (10mM PIPES pH 7.5, 0.5mM MgCl2, 0.05mM CaCl2 and complete protease inhibitor) and resuspended in 2.5ml of digestion buffer. Combine nuclei from the two liters culture and pre-warm at 37°C for 10min. Add MNase (Worthington) to 30U/ml and incubate at 37°C for 10min. Stop reaction with the addition of 10mM EDTA and spun. The supernatant, which contains mostly mononucleosomes, was aliquoted and store at -80°C.

**Nucleosome and histone pull-downs**

For nucleosome pull-down assays, one liter of yeast culture (Spt16-TAP, OD=1.0) was collected, washed and lysed with glass beads in E-buffer (40 mM HEPES pH 7.5, 0.1% Tween20, 200 mM NaCl, 10% Glycerol, 1mM PMSF, 2ug/ml leupeptin, 2ug/ml pepstatin A). After clarification of the lysate with centrifugation, TAP-tagged proteins were bound to immunoglobulin G-Sepharose beads (IgG-sepharose fast flow, GE healthcare) overnight at 4°C. The beads were washed 3 times in binding buffer (20mM Tris pH 7.4, 200mM NaCl, 1mM MgCl2, 0.1% Triton X-100, 10% glycerol, 0.5mM DTT, 0.5mM PMSF). Aliquots of beads containing 500ng of FACT were washed in binding containing 500mM NaCl for 20min to reduce the amount of endogenous H2A/H2B co-purified with FACT. The beads were then washed once in binding buffer (200mM NaCl), and incubated with 500ng of wild type or ΔHBR nucleosomes for 1hr at 4°C. FACT-IgG beads were washed 3 times in binding buffer (200mM NaCl). 3xSDS loading buffer was
added before boiling. Nucleosomes associated with FACT were then analyzed by western blotting using H3 antibody.

For H2A/H2B dimer pull-down assays, 500ng purified FACT complex were incubated with 500ng of H2A/H2B or H2A/H2BΔHBR dimer in binding buffer (20mM Tris pH 7.4, 150mM NaCl, 1mM MgCl₂, 2mM CaCl₂, 0.1% Triton X-100, 10% glycerol, 0.5mM DTT, 0.5mM PMSF) for 20min at RT. 10μl calmodulin-Sepharose 4B (GE healthcare) were then added to reaction and let bind for another 20min at RT. The resin was washed 3 times in binding buffer and 10μl of 3x SDS-load buffer was added before boiling. The proteins bound to beads were analysis by commassie blue staining.

Detection of non chromatin-bound histones associated with FACT and Asf1

One liter of yeast culture carrying Spt16-myc or Asf1-myc was collected, washed and lysed with glass beads in E-buffer (40 mM HEPES pH 7.5, 0.1% Tween20, 200 mM NaCl, 10% Glycerol, 1mM PMSF, 2ug/ml Leupeptin, 2ug/ml Pepstatin). Spt16-myc or Asf1-myc was immunoprecipitated with IgG-Sepharose beads (IgG-sepharose fast flow, GE healthcare) overnight at 4°C. The immunoprecipitated material was washed three times in IPP150 buffer (10mM Tris-cl pH 8.0, 150mM NaCl, 0.1% NP40), resolved on 15% PAGE-SDS gels and analyzed by western blotting using H3, H2A, and 9E10 antibodies.
Table 1: strains used in this study

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<td>PY021 with Spt16-myc::KanMX</td>
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<td>JR1262</td>
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<td>PY014 with Asf1-myc::KanMX</td>
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<td>H2B Δ30-37, Asf1-Myc</td>
<td>PY020 with Asf1-myc::KanMX</td>
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<td>H2A Δ3-37, Asf1-Myc</td>
<td>PY021 with Asf1-myc::KanMX</td>
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Table 2: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>Gal1 Pro 3 FWD</td>
<td>5’- CGTCAAGGAGAAAAAACTATAATGACTAAATC</td>
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<td>Gal1 Pro 3 REV</td>
<td>5’- TCGGCCAATGGTCTTGGTAA</td>
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<tr>
<td>Gal1 ORF FWD</td>
<td>5’- ACCAAACTGGCAGTGGATTGTCT</td>
</tr>
<tr>
<td>Gal1 ORF REV</td>
<td>5’- GGGCCCATATTCGCTTTAACA</td>
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<td>PYK1 ORF1 FWD</td>
<td>5’- AGGACAAGGAAGATTTGAGATTCG</td>
</tr>
<tr>
<td>PYK1 ORF1 REV</td>
<td>5’- CATCGTTGGCGGTCTGAT</td>
</tr>
<tr>
<td>PMA1 ORF1 FWD</td>
<td>5’- CCGGTACTTTGACCAAGAACAG</td>
</tr>
<tr>
<td>PMA1 ORF1 REV</td>
<td>5’- TCAACATCAAGTCGTCTGGAGAA</td>
</tr>
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<td>RNR3 PRO FWD</td>
<td>5’- TGTCAGCGTTTTATATTGTTTCT</td>
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<td>RNR3 PRO REV</td>
<td>5’- GATGAGAGAAAGGCAAGGAAGAAG</td>
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<tr>
<td>scR1 ORF FWD</td>
<td>5’- TGTCCTGGCAGAGCTGTCT</td>
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<td>scR1 ORF REV</td>
<td>5’- CCCGCAAAGATCGATTTATTATAGC</td>
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Appendix

YEAST RAP1 CONTRIBUTES TO GENOMIC INTERGRITY BY ACTIVATING DNA DAMAGE REPAIR GENES
Abstract

Rap1 (repressor-activator protein 1) is a multifunctional protein that controls telomere function, silencing and the activation of glycolytic and ribosomal protein genes. We have identified a novel function for Rap1, regulating the ribonucleotide reductase (RNR) genes that are required for DNA repair and telomere expansion. Both the C terminus and DNA-binding domain of Rap1 are required for the activation of the RNR genes, and the phenotypes of different Rap1 mutants suggest that it utilizes both regions to carry out distinct steps in the activation process. Recruitment of Rap1 to the RNR3 gene is dependent on chromatin remodeling by SWI/SNF, suggesting that Rap1 acts after remodeling to prevent the repositioning of nucleosomes back to the repressed state. Furthermore, the recruitment of Rap1 requires TAF11S, indicating a role for TFIID in stabilizing activator binding in vivo. We propose that Rap1 acts as a rheostat, controlling nucleotide pools in response to shortened telomeres and DNA damage, providing a mechanism for fine-tuning the RNR genes during checkpoint activation.
**Introduction**

Rap1 (repressor-activator protein 1) is an essential DNA binding protein that regulates transcriptional activation, silencing and the maintenance of telomeres (Morse, 2000). Its ability to maintain telomeres and repress the silent mating type loci is dependent on the recruitment of co-repressors to these regions, which includes the histone deacetylase Sir2 and structural proteins to form heterochromatic-like states (Grunstein, 1997; Shore, 2001). Thus, Rap1 is implicated in regulating chromatin structure at repressed loci. It binds to approximately 5% of yeast genes under optimal growth conditions and is enriched at glycolytic enzyme and ribosomal protein (RP) genes (Lieb et al, 2001). Rap1 binds to a wide variety of DNA binding sites, and it is difficult to identify sites based on sequence information alone. This property is believed to be attributed to its functioning with other DNA binding proteins and cofactors to stimulate transcription in a context-dependent manner (Del Vescovo et al, 2004; Lieb et al, 2001; Pina et al, 2003).

*RNR3* (ribonucleotide reductase 3) encodes one of the subunits of RNR, and it is strongly induced upon DNA damage (Huang et al, 1998; Li & Reese, 2000). In the absence of DNA damage, the sequence-specific DNA binding protein Crt1 (constitutive RNR transcription 1) binds to three binding sites (X-boxes) located in the upstream repression sequences (URS) of the gene (Huang et al, 1998). Crt1 represses transcription by recruiting the Ssn6–Tup1 co-repressor complex to promoter. Checkpoint-dependent phosphorylation and release of Crt1 allows general transcription machinery access to the promoter and activates *RNR3* (Huang et al, 1998; Zhang & Reese, 2004a; Zhang & Reese, 2004b; Zhang & Reese, 2005). Crt1 also has a role in activation through recruiting TFIID
to the promoter (Zhang & Reese, 2005). Thus, Crt1 acts as a transient activator that allows the initial access of transcription factors to the promoter, and disassociates after the promoter is remodeled.

A rate-limiting step in the assembly of pre-initiation complex is the delivery of the TATA-binding protein (TBP) to core promoter (Pugh, 2000; Thomas & Chiang, 2006). Computational analysis of gene expression profiles in TAFII mutants found that a fraction of the genome utilizes TFIID (TAFII8) to deliver TBP to promoters, whereas the others utilizes the TAFII-containing SAGA histone acetyltransferase complex (Huisinga & Pugh, 2004; Lee et al, 2000). RNR3 is characterized as a TFIID dependent gene. Its expression strictly depends upon the TAFII subunits of TFIID, which are required for the recruitment of SWI/SNF and chromatin remodeling (Li & Reese, 2000). However, it contains a consensus TATA-box, a feature of SAGA-regulated core promoters, suggesting that it may utilize both pathways to facilitate the full activation of the gene (Zhang et al, 2008).

In this study I characterized the domains of Rap1 required for the activation of the RNR3 as well as how it coordinates with TAFII8, chromatin remodelers and SAGA to activate transcription. My results show that the DNA binding domain (DBD), and the C-terminal domain of Rap1 are required for RNR3 transcription. Rap1 has multiple binding sites at the Damage Response Element (DRE) of RNR3 promoter; Recruitment of Rap1 to DRE depends on chromatin remodeling activity of Swi/Snf and the integrity of SAGA acetyltransferase complex. Combined with previous data from the lab, we propose a model to show how Rap1 orchestrate multiple steps in activation. We suggest that through interaction with TFIID, Rap1 brings Swi/Snf to the RNR3 promoter to remodel
chromatin. After removal of promoter nucleosomes, Rap1 engages on the remodeled promoter, and acts as a place holder to prevent the reassembly of nucleosomes over the promoter.
Results

The C terminus of Rap1 is required for activation of RNR3

Rap1 contains a number of functional domains, including a BRCT (BRCA1 C Terminus) domain at the N terminus, a myb-like DBD (DNA Binding Domain) in the central third of the protein, and an activation and a silencing domain locating at the C terminus (Figure 7-1A) (Graham et al., 1999; Morse, 2000; Pina et al., 2003). The BRCT domain has been shown to facilitate the transcription activation of glycolytic genes in yeast (Mizuno et al., 2004). The myb-like DNA binding domain is required for the chromatin opening functions of Rap1 (Yu & Morse, 1999) and point mutation within this region greatly impaired the activation of RNR3 (data not shown). The ACT domain, when fused to the DBD of LexA or Gal4, can activate transcription from a minimal TATA containing promoter (Hardy et al., 1992). Since multiple domains of Rap1 are implicated in transcription activation and chromatin remodeling, we analyzed the domain(s) of Rap1 required for the expression of RNR3 by Northern blot analysis. We found that deleting the N terminus of Rap1 containing the BRCT domain (A43–279) had little to no effect on the activation of RNR3 (Figure 7-1B). Thus, the BRCT domain, which is required for the transcription activation of glycolytic genes, is not essential for the expression of DNA damage-inducible genes. However, deleting the C terminus clearly impaired activation of RNR3 (rap1 1-716 and rap 1-695 mutants). The level of activation in the C-terminal mutants was about 20% of that of wild-type cells. This was not due to reduced protein levels because western blotting of extracts from these mutants indicated that the Rap1 mutants were expressed to similar levels as wild-type Rap1 (data not shown). Further
deletion in Rap1 is lethal to yeast (Morse, 2000). These results indicate that the DBD and C terminus of Rap1 are required for the activation of RNR3, while the N terminus is not.

**Rap1 has multiple binding sites at the DRE of RNR3**

The DNA binding property of Rap1 is required for its function in repression or transcription activation. Since Rap1 is required for the activation of RNR3, we sought to determine its binding site at the RNR3 promoter. Examination of the promoter region of RNR3 revealed only one potential binding site for Rap1, located a few base pairs upstream of the translation start codon. However, mutation of that site did not affect RNR3 expression or Rap1 recruitment (data not shown). As the DRE (Damage Response Element) confers DNA damage-dependent transcription to RNR3 (Endo-Ichikawa et al, 1996), we focused on this region. Promoter constructs lacking the entire DRE region or the 5' and 3' halves were constructed and integrated into the RNR3 locus (Figure 7-2A). ChIP assays were conducted and the results show that deleting the entire DRE severely compromised the recruitment of Rap1, TBP, and the activation of RNR3 (Figure 7-2B,C and D). Deleting either the 5' or 3' half of the DRE only partially reduced Rap1 crosslinking, suggesting that Rap1 may bind to multiple regions with this region. Interestingly, deleting the 5' end of the DRE essentially abolished TBP recruitment and the activation of RNR3, yet some crosslinking of Rap1 was detected. Thus, full Rap1 occupancy is required for activation.
Rap1 binding is not required for TAFII\textsubscript{11} recruitment

Mutating the Rap1 binding site strongly reduced the MMS-induced crosslinking of TBP to the promoter, and the level of PIC formation correlated well with the levels of \textit{RNR3} mRNA in each mutant, suggesting that Rap1 plays a role in PIC formation. TBP is not recruited to \textit{RNR3} in the promoter mutants, which abolished Rap1 binding, suggesting that TAFII\textsubscript{11}s are not recruited. However, TAFII\textsubscript{11}s can be recruited to Rap1 sites in the absence of functional TBP (Li et al, 2002b; Mencia et al, 2002). To determine if Rap1 is required for TAFII\textsubscript{11} recruitment, we examined the crosslinking of two TFIID-specific TAFII\textsubscript{11}s to \textit{RNR3} in the Rap1 mutants which impairs \textit{RNR3} transcription (Figure 7-3).

\textit{rap1-2} bears a point mutation within the DBD, while the \textit{rap1-716} has the C terminal of Rap1 deleted. \textit{RNR3} transcription was reduced to less than 20\% of the wild type level in either mutant. Remarkably, TAFII\textsubscript{11} recruitment was only weakly, if at all, affected in these mutants. This was unexpected given the belief that Rap1 recruits TFIID (Garbett et al, 2007; Li et al, 2002b; Mencia et al, 2002). As a control, we examined TAFII\textsubscript{11} recruitment to \textit{RPS11B}, a ribosomal gene in the Rap1 mutants and found that TAFII\textsubscript{11} recruitment was not affected at this bona fide target gene in the mutants. These results suggest that although Rap1 sites are required for TAFII\textsubscript{11} recruitment to promoters, the requirement for Rap1 itself is unclear.

SWI/SNF-dependent recruitment of Rap1

The TAFII\textsubscript{11} subunits of TFIID are required for the recruitment of SWI/SNF and chromatin remodeling at \textit{RNR3} (Sharma et al, 2003). We asked if Rap1 recruitment requires chromatin remodeling. ChIP assays were carried out in a \textit{Δsnf2} mutant, which
was defective for remodeling of RNR3 (Sharma et al, 2003). Figure 7-4 clearly showed that SWI/SNF and chromatin remodeling are required for Rap1 recruitment. Since RNR3 promoter contains a consensus TATA-box, a feature of SAGA-regulated core promoters, we examined the requirement for SAGA by analyzing the recruitment of Rap1 in Δgen5 and Δspt7 mutants. SAGA acetylates nucleosomes through its catalytic subunit Gcn5, while Spt7 is involved in proper assembly of the complex (Sterner et al, 1999; Wu et al, 2004). SPT7 deletion strongly reduced the crosslinking of Rap1 to RNR3 promoter, while GCN5 did not (Figure 7-4). SPT7, and thus SAGA integrity, is required for the recruitment of Rap1 to RNR3. Further, Rap1 recruitment is slightly higher in the Δgen5 mutant compared with the other strains. Interestingly, we have previously observed increased SWI/SNF recruitment to RNR3 in the Δgen5 mutant, which suggests a striking correlation between SWI/SNF and Rap1 recruitment.

Crt1 and Rap1 are both required for the activation of RNR3. We characterized the interplay between these two proteins by examining the requirement for the activation function of Crt1 in Rap1 recruitment. Previous study from the lab has identified derepression-defective Crt1 mutants that are repression competent but fail to activate transcription and recruit TFIID and SWI/SNF (Zhang & Reese, 2005). The recruitment of Rap1 was analyzed in one of these mutants, crt1Δ162–172, and it was found to be blocked in this mutant (Figure 7-4). This suggests that the activation function of Crt1 is required for Rap1 recruitment. Together, these data show that chromatin remodeling is required for Rap1 recruitment, suggesting that Rap1 binds after remodeling has occurred. The expression of Rap1 was not significantly altered in any of the regulatory mutants.
Discussion

Rap1 orchestrates multiple steps in activation

Rap1 is one of the most abundant and ubiquitous transcription factors in yeast. Most of its functions are related to controlling chromatin structure. Rap1 has also been shown to specify the use of TFIID at promoters, although how it functions with TFIID at repressed genes to achieve a remodeled, active state is not known. Here we used the repressed and highly inducible RNR3 gene to characterize the roles of Rap1, TFIID and the chromatin remodeling machinery in transcription. We provide evidence that Rap1 utilizes multiple domains to carry out distinct steps in opening chromatin at the promoter and maintaining this state to allow for high levels of RNR3 transcription. Based on our work to date, we propose a complete picture of how RNR3 is activated (Figure 7-5): (1) the binding of the Crt1–Ssn6–Tup1 complex establishes a repressive chromatin state, and DNA damage signals convert Crt1 to a form that causes the release of the corepressor, possibly by phosphorylation; (2) the activation domain of Crt1 is exposed, recruiting TFIID and the SWI/SNF complex; (3) remodeling occurs, and Rap1 is then able to engage the promoter; (4) Rap1 and TFIID undergo reciprocal interactions to maintain SWI/SNF recruitment and PIC assembly. The C terminus of Rap1 is required for PIC formation and transcription. The DBD of Rap1, additionally, maintains the remodeled state by binding to the promoter to resist the reassembly of nucleosomes over its binding site and to recruit SWI/SNF. The first and second steps of this model are supported by previous work from our lab identifying the derepression-defective mutants of Crt1 and the proof of a physical interaction between Crt1 and the TFIID and SWI/SNF complexes.
(Zhang & Reese, 2005). The third step is suggested by our data showing that Rap1 recruitment is impaired in the derepression-defective Crt1 mutant that does not remodel the promoter, and in mutants of SWI/SNF and SAGA. The final part of the model is consistent with our characterization of the roles of the functional domains of Rap1 in \textit{RNR3} activation.
Acknowledgement

We are grateful to Raghuvir Tomer for initial characterization of the Rap1 mutants. We thank Dr. David Shore for providing the Rap1 mutants and discussions. Members of the Reese lab and the Center for Eukaryotic Gene Regulation at The Pennsylvania State University are acknowledged for advice and comments on this work. Marta Mendoza and Jeong-Seon Kim are acknowledged for constructing promoter mutants in Figure 7-2 and Deborah Brunke-Reese is acknowledged for performing Northern Blot in Figure 7-2D. This research was supported by funds provided by the National Institutes of Health (GM58672) to JCR.
Figure Appendix-1: The C-terminal domain of Rap1 is required for activation of DNA damage-inducible genes.

(A) A schematic of the known protein domains in Rap1. BRCT is the Breast cancer susceptible gene C Terminal domain, a phospho-protein binding domain found in many DNA damage response proteins; DBD is the myb-like DNA binding domain; TOX is the region identified to relieve the toxicity caused by Rap1 overexpression; ACT is the domain that can activate transcription when fused to the DBD of LexA. SIL is the silencing domain, which is known to bind Sirs and Rif1/2;

(B) Northern blotting for RNR3 mRNA. Wild type, Rap1 truncation and C terminal deletion mutants were grown and harvested at different time points after exposure to 0.02% MMS. mRNA levels were detected by northern blot and corrected for a loading control scR1.
Figure Appendix-2. The DRE mediates Rap1 recruitment.

(A) Promoter constructs containing deletions in the DRE were constructed in vitro and used to replace the endogenous RNR3 locus. The black ovals indicate the positions of the X-boxes that bind the repressor Crt1. With the start site of translation=+1 and the TATA box ('T') at -126, the deletions are as follows: ΔDRE, Δ-187/-548; ΔAM, Δ-187/-259; ΔMA, Δ-259/-548.

(B) ChIP assay in untreated (MMS-, white bars) and MMS-treated (MMS+, black bars) strains containing Rap1-myc. The immunoprecipitated DNA was amplified using primers directed to the promoter of RNR3. The promoter was not altered by the deletions in the URS region.

(C) Same as (B) except TBP recruitment was examined over the promoter.
Figure Appendix-3. TAF\textsubscript{II} recruitment to \textit{RNR3} was not affected in RAP1 mutants. 
(A and B) Cells were treated or not with MMS for 2.5 h before crosslinking with formaldehyde. TAF1 (A) and TAF4 (B) recruitment were measured over \textit{RNR3} promoter in Rap1 mutants indicated below the panel. 
(C) Crosslinking of TFIID-specific TAF\textsubscript{II}s to RPS11B. Polyclonal antisera to TAF1 (white bars) and TAF4 (black bars) were used in IP. Primers flanking the UAS of \textit{RPS11B} were used to amplify the immunoprecipitated DNA.
Figure Appendix-4. Chromatin remodeling is required for Rap1 recruitment. Recruitment of Rap1 to RNR3 promoter in SAGA, SWI/SNF mutants and a derepression-defective mutant of Crt1. Polyclonal antiserum (yN-18) to Rap1 was used in the IP.
Figure Appendix-5. Proposed model of Rap1 functions with Crt1 to open promoters. 

1) The binding of the Crt1–Ssn6–Tup1 complex establishes a repressive chromatin state, and DNA damage signals convert Crt1 to a form that causes the release of the corepressor; 
2) the activation domain of Crt1 is exposed, recruiting TFIID and the SWI/SNF complex; 
3) remodeling occurs, and Rap1 is then able to engage the promoter. Rap1 and TFIID undergo reciprocal interactions to maintain SWI/SNF recruitment and prevent the reassembly of nucleosome over promoter; 
4) PIC assemble and transcription starts. The C terminus of Rap1 is required for PIC formation and transcription.
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