ANALYSIS OF THE HISTONE N-TERMINI IN TRANSCRIPTIONAL REGULATION

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by

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ABSTRACT

The genetic material of eukaryotic cells is packaged into chromatin. DNA is wrapped around histone proteins to form nucleosomes, the basic repeating unit of chromatin. The histone N-termini protrude from the nucleosomal core and are extensively modified during transcription. Many transcription factors harbor domains that will interact with the modified histone N-termini. Additionally, modification of the N-termini can alter the charge of the basic residues resulting in the unfolding of higher order chromatin structures. In S. cerevisiae, the histone N-termini are nonessential and this has allowed for investigations into how N-termini mutations affect the regulation of transcription. Initial characterizations of histone N-termini mutants revealed transcription defects; however, the mechanism of these defects has not been examined. In this study, we explore roles for the histone N-termini in transcription initiation, elongation, termination, and mRNA processing.

Using the DNA damage-inducible \textit{RNR3} gene, we explore how deletions and lysine to glutamine substitutions would affect transcription induction. We establish that the H2A, H3, and H4 N-termini are important for \textit{RNR3} activation. Interestingly, we observe that these transcription defects are the results of different mechanisms. For example, we demonstrate that the H4 N-terminus, specifically, is required for the release of the Crt1-Ssn6-Tup1 corepressor upon DNA damage. Additionally, deletion of the H4 N-terminus results in constitutive SWI/SNF recruitment in a mechanism that likely involves the loss of Isw2 function. Deletion of the H2A N-terminus results in a significant reduction of H3 lysine 4 tri-methylation. The methylation loss in this mutant is suggestive of another form of trans-tail regulation of histone modifications. Our characterization of the histone N-termini in transcription initiation revealed possible roles for the N-termini in transcription elongation. We demonstrate that deletion of the H3 N-terminus or
substitution of the H4 lysine to glutamines result in a shift of RNA polymerase density that is indicative of a transcription elongation defect.

In the second part of this study, we explore how mutations to the H4 N-terminus affect transcription elongation. Substituting all of the H4 N-terminal lysine residues for glutamines results in genetic interactions with elongation factors and suggests that the H4 lysines have roles in elongation. Interestingly, mutation of only three lysines did not result in growth defects. This implies redundancy among the acetylable lysines. We also demonstrate that the H4 N-terminus is important for the repression of intragenic transcription at *FLO8*. This repression mechanism is separate from the Set2-Rpd3 pathway and likely involves the elongation factors Spt6 and Spt16. Further examination revealed novel roles for the H4 N-termini in transcription termination at snoRNAs as well as mRNA splicing. These results suggest that the H4 N-terminus is an important regulator throughout transcription.

The final section of this study involves the characterization of novel intra-tail regulation of Set2 by the H3 N-terminus. Deletion of the H3 N-terminus, or substitution of the lysine residues within the tail for glutamine, results in significant defects in H3 lysine 36 tri-methylation. We demonstrate that the H3 N-terminus is not required for the recruitment of Set2, but does stimulate Set2 activity. Furthermore, we demonstrate that the charge of the H3 N-terminus is important for Set2 activity. We propose a mechanism by which Set2 activity is regulated by novel intra-tail interactions within H3, which can be regulated by modifications to the tail.

It has been the goal of this study to better understand the mechanisms by which the histone N-termini regulate transcription. Initially, we expected the histone N-termini to regulate transcription initiation predominantly. However, our characterization of initiation led us to the hypothesis that the histone N-termini regulate multiple facets of transcription. Experiments to test this hypothesis revealed novel roles for the H4 N-terminus in elongation, termination, and mRNA
splicing. Additionally, we identified novel intra-tail regulation of Set2 activity and characterized the mechanism.
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Chapter 1

TRANSCRIPTIONAL REGULATION BY CHROMATIN STRUCTURE AND MODIFICATION
I. Chromatin Dynamics Regulate Transcription Initiation

Eukaryotic cells package their genetic material into chromatin. Chromatin must therefore act as the backdrop for DNA replication, repair, recombination, and transcription. The factors responsible for these processes depend on access to DNA, which is modulated by the dynamics of chromatin structure. The dynamics of chromatin structure during transcription are altered through the concerted actions of chromatin remodeling complexes, chromatin modifying complexes, and elongation factors.

Chromatin Remodeling

Chromatin remodeling and nucleosome positioning are performed by a variety of enzyme complexes that use the energy of ATP to break histone-DNA contacts. Chromatin remodeling complexes have been classified into four major categories based on the homology of their ATPase subunits. These categories are the SWI/SNF family, the Imitation Switch (ISWI) family, the INO80/SWR family, and the Chd/Mi2 family (Becker & Horz, 2002, Kingston & Narlikar, 1999, Krogan et al, 2004). The SWI/SNF and ISWI families of chromatin remodeling complexes are the most ubiquitous and thus have been studied more extensively.

The classical example of the SWI/SNF family is the yeast SWI/SNF complex. Yeast SWI/SNF is composed of 8-15 subunits that were originally identified for defects in mating type switching (Peterson et al, 1994, Smith et al, 2003). SWI/SNF remodels nucleosomes by breaking histone-DNA contacts, which causes DNA loop formation and histone eviction and results in greater access to DNA for transcription factors (Martens & Winston, 2003). SWI/SNF is recruited to promoters by upstream activators and has been shown to stimulate transcription both in vivo and in vitro (Cote et al, 1994, Neely et al, 1999, Yudkovsky et al, 1999). Furthermore, SWI/SNF
is co-purified with the RNA polymerase II (RNAPII) holoenzyme (Wilson et al, 1996). While SWI/SNF components are nonessential in \textit{S. cerevisiae}, the complex is required for the transcription of a subset of genes including the DNA damage inducible \textit{RNR3} (Holstege et al, 1998, Sharma et al, 2003). The SWI/SNF ATPase containing subunit Swi2 also contains a bromodomain (Vignali et al, 2000). Bromodomains are thought to bind acetylated lysines on the histone N-termini, a modification often associated with activation. It has been demonstrated \textit{in vitro} that SWI/SNF can remodel tailless nucleosomes as well as wild type nucleosomes (Logie et al, 1999). However, histone eviction from tailless nucleosome arrays was less efficient (Logie et al, 1999).

The second major category of chromatin remodeling complexes is the ISWI family. This family was serendipitously identified and characterized in \textit{D. melanogaster} and given the name Imitation Switch based on its similarity to the \textit{D. melanogaster} SWI/SNF (Elfring et al, 1994). \textit{S. cerevisiae} have two genes that code for ISWI family members: \textit{ISW1} and \textit{ISW2} (Tsukiyama et al, 1999). Isw1 is found in two distinct complexes, Isw1a and Isw1b, which have distinct functions in the cell presumably due to differences in their ability to bind DNA and nucleosome substrates (Vary et al, 2003). Most evidence points to repressive roles for the ISWI family of chromatin remodelers. Deletion of either ISW1 or ISW2 results in derepression of genes and this has been the prevailing view of the ISWI family (Mellor & Morillon, 2004). More recently however, positive roles in transcription initiation, elongation, and termination have been identified for Isw1 (Morillon et al, 2003, Morillon et al, 2005, Santos-Rosa et al, 2003). The mechanism of Isw2 chromatin remodeling is thought to involve sliding of nucleosomes, which results in a translational shift in position (Fazzio & Tsukiyama, 2003). A small basic patch on the H4 N-terminus is important for Isw2 activity and this links the H4 N-terminus to Isw2 mediated chromatin structure (Dang et al, 2006, Fazzio et al, 2005).
Chromatin remodeling complexes modulate the access of the general transcription machinery to the promoters of genes. Promoter sequences can be wrapped within nucleosomes which can form higher order repressive chromatin structures that preclude the recruitment of the general transcription machinery. Alternatively, promoter sequences can lie within nucleosome free regions that allow for uninhibited factor binding. Chromatin remodeling complexes create and alter these environments. Their activities are recruited by a number of mechanisms. Sequence specific transcription activators and repressors can function to recruit chromatin remodeling complexes. SWI/SNF, for example, is targeted for recruitment by several activators (Cote et al, 1994, Neely et al, 1999, Yudkovsky et al, 1999). Another mechanism for recruitment involves histone modifications. SWI/SNF harbors a bromodomain that is capable of binding acetylated histone tails and this interaction allows SWI/SNF to remain bound to the promoter even after the activator is no longer present (Hassan et al, 2001). The final method for chromatin remodeling complex recruitment involves global untargeted associations. Isw2 is pervasive in the cell and can be found, by chromatin immunoprecipitation, at genomic loci that do not strictly depend on Isw2 for chromatin structure. This has led to the hypothesis that in addition to targeted activity, Isw2 transiently scans the genome for targets until one is found (Gelbart et al, 2005). We present data here that suggests the untargeted recruitment of SWI/SNF to RNR3 in the absence of DNA damage, when the H4 N-terminus is deleted (Tomar et al, 2009).

Chromatin Modifications during Initiation

Histone modifications involve covalent chemical additions to amino acid side chains. Histone acetylation and methylation were first identified over 45 years ago (Allfrey et al, 1964). Since then, histone modifications have been studied extensively and the variety of modifications has grown significantly to include phosphorylation, ubiquitylation, sumoylation, ADP
ribozymylation, deimination, and proline isomerization in addition to acetylation and methylation (Kouzarides, 2007). These modification types have all been associated with transcription and some are also associated with DNA repair, replication, and chromatin condensation. With regard to transcription, the same type of modification can be associated with transcriptionally active genes or transcriptionally silent genes based on which histone residue is modified. For instance, histone H3 lysine 4 methylation is found at active promoter regions while H3 lysine 9 and 27 is found to be predominantly repressive (Fischle et al, 2003, Strahl et al, 1999). The possible permutations of histone modifications are staggering considering the variety of modification sites and types. This has led to the proposal of a histone code, which suggests that transcription regulatory factors recognize specific histone modifications and are then recruited to the sites of modification (Jenuwein & Allis, 2001). While the rules for such a code are unlikely to be absolute, great progress has been made in understanding the roles of histone modifications in transcriptional regulation.

Lysine acetylation is the best characterized histone modification and is closely associated with transcription activation. Its roles in activation are twofold: the recruitment of transcription factors that recognize the acetylated lysine residues, and the disruption of repressive chromatin structure. Bromodomains have been shown to bind acetylated lysines on the histone N-termini and these domains are prevalent in transcriptional regulatory proteins. SWI/SNF, the SAGA histone acetyltransferase (HAT), and Bdf1 all harbor bromodomains that are important for the functions of these proteins (Durant & Pugh, 2007, Li & Shogren-Knaak, 2009, Syntichaki et al, 2000, Vignali et al, 2000). SWI/SNF, SAGA, and Bdf1 represent three different categories of proteins involved in transcription activation. This underscores the importance of lysine acetylation in the coordination of transcription initiation.

In addition to the recruitment of bromodomain containing factors, lysine acetylation serves to open chromatin. The positively charged histone tails are involved in the formation of
higher order chromatin structure that is repressive to transcription (Kan et al, 2009, Wang & Hayes, 2008, Zheng & Hayes, 2003). Furthermore, in vitro acetylation mimics inhibit the formation of higher order structure (Wang & Hayes, 2008). Between breaking the interactions required for higher order structure and the recruitment of transcription factors, histone lysine acetylation is a powerful mechanism to activate and maintain transcription.

H3 lysine 4 methylation is another well characterized histone modification that is associated with transcription initiation. This modification is catalyzed by the Set1 methyltransferase that is part of the COMPASS complex (Krogan et al, 2002a, Miller et al, 2001). H3 lysine 4 methylation is found predominantly at the promoter and 5’ ends of genes. In S. cerevisiae, this mark is important for the recruitment of Isw1 (Santos-Rosa et al, 2003). In higher eukaryotes, H3 lysine tri-methylation recruits the Chd1 chromatin remodeling complex and is important for mRNA processing (Sims et al, 2007).

The activity of COMPASS is extensively regulated. Unlike most histone acetyltransferases which can promiscuously acetylate multiple lysine residues, COMPASS can only methylate H3 lysine 4. Furthermore, the ability of COMPASS to tri-methylate is dependent upon another histone modification, H2B lysine 123 ubiquitylation (Dover et al, 2002, Lee et al, 2007). The Cps35 subunit of COMPASS specifically recognizes the mono-ubiquitylated H2B and this interaction results in COMPASS being tri-methylation competent (Lee et al, 2007). This type of regulation is an example of histone “crosstalk” or trans-tail regulation. Also regulating COMPASS activity is a form of negative intra-tail regulation. H3 arginine 2 methylation and lysine 4 methylation are mutually exclusive (Kirmizis et al, 2007). It is believed that methylation of H3 arginine 2 obstructs the COMPASS active site from lysine 4 (Kirmizis et al, 2007). This extensive regulation of histone modification is a theme that we explore.
II. Transcription Elongation and Chromatin Structure

Transcription elongation, like initiation, involves dynamic changes in chromatin structure. With each passage of RNA polymerase II, chromatin structure must be disrupted before, and reassembled afterward. These events are coordinated by differential phosphorylation of the RNA polymerase II C-terminal domain. These phosphorylation events serve to recruit elongation factors as well as histone modifying enzymes that are involved in the regulation of transcription elongation.

RNA Polymerase II C-terminal Domain Phosphorylation

The large subunit of RNA polymerase II (RNAPII), Rpb1, has a large C-terminal domain (CTD) that extends out from the polymerase core. This CTD is composed of consensus heptad repeats, 26 in yeast and 52 in humans, that harbor serine, threonine, and tyrosine residues that are the sites of phosphorylation (Phatnani & Greenleaf, 2006). In *S. cerevisiae*, phosphorylation of serine 5 (Ser5) and serine 2 (Ser2) are the best characterized and the differential patterning of these phosphorylation events is known to regulate a variety of elongation processes (Phatnani & Greenleaf, 2006).

Once RNAPII is loaded onto the promoter, the Kin28 subunit of TFIIH begins to phosphorylate Ser5 of the RNAPII CTD (Dahmus, 1995, Phatnani & Greenleaf, 2006, Rodriguez et al, 2000). Once approximately 30 base pairs have been transcribed, the transition from initiation to elongation has occurred. As elongation continues, Ctk1 begins to phosphorylate Ser2. In addition to the Kin28 and Ctk1 kinases, RNAPII CTD phosphorylation patterns are affected by the Ssu72 and Fcp1 CTD phosphatases (Cho et al, 2001, Krishnamurthy et al, 2004, Reyes-Reyes
RNAPII CTD Ser5 phosphorylation is important for pre-mRNA processing (Shuman, 1997). Additionally, Ser5 and Ser2 phosphorylation are important for the interaction with the Polymerase Associating Factors (PAF) complex (Hampsey & Reinberg, 2003). PAF then coordinates both Set1 and Set2 mediated methylation of histone H3. RNAPII CTD kinases and phosphatases are also important for transcription termination events (Egloff et al, 2007, Ganem et al, 2003, Sheldon et al, 2005). In addition to Ser5 and Ser2, Ser7 phosphorylation has recently been demonstrated to coordinate the recruitment of snoRNA specific termination factors (Egloff et al, 2007). The patterns of RNAPII CTD phosphorylation across genes serve to synchronize transcription with chromatin structure and mRNA processing.

Transcription Elongation Factors

Transcription elongation factor is a broad term that describes proteins which facilitate elongation. Several such factors were first identified as high copy suppressors of the Ty and delta retrotransposons (Clark-Adams & Winston, 1987, Malone et al, 1991, Swanson et al, 1991). Spt4, Spt5, Spt6, and Spt16 are all known to be important for transcription elongation and roles in initiation, termination, and mRNA processing have been reported as well (Adkins & Tyler, 2006, Hartzog et al, 1998, Kaplan et al, 2000, Malone et al, 1991). The PAF complex is another elongation factor with roles throughout transcription and was originally identified through its association with RNAPII and Spt16 (Krogan et al, 2002b).

After its original identification as a high copy suppressor of the Ty and delta retrotransposons, Spt16 was found to interact with Pob3 to form the Facilitates Chromatin Transcription complex (FACT) complex (Belotserkovskaya et al, 2003, Orphanides et al, 1999). The major role of FACT is thought to involve the disassembly and reassembly of chromatin during transcription and FACT interacts with RNAPII (Krogan et al, 2002b). In vitro FACT will
bind nucleosomes as well as H2A/H2B dimers, but not the H3/H4 tetramer (Belotserkovskaya et al, 2003). This has supported a model in which FACT removes and then replaces the H2A/H2B dimer during transcription. Spt16 conditional mutants result in cryptic intragenic transcripts presumably due to the inability to reassemble repressive chromatin after the passage of RNAPII (Kaplan et al, 2003). Although FACT is thought to interact with the H2A/H2B dimer, mutation to the core domain of H3 results in altered Spt16 crosslinking throughout the coding region, suggesting a direct and physical interaction between FACT and H3 (Duina et al, 2007).

Spt6, like the FACT complex, is important for maintaining chromatin structure during elongation and associates with RNAPII (Hartzog et al, 1998, Kaplan et al, 2000, Krogan et al, 2002b). Human Spt6 stimulates RNAPII transcription \textit{in vitro} (Endoh et al, 2004). Like FACT, Spt6 is thought to be important for the disassembly and reassembly of chromatin during elongation. Spt6 interacts with H3 and functions as a chaperone during elongation (Bortvin & Winston, 1996). More recently, mammalian Spt6 has also been shown to interact with the Ser2 phosphorylated RNAPII CTD; although functions for Spt6 in initiation, in the absence of Ser2 phosphorylation, have also been identified (Adkins & Tyler, 2006, Yoh et al, 2007). Yeast Spt6 interacts with RNAPII as well; however, it is unclear whether this interaction is dependent upon CTD phosphorylation (Krogan et al, 2002b). The interaction of Spt6 with RNAPII is likely to mediate its roles in Set2 activity, in transcription termination at the GAL10-GAL7 locus, and in splicing in higher eukaryotes (Kaplan et al, 2005, Yoh et al, 2007, Youdell et al, 2008).

DRB Sensitivity Inducing Factor (DSIF) is composed of Spt4 and Spt5 (Hartzog et al, 1998, Wada et al, 1998). Unlike FACT and Spt6, DSIF does not act directly on chromatin structure. It is however involved in the recruitment of the PAF complex which modulates histone methylation (Qiu et al, 2006). DSIF is involved in coordinating a variety of cotranscriptional processes in addition to PAF recruitment. Coimmunoprecipitation experiments reveal that Spt5 interacts with FACT, Spt6, TFIIS, and mRNA capping enzymes (Lindstrom et al, 2003).
Furthermore, DSIF is found in three distinct RNAPII complexes (Lindstrom et al, 2003). These interactions suggest that DSIF functions to coordinate different aspects and stages of transcription. Spt4/Spt5 mutants result in elongation, splicing, and termination defects (Reyes-Reyes & Hampsey, 2007, Sheldon et al, 2005, Xiao et al, 2005).

Originally, the PAF complex was identified through its interactions with RNAPII (Krogan et al, 2002b). It is composed of five subunits: Paf1, Rtf1, Leo, Ctr9, and Cdc73 (Krogan et al, 2002b). Most notably, PAF coordinates patterns of histone methylation during transcription elongation (Chu et al, 2007a, Laribee et al, 2005). At the 5′ ends of genes, PAF stimulates H2B lysine 123 ubiquitylation, a prerequisite for H3 lysine 4 methylation (Laribee et al, 2005). Within the gene, the PAF complex is important for H3 lysine 36 tri-methylation (Chu et al, 2007a). In addition to histone methylation during elongation, PAF has been implicated in transcription termination of snoRNAs in a mechanism not involving methylation. PAF associates with, and is required for, the recruitment of the Nrd1 termination factor (Sheldon et al, 2005). This establishes roles for the PAF complex ranging from histone modification at the promoter to transcription termination.

**Chromatin Modification during Elongation**

The chromatin modification most closely associated with transcription elongation is methylation of H3 lysine 36. This mark is catalyzed by the Set2 methyltransferase during transcription elongation (Strahl et al, 2002). Set2 was originally characterized as a nucleosome selective methyltransferase (Strahl et al, 2002). This observation implies that nucleosomal structure plays an important role in Set2 activity. Subsequent reports have indeed demonstrated this importance. In an example of trans-tail regulation, a region of the Set2 SET domain interacts with a region of the histone H4 core domain and this region of H4 is important for Set2 activity.
set2 activity depends on the isomerization state of h3 proline 38; this provides an example of intra-tail regulation (nelson et al, 2006). this evidence emphasizes the structure of the nucleosome as a regulator of set2 activity.

set2 is directed to coding regions through an interaction with the rnapii ctd (morillon et al, 2005, morris et al, 2005, rao et al, 2005, xu et al, 2008). ctk1 mediated ser2 phosphorylation results in the recruitment of set2 to the middle and 3` ends of genes (keogh et al, 2005, kizer et al, 2005, krogan et al, 2003). the set2 c-terminal sri (set2-rnapii interaction) domain is important for this interaction (kizer et al, 2005). ctk1 has also been reported to be important for set2 stability, although a conflicting report exists (du et al, 2008, youdell et al, 2008). in the former case, the authors demonstrate that a Δctk1 strain results in the destabilization of set2 and a loss of h3 lysine 36 methylation (youdell et al, 2008). they hypothesize that without the sri domain-rnapii ctd interaction, set2 is rapidly degraded. these results provide an interesting mechanism whereby set2 activity is regulated two-fold by ctk1. the phosphorylation state of rnapii determines the location of set2 activity, as well as the amount of cellular set2. in addition to rnapii ctd phosphorylation, set2 activity depends on the paf complex and sp6, two elongation factors that associate with the rnapii ctd (chu et al, 2007a, youdell et al, 2008).

h3 lysine 36 tri-methylation is found predominantly within the middle and 3` ends of genes. the role of this mark in the repression of intragenic transcription is well established (carrozza et al, 2005, joshi & struhl, 2005, keogh et al, 2005, li et al, 2007b). during transcription elongation, nucleosomes are acetylated within orf regions in order to disrupt chromatin ahead of rnapii. if the histones are left acetylated, chromatin structure remains disrupted and transcription factors are able to bind within genes. this can lead to cryptic intragenic transcription. h3 lysine 36 tri-methylation is recognized by the rpd3 histone
deacetylase complex (Carrozza et al, 2005, Li et al, 2007b). Following RNAPII passage, Rpd3 will deacetylate nucleosomes and repressive chromatin can be restored.

In addition to repressing intragenic transcription, Set2 has been shown to repress the spreading of heterochromatin in *S. cerevisiae* by a mechanism independent of Rpd3; however, much has yet to be elucidated about this pathway (Tompa & Madhani, 2007). In higher eukaryotes, Set2 mediated H3 lysine 36 methylation levels are found to occur in peaks and troughs (Kolasinska-Zwierz et al, 2009). The peaks correspond to exons and the troughs to introns. Interestingly, constitutive exons have higher levels of methylation than alternative exons (Kolasinska-Zwierz et al, 2009). These results suggest that chromatin modifications may be important for splicing or the determination of splice variants.

H3 is also methylated at lysine 79 during transcription elongation. The Dot1 methyltransferase catalyzes this reaction throughout the coding region of actively transcribed genes (Lacoste et al, 2002, Ng et al, 2002). Mutation of Dot1 results in the disruption of telomere silencing and it was named for this phenotype (Ng et al, 2002). Methylation of lysine 79 inhibits the binding of Sir proteins which are responsible for maintaining heterochromatin at the telomeres (Martino et al, 2009). In the absence of Dot1, Sir proteins will bind throughout the genome (Lacoste et al, 2002). It is this diffusion away from the telomeres that is responsible for the loss of silencing. Dot1 methylation is also involved in cell cycle checkpoints and the DNA damage response (Lacoste et al, 2002, Martino et al, 2009, Ng et al, 2002). H3 lysine 79 methylation is regulated by two trans-tail regulatory pathways. Similar to Set1, Dot1 tri- and di-methylation requires H2B lysine 123 ubiquitylation (Lee et al, 2007, Shahbazian et al, 2005). Furthermore, Dot1 activity requires an interaction with the H4 N-terminus of a neighboring nucleosome (Fingerman et al, 2007).
III. Transcription Termination Events

The regulation of transcription termination is important in order to avoid interference with downstream genes and properly process the 3’ ends of the nascent RNA. RNAPII is responsible for the transcription of several classes of RNAs including mRNAs, sn- and snoRNAs (small nuclear and small nucleolar), and CUTs (Cryptic Unstable Transcripts). The transcription termination processes differ for each of these classes of RNAs (Kim et al, 2006, Lykke-Andersen & Jensen, 2007). While some of the components of the termination machinery are shared, there are factors specific for the different mechanisms (Kim et al, 2006, Lykke-Andersen & Jensen, 2007). The major differences involve the decision of how to process the 3’ end of the RNA: with or without poly-adenylation.

During mRNA transcription, the termination decision is based on the sequence of the nascent RNA. As the polyadenylation signal emerges from RNAPII, the cleavage and polyadenylation factor (CPF), cleavage factor IA (CFIA), and cleavage factor IB (CFIB) recognize the signal (Birse et al, 1998, Dichtl et al, 2002, Kim et al, 2006). As a result, the RNA is cleaved and polyadenylated. Termination of transcription occurs shortly after this event and there are two proposed models for transcription termination: the allosteric model and the torpedo model. The allosteric model involves Pcf11, a component of CFIA, dismantling RNAPII by causing conformational changes (Sadowski et al, 2003, Zhang et al, 2005, Zhang & Gilmour, 2006). This results in the slowing and eventual termination of transcription. The torpedo model suggests that after RNA cleavage, the exposed 5’ end of the RNA can be quickly degraded by the Xrn2 exonuclease (Bentley, 2005, Buratowski, 2005). This results in RNAPII dismantling. These models are not mutually exclusive, and in either case, the termination events depend on the recognition of the polyadenylation signal.
The termination events and machinery at sn- and snoRNAs loci are different from mRNAs (Kim et al, 2006). These RNAs have a Nrd1 terminator element as opposed to a polyadenylation signal (Conrad et al, 2000, Steinmetz et al, 2001). The Nrd1 terminator can consist of several Nrd1/Nab3 binding sites (Conrad et al, 2000, Steinmetz et al, 2001). The Nrd1/Nab3 complex interacts with the phosphorylated RNAPII CTD and will recognize the Nrd1 terminator (Carroll et al, 2004, Carroll et al, 2007, Steinmetz & Brow, 1998). Presumably, this RNA-Nrd1/Nab3-CTD bridge results in RNAPII termination. At this point, the nuclear RNA exosome degrades the ends of the sn- and snoRNAs until it reaches the Nrd1/Nab3 complex which protects the 3’ end (Allmang et al, 1999, van Hoof et al, 2000). The Nrd1/Nab3 complex acts to protect the RNA similarly to poly-A binding proteins for mRNAs. CUTs, which have recently been implicated in transcription interference, follow a similar pathway of termination as sn- and snoRNAs (Lykke-Andersen & Jensen, 2007). However, as their name indicates, they are unstable due to a lack of protection afforded to sn- and snoRNAs.

Several of the factors involved in these distinct termination mechanisms are found at mRNAs as well as sn- and snoRNAs. Pcf11 and Nrd1 are two examples (Kim et al, 2006, Steinmetz et al, 2006b). Additionally, reports that suggest at least some snoRNAs are transiently polyadenylated blur the lines between mRNA and snoRNA termination. This has led to the hypothesis that RNAPII carries with it the machinery for both types of termination and that there is an ability to choose one versus the other (Kim et al, 2006, Steinmetz et al, 2006a). Recently, it has been proposed that the length of the RNA will determine which termination pathway it will follow: shorter RNAs use the Nrd1/Nab3 pathway while longer RNAs utilize polyadenylation. The phosphorylation status of Ser5 on the RNAPII CTD is another determinant of termination events (Ganem et al, 2003, Steinmetz & Brow, 2003).

Termination is the stage of transcription that is least associated with chromatin regulation. While roles for chromatin structure are abundant in initiation and elongation, roles in
termination are scarce. The chromatin remodeling complexes Isw1b and Chd1 have both been implicated in transcription termination (Alen et al, 2002, Morillon et al, 2003). The authors suggest that chromatin structure may affect RNAPII pausing and thus termination (Alen et al, 2002, Morillon et al, 2003). Alternatively, it is possibility is that chromatin or chromatin remodelers may recruit certain termination factors. There is precedent for this mechanism: the elongation factor Spt2 is involved with the recruitment of CPF and mutation to Spt2 results in inefficient polyadenylation (Hershkovits et al, 2006). Spt2 is important for the maintenance of chromatin integrity during transcription elongation and its binding is enriched directly upstream of the polyadenylation signal (Hershkovits et al, 2006). Spt2 is also known to interact with SWI/SNF and histone acetyltransferases (Hershkovits et al, 2006). These interactions provide a direct link between chromatin structure and termination.
IV. Roles for Chromatin in Cotranscriptional mRNA Processing

Emerging evidence suggests that mRNA processing is tightly coupled to transcription. Three transcriptional phases, promoter clearance, elongation, and termination, are tightly coupled to three aspects of mRNA processing, 5’ mRNA capping, splicing, and 3’ end processing, respectively (Bentley, 2005, Buratowski, 2005, Saunders et al, 2006). These transcriptional phases are coupled to these mRNA processes by the differential phosphorylation of the RNAPII CTD (Phatnani & Greenleaf, 2006). In addition to chromatin alterations, phosphorylation of the RNAPII CTD coordinates the sequential processing of mRNAs cotranscriptionally.

mRNA 5’ end capping events occur early on in transcription in order to protect the nascent mRNA from degradation. The capping enzymes are recruited to the Ser5 phosphorylated form of RNAPII that is found predominantly at the 5’ ends of genes (Komarnitsky et al, 2000, Schroeder et al, 2000). Once Ser2 phosphorylation occurs, several splicing factors are recruited by the Ser2 and Ser5/Ser2 phosphorylated CTD heptads (Morris & Greenleaf, 2000, Phatnani et al, 2004). As RNAPII approaches the end of the gene, 3’ end processing factors are recruited by the Ser2 phosphorylated CTD and termination is triggered (Buratowski, 2005).

There are several reasons for the tight coupling of mRNA processing to transcription. mRNA processing protects the nascent RNA from exonucleases (Moore & Proudfoot, 2009). The 5’ cap and 3’ end polyadenylation both serve this function. Additionally, the actions of 3’ end formation actually stimulate termination (Buratowski, 2005). Without proper cleavage and polyadenylation, transcription termination would be deregulated.

Why splicing occurs cotranscriptionally is less clear. The original characterizations of mRNA splicing were conducted in vitro independent of transcription (Mayeda & Krainer, 1999). These studies led to a great number of advances in the field; however, more recently it has been
demonstrated that RNAPII stimulates splicing, leading to the hypothesis that splicing occurs cotranscriptionally because it is more efficient (Das et al, 2006, Hicks et al, 2006, Hirose et al, 1999). One of the major differences between cotranscriptional splicing and uncoupled splicing is the sequential recognition of splice sites (de la Mata et al, 2003, Dye et al, 2006, Howe et al, 2003). This is especially relevant in higher eukaryotes where alternative splicing occurs frequently. A full-length pre-mRNA will harbor many splice sites with varying strengths of recognition. This may not result in the same mature transcript as if the pre-mRNA were cotranscriptionally spliced. The current model of splice site selection involves kinetics and suggests that weak splice sites can only be selected when RNAPII transcription slows down to allow time for their selection (Allemand et al, 2008, de la Mata et al, 2003, Howe et al, 2003). Since transcription rates are thought to be related to the chromatin environment, this provides a possible link between chromatin structure and splicing.

Along these lines, yeast Spt5 and human Spt6 have roles in splicing (Xiao et al, 2005, Yoh et al, 2007). This further implicates transcription efficiency in the regulation of splicing. Spt6 is an important determinant of chromatin structure during elongation; however, its roles in chromatin structure have not been directly linked to its roles in splicing (Yoh et al, 2007). As described earlier, exons are preferentially marked by histone H3 lysine 36 tri-methylation in higher eukaryotes (Kolasinska-Zwierz et al, 2009). Furthermore, constitutive exons exhibit higher levels of tri-methylation than alternative exons. Based on studies that demonstrate the recruitment of Rpd3 by the H3 lysine 36 tri-methyl mark, constitutive exons would exhibit lower levels of histone acetylation, a modification known to facilitate transcription (Li et al, 2007b, Li et al, 2009). Perhaps these patterns of methylation serve to alter local chromatin environments and regulate splice site selection.

Another, more speculative link between chromatin and splicing is the nascent mRNA sequestration model (Allemand et al, 2008). This model suggests that the positively charged
histone N-termini interact with the negatively charged nascent mRNA as it exits RNAPII.

According to this model, the sequestration has two purposes: the interaction with the histone N-termini protects the nascent mRNA until RNA binding factors can bind, and the interaction serves to modulate the rate of transcription by “pulling” on RNAPII (Allemand et al, 2008). This model has not yet been tested experimentally, but is based on the ability of the histone N-termini to bind nucleic acids as well as the role of transcription rates on alternative splice site selection.
Chapter 2

EXAMINATION OF ROLES FOR THE HISTONE N-TERMINI IN TRANSCRIPTION INITIATION
Abstract

Chromatin structure is dynamic and regulates all stages of transcription. Chromatin remodeling and modifications are required for transcription initiation. These complexes alter the local chromatin environment of promoters and allow for the general transcription machinery to bind. The histone N-termini protrude from the nucleosome core and are the locations of a variety of histone modifications. Furthermore, both chromatin remodeling complexes and chromatin modifying complexes harbor domains that interact with the histone N-termini. In *S. cerevisiae*, the histone N-termini are nonessential and early characterization of histone mutants has revealed important roles for the N-termini in transcription. However, the mechanism of these transcription defects has not been established. Using the DNA damage-inducible *RNR3* gene, we explore the roles of the histone N-termini in transcription initiation. We find that the H2A, H3, and H4 N-termini are important for *RNR3* induction. Deletion of the H4 N-terminus results in the inability to release the Crt1-Ssn6-Tup1 corepressor upon induction. This mutant also exhibits constitutive SWI/SNF recruitment and partial remodeling of the promoter even in the absence of DNA damage. This is likely due to an inability to form repressive chromatin structure. Deletion of the H2A N-terminus causes defects in H3 lysine 4 methylation revealing another trans-tail regulatory pathway for Set1 methylation. Finally, deletion of the H3 N-terminus, or substitution of the H4 lysine residues for glutamines, both cause changes in RNAPII density across *RNR3*, suggesting that these mutations affect transcription elongation.
Introduction

Eukaryotic DNA is packaged into chromatin. Compact chromatin structure will inhibit the ability of transcription, DNA repair, replication, and recombination factors to access DNA. Conversely, open chromatin structure, or DNA regions deficient of nucleosomes, provide access to these factors. The dynamics of chromatin structure are vital to the regulation of these processes. The chromatin dynamics of transcriptional regulation have been intensely studied. Chromatin remodelers and modifying enzymes alter the chromatin environment at promoters to allow for transcription initiation. This allows for the recruitment of the general transcription machinery and RNA polymerase II (RNAPII). Once RNAPII is loaded onto the promoter, transcription is initiated.

Chromatin remodeling complexes use ATP hydrolysis to alter nucleosome structure. These remodelers break DNA-histone contacts, having several consequences: the translational sliding of nucleosomes, the formation of DNA loops, eviction of histones, and greater access to DNA for transcription factors. Nucleosome sliding involves the translational movement of the histone octamer to a different position on the DNA. This action can either expose or hide DNA elements important for transcription initiation. The ISWI family of chromatin remodeling complexes is thought to predominantly utilize nucleosome sliding (Fazzio & Tsukiyama, 2003, Tsukiyama et al, 1999). Histone eviction involves the removal of histones from the octamer. The H2A/H2B dimer is easily exchanged and the entire octamer can also be evicted, although this is observed less frequently. The SWI/SNF family of chromatin remodeling complexes is known to remodel by eviction (Cote et al, 1998, Logie et al, 1999).

Chromatin modifications involve the covalent addition of small chemical groups to histones. The histone N-termini are especially enriched for histone modifications (Kouzarides,
These highly conserved modifications have been associated with both transcriptional activation and repression. The two modifications most associated with transcription initiation are histone acetylation and H3 lysine 4 tri-methylation (K4me3). Histone acetylation is thought to have multiple roles in altering chromatin dynamics. The histone N-termini are highly basic and interact strongly with DNA. These contacts are inhibitory to transcription. Acetylation can neutralize the positively charged histone N-termini and weaken the interaction between the histones and DNA. Additionally, several transcription factors harbor bromodomains that interact with acetylated lysines residues on the histone tails (Durant & Pugh, 2007, Hassan et al, 2002, Marmorstein & Berger, 2001, Syntichaki et al, 2000). The chromatin remodeling complex SWI/SNF is one example. H3 K4me3 is catalyzed by the COMPASS complex and is found predominantly at the promoter and 5’ ends of genes (Dover et al, 2002, Morillon et al, 2005). The H3 K4me3 mark is recognized by the PHD finger of Yng1, part of the NuA3 histone acetyltransferase (HAT) (Taverna et al, 2006). In higher eukaryotes, H3 K4me3 is recognized by the PHD finger in the NURF chromatin remodeling complex (Wysocka et al, 2006). The interactions between chromatin remodeling complexes and chromatin modifying complexes demonstrate the coordination necessary to properly regulate chromatin dynamics.

Transcription initiation requires the binding of the general transcription machinery and RNAPII. Chromatin remodeling and modifying enzymes are responsible for opening chromatin to allow access for these factors. Once TFIID and the other general transcription factors bind, RNAPII is loaded onto the promoter and preinitiation complex (PIC) formation is complete. The RNAPII C-terminal domain (CTD) is phosphorylated by TFIIH and once RNAPII has transcribed approximately 30 base pairs, the transition from initiation to elongation occurs (Feaver et al, 1994, Rodriguez et al, 2000).

In *S. cerevisiae*, the histone N-termini are non-essential. This has enabled investigations into the roles of N-termini in transcriptional regulation. Early predictions about the histone N-
termini suggested a predominantly repressive role in transcription (Edmondson et al, 1996, Wan et al, 1995). In vitro transcription assays revealed that RNAPII more readily transcribed “tailless” chromatin templates than wild type chromatin templates (Georges et al, 2002). Furthermore, strains harboring a deletion of the H3 N-terminus resulted in the derepression of the GAL1 gene (Wan et al, 1995). Conversely, deletion of the H4 N-terminus significantly inhibited GAL1 induction (Wan et al, 1995). These observations provided evidence that the histone N-termini are involved in both transcriptional activation and repression and that the roles of individual tails may differ from or antagonize the others. More recently, genome wide expression studies have revealed a more complex scenario. The H3 and H4 N-termini have roles in both activation and repression and the deletion of either significantly affects the global mRNA expression profile (Sabet et al, 2003, Sabet et al, 2004).

In this study, we examine the roles of the histone N-termini in transcription initiation of the DNA damage-inducible gene RNR3. We demonstrate that the H2A, H3, and H4 N-termini are required for full activation of RNR3. Upon further examination, we have determined that the mechanisms of these transcription defects are different for each of the histone mutants. Deletion of the H2A tail results in a significant loss of H3 K4me3. Deletion of the H3 tail causes a defect in RNAPII recruitment, but also a shift in RNAPII density across RNR3 that suggests additional elongation defects. Deletion of the H4 tail and mutation of the lysine residues within the tail both caused significant activation defects; however, these two mutants exhibit different defects in initiation. The H4 tail deletion resulted in the inability to release the Crt1-Ssn6-Tup1 corepressor as well as the constitutive recruitment of the SWI/SNF chromatin remodeling complex. On the other hand, mutation of the H4 lysine residues resulted in the inability to transition from initiation to elongation. We report several roles for the histone N-termini in transcription initiation as well as identify possible roles in transcription elongation.
Results

The Histone N-termini are required for full activation of RNR3

While the effects of histone N-termini deletions on mRNA expression have been studied extensively, the mechanisms of their effects is not well understood. We sought to investigate these mechanisms in order to better understand transcriptional regulation. We first examined the effects of histone N-terminal deletions on the induction of the DNA damage inducible RNR3 gene (Figure 2-1A). The various histone mutant strains were constructed using different strategies. Some of the mutations are integrated while others are maintained on plasmids; therefore, there are multiple wild type strain backgrounds throughout this study. In each experiment, the mutants are compared to their cogenic wild type to account for any strain differences. To examine the induction of RNR3, northern blotting was performed on RNA isolated from cells that were treated, or not, with methyl methane-sulfonate (+MMS). To account for loading differences, RNR3 mRNA levels were corrected for scR1 levels. scR1 is an abundant RNA polymerase III transcribed non-coding RNA that is part of the signal recognition particle (Dieci et al, 2002, Hann & Walter, 1991). Under normal growth conditions RNR3 is repressed, and surprisingly none of the N-terminal deletions (H2A Δ1-20, H2B Δ1-32, H3 Δ1-28, or H4 Δ2-26) resulted in significant derepression of RNR3 (Figure 2-2A). On the other hand, MMS-induced activation of RNR3 was severely compromised in the H2A, H3, and H4 N-terminal deletion strains. The wild type strains exhibit 60-80 fold induction of RNR3 2.5 hours post induction. In contrast, the H2A Δ1-20, H3 Δ1-28, and H4 Δ2-26 strains exhibited only 20-25 fold induction (Figure 2-2A). Deletion of the H2B N-terminus did not significantly alter RNR3 mRNA levels, suggesting that RNR3 induction can occur independent of the H2B N-terminus.
The histone H3 and H4 N-termini contain several lysine residues that are sites for post-translational acetylation or methylation. These modifications are associated with active transcription (Grant et al, 1998, Morillon et al, 2005, Strahl et al, 1999, Wang et al, 1997) and since we found that deletion of the H3 and H4 N-termini reduced \textit{RNR3} expression, we sought to determine if the lysine residues within these regions were also important. We obtained several strains with lysine to glutamine mutations within the tail regions (Figure 2-1A). Substituting glutamines for lysines at residues 4, 9, 14, 18, 23, and 27 on the H3 tail (H3 K->Q hereafter), or residues 5, 8, 12, and 16 on the H4 tail (H4 K->Q hereafter) both resulted in a significant reduction of \textit{RNR3} expression (Figure 2-2B). This suggests that the H3 and H4 N-termini, and specifically the lysines within the tail region, are important for \textit{RNR3} induction. We were also able to further dissect the importance of the individual H4 lysine residues. We examined the expression \textit{RNR3} in mutants harboring three lysine to glutamine mutations in the H4 N-terminus (Figure 2-1A). This allowed us to explore whether a single remaining lysine residue could restore transcription of \textit{RNR3}. Interestingly, we found that three of the triple lysine to glutamine mutants had a significant reduction of RNR3 expression resembling the defect observed in the H4 all K->Q mutant (Figure 2-2B). These triple mutant strains all harbored the K16Q mutation, on the other hand, the H4 K5,8,12Q mutant strain exhibited a slight, but reproducible, increase in \textit{RNR3} induction compared to wild type (Figure 2-2B). This observation suggests that H4 K16, and possibly the dynamic acetylation of this residue, is important for activation of \textit{RNR3}.

To further dissect the role of the H4 lysine residues in the activation of \textit{RNR3}, we next examined other lysine substitution mutants (Figures 2-2C and 2-2D). Lysine to glutamine (K->Q) substitutions have been used to mimic lysine acetylation, while lysine to arginine (K->R) substitutions have been used to mimic “unacetylable” lysines. In Figure 2-2C, we demonstrate that the H4 K->R, like the H4 K->Q, substitution results in a ~70% loss of \textit{RNR3} activation. While this result does not elaborate on the mechanisms of the defect, it does support the
proposition that dynamic modification of H4 lysine residues is important for RNR3 activation. We further explored the role of lysine 16 in RNR3 activation by examining two strains that harbor substitutions at two of the H4 lysines: H4 K8,12Q and H4 K12,16Q (Figure 2-2D). In support of our hypothesis that lysine 16 is especially important to RNR3 induction, the H4 K12,16Q mutation results in a ~60% loss of transcription (Figure 2-2D). Conversely, the H4 K8,12Q mutation did not alter RNR3 induction. We also examined how the H4 K16R mutation would affect RNR3 induction. We demonstrate that substitution of K16 alone results in a ~40% loss of induction. While this result further stresses the importance of lysine 16, the magnitude of the defect cannot be directly compared to the K->Q mutations because glutamine and arginine substitutions are not equivalent. Rather than constructing more histone mutants, we chose to focus our efforts on examining the mechanisms involved in these defects.

We have identified the histone H2A, H3, and H4 N-termini as important for the activation of RNR3 and we proceeded to explore the mechanism of their roles. Under normal growth conditions, RNR3 is repressed in part by the Crt1-Ssn6-Tup1 corepressor complex (Li & Reese, 2001, Zhang & Reese, 2004a, Zhang & Reese, 2004b). When DNA damage is sensed by the cell, the Rad53-Mec1-Dun1 pathway is activated and results in the release of Crt1-Ssn6-Tup1 from the RNR3 DNA damage response element (DRE) (Zhou & Elledge, 1993). This is the initial step in RNR3 activation and we began our mechanistic investigation here. We used chromatin immunoprecipitation (ChIP) with antiserum to Crt1 to examine its release from the RNR3 DRE after 2.5 hours of induction with MMS. Crt1 release demonstrates that the DNA damage signal has reached the RNR3 upstream activation sequence. In the wild type strains, there is an approximate 5-fold reduction in Crt1 crosslinking (Figure 2-3A). This fold loss is also observed in the H2A Δ1-20, H2B Δ1-32, and H3 Δ1-28 strains, indicating that the DNA damage response has been activated (Figure 2-3A). The H4 Δ2-26 mutation results in the inability to release Crt1. This could be due to an interruption in the DNA damage signaling pathway. Previously, it has
been shown that phosphorylation of H4 serine 1 plays a role in the DNA damage response (Cheung et al, 2005). Although the H4 Δ2-26 mutant does have an intact serine 1, its altered local environment could inhibit the phosphorylation event or recognition of the modification. However, this possibility has not been directly examined.

The lysine residues within H4 are important for RNR3 transcription (Figure 2-2B). We sought to determine if mutation of the H4 lysine residues also resulted in the inability to release Crt1 similar to the H4 Δ2-26 strain. Once again, we observed a 4-5 fold release of Crt1 in the wild type strain upon induction with MMS (Figure 2-3B). Crt1 release is unaffected in the H4 lysine to glutamine mutants. Since the H4 all K->Q mutant is able to release Crt1, it would suggest that the Crt1-release defect in the H4 Δ2-26 strain is not due to the inability to dynamically acetylate lysine residues. Furthermore, the transcriptional defect(s) in the H4 point mutants occur after the release of the RNR3 corepressor.

Tup1, part of the Crt1-Ssn6-Tup1 corepressor complex, is also released from the RNR3 DRE upon DNA damage. Tup1, in conjunction with Isw2, serves to position nucleosomes in a highly ordered arrangement that represses RNR3 (Li & Reese, 2001, Zhang & Reese, 2004a, Zhang & Reese, 2004b). We were interested in Tup1 crosslinking at the RNR3 DRE because it has been shown to interact with the H4 N-terminus (Edmondson et al, 1996). Upon DNA damage, there is a 3-fold loss of Tup1 in the wild type strains (Figure 2-4A). Our analysis reveals that deletion of the H2A or H2B N-termini does not affect the overall levels of Tup1 crosslinking or its release upon MMS treatment (Figure 2-4A). Deletion of the H3 N-terminus results in increased crosslinking of Tup1 both before and after MMS treatment compared to wild type; however, the approximate 3-fold loss upon induction is still observed. Tup1 crosslinking in the H4 Δ2-26 strain mirrors that of Crt1 crosslinking. Prior to induction, Tup1 levels are equal to wild type. However, like Crt1, Tup1 is inefficiently released from the DRE in the H4 Δ2-26 strain.
upon induction and these two defects are likely linked. If this were true, we postulated that the H4 lysine to glutamine mutants would exhibit wild type levels of Tup1 release which would mirror that of Crt1 release. This is what we observed (Figure 2-4B), further emphasizing a role for the H4 N-terminus, but not specifically the lysines within that region, in the release of the Crt1-Ssn6-Tup1 corepressor from the RNR3 DRE.

The H4 N-terminus antagonizes SWI/SNF recruitment

We have established that the H2A, H3, and H4 N-termini are required for full activation of the DNA damage inducible gene, RNR3. Our characterization of these strains has revealed that the H4 N-terminus is important for the release of the Crt1-Tup1-Ssn6 corepressor complex. This is not the only transcriptional defect in this strain; many genes whose regulation is independent of Crt1-Tup1-Ssn6 are also affected by deletion of the H4 N-terminus (Sabet et al, 2004). Therefore, we continued to explore other aspects of transcription regulation in which the histone N-termini may be involved. After Crt1-Ssn6-Tup1 release, the ordered state of chromatin over the RNR3 promoter must be disrupted to allow for PIC formation. This disruption is strictly dependent upon the SWI/SNF chromatin remodeling complex (Sharma et al, 2003). SWI/SNF contains two subunits with domains that recognize histone tails and/or modifications of the tails; Snf2 contains a bromodomain and Swi3 contains a SANT domain (Awad & Hassan, 2008, Hassan et al, 2006, Smith et al, 2003). We hypothesized that modification of the histone N-termini may act to recruit SWI/SNF to the RNR3 promoter and that deletion of the tails may result in the inability to recruit SWI/SNF. Our lab has previously shown that deletion of Swi2, the ATPase subunit of SWI/SNF, results in the inability to remodel chromatin, the loss of PIC formation, and thus the loss of RNR3 transcription (Sharma et al, 2003). Here we examine the recruitment of SWI/SNF to RNR3 (Figure 2-5A). Swi2 recruitment to the RNR3 promoter increases greater than 2-fold after MMS
treatment in the wild type strains. Contrary to our expectations, none of the histone N-termini are required for Swi2 recruitment. In fact, deletion of the H4 N-terminus results in constitutive recruitment of Swi2 (Figure 2-5A).

This result was unexpected; therefore, we further explored chromatin structure over the \textit{RNR3} promoter by micrococcal nuclease (MNase) nucleosome mapping (Figure 2-5B). The wild type strain exhibits well positioned nucleosomes over the promoter and throughout the gene prior to induction with MMS (Figure 2-5B and (Li & Reese, 2001, Sharma et al, 2003, Zhang & Reese, 2004b)). Upon induction, chromatin is remodeled and a doublet of hypersensitivity is observed near the TATA element (Figure 2-5B, denoted by the asterisk). In the H4 $\Delta$2-26 strain prior to induction, the hypersensitive doublet is observed, as well as a broadening of the hypersensitive site between the +1 and +2 nucleosomes (Figure 2-5B, doublet denoted by the asterisk and the broadened nucleosome designated by bars). While the chromatin structure of the H4 $\Delta$2-26 strain does not appear to be fully remodeled prior to induction, it is clear that the constitutive SWI/SNF recruitment results in partial depositioning of nucleosomes. SWI/SNF remodeling results in the eviction of nucleosomes at the \textit{RNR3} promoter. We examined the density of nucleosomes at the \textit{RNR3} promoter using an antibody to H3 (Figure 2-5C). In the uninduced state (-MMS), the density of nucleosomes is not affected by the H4 tail deletion (Figure 2-5C). Nucleosomes are disrupted, but not evicted. This is also observed in an $\Delta$isw2 mutant (Tomar et al, 2009). There was, however, a reduction in the ability to evict nucleosomes upon MMS induction in the H4 $\Delta$2-26 strain (Figure 2-5C). This reduction correlates with reduced Tup1 release (Figure 2-4A). Based on the constitutive SWI/SNF crosslinking and the mild H3 eviction defect, it is likely that nucleosomes are neither fully positioned, nor evicted in the H4 $\Delta$2-26 mutant. This data demonstrates that the H4 N-terminus is important for the maintenance of repressive chromatin structure, but not the recruitment of SWI/SNF.
The histone N-termini are important for H3 acetylation and H3 K4 tri-methylation

In addition to chromatin remodelers, chromatin structure is also modulated by histone modifications. Histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs) all alter the chromatin environment and have domains that recognize the histone N-termini. Previously, our lab has shown that transcription of RNR3 is highly dependent on histone acetylation (Sharma et al, 2003, Zhang et al, 2008). Upon induction, an increase in H3 acetylation is observed at the RNR3 promoter. Deletion of Gcn5, the acetyltransferase subunit of the SAGA coactivator, results in a complete loss of H3 acetylation and significant defects in transcription at RNR3 (Zhang et al, 2008). We examined the levels of H3 K9,14 acetylation by ChIP to determine if the H2A, H2B, or H4 N-termini were important for H3 acetylation (Figure 2-6A). Upon induction, we observe a 3.5-fold increase in H3 acetylation in the wild type strains. This is also observed in the H2A tail deletion. The H2B tail deletion exhibited even greater levels of acetylation. The reason for this increase is unclear, but does not seem to influence mRNA levels (Figure 2-2A). Deletion of the H4 tail did result in an H3 acetylation defect. Acetylation levels in this strain were about 60% of wild type levels. Tup1 has been shown to recruit histone deacetylases (HDACs) and since the H4 Δ2-26 strain exhibits a partial inability to release Tup1 (Figure 2-4A), the reduced H3 acetylation may be the result of increased HDAC activity. Interestingly, during normal growth conditions (-MMS), the H4 Δ2-26 strain exhibits chromatin remodeling but not histone acetylation at RNR3.

To determine if the defects in H3 acetylation in the H4 Δ2-26 strain were due to the loss of acetylatable H4 lysine residues, we examined H3 acetylation in the H4 lysine to glutamine mutants. Interestingly, none of the H4 lysine to glutamine mutants exhibited more than a 20-25% loss of H3 acetylation (Figure 2-7B). Unlike the H4 N-terminal deletion strain, the H4 lysine to
glutamine mutants were able to efficiently release Tup1. This supports the hypothesis that the acetylation defect in the H4 Δ2-26 strain is due to increased HDAC activity.

Another histone modification closely related to transcription initiation and the early stages of elongation is H3 lysine 4 tri-methylation (K4me3). This modification is dependent upon ubiquitylation of H2B lysine 123 (K123Ub) and this represents one of the better characterized trans-tail regulatory pathways of histone modifications (Lee et al, 2007, Shahbazian et al, 2005). We explored the possibility that the N-termini of H2A, H2B, or H4 may also play a role in H3 K4me3. This modification has been characterized at several other genes and is known to be found primarily at the promoters and 5’ ends of genes. For this reason, we used additional primers to determine the pattern of H3 K4me3 across RNR3 (Figure 2-1B for primer schematic and 2-8). In the wild type strains, we observed the characteristic pattern of H3 K4me3; an increase is observed upon induction at the promoter and ORF B regions, but there is no increase at ORF C (Figure 2-8 A, B, and C respectively). Deletion of the H2A N-terminus significantly reduces H3 K4me3 at both the RNR3 promoter and ORF B. This is in striking contrast to the H2B N-terminal deletion which exhibits no adverse effects on H3 K4me3. Deletion of the H4 N-terminus results in a minor H3 K4me3 defect at the promoter which is slightly enhanced within the gene at ORF B. This was unexpected because Crt1 and Tup1 release is defective in this strain and mRNA expression is reduced. Perhaps RNR3 transcription is active at low levels in this strain even without efficient release of Crt1 and Tup1. This would be supported by the fact that mRNA levels are not abolished (Figure 2-2A). The low levels of transcription may be enough to mark the chromatin environment with H3 K4 methylation. Deletion of the H4 N-terminus is not nearly as devastating to H3 K4me3 as deletion of the H2A N-terminus. The effect of the H2A tail deletion on H3 K4me3 suggests the existence of another trans-tail regulatory pathway and the mechanism behind this defect has become the major project of another member of our lab.
We next examined whether the H4 lysine residues were important for H3 K4me3. We had already observed that deletion of the H4 N-termini resulted in a 25% loss of H3 K4me3 at the RNR3 promoter and a 40% loss at ORF B (Figure 2-8 A and B). While the H4 lysine to glutamine triple mutants exhibited wild type levels of H3 K4me3, the H4 all K->Q mutation results in a 35% loss at the RNR3 promoter and 50% loss at ORF B (Figure 2-8 D and E). The pattern of this defect clearly mirrors the defect observed in the H4 Δ2-26 strain. Perhaps the availability of a single acetylatable lysine on H4 is important for H3 K4me3. Alternatively, this modification is transcription dependent and decreased transcriptionally active RNAPII at RNR3 could result in less H3 K4me3.

**RNAPII, but not TBP, recruitment is partially dependent on the histone N-termini**

In order to determine how mutations to the histone N-termini affect transcription initiation and more specifically PIC formation, we examined (TATA binding protein) TBP recruitment to the RNR3 promoter (Figure 2-8). Recruitment of TBP and the general transcription factors are essential for RNR3 induction. In the wild type strains, we observe a 4.5-6.5 fold increase in TBP crosslinking upon induction. None of the histone N-termini deletion strains exhibit significant defects in TBP recruitment (Figure 2-8A). The defects are mild and not proportional to the mRNA expression loss observed in these strains (Figures 2-8A and 2-2A, respectively). Similar results were observed when we examined the H4 lysine to glutamine mutants; TBP recruitment was unaffected (Figure 2-8B). These results suggest that the transcription defects caused by mutating the histone N-termini occur after TBP recruitment possibly indicating an RNAPII recruitment defect or post-initiation defect.
We next explored whether the histone N-termini were required for RNAPII recruitment. RNAPII recruitment to the RNR3 promoter was unaffected in the H2B Δ1-32 strain (Figure 2-9A). However, the H2A Δ1-20 mutation resulted in a 30% loss of RNAPII recruitment (Figure 2-9A). While this reduction in RNAPII recruitment is reproducible, the magnitude is not as great as the loss of RNR3 mRNA or the loss of H3 K4me3 (Figures 2-9A, 2-2A, and 2-8A). This suggests that the H2A N-terminus plays important post-initiation transcriptional roles. Furthermore, the defect in H3 K4me3 is not exclusively a result of reduced RNAPII.

Deletion of the H3 or H4 N-termini results in significantly lower levels of RNAPII recruitment to the RNR3 promoter upon induction (Figure 2-9A). Recruitment in the H3 Δ1-28 strain is 50% less than wild type. This suggests that although initiation events leading up to RNAPII recruitment are not directly dependent on the H3 tail, the recruitment of RNAPII is at least partially dependent. The H4 Δ2-26 mutation results in a 60% loss of RNAPII recruitment. This correlates well with the lower levels of H3 acetylation and H3 K4me3 even though TBP recruitment is only slightly affected (Figures 2-7, 2-8 and 2-10). It is unclear whether the RNAPII recruitment defect causes, or is caused by, the decreased histone acetylation and methylation.

We also sought to determine if the H4 lysine residues were important for RNAPII recruitment. None of the H4 lysine to glutamine mutations resulted in significant RNAPII recruitment defects (Figure 2-9B). This was quite surprising since mRNA induction is so significantly reduced in three of the triple mutants and the H4 all K->Q mutant (compare RNAPII recruitment in Figure 2-9B to mRNA induction in Figure 2-2B). These observations strongly suggest that the H4 lysine residues are playing an important post-initiation role in transcription.

One common indicator of transcription elongation competence is RNAPII density throughout a gene. Mutations to factors involved in elongation have been shown to alter RNAPII density. We first investigated whether any of the histone N-terminal deletions result in changes to
RNAPII density (Figures 2-1B for primer schematic). We hypothesized that the H2A N-terminus may be involved in elongation since we observed only mild defects in RNAPII recruitment, but significant defects in mRNA expression in this mutant. Therefore, we thought that RNAPII density may be different than in the wild type. This is not the case: while RNAPII is recruited to slightly lower levels in the H2A Δ1-20 strain, it is consistently lower across RNR3, indicating that there is not a shift in RNAPII density (Figure 2-10A).

Even though our initial characterization of the H3 and H4 N-termini deletion strains did not suggest elongation defects, we did analyze RNAPII density across RNR3 in these strains. While recruitment of RNAPII to the RNR3 promoter is only 50% of the wild type in the H3 Δ1-28 mutant, RNAPII crosslinking within the gene reaches wild type levels (Figure 2-10B). In fact, RNAPII seems to be “piling up” within the gene. Assuming that a certain amount of RNAPII is loaded onto the promoter, an increase in the density of polymerase within the gene would indicate inefficient transcription elongation. These observations are examined further in Chapter 4.

Deletion of the H4 N-terminus does not significantly alter the pattern of RNAPII density across RNR3 relative to wild type. The H4 Δ2-26 mutant results in significantly lower levels of RNAPII recruitment, and similarly, polymerase density is low across the gene (Figure 2-10C). We observe no shift in density which would suggest the defects observed in this mutant are primarily affecting initiation at RNR3. However, it is possible that the RNAPII recruitment defect in the H4 Δ2-26 strain is masking possible elongation defects.

Because we observed a disproportionate loss of RNR3 expression relative to the RNAPII found at the promoter, we also examined RNAPII density across RNR3 in the H4 lysine to glutamine mutants (Figure 2-10D). Depicted is the fold enrichment of RNAPII after induction (+MMS). As seen previously, RNAPII recruitment to the promoter is relatively unaffected in the H4 all K->Q mutant. However, at ORF A, there is a striking difference between the wild type and
H4 all K->Q strain. While the wild type strain exhibits a 10-fold increase in RNAPII crosslinking, the H4 all K->Q mutant exhibits only a 3-fold increase (Figure 2-10D). We believe that the difference in RNAPII crosslinking between the promoter and ORF A is indicative of an elongation defect, possibly a defect in the transition from initiation to elongation. The H4 K5,8,12Q strain that exhibits slightly greater levels of RNR3 induction also exhibits slightly higher levels of RNAPII density (Figures 2-2B and 2-10D). This may suggest that the H4 K5,8,12Q mutation enhances transcription elongation and results in greater mRNA expression. The data presented here demonstrate that the H4 tail lysine residues are important for transcription elongation. For this reason, we further explored the roles of the H4 N-termini in post initiation transcriptional regulation in Chapter 3.
Discussion

**Roles for the histone N-termini in the expression of RNR3**

The histone H2A, H3, and H4 N-termini are required for normal induction of the DNA damage inducible gene, *RNR3*. We demonstrate that deletion of these N-termini, or mutation of the lysine residues within the H3 or H4 N-termini, significantly reduces the induction of *RNR3*. The latter observation suggests that the ability to dynamically acetylate H3 and H4 lysine residues is essential for transcription of *RNR3*. Additional exploration into the mechanistic details of these deficiencies revealed that the defects observed in one histone mutant differ from the other mutants. Furthermore, the fact that the H4 Δ2-26 mutation resulted in phenotypes different from the H4 all K->Q mutation suggests that structures within the tail, in addition to the acetylatable lysines, are important.

The function of the H4 lysine residues was examined more closely through the use of triple lysine to glutamine mutants. Of particular interest was the importance of H4 lysine 16. Any of the triple lysine to glutamine mutants that include the K16Q substitution are significantly defective for *RNR3* expression. However, the H4 K5,8,12Q strain actually exhibited enhanced expression. While this mechanism is unclear, others have observed the importance of lysine 16 in gene regulation (Dion et al, 2005). For some time the dynamic acetylation of lysine 16 has been implicated in the maintenance of heterochromatin (Suka et al, 2002). More recently, the importance of these dynamics in higher order chromatin folding has been established (Shogren-Knaak et al, 2006). Acetylation of lysine 16 prevents the formation of the 30nm chromatin fiber and the mobilization of nucleosomes by the ATP dependent chromatin remodeler ACF (Shogren-
Knaak et al, 2006). The mechanism of how lysine 16 is regulating *RNR3* expression is not completely resolve, but its significance is clear.

One of the most fascinating principles that have arisen from these studies is that the histone N-termini individually are not required for chromatin remodeling and the recruitment of TBP. This suggests that there exists some level of redundancy for the histone N-termini in transcription initiation, or alternatively, a lack of requirement for the tails in initiation. The various histone modifications, and domains within chromatin remodelers that recognize the tail regions, point to important roles for the N-termini. It may be that these interactions are so vital, that evolution has built in redundancy. This could explain the modest defects in TBP recruitment and H3 acetylation.

**Roles for the histone H4 N-termini in maintenance of repressive chromatin structure over *RNR3***

The SWI/SNF chromatin remodeling complex contains both a bromodomain and a SANT domain. These domains are thought to interact with histone tails and help recruit or execute the function of SWI/SNF (Hassan et al, 2001, Hassan et al, 2006, Smith et al, 2003, Syntichaki et al, 2000). Our lab has previously demonstrated that *RNR3* expression is dependent on SWI/SNF nucleosome remodeling (Sharma et al, 2003). Therefore, we thought that deletion of one or more of the histone N-termini would result in the inability to recruit SWI/SNF. Contrary to this expectation, we observed that each tail deletion strain exhibited wild type levels of Swi2 recruitment after induction (Figure 2-5A). Interestingly, the H4 Δ2-26 strain displayed constitutive recruitment of SWI/SNF. Initially, this result was perplexing; however, in conjunction with other results from our lab, we were able to build a model for another level of *RNR3* repression. Isw2 acts to position nucleosomes across *RNR3* (Zhang & Reese, 2004b). The
activity of Isw2 is dependent upon an interaction with a small basic patch on the H4 N-terminus (Fazzio et al, 2005). Furthermore, an Δisw2 strain, similar to the H4 Δ2-26 strain, results in constitutive recruitment of Swi2 (Figure 2.5A and (Tomar et al, 2009)). Neither the Δisw2 nor the H4 Δ2-26 strains result in uninduced PIC formation. We propose that Isw2 and the H4 N-terminus are important for building higher order chromatin structure across RNR3. This structure serves to maintain a repressive environment and exclude SWI/SNF from binding to the promoter. In the absence of the higher order structure, SWI/SNF can scan, bind, and remodel chromatin, but, the DNA damage signal is required for PIC formation and the induction of transcription.

**Potential novel trans-tail regulation of H3 K4me3**

Tri-methylation of H3 lysine 4 depends on H2B lysine 123 ubiquitylation. This trans-tail regulatory pathway is well characterized. The Rad6/Bre1 complex is recruited to actively transcribed regions and catalyzes the ubiquitylation of H2B K123 (Dover et al, 2002, Lee et al, 2007). Cps35, a subunit of the H3 K4 methyltransferase COMPASS, will interact with the H2B K123Ub moiety and will result in COMPASS being tri-methylation competent (Lee et al, 2007). The H2A N-terminal deletion strain exhibits significant defects in H3 K4me3. There is a slight reduction in the recruitment of RNAPII to the RNR3 promoter in this strain. It is unlikely that this small reduction is responsible for the 75% reduction in H3 K4me3. Therefore, we hypothesize that the H2A N-terminus is either required for 1) COMPASS recruitment or activity, or for 2) Rad6/Bre1 recruitment or activity. In either case, these observations reveal novel trans-tail regulation and the mechanism of this defect is being pursued by another member of our lab.
The histone N-termini modulate transcription elongation

Many histone remodeling and modifying complexes are known to have domains that interact with the histone N-termini. Our prediction was that the mechanisms of the transcription defects observed in these histone mutants would involve initiation defects. Deletion of the H4 N-terminus did result in several initiation related phenotypes, including the inability to release the Crt1-Ssn6-Tup1 corepressor complex, constitutive SWI/SNF recruitment, and an RNAPII recruitment defect. On the other hand, the H4 all K->Q mutation, which also significantly reduced RNR3 transcription, did not result in initiation defects. This suggested that the H4 lysine residues may play a role in post-initiation events. Furthermore, the RNAPII recruitment defect in the H4 tail deletion may mask possible roles in elongation. These results, along with the observation that RNAPII density is altered in some of the histone mutants, suggest post-initiation roles for the histone N-termini. While there are histone modifications associated with transcription elongation, an in-depth examination into the roles of the histone tails per se, in elongation has not been performed.

Here we report that deletion of the H3 N-terminus results in changes to RNAPII density across RNR3. Deletion of the H3 N-terminus results in an RNAPII recruitment defect; however, we observe increased levels of RNAPII within the gene. This recruitment defect indicates that RNAPII is being loaded onto the promoter more slowly than in the wild type strain. The increased RNAPII density within the gene suggests that transcription elongation is less efficient and that RNAPII is “piling-up” as a result. Slowed or stalled RNAPII within a gene will result in greater density in the ORF than at the promoter. The H3 N-terminus is likely to be involved in both the recruitment of polymerase and transcription elongation. Surprisingly, deletion of the H3 N-terminus did not significantly affect the events that lead up to RNAPII recruitment. In Chapter 4, the role of the H3 N-terminus in transcription elongation is further explored.
Substituting the four H4 lysine residues for glutamines (H4 all K->Q) also altered RNAPII density across *RNR3*, but in a very different pattern. Here we observe RNAPII recruitment to the promoter at 75% of wild type; however, at ORF A polymerase crosslinking is only 30% of wild type. This implies that while RNAPII is able to be brought to the promoter upon induction, there are defects in the transition from initiation to elongation in the H4 all K->Q mutant. Whether or not the H4 Δ2-26 strain also exhibits this defect in the transition from initiation to elongation could not be resolved due to the RNAPII recruitment defect. Deletion of the H4 N-terminus results in the inability to release Crt1-Ssn6-Tup1. This is the first step in *RNR3* activation and thus all subsequent steps are blocked to some degree. While TBP recruitment is mostly unaffected, constitutive Tup1 crosslinking will maintain a repressive environment. This is evidenced by the reduced H3 acetylation and RNAPII recruitment. It is clear that deletion of the H4 N-terminus results in some transcription initiation defects. Based on the changes in RNAPII density observed in the H4 all K->Q strain, additional roles for the H4 N-terminus in elongation are apparent. Roles for the H4 N-terminus in elongation are examined in Chapter 3.

Mutations that affect transcription elongation often alter the pattern of RNAPII density. These changes can be the result of slowed or stalled polymerase, premature termination, or the inability to shift from one phase of transcription to the next (the transition from initiation to elongation, for example). The histone N-termini possibly play integral roles in transcription elongation. Elongation involves the disruption and reassembly of chromatin as RNAPII passes. It is likely that the histone N-termini are involved in these chromatin dynamics. While factors that facilitate elongation have been identified, the roles of the histone N-termini in elongation are unresolved.
Materials and Methods

Strains and Media

The strains used in this study are listed in Table 2-1. Gene deletions and tagging were carried out by standard one step replacements using PCR generated cassettes (Brachmann et al, 1998, Longtine et al, 1998). Cells were grown in 2% peptone, 1% yeast extract, 20μg/mL adenine sulfate, 2% dextrose at 30°C.

Northern Blotting

Yeast culture was grown to (OD$_{600}$=0.7), and 15mL of cells were harvested for total RNA extraction as previously described (Reese & Green, 2003, Walker et al, 1996). Where indicated, cells were treated with 0.02% MMS for 1 or 2.5h prior to harvesting. RNA was liberated with bead beating in the presence of phenol/chloroform, extracted again with phenol/chloroform, precipitated with ethanol, and resuspended in DEPC-treated water(Walker et al, 1996). Twenty micrograms of total RNA was separated on 1.2% agarose gels containing formaldehyde and transferred to Hybond-XL membrane (GE Biosciences, Piscataway NJ) by capillary blotting overnight. Membranes were UV crosslinked using a Stratalinker and incubated in prehybridization solution for more than 4 hours at 65°C. Probes were added for overnight incubation. Membranes were washed and signals were detected using a phosphoimager. The phosphoimager screens were scanned with the Typhoon System (Molecular Dynamics), and quantified by ImageQuant. Probes for RNR3 and Scr1 were prepared by the polymerase chain
reaction. The seR1 signal of each sample was used to correct for loading of RNA. All data represents at least three independent experiments. Error bars represent the standard error.

**Chromatin Immunoprecipitation (ChIP)**

ChIP assays were performed as previously described, with minor changes (Sharma et al, 2007). Yeast cultures (100mL) were grown in YPAD medium to an OD600 of 0.6 to 0.8. The cells were cross-linked with 1% (vol/vol) formaldehyde at room temperature for 15 min. The formaldehyde was quenched by the addition of 125mM glycine. MMS-induced cells were treated with 0.02% MMS and incubated for 2.5 h prior to formaldehyde treatment. Whole cell extracts were prepared by glass bead disruption, and sheared into fragments averaging 200-600 base pairs in size using a Bioruptor (Diagenode, Philadelphia PA). Whole cell extracts were clarified by centrifugation. Clarified extracts were immunoprecipitated (IP) overnight at 4°C with antibodies indicated throughout the text. For antibody information see Table 2-2. Immune complexes were precipitated with 20μL of protein A sepharose. After purification, the precipitated and input DNA was analyzed by semiquantitative PCR. Primer regions are depicted in Figure 2-1B. PCR products were analyzed by electrophoresis, ethidium bromide staining, scanned with the Typhoon System (Molecular Dynamics), and quantified by ImageQuant. The % IP represents the (IP signal / input signal) x100. All data represents at least three independent experiments from multiple extracts. Error bars represent the standard error.

**Micrococcol Nuclease (MNase) Nucleosome Mapping**

Nuclei preparation was carried out essentially as described previously (Li & Reese, 2001). Briefly, 1 liter of cells was grown in YPAD to an OD600 of around 0.8, induced with
0.02% MMS, harvested, washed twice with 30mLs sorbitol buffer (1.4M sorbitol, 40mM HEPES pH 7.5, 0.5mM MgCl₂, 10mM β–ME, and imM PMSF), and centrifuged at 5000 rpm for 5 minutes in a Sorvall HB6 rotor. Cell pellets were weighed and resuspended in 4 times their weigh in volume of SB (1g of cells = 4mL of SB). The cell suspension was warmed to 30 °C for five minutes before digestion with 0.5mg/mL Zymolyase T100 (Seikagaku) for ~25 minutes. The nuclei were washed twice more with cold SB, resuspended in 20mL Ficoll buffer (18% Ficoll 400, 20mM PIPES pH 6.5, 0.5mM MgCl₂, 1mM PMSF), homogenized by a machine pestle, and laid over glycerol buffer (7% Ficoll 400, 20% glycerol, 20mM PIPES pH 6.5, 0.5mM MgCl₂, 1mM PMSF). Nuclei were isolated by differential centrifugation and finally resuspended in digestion buffer (10mM HEPES pH 7.5, 0.5mM MgCl₂, 0.05 CaCl₂). Nuclei were digested by 0, 2, and 4 units/ml of micrococcal nuclease (MNase, Worthington) for 10 min at 37 °C. Reactions were stopped by the addition of 0.5M EDTA and treated with RNase A and proteinase K. The purified DNA was digested with PstI restriction enzyme, purified and separated on a 1.6% agarose gel. DNA was transferred to Hybond-XL membrane (GE Biosciences, Piscataway NJ) by capillary blotting overnight. Membranes were UV crosslinked using a Stratalinker and incubated in prehybridization solution for more than 4 hours at 65°C. Probe was added for overnight incubation. Membranes were washed and signals were detected using a phosphoimager. The phosphoimager screens were scanned with the Typhoon System (Molecular Dynamics), and quantified by ImageQuant. The probe for RNR3 corresponds to +486 to +725 relative to the translational start site. The PstI site is at +731.
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 Dr. Mitch Smith for histone mutant strains. 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 N-termini mutants examined in Chapter 2. (B) Schematic of primers used in ChIP experiments for Chapter 2.
2-1 A

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2-1 B

![Diagram of RNR3 - 2.6kb ORFs](image)
Figure 2-2: The histone N-termini are required for full activation of *RNR3*

(A) Northern blotting for *RNR3* mRNA. Wild type and N-termini deletion strains were grown and harvested before and after exposure to 0.02% MMS. mRNA levels were detected by northern blot and corrected for a loading control (scR1). (B) Northern blotting for *RNR3* mRNA. Wild type and H4 lysine to glutamine mutant strains were grown and harvested before and after exposure to 0.02% MMS. mRNA levels were detected by northern blot and corrected for a loading control (scR1). (C) Northern blotting for *RNR3* mRNA. Wild type (PYK501), H4 lysine to glutamine (LDY107), and H4 lysine to arginine mutant (LDY722) strains were grown and harvested before and after exposure to 0.02% MMS. mRNA levels were detected by northern blot and corrected for a loading control (scR1). (D) Northern blotting for *RNR3* mRNA. Wild type and H4 lysine mutant strains were grown and harvested before and after exposure to 0.02% MMS. mRNA levels were detected by northern blot and corrected for a loading control (scR1).
2-2 A

RNR3 mRNA Fold Expression

WT H2A Δ1-20 H2B Δ1-32 WT H3 Δ1-28 WT H4 Δ2-26

0h 1h +MMS 2.5h +MMS

2-2 B

RNR3 mRNA Fold Expression

WT H4 K5,8,12Q H4 K5,8,16Q H4 K5,12,16Q H4 all K→Q H3 all K→Q
Figure 2-3: Deletion of the H4 N-terminus results in the inability to release Crt1

(A) Levels of Crt1 loss at the RNR3 DRE. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for Crt1. The data is represented as fold loss relative to the untreated (-MMS) wild type cells. (B) Levels of Crt1 loss at the RNR3 DRE. Wild type and H4 lysine to glutamine mutant strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for Crt1. The data is represented as fold loss relative to the untreated (-MMS) wild type cells.
Figure 2-4: Deletion of the H4 N-terminus results in the inability to release Tup1

(A) Levels of Tup1 loss at the RNR3 DRE. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for Tup1. The data is represented as fold loss relative to the untreated (-MMS) wild type cells. (B) Levels of Tup1 loss at the RNR3 DRE. Wild type and H4 lysine to glutamine mutant strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for Tup1. The data is represented as fold loss relative to the untreated (-MMS) wild type cells.
Figure 2-5: Deletion of the H4 N-terminus results in constitutive recruitment of SWI/SNF

(A) Levels of SWI/SNF recruitment to the *RNR3* promoter. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for Swi2. The data is represented as fold increase relative to the untreated (-MMS) wild type cells. (B) Micrococcol nuclease map over *RNR3*. Wild type and H4 Δ2-26 strains were grown and treated or not with 0.02% MMS for 2.5hr. MNase nucleosome mapping was performed. The asterisk denotes the appearance of a hypersensitive double near the *RNR3* TATA region. The bars denote the hypersensitive site between the +1 and +2 nucleosomes. (C) Levels of H3 loss at the *RNR3* promoter upon induction. Wild type and H4 Δ2-26 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for the H3 C-terminus. The data is represented as fold loss relative to the untreated (-MMS) wild type cells.
2-5 C
Figure 2-6: Mutations to the H2A, H2B, and H4 N-termini do not significantly reduce H3 acetylation levels

(A) Levels of H3 acetylation (H3 K9,14Ac) enrichment at the RNR3 promoter. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K9,14Ac. The data is represented as fold increase relative to the untreated (-MMS) wild type cells. (B) Levels of H3 K9,14Ac enrichment at the RNR3 promoter. Wild type and H4 lysine to glutamine mutant strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K9,14Ac. The data is represented as fold increase relative to the untreated (-MMS) wild type cells.
2-6 A

2-6 B
Figure 2-7: The H2A N-terminus is important for H3 K4me3

(A) Levels of H3 lysine 4 tri-methylation (H3 K4me3) enrichment at the \textit{RNR3} promoter. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K4me3. The data is represented as % IP/Input. Primer regions for all experiments in this figure are depicted in Figure 2-1B. (B) Levels of H3 K4me3 enrichment at \textit{RNR3} ORF B. Same as Figure 2-7A, except primers for ORF B were used. (C) Levels of H3 K4 me3 enrichment at \textit{RNR3} ORF D. Same as Figure 2-7A, except primers for ORF D were used. (D) Levels of H3 lysine 4 tri-methylation (H3 K4me3) enrichment at the \textit{RNR3} promoter. Wild type and H4 lysine to glutamine mutant strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K4me3. The data is represented as % IP/Input. Primer regions for all experiments in this figure are depicted in Figure 2-1B. (E) Levels of H3 K4 me3 enrichment at \textit{RNR3} ORF B. Same as Figure 2-7D, except primers for ORF B were used. (F) Levels of H3 K4 me3 enrichment at \textit{RNR3} ORF D. Same as Figure 2-7D, except primers for ORF D were used.
2-7 A

2-7 B

2-7 C
2-7 D

Promoter

H3K4me3 %/Input

WT

H4 K5,8,12Q

H4 K5,8,16Q

H4 K5,12,16Q

H4 K8,12,16Q

H4 all K->Q

2-7 E

ORF B

H3K4me3 %/Input

WT

H4 K5,8,12Q

H4 K5,8,16Q

H4 K5,12,16Q

H4 K8,12,16Q

H4 all K->Q

2-7 F

ORF C

H3K4me3 %/Input

WT

H4 K5,8,12Q

H4 K5,8,16Q

H4 K5,12,16Q

H4 K8,12,16Q

H4 all K->Q
Figure 2-8: The histone N-termini are dispensable for TBP recruitment to the RNR3 promoter

(A) Levels of TBP recruitment to the RNR3 promoter. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for TBP. The data is represented as fold increase relative to the untreated (-MMS) wild type cells. (B) Levels of TBP recruitment to the RNR3 promoter. Wild type and H4 lysine to glutamine mutant strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for TBP. The data is represented as fold increase relative to the untreated (-MMS) wild type cells.
Figure 2-9: Mutations to the histone N-termini result in slight defects in RNAPII recruitment to the RNR3 promoter

(A) Levels of RNAPII recruitment to the RNR3 promoter. Wild type and N-termini deletion strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for RNAPII. The data is represented as fold increase relative to the untreated (-MMS) wild type cells. (B) Levels of RNAPII recruitment to the RNR3 promoter. Wild type and H4 lysine to glutamine mutant strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for RNAPII. The data is represented as fold increase relative to the untreated (-MMS) wild type cells.
2-9 A

RNAPII Fold Recruitment

-MMS
+MMS

2-9 B

RNAPII Fold Recruitment

-MMS
+MMS
Figure 2-10: Mutations to the H3 and H4 N-termini result in changes in RNAPII density over RN3

(A) Levels of RNAPII crosslinking across RN3. Wild type, H2A Δ1-20, and H2B Δ1-32 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for RNAPII. The data is represented as fold increase relative to the untreated (-MMS) wild type cells. Primer regions for all experiments in this figure are depicted in Figure 2-1B. (B) Levels of RNAPII crosslinking across RN3. Essentially the same as Figure 2-10A, except the H3 Δ1-28 strain was examined. (C) Levels of RNAPII crosslinking across RN3. Essentially the same as Figure 2-10A, except the H4 Δ2-26 strain was examined. (D) Levels of RNAPII crosslinking across RN3. Essentially the same as Figure 2-10A, except the H4 lysine to glutamine mutant strains were examined. Also, only the induced fold (+MMS) is depicted.
Table 2-1: List of Strains
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Table 2-2: List of Antibodies
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Chapter 3

ROLES FOR THE H4 N-TERMINUS IN TRANSCRIPTION
ELONGATION, TERMINATION, AND mRNA PROCESSING
Abstract

Post-initiation transcription events include elongation, termination, and mRNA processing. Roles for the H4 N-terminus in these post-initiation events are not well understood. Transcription elongation involves the disruption and reassembly of chromatin before and after RNAPII passes. Furthermore, cotranscriptional histone modifications are important for elongation. Since chromatin structure is integral to the regulation of elongation the H4 N-terminus may have roles in elongation; however, no elongation specific H4 modifications or functions have been identified. Previously, we have demonstrated that mutations to the H4 lysine residues alter RNAPII density at \textit{RNR3}; this is suggestive of an elongation defect. Here, we observe genetic interactions between the H4 lysine-5,8,12,16-glutamine mutation and deletions of elongation factors. Furthermore, we demonstrate that deletion of the H4 N-terminus results in decreased Spt6 and Spt16 recruitment and this leads to cryptic intragenic transcription at \textit{FLO8}. Currently, the role of chromatin in transcription termination and mRNA processing is poorly understood although many of the factors necessary for these events have been identified. Here we report that the H4 lysine residues are important for transcription termination at snoRNA genes. We also demonstrate that the deletion of the H4 N-terminus, or mutation of the lysine residues within the tail, results in mRNA splicing defects. These observations point to a model whereby the H4 N-terminus facilitates multiple stages of transcription.
Introduction

Although transcription initiation has been studied more intensively than post-initiation events, interest in transcription elongation, termination, and mRNA processing has developed more recently. Transcription elongation involves the coordination of chromatin disruption ahead of RNAPII as well as chromatin reassembly after the passage of RNAPII. In the absence of transcription, DNA is packaged tightly into chromatin in order to prevent the unintended binding of factors to the DNA. For this reason, the dynamics of disruption and reassembly during elongation are necessary. The nucleosome is an inherently repressive structure that will inhibit the passage of RNAPII. *In vitro* studies demonstrate that deletion or removal of the histone N-termini can partially alleviate this inhibition, suggesting that the tails provide an added level of repression (Georges et al, 2002). In order to overcome the nucleosomal barrier, RNAPII has evolved to recruit transcription elongation factors that facilitate chromatin disruption and reassembly, as well as histone modifications that allow for a more fluid passage.

The coordination of elongation is dependent on the dynamic phosphorylation state of the RNAPII C-terminal domain (CTD) repeats. In *S. cerevisiae*, Kin28, a subunit of TFIIH, is the major serine 5 kinase. Serine 5 phosphorylation is observed at the 5` ends of genes and occurs shortly after RNAPII is loaded onto the promoter (Komarnitsky et al, 2000, Rodriguez et al, 2000). Serine 2 phosphorylation is catalyzed by Ctk1 and is mostly observed in the middle and 3` ends of genes (Lee & Greenleaf, 1991, Xiao et al, 2003). Ssu72 and Fep1 are the major serine 5 and 2 phosphatases respectively and help regulate the patterns of RNAPII CTD phosphorylation (Cho et al, 2001, Ganem et al, 2003, Krishnamurthy et al, 2004). Additionally, other complexes have been reported to play a role in CTD phosphorylation including Bur1/Bur2 and the Srb10 subunit of Mediator (Kuchin & Carlson, 1998, Yao & Prelich, 2002). These phosphorylation
events have been implicated in the recruitment of elongation factors, histone modifying enzymes, and mRNA processing factors.

The RNAPII CTD is phosphorylated during active transcription. These phosphorylation events play a role in the recruitment of PAF, DSIF, FACT, and Spt6. While phosphorylation of the RNAPII CTD coordinates the recruitment of these elongation factors, these factors then coordinate histone modifications as well as the chromatin disruption and reassembly. The PAF (Polymerase Associating Factor) complex interacts directly with RNAPII and coordinates cotranscriptional histone methylation. The PAF complex is composed of five subunits: Paf1, Cdc73, Leo1, Rtf1, and Ctr9. The subunits of PAF have individual and overlapping functions in a variety of transcription elongation events (Chu et al, 2007a, Hampsey & Reinberg, 2003, Laribee et al, 2005, Sheldon et al, 2005). DSIF (DRB Sensitivity Inducing Factor) is composed of Spt4 and Spt5. In vitro and in vivo analyses have demonstrated that DSIF is an elongation factor that interacts with RNAPII (Kaplan et al, 2000, Qiu et al, 2006, Wada et al, 1998). DSIF is involved in both repression and stimulation of transcription (Kaplan et al, 2000, Qiu et al, 2006, Wada et al, 1998). The FACT (Facilitates Chromatin Transcription) complex is composed of Spt16 and Pob3; it is another complex whose recruitment is dependent upon the phosphorylation of the RNAPII CTD (Krogan et al, 2002b). FACT is known to interact with and partially evict the H2A-H2B dimer during transcription elongation (Belotserkovskaya et al, 2003). FACT is also important for the repression of intragenic transcription and therefore is involved with the reassembly of chromatin after RNAPII passage (Kaplan et al, 2003). Spt6 is an elongation factor in and of itself and has been implicated in a variety of transcription events (Endoh et al, 2004, Kaplan et al, 2000, Yoh et al, 2007). In S. cerevisiae, Spt6 is known to be involved in chromatin disruption and reassembly as well as Set2 methylation (Youdell et al, 2008). In higher eukaryotes, Spt6 has been shown to interact directly with the phosphorylated CTD as well as play roles in mRNA splicing (Endoh et al, 2004, Kaplan et al, 2000, Yoh et al, 2007). These factors play
essential roles in transcription elongation and attest to the complexity and regulation involved in elongation.

Some of the factors described above are important for the regulation of cotranscription histone modifications. Histone methylation patterns during elongation are well characterized. Histone H3 lysine 4 tri-methylation (K4me3) is found predominantly at the promoter and 5’ ends of genes and is involved with mRNA capping. H3 K4me3 and histone acetylation are known to be cooperative. H3 K4 methylation is dependent upon H2B lysine 123 ubiquitylation (K123Ub) which also stimulates H3 lysine 79 methylation (K79me) (Dover et al, 2002, Giannattasio et al, 2005, Lee et al, 2007). H2B K123Ub is catalyzed by Rad6/Bre1 and is dependent upon phosphorylation of the RNAPII CTD (Dover et al, 2002, Laribee et al, 2005, Wysocki et al, 2005). The PAF complex links RNAPII CTD phosphorylation to these histone modifications (Hampsey & Reinberg, 2003, Laribee et al, 2005). Additionally, PAF is important for H3 lysine 36 tri-methylation (K36me3). H3 K36me3 is catalyzed by Set2 and is found predominantly in the middle and 3’ ends of genes (Krogan et al, 2003, Li et al, 2003, Rao et al, 2005, Strahl et al, 2002). Set2 directly interacts with the dually phosphorylated RNAPII CTD and the pattern of CTD phosphorylation directly regulates the pattern of Set2 methylation across a gene (Kizer et al, 2005, Krogan et al, 2003, Li et al, 2003). Spt6 is also important for Set2 methylation further, establishing a complex network of cotranscriptional events (Youdell et al, 2008).

Histone acetylation is found predominantly at the promoters of genes; however, cotranscriptional histone acetylation by SAGA has been recently identified (Govind et al, 2007). Presumably, these acetylation events aid to disrupt histone tail interactions with nucleosomal DNA. However, to prevent spurious intragenic transcription from initiating, Set2 mediated H3 K36me3 serves to recruit the Rpd3 histone deacetylase (HDAC) complex (Carrozza et al, 2005, Joshi & Struhl, 2005, Keogh et al, 2005, Li et al, 2007b). This in turn removes the acetyl marks from nucleosomes within the gene. Mutations to components of the Set2-Rpd3 pathway, or any of
the elongation factors involved in H3 K36me3, result in intragenic transcription (Carrozza et al, 2005). Mutations to the Spt6 and Spt16 elongation factors also result in intragenic transcription because chromatin is not properly reassembled without these factors (Kaplan et al, 2003).

RNAPII CTD phosphorylation is also important for the transition from elongation to termination. Transcription termination is a complex process dependent on the recruitment and function of termination factors. There exist two distinct pathways of termination for transcription of mRNAs and snoRNAs (Kim et al, 2006). Termination of mRNA transcripts involves the cleavage and polyadenylation of the nascent mRNA and these events are signaled by cis acting elements (Kim et al, 2006). Termination of snoRNAs is slightly different. The original models of snoRNAs termination involved the cleavage of the nascent transcript, but not polyadenylation (Kim et al, 2006). However, the polyadenylation machinery was found to crosslink at the 3’ ends of snoRNAs but mutations that inhibited polyadenylation did not affect snoRNA termination (Grzechnik & Kufel, 2008, Lykke-Andersen & Jensen, 2007). This led to the hypothesis that RNAPII transcription kept both termination pathways available and that although the machinery is in place, snoRNAs do not require polyadenylation. More recently, it was demonstrated that snoRNAs are transiently polyadenylated and that the nuclear exosome, Rrp6, rapidly removes the poly-A tail from snoRNAs (Grzechnik & Kufel, 2008). Nrd1, Nab3, and Sen1 are all termination factors that are important for termination of snoRNAs (Carroll et al, 2004). In addition to these termination factors, RNAPII CTD phosphorylation, DSIF, and the PAF complex are important for snoRNA termination, linking transcription elongation to termination (Sheldon et al, 2005).

mRNA splicing is another component in the production of mature mRNAs. The splicing machinery was initially characterized using mostly in vitro systems. One of the limitations of these studies has been that most of these in vitro systems uncouple splicing from transcription. More recent experiments suggest that in higher eukaryotes, most splicing occurs cotranscriptionally while the RNA is still emerging from RNAPII (Das et al, 2006, de la Mata et
alt, 2003, Hicks et al, 2006, Hirose et al, 1999, Morris & Greenleaf, 2000). These observations are supported by the fact that RNAPII CTD phosphorylation and transcription elongation factors, Spt5 and Spt6, are important for splicing (Dye et al, 2006, Hirose et al, 1999, Lindstrom et al, 2003, Morris & Greenleaf, 2000, Sims et al, 2007, Yoh et al, 2007). In *S. cerevisiae*, cotranscriptional splicing is thought to occur only at genes with long second exons (Tardiff et al, 2006). In higher eukaryotes, where cotranscriptional splicing is more prevalent than in *S. cerevisiae*, chromatin remodeling complexes such as hSWI/SNF and chromatin modifying complexes such as PRMT1, PRMT4, and PRMT5 have been implicated in splicing (Allemand et al, 2008). This data suggests that chromatin structure and modifications may be an important regulator of cotranscriptional splicing. One model suggests that the highly basic histone N-termini sequester nascent mRNA until the RNA binding factors bind (Allemand et al, 2008). This model also invokes the possibility that histone modifications can alter the mRNA-histone tail interaction and thus regulate splicing. While the mechanism of this model is partially speculative, roles for the histone N-termini in splicing could be revealed through studies in a model system like *S. cerevisiae*, in which the histone tails are nonessential.

In this study, we examine the histone H4 for roles in transcription elongation, termination, and mRNA splicing. Transcription elongation is tightly coupled to chromatin structure, but specifically how the histone tails regulate transcription elongation is unclear. Here we report that substitution of the H4 lysine residues for glutamines results in genetic interactions with elongation factors. Furthermore, deletion of the H4 N-terminus results in intragenic transcription at *FLO8*. This defect is likely the result of an inability to recruit Spt6 and Spt16 and implicates the H4 tail in the maintenance of repressive chromatin structure. Roles for chromatin structure and the histone N-termini in transcription termination and mRNA splicing are less clear. We demonstrate that the H4 N-terminal lysine to glutamine mutation results in termination defects at snoRNAs. We also demonstrate that deletion of the H4 N-terminus, or mutation of the
lysine residues within the tail, results in splicing defects at \textit{ACT1} and \textit{CYH2}, two genes thought to be cotranscriptionally spliced.
Results

Mutations to the H4 N-terminus result in genetic interactions with elongation factors

We have established that mutations to the histone H4 N-terminus result in changes in RNAPII density across RNR3. This led to the hypothesis that the H4 N-terminus may be involved in transcription elongation. To further explore this hypothesis, we examined the effects of 6-azauracil (6-AU) on the growth of H4 mutants (Figure 3-2A). 6-AU is a drug that inhibits IMP dehydrogenase. As a result GTP levels are significantly reduced and the limiting pool of free GTP inhibits transcription elongation. Mutations to elongation factors are known to show altered sensitivity to 6-AU. We examined the effects of 6-AU on the growth of the H4 lysine to glutamine mutants as well as the H4 N-terminal deletion (Figure 3-2). While the H4 all K->Q and H4 Δ2-26 strains grew more slowly than the wild type on untreated media, the triple K->Q mutants grew similar to wild type. When treated with 6-AU, none of the H4 mutants showed significant sensitivity to the drug. The H4 K5,8,12Q, H4 K5,8,16Q, and H4 K5,12,16Q mutants exhibited very slight, but reproducible, resistance to 6-AU. Mutations to negative elongation factors can result in resistance to 6-AU by enhancing transcription elongation. This may suggest that the H4 K5,8,12Q, H4 K5,8,16Q, and H4 K5,12,16Q mutations are also enhancing transcription elongation.

We next sought to examine whether mutations to the histone tails would genetically interact with elongation factors. This approach has been utilized extensively in order to help determine functions of proteins. The strains we examined are the wild type, the H4 K5,8,12Q, the H4 K8,12,16Q, and the H4 all K->Q. In addition to the H4 all K->Q, we chose to examine the H4 triple mutant with increased RNR3 expression (H4 K5,8,12Q) as well as the H4 triple mutant with
the most significant \textit{RNR3} expression defect (H4 K8,12,16Q). The elongation factors that we sought to delete in these strains were Srb10, Eaf3, Set1, and Dst1. Srb10 is a component of mediator and is involved in the phosphorylation of the RNAPII CTD (Kuchin & Carlson, 1998). Eaf3 is a component of the Rpd3 HDAC complex that is important for Set2 mediated repression of intragenic transcription (Carrozza et al, 2005, Joshi & Struhl, 2005, Keogh et al, 2005, Li et al, 2007b). Set1, the methyltransferase subunit of COMPASS, mediates H3 K4 methylation which has been implicated in the recruitment of factors for chromatin remodeling and mRNA capping (Hampsey & Reinberg, 2003, Krogan et al, 2002a, Santos-Rosa et al, 2003). DST1 codes for TFIIS, an elongation factor which is important for the release of stalled polymerases (Kipling & Kearsey, 1993). We felt that these elongation factors encompass a wide variety of functions during transcription elongation and therefore would provide a well rounded examination.

We began our genetic interaction studies by transforming each of the histone mutant strains, wild type included, with a plasmid harboring the URA3+ marker and a wild type copy of the histone genes. We used these strains, now expressing both the wild type (plasmid) and the mutant (integrated) histones, to knock-out our elongation factors of interest. Once these double mutants were constructed, we grew cultures and spotted serial dilution on synthetic complete (SC) or SC+5-FOA media (Figure 3-3). In order to grow on 5-FOA, the strains must lose the plasmid harboring the URA3+ marker and the wild type copy of the histone genes. Colonies growing on the 5-FOA plates will then be left with only their integrated histone genes. Growth defects on the 5-FOA plates are suggestive of genetic interactions between the histone mutation and the elongation factor mutation. The H4 all K->Q strain exhibited striking synthetic lethality with each of the elongation factor deletions (Figure 3-3, compare SC plates to SC+5-FOA plates). Interestingly, neither of the H4 triple lysine to glutamine mutants resulted in slow growth defects when combined with any of the deleted elongation factors (Figure 3-3, compare SC plates to SC+5-FOA plates). This was somewhat unexpected considering the different patterns of RNAPII
density at RNR3 upon induction in these strains. Unlike RNR3 expression, where the H4 K8,12,16Q mutation resulted in a phenotype similar to the H4 all K->Q strain, the genetic interactions with elongation factors seems to require all four of the lysines to be mutated.

The fact that the H4 triple lysine to glutamine mutants did not genetically interact with the elongation factors was surprising. To further examine these H4/elongation factor double mutants, we challenged their growth with 6-AU. We were unable to further explore the double mutants that harbored the H4 all K->Q mutation because growth could not be sustained after passage on 5-FOA. We therefore focused on the strains with the triple lysine to glutamine mutations. Each of the strains examined was passed several times on 5-FOA plates to ensure the removal of the wild type histone plasmid prior to performing the spot tests. The H4 K5,8,12Q and H4 K8,12,16Q mutations do not exhibit growth defects on –URA media (Figure 3-4A-D). Furthermore, in strains in which these mutations are combined with elongation factors deletions, growth defects are still not observed (Figures 3-3 and 3-4A-D) Previously it has been shown that deletion of SRB10 or EAF3 confers slight resistance to 6-AU while deletion of SET1 or DST1 results in 6-AU sensitivity. We also observe these phenotypes and use them as controls (Figure 3-4A-D). As noted earlier, the H4 K5,8,12Q mutation confers slight resistance to 6-AU, but the H4 K8,12,16Q mutation results in neither resistance nor sensitivity to 6-AU (Figure 3-2 and 3-4 A-D). In fact, the H4 K8,12,16Q mutation does not alter growth on 6-AU even in the presence of the elongation factor deletions (Figure 3-4A-D). This again was unexpected since this histone mutation so strongly affects RNR3 transcription. Perhaps some level of redundancy for the histone tails is prevalent throughout the cell but not specifically at RNR3.

The H4 K5,8,12Q/Δsrb10 and H4 K5,8,12Q/Δeaf3 double mutants do not result in greater resistance than any of the mutations alone (Figure 3-4 A-B). Deletion of SRB10 or EAF3 in the H4 K5,8,12Q background did not increase the 6-AU resistance. While it is possible that the H4 K5,8,12Q, Δsrb10, and Δeaf3 mutations are all affecting the same pathway, it is also possible that
there is a limit to the amount of resistance to 6-AU that can be conferred by mutations. Therefore, while we cannot determine the pathway by which the H4 K5,8,12Q mutation is mediating its effects, the evidence suggests that it is enhancing transcription elongation.

The additional deletion of SET1 in the H4 K5,8,12Q strain did not significantly diminish the resistance to 6-AU that this H4 mutant exhibits. The H4 K5,8,12Q/Δset1 mutant is still resistant to 6-AU, suggesting that the resistance observed in the H4 K5,8,12Q strain is independent of Set1 methylation. Conversely, the H4 K5,8,12Q/Δdst1 strain is slightly more sensitive to 6-AU than the Δdst1 strain and significantly more sensitive than the H4 K5,8,12Q strain. This may imply that the resistance to 6-AU observed in the H4 K5,8,12Q is mediated by enhanced interactions with TFIIS.

The H4 N-terminus suppresses intragenic transcription in a pathway separate from Set2-Rpd3

Transcription elongation involves the disruption of chromatin structure ahead of RNAPII as well as the reassembly of chromatin structure after RNAPII passes. This disruption and reassembly is mediated by several elongation factors and chromatin modifications. Mutations to the factors and chromatin modifiers involved in the reassembly process can result in intragenic transcription. This occurs when RNAPII inappropriately initiates in the middle of a gene because repressive chromatin structure has not been reset. This results in the production of aberrant transcripts. FLO8 and STE11 are two genes that exhibit short intragenic transcripts when certain elongation factors are mutated or deleted.

We sought to determine if the H4 N-terminus was important for the repression of intragenic transcription at FLO8 (Figures 3-5A and 3-5B). Initially we screened the H4 tail
deletion as well as the H4 lysine to glutamine mutants (Figure 3-5A). As controls, we examined \( \Delta \text{Set1}, \Delta \text{Set2}, \Delta \text{Eaf3}, \text{and} \Delta \text{Ctk1} \) strains. Set1 is not involved with the repression of intragenic transcription and acted as a negative control; however, Set2, Eaf3, and Ctk1 are all involved in the Set2-Rpd3 pathway that represses intragenic transcription. Set2 methylates H3 lysine 36 and this mark serves to recruit the Rpd3 HDAC complex (Carrozza et al, 2005, Joshi & Struhl, 2005, Keogh et al, 2005). Rpd3 deacetylates histones within the coding region to maintain repressive chromatin (Carrozza et al, 2005, Joshi & Struhl, 2005, Keogh et al, 2005). We observe that deletion of any of these factors results in intragenic transcription. The H4 \( \Delta 2-26 \) mutation results in significant intragenic transcription at \( \text{FLO8} \) (Figure 3-5A). The H4 all K->Q strain does as well, but to a lesser degree. None of the H4 lysine to glutamine triple mutants exhibited intragenic transcription at \( \text{FLO8} \). Similar to the genetic interaction analysis, this suggests redundancy for the H4 tail lysine residues.

The chromatin modification pathway of repression of intragenic transcription is dependent upon Set2 mediated H3 K36me3. To determine if the H4 N-terminus is important for this pathway we performed western blotting to examine the global levels of H3 K36me3 in the wild type and H4 \( \Delta 2-26 \) strains (Figure 3-6A). Deletion of the H4 N-terminus does not result in global H3 K36me3 defects and there may even be a slight enhancement of methylation in this strain. We next examined H3 K36me3 levels by ChIP at two constitutively active genes, \( \text{PMA1} \) and \( \text{PYK1} \), and once again observed no H3 K36me3 defect (Figure 3-6B). These results suggest that the H4 N-terminus is important for the repression of intragenic transcription independent of the Set2-Rpd3 pathway.

In strains harboring mutations to factors responsible for maintaining repressive chromatin, \( \text{FLO8} \) intragenic transcription results in two short transcripts presumably from two cryptic promoters. We note that there exists a third band in the H4 \( \Delta 2-26 \) mutant that appears just
below the full length transcript and often appears as a doublet. Whether or not this is a third cryptic transcript from a previously unidentified cryptic promoter is unclear. The major intragenic transcript that results from the H4 mutations is the smaller of the two short transcripts (Figure 3-5A). This differs from what is observed in the Δset2, Δeaf3, and Δctk1 strains and this also supports the hypothesis that the mechanisms of these defects are different. Two different mechanisms of repression exist: the chromatin modification pathway (Rpd3-Set2) and the chromatin remodeling pathway (Spt6 and Spt16). One type of defect may favor initiation from one promoter over the other. Significantly, when we examined cryptic transcription at STE11, we observed no defect in the H4 mutants (Figure 4-5A). Previously, it has been established that Spt6 inhibits intragenic transcription at both FLO8 and STE11. At STE11, the intragenic transcription defect in an spt6-1004 mutant can be suppressed through overexpression of Set2 (Youdell et al, 2008). This is not the case at FLO8, suggesting that Spt6 represses intragenic transcription in a pathway separate from Set2-Rpd3 at FLO8 (Youdell et al, 2008). The H4 N-termini may also be involved in this separate pathway.

The FACT (Facilitates Chromatin Transcription) complex is another elongation factor known to repress intragenic transcription. Spt6, one of two FACT subunits, and Spt16 are both essential so we utilized temperature sensitive mutants for our investigation. We harvested spt6-1004 and spt16-197 cells that were grown at 30°C and shifted to 37°C for 1.5 hours in order to compare the intragenic transcription in these temperature sensitive strains to those in the H4 mutants. Prior to heat shock, we observe mild intragenic transcription in the spt6-1004 and spt16-197 strains (Figure 3-5B). After the shift to 37°C, there is a significant increase in intragenic transcription especially with regard to the smaller of the two intragenic transcripts. This pattern clearly resembles that observed in the H4 Δ2-26 strain and may indicate a role for the H4 N-terminus in the recruitment or activity of Spt6 and FACT (Figure 3-5B).
The H4 N-terminus is important for the recruitment of elongation factors

To further pursue the role of the H4 N-terminus in the recruitment of elongation factors, we constructed strains that harbored the H4 Δ2-26 or the H4 all K->Q mutations as well as myc-tagged elongation factors. In addition to Spt6-myc and Spt16-myc, we also examined the recruitment of Spt5-myc and Paf1-myc. The recruitment of these factors is dependent upon transcription and therefore defects in crosslinking must take into account RNAPII density. The fact that RNAPII levels are so low within RNR3 in the H4 Δ2-26 and H4 all K->Q mutant strains makes detecting elongation defects difficult. For this reason, we sought other well characterized genes in which RNAPII density is not as significantly reduced. PMA1 and PYK1 are constitutively transcribed and we hypothesized that using these model genes would reduce the effects of activation defects that may be observed at RNR3. We examined RNAPII density in the ORF regions of PYK1 and PMA1 and observed lesser, although detectable, defects in the H4 mutants relative to RNR3. The H4 Δ2-26 mutation resulted in 80% and 65% of wild type RNAPII density at PYK1 and PMA1 respectively (Figure 3-7A). The H4 all K->Q mutation resulted in 50% of wild type RNAPII density at both at PYK1 and PMA1 (Figure 3-7A). PYK1 and PMA1 therefore provide better models to examine transcription elongation defects because it avoids the complication of transcription initiation defects.

We next examined the crosslinking of Spt5, Spt6, Spt16, and Paf1 (Figures 3-7B, C, D, and E respectively) to the ORFs of PYK1 and PMA1. To account for differences in RNAPII density, we have normalized the levels of these elongation factors to RNAPII and set the wild type ratio to 1. Mutation of the H4 lysines to glutamine did not reduce the crosslinking of these elongation factors. In fact, Spt6 and Spt16 crosslinking is enhanced relative to RNAPII (Figure 3-7B and C). This suggests that the H4 lysine residues are not required for the recruitment of these factors. Distinct from the lysine to glutamine mutant, deletion of the H4 tail significantly reduces
Spt6, Spt16, Spt5, and Paf1 recruitment (Figures 3-7B, C, D, and E respectively). The crosslinking of these factors relative to RNAPII levels is only 40-50% of the wild type. This further implicates the H4 N-terminus in transcription elongation and reveals an important distinction between the H4 tail deletion and the H4 all K->Q mutation. Furthermore, the loss of Spt6 and Spt16 crosslinking provides insight into the role of the H4 N-terminus in repression of cryptic transcription at *FLO8*. Both of these factors are important for maintaining repressive chromatin structure. It is likely that the H4 N-terminus is also involved in the chromatin remodeling pathway of repression, possibly through the recruitment or function of Spt6 and Spt16.

Deletion of the H4 N-terminus results in recruitment defects of several elongation factors to the *PYK1* and *PMA1* genes (Figure 3-7). We have established that the H4 Δ2-26 mutation results in a pattern of intragenic transcription at *FLO8* similar to the patterns observed in the spt6-1004 and spt16-197 temperature sensitive mutants (Figure 3-5B). We next sought to determine how the H4 Δ2-26, spt6-1004, and spt16-197 mutants affected *PYK1* and *PMA1* expression. We reasoned that if Spt6 and Spt16 were important for *PYK1* and *PMA1* expression so would the H4 N-terminus. We examined the expression by northern blots and found that when either Spt6 or Spt16 was inactivated by heat shock, both *PYK1* and *PMA1* mRNA levels were reduced (Figures 3-8A and B respectively). The effects on *PMA1* were more pronounced than on *PYK1*. Deleting the H4 N-terminus also resulted in lower levels of *PYK1* and *PMA1* expression. However, the expression levels, 0.8 and 0.6-fold wild type respectively, very closely mirror the levels of RNAPII density within the ORFs (Figures 3-7A and 3-8). The H4 Δ2-26 strain has significantly reduced levels of Spt6 and Spt16 in the ORFs of *PYK1* and *PMA1*. While expression of these genes is dependent upon Spt6 and Spt16, the H4 Δ2-26 strain expresses these genes at levels that correlate well with RNAPII density. On the other hand, the H4 Δ2-26 strain does exhibit
intragenic transcription similar to the spt6-1004 and spt16-197 mutants. This may suggest that deletion of the H4 N-terminus alleviates the requirement for elongation factors in the disruption of chromatin structure. Alternatively, it is possibly that the reduced levels of Spt6 and Spt16 are enough to disrupt chromatin. In either case, it is clear that the reduced Spt6 and Spt16 recruitment results in the inability for these elongation factors to reassemble chromatin structure after RNAPII passes.

**Roles for the H4 N-terminus in transcription termination and mRNA splicing**

The process of transcription involves initiation, elongation, termination, and cotranscriptional mRNA splicing. Emerging evidence has implicated elongation factors in transcription termination and mRNA splicing. Spt5 and Paf1 mutants result in termination defects at snoRNA genes (Sheldon et al, 2005). Additionally, in *S. cerevisiae* Spt5 is important for the splicing of a subset of genes (Lindstrom et al, 2003, Xiao et al, 2005). Recent evidence also suggests that Spt6 has roles in mRNA splicing in humans, although this has not been examined in *S. cerevisiae* (Yoh et al, 2007). We have already demonstrated that deletion of the H4 N-terminus results in reduced crosslinking of these elongation factors. For this reason, the H4 tail may be important for termination and mRNA processing events. Recently snoRNAs have been used as a model to study transcription termination (Ganem et al, 2003, Sheldon et al, 2005). snoRNAs are particularly sensitive to mutations that affect termination. The RNAPII CTD kinases and phosphatases play important roles in the termination of snoRNAs as do the PAF complex and DSIF (Ganem et al, 2003, Sheldon et al, 2005). Mutations to these factors lead to transcription readthrough of SNR13 and result in a SNR13-TRS31 fusion transcript (Figure 3-9A). We used a probe for TRS31 to determine if mutations to the H3 or H4 N-termini result in transcription readthrough (Figure 3-9B). Mutations to the H3 N-terminus did not result in a termination defect.
Since deletion of the H4 N-terminus results in reduced Paf1 and Spt5 recruitment, we also expected a snoRNA termination defect in this strain. This was not the case. The H4 Δ2-26 mutant exhibits only minor amounts of transcription readthrough (Figure 3-9B). On the other hand, The H4 all K->Q strain did exhibit a significant level of transcriptional readthrough similar to the Δctk1 strain that was used as a positive control. Since the H4 lysine residues seemed to be of particular importance for transcription termination, we probed the H4 triple lysine to glutamine mutants for readthrough at SNR13 (Figure 3-9C). None of the triple mutants exhibit termination defects. Previously we had observed strong synthetic lethality when the H4 all K->Q mutation was combined with mutations to elongation factors. The H4 lysine triple mutants did not result in synthetic lethality. The specificity for the H4 all K->Q is once again observed for transcription termination defects. This may indicate redundant functions for the H4 lysine residues in both elongation and termination.

Cotranscriptional mRNA splicing is another process that the histone N-termini may help coordinate. Although mRNA splicing occurs less frequently in *S. cerevisiae* than in higher eukaryotes, the splicing machinery and several genes that require splicing are essential. We have demonstrated that deletion of the H4 N-terminus results in reduced recruitment of Spt6 and Spt5, two factors known to play roles in splicing (Lindstrom et al, 2003, Xiao et al, 2005, Yoh et al, 2007). Furthermore, it has been proposed that the histone tails themselves sequester the nascent mRNA until RNA binding and splicing factors can bind (Allemand et al, 2008). This is speculative and has not been examined in a system which allows for histone tail mutations.

We used northern blotting probes for the *ACT1* intron, full length *ACT1*, and a *CYH2* intron/exon junction to examine splicing in the histone mutants. We then calculated the ratio of unspliced to spliced transcript. Mutations to the H3 N-termini did not change the ratio by more than 10% suggesting that the H3 tail is not required for splicing (Figure 3-10A and B). Conversely, both the H4 Δ2-26 and H4 all K->Q mutations resulted in an increase in the ratio of
unspliced to spliced transcript (Figures 3-10A and B). The H4 tail deletion increased the $ACTI$ ratio by 50% and the $CYH2$ ratio by 30%. The H4 lysine to glutamine mutation increased the $ACTI$ ratio by 40% and the $CYH2$ ratio by 80%. These observations suggest that the H4 N-terminus plays an important role in mRNA splicing either through the recruitment of factors or by mediating mRNA sequestration. If the histone tail-mRNA sequestration model were true, our results would suggest that the H4 tail specifically regulates this process.
Discussion

Genetic interactions implicate the H4 N-terminus in transcription elongation

Substitution of the histone H4 lysine residues for glutamines resulted in altered RNAPII density across RNR3. This provided the first evidence of elongation defects in this mutant and helped explain the disparity between nearly wild type PIC formation as evidenced by TBP crosslinking (Figure 2-9B) and significantly reduced mRNA expression levels (Figure 2-2B). We further examined possible roles for the H4 N-terminus in elongation by exploring whether or not mutations to H4 genetically interact with elongation factors. Constructing double mutants and probing for changes in growth phenotypes is a classical genetic approach that has been used to ascertain the function of unknown proteins. While the function of histones in chromatin structure is well established, roles in the regulation of transcription elongation are underdeveloped. Here we constructed a series of H4 mutants in combination with deletions of several elongation factors and probed for synthetic lethality. We chose to delete SRB10, SET1, EAF3, and DST1 in the H4 mutants because these genes represent integral parts of four separate elongation processes. The most striking result from this analysis was the synthetic lethality that resulted in each of the H4 all K->Q double mutants. This has led to two conclusions. The first is that mutation of all four acetylatable lysines on H4 is necessary to observe these genetic interactions, suggesting a high level of redundancy among the H4 lysine residues. The second, more significant conclusion, is that the H4 N-terminus may regulate transcription elongation on many levels. SRB10, SET1, EAF3, and DST1 represent different aspects of elongation and the H4 all K->Q strain genetically interacts with each of these gene deletions.
The combination of elongation factor deletions and H4 lysine to glutamine triple mutants did not result in synthetic growth defects. This result was unanticipated based on the differences in RNR3 expression and RNAPII density between the H4 K5,8,12Q and H4 K8,12,16Q strains. Using a global approach like genetic interactions, there seems to be redundancy in the roles of the H4 lysine residues in elongation. On the other hand, at RNR3 specifically, lysine 16 seems to be crucial and its mutation results in a significant transcription defect. We challenged these double mutants with 6-AU to explore the possibility that the elongation inhibitor may enhance subtle phenotypes that previously had been indiscernible. The most interesting observation from this analysis was the difference between the H4 K5,8,12Q/Δset1 and the H4 K5,8,12Q/Δdst1 strains. While the H4 K5,8,12Q/Δset1 strain remained resistant to 6-AU even though Δset1 confers sensitivity, the H4 K5,8,12Q/Δdst1 strain was very sensitive to 6-AU. These observations point to a mechanism whereby the 6-AU resistance observed in the H4 K5,8,12Q strain is strictly dependent upon DST1 and independent of SET1. Accordingly, we have previously observed that the H4 K5,8,12Q mutation did not result in changes to H3 K4me3 levels.

The H4 N-terminus mediates repressive chromatin during transcription elongation

Transcription elongation involves the disruption and reassembly of ordered chromatin before and after RNAPII passes. The inability to disrupt chromatin would inhibit transcription elongation due to the blockage created by the nucleosome. Additionally, defects in the reassembly of chromatin result in intragenic transcription. In order to maintain the proper dynamics of transcription elongation, the coordination of disruption and reassembly must be seamless. This coordination involves the recruitment of elongation factors such as Spt6 and FACT, which are known to facilitate these processes. While the recruitment of these factors is
dependent upon RNAPII, we hypothesized that the histone N-termini may also be important for their recruitment or function.

In order to gain better insight into the roles of the histone H4 in the disruption and reassembly of chromatin, we first examined the H4 mutants for defects in the repression of intragenic transcription at \textit{FLO8}. Deletion of the H4 N-terminus resulted in significant levels of \textit{FLO8} intragenic transcription (Figure 3-5A). The H4 all K->Q strain also exhibited this phenotype, but to a lesser degree. Initially, our positive controls for this experiment were deletions to factors involved in the Set2-Rpd3 pathway. However, the pattern of intragenic transcription we observed in the H4 $\Delta$2-26 strain was different from the pattern observed in the $\Delta$set2 strain (Figure 3-5A). Furthermore, the H4 N-terminus is not required for Set2 mediated methylation of H3 K36 (Figure 3-6). This suggests that the mechanism of repression by the H4 tail was different from the Set2-Rpd3 pathway. Spt6, part of the chromatin remodeling pathway, has been shown to repress intragenic transcription at \textit{FLO8}, but not \textit{STE11}, independent of Set2-Rpd3 (Youdell et al, 2008). We hypothesized that the H4 N-terminus may also be part of the chromatin remodeling pathway. Indeed, we do not observe intragenic transcription at \textit{STE11} in the H4 $\Delta$2-26 strain (Figure 4-5A). Next we compared the H4 $\Delta$2-26 strain to Spt6 and Spt16 temperature sensitive mutants. The patterns of intragenic transcription in these mutants are very similar which suggests these elongation factors are involved in the same pathway as the H4 N-terminus.

Deletion of the H4 N-terminus results in significantly lower levels of Spt6 and Spt16 recruitment to the constitutively expressed \textit{PYK1} and \textit{PMA1} genes (Figure 3-7). Spt6 and Spt16 are known to be important for the repression of intragenic transcription; this data correlates well with the fact that the H4 $\Delta$2-26 strain results in greater levels of intragenic transcription than the H4 K->Q strain. Unexpectedly, we found that the reduced levels of elongation factors within
PYK1 and PMA1 did not adversely affect expression of these genes relative to the spt6-1004 and spt16-197 strains. This suggests that the levels of Spt6 and Spt16, although reduced, are enough to stimulate transcription at PYK1 and PMA1. It is possible that RNAPII’s requirement for these elongation factors is reduced in the H4 tail deletion. In vitro experiments reveal that deletion of the histone N-termini alleviate the requirement for coactivators on chromatin templates (Georges et al, 2002). Repressive chromatin cannot be formed at FLO8 in the H4 Δ2-26 strain and this is presumably the case genome wide. Is possible that RNAPII can more easily transcribe through the open chromatin environment, and that the stringent requirements for Spt6 and Spt16 may be alleviated.

**Coordination of transcription by the H4 N-terminus**

In addition to transcription initiation and elongation, we also explored roles for the H4 N-terminus in termination and mRNA splicing. snoRNAs have been a particularly useful model for studying termination defects. Termination of snoRNAs involve a similar but distinct pathway to the termination of mRNAs (Kim et al, 2006). For this reason, it is possible observe termination defects at snoRNAs even in the absence of termination defects at mRNAs, such as RNR3. The termination events at snoRNAs are dependent upon the RNAPII CTD phosphorylation status. CTD kinase or phosphatase mutants exhibit transcription readthrough due to termination defects (Ganem et al, 2003). Additionally, DSIF and the PAF complex have been shown to be important for termination (Sheldon et al, 2005). We hypothesized that the H4 mutants, in particular the H4 tail deletion, may also result in transcriptional readthrough based on the ChIP data demonstrating reduced levels of Spt5 and Paf1 in this strain. This was not the case. We observed that the H4 all K->Q mutation results in significant amounts of transcription readthrough and that the H4 Δ2-26 mutation results in only a minor phenotype (Figure 3-9). Additionally, we note that none of the
H4 lysine to glutamine triple mutants exhibit termination defects. This phenomenon is similar to that observed with the genetic interaction studies and again suggests redundancy in the H4 acetylatable lysine residues, whether the role is in transcription elongation or termination. It is interesting that the H4 all K->Q mutation resulted in more significant termination defects. Although Spt5 and Paf1 recruitment is unaffected in this mutant, it is possible that the binding of snoRNA specific termination factors is inhibited. Nrd1, Nab3, and Sen1 are all important for snoRNA termination and the H4 all K->Q mutation may inhibit their recruitment. The H4 Δ2-26 mutation significantly reduces Paf1 and Spt5 recruitment; however, we observe only mild readthrough of snoRNAs in this strain. Perhaps the limited amount of Paf1 and Spt5 is enough to stimulate proper termination more often than not.

The final aspect of transcription that we examined was mRNA splicing. mRNA splicing occurs less frequently in *S. cerevisiae* than in higher eukaryotes, yet it is still essential. Splicing events begin cotranscriptionally and we sought to determine whether the histone N-termini are involved in the coordination of splicing. We examined the unspliced and spliced levels of *ACT1* and *CYH2* mRNAs by northern blotting and found that mutation of the H4, but not the H3, N-terminus resulted in an increase in the ratio of unspliced to spliced transcripts. This shows specificity for the H4 tail in mRNA splicing and suggests that it may be required for the recruitment of splicing factors cotranscriptionally. Spt5 and Spt6 have been implicated in splicing and we have demonstrated that deletion of the H4 N-terminus significantly reduces the recruitment of these factors. Furthermore, the histone tails have been proposed to sequester nascent RNA until splicing factors can bind (Allemand et al, 2008). This model suggests that the basic histone tails interact with the negatively charged mRNA. Deletion or mutation of the positively charged lysine residues would significantly inhibit this interaction and thus splicing. While the exact mechanism of these splicing defects remains unclear, this data provides evidence
that the H4 N-terminus specifically is important for several post-initiation transcriptional processes.
Materials and Methods

Strains and Media

The strains used in this study are listed in Table 2-1. Gene deletions and tagging were carried out by standard one step replacements using PCR generated cassettes (Brachmann et al, 1998, Longtine et al, 1998). Cells were grown in 2% peptone, 1% yeast extract, 20μg/mL adenine sulfate, 2% dextrose at 30°C. For heat shock of temperature sensitive strains, cultures were shifted to 37°C for 90 min. For 6-AU sensitivity, 3-fold dilutions of cultures transformed with a URA3+ plasmid were spotted onto either SC–URA media or SC-URA + 100μg/ml 6-azauracil and grown at 30°C.

Northern Blotting

Yeast culture was grown to (OD_{600}=0.7), and 15mL of cells were harvested for total RNA extraction as previously described (Reese & Green, 2003, Walker et al, 1996). RNA was liberated with bead beating in the presence of phenol/chloroform, extracted again with phenol/chloroform, precipitated with ethanol, and resuspended in DEPC-treated water(Walker et al, 1996). Twenty micrograms of total RNA was separated on 1.2% agarose gels containing formaldehyde and transferred to Hybond-XL membrane (GE Biosciences, Piscataway NJ) by capillary blotting overnight. Membranes were UV crosslinked using a Stratalinker and incubated in prehybridization solution for more than 4 hours at 65°C. Probes were added for overnight incubation. Membranes were washed and signals were detected using a phosphoimager. The phosphoimager screens were scanned with the Typhoon System (Molecular Dynamics), and
quantified by ImageQuant. Probes for TRS31, FLO8, PMA1, PYK1, Scr1, and STE11 were prepared by the polymerase chain reaction. The $\text{scR1}$ signal of each sample was used to correct for loading of RNA.

**Chromatin Immunoprecipitation (ChIP)**

ChIP assays were performed as previously described, with minor changes (Sharma et al, 2007). Yeast cultures (100mL) were grown in YPAD medium to an OD$_{600}$ of 0.6 to 0.8. The cells were cross-linked with 1% (vol/vol) formaldehyde at room temperature for 15 min. The formaldehyde was quenched by the addition of 125mM glycine. MMS-induced cells were treated with 0.02% MMS and incubated for 2.5 h prior to formaldehyde treatment. Whole cell extracts were prepared by glass bead disruption, and sheared into fragments averaging 200-600 base pairs in size using a Bioruptor (Diagenode, Philadelphia PA). Whole cell extracts were clarified by centrifugation. Clarified extracts were immunoprecipitated (IP) overnight at 4°C with antibodies indicated throughout the text. For antibody information see Table 2-2. Immune complexes were precipitated with 20μL of protein A sepharose. After purification, the precipitated and input DNA was analyzed by semiquantitative PCR. Primer regions are depicted in Figure 2-1B. PCR products were analyzed by electrophoresis, ethidium bromide staining, scanned with the Typhoon System (Molecular Dynamics), and quantified by ImageQuant. The % IP represents the (IP signal / input signal) x100. For histone modifications ChIPs, the data is represented by the (%IP modified / %IP total H3) in order to account for variations in histone levels. All data represents at least three independent experiments from multiple extracts. Error bars represent the standard error.
**Western Blotting**

Yeast cultures (25mL) were grown to an OD$_{600}$ of 0.6 to 0.8. Whole cell extracts were prepared by glass bead disruption. Extracts were clarified by centrifugation. The supernatant was recovered and 1 volume 3x SDS-load buffer was added before boiling. Extracts were separated by 17% SDS-PAGE and transferred to nitrocellulose membranes (Whatman) with a semi-dry transfer apparatus. Membranes were blocked in 5% milk/TBST for 1 hour at 25°C and transferred to primary antibody. Membranes were probed with antibodies indicated in the text overnight at 4°C. Membranes were washed in 2% milk/TBST (2 times) and incubated with secondary antibody for 1-2 hours at 25°C before being washed again in 2% milk/TBST (2 times). Detection was by enhanced chemiluminescence (Peirce). For antibody information see Table 2-2.
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 thank Suting Zheng for constructing the Spt5-myc, Spt6-myc, Spt16-myc, and Pafl-myc strains. We are grateful to Dr. Mitch Smith for histone mutant strains. This research was supported by funds from National Institutes of Health (GM58672) to J.C.R.
Figure 3-1: Schematic of strains and primer pairs used in Chapter 3

(A) Schematic of histone N-termini mutants examined in Chapter 3. (B) Schematic of primers used in ChIP experiments for Chapter 3.
3-1 A

\[
\begin{align*}
\text{ARTKQTARKSTGGAAPRKQLRASKARSAPSTGGVKKPH} & \quad \text{H3 WT} \\
& \quad \text{aa 40-138} \\
\text{APSTGGVKKPH} & \quad \text{H3 \( \Delta 1-28 \)} \\
& \quad \text{aa 40-138} \\
\text{ARTQQTARQSTGGQAPRQGLRASKARQSAPSTGGVKKPH} & \quad \text{H3 K->Q} \\
& \quad \text{aa 40-138} \\
\text{SGRGKGGKGLGKGGAKRHRKLRDNIQGI} & \quad \text{H4 WT} \\
& \quad \text{aa 30-103} \\
\text{SQGI} & \quad \text{H4 \( \Delta 2-26 \)} \\
& \quad \text{aa 30-103} \\
\text{SGRGQGGQGLGGQAQRAHRKLRDNIQGI} & \quad \text{H4 K->Q} \\
& \quad \text{aa 30-103} \\
\text{SGRGQGGQGLGGQAQRAHRKLRDNIQGI} & \quad \text{H4 K5,8,12Q} \\
& \quad \text{aa 30-103} \\
\text{SGRGQGGQGLGGQAQRAHRKLRDNIQGI} & \quad \text{H4 K5,8,16Q} \\
& \quad \text{aa 30-103} \\
\text{SGRGQGGQGLGGQAQRAHRKLRDNIQGI} & \quad \text{H4 K5,12,16Q} \\
& \quad \text{aa 30-103} \\
\text{SGRGQGGQGLGGQAQRAHRKLRDNIQGI} & \quad \text{H4 K9,12,16Q} \\
& \quad \text{aa 30-103}
\end{align*}
\]

3-1 B

\begin{align*}
\text{Pro} & \quad \text{ORF 1} & \quad \text{ORF 2} \\
\text{PYK1 - 1.5kb} & \quad & \quad \\
\text{Pro} & \quad \text{ORF} \\
\text{PMA1 - 2.7kb}
\end{align*}
Figure 3-2: H4 mutations do not result in 6-AU sensitivity

6-AU sensitivity of histone H4 mutants. Serial dilutions of wild type and mutant strains were spotted onto –URA or -URA+6-AU media and incubated at 30°C. Plates lacking and containing 6-AU were grown for 36 and 48 hrs, respectively.
Figure 3-3: The H4 N-terminus genetically interacts with elongation factors

Examination of growth defects in H4/elongation factor double mutant strains. Serial dilutions of wild type and mutant strains were spotted onto SC or SC+5-FOA media and incubated at 30°C. Plates were grown for 36 hours. The top left, top right, bottom left, and bottom right panels depict the genetic interactions of the H4 tail with Δsrb10, Δeaf3, Δset1, and Δdst1, respectively.
Figure 3-4: Histone H4/elongation factor double mutants exhibit altered 6-AU sensitivity

(A) 6-AU sensitivity of histone H4/Δsrb10 double mutants. Serial dilutions of wild type and mutant strains were spotted onto –URA or -URA+6-AU media and incubated at 30°C. Plates lacking and containing 6-AU were grown for 36 and 48 hrs, respectively. (B) 6-AU sensitivity of histone H4/Δeaf3 double mutants. The same as Figure 3-4A, except the H4/Δeaf3 double mutants were examined. (C) 6-AU sensitivity of histone H4/Δset1 double mutants. The same as Figure 3-4A, except the H4/Δset1 double mutants were examined. (D) 6-AU sensitivity of histone H4/Δdst1 double mutants. The same as Figure 3-4A, except the H4/Δdst1 double mutants were examined.
3-4 A

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3-4 C

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3-4 D

| DST1        | Δdst1       | DST1         | Δdst1       |
Figure 3-5: Deletion of the H4 N-terminus results in intragenic transcription at *FLO8*

(A) Northern blot of *FLO8* mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. *FLO8* full-length and short transcripts are labeled. scR1 mRNA was probed for a loading control. (B) Northern blot of *FLO8* mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. Cultures were heat shocked where indicated. *FLO8* full-length and short transcripts are labeled. scR1 mRNA was probed for a loading control.
3-5 A

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<th>H4 K5,8,12Q</th>
<th>H4 K8,12Q</th>
<th>H4 K&gt;Q</th>
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<th>ΔSET2</th>
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- FLO8 full-length
- short
- short

ScR1

3-5 B

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<th>H4 K&gt;Q</th>
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- 37°C heat shock
- FLO8 full-length
- short
- short

ScR1
Figure 3-6: The H4 N-terminus is not required for H3K36me3

(A) Western blot analysis of H3 K36me3. Western blots were performed on whole cell extracts of wild type and H4 Δ2-26 strains. Blots were probed with αH3 K36me3 and αH3 core antibodies. The gradient indicates a decreasing amount of extract loaded unto the gel. (B) Levels of tri-methylated K36 (K36me3) across *PYK1* and *PMA1*. Wild type and H4 Δ2-26 strains were grown and harvested. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. Primer regions for all experiments in this figure are depicted in Figure 3-1B.
Figure 3-7: Deletion of the H4 N-terminus results in reduced levels of elongation factors at *PYK1* and *PMA1*. 

(A) Levels RNAPII crosslinking at *PYK1* and *PMA1*. Wild type, H4 Δ2-26, and H4 all K->Q strains were grown and harvested. ChIP was performed for 8WG16. The data is represented as % IP/Input. Primer regions for all experiments in this figure are depicted in Figure 3-1B. (B) Levels of Spt6 recruitment relative to RNAPII at *PYK1* and *PMA1*. Wild type, ΔH4 2-26, and H4 all K->Q strains harboring Spt6-myc were grown and harvested. ChIP was performed for anti-myc. The % IP/Input of Spt6-myc was plotted relative to RNAPII density by conducting ChIP using 8WG16. The wild type Spt6-myc/8WG16 ratio was set to 1. (C) Levels of Spt16 recruitment relative to RNAPII at *PYK1* and *PMA1*. The same as in Figure 3-7B, except the strains harbored Spt16-myc. (D) Levels of Spt5 recruitment relative to RNAPII at *PYK1* and *PMA1*. The same as in Figure 3-7B, except the strains harbored Spt5-myc. (E) Levels of Paf1 recruitment relative to RNAPII at *PYK1* and *PMA1*. The same as in Figure 3-7B, except the strains harbored Paf1-myc.
Figure 3-8: Mutation of the H4 N-terminus does not significantly alter the mRNA levels of *PYK1* or *PMA1*

(A) Northern blot of *PYK1* mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. Cultures were heat shocked where indicated. A probe for *PYK1* was used. *scR1* mRNA was probed for a loading control. The fold expression relative to wild type is displayed below the *PYK1* blot. (B) Northern blot of *PMA1* mRNA. The same as Figure 3-8A, except the blot was probed for *PMA1*. 
Figure 3-9: H4 lysine to glutamine mutations result in a termination defect at SNR13

(A) Schematic of transcription readthrough at the SNR13 locus. (B) Northern blot of TRS31 mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. A probe for TRS31 was used to detect the both the TRS31 transcript as well as the SNO13-TRS31 readthrough transcript. The SNO13-TRS31 and TRS31 are labeled to the left of the blots. scR1 mRNA was probed for a loading control. (C) Northern blot of TRS31 mRNA. The same as Figure 3-9B, except different mutants were analyzed.
Figure 3-10: The H4 N-terminus is important for splicing of ACT1 and CYH2

(A) Northern blot of ACT1 mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. A probe for the ACT1 intron was used to detect the unspliced form and a probe for full-length ACT1 was used to detect the spliced form. The ratio of unspliced/spliced transcripts is displayed below the ACT1 blot. (B) Northern blot of CYH2 mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. A probe for the CYH2 intron/exon junction was used. The unspliced and spliced forms are labeled. The ratio of unspliced/spliced transcripts is displayed below the CYH2 blot.
3-10 A

WT  H3 Δ1-28  H3 K->Q  WT  H4 Δ2-26  H4 K->Q  ΔCTK1  ΔDST1

ACT1 unspliced

ACT1 spliced

1.0  1.1  1.1  1.0  1.5  1.4  1.3  0.9  ratio unspliced / sliced

3-10 B

WT  H3 Δ1-28  H3 K->Q  WT  H4 Δ2-26  H4 K->Q  ΔCTK1  ΔDST1

CYH2 unspliced

CYH2 spliced

1.0  0.9  1.0  1.0  1.3  1.8  0.7  0.7  ratio unspliced / sliced
Chapter 4

SET2-DEPENDENT K36 METHYLATION IS REGULATED BY NOVEL INTRA-TAIL INTERACTIONS WITHIN H3
Abstract

The histone tails provide sites for many post-translational modifications, which have been associated with transcription repression and activation. To further understand the roles of the histone tails in transcription activation, we examined the effects of histone H3 tail mutations on transcription. The H3 N-terminus is required for full activation of RNR3, a DNA damage inducible gene. Mutating the H3 tail predominantly affects elongation, and not remodeling or initiation. Our characterization of the H3 mutants revealed that the first 20 or 28 residues, and the lysine residues specifically, are required for K36 methylation (K36me) by Set2. Importantly, these residues within H3 are predicted not to occupy the catalytic site of Set2. Consistent with defects in K36me, the H3 mutants display similar phenotypes to a set2Δ mutant. Surprisingly, the recruitment of Set2 to genes is unaffected by mutations in the tail, but in vitro methyltransferase and nucleosome binding assays using recombinant nucleosomes demonstrate that the tail is required for the catalytic activity of Set2. We propose a mechanism by which Set2 activity is regulated by novel intra-tail interactions within H3, which can be regulated by modifications to the tail, providing control over the dynamics and localization of K36me during the elongation of transcription.
Introduction

Understanding how the histone code is regulated has been of great interest. Several instances of trans-tail regulation have been observed, where one histone modification is regulated by the interaction between the histone modifying enzyme and residues in another histone in the nucleosome. For example, Dot1 methylation of H3 lysine 79 requires an interaction between Dot1 and a small basic patch on the H4 tail (Fingerman et al, 2007). There also exist more complex relationships in which one histone modification precedes and is required for the second modification. Mono-ubiquitylation of H2B lysine 123 precedes tri-methylation of H3 lysine 4 by COMPASS (Dover et al, 2002, Lee et al, 2007). Histone modifications are regulated by intra-tail mechanisms also, where residues within the same histone control the modification at another site. Methylation of H3 arginine 2 inhibits COMPASS methylation (Guccione et al, 2007, Kirmizis et al, 2007), and similarly, phosphorylation of H3 serine 10 inhibits methylation of lysine 9 (Rea et al, 2000). Conversely, serine 10 phosphorylation stimulates GCN5 mediated acetylation of lysine 14 (Cheung et al, 2000, Lo et al, 2000). Generally, examples of intra-tail regulation described thus far involve interactions between residues in close proximity to the modification site and modifications that are discriminated by the structure of the active site of the enzyme (Couture et al, 2008).

enzyme responsible for H3 lysine 36 methylation, interacts with the transcribing RNA polymerase II, RNAPII (Kizer et al., 2005, Li et al., 2003). In the wake of RNAPII elongation, chromatin structure is disrupted and must be reset to a compact state. Lysine 36 methylation serves to recruit the Rpd3 histone deacetylase (HDAC) complex to genes (Carrozza et al., 2005, Joshi & Struhl, 2005, Li et al, 2007b). Rpd3 deacetylates both H3 and H4, preventing intragenic transcription. Set2 methylation, independent of the Rpd3 pathway, has also been shown to play a role in the prevention of heterochromatin spreading in a mechanism that is understood to a lesser degree (Tompa & Madhani, 2007). These observations suggest that lysine 36 methylation serves multiple functions in writing and interpreting the histone code.

Several levels of regulation have been observed for Set2 activity. Set2 is recruited to actively transcribed regions through an interaction between its SRI domain and serine 2 phosphorylated C-terminal domain (CTD) of RNAPII (Kizer et al., 2005, Li et al., 2003, Xiao et al, 2003). Furthermore, deletion of Ctk1, the kinase responsible for Ser2-phosphorylation, results in significantly lower cellular levels of Set2 (Youdell et al., 2008). Another level of regulation is the structure of the nucleosome. Set2 was first characterized as a “nucleosome selective” histone methyltransferase (Strahl et al., 2002). However, lower levels of activity on H3 alone and on H3 peptides have been observed (Du et al, 2008, Nelson et al, 2006). 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 (Du et al, 2008). While the authors demonstrate that H4 lysine 44 interacts with Set2, it remains unclear how this interaction occurs and stimulates methylation in vitro when transcription and nucleosome disruption does not occur. It is also possible that mutation of lysine 44 disrupts nucleosome structure and therefore methylation by Set2. In this scenario, the interaction between Set2 and lysine 44 would be coincidental and irrelevant. In either case, the
importance of H4 lysine 44 provides another example of trans-tail regulation of histone modifications and further highlights nucleosomal structure as a regulator of Set2 activity.

To further understand how histone tails regulate transcription, we characterized the transcription activation defects in H3 tail mutants. We found that residues within the H3 tail, predicted to be distant from those expected to occupy the catalytic site of Set2, are required for Set2-directed K36 methylation \textit{in vivo}. Furthermore, these mutants display all the hallmark phenotypes of a \textit{set2Δ} strain, including 6-AU resistance and production of cryptic transcripts from within ORFs (Carrozza et al, 2005, Krogan et al, 2003). Biochemical analyses using recombinant components suggest that the tail is required to stimulate Set2 activity. Together, these results support a model by which Set2 methylation is regulated by intra-tail interactions within H3, which have the potential to be regulated by the modification of residues or by the recently described proteolytic cleavage of the tail.
Results

Mutations to the H3 N-termini result in altered RNAPII density and 6-AU resistance

In *S. cerevisiae*, the histone N-termini are nonessential, enabling investigations into their roles in transcription regulation (Morgan et al, 1991). Historically, deletion of the H3 tail has been associated with derepression of transcription (Wan et al, 1995). However, recent gene expression profiling of H3 tail mutants revealed it is required for activation and repression *in vivo* (Sabet et al, 2003, Sabet et al, 2004). Whether or not the activation defects were direct, and the mechanisms behind the activation defect, were not examined. Specifically, the effects of the mutations on chromatin remodeling, initiation, and elongation were not established. The DNA damage-inducible *RNR3* gene was used as a model to examine the role of the histone N-termini in transcription activation (Li & Reese, 2001, Zhang & Reese, 2007). We examined its activation in strains with a deletion or lysine to glutamine substitutions within the N-terminal tail of H3 (Figure 4-1A and 4-2A). Despite the well known role of the tails in repression, neither the H3 Δ1-28 nor the H3 K-4,9,14,18,23,27-Q (H3 K->Q hereafter) mutant displayed strong derepression of *RNR3* in the uninduced condition (-MMS); however, both mutants had significantly reduced levels of induced *RNR3* transcription (Figure 4-2A). The H3 K->Q mutant exhibited a defect in transcription similar to the H3 Δ1-28 strain, suggesting that the modifiable lysines on the H3 N-terminus are of particular importance for high levels of induced transcription.

To determine which steps in the activation of *RNR3* were affected by the histone tail mutations, we first examined preinitiation complex (PIC) formation in the mutants by measuring the recruitment of TBP to *RNR3*. In the wild type strain there is a 4- to 5-fold increase in TBP recruitment upon induction (Figure 4-2B). Surprisingly, neither histone tail mutation displayed
significant reduction in TBP recruitment. This is in contrast to the 60-70% loss of mRNA under the same conditions (Figure 4-2A). TBP recruitment is one of the first steps in PIC formation, so we next analyzed the recruitment of RNAPII to the promoter in the histone mutants (Figure 4-2C). RNAPII recruitment to the promoter was ~45-50% lower in the H3 Δ1-28 strain compared to that observed in the wild type; however, the loss was not proportional to the loss of RNR3 expression. Interestingly, RNAPII recruitment to the promoter was unaffected in the H3 K->Q strain, yet mRNA levels were greatly reduced in this mutant (Figure 4-2A). These results suggest that the transcription defects in this mutant predominantly occur after PIC formation, possibly at the elongation step.

Next, we characterized the H3 tail mutants to identify defects in transcription elongation, first examining the density of RNAPII across the gene. We demonstrate in Figure 2-10B that RNAPII seems to be “piling-up” in the H3 Δ1-28 strain. To better illustrate the difference in the distribution of RNAPII between the wild type and the H3 Δ1-28 mutant, we plotted its density across RNR3 in each strain, relative to that recruited to the promoter (Figure 4-2D). The wild type strain showed little variation in RNAPII density across the RNR3, until it dropped off at the UTR near the transcription termination site, indicating polymerase was distributed equally. On the other hand, RNAPII density increased progressively further downstream in H3 Δ1-28 cells, 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. Theoretically, the increase in RNAPII within the body of the gene could be caused by internal initiation from cryptic promoters. However, we failed to detect cryptic transcripts originating from RNR3 in the H3 tail mutants, even when RRP6, a subunit of the exosome, was deleted (Figure 4-2E).
To provide further evidence for elongation defects we examined the sensitivity of the mutants to 6-azauracil (6-AU), an inhibitor of transcription elongation (Exinger & Lacroute, 1992, Riles et al, 2004). The H3 Δ1-28 and H3 K->Q strains were spotted below their respective wild types. Sensitivity to 6-AU is thought to vary in different strain backgrounds and therefore each mutant must be compared directly to its parent strain. Interestingly, instead of sensitivity, we found that both the H3 Δ1-28 and H3 K->Q strains displayed resistance to 6-AU relative to their cogenic wild type strain (Figure 4-2F). The resistance to 6-AU is very obvious in the H3 K->Q mutant, which grows well on –uracil medium, but grows better than the wild type on media containing 6-AU. While the growth of the H3 Δ1-28 on media containing 6-AU was similar to that of the wild type strain, this strain displays a significantly slowed growth phenotype on media lacking the drug (Figure 4-2F). Thus, the relative growth rate of the H3 Δ1-28 mutant on media lacking versus media containing elongation inhibitors indicates a resistance to 6-AU. set2Δ strains are among the few elongation mutants known to show resistance to 6-AU (Biswas et al, 2006, Keogh et al, 2005, Kizer et al, 2005). We compared the sensitivity of a set2Δ mutant constructed in the MSY590 genetic background and found it displayed resistance to the drug similar to the histone mutants (Figure 4-2F).

An intact H3 N-terminus is required for H3 K36 tri-methylation

Since finding that mutations to the H3 tail resulted in 6-AU resistance similar to a Δset2 strain, we sought to determine if mutating the N-terminus of H3 affected Set2 mediated H3 K36 tri-methylation (me3). In wild type cells, we observed a transcription dependent increase in H3 K36me3 levels towards the 3’ end of the open reading frame, where the levels peak over the region described as ORF D (Figures 4-3A, 4-3B, 4-3C and 4-3D). A similar pattern has been
reported at other actively transcribed genes (Bannister et al, 2005, Krogan et al, 2003, Pokholok et al, 2005). Furthermore, the increase in H3 K36me3 was dependent upon SET2 (Figure 4-3D). Significantly, both the H3 Δ1-28 and H3 K->Q mutations caused a strong reduction in the level of H3 K36me3 across RNR3 (Figures 4-3A and 4-3B). These observations are not trivial because 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. In fact, a histone peptide containing only amino acids 29-41 is capable of being methylated by Set2 (Nelson et al, 2006). Furthermore, a H3 Δ1-20 mutant also resulted in a significant (~40%) reduction of K36me3 across RNR3 (Figure 4-3C). Thus, residues >15 amino acids away from K36 are playing a significant role in methylation by Set2.

Acetylation of H3 K36 (H3 K36Ac) is another highly conserved histone modification (Morris et al, 2007). Increased H3 K36Ac levels could preclude tri-methylation in the H3 tail mutants. However, we examined H3 K36Ac at RNR3 by ChIP and found that mutating the H3 tail did not cause an increase in K36 acetylation compared to wild type cells, ruling out a trivial explanation for our results (Figure 4-3E).

In order to determine if the K36 methylation defect observed in the H3 mutants was specific for RNR3, we examined the level of H3 K36me3 at PMA1 and PYK1, two constitutively expressed genes that have been used extensively as models to study cotranscriptional histone modifications (Chu et al, 2007a, Krogan et al, 2003). As reported previously, there is a high level of H3 K36me3 within the 3’ ORF regions of both PMA1 and PYK1 in the wild type strain (Figure 4-3F). Deletion of the H3 N-terminus resulted in a significant decrease in H3 K36me3 across both PMA1 and PYK1. Thus, the same phenotype is observed at constitutively transcribed and highly induced genes. To test if H3 K36me3 is reduced across the genome, we examined its levels in bulk chromatin from wild type, H3 Δ1-28, H3 K->Q, H4 Δ2-26 (control), and set2Δ strains (Figure 4-3G). Antibody recognizing the H3 C-terminus was used to estimate the amount of H3 on the blot. Consistent with the ChIP results, K36me3 levels were lower in the H3 tail
mutants than wild type cells (Figure 4-3G). As a control, we analyzed a strain containing a deletion of the H4 tail. The H4 Δ2-26 mutant showed no defect in H3 K36me3, suggesting specificity for the requirement of the N-terminus of H3 in Set2 mediated methylation.

H3 K36 di-methylation (me2) is also associated with transcriptionally active regions, although this mark is more broadly distributed across the genome and does not correlate as well with the level of transcriptional activity as the tri-methylated form of K36 (Bannister et al, 2005, Pokholok et al, 2005). We first tested the levels of K36m2 in bulk chromatin. Surprisingly, we observed a small, by reproducible, reduction in K36me2 in the H3 Δ1-28 mutant, and no detectable decrease in the H3 K->Q mutant, after correcting for the load of histones (Figure 4-4A). We reasoned that since K36me2 is more widely distributed throughout the genome, reduction in its levels may be restricted to actively transcribed regions, thus we examined K36me2 levels at RNR3 by ChIP. Here, we observe a significant reduction in K36me2 in both mutants over the 3’ end of the ORF (ORFD) of RNR3 under the induced condition (Figures 4-4B and 4-4C). However, reduction in K36me2 was observed across RNR3 in the H3 Δ1-28 mutant, including the promoter and 3’ UTR, while reduction was more restricted to the ORF region in the H3 K->Q mutant. This suggests that the H3 K->Q mutation is having a stronger effect on K36me2 over the middle of active genes. Presently, the reason for this is unclear. The quality and selectivity of the commercial K36me1 prevented us from examining this modification.

We also explored the possibility that mutations to the H3 N-terminus would affect other histone methylation marks associated with elongation. We could not examine K4 methylation because the mutants lacked K4, or have a glutamine substitution at that residue. However, we examined Dot1-mediated H3 K79 methylation by western blotting, and found that mutations to the N-terminus of H3 did not significantly affect K79me2 levels (Figure 4-4D). This result suggests that the H3 tail plays a specific role in Set2 methylation.
The H3 N-terminus suppresses intragenic transcription at STE11 and FLO8

K36 methylation is required to recruit the Rpd3S HDAC complex, which in turn deacetylates nucleosomes within ORFs of genes (Carrozza et al, 2005, Joshi & Struhl, 2005, Keogh et al, 2005, Li et al, 2007b). Deleting SET2, or other effectors of this pathway, results in cryptic intragenic transcription within certain genes, including STE11 (Carrozza et al, 2005). We used Northern blotting to probe for short STE11 transcripts in the H3 mutants (Figure 4-5A). As expected, deletion of SET2, EAF3, or CTK1 results in short intragenic STE11 transcripts. This phenotype is observed in the H3 Δ1-28 and H3 K->Q strains, as well as in a strain harboring a smaller deletion of the N-terminus, H3 Δ1-20 (Figure 4-5A). The amounts of intragenic transcription in the histone tail mutants (3-4 fold increase) were not as high as those observed in set2Δ or ctk1Δ mutants (6-7 fold), which is expected because the H3 mutations do not completely abolish Set2 methylation (Figures 4-2A and 4-2B). Deleting or mutating the H4 N-terminal tail did not result in intragenic transcription at STE11, which is consistent with the data showing K36me3 is not reduced in these mutants (Figure 4-5A and Figure 4-3D). We verified that the intragenic transcripts arose due to a loss of H3 K36 methylation. Both H3 mutants displayed less than 50% of the levels of H3 K36me3 and K36me2 at the 3’ORF compared to wild type cells (Figure 4-5B and 4-5C). Once again, even though a significant loss of K36me2 is not detected in bulk chromatin, the reduction in K36me2 is quite obvious. Recently it was shown that H3 K36me2, in the absence of H3 K36me3, is sufficient to recruit the Rpd3S complex and repress intragenic transcription (Li et al, 2009, Youdell et al, 2008), so the appearance of intragenic transcripts in the H3 mutants is consistent with a defect in K36me2.

In addition to the STE11 locus, we probed for intragenic transcription at FLO8 (Figure 4-5D). The H3 mutants again exhibit intragenic transcription and this is consistent with an H3 K36 methylation defect. Of note, we do observe an increase in the smaller of the two short intragenic...
transcripts that is reminiscent of the H4 Δ2-26 phenotype. We have previously suggested that the H4 Δ2-26 phenotype is due to an inability to recruit Spt6; however, the H3 mutants are not defective for the recruitment of Spt6 (Figure 4-7F and 4-7G). It is possible that the H3 N-terminus is required for the activity, but not the recruitment, of Spt6 or Spt16. Alternatively, the H3 N-terminus may be part of a third pathway involved in the repression of intragenic transcription in addition to its roles in regulating H3 K36me3.

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

Having established that mutating the N-terminus of H3 results in significantly lower levels of H3 K36 methylation, we next turned to determining the mechanism of how the tail regulates Set2 activity. Set2 is recruited to genes by the phosphorylation of Ser2 (S2) on the CTD of RNAPII by Ctk1, and results in the cotranscriptional methylation of histone H3 (Kizer et al, 2005, Li et al, 2003, Xiao et al, 2003). The N-terminal tail of H3 could play a role in Ctk1 mediated phosphorylation of RNAPII, which would cause the reduced recruitment of Set2 and adversely affect its activity on nucleosomes during elongation. This was tested by measuring the levels of S2 phosphorylation of RNAPII at \textit{RNR3} (Figures 4-6A, 4-6B, and 4-6C). Upon induction with MMS, an increase in S2 phosphorylation was observed at the 3’ end of \textit{RNR3} (ORF D and the 3` end). The peak of S2 modification at ORF D is consistent with reports that it occurs predominantly in the middle and 3’ ends of genes. The low level of S2 phosphorylation observed near the promoter of \textit{RNR3} is likely due to cross reactivity of the antibody with differently modified forms of RNAPII, because the same level of crosslinking is observed in a \textit{Δctk1} strain (Figure 4-6C). In contrast, the increase in MMS-induced level of S2 phosphorylation at the 3’ end of \textit{RNR3} was strongly reduced in the \textit{ctk1Δ} mutant. Neither the H3 Δ1-28 nor the H3
K->Q mutations affected the level of S2 phosphorylation (Figure 4-6A and 4-6B). Thus, the lack of K36 methylation is not caused by reduced Ser2 phosphorylation of the CTD of RNAPII.

It is possible that the N-terminus of H3 plays a role, either directly or indirectly (such as the recruitment of the PAF complex), in the recruitment of Set2 to genes. In order to investigate this possibility, we examined the recruitment of Set2 (Set2-myc) to RNR3 and PYK1 (Figures 4-7A, 4-7B, and 4-7C). In the wild type strain, Set2-myc recruitment increased almost 3-fold upon induction of transcription at ORF D, the same location of the peak of K36me3 (Figure 4-2A). We also observed recruitment of Set2 to ORF B and the 3` end of RNR3, but to a lesser degree. Importantly, neither the H3 Δ1-28 nor the H3 K->Q mutations reduced Set2 recruitment, and in fact, Set2 crosslinking was actually slightly higher in the H3 mutants than the wild type strain. Furthermore, Set2-myc recruitment to PYK1 was equal in wild type and mutant cells (Figure 4-7C). Collectively, this data indicates that the N-terminus of H3 is not required for Set2 recruitment, suggesting that the tail regulates Set2 activity at a post-recruitment step.

The PAF complex interacts with the CTD of RNAPII and regulates both H3 K4 and K36 methylation (Chu et al, 2007b, Laribee et al, 2005). Deletion of PAF complex components PAF1 or CTR9 nearly eliminates H3 K36me3 and significantly reduces me2 at genes, presumably by reducing Set2 recruitment (Chu et al, 2007b, Nakanishi et al, 2008). While we do not observe a Set2 recruitment defect in the H3 mutants, it is possible that the PAF complex regulates Set2 at a post recruitment step as well, such as by stimulating its activity. We examined the recruitment of an epitope-tagged version of Paf1 to RNR3 (Figures 4-7D and 4-7E). In the wild type strain, Paf1-myc recruitment increased ~5-fold at ORF B and ~3-fold at the RNR3 promoter, ORF D, and 3` end in the induced condition (+MMS). The peak of Paf1-myc recruitment occurs at ORF B, a region where H3 K4me3 and K36me3 methylation overlap considerably upon induction of RNR3 (Figures 4-7D, 4-7E, 4-2A, and unpublished data). Neither the H3 Δ1-28 nor the H3 K->Q mutations affected the recruitment of Paf1-myc to most regions of RNR3 (Figures 4-7D and 4-
7E). At ORF B, Paf1-myc recruitment was ~50% less than the wild type in the H3 Δ1-28 mutant and ~35% less in the H3 K->Q mutant. Since Paf1 recruitment to ORF D, the location of the peak of H3 K36 methylation and Set2 recruitment, 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 to genes. It was recently reported that substituting K14 for alanine reduced K4me3 (Nakanishi et al, 2008). The mechanism behind this was not known; but our data suggests that the N-terminus of H3 may be required to recruit PAF to the 5’ ends of genes, which could explain the reduced K4me3 in the K14A mutant.

Spt6 is another elongation factor recently identified as a regulator of Set2 activity. Inactivation of the conditional spt6-1004 mutant results in a loss of H3 K36me3 (Youdell et al, 2008). The spt6-1004 mutation results in lower levels of cellular Set2 (Youdell et al, 2008). However, K36me3 levels are still reduced when Set2 levels are restored by overexpression (Youdell et al, 2008). This suggests a second role for Spt6 in regulating Set2 activity, in addition to its ability to regulate Set2 protein levels. Thus, it is possible that mutating the tail affects Set2 activity even though we do not observe a reduction in Set2 recruitment to genes. The crosslinking of Spt6-myc to RNR3 was examined before and after induction with MMS (Figures 4-7F and 4-7G), and its recruitment increased across RNR3 upon induction with MMS (Figures 4-7F and 4-7G). The largest increase (~8-fold) was observed at the ORF B region (Figures 4-7F and 4-7G). Neither the H3 Δ1-28 nor H3 K->Q mutations adversely affected Spt6 recruitment to RNR3, and the pattern of Spt6 recruitment in the H3 mutant strains mirrors that of the wild type. Therefore, the reduced levels of K36me in the H3 mutants cannot be explained by impaired Spt6 recruitment. The experiments described in Figures 4-6 and 4-7 tested all known factors that regulate Set2 activity in vivo, and we found these parameters to be intact. These observations suggest that the H3 tail regulates Set2 activity through a novel mechanism.
The N-terminus of H3 is required to stimulate Set2 activity

We have shown that mutating the H3 N-terminus does not affect Set2 recruitment, but does significantly reduce the levels of H3 K36 methylation \textit{in vivo}. We hypothesized that the N-terminus of H3 might be required to stimulate the enzymatic activity of Set2 or the binding of Set2 to nucleosomes. These possibilities were tested using an \textit{in vitro} methyltransferase assay with recombinant Set2 and nucleosomes. A GST-tagged version of the SET domain of Set2 (residues 1-261) was expressed in \textit{E. coli} and purified (Figure 4-8A), and the GST portion removed by protease digestion. \textit{In vitro} methyltransferase assays were performed using recombinant Set2 and either wild type recombinant \textit{Xenopus} nucleosomes or mutant versions containing a deletion of the H3 or H4 tail. Activity was measured by the incorporation of $^{3}$H-S-adenosyl-methionine (SAM) in nucleosomes. The data shows that Set2 was significantly more active on wild type nucleosomes versus the version lacking the H3 N-terminus (Figure 4-8B). Importantly, nucleosomes lacking the H4 tail were at least as good of a substrate for Set2 as wild type nucleosomes, which correlates well with other data showing that K36 methylation is unaffected by a deletion of the H4 tail \textit{in vivo} (Figure 4-3D).

The reduced activity of Set2 on the mutant nucleosomes can be caused by reduced binding of the enzyme to the substrate or by reduced catalytic activity. In the former case, Set2 should interact better with wild type nucleosomes than H3 mutant nucleosomes. To determine how deletion of the H3 N-terminus affects the interaction of Set2 with nucleosomes, we used immobilized Set2-FLAG protein in nucleosome pulldown assays (Figure 4-8A). Either wild type or H3 Δ1-26 nucleosomes were incubated with recombinant Set2-FLAG bound to α-FLAG resin and the immunoprecipitated material was analyzed by western blotting. Both wild type and H3 Δ1-26 nucleosomes were pulled down by Set2-FLAG but not the FLAG resin alone (Figure 4-8C). Interestingly, this initial assay suggested that the H3 Δ1-26 nucleosomes interacted more
strongly with Set2-FLAG than the wild type nucleosomes (Figure 4-8C, compare lanes 3 and 4). To further confirm this observation, the assays were repeated while titrating the amount of nucleosome added to each pulldown. The results confirmed that Set2 pulled down more H3 Δ1-26 nucleosomes than the wild type nucleosomes (Figure 4-8D, compare lanes 1-3 with 4-6). This data clearly indicates that mutation of the H3 N-terminus does not reduce the ability of Set2 to bind or recognize the nucleosome, suggesting that mutation of the tail affects the catalytic activity of Set2.

The enhanced binding of the mutant nucleosomes to Set2 was unexpected, and required further study. We hypothesized that the enhanced binding to the mutant nucleosomes could be the result of the reduced activity of Set2 on these nucleosomes. Catalytically active Set2 would modify the tail, and Set2 is likely to interact less well with the newly modified nucleosome. Exogenous SAM was not added to the binding reactions. However, Set7/9 methyltransferase co-purifies with SAM when expressed in *E. coli* and raises the possibility that all the necessary components for methylation are present in the binding reaction (Couture et al, 2006, Wilson et al, 2002). Co-purification of SAM with rSet2 could cause nucleosome methylation and reduced binding to the substrate. If true, we expect that a catalytically inactive version of Set2 would bind better to nucleosomes, and the enhanced binding of Set2 to the mutant nucleosomes, relative to wild type nucleosomes, would be reduced. We repeated the assays using a catalytically inactive (R195G) version of Set2 that has been characterized previously (Strahl et al, 2002). Indeed, in Figure 4-8E, we show that the R195G Set2-FLAG interacts equally well with both wild type and H3 Δ1-26 nucleosomes. Importantly, the equal binding is not caused by a reduction in binding of Set2 R195G to the mutant nucleosomes, but rather a greater increase in the binding of the R195G mutant to the wild type nucleosomes (compare Figure 4-8E top panel lanes 1-6 against Figure 4-8D lanes 1-6). This suggests that the binding of Set2 to nucleosomes is inversely correlated to its ability to modify K36, and that the turnover of nucleosomes by Set2 may be important for the
processivity of the elongation complex during transcription. In this scenario, we speculate that modification of the nucleosome by Set2 may promote the release of Set2 from modified tails so that it can move with RNAPII to hit unmodified tails downstream.

**H3 lysine to arginine mutations do not result in a K36me3 defect**

We observed global and gene specific defects in H3 K36me3 when the H3 tail is deleted or when the lysine residues within the tail are mutated to glutamine. Lysine to glutamine mutations have been used to mimic lysine acetylation and have been shown to have similar effects on chromatin folding *in vitro* (Wang & Hayes, 2008). This raises the possibility that the charge of the H3 tail may be important for Set2 methylation. Alternatively, mutating six lysine residues may cause the nucleosome to be unrecognizable by Set2. These possibilities can be discerned by analyzing the methylation levels in strains containing arginines substituted for the lysine residues. Bulk levels of K36me3 were examined in a strain containing all six lysines replaced by arginines (Figures 4-1A and 4-9A). Unlike the H3 K->Q mutant, the H3 K->R mutant does not result in a noticeable reduction in global H3 K36me3 levels (Figures 4-9A and 4-3D). In fact, methylation may be slightly enhanced.

This was further investigated by examining the levels of K36me3 at specific genes by ChIP. In this case, we normalized the level of K36me3 to the amount of RNAPII at the gene because the H3 K->R mutant displays less RNAPII over *RNR3, PYK1*, and *PMA1*. Since Set2 is recruited to genes through an interaction with the RNAPII CTD (Kizer et al, 2005, Li et al, 2003), normalization to RNAPII levels provides a better assessment of the effects of the tail mutants on K36me3. This was repeated for the K->Q in parallel. When H3 K36me3 is plotted relative to RNAPII density, the H3 K->Q mutant clearly exhibits a Set2 methylation defect, equal to that when not normalized to RNAPII (Figures 4-9B, 4-9C, and 4-9D). However, the H3 K->R mutant
results in a mild enhancement of Set2 methylation at \textit{RNR3}, \textit{PYK1}, and \textit{PMA1} (Figure 4-9B, 4-9C, and 4-9D). To provide additional evidence that the H3 K-R mutant does not exhibit an H3 K36me3 defect, we examined the 6-AU sensitivity and the appearance of intragenic transcription in the strain. The results show that although the mutant grows more slowly on all media tested, the relative growth compared to wild type cells on media lacking or containing 6-AU was the same, indicating that the strain is no more resistant to the drug (Figure 4-9E). Finally, northern blotting for \textit{STE11} shows that the H3 K->R strain does not exhibit intragenic transcription like the H3 K-Q and \textit{Δ}set2 strains (Figure 4-9F). There is a small increase in one of the two shorter products, but the amount of intragenic transcription is less. The small increase could be caused indirectly by other mechanisms independent of K36me3. Collectively, the data supports the hypothesis that the glutamine mutations are not merely making the tail unrecognizable, but that the charge of the tail is important for Set2 activity.
Discussion

Novel intra-tail regulation of Set2

As part of the histone code, intra-tail regulation of histone modifications within H3 has been described. The existing paradigms, however, involve the control of one modification by the modification state of another residue within close proximity and within the active site of the enzyme (Guccione et al, 2007, Lo et al, 2000, Nelson et al, 2006). However, we demonstrate a novel mechanism where H3 K36me3 is regulated by residues of H3 predicted to be located outside of the catalytic site of Set2. The crystal structure of most SET domains with histone peptides reveal no more than 9 total amino acids within the active site of the protein, and contacts with residues on each side of the modification site are observed (Dillon et al, 2005). More relevant to our story, Set2 can methylate a histone peptide containing only amino acids 29-41 (Nelson et al, 2006). This suggests that the reduction in Set2 activity observed in the H3 mutants examined here is not merely due to an alteration of the interaction of residues within the active site of Set2. To our knowledge, the stimulatory effect of the H3 tail on Set2 activity is unique to these other forms of intra-tail regulation.

The H3 N-terminus stimulates Set2 activity

Our data is fully consistent with the tail affecting Set2 catalytic activity. Deleting the tail has no effect on the interaction between Set2 and nucleosomes, once the differences in the turnover of nucleosomes were corrected for by using the catalytic mutant of Set2 (Figure 4-8E). The binding of the tail to Set2 could affect its activity through an allosteric mechanism, or it
could position K36 within the active site of Set2. In the latter case, the H3 N-terminus interacts with Set2 to position lysine 36 next to the catalytic residues, or restricts movement of the tail in the active site of Set2 (Figure 4-11, left panel). Deleting the H3 tail or mutating the lysines to neutral glutamines could result in the misalignment of K36 within the active site, reducing the modification of the residue (Figure 4-11, middle and right panels, respectively). We favor this scenario, versus the allosteric mechanism, based on the previously described regulation of K36me by the isomerization of an adjacent proline residue, P38, by the prolyl-isomerase Frp4 (Nelson et al, 2006). According to this model, Set2 can methylate lysine 36 only when P38 is in the trans conformation, which orients K36 in the active site.

The region within Set2 that interacts with the H3 tail is not yet fully characterized. We demonstrate that the SET domain of Set2 (aa 1-261) has greater activity on wild type nucleosomes than those lacking the H3 tail (Figure 4-8B). From this, we conclude that the intra-tail regulatory interaction occurs within the first 261 amino acids of Set2. Since deleting the tail or mutating the lysines to glutamine results in similar phenotypes, this suggests that the charge of the H3 tail is important for mediating the interaction with Set2. Accordingly, we show that lysine to arginine mutations do not negatively affect K36me3. The pI of the SET domain of Set2 (aa 1-261) is 4.94, and it is possible that an acidic patch in Set2 could act as the interaction partner for the basic H3 tail. Examining the structure of the SET domain of Set2 could provide further evidence for this, but this structure has not been resolved.

**Regulation of Set2 activity by H3 acetylation or cleavage**

An intriguing possibility is that modification of residues within the tail of H3, such as acetylation, can regulate the levels of K36me3. Histone H3 acetylation, found predominantly at promoter regions where H3 K36 methylation is low (Li et al, 2007a), also neutralizes the charge
of H3 lysine residues similar to the glutamine mutations. It is possible that acetylation of H3 helps create a barrier between the promoter and ORF regions by limiting H3 K36 methylation. This might be especially important when the end of one gene is in close proximity to the promoter of another gene. Alternatively, cotranscriptional acetylation within genes may serve to dampen Set2 activity. Histone H3 acetylation and Set2 methylation may negatively regulate the activity of each other to strike the proper balance of histone modifications. We attempted to directly test this model \textit{in vivo}, by deleting Gcn5, the HAT responsible for the DNA damage induced H3 acetylation at \textit{RNR3}, and examining H3K36me3 patterns. While we do observe an increase in H3 K36me3 relative to RNAPII density in the Δgcn5 mutant at \textit{RNR3}, we also observe a loss of H3 K36Ac and cannot exclude the possibility that the H3 K36me3 increase is the result of more “methylatable” K36 residues (Figures 4-10A, 4-10B, and 4-10D).

In addition to regulation by modification of the tail, Set2 methylation could be regulated by proteolytic cleavage of the tail. Recent evidence has suggested that programmed “clipping” of the H3 tail at residue 21 occurs \textit{in vivo} in \textit{S. cerevisiae} within nucleosomes at the promoters of activated sporulation control genes (Santos-Rosa et al, 2009). Clipping the H3 tail at promoters could function to inhibit Set2 methylation, a mark inhibitory in nature by mediating the recruitment of HDACs, near the promoters of genes that are being activated. Furthermore, cleavage of the H3 tail has been identified in mouse embryonic stem cells during differentiation (Duncan et al, 2008). While the purpose of this cleavage is not well understood, the authors found that histone acetylation inhibited H3 proteolysis. Given that histones are found to be mostly deacetylated in differentiating cells, cleavage of the H3 tail may provide another mechanism to regulate lysine 36 methylation patterns during differentiation or under circumstances when changes in acetylation alone are insufficient. It is possible that cells have developed multiple mechanisms to regulate Set2 methylation in order to accommodate diverse growth conditions and phases.
Antagonistic relationship between H3 K36me3 and H3 acetylation

In this study, we report that the H3 tail plays an important role in H3 K36 methylation. This novel intra-tail regulation provides insight into the depth of the regulatory mechanisms controlling histone modifications and may reveal a consequence of the recently described programmed proteolysis of H3. Given the antagonistic nature between H3 acetylation and H3 K36me3, questions remain about which regulation evolved first. Significantly, the mechanisms of antagonism are rather different: H3 acetylation acts directly by inhibiting Set2 activity, while Set2 methylation acts indirectly by recruiting an enzyme responsible for removing acetyl marks.
Materials and Methods

Strains and Media

The strains used in this study are listed in Table 2-1. Gene deletions and tagging were carried out by standard one step replacements using PCR generated cassettes (Brachmann et al, 1998, Longtine et al, 1998). Cells were grown in 2% peptone, 1% yeast extract, 20μg/mL adenine sulfate, 2% dextrose at 30°C. For heat shock of temperature sensitive strains, cultures were shifted to 37°C for 90 min. For 6-AU sensitivity, 3-fold dilutions of cultures transformed with a URA3+ plasmid were spotted onto either SC–URA media or SC-URA + 100μg/ml 6-azauracil and grown at 30°C.

Northern Blotting

Yeast culture was grown to (OD$_{600}$=0.7), and 15mL of cells were harvested for total RNA extraction as previously described (Reese & Green, 2003, Walker et al, 1996). Where indicated, cells were treated with 0.02% MMS for 1 or 2.5h prior to harvesting. RNA was liberated with bead beating in the presence of phenol/chloroform, extracted again with phenol/chloroform, precipitated with ethanol, and resuspended in DEPC-treated water(Walker et al, 1996). Twenty micrograms of total RNA was separated on 1.2% agarose gels containing formaldehyde and transferred to Hybond-XL membrane (GE Biosciences, Piscataway NJ) by capillary blotting overnight. Membranes were UV crosslinked using a Stratalinker and incubated in prehybridization solution for more than 4 hours at 65°C. Probes were added for overnight incubation. Membranes were washed and signals were detected using a phosphoimager. The
phosphoimager screens were scanned with the Typhoon System (Molecular Dynamics), and quantified by ImageQuant. Probes for TRS31, FLO8, PMA1, PYK1, Scr1, and STE11 were prepared by the polymerase chain reaction. The scR1 signal of each sample was used to correct for loading of RNA.

**Chromatin Immunoprecipitation (ChIP)**

ChIP assays were performed as previously described, with minor changes (Sharma et al, 2007). Yeast cultures (100mL) were grown in YPAD medium to an OD600 of 0.6 to 0.8. The cells were cross-linked with 1% (vol/vol) formaldehyde at room temperature for 15 min. The formaldehyde was quenched by the addition of 125mM glycine. MMS-induced cells were treated with 0.02% MMS and incubated for 2.5 h prior to formaldehyde treatment. Whole cell extracts were prepared by glass bead disruption, and sheared into fragments averaging 200-600 base pairs in size using a Bioruptor (Diagenode, Philadelphia PA). Whole cell extracts were clarified by centrifugation. Clarified extracts were immunoprecipitated (IP) overnight at 4°C with antibodies indicated throughout the text. For antibody information see Table 2-2. Immune complexes were precipitated with 20μL of protein A sepharose. After purification, the precipitated and input DNA was analyzed by semiquantitative PCR. Primer regions are depicted in Figure 2-1B. PCR products were analyzed by electrophoresis, ethidium bromide staining, scanned with the Typhoon System (Molecular Dynamics), and quantified by ImageQuant. The % IP represents the (IP signal / input signal) x100. For histone modifications ChIPs, the data is represented by the (%IP modified / %IP total H3) in order to account for variations in histone levels. All data represents at least three independent experiments from multiple extracts. Error bars represent the standard error.
Western Blotting

Yeast cultures (25mL) were grown to an OD$_{600}$ of 0.6 to 0.8. Whole cell extracts were prepared by glass bead disruption. Extracts were clarified by centrifugation. The supernatant was recovered and 1 volume 3x SDS-load buffer was added before boiling. Extracts were separated by 17% SDS-PAGE and transferred to nitrocellulose membranes (Whatman) with a semi-dry transfer apparatus. Membranes were blocked in 5% milk/TBST for 1 hour at 25°C and transferred to primary antibody. Membranes were probed with antibodies indicated in the text overnight at 4°C. Membranes were washed in 2% milk/TBST (2 times) and incubated with secondary antibody for 1-2 hours at 25°C before being washed again in 2% milk/TBST (2 times). Detection was by enhanced chemiluminescence (Peirce). For antibody information see Table 2-2.

Set2 Expression, Methyltransferase Assays, and Nucleosome Pulldowns

A DNA fragment of Set2 encoding amino acids 1-261 was cloned into pGEX6P by standard molecular biology techniques. Full-length wild type and R195G Set2-FLAG were described previously (a gift from Brian Strahl) (Strahl et al, 2002). Constructs were transformed into *E. coli* BL21(DE3 LysS) and proteins purified using glutathione-agarose beads (GST-Set2 1-261) or α-FLAG resin (Set2-FLAG) according to the manufacturer's recommended conditions (Amersham-Pharmacia and Sigma). Set2 1-261 was released from the glutathione beads with PreScission protease (GE Healthcare). Protein quality and quantity were determined by SDS-PAGE with Coomassie blue staining. Methyltransferase assays were performed as described previously with minor alterations (Strahl et al, 2002). Briefly, varying amounts of Set2 1-261 were incubated with 4µg of recombinant Xenopus nucleosomes and 0.6µCi $^3$H S-adenosylmethionine for 30min. at 30°C. For nucleosome pulldown assays, 1µg of wild type or
R195G Set2-FLAG was incubated with varying amounts of recombinant Xenopus nucleosomes in binding buffer (10mM Tris pH 7.4, 50mM NaCl, 1mM MgCl$_2$, 1% Triton X-100, 1% Ficoll, 0.5mM PMSF) for 1.5hr at 4°C. Set2-FLAG resin was washed 3 times in binding buffer and 20µl of 3x SDS-load buffer was added before boiling. Nucleosome pulldown assays were then analyzed by western blotting using αH3 C-terminus and α-FLAG.
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 thank Suting Zheng for constructing the Set2-myc, Paf1-myc, and Spt6-myc strains as well as performing the ChIP experiments with these strains. We are grateful to Dr. Song Tan for recombinant *Xenopus* nucleosomes. We are grateful to Dr. Mitch Smith and Dr. LeAnne Howe for histone mutant strains. We are grateful to Brian Strahl for the Set2-FLAG constructs. We thank Bing Li for comments. This research was supported by funds from National Institutes of Health (GM58672) to J.C.R.
Figure 4-1: Schematic of strains and primer pairs used in Chapter 4

(A) Schematic of histone N-termini mutants examined in Chapter 4. (B) Schematic of primers used in ChIP experiments for Chapter 4.
Figure 4-2: Mutations to the H3 N-terminus alter RNAPII density and result in 6-AU resistance

(A) Northern blotting for $RNR3$ mRNA. Wild type, H3 Δ1-28, and H3 K->Q strains were grown and harvested before and after exposure to 0.02% MMS. mRNA levels were detected by northern blot and corrected for a loading control (scR1). The cogenic wild type for the H3 Δ1-28 strain is MSY552 and the cogenic wild type for the H3 K->Q strain is MSY590. (B) ChIP analysis of TBP crosslinking to the $RNR3$ promoter. Wild type, H3 Δ1-28, and H3 K->Q strains were grown were grown and treated or not with 0.02% MMS for 2.5hr prior to crosslinking. The data is represented as fold recruitment relative to the untreated (-MMS) wild type cells. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (C) ChIP analysis of RNAPII crosslinking to the $RNR3$ promoter. Wild type, H3 Δ1-28, and H3 K->Q strains were grown were grown and treated or not with 0.02% MMS for 2.5hr prior to crosslinking. The data is represented as fold recruitment relative to the untreated (-MMS) wild type cells. (D) ChIP analysis of RNAPII crosslinking across $RNR3$ relative to the promoter. Essentially the same data as Figure 2-10BD; however, the RNAPII density at the promoter in each strain is set to 1.0 and only the induced levels are depicted. (E) Northern blotting for $RNR3$ mRNA. Wild type, H3 Δ1-28, ΔRRP6 and H3 Δ1-28/ΔRRP6 strains were grown and harvested before, 1, and 2.5 hrs after treatment with 0.02% MMS. The full length $RNR3$ transcript is labeled. No short intragenic transcripts are observed. (F) Spot tests for growth on 6-AU. Serial dilutions of wild type and mutant strains were spotted onto –URA or -URA+6-AU media and incubated at 30°C. Plates lacking and containing 6-AU were grown for 36 and 48 hrs, respectively.
4-2 E

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hours post induction

RNR3 full-length

4-2 F

-URA

MSY552 WT
H3Δ1-28
MSY590 WT
H3 K->Q
Δset2

-URA + 100μg/mL 6-AU
Figure 4-3: Mutations to the H3 N-terminus result in H3 K36me3 defects at RNR3

(A) Levels of tri-methylated K36 (K36me3) across RNR3. Wild type and H3 Δ1-28 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (B) Levels of tri-methylated K36 (K36me3) across RNR3. Wild type and H3 K->Q strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. (C) Levels of tri-methylated K36 (K36me3) across RNR3. Wild type and H3 Δ1-20 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. (D) Levels of tri-methylated K36 (K36me3) across RNR3. Wild type and Δset2 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. (E) Levels of acetylated K36 (K36Ac) across RNR3. Wild type and H3 Δ1-28 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36Ac. K36Ac signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. (F) Levels of tri-methylated K36 (K36me3) across PYK1 and PMA1. Wild type and H3 Δ1-28 strains were grown and harvested. ChIP was performed for H3 K36me3. The data is represented as the % IP/Input. (G) Western blot analysis of H3 K36me3. Western blots were performed on whole cell extracts of wild type, H3 Δ1-28, H3 K->Q, and H4 all K->Q strains. Blots were probed with αH3 K36me3 and αH3 core antibodies. The gradient indicates a decreasing amount of extract loaded unto the gel.
4-3 F

![Graph showing H3K36me3 %/input for various conditions]

4-3 G

![Images showing Western blots for various proteins under different conditions]

- αH3 K36me3
- αH3 C-term
- Δset2 WT H3 Δ1-28
- Δset2 WT H3 K->Q
- Δset2 WT H4 Δ2-26
Figure 4-4: H3 K36me2 in the H3 mutants

(A) Western blot analysis of H3 K36me2. Western blots were performed on whole cell extracts of wild type, H3 Δ1-28, and H3 K->Q strains. Blots were probed with αH3 K36me2 and αH3 core antibodies. The gradient indicates a decreasing amount of extract loaded unto the gel. (B) Levels of di-methylated K36 (K36me2) across RNR3. Wild type and H3 Δ1-28 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36me2. K36me2 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (C) Levels of di-methylated K36 (K36me2) across RNR3. Wild type and H3 K->Q strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed for H3 K36me2. K36me2 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3. (D) Western blot analysis of H3 K79me2. Western blots were performed on whole cell extracts of wild type, H3 Δ1-28, and H3 K->Q strains. Blots were probed with αH3 K79me2 and αH3 core antibodies. The gradient indicates a decreasing amount of extract loaded unto the gel.
Figure 4-5: The H3 N-terminus suppresses intragenic transcription at *STE11* and *FLO8*

(A) Northern blot of *STE11* mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. *STE11* full-length and short transcripts are labeled. The ratio of short intragenic/full-length transcripts is displayed below the *STE11* blot. scR1 mRNA was probed for a loading control. (B) Levels of tri-methylated K36 (K36me3) across *STE11*. Wild type, H3 Δ1-28, and H3 K->Q strains were grown and harvested. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3 as described in the Methods section. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (C) Levels of di-methylated K36 (K36me2) across *STE11*. Wild type, H3 Δ1-28, and H3 K->Q strains were grown and harvested. ChIP was performed for H3 K36me2. K36me2 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3 as described in the Methods section. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (D) Northern blot of *FLO8* mRNA. Wild type and mutant cells were grown and harvested as in the Methods section. *FLO8* full-length and short transcripts are labeled. scR1 mRNA was probed for a loading control.
Figure 4-6: The H3 N-terminus is dispensable for RNAPII CTD phosphorylation

(A) ChIP analysis of RNAPII CTD Ser2 phosphorylation levels at *RNR3*. Wild type and H3 Δ1-28 strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed with an antibody to RNAPII CTD Ser2 phosphorylation. Ser2 phosphorylation signals are represented as the % IP/Input. Induced levels (+MMS) are represented. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (B) ChIP analysis of RNAPII CTD Ser2 phosphorylation levels at *RNR3*. Wild type and H3 K->Q strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed with an antibody to RNAPII CTD Ser2 phosphorylation. Ser2 phosphorylation signals are represented as the % IP/Input. Induced levels (+MMS) are represented. (C) ChIP analysis of RNAPII CTD Ser2 phosphorylation levels at *RNR3*. Wild type and Δ*ctk1* strains were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed with an antibody to RNAPII CTD Ser2 phosphorylation. Ser2 phosphorylation signals are represented as the % IP/Input. Induced levels (+MMS) are represented.
4-6 A

4-6 B

4-6 C
Figure 4-7: The H3 N-terminus is dispensable for Set2, Paf1, and Spt6 recruitment

(A) Set2-myc recruitment across *RNR3*. Wild type and H3 Δ1-28 strains with Set2-myc were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed with anti-myc. Set2-myc signals are represented as the % IP/Input. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (B) Set2-myc recruitment across *RNR3*. Wild type and H3 K->Q strains with Set2-myc were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed with anti-myc. Set2-myc signals are represented as the % IP/Input. (C) Set2-myc recruitment across *PYK1*. Wild type and H3 Δ1-28 strains with Set2-myc were grown and harvested. IPs were performed with anti-myc antiserum. Set2-myc signals are represented as the % IP/Input. (D) Paf1-myc recruitment across *RNR3*. Wild type and H3 Δ1-28 strains with Paf1-myc were grown and treated or not with 0.02% MMS for 2.5hr. IPs were performed with anti-myc antiserum. Paf1-myc signals are represented as the % IP/Input. (E) Paf1-myc recruitment across *RNR3*. Wild type and H3 K->Q strains with Paf1-myc were grown and treated or not with 0.02% MMS for 2.5hr. ChIP was performed with anti-myc. Paf1-myc signals are represented as the % IP/Input. (F) Spt6-myc recruitment across *RNR3*. Wild type and H3 Δ1-28 strains with Spt6-myc were grown and treated or not with 0.02% MMS for 2.5hr. IPs were performed with anti-myc antiserum. Spt6-myc signals are represented as the % IP/Input. (G) Spt6-myc recruitment across *RNR3*. Wild type and H3 K->Q strains with Spt6-myc were grown and treated or not with 0.02% MMS for 2.5hr. IPs were performed with anti-myc antiserum. Spt6-myc signals are represented as the % IP/Input.
Figure 4-8: The H3 N-terminus stimulates Set2 activity

(A) Schematic of the recombinant Set2 proteins. (B) *in vitro* nucleosome methyltransferase assay. Increasing amounts of Set2 (1-261) were incubated with recombinant Xenopus nucleosome core particles (wild type, H3 Δ1-26, or H4 Δ1-22) and 3H S-adenosyl-methionine (SAM). The amount of 3H SAM incorporated into nucleosomes was detected by scintillation counting. The bottom panel is a coomassie stained gel of a representative methyltransferase assay that shows equal addition of nucleosomes to each reaction. The dark triangles represent increasing amounts of Set2. The histone bands are labeled. (C) Set2 nucleosome pulldowns. Set2-FLAG or FLAG resin was incubated with wild type or H3 Δ1-26 nucleosomes. Interactions were examined by western blotting: blots were probed for αH3 core or αFLAG. 5% of the nucleosome input is shown in lanes 5 and 6. The asterisk designates a commonly observed truncated form of the histone H3. Although the western blot gives the impression that significant cleavage has occurred, this is because the film was deliberately exposed to detect the histones in the wild type pulldown assays. Shorter exposures reveal that less than 10% of the histone H3 is truncated (not shown). (D) Wild type Set2 nucleosome pulldown titration. Same as in Figure 4-8C, except nucleosome amounts used in the binding assays were titrated (3-fold serial dilutions). 5% of the nucleosome input corresponding to the assays in lanes 1 and 4 are shown in lanes 8 and 9 respectively. (E) R195G Set2 nucleosome pulldown. Same as in Figure 4-8D, except the R195G Set2-FLAG was used.
4-8 C

4-8 D

4-8 E
Figure 4-9: H3 lysine to arginine mutations do not result in an H3 K36me3 defect

(A) Western blot analysis of H3 K36me3. Western blots were performed on whole cell extracts of wild type and H3 K->R strains. Blots were probed with αH3 K36me3 and αH3 core antibodies. The gradient indicates a decreasing amount of extract loaded unto the gel. The asterisk indicates a non-specific band also observed in the Δset2 strain. (B) Levels of tri-methylated K36 (K36me3) relative to RNAPII at RNR3 ORF D. Wild type, H3 K->Q, and H3 K->R strains were grown and treated or not with 0.02% MMS for 2.5 hours. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3 and plotted relative to RNAPII density by conducting ChIP using 8WG16. The wild type (H3K36me3/H3)/8WG16 ratio was set to 1. Induced levels (+MMS) are depicted. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (C) Levels of tri-methylated K36 (K36me3) relative to RNAPII at PYK1. As in Figure 4-9B, except primers to the PYK1 ORF1 were used and cells were not treated with MMS. (D) Levels of tri-methylated K36 (K36me3) relative to RNAPII at PMA1. As in Figure 4-9C, except primers to the PYK1 ORF1 were used. (E) Spot tests for growth on 6-AU. Serial dilutions of wild type and H3 K->R strains were spotted onto –URA or -URA+6-AU media and incubated at 30°C. Plates lacking and containing 6-AU were grown for 36 and 48 hours respectively. (F) Northern blot of STE11 mRNA. STE11 full-length and short transcripts are labeled. scR1 mRNA was probed for a loading control.
Figure 4-10: Deletion of Gcn5 results in increased H3 K36me3 and decreases H3 K36Ac

(A) Levels of tri-methylated K36 (K36me3) across RNR3. Wild type and Δgcn5 strains were grown and treated or not with 0.02% MMS for 2.5hr for the experiments in this figure. ChIP was performed for H3 K36me3. K36me3 signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3 as described in the Methods section. Primer regions for all experiments in this figure are depicted in Figure 4-1B. (B) Levels of RNAPII across RNR3. ChIP was performed for RNAPII in wild type and Δgcn5 strains. The data is represented at the % IP/Input as described in the Methods section. (C) Levels of H3 K9,14 acetylation (H3 K9,14Ac) across RNR3. ChIP was performed for H3 K9,14Ac in wild type and Δgcn5 strains. K9,14Ac signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3 as described in the Methods section. (D) Levels of H3 K36 acetylation (H3 K36Ac) across RNR3. ChIP was performed for H3 K36Ac in wild type and Δgcn5 strains. K36Ac signals were corrected for nucleosome density by conducting ChIP using an antibody to the core domain of H3 as described in the Methods section.
The H3 tail interacts with Set2 to properly position lysine 36 in the Set2 active site. Deletion of the H3 tail or mutation of the charged lysine residues within the tail results in the inability of Set2 to methylate lysine 36. The importance of charge suggests that modification of lysine residues within the H3 tail may regulate Set2 activity.
Chapter 5

TRANSCRIPTIONAL REGULATION BY THE HISTONE N-TERMINI
CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS
Roles for the H4 N-terminus throughout Transcription

Transcription can be divided into three basic stages: initiation, elongation, and termination. In addition to these events, cotranscriptional mRNA processing occurs. Chromatin structure, which is the backdrop for these processes, plays important regulatory roles in transcription. The participation of chromatin dynamics in initiation and elongation has been studied extensively. During initiation, chromatin is remodeled and modified in order to open the promoter region for the binding of transcription factors. Throughout elongation, chromatin structure must be disrupted to allow RNAPII passage and then reassembled to protect the DNA from inadvertent binding of factors. While these changes in chromatin dynamics have been well characterized, the mechanisms underlying the dynamics are still unclear. The histone N-termini protrude from the nucleosome core, are extensively modified, and provide binding sites for transcription factors and chromatin remodelers. These roles are most closely associated with initiation and to a lesser degree elongation. How the N-termini function in termination and mRNA processing is poorly understood. We utilized the powerful genetic system of \textit{S. cerevisiae} to examine how mutations to the histone N-termini affect transcription regulation.

Our studies have identified novel roles for the histone H4 N-terminus throughout multiple stages of transcription. In addition to roles in transcription initiation, we demonstrate that deletion of the H4 N-terminus results in cryptic intragenic transcription at \textit{FLO8}. We provide evidence that the H4 tail is involved in a pathway with Spt6 and Spt16, but separate from Set2-Rpd3 (Figure 5-1A). Accordingly, the H4 tail deletion results in reduced crosslinking of Spt6 and Spt16. The mechanistic interplay between the H4 N-terminus and Spt6 and Spt16 is unclear. Specifically, the question arises as to whether the H4 N-terminus is required only for the reassembly of chromatin, or for both disruption and reassembly. Previously, Spt6 and Spt16 have
both been shown to stimulate transcription \textit{in vitro} (Belotserkovskaya et al, 2003, Endoh et al, 2004). By examining the ability of these factors to stimulate transcription on wild type and H4 tailless chromatin templates, the requirements and roles for the H4 N-terminus might be discerned.

Substitution of the H4 lysine residues for glutamines results in a termination defect at snoRNAs. The termination pathway for snoRNAs differs slightly from mRNAs and since we have not observed termination defects at \textit{RNR3} or other mRNA encoding genes, snoRNA specific termination events may be defective (Kim et al, 2006). Nrd1 and Nab3 are two factors involved in snoRNA termination (Carroll et al, 2004). The H4 K->Q mutation may cause termination defects due to defective recruitment or activity of Nrd1 or Nab3.

Deletion of the H4 N-terminus or mutation of the lysines within the tail both result in splicing defects. The H4 tail or modifications of the tail may act as a signal or platform for splicing factor recruitment. Examining the recruitment of these factors could better establish the mechanism of the splicing defects in these mutants. Furthermore, an examination into the histone tail-mRNA sequestration model could be conducted. If the H4 all K->Q splicing defect is due to the loss of charge, and in effect the inability to sequester negatively charged mRNA, then an H4 all K->R mutant may not result in a splicing defect.

We provide clear evidence that mutations to the H4 N-terminus cause transcriptional defects at multiple stages. As the template and backdrop for transcription, chromatin is a potent regulator. Specifically, the H4 N-terminus functions throughout transcription to regulate these multiple processes. The questions discussed in this section are aimed at further exploration into the mechanisms of the defects we observe throughout transcription in the H4 mutants.
The Prevalence of Redundancy throughout Transcription

A major reoccurring theme of this work is the prevalence of redundancy throughout transcription regulation. The most striking examples of this are found within the lysine residues of the H4 N-terminus. Our initial analysis of transcription initiation at RNR3 suggested that H4 lysine 16 is of particular importance for expression. The importance of this residue has been identified elsewhere (Dion et al, 2005). However, when we further probed into the roles of the H4 tail in post-initiation events, we found that the H4 acetylatable lysines exhibited redundancy. Only the H4 all K->Q mutation exhibited synthetic lethality when combined with elongation factor deletions. The two triple K->Q mutations exhibited no additional growth defects. Furthermore, we demonstrated that the H4 all K->Q mutation resulted in termination and splicing defects. While the triple K->Q mutants did not exhibit these phenotypes. This suggests redundancy for the H4 lysine residues in transcription elongation, termination, and mRNA splicing. The theme of redundancy for H4 lysine residues does not end with transcription. Strains harboring the H4 all K->Q mutation are defective for nonhomologous end joining DNA repair (Bird et al, 2002). Interestingly, in strains that express an ectopic lysine at residue 3 on the H4 N-terminus in addition to the K5,8,12,16Q mutation, this defect is suppressed which once again suggests redundancy of function (Bird et al, 2002).

Overlapping functions have been identified in biology and throughout transcription. The TFIID and SAGA complexes have overlapping functions in initiation (Lee et al, 2000, Santisteban et al, 2000). Different transcription factors that bind the same DNA sequences and exert similar effects provide another example of overlapping function (Hollenhorst et al, 2007). The obvious explanation for the prevalence of redundancy is that transcriptional regulation is so important to the cell that multiple paths to the same end are evolutionarily advantageous. The
level of redundancy we observe throughout transcription for the H4 tail lysine residues stresses their importance in transcriptional regulation.

**Histones as Regulators of Histone Modifications**

The histone code was formally proposed many years ago (Jenuwein & Allis, 2001). Numerous trans-tail and intra-tail regulatory pathways have been identified both before and after this proposal (Cheung et al, 2000, Dover et al, 2002, Du et al, 2008, Fingerman et al, 2007, Guccione et al, 2007, Kirmizis et al, 2007, Lee et al, 2007). This body of evidence suggests that the structure of the nucleosome is vital to reading and writing the histone code. In this study, we identify two novel regulatory pathways. During the characterization of the histone N-termini in transcription initiation, we demonstrate that the H2A N-terminus is important for H3 lysine 4 methylation. This provides evidence for additional trans-tail regulation of Set1. Determining the mechanism of this defect has become the major project of another graduate student.

We have also identified and characterized the mechanism of novel intra-tail regulation of Set2 by the H3 N-terminus. We demonstrate that the H3 N-terminus directly stimulates Set2 methylation and that the positive charge of the tail is important (Figure 4-10 and 4-11). It remains unclear exactly which regions within Set2 are interacting with the H3 tail. The stimulatory effect observed in the histone methyltransferase assays is observed with the Set2 SET domain (amino acid 1-261) and therefore it is likely that the H3 tail interacts with a region of the SET domain. The pI of this region is 4.94 and it is possible that an acidic patch within Set2 interacts with the positively charged tail. Unfortunately, a crystal structure of Set2, or any of its homologues, has not been determined and therefore modeling cannot be done at this time. *In vitro* crosslinking studies with a labeled H3 N-terminus could shed light onto which regions of Set2 are important for this interaction.
The novel intra-tail regulation of Set2 that we describe here is distinct from previously described intra-tail regulation. Most intra-tail regulation involves the regulation of one modification by another in close proximity (Cheung et al, 2000, Guccione et al, 2007, Kirmizis et al, 2007, Lo et al, 2000, Nelson et al, 2006). For example, Set2 activity is regulated by H3 proline 38 isomerization (Nelson et al, 2006). This residue is quite close to lysine 36 and prolyl isomerization is likely to alter the environment of the active site. We demonstrate that regions of H3 as far as 15 amino acids away can influence Set2 methylation. Regulatory effects over this distance within the same histone have not been reported to our knowledge. The other distinction of this pathway is the availability of a gradient. Whereas the isomerization of a proline residue is either on or off, six modifiable lysine residues will provide the option of a gradient. This could allow for subtle changes in Set2 activity that may be important in higher eukaryotes. We are unaware of any other histone crosstalk that involves the possibility of a regulatory gradient that allows for fine tuning of histone modifications.

Relevance and Implications of Intra-tail Regulation of Set2

In our analysis of the H3 N-terminal deletion, we demonstrate the importance of the H3 tail on Set2 methylation. Recently, the use of the H3 tail deletion strain has increased in biological relevance. Two reports, one in *S. cerevisiae* and one in mouse embryonic stem (MES) cells, demonstrate that the H3 N-terminus undergoes programmed cleavage (Duncan et al, 2008, Santos-Rosa et al, 2009). In yeast, the programmed “clipping” of the H3 N-terminus occurs at the promoters of sporulation genes upon activation (Santos-Rosa et al, 2009). *GAL1* is repressed by the transcription of a noncoding RNA that results in H3 K36me3 over the *GAL1* promoter (Houseley et al, 2008). It is unknown whether sporulation genes are also regulated similarly, but if they were, perhaps “clipping” of the H3 N-terminus results in decreased H3 K36me3 which in
turn leads to increased acetylation and enhanced activation. Additionally, proteolytic cleavage of H3 occurs in MES cells (Duncan et al, 2008). The purpose of the cleavage is unknown, but the authors note that histone acetylation is relatively low in MES cells. In the absence of acetylation, H3 cleavage may provide an important and alternative regulation of H3 K36me3.

Our model of intra-tail regulation of Set2 by the H3 N-terminus suggests a dually antagonistic relationship between histone H3 acetylation and H3 lysine 36 methylation (Figure 5-1B). The mechanism of the Set2-Rpd3 pathway is well established (Carrozza et al, 2005, Joshi & Struhl, 2005, Li et al, 2007b). Set2 methylation results in the recruitment of Rpd3 which deacetylates both H3 and H4 within the gene. Our model suggests that H3 acetylation inhibits Set2 methylation and implies that nucleosomes deacetylated by Rpd3 would then become better substrates for the next round of Set2 methylation; Set2 indirectly stimulates the activity of the next round of Set2 methylation, further propagating the H3 K36me3 mark.

Given the circular nature of the antagonistic relationship between H3 acetylation and H3 K36me3, questions remain about which regulation evolved first. The mechanisms of antagonism are rather different: H3 acetylation acts directly by inhibiting Set2 activity while Set2 methylation acts indirectly by recruiting an enzyme responsible for removing acetyl marks. The antagonism of our model is not completely reciprocal. While Set2 methylation results in the deacetylation of both H3 and H4, only the acetylation status of H3 can regulate Set2 activity (Figure 5-1B). H4 acetylation status has no bearing on Set2 activity, but is likely involved in other transcription processes such as the repression of intragenic transcription and possibly splicing (Figures 5-1A and 5-1B).

The fine tuning of this relationship likely has consequences beyond the repression of intragenic transcription. In higher eukaryotes, discrete peaks and troughs of H3 K36me3 are observed across genes (Kolasinska-Zwierz et al, 2009) (also depicted in Figure 5-1B). Interestingly, the peaks of H3 K36me3 correspond to exons and the troughs to introns.
(Kolasinska-Zwierz et al, 2009). Furthermore, constitutively spliced exons have higher levels of H3 K36me3 than alternatively spliced exons (depicted in Figure 5-1B). Kolasinska and colleagues speculate that the marked exons may facilitate the recruitment of splicing factors, or conversely that splicing factors may regulate Set2 activity (Kolasinska-Zwierz et al, 2009). Alternatively, variations in Set2 methylation will affect the levels of H4 acetylation via the recruitment of Rpd3. We have established that the H4 N-terminus is important for splicing in S. cerevisiae (Figure 3-10 and depicted in Figure 5-1B) and others have proposed that the histone N-termini act to sequester nascent mRNA until splicing factors are able to bind (Allemand et al, 2008). A provocative model is that H3 acetylation levels regulate Set2 activity, lysine 36 methylation then regulates H4 acetylation levels, and finally H4 acetylation affects splicing efficiency. Although we have established a role for H4 in splicing, further studies are necessary to provide a link between H4 acetylation and splicing (Figure 5-1B). This model is highly speculative, but demonstrates the possible complexities of transcriptional regulation.
(A) Both the histone H3 and H4 N-termini have roles in the repression of intragenic transcription. The H4 N-terminus is important for the chromatin remodeling pathway likely through the recruitment of Spt6 and Spt16. The H3 N-terminus stimulates Set2 activity and is important for the chromatin modification pathway. (B) H3 acetylation inhibits Set2 methylation. Others have established that Set2 methylation recruits Rpd3 to deacetylate both H3 and H4 (Carrozza et al, 2005, Joshi & Struhl, 2005, Li et al, 2007b). This antagonistic relationship likely creates a delicate balance of histone modifications important for transcription elongation and splicing.
Diagram 5-1 A:

- Spt6 & Spt16
- Remodeling Pathway
- Intragenic Transcription
- H4 tail
- H3 tail
- Set2
- Modification Pathway

Diagram 5-1 B:

- Rpd3
- H3 K36me3
- H3/H4 acetylation
- Constitutive Exon
- Alternative Exon
- H3 Ac
- H4 Ac
- Splicing efficiency
- H4 Tail

Note: intra-tail regulation
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