EXAMINING THE CONTRIBUTION OF CHROMATIN AND CHROMATIN ASSOCIATED FACTORS TO TRANSCRIPTION REGULATION

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Regulation of gene expression is complex, involving the coordinated effect of a large number of proteins with a wide range of activities that control the recruitment and activity of RNA polymerases. Eukaryotic genomes are packaged into chromatin, where positioned nucleosomes are used to control transcriptional output. More recently, transcription of ncDNA has been shown to be pervasive and act as a regulator of gene expression as well.

Previous studies in *Saccharomyces cerevisiae* described a gene repression mechanism whereby transcription of intergenic ncDNA (*SRG1*) over the promoter of the adjacent *SER3* gene interferes with the binding of transcription factors. In this work, I contributed evidence to support a mechanism whereby *SRG1* transcription represses *SER3* by controlling chromatin. In the presence of serine, transcription of *SRG1* ncDNA is initiated upstream of the adjacent *SER3* gene and extends across the *SER3* promoter. As RNA pol II transcribes *SRG1*, the accompanying Spt6 and Spt16 histone chaperones reassemble nucleosomes over the *SER3* promoter, which then interfere with transcription factor binding resulting in *SER3* repression. In response to serine starvation, *SRG1* transcription is reduced, causing nucleosome depletion over the *SER3* promoter, which in turn allows transcription factors to bind and activate *SER3* transcription.

I then use this system of gene regulation to identify and characterize mutant versions of the Spt16 histone chaperone and histone H3 and H4 proteins that are defective for transcription-coupled nucleosome assembly. These studies identify single amino acid substitutions in these

proteins that cause a loss of nucleosome occupancy specifically over highly transcribed regions, revealing regions of the proteins that play critical roles in orchestrating transcription-coupled nucleosome assembly.

Furthermore, I provide evidence that a subset of the amino acids in histone H3 function to control chromatin dynamics, and may function as a binding site for histone chaperones Spt6 and Spt16. Therefore, when the residues are mutated, these factors can no longer bind chromatin, resulting in slowed nucleosome reassembly over transcribed regions. Together, my work further elucidates the mechanism of *SER3* regulation by transcription of *SRG1* and reveals a set of novel residues working to regulate this mechanism and transcription-coupled nucleosome dynamics in general.

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PREFACE

My deepest thanks goes to the members of my thesis committee, and I hope that my words here might express the gratitude I feel for their enduring support and kindness. It would be difficult to find an advisor more dedicated, thoughtful, and generous than Joe Martens. His guidance and insights have shaped me as a scholar, and in him I see the academic community at its best. I find myself inspired by Joe's passion for the field; he is a model of the type of teacher and thinker that I hope to one day become. Karen Arndt was, from my first days as a graduate student at the University, a supremely encouraging force in my life. Always friendly and always shrewd, she was able to nuance my thinking and at the same time ease my scholarly anxieties. Jeff Brodsky's enthusiasm, kindness, and thought-provoking perspectives have made this entire experience all the more worthwhile. Jeff Hildebrand's insightful, encouraging, and challenging comments and questions have been key to shape my dissertation to what it is today. I was fortunate that Bino John agreed to serve as my external committee member; his support in this project and helpful comments have been invaluable.

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1.0 INTRODUCTION

The precise and highly coordinated action of regulating gene expression is an important biological process as it increases versatility and adaptability of an organism by allowing the cell to express various RNA and protein species when necessary. It is not surprising, then, that mechanisms that regulate gene expression are very complex and diverse. In eukaryotes, the coordination of transcription and chromatin dynamics is fundamental to the process of gene regulation. Interestingly, this process has become much more complex due to the recent understanding that the transcriptome generates many more RNAs species than originally suspected – in fact the majority of transcription which occurs in the cell is in the form of non-protein coding transcripts. As my thesis research investigates the role of chromatin and chromatin associated factors during transcription, this chapter primarily focuses on regulatory events during transcription, as well as on factors involved in regulating chromatin during the transcription process.

1.1 TRANSCRIPTION IN EUKARYOTES REQUIRES MANY FACTORS TO PERMIT EFFICIENT TRAVERSAL OF RNA POLYMERASE II AND PROMOTE GENE EXPRESSION

Transcription of DNA to RNA is an important biological process in all cells. The enzymes responsible for performing this function in organisms are DNA dependent RNA polymerases. The coordination of proper recruitment, regulation, and traversal of these polymerases is a dynamic process that requires a number of transcription factors. For the purposes of this dissertation, I will focus my introduction on R NA polymerase II (RNA pol II) transcription exclusively.

1.1.1 Overview of DNA transcription

A typical RNA pol II transcription cycle begins with the binding of activators upstream of the core promoter, which includes a TATA box and transcription start site (Figure 1A). Activator binding leads to the recruitment of adaptor complexes such as SAGA (reviewed in (GREEN 2005)), or Mediator (reviewed in (CHEN and ROEDER 2011; RIES and MEISTERERNST 2011)), both of which facilitate binding of general transcription factors (GTFs) (THOMAS and CHIANG 2006). RNA pol II is positioned at the promoter by a combination of TFIID, TFIIA, and TFIIB to form the closed form of the preinitiation complex (PIC). TFIIH then melts 11-15bp of DNA to position the single strand template in the RNA pol II cleft to initiate RNA synthesis. The carboxy-terminal domain (CTD) of RNA pol II is phosphorylated by the TFIIH subunit during the first 30bp of transcription and loses its contacts with GTFs before it proceeds onto the elongation phase. Meanwhile, the phosphorylated CTD begins to recruit the factors that are

important for productive elongation and mRNA processing (Figure 1B). The termination of transcription, 3' end processing of the transcript, and release of RNA pol II, is precisely coordinated in order to ensure the proper production of the RNA product (reviewed in (KUEHNER *et al.* 2011; RICHARD and MANLEY 2009)). RNA pol II pauses once it has transcribed the poly(A) track at the 3' end of the gene, resulting in the recruitment of the termination machinery, including Rtt103, Rat1, Ssu72, and cleavage and polyadenylation factor (CPF) (Figure 1C).

1.1.2 RNA pol II is a dynamic enzyme responsible for transcribing many regions of the genome

The eukaryotic core RNA pol II catalyzes the transcription of DNA to mRNA and many snRNA and microRNA species (reviewed in (KORNBERG 1999; SIMS *et al.* 2004)). RNA pol II was first purified using transcription assays (SAWADOGO and SENTENAC 1990). This 551kDa complex contains 12 subunits in yeast: Rpb1-3, 5, 6, 8-12 form the core highly conserved portion of this complex, while Rpb4,7 are able to detach from the core complex readily (CRAMER *et al.* 2008). *In vitro* biochemical experiments have shown that the assembly of RNA pol II involves the formation of an Rpb2,3 subcomplex, which forms immediately after their synthesis, and subsequently interacts with Rpb1 (ACKER *et al.* 1997; KOLODZIEJ and YOUNG 1991). Mutational experiments in specific subunits of RNA pol II have shown that Rpb3,5,7 are able to interact among themselves to form homodimers (KOLODZIEJ and YOUNG 1991). However, strong binding of all the subunits does not occur until Rpb1 enters this complex. Once these subunits have initiated assembly, the remaining subunits will assemble to form the entire complex. Once the complex is formed, the two largest subunits, Rpb1 and Rpb2 form opposite sites of the active site cleft, which is a flexible domain that can change conformation during transcription

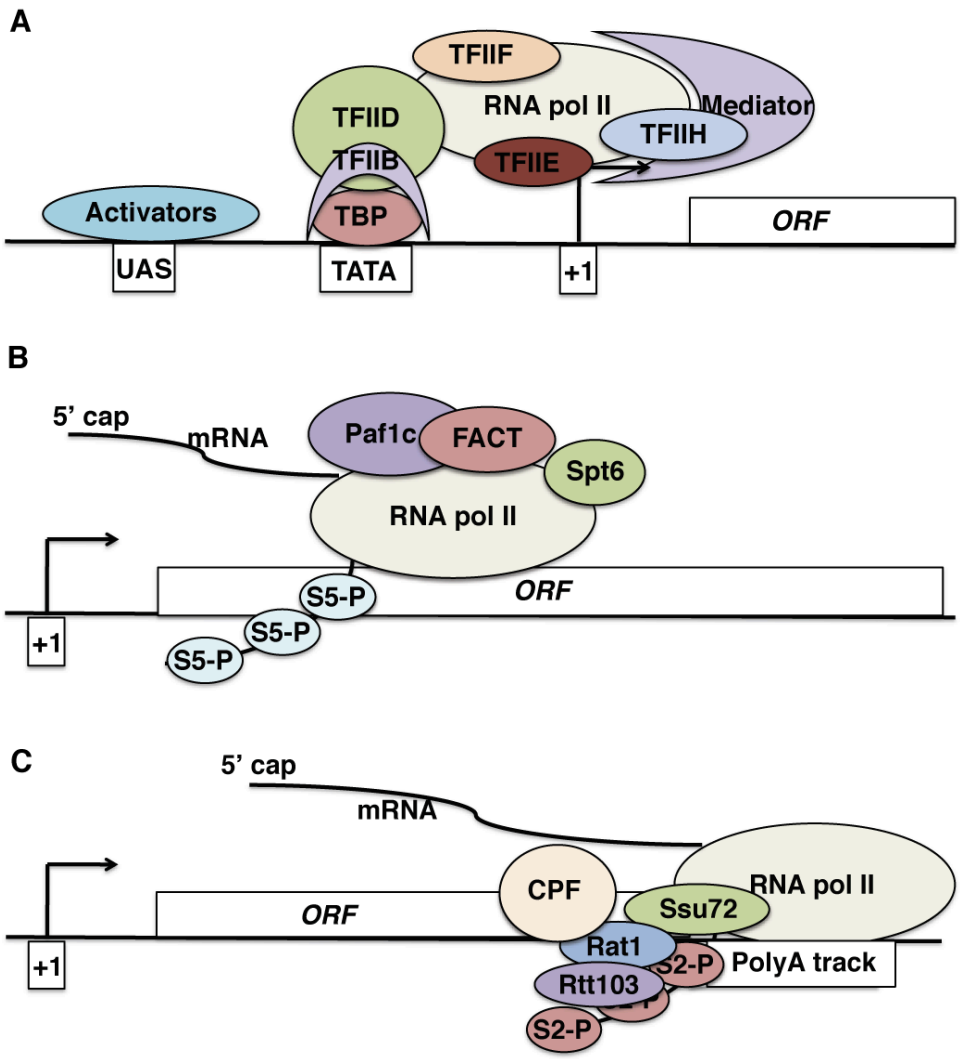


Figure 1. Transcription of protein coding genes by RNA pol II.

Schematic of the transcription cycle. A) Transcription initiation occurs when RNA pol II and the preinitiation complex (PIC) occupies the promoter of a gene. B) Once RNA pol II has traversed through the first ~30bp of DNA, the PIC diassembles and transcription elongation can begin. Elongation requires many factors (only a few of which are shown) for successful traversal of RNA pol II and production of an mRNA transcript. C) Poly(A)-dependent transcription termination occurs once RNA pol II has traversed the entire ORF and paused over the poly(A) track. Many factors are recruited to promote the 3' end processing cleavage event.

(CRAMER *et al.* 2000; CRAMER *et al.* 2001; GNATT *et al.* 2001). In this cleft, a magnesium ion is found that is required for the enzymatic activity of the complex. In order to transcribe DNA, duplexed DNA is able to enter the cleft and begins to unwind. DNA is quickly transcribed into RNA, where a brief DNA-RNA hybrid is created until the RNA is able to exit the cleft after splitting the phosphodiester bonds made between the DNA molecule. Exiting of the RNA occurs through an exit channel, by the C-terminal domain of Rpb1 (GNATT *et al.* 2001; KETTENBERGER *et al.* 2004).

1.1.3 Transcription initiation and promoter proximal pausing of RNA pol II

Transcription initiation begins when the TATA-binding protein (TBP), a subunit of TFIID, binds to the promoter, often times at a TATA consensus sequence, and triggers the assembly of the preinitiation complex (reviewed in (SIKORSKI and BURATOWSKI 2009)) (Figure 1A). This preinitiation complex, consisting of RNA pol II and general transcription factors, is positioned at the transcription start site, where TFIIE and TFIIH can unwind DNA. Interestingly, many promoters do not contain the canonical TATA sequence, yet TBP is still able to bind to these promoters (CARNINCI *et al.* 2006). Once DNA is unwound, RNA pol II is permitted to begin transcribing over the beginning sequences. However, recently it has been discovered that downstream of many transcription start sites, RNA pol II will pause (ADELMAN *et al.* 2005). While this promoter-proximal pausing of RNA pol II is not found at every gene, it is found at a number of genes which are required for rapid induction, and therefore serves as an important regulatory mechanism for many genes (ADELMAN *et al.* 2005; ALEXANDER *et al.* 2010; GILCHRIST *et al.* 2008; LEE *et al.* 2008; MUSE *et al.* 2007; RADONJIC *et al.* 2005).

RNA pol II pausing has been observed in many eukaryotic organisms, ranging from

Saccharomyces cerevisiae to mammalian cells, suggesting a conserved mechanism of gene regulation throughout eukaryotes, where the polymerase is paused for rapid induction of a subset of genes (ALEXANDER *et al.* 2010; GILCHRIST *et al.* 2008; LEE *et al.* 2008; MUSE *et al.* 2007; RADONJIC *et al.* 2005). Consistent with this, the majority of the genes that exhibit RNA pol II pausing are those that are briefly required during development (WANG *et al.* 2007; ZEITLINGER *et al.* 2007), or those that respond to environmental stimuli (MUSE *et al.* 2007). Since the discovery of paused polymerase, it has been found to be involved in additional cellular processes, such as facilitating co-transcriptional RNA splicing (ALEXANDER *et al.* 2010).

There are many factors involved in the regulation of paused RNA pol II, which can either positively or negatively regulate the pausing activity. Three of these factors are DRB Sensitivity-Inducing Factor (DSIF) (WADA *et al.* 1998), the Negative ELongation Factor (NELF) complex (NARITA *et al.* 2003), and Positive Transcription Elongation Factor b (P-TEFb) (KIM and SHARP 2001). RNA pol II and DSIF are able to recruit NELF to transcribed regions, and together, DSIF and NELF promote polymerase pausing (AIDA *et al.* 2006; CHENG and PRICE 2007; RENNER *et al.* 2001; WU *et al.* 2005; WU *et al.* 2003). There have been two models proposed for how NELF is able to inhibit transcription elongation through polymerase pausing. In the first hypothesis, it has been suggested that NELF is able to bind to RNA pol II, and in binding to a specific clamp domain, change the active site, which would result in pausing during active transcription (YAMAGUCHI *et al.* 2001; YAMAGUCHI *et al.* 2007). Alternatively, it has been proposed that the RNA recognition motif of a subunit of NELF is able to bind to the nascent mRNA from RNA pol II, preventing further elongation (RAO *et al.* 2008; YAMAGUCHI *et al.* 2002). In contrast to the functions of NELF, P-TEFb is able to promote transcription elongation through releasing paused RNA pol II (PETERLIN and PRICE 2006).

1.1.4 Release of paused RNA pol II and transcription elongation

P-TEFb is able to release paused RNA pol II through the phosphorylation of both NELF and DSIF (IVANOV *et al.* 2000; KIM and SHARP 2001; PETERLIN and PRICE 2006). Phosphorylation of NELF results in its dissociation from RNA pol II. While the role of DSIF phosphorylation by P-TEFb is not clear, DSIF does remain associated with RNA pol II and influences transcription at later stages (ANDRULIS *et al.* 2000; KAPLAN *et al.* 2000; WADA *et al.* 1998). In addition to phosphorylating NELF and DSIF, P-TEFb also phosphorylates the C-terminal domain (CTD) of Rpb1, the largest subunit of RNA pol II, to promote transcription elongation (PETERLIN and PRICE 2006; QIU *et al.* 2009). The CTD of RNA pol II contains 26 heptapeptide repeats (YSPTSPS) in yeast or 52 heptapeptide repeats in human cells which is phosphorylated on serines in position 2, 5, and 7, and has recently been found to be phosphorylated on threonine at position 4 and tyrosine at position 1 of the heptapeptide repeats (reviewed in (BURATOWSKI 2009)) (BASKARAN *et al.* 1993; HINTERMAIR *et al.* 2012; HSIN *et al.* 2011; MAYER *et al.* 2012). Differential phosphorylation of the CTD occurs throughout the stages of transcription, resulting in recruitment of factors required for proper transcription (reviewed in (BURATOWSKI 2009)).

RNA pol II is hypophosphorylated during transcription initiation, and phosphorylation of the CTD transitions during transcription elongation. Immediately following transcription, during early elongation, the CTD is phosphorylated on serine 5 (Ser^5) by Ctk7/Kin28 in yeast (KOMARNITSKY *et al.* 2000). This form of phosphorylated CTD is recognized by the mRNA capping enzyme, and after the mRNA cap is removed by Ssu72 in yeast (FABREGA *et al.* 2003), Ser^5 phosphorylation begins to decrease (KRISHNAMURTHY *et al.* 2004). Serine 7 (Ser^7) is also phosphorylated by Ctk7/Kin28 during similar stages as Ser^5 phosphorylation, however the function of Ser^7 phosphorylation has not yet been uncovered (AKHTAR *et al.* 2009; CHAPMAN *et*

al. 2007; EGLOFF *et al.* 2007; KIM *et al.* 2009). Next, serine 2 (Ser^2) is phosphorylated on the CTD by Ctk1 in yeast, and this event is coordinated by P-TEFb in mammalian cells (CHO *et al.* 2001; KEOGH *et al.* 2003; PATTURAJAN *et al.* 1999; PRICE 2000; QIU *et al.* 2009). Phosphorylation of Ser^2 leads to the recruitment of cleavage and polyadenylation factors to the 3' end of genes, leading to 3' end processing of mRNA (AHN *et al.* 2004). Threonine 4 (Thr^4) phosphorylation occurs at the 3' end of open reading frames (ORFs) in both yeast and mammalian cells, by Cdk9 in yeast and Plk3 in mammalian cells (HINTERMAIR *et al.* 2012; HSIN *et al.* 2011). While the phosphorylation of Thr^4 has been found to occur at similar times as Ser^2 phosphorylation, the function of this modification is not yet known. Finally, the phophorylation on a fifth residue on the CTD, tyrosine 1 (Tyr^1), while discovered many years ago (BASKARAN *et al.* 1993), has only very recently been assigned a function (MAYER *et al.* 2012). Tyr^1 phosphorylation is enriched over the ORF of transcribed genes, similar to the pattern of Ser^2 phosphorylation, however Tyr^1 phosphorylation decreases before the end of the ORF. Interestingly, Tyr^1 phosphorylation is able to promote the recruitment of Spt6, a transcription elongation factor, and prevent the recruitment of termination factors, and therefore by decreasing this modification before the polyadenylation site, the termination factors are excluded from the ORF but permitted to bind the very 3' end of the ORF (MAYER *et al.* 2012).

Another key player in the orchestration of transcription elongation is the polymerase associated Paf1 complex (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). RNA pol II CTD Ser^5 phosphorylation permits the recruitment of the Bur1 kinase in yeast, which phosphorylates the elongation factors Spt4 and Spt5. Phosphorylation of these factors leads to the recruitment of the Paf1 complex (Paf1c), which is composed of Paf1, Rtf1, Cdc73, Ctr9, and Leo1 (MUELLER and JAEHNING 2002; SHI *et al.* 1997; SQUAZZO *et al.* 2002). Paf1 assists the

recruitment of Rad6 and Bre1, a ubiquitin conjugase and ligase, respectively, which ubiquitylate histone H2B at K123 (CHU *et al.* 2007; NG *et al.* 2003; NG *et al.* 2002; WOOD *et al.* 2003). Paf1c is also required for subsequent methylation marks on histone H3, by the Set1 and Dot1 methyltransferases (PIRO *et al.* 2012a; PIRO *et al.* 2012b; SHAHBAZIAN *et al.* 2005; SUN and ALLIS 2002; WARNER *et al.* 2007; WOOD *et al.* 2003). The dynamics that occur between transcription and chromatin to coordinate efficient gene expression are discussed below in Chapter 1.3.

1.2 HISTONE PROTEINS COMPACT DNA INTO CHROMATIN IN EUKARYOTES

1.2.1 Packaging of eukaryotic DNA into chromatin acts as a barrier to transcription elongation

In eukaryotes, genomic DNA is packaged with proteins to form chromatin: a repeating array of nucleosomes that contain 147bp of DNA wrapped 1.65 times around an octamer of histone proteins composed of a tetramer of H3 and H4 and two H2A and H2B dimers, making 14 important contact points with residues of the histone proteins (LUGER *et al.* 1997) (Figure 2). DNA must wrap around these proteins in order to fit into the organelle provided for DNA in the cell, the nucleus. In general, this stable association of DNA and histone proteins poses a significant obstacle to many cellular processes that rely on proteins being able to interact with DNA, including transcription, DNA replication, and DNA repair (reviewed in (BAI and MOROZOV 2010; DUINA 2011; LI *et al.* 2007a; LUGER 2006)). Histones are small, highly conserved, positively charged proteins consisting of a folded domain which forms the

nucleosome globular core and highly unstructured N- and C-terminal tails that extend out from the core. Nucleosomes are repeated along the length of DNA, with approximately 100 to 200bp between each nucleosome, forming a chromatin template (KORNBERG 1974). These nucleosomes are able to stack and fold upon each other, with the help of an accessory histone protein H1, to progressively create higher order chromatin structures, which eventually condense into chromosomes during mitosis (Figure 2). Surprisingly, little is known about the specific role of histone proteins during DNA templated processes.

1.2.2 Previously characterized amino acid histone substitutions have provided helpful insight into the function of chromatin

Examination of the effects of amino acid substitutions in histone has proven to be a successful strategy to help define the role of chromatin (DAI *et al.* 2008; DU and BRIGGS 2010; DU *et al.* 2008; DUINA and WINSTON 2004; HECHT *et al.* 1995; HIRSCHHORN *et al.* 1992; IWASAKI *et al.* 2011; KRUGER *et al.* 1995; ZHENG *et al.* 2010). The N-terminal tails of histones have been the focus of many mutational analyses (HECHT *et al.* 1995; SABET *et al.* 2003), and this work has lead to a working histone code model where certain posttranslational modifications and crosstalk between these modifications provides a pattern of information for proper transcriptional regulation and gene expression (BERGER 2007; JENUWEIN and ALLIS 2001; STRAHL and ALLIS 2000) (discussed in Chapter 1.3.2.2). The globular domains of histones have also been examined for residue contributions to transcriptional regulation. Recent work has revealed many post-translational modifications within the structured region of the nucleosome, which have been proposed to increase the mobility of nucleosomes (COCKLIN and WANG 2003; ZHANG *et al.* 2002; ZHANG *et al.* 2003). Specific mutations within the globular domain of H2A and H4 have

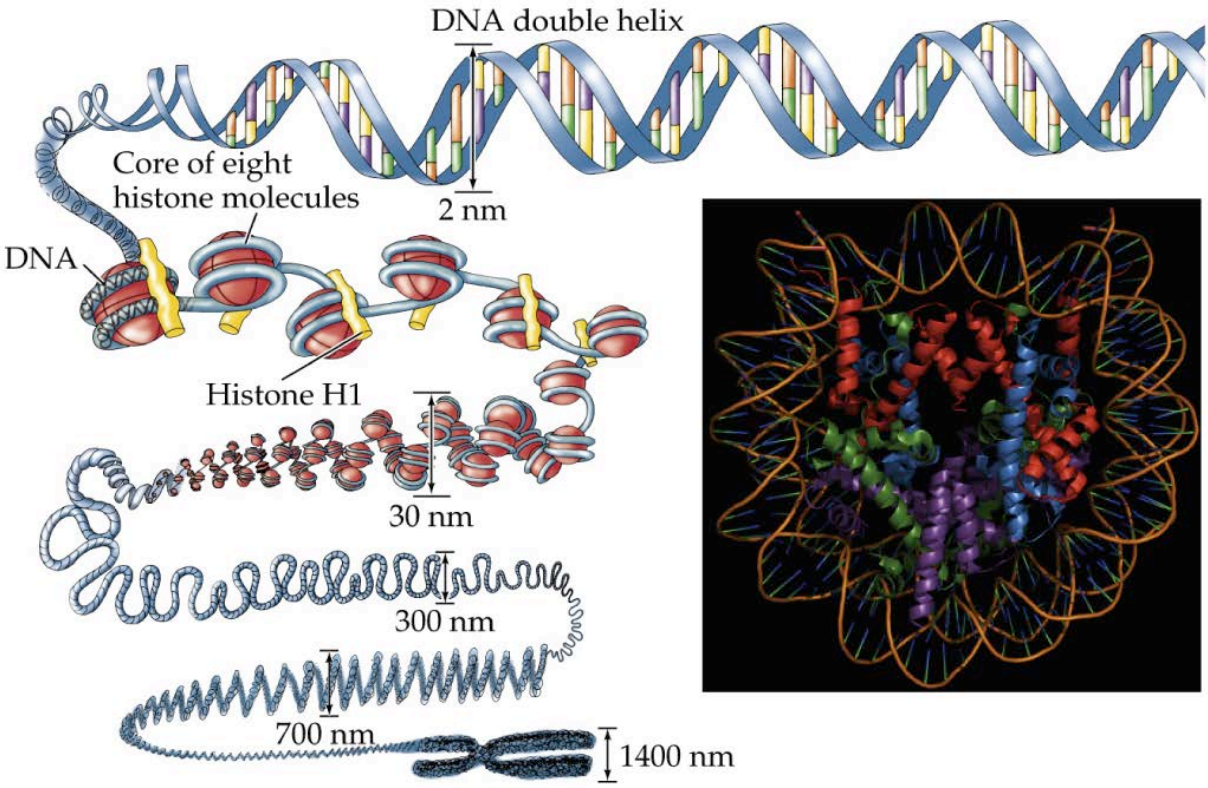


Figure 2. DNA compaction by nucleosomes and crystal structure of the yeast nucleosome core particle.

Left image: DNA compaction by nucleosomes. This figure was reprinted from Sinauer Associates, copyright 2001. Right image: cartoon of the X-ray crystal structure of the yeast nucleosome core particle, viewed down the superhelical axis (WHITE *et al.* 2001). Histone chains are colored red for H3, blue for H4, purple for H2B, and green for H2A. The DNA is shown in orange, with bonds in blue and green. This image was created on Pymol with PDB access number 1ID3.

been shown to affect transcription (HIRSCHHORN *et al.* 1995; SANTISTEBAN *et al.* 1997). Additionally, genetic experiments have identified a nucleosomal surface that is required to maintain proper transcriptional silencing (PARK *et al.* 2002) and residues in histone H3 and H4 globular domains that are required for normal levels of transcriptional silencing (SMITH *et al.* 2002; THOMPSON *et al.* 2003).

A well described set of histone residue substitutions are those that suppress transcriptional defects caused by the loss of a component of the Swi/Snf chromatin remodeling complex (HIRSCHHORN *et al.* 1992; HSIEH *et al.* 2010; KRUGER *et al.* 1995; KURUMIZAKA and WOLFFE 1997; RECHT and OSLEY 1999). The *sin* mutations (Swi/Snf independent) identified within the histone H3 and H4 genes had the following amino acid substitutions: H3 T118I, H3 R116H, H3 D123, H3 E105K, H4 R45H/C, and H4 V43I. These *sin* mutations specifically affect the strong DNA-histone interactions over the nucleosome dyad and result in increased mobility along DNA and reduced stability (MUTHURAJAN *et al.* 2004). More recently, it was shown that the *sin* mutants do no t pose as significant nucleosomal barrier for RNA pol II during transcription, likely because the *sin* mutants have a greater tendency to dissociate completely from DNA during traversal of RNA pol II (HSIEH *et al.* 2010).

1.3 CHROMATIN DYNAMICS DURING TRANSCRIPTION REGULATE GENE EXPRESSION

In general, most yeast promoters are nucleosome-free regions (NFR), which permits binding of transcription factors and successful transcription initiation (LEE *et al.* 2004; LEE *et al.* 2007). However, transcription initiation can be hindered when promoter DNA is wrapped into a

nucleosome, which can no longer be easily recognized by DNA binding factors (KORNBERG and LORCH 1999). Transcription elongation can also be physically hindered by nucleosome occupancy in that transcription rates of RNA pol II are slowed due to increased pausing and backtracking (CHURCHMAN and WEISSMAN 2011; IZBAN and LUSE 1991; LEE *et al.* 2004). Therefore, the mechanisms through which eukaryotes regulate chromatin dynamics are utilized during all stages of transcription to successfully regulate gene expression.

Changes in transcription of genes are tightly correlated with changes in chromatin structure (FIELD *et al.* 2008; RADMAN-LIVAJA and RANDO 2010; SCHWABISH and STRUHL 2004; SHIVASWAMY and IYER 2008; WEINER *et al.* 2010; ZAWADZKI *et al.* 2009). By comparing highly transcribed genes to lowly transcribed genes in a single growth condition, or comparing a gene that is activated or repressed in response to different stimuli, this can be especially appreciated. For example, when genes are highly transcribed, the -1 nucleosome relative to the TSS (where the -1 nucleosome is the nucleosome immediately 5' of the NFR in most promoter regions and the +1 nucleosome is the nucleosome immediately 3' of the NFR, by the TSS) is evicted, generating an increased NFR (SHIVASWAMY and IYER 2008; ZAWADZKI *et al.* 2009). Conversely, genes that are not expressed, or lowly expressed, have an existing NFR that is not altered until the gene is upregulated. Additionally, over the coding regions of the genes, nucleosome occupancy decreases during high rates of transcription, whereas over lowly transcribed genes, the nucleosome occupancy is not significantly disrupted.

Changes such as those mentioned above occur through many factors, such as chromatin remodeling factors, histone modifying enzymes, and histone chaperones, many of which are recruited by RNA pol II and general transcription factors. However, RNA pol II itself is also responsible for some of the alterations which occur to nucleosome architecture. *In vitro*, RNA

pol II is able to transcribe chromatinized DNA without evicting the nucleosome, or by creating subspecies of nucleosomes, such as hexasomes (KULAEVA *et al.* 2010; STUDITSKY *et al.* 1994; STUDITSKY *et al.* 1997). Additionally, inactivation of RNA pol II in yeast has been shown to increase the -1 nucleosome occupancy, lending more support to the role of RNA pol II in regulating the chromatin environment of genes (WEINER *et al.* 2010).

1.3.1 DNA sequence plays a role in chromatin dynamics during transcription

As histone proteins have always been assumed to be general packaging factors, little sequence preference was ever appreciated for these proteins. However, due to the properties in which nucleosomes are created – bending genomic sequence to wrap tightly around a small octamer of proteins – it would be logical to assume that the ability to bend the DNA would partially depend on the sequence of the DNA. Based on this hypothesis, specifically spaced A/T dinucleotide sequences were found to bind the histone octamer with higher affinity than a random sequence (SEGAL *et al.* 2006). Further investigation into this sequence requirement has revealed that most promoter sequences, which are generally nucleosome depleted, are enriched for long poly(dA:dT) runs, supporting the possibility of sequence preference for nucleosome positioning over the genome (SEGAL and WIDOM 2009a; SEKINGER *et al.* 2005; THASTROM *et al.* 1999). Further experimental and computational research into the role for sequence in directing chromatin structure over the genome has revealed that, in some cases, the sequence can predict the probability of nucleosome occupancy (BROGAARD *et al.* 2012; KAPLAN *et al.* 2009; ZHANG *et al.* 2009). However, these studies are not able to completely predict the positioning of *in vivo* nucleosomes, as *trans*-acting factors will redistribute nucleosomes, as necessary.

In addition to sequence preferences for histone octamers, statistical positioning of

nucleosomes is another *cis*-determinant of nucleosome positioning over the genome. Statistical positioning predicts that well-positioned nucleosomes can lead to adjacently positioned nucleosomes, as space constraints restrict movement between adjacent proteins (KORNBERG 1981). Therefore, the very well positioned nucleosome immediately 3' of the promoter NFR (the +1 nucleosome) results in positioned nucleosomes immediately 3' of its position, resulting in delocalized ("fuzzy") nucleosomes as nucleosomes go more distal from this first nucleosome (YUAN *et al.* 2005).

1.3.2 *Trans* regulatory factors

Not surprisingly, eukaryotes express an array of proteins with a range of activities that contribute to the reorganization of chromatin to facilitate these processes, including chromatin remodeling factors, histone modifying enzymes, and histone chaperones. In addition to these factors, which are discussed below, there are a n umber of transcription factors that are able to establish nucleosome positioning, such as Abf1 and Rap1 (YARRAGUDI *et al.* 2007). Upon depletion of these factors, increased nucleosome occupancy has been observed over protein coding promoter regions (GANAPATHI *et al.* 2011; HARTLEY and MADHANI 2009). This, along with strong *in vitro* data, supports the role of these factors in maintaining regulatory sequences in a nucleosome free state.

1.3.2.1 Chromatin remodeling complexes regulate chromatin dynamics in an ATP-dependent manner

Chromatin remodeling factors are protein complexes which use the energy from ATP hydrolysis to reposition (FAZZIO and TSUKIYAMA 2003; LOMVARDAS and THANOS 2001) or remove

nucleosomes (BOEGER *et al.* 2004; CAIRNS 2005) by altering the histone-DNA contacts. Eukaryotes possess four types of chromatin remodeling complex families, and the yeast complexes are: Swi/Snf (composed of Swi/Snf and RSC), Iswi (composed of ISWIa, ISWIb, and ISW2), Chd1 (composed of Chd1), and Ino80 (composed of Ino80 and Swr1) (reviewed in (CLAPIER and CAIRNS 2009; RANDO and WINSTON 2012)). While these chromatin remodeling families share the same basic mechanism, they vary greatly in their effects on nucleosome stability and position. The function of each chromatin remodeler is depicted in Figure 3. Swi/Snf and RSC destabilize nucleosomes, Isw chromatin remodelers function to slide nucleosomes laterally, and other remodeling complexes, such as Ino80, alter H2A/H2B dimer exchange (YEN *et al.* 2012).

Additionally, certain chromatin remodelers have been associated with transcriptional activation or repression, based on the mechanism of altering accessibility of nucleosomal DNA to other regulatory proteins, such as transcription factors. For example, members of the Swi/Snf family have functions associated with nucleosome disorganization, through sliding and ejection of nucleosomes and, therefore, are thought to promote transcription (reviewed in (RANDO and WINSTON 2012)). Alternatively, members of the Isw1 family have been shown to remodel and organize nucleosomes over transcriptionally silent regions (TIROSH *et al.* 2010).

1.3.2.2 Histone post-translational modifications influence transcription elongation

Histone proteins are composed of globular domains and N- and C-terminal tails, which are intrinsically disordered (LUGER *et al.* 1997). Both the globular domains and tails of histone proteins are subject to a vast array of post-translational modifications. Chromatin modifiers attach covalent modifications, including methylation, acetylation, phosphorylation,

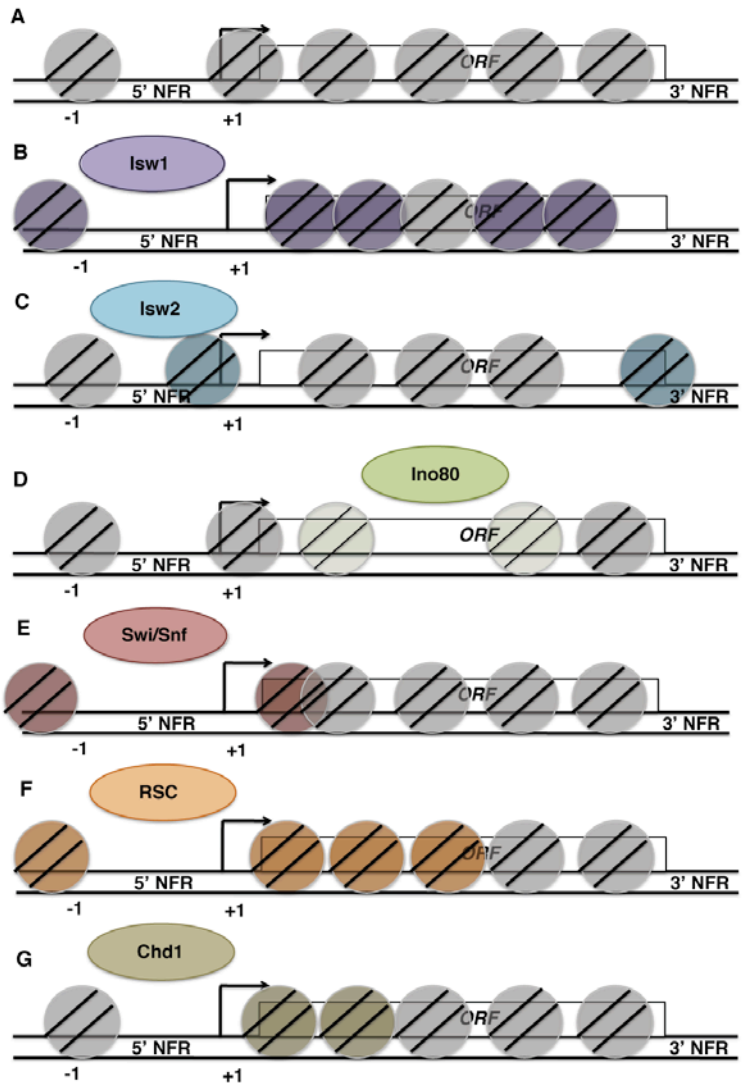


Figure 3. Effect of chromatin remodelers on nucleosome occupancy.

Diagram of the role of a subset of chromatin remodelers on nucleosome occupancy of a traditional gene. A) Traditional gene which has nucleosomes (+1 and -1) flanking the promoter region, leaving this region nucleosome free (5'NFR). The ORF has nucleosomes over the entire coding region, and the 3' UTR is generally nucleosome free (3' NFR). B-G) Effect of specific chromatin remodeling complexes on nucleosome locations. In each panel, the specific chromatin remodeler of interest is colored and the nucleosomes that were remodeled are colored to match the remodeler. Ino80 (D) is an exception, as it causes the loss of nucleosomes in the middle of the ORF, resulting in either the absence of or lighter coloring of the nucleosomes, to depict depletion.

ubiquitylation, and sumolytion, to specific residues on histones (reviewed in (FUCHS *et al.* 2009; SMITH and SHILATIFARD 2010)). These modifications function to regulate gene expression by affecting chromatin structure through altering DNA-histone interactions and recruiting additional regulatory proteins that can influence nucleosome function and dynamics.

Lysine residues are the most heavily modified amino acid in histone proteins. Acetylation is thought to loosen DNA-histone contacts by neutralizing the basic charge of the lysine residue, and therefore acetylation at gene promoters is typically associated with active transcription (POKHOLOK *et al.* 2005). Histone acetyltransferases (HATs), which covalently attach acetyl groups to amino acids, and histone deacetylases (HDACs), which remove the acetyl marks, are able to continuously turn over the acetylation (CARROZZA *et al.* 2005; CLOSE *et al.* 2006; GILBERT *et al.* 2004; GOVIND *et al.* 2007; KEOGH *et al.* 2005). Both histone acetylation and ubiquitylation have recently been shown to interfere with the formation of higher order chromatin structure (FIERZ *et al.* 2011). Mono-ubiquitylation of histone H2B K123 (K120 in humans) is catalyzed by the ubiquitin conjugase Rad6, and the ubiquitin ligase Bre1 in yeast (HWANG *et al.* 2003; ROBZYK *et al.* 2000; WOOD *et al.* 2003). Ubiquitylation of K123 on H2B is found on most gene promoters and coding regions (KAO *et al.* 2004; XIAO *et al.* 2005), and has been shown to have both positive and negative effects on transcription (HENRY *et al.* 2003). H2B ubiquitylation is required for subsequent methylation on histone H3 K4 and K79 by the histone methyltransferases Set1 and Dot1, respectively (BRIGGS *et al.* 2002; DOVER *et al.* 2002; SUN and ALLIS 2002).

Both the addition and removal of specific post-translational modifications are associated with either active transcription, such as H3K4 methylation, or with inactive regions, such as H3 K27 methylation. During transcription elongation, alterations to the pattern of histone

modifications occur as chromatin is being disassembled and reassembled around the transcribing polymerase. Also, the modification state of histones is both spatially and temporally controlled, forming a "histone code" that can signal for distinct states of DNA (STRAHL and ALLIS 2000). The location of a modification over a genes locus is regulated and critical for its proper expression. One example is Set2-mediated H3 K36 methylation which is required for proper transcription elongation, and prevention of intragenic cryptic transcription (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KIM and BURATOWSKI 2009; POKHOLOK *et al.* 2005). The mechanism for preventing cryptic transcription has been described in detail, where the methylation of H3 K36 recruits and activates the Rpd3S complex that subsequently deacetylates histone H4, preventing internal initiation (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). However, there is contention over whether H3 K36me2 actually recruits Rpd3S (GOVIND *et al.* 2010).

1.3.2.3 Histone variant incorporation alters chromatin structure

Replacement of canonical histones with histone variants, such as the replacement of H2A with H2A.Z over promoters (SANTISTEBAN *et al.* 2011; WAN *et al.* 2009; ZHANG *et al.* 2005), plays an important role in chromatin dynamics during transcription. Many different variant forms of histones exist throughout different organisms (reviewed in (KAMAKAKA and BIGGINS 2005)). Histone variants are distinguished from canonical core histones mainly by the fact that they are expressed outside of S phase and incoporated into chromatin in a DNA replication-independent manner. Studies suggest that H2A.Z can be deposited into a nucleosome either through ATP-dependent histone exchange reactions (MIZUGUCHI *et al.* 2004) or with the help of replication-independent histone chaperones, such as Nap1 (PARK *et al.* 2005).

Differences between histone variants and canonical histones are found in many regions of the histone proteins: either in the terminal tails, the globular fold domains, or in a few important amino acid residues (DOYEN *et al.* 2006a; DOYEN *et al.* 2006b; HENIKOFF and AHMAD 2005). While their incorporation impacts chromatin structure in various ways, many sites of post-translational modification are conserved between variants and canonical histones (MCKITTRICK *et al.* 2004). Therefore, interchanging the canonical histones with these variants may not alter nucleosome recognition by various chromatin-regulatory proteins.

1.3.2.4 Histone chaperones regulate chromatin dynamics

During transcription initiation and elongation, nucleosomes are commonly evicted from promoters and coding regions. Histone chaperones, such as Asf1, Spt6, and FACT, are factors which have been implicated in this process through evidence which shows they interact with nucleosomes *in vitro*, associate with chromatin *in vivo*, and facilitate histone deposition, exchange, or eviction from chromatin (reviewed in (AVVAKUMOV *et al.* 2011; DAS *et al.* 2010; EITOKU *et al.* 2008; PARK and LUGER 2008)). Removal of nucleosomes from promoters is essential for proper recruitment of RNA pol II and other initiation factors. Furthermore, the removal of nucleosomes ahead of polymerase is essential for efficient transcription elongation, while the replacement of nucleosomes behind transcribing polymerase is essential to prevent initiation of intragenic cryptic transcripts (BELOTSERKOVSKAYA *et al.* 2003; FORMOSA *et al.* 2002; JAMAI *et al.* 2009; ORPHANIDES *et al.* 1999; SCHWABISH and STRUHL 2004; STUWE *et al.* 2008; VANDEMARK *et al.* 2008). Each histone chaperone has specificity for particular histones or portion of the nucleosome and facilitate different steps in the assembly, disassembly, or exchange of histones (reviewed in (EITOKU *et al.* 2008)).

When transcription is not occurring, there is also active histone exchange occurring (KATAN-KHAYKOVICH and STRUHL 2011; PARK and LUGER 2008). Interestingly, much of this turnover of histones occurs in a replication-independent manner, and rather occurs over many regions of the genome, including promoters, intergenic regions, and coding regions, during all stages of the cell cycle (DION *et al.* 2007; JAMAI *et al.* 2007; RUFIANGE *et al.* 2007). Exchange of nucleosomal histones with those from a pool of free histones, unattached to DNA, occurs with the help of histone chaperones, such as Asf1 (OSADA *et al.* 2001; RUFIANGE *et al.* 2007).

1.4 FACT IS AN IMPORTANT HISTONE CHAPERONE COMPLEX WHICH PERFORMS VARIOUS FUNCTIONS IN THE CELL

1.4.1 Components of the FACT complex

In eukaryotes, FACT (Facilitates Chromatin Transactions) is a highly conserved complex that plays important roles in several nuclear processes including DNA replication, DNA repair, and transcription initiation and elongation (reviewed in (DUINA 2011; FORMOSA 2008; FORMOSA 2011)). In yeast FACT is composed of Spt16 and Pob3 and is joined by the HMGB-like protein Nhp6 (FORMOSA *et al.* 2001; WITTMEYER and FORMOSA 1997). In higher organisms, FACT is a heterodimer composed of Spt16 and SSRP1, which contains the functional domains of both Pob3 and Nhp6 (ORPHANIDES *et al.* 1998; ORPHANIDES *et al.* 1999). Spt16 was independently discovered in two genetic screens, the first looking for proteins involved in transcription initiation (providing an *SPT*⁻ phenotype) and the second for factors controlling cell division cycle progression (which gave Spt16 its alternative name, Cdc68) (CLARK-ADAMS *et al.* 1988;

MALONE *et al.* 1991; PRENDERGAST *et al.* 1990; ROWLEY *et al.* 1991). Pob3 was first identified as a Pol1-binding factor that associated with DNA polymerase α in yeast (BREWSTER *et al.* 2001; WITTMEYER and FORMOSA 1997). Since then, FACT has been shown to have many important roles in nucleosome dynamics based on its ability to reorganize chromatin structure during all processes involving a nucleosomal template. In yeast, the additional factor Nhp6, has been shown to provide the DNA binding activity, which in humans is provided by SSRP1 (FORMOSA *et al.* 2001; RUONE *et al.* 2003; STILLMAN 2010).

1.4.2 Functional domains of the FACT complex

Spt16 has been characterized as having four domains: the N-terminal (NT), dimerization (D), middle (M), and C-terminal (C) domains, while Pob3 has been shown to have three separate domains: the N-terminal/dimerization (NT/D), middle (M) and C-terminal (C) domains (Figure 4). The NT domain of Spt16 can be removed in yeast and cells are viable, however if this is combined with a mutated version of Pob3, synthetic lethality is observed (O'DONNELL *et al.* 2004; VANDEMARK *et al.* 2008). The NT domain structure has been solved, and was found to resemble aminopeptidases, but no peptidase activity has been observed (STUWE *et al.* 2008; VANDEMARK *et al.* 2008). However, the Spt16 NT domain does bind peptides and an interesting hypothesis is that the NT domain binds histone N-terminal tails (STUWE *et al.* 2008; VANDEMARK *et al.* 2008). The D domain of Spt16 is important for dimerization with the NT/D domain of Pob3. Very recently, evidence has been provided for the Spt16-M domain being important for interactions with histone H3 (MYERS *et al.* 2011). In this work, the authors provided evidence for the M-domain in directing histone deposition during transcription elongation, and in controlling FACT dissociation from chromatin at the 3' end of genes. The

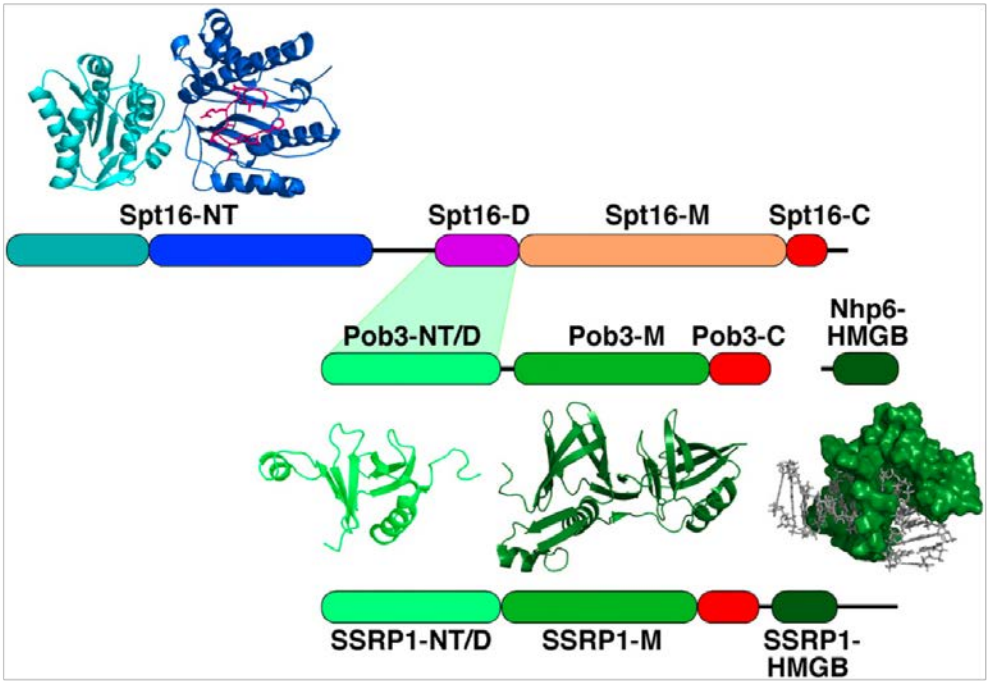


Figure 4. Domain structure of FACT protein components.

Depicted are schematics of the domain architecture of yeast (Spt16, Pob3, and Nhp6) and human (Spt16 and SSRP1) member of the FACT complexes. Also shown are the ribbon diagrams of crystallographic structure of those domains that have been solved (MASSE *et al.* 2002; VANDEMARK *et al.* 2006; VANDEMARK *et al.* 2008). This figure is reprinted from (FORMOSA 2012) with permission from BBA Press, copyright 2012.

structure of the M domain for Pob3 has been solved and shows a double pleckstrin homology (PH) fold and, interestingly, this domain has sequence homology for a H3-H4 histone chaperone, Rtt106, agreeing with the above suggestion that Pob3/SSRP1 can act as an H3-H4 histone chaperone (LIU *et al.* 2010; VANDEMARK *et al.* 2006). Finally, the C-terminal domains of both Spt16 and Pob3 are highly acidic, however the function for these domains is unknown. Very recently, *in vitro* analysis of the C-terminal domain of human Spt16 has been performed, where it was found that the C-terminal domain is required for active displacement of nucleosomal DNA during nucleosome reorganization (WINKLER *et al.* 2011).

1.4.3 FACT functions as a histone chaperone during transcription

In human cells, FACT was initially identified by its ability to allow RNA pol II to transcribe through nucleosomal DNA (ORPHANIDES *et al.* 1998). Additionally, human FACT binds histone H2A-H2B dimers while human SSRP1 and *Schizzosaccharomyces pombe* Spt16 can both bind H3-H4 (BELOTSERKOVSKAYA *et al.* 2003; RANSOM *et al.* 2010; STUWE *et al.* 2008). Taken together, this indicates that FACT can act as both an H2A-H2B and H3-H4 histone chaperone. Studies have led to two models for FACT function during transcription elongation (reviewed in (FORMOSA 2012)). The first begins when Nhp6 binds to the DNA at the surface of a nucleosome, causing bending of DNA and destabilization of DNA-histone contacts. Reorganization of the nucleosomes involves disruption of contacts between H2A-H2B and H3-H4, where the nucleosome components can remain bound together by FACT, but H2A-H2B dimers are readily lost. The second model involves more active dimer displacement in which FACT actively displaces H2A-H2B dimers to form a hexasome. In this model, FACT may function to chaperone

other H2A-H2B dimers into the hexasome, where it is believed that the C-terminal tail of H2A forms a docking domain responsible for the dimer/tetramer stability.

FACT activity is also required for proper regulation of transcription initiation. Studies have indicated that Spt16 nucleosome reassembly occurs over certain gene promoters and this activity is required for proper transcriptional repression of these genes (ADKINS and TYLER 2006). In addition to contributing to transcription initiation and elongation, FACT has also been shown to participate in the process of mRNA nuclear export (HAUTBERGUE *et al.* 2009; HEROLD *et al.* 2003). Together these studies have shown how important the FACT complex is in regulating different stages of transcription and mRNA processing.

1.4.4 FACT functions outside of transcription

During replication, evidence supports a role for FACT in the disassembly of nucleosomes to stimulate MCM helicase activity and their reassembly on newly replicated DNA (MAIORANO *et al.* 2006; TAKAHASHI *et al.* 2005; TAN *et al.* 2006; WITTMEYER and FORMOSA 1997). During the repair of damaged DNA, evidence supports a role for FACT in the physical exchange of histones involving the H2AX histone variant (HEO *et al.* 2008). Finally, Spt16 is required for proper splicing of some genes in the cell (BURCKIN *et al.* 2005).

1.5 TRANSCRIPTION OF NON-CODING REGIONS OF THE GENOME IS ABUNDANT AND CAN REGULATE EXPRESSION OF PROTEIN-CODING GENES

1.5.1 Discovery of ncRNAs

The first non-coding RNA (ncRNA) identified was an alanine tRNA in *S. cerevisiae* (HOLLEY *et al.* 1965). This 80 nucleotide tRNA was purified from *S. cerevisiae*, which was then sequenced by digestion with ribonuclease. Identification of the 5' and 3' ends helped arrange the digested fragments and establish the RNA sequence. Three structures were originally proposed for this $tRNA^{Ala}$ (HOLLEY *et al.* 1965), however the cloverleaf structure was independently proposed by subsequent publications (DUDOCK *et al.* 1969; MADISON *et al.* 1966; ZACHAU *et al.* 1966) and finally confirmed when the X-ray crystal structure was solved (KIM *et al.* 1973). Ribosomal RNAs were the next ncRNAs to be discovered even though their existence had been known for many years (reviewed in (SCHERRER 2003)).

Soon after, several abundant, small ncRNAs, other than rRNA and tRNA, were detected and isolated biochemically: among them the uridine (U)-rich U RNAs (BUSCH *et al.* 1982; ZIEVE 1981). Many of these small RNAs are associated with proteins to form ribonucleoprotein (RNP) complexes. Many of the abundant small RNPs precipitated by antisera of patients with autoimmune diseases, such as U1, U2, U4, U5 and U6 small nuclear RNA (snRNA), which turned out to be components of the spliceosome, involved in splicing mRNAs (reviewed in (KARIJOLICH and YU 2010; VALADKHAN 2010)). The discovery of these snRNAs lead to the identification of many other snRNAs, such as *7SK* and *B2* ncRNAs which regulate transcription factors and RNA pol II, respectively (reviewed in (GOODRICH and KUGEL 2006)). Many other small RNAs were isolated biochemically. Sometimes these isolations were deliberate, such as the

isolation of numerous, small nucleolar RNAs (snoRNAs) from nucleoli (reviewed in (ELICEIRI 1999)). In other cases, biochemical fractions were unexpectedly found to contain ncRNAs, as in the case of RNaseP, the first ribozyme discovered (reviewed in (FRANK and PACE 1998)).

ncRNAs have continued to appear throughout history. Among the more fascinating stories is the discovery that RNAs have roles in chromatin structure. A well-known example is the human Xist (X(inactive)-specific transcript) RNA, a 17-kb ncRNA with a key role in dosage compensation and X-chromosome inactivation (reviewed in (AUGUI *et al.* 2011; LEE 2010)). *Drosophila melanogaster* also seems to control dosage compensation using small chromatin-associated roX (RNA on the X) RNAs (reviewed in (ILIK and AKHTAR 2009)). Another class of exciting ncRNAs include microRNAs (miRNAs) which are post-transcriptional regulators that bind to complementary sequences on target mRNAs, usually resulting in translational repression or target degradation and gene silencing (reviewed in (BARTEL 2009; SUNKAR *et al.* 2007)). miRNAs were discovered in 1993 during a study of the gene *lin-14* in *C. elegans* development (LEE *et al.* 1993).

Once the above listed ncRNAs, along with others, were discovered through standard biochemical and genetic approaches, there was a number of screens for ncRNAs which exploited comparative genome analysis to identify more miRNAs in *C. elegans* (LEE and AMBROS 2001) as well as ncRNAs in *E. coli* (ARGAMAN et al. 2001; RIVAS et al. 2001; WASSARMAN et al. 2001). These screens ranged in complexity and were the first real successful computational genome-wide search for ncRNAs. In addition to computational approaches, the genomic search was complemented by cDNA cloning strategies enriching for ncRNAs to expand on the discovery of novel ncRNAs (HUTTENHOFER *et al.* 2001). Finally, with the production of microarrays, novel transcripts were beginning to be discovered on low-resolution whole-genome

chips (SELINGER *et al.* 2000). With the rapid improvement on microarray techniques and implementation of genomic sequencing as a tool, the number of ncRNAs discovered has exploded in the past decade, leading to an entirely new perception on the extent to which genomes are transcribed and, of this transcription, the function.

1.5.2 Transcription of non-coding regions of the genome accounts for the majority of transcriptional activity in the cell

Genome-wide expression studies of multiple organisms, ranging from bacteria to humans, have revealed that transcription of ncDNA accounts for a major portion of the transcriptional activity observed in cells (reviewed in (BERRETTA and MORILLON 2009; COLIN *et al.* 2011; GOODRICH and KUGEL 2009; HAINER and MARTENS 2011b; KUGEL and GOODRICH 2012; WILUSZ *et al.* 2009)). This activity not only yields a group of well-studied functional ncRNAs that include ribosomal RNAs, transfer RNAs, small nuclear RNAs, and small nucleolar RNAs, but also an amazing array of previously uncharacterized ncRNAs that range in size from 18 nucleotides to many kilobases (CARTHEW and SONTHEIMER 2009; JACQUIER 2009; MERCER *et al.* 2009). An emerging theme is that many of these ncRNAs play important roles in regulating gene expression.

1.5.2.1 Yeast ncRNAs: SUTs, CUTs, and XUTs

In yeast, ncRNAs have been divided into three classes based on their stability: 1) stable unannotated transcriptions (SUTs), as their name implies, are stable in wild-type strains (NEIL *et al.* 2009; XU *et al.* 2009); 2) cryptic unstable transcripts (CUTs) are degraded by the nuclear exosome and/or TRAMP complex and are therefore only detectable in strains deleted for

members of these complexes (CALLAHAN and BUTLER 2010; DAVIS and ARES 2006; LACAVA *et al.* 2005; VANACOVA *et al.* 2005; WYERS *et al.* 2005); and 3) Xrn1-sensitive unstable transcripts (XUTs) are degraded by Xrn1, a cytoplasmic endonuclease, and are therefore only detectable in a strain deleted for Xrn1 (VAN DIJK *et al.* 2011). Deletions of either *RRP6*, the exonuclease component of the exosome, or *TRF4*, a member of the TRAMP complex, permit the detection of CUTs (CALLAHAN and BUTLER 2010; LACAVA *et al.* 2005; VANACOVA *et al.* 2005; WYERS *et al.* 2005). Interestingly, the biogenesis of CUTs has been fairly well determined. Nab3 and Nrd1 are required for transcription termination of CUTs where they recruit the TRAMP complex (ARIGO *et al.* 2006; THIEBAUT *et al.* 2006). Recruitment of the TRAMP complex leads to the addition of a poly-A tail on the CUT, which signals CUTs for degradation by the nuclear exosome (CALLAHAN and BUTLER 2010; LACAVA *et al.* 2005; VANACOVA *et al.* 2005; WYERS *et al.* 2005). Less is known about the biogenesis of XUTs, where these polyadenylated transcripts are specifically degraded by the Xrn1 exonuclease (THOMPSON and PARKER 2007; VAN DIJK *et al.* 2011).

SUTs, CUTs, and XUTs account for at least 12% of the transcripts produced in yeast and are transcribed in either the sense or antisense direction relative to protein coding regions. In fact, at least 55% of SUTs and 66% of XUTs are transcribed in the antisense direction relative to their neighboring coding region (VAN DIJK *et al.* 2011; XU *et al.* 2009). The majority of all three types of transcripts overlap the nucleosome free promoter region of protein coding genes (NEIL *et al.* 2009; VAN DIJK *et al.* 2011; XU *et al.* 2009). The second most common location for these transcripts is 3' of protein coding genes, also in the nucleosome free region of these genes (NEIL *et al.* 2009; VAN DIJK *et al.* 2011; XU *et al.* 2009).

1.5.2.2 Mammalian long ncRNAs: PROMPTs, PALRs, eRNAs, and lincRNAs

Similar to CUTs found in yeast, PROMoter uPstream Transcripts (PROMPTs) were identified as promoter-associated unstable human transcripts that can be stabilized by depletion of exosome components (PREKER *et al.* 2008). PROMPTs are transcribed from both sense and antisense strands, about 500 to 2500bp upstream of the transcription start site of coding genes. Stable Promoter-Associated Long ncRNAs (PALRs) were also identified as polyadenylated transcripts, longer than 200bp (KAPRANOV *et al.* 2007). Although many PROMPTs and PALRs have been identified, few have been investigated for their mechanism of regulation. These promoter associated RNAs may function as a recognition motif for siRNAs that would direct silencing complexes to corresponding targeted promoters, as has been shown for the EF1a promoter in human cells (HAN *et al.* 2007). In addition to promoter regions, enhancers have been shown to have bi-directional transcription occurring that produce another class of ncRNAs termed enhancer RNAs (eRNAs) (KIM *et al.* 2010). While little is known about eRNA mechanism, they are not polyadenylated and have been suggested to be involved in enhancer function (OROM *et al.* 2010). Finally, the majority of mammalian ncRNAs are transcribed from intergenic regions and are therefore named long intergenic noncoding RNAs. Computational approaches analyzing the chromatin map of mammalian cells have identified 3300 putative lincRNAs (GUTTMAN *et al.* 2009), and further extrapolations have estimated a total of 4500 human lincRNAs (KHALIL *et al.* 2009).

1.5.2.3 Noncoding RNAs can regulate coding gene expression *in trans*

Trans regulatory mechanisms have been well documented including microRNAs, which inhibit translation or target mRNAs for degradation, and longer ncRNAs, such as mammalian *Xist*,

HOTAIR, and antisense *Kcnq1ot1* RNAs and *Drosophila roX* RNAs, which interact with protein complexes to modify chromatin structure (Figure 5).

Xist was the first long regulatory ncRNA to be identified in mammals, and its role in the initiation of X-chromosome inactivation has been extensively studied (reviewed in (LEE 2009)) (Figure 5A). It acts by coating the inactive X-chromosome and recruiting polycomb group proteins that establish a heterochromatin state and subsequent transcriptional silencing at the level of an entire chromosome. *Xist* itself is negatively and positively controlled by three other noncoding RNAs: the antisense *Tsix* RNA (LEE *et al.* 1999) and the *Jpx* RNA (TIAN *et al.* 2010) and the *RepA* ncRNA (ZHAO *et al.* 2008). An added complexity with *Xist* regulation has been identified in that the antisense *Tsix* RNA is regulated by another ncRNA, *Xite*, which acts as an enhancer to increase *Tsix* transcription (OGAWA and LEE 2003; STAVROPOULOS *et al.* 2005).

Dosage compensation in *Drosophila* is different from that in mammals, although interestingly, it also involves ncRNAs. *Drosophila* dosage compensation involves the upregulation of a single male X chromosome through the binding of the male specific lethal (MSL) complex, of which two ncRNAs, RNA on the X1 and X2 (*roX1* and *roX2*) are a part (DENG and MELLER 2006). *roX1* and *roX2* are functionally redundant, but dosage compensation and the proper binding of MSL to the X chromosome requires either one. Interestingly, there is little similarity between *roX1* and *roX2*, in that *roX1* is 2.7kB and *roX2* is predominantly 0.5kB, and the sequence similarity is low (DENG *et al.* 2005; STUCKENHOLZ *et al.* 2003). Both ncRNAs are expressed from the X chromosome in males, and act *in trans* to activate transcription of the X chromosome.

A more recently described example of a *trans*-acting lincRNA is *HOTAIR*, which is transcribed from the *HOXC* locus and silences the *HOXD* locus, located on another chromosome,

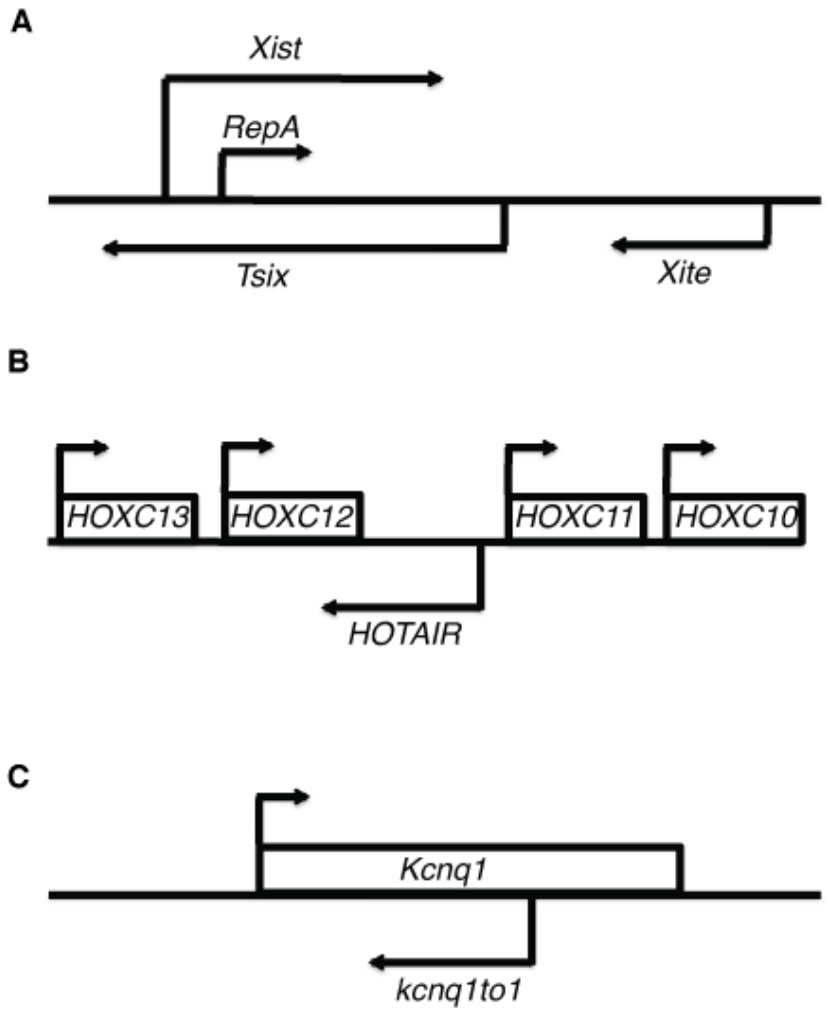


Figure 5. Diagram of gene loci with ncRNA transcripts upregulating gene expression *in trans*.

Diagram of genes and/or ncRNAs transcribed from the X-inactivation center (A), the *HOXC* locus (B), and the *Kcnq1* locus (C). The ncRNAs produced (indicated by arrows not attached to a gene, which is indicated by a box) act *in trans* through various mechanisms.

and deposits a repressive chromatin environment (RINN *et al.* 2007) (Figure 5B). *HOTAIR* serves as a scaffold for two histone modification complexes, PRC2 and CoREST/REST, which direct H3 K27 trimethylation and H3 K4 dimethylation, respectively (TSAI *et al.* 2010).

Trans-acting lincRNAs can also inhibit gene expression through interaction with repressive DNA-binding factors. This has been shown for the lincRNA-p21 that is involved in the p53-mediated repression of genes involved in apoptosis (HUARTE *et al.* 2010). When p53 is induced, lincRNA-p21 is expressed and interacts with heterogeneous nuclear ribonucleoprotein K (hnRNP-K), which is a component of a repressor complex acting in the p53 pathway. This interaction leads to transcriptional repression of a specific set of target genes of the p53 pathway (HUARTE *et al.* 2010).

The antisense *Kcnq1ot1* ncRNA has been functionally involved in the silencing of imprinted clusters of genes in the *Kcnq1* domain (PANDEY *et al.* 2008) (Figure 5C). *Kcnq1ot1* interacts with the H3 K9 histone methyltransferase (HMT) G9a and the H3 K27 HMT PRC2, presumably leading to the establishment of lineage-specific transcriptional silencing of genes in the *Kcnq1* domain.

1.5.2.4 Noncoding RNAs can regulate gene expression *in cis*

In many cases, ncRNA sequence is not conserved, but the promoters and transcription start sites are, which is consistent with the hypothesis that transcription of ncDNA regions plays a role in protein coding gene expression. Particularly, there are a number of *cis* acting ncDNA sequences which can regulate gene expression through a number of methods. Initially, promoter occlusion and transcription interference by RNA polymerases transcribing ncRNAs were the only two proposed mechanisms through which *cis* regulation of ncRNAs could occur (YAZGAN and

KREBS 2007), however, there have been an increasing number of examples through which transcription of ncRNA can regulate gene expression (Figure 6).

Promoter occlusion by ncDNA transcription represses gene expression

Recently, two ncRNAs have been found to control the variegated expression of the *S. cerevisiae* gene *FLO11* (BUMGARNER *et al.* 2009). *FLO11* encodes a cell-wall glycoprotein involved in cell adhesion, and its expression has been shown to be variegated in a cell population. Interestingly, two ncRNAs transcribed over the *FLO11* locus regulate the gene in alternative ways: one activates and one represses *FLO11* expression, and the ncRNAs themselves are expressed differently in the cell population (BUMGARNER *et al.* 2009) (Figure 6A). *ICR1* is transcribed in the sense direction with *FLO11* over its regulatory region and results in *FLO11* repression through a promoter occlusion model. Alternatively, *PWR1* is transcribed antisense to both *FLO11* and *ICR1*, initiating in the regulatory region of *FLO11*, and results in activation of *FLO11*. Interestingly, *PWR1* expression is controlled by two transcription factors, an activator (Flo8) and a repressor (Sfl1), where activation of *PWR1* through Flo8 prevents *ICR1* transcription, allowing *FLO11* to be expressed or, conversely, repression of *PWR1* through Sfl1 permits *ICR1* transcription, preventing *FLO11* activation (BUMGARNER *et al.* 2009). This analysis has shown a dynamic interplay between two ncRNAs in the regulation of a protein coding gene.

Transcription of ncDNA repositions nucleosomes over promoter regions

Chromatin dynamics at promoters play a key role during transcription regulation in eukaryotes. Many genes require the action of chromatin remodeling factors to remove or slide nucleosomes from their promoters to facilitate transcription factor binding and transcription initiation. Three

studies have determined that transcription of ncDNA across gene promoters effectively alters the chromatin dynamics at these regions, leading to dramatic changes in gene expression (HIROTA *et al.* 2008; LEFEVRE *et al.* 2003; UHLER *et al.* 2007).

In *Schizosaccharomyces pombe,* transcription of the *fbp1*$^{+}$ gene is strongly induced in response to glucose starvation. A recent study detected several species of ncRNAs that are transiently expressed in response to glucose starvation prior to the production of *fbp1*$^{+}$ mRNA (HIROTA *et al.* 2008). These transcripts initiate from several sites within the intergenic region 5' of *fbp1*$^{+}$ and extend across its promoter to the 3' end of this gene (Figure 6B). MNase accessibility assays indicated a step-wise remodeling of chromatin – converting chromatin to a more open conformation – that parallels the appearance of the ncRNAs in response to glucose starvation. Blocking intergenic transcription by the insertion of a transcription terminator prevents this chromatin remodeling event and *fbp1*$^{+}$ activation is severely attenuated. In this case, transcription of intergenic ncDNA remodels promoter nucleosomes to increase the accessibility of the DNA to RNA pol II and transcriptional activators.

Transcription of ncDNA has also been reported to facilitate induction of the chicken lysozyme gene (LEFEVRE *et al.* 2008) (Figure 6C). In this case, transcription-dependent chromatin remodeling inhibits the binding of a repressor. Transcription of the lysozyme gene is induced in response to lipopolysaccharide (LPS), which parallels changes in the DNase I hypersensitive sites within the regulatory regions upstream of the gene. MNase accessibility assays revealed that this change in DNase I hypersensitivity is the result of repositioned nucleosomes that then interefere with the binding of CTCF, a sequence specific transcriptional repressor (KONTARAKI *et al.* 2000; LEFEVRE *et al.* 2003). Using strand-specific RT-PCR, a non-coding transcript termed LINoCR, initiating 5' of the lysozyme gene promoter and extending

across several *cis*-regulatory elements in an antisense direction, was detected in response to LPS treatment (LEFEVRE *et al.* 2008). ChIP and nucleosome scanning assays in the presence and absence of a transcription elongation inhibitor indicated that LINoCR transcription is responsible for repositioning the nucleosomes that inhibit CTCF binding leading to increased lysozyme gene expression (LEFEVRE *et al.* 2008).

In the case of the *S. cerevisiae PHO5* gene, the act of ncDNA transcription is also responsible for regulating nucleosome occupancy over protein-coding gene promoter sequences, and in this case the transcription of the ncRNA activates *PHO5* expression (UHLER *et al.* 2007) (Figure 6D). A 2.4 kb antisense ncRNA to *PHO5* originates near the 3' end of the *PHO5* gene, and regulates the protein-coding gene expression *in cis*. The act of transcribing this ncRNA affects the local rate of nucleosome exchange and/or turnover, which permits nucleosome eviction over the *PHO5* promoter. This allows RNA pol II access to the promoter, therefore permitting *PHO5* expression in the absence of phosphate (UHLER *et al.* 2007).

Transcription of ncDNA alters post-translational modifications of histones within promoter nucleosomes

Recent studies have shown that transcription of ncDNA at the promoters of protein-coding genes can regulate the expression of these genes by altering post-translational modifications of histones that occupy these promoters (CAMBLONG *et al.* 2007; HADDAD *et al.* 2010; HOUSELEY *et al.* 2008; KANHERE *et al.* 2010; KIM *et al.* 2007a; PINSKAYA *et al.* 2009). In some cases, the act of transcribing these regions brings about changes in histone modifications that reflect this activity, while other cases are dependent on the ncRNA product for recruitment of histone modifying enzymes.

In *S. cerevisiae,* two recent studies identified a ncRNA transcript (*GAL10* ncRNA or *GALucut*) that initiates near the 3' end of *GAL10* and extends across the promoter region shared by the divergent *GAL1* and *GAL10* genes into the *GAL1* coding sequence (HOUSELEY *et al.* 2008; PINSKAYA *et al.* 2009) (Figure 6E). Both groups provided evidence that transcription across the *GAL1-10* promoter, not the ncRNA product, attenuates expression of these genes using a mechanism similar to what has been described for the repression of aberrant transcription from cryptic promoters located within protein coding sequences (LEE and SHILATIFARD 2007). Histone modifications, including histone H3 K4 and K36 methylation and H4 deacetylation, which are hallmarks of transcriptional activity, correlate with ncDNA transcription across the repressed *GAL1-10* locus. Mutations in the genes encoding subunits of the Rpd3S complex – the complex responsible for transcription-dependent deacetylation of histone H4 – alter the expression of *GAL1* and *GAL10* to a degree similar to that observed in the absence of ncDNA transcription. Taken together, these data indicate that transcription of ncDNA across the *GAL1-10* promoter directs a cascade of histone modifications thus creating a chromatin environment that inhibits the binding of transcription factors.

Recently, three studies in human cells have revealed a r ole for antisense ncDNA in regulating coding gene expression through altering histone modifications over the promoter (HAWKINS and MORRIS 2010; MORRIS *et al.* 2008; YU *et al.* 2008). Expression of the pluripotency-associated factor Oct4 has been shown to be epigenetically regulated through the recruitment of chromatin modifying factors by a long ncRNA (lncRNA) transcribed antisense through the promoter of the gene (HAWKINS and MORRIS 2010). Through strand-specific RT-PCR, antisense transcripts were identified, which overlap the promoter and coding regions of the Oct4 pseudogene 5 (Oct4-pg5) and Oct4 (referred to as asOct4-pg5 and asOct4, respectively). To

investigate the role of these antisense lncRNAs in gene regulation, siRNA was used to knockdown the expression of various histone modifying enzymes and ChIPs were performed for these enzymes and their modifications when the antisense lncRNA was knocked-down. These experiments demonstrate that the asOct4-pg5 plays a role in recruiting the histone methyl transferase Ezh2 and G9a to the Oct4 promoter and the subsequent histone modifications are required to maintain Oct4 in a repressed state. However, it is not clear whether it is the ncRNA product or transcription of the ncDNA which is required for recruitment of these factors. Similar experiments were performed on antisense transcripts identified at the *p15* and *p21* tumor suppressor genes. As was observed for Oct4, histone modifications associated with heterochromatin formation (such as decreased H3 K4 dimethylation and increased H3 K9 dimethylation and H3 K27 trimethylation) are induced over the promoter of *p15* and *p21* due to the antisense ncDNA transcription leading to gene silencing.

Two more examples of antisense noncoding transcription regulating a coding gene through a histone modification pathway include: an antisense transcript to *PHO84* which recruits an HDAC to inhibit *PHO84* transcription (CAMBLONG *et al.* 2007) and ANRIL, which is transcribed antisense to *INK4* and changes chromatin modifications to regulate this gene cluster (KOTAKE *et al.* 2011; YAP *et al.* 2010) (Figure 6F).

Transcription start site selection can be mediated by ncDNA transcription

A different type of regulation involves the transcription of a CUT and an mRNA in tandem that has been described at the *S. cerevisiae IMD2* locus. *IMD2* expression is regulated by intracellular guanine nucleotides, and regulation has been shown to be controlled through the choice of alternative transcription start sites based on the production of a CUT immediately upstream of *IMD2* (JENKS *et al.* 2008; KUEHNER and BROW 2008). Both the CUT and the mRNA have the

same promoter but originate from different transcription start sites (Figure 6G). The start site selection is dictated by the intracellular guanine nucleotide levels available in the cell, and these start sites compete for the recruitment and formation of a preinitiation complex, including RNA pol II (JENKS *et al.* 2008). While this is the only start site selection-dependent mode of regulation described to date, this method of regulation, along with transcription interference, will likely be widespread, as a number of yeast promoters transcribe a ncRNA immediately upstream from a protein-coding DNA, in the same orientation. A similar mechanism has been suggested for three additional genes in *S. cerevisiae* that are all involved in nucleotide biosynthetic pathways: *URA2*, *URA8*, and *ADE12* (THIEBAUT *et al.* 2008).

Transcription of ncDNA from within protein coding genes to regulate gene expression

There have been surprisingly few examples of ncRNAs that arise from within a protein-coding region of a gene and act to regulate that genes' expression. However, the reason for such few examples could be due to an inability to identify transcripts through the genome-wide analysis that arise from within coding fragments. In fact, the examples that have been identified were not found through genome-wide studies, but rather through other means of identification. The first example of a ncRNA transcribed from within a protein coding region is the ncRNA which regulates the beta-globin gene to keep the region open to transcription (GRIBNAU *et al.* 2000; KIM *et al.* 2007b). Two more examples have arisen since then, including the regulation of *ASP3* (HUANG *et al.* 2010) (Figure 6H) in *S. cereviaise*, and the unique regulation of SRA where an alternative splice of the SRA gene creates a ncRNA which, when expressed, disrupts the protein coding gene expression (COLLEY and LEEDMAN 2011; EMBERLEY *et al.* 2003; KAWASHIMA *et al.* 2003).

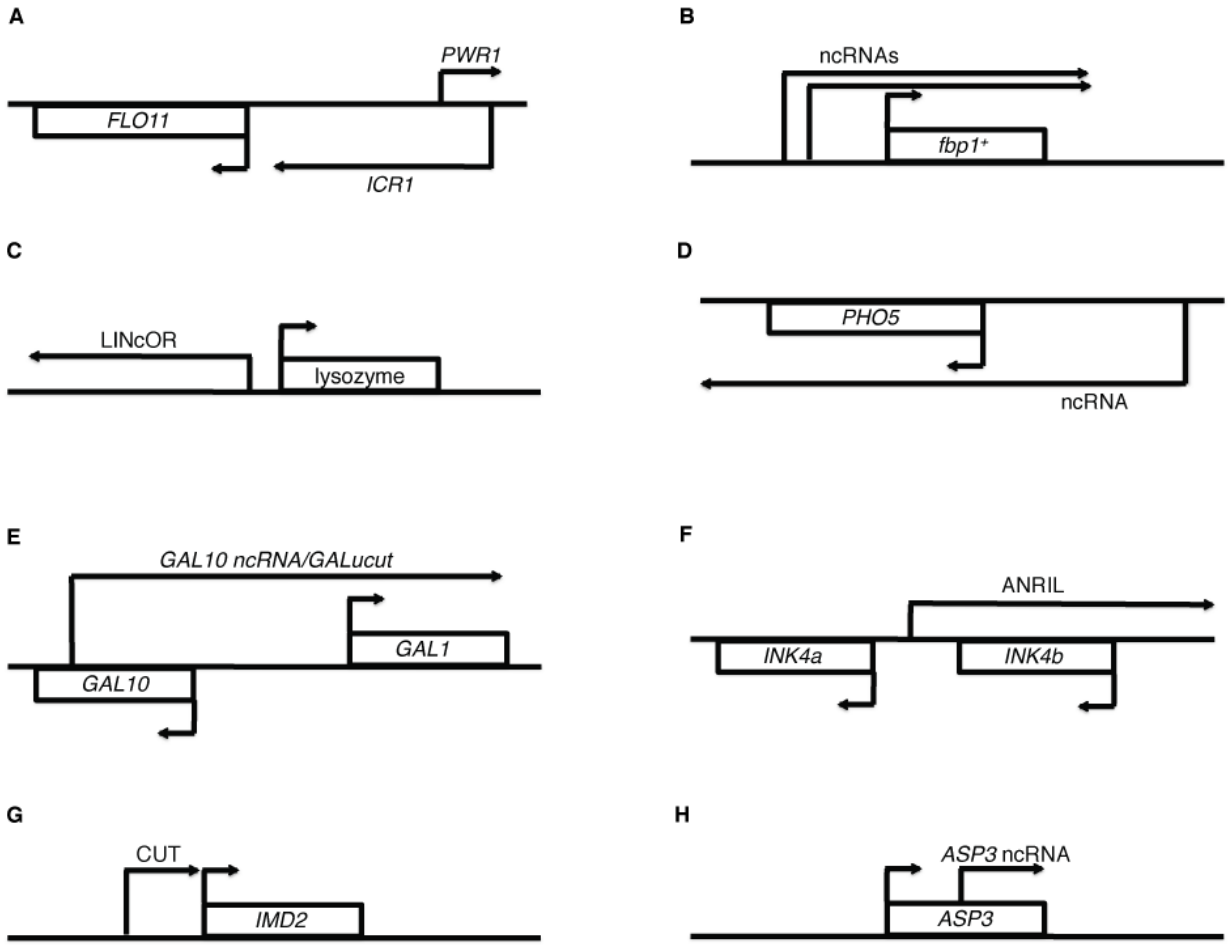


Figure 6. Diagram of gene loci with ncRNA transcription regulating gene expression *in cis*.

A) The *S. cerevisiae FLO11* locus, B) the *S. pombe fbp1+* locus, C) the chicken lysozyme locus, D) the *S. cerevisiae PHO5* locus, E) the *S. cerevisiae GAL1/10* locus, F) the mammalian *INK4* locus, G) the *S. cerevisiae IMD2* locus, and H) the *S. cerevisiae ASP3* locus are depicted as examples of protein coding regions controlled by *cis*-acting ncRNA, through various mechanisms.

Regulation of genes through transcription interference

While transcriptional interference has been a well described mode of regulation for protein coding genes through ncRNA transcription (YAZGAN and KREBS 2007), the majority of ncRNAs which were originally classified as regulating an ORF through this mechanism have been examined in more detailed and revealed a more complex mode of gene regulation. In a true transcription interference mechanism, the act of transcribing ncDNA over promoter sequences causes the arrival of a transcribing complex which initiated at a distant promoter to a protein coding promoter (SHEARWIN *et al.* 2005). The arrival of this transcript prevents activation of the protein coding gene.

1.6 TRANSCRIPTION OF THE NON-CODING RNA *SRG1* REGULATES *SER3* EXPRESSION IN A SERINE DEPENDENT MANNER

The *S. cerevisiae SER3* gene encodes a phosphoglycerate dehydrogenase, an enzyme required for serine biosynthesis. Interestingly, *SER3* is an isozyme to *SER33*, which encodes for the same enzyme in the serine biosynthesis pathway. However, *SER3* has been found to have a unique mechanism of gene regulation from *SER33*, where transcription of ncDNA across its promoter sequences represses the protein-coding gene expression. With my thesis work, my aim was to determine the contribution of chromatin dynamics to gene regulation, and to that end, I characterized and utilized the regulation of *SER3* as a model for transcription-coupled chromatin dynamics.

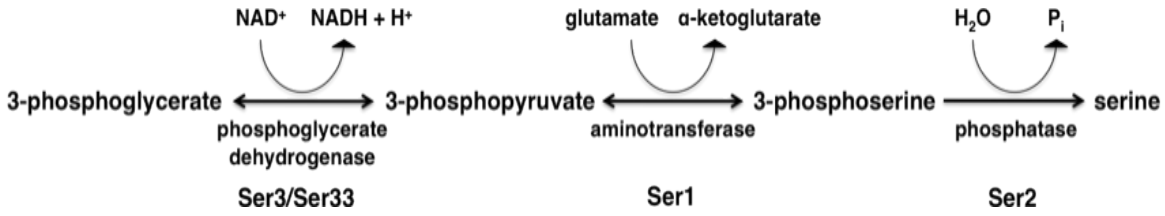


Figure 7. Serine biosynthetic pathway in yeast.

Schematic of serine biosynthesis. First, 3-phosphoglycerate is converted to 3-phosphopyruvate by the redundant phosphoglycerate dehydrogenases, Ser3 and Ser33. The product from this reaction is then converted to 3-phosphoserine by the aminotransferase Ser1. Finally, 3-phosphoserine is converted to serine by the phosphoserine phosphatase Ser2.

1.6.1 *SER3* and *SER33* encode redundant enzymes for the biosynthesis of serine in yeast

In *S. cerevisiae,* there are four genes which encode for enzymes required for the biosynthesis of serine (Figure 7). The first step in the synthesis of serine converts 3-phosphoglycerate to 3-phosphopyruvate catalyzed by the activity of two redundant proteins, Ser3 and Ser33, which are phosphoglycerate dehydrogenases that are 92% identical (ALBERS *et al.* 2003). The second and third step of serine biosynthesis convert 3-phosphopyruvate to 3-phosphoserine by Ser1, and this product to serine by Ser2, respectively. However, these two proteins do not have redundantly encoded enzymes. Due to the redundancy of *SER3* and *SER33*, deletion of either of these genes is permissive for growth in the absence of serine in yeast, however deleting the two genes in combination results in an inability for these yeast strains to grow in the absence of serine (ALBERS *et al.* 2003). Interestingly, of the four genes expressing the enzymes required for serine biosynthesis, *SER3* is the only one whose expression is regulated by the serine levels in the cell. *SER1, SER2*, and *SER33* are constitutively expressed genes, while *SER3* is only expressed in the absence of serine through a mechanism that was, as of then, uncharacterized (ALBERS *et al.* 2003).

1.6.2 Transcription of ncDNA across the *SER3* promoter occurs to repress *SER3* in a transcription interference mechanism

Genome-wide analysis characterizing the role of the Swi/Snf chromatin remodeling complex in gene regulation was the first insight into the regulation of *SER3* (HOLSTEGE *et al.* 1998; SUDARSANAM *et al.* 2000). Swi/Snf was shown to activate many genes, however, many genes were also revealed to be repressed by the chromatin remodeling complex, including *SER3*.

Initial analysis into the mechanism of Swi/Snf regulation of *SER3* revealed that its repression was mostly dependent on the direct contribution of the Snf2 subunit, whereas activation of genes by Swi/Snf requires the majority of the subunits (MARTENS and WINSTON 2002).

Based on the identification of the requirement for Swi/Snf to regulate *SER3*, additional factors were examined for their occupancy at this locus in both repressing (presence of serine) and activating (absence of serine) conditions. Surprisingly, a number of factors required for gene activation, including RNA pol II and TBP, were found to associate upstream of *SER3* in repressing conditions (MARTENS *et al.* 2004). Further investigation into this result revealed that RNA pol II transcribes the intergenic region upstream of *SER3* and downstream of the adjacent gene, *AIM9* (previously *YER80W*) in *SER3* repressing conditions (MARTENS *et al.* 2004). The intergenic transcripts were found to initiate from an independent promoter approximately 475bp upstream of the *SER3* translational start site, containing a conserved TATA box and activating sequences, and transcribe in the same direction as both *AIM9* and *SER3* (MARTENS *et al.* 2004). Interestingly, further examination into the intergenic transcripts revealed that there were three independent transcripts produced from the same promoter, however their termination sites differed where two of the transcripts terminate immediately adjacent to the *SER3* start site (75bp 5' and 25bp 3' of the start site) while the remaining transcript is a read-through to the end of the *SER3* genic sequence (MARTENS *et al.* 2004; THOMPSON and PARKER 2007).

As mentioned above, noncoding transcripts in yeast are divided into three types: SUTs, CUTs, and XUTs. The transcripts upstream of *SER3* are considered SUTs, in that they are capped and polyadenylated like mRNAs, however, they are also regulated by RNA degradation pathways. Unlike CUTs, visualizing the production of these noncoding transcripts does not require the deletion of subunits of the exosome or TRAMP complexes, although loss of members

of these complexes does somewhat stabilize the transcripts. Interestingly, the two small transcripts are able to be degraded by the exosome in the nucleus, but are largely degraded by canonical decapping and exonucleolyic degradation in the cytoplasm (DAVIS and ARES 2006). The largest product, which is a read-through from the intergenic promoter through the *SER3* gene, is degraded through a separate pathway in the cytoplasm, the nonsense mediated decay pathway (THOMPSON and PARKER 2007).

Since *SER3* was known to be regulated by the availability of serine in the cell, Northern blot analysis was performed to analyze the effect of serine availability on the intergenic transcript (MARTENS *et al.* 2004; MARTENS *et al.* 2005). This analysis revealed that the intergenic transcript is transcribed in the presence of serine and attenuated in the absence of serine, whereas it was previously found that *SER3* expression was turned on in the absence of serine (Figure 8). Based on the inverse relationship between transcription of the intergenic transcripts and *SER3,* the regulation of *SER3* by the intergnic transcripts was examined more closely and it was found that *SER3* is entirely regulated by these transcripts and they were therefore named *SRG1* for *SER3* Regulatory Gene 1 (MARTENS *et al.* 2004).

Three potential mechanisms of gene regulation for *SER3* were proposed: 1) the intergenic promoter could compete away transcription factors from the *SER3* promoter in a promoter competition mechanism; 2) the ncRNA product, rather than the act of ncDNA transcription, could be responsible for regulating *SER3*; 3) transcription of the ncDNA could interfere with transcription factor binding to the *SER3* promoter in a transcription interference mechanism (MARTENS *et al.* 2004). Numerous experiments were performed to determine through which mechanism *SRG1* regulates *SER3*, including a *cis/trans* test to determine if the RNA product or the act of transcribing the ncDNA was regulating *SER3* and tests to determine the effect of *SRG1*

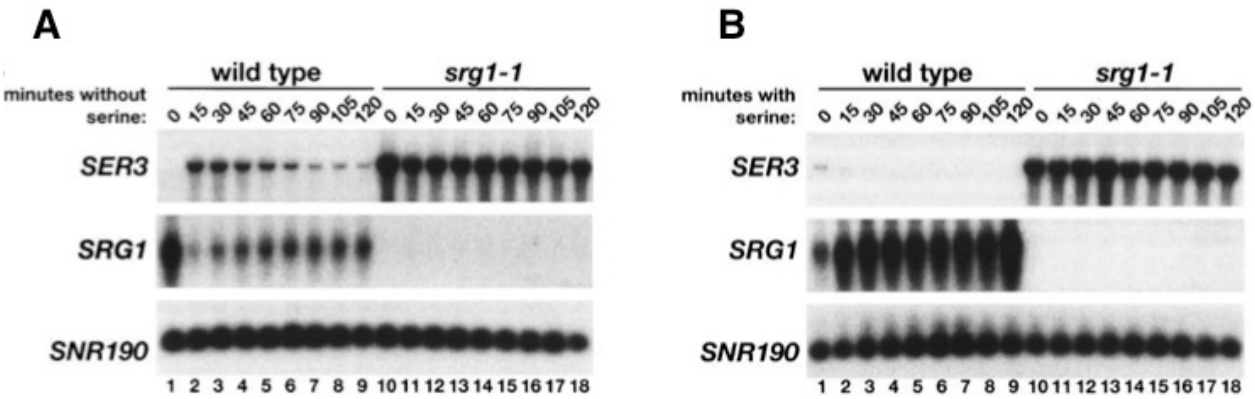


Figure 8. Effect of serine on *SER3* and *SRG1* expression.

A) Northern analysis of *SER3*, *SRG1*, and *SNR190* (loading control) was performed on wild-type and *srg1-1* strains. Cells were grown at 30°C in SC+serine minimal media (1mM serine) and then shifted to SC-serine media for the indicated number of minutes. B) Northern analysis of *SER3*, *SRG1*, and *SNR190* (loading control) was performed on wild-type and *srg1-1* strains. Cells were grown at 30°C in SC-serine minimal media and then shifted to SC+serine minimal media (1mM serine) for the indicated number of minutes. This figure is reprinted from (MARTENS *et al.* 2005) with permission from Cold Spring Harbor Laboratory Press, copyright 2005.

transcription on transcription factor binding over the *SER3* promoter to determine if *SRG1* was competing for transcription factors or if the transcription itself reduced transcription factor occupancy (MARTENS *et al.* 2004). These experiments support a transcription interference model in which the act of transcribing *SRG1* over *SER3* activator sequences represses *SER3* transcription.

1.6.3 Serine dependent control of *SER3* expression through *SRG1* transcription regulation

As indicated above, *SER3* expression is regulated by the level of serine in the cell: in high levels of serine, *SER3* is not expressed and in low levels of serine, *SER3* is expressed (ALBERS *et al.* 2003; MARTENS *et al.* 2005). The inverse relationship between *SER3* and *SRG1* expression in serine conditions lead to the discovery that *SRG1* transcription regulates *SER3* expression (MARTENS *et al.* 2004). Therefore, the regulation of *SRG1* is paramount to the regulation of *SER3*. The physiological regulation of *SRG1* shows that *SRG1* is induced in high serine conditions through the activity of the serine responsive activator Cha4. Therefore, the direct regulation of *SRG1* by Cha4 in response to serine indirectly regulates *SER3* in a serine dependent manner. Interestingly, Cha4 is required for the regulation of another serine responsive gene, *CHA1*, which encodes an enzyme required for serine catabolism in yeast. Cha4 activates *CHA1* in the presence of serine and no longer activates *CHA1* in the absence of serine (HOLMBERG and SCHJERLING 1996). Therefore, the combined regulation of *SRG1* and *CHA1* by Cha4 regulates the serine levels in cells. In high serine conditions, Cha4 upregulates transcription of both *CHA1* and *SRG1* in order for Cha1 to catabolize the serine present in the cell and for *SRG1* transcription to turn off *SER3* expression. In serine starvation conditions, Cha4 no longer activates *CHA1* or

SRG1 transcription, and therefore the amount of Cha1 decreases in the cells, while lower *SRG1* transcription permits transcription of *SER3* and therefore the amount of Ser3 in the cell is increased, increasing the level of serine in the cell (Figure 8) (MARTENS *et al.* 2005).

Having previously found a repressive role for the Swi/Snf chromatin remodeling complex in regulating *SER3* expression (MARTENS and WINSTON 2002), the role of Swi/Snf and other regulatory factors was examined in the regulation of *SER3* indirectly through regulating *SRG1* (MARTENS *et al.* 2005). Both Swi/Snf and the histone acetyltransferase SAGA were found to facilitate Cha4 activation of *SRG1* in the presence of serine. The individual contribution of the subunits of both these complexes were determined through deletions of each individual gene to gain a deeper understanding into the regulation (MARTENS *et al.* 2005).

Together, these data developed a model for serine dependent regulation of *SRG1* (Figure 9). In the presence of serine, the serine responsive activator Cha4 recruits two coactivator complexes, SAGA and Swi/Snf, and together these factors activate *SRG1* transcription in which this transcription acts to prevent recruitment of activators to the *SER3* activating sequences therefore repressing *SER3*. In the absence of serine, the Cha4 activator is still bound to the *SRG1* activating sequences, but is unable to recruit the coactivator complexes to promote transcription of *SRG1*. With decreased *SRG1* transcription, activators are able to bind the *SER3* activating sequences, and therefore *SER3* expression is upregulated (MARTENS *et al.* 2004; MARTENS *et al.* 2005). Although the identification of a sequence specific activator of *SER3* transcription remains elusive, *SRG1* transcription has been shown to inhibit both TBP binding to the *SER3* promoter and the binding of Gal4p when binding sites for this protein are placed within the *SER3* promoter (HAINER *et al.* 2011; MARTENS *et al.* 2005). Also, while serine dependent regulation of coactivator recruitment of SAGA and Swi/Snf by Cha4 is still not described, similar mechanisms

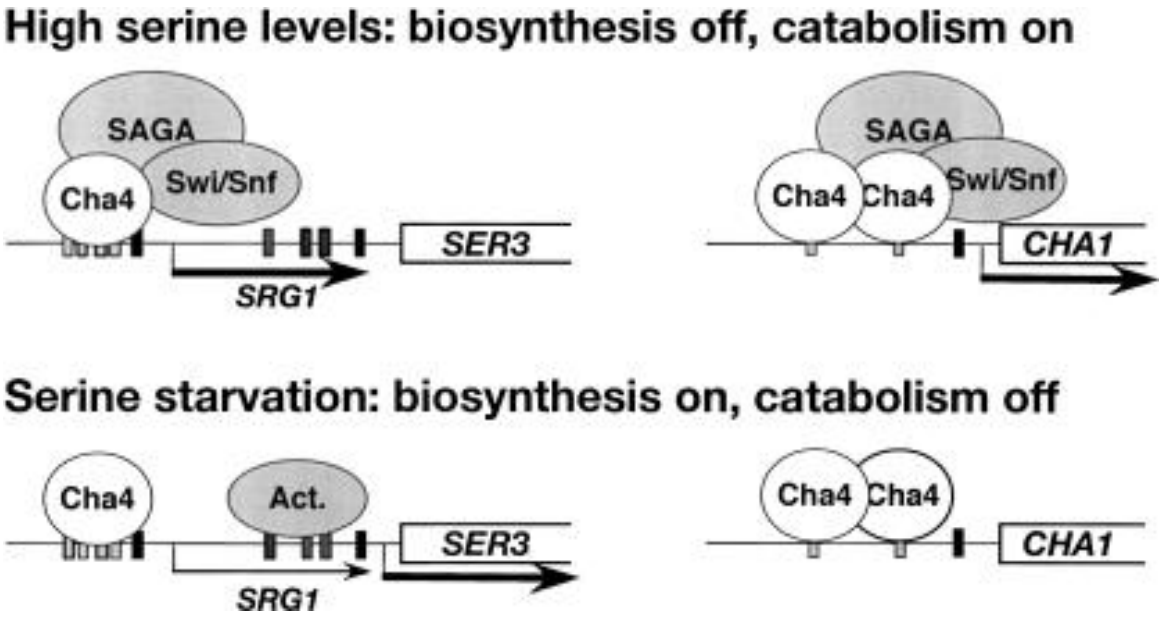


Figure 9. Model for coordinated regulation of *SER3* and *CHA1* by the serine responsive activator Cha4.

In the presence of serine (top panel), Cha4 indirectly represses the serine biosynthetic *SER3* gene through activation of *SRG1* and directly activates the serine catabolic gene *CHA1*. In serine starvation conditions (bottom panel), when Cha4 is no longer able to recruit Swi/Snf1 or SAGA, the expression states of *SER3* and *CHA1* are reversed. In this model, the activation of *SER3* also requires a putative activator (Act.) that binds to the *SER3* activating sequences. Cha4 is able to act as both an activator and repressor in response to serine. This figure is reprinted from (MARTENS *et al.* 2005) with permission from Cold Spring Harbor Laboratory Press, copyright 2005.

have been described for other yeast promoters (SELLICK and REECE 2005).

1.7 THESIS AIMS

Chromatin dynamics is a well-known factor in regulating gene expression in eukyarotes. However, upon t he commencement of my dissertation research, we were just beginning to appreciate the role of chromatin in regulating a novel mechanism of gene regulation that had been described in *S. cerevisiae*. While it was known that chromatin was important in regulating *SER3* expression (KAPLAN *et al.* 2003; LEE *et al.* 2007; PRUNESKI 2011; WYRICK *et al.* 1999), how chromatin dynamics contributed to this process was unknown.

Therefore, my thesis research was aimed at determining the contribution of chromatin dynamics to the regulation of *SER3*. There were a number of unanswered questions that were immediately obvious about the contribution of chromatin to *SER3* regulation. How does chromatin repress *SER3*? What chromatin associated factors are important for this process? What is the contribution of individual histone proteins to this regulation? Can the characterization of *SER3* regulation be used to help understand transcription dynamics in general? Fortunately, my thesis research was successful at answering all of these questions and provides a broader understanding of chromatin dynamics.

To initiate these studies, I assisted in determining the mechanism through which chromatin occupancy represses *SER3*. These studies revealed that the role of *SRG1* transcription over the *SER3* promoter was to maintain a repressive chromatin structure over this region to prevent factors from binding and initiating *SER3* transcription. From this work, Spt6 and Spt16,

along with histone proteins, were identified as important regulators of this processes, and this lead to my deeper characterization of how both Spt16 and histones are functioning during this mechanism of gene repression. In order to perform these study, I utilized our now well-described model system as a method to identify specific amino acids in Spt16 and histones H3 and H4 which are important for regulating both *SER3* expression, but also important for transcription-coupled nucleosome dynamics in general. These studies provided not only a novel molecular toolbox for transcription research, but also uncovered a deeper understanding of the roles of both Spt16 and histone proteins during transcription dynamics. Specifically, my analysis revealed a previously unappreciated patch of histone residues that are required for maintenance of nucleosome occupancy during high rates of transcription, likely due to its role in interacting with histone chaperones. Cumulatively, my thesis research has provided a detailed characterization of a number of factors involved in the dynamic process of gene regulation.

2.0 *SRG1* TRANSCRIPTION REGULATES *SER3* EXPRESSION THROUGH MAINTAINING NUCLEOSOME OCCUPANCY OVER THE *SER3* PROMOTER

The work discussed in this Chapter has been adapted from published material (HAINER *et al.* 2011) and is reprinted, with alterations, with permission from Cold Spring Harbor Laboratory Press, copyright 2011. This project was a collaborative effort involving members of the Martens Lab. I performed the Northern analysis in Figures 12 and 15 and the nucleosome scanning assay in Figure 18. Justin Pruneski performed the ChIP experiments in Figures 12, 13, and 14 and Joe Martens performed the nucleosome scanning experiments in Figures 10, 11, and 13. Robin Monteverde and Rachel Mitchell constructed strains and contributed to control experiments in Figures 14 and 17.

2.1 INTRODUCTION

Previously, we showed that serine-dependent transcription of ncDNA (*SRG1*) in *S. cerevisiae* represses expression of the adjacent *SER3* gene (MARTENS *et al.* 2004; MARTENS *et al.* 2005). In the presence of serine, transcription of *SRG1* extends across the promoter of the adjacent *SER3* gene, yielding two short transcripts that terminate 75bp 5' and 25bp 3' of the *SER3* translational start (THOMPSON and PARKER 2007), and a minor *SRG1–SER3* readthrough transcript that extends to the end of *SER3* (MARTENS *et al.* 2004; THOMPSON and PARKER 2007). We provided

evidence that it is the act of transcribing *SRG1* across the *SER3* promoter, rather than the *SRG1* RNA products, that represses *SER3* (Martens et al. 2004). In this study, we elucidate the mechanism whereby serine-dependent transcription of ncDNA (*SRG1*) in *S. cerevisiae* represses expression of the adjacent *SER3* gene. We show that *SER3* repression correlates with a broad region of strong micrococcal nuclease (MNase) protection spanning the entire *SRG1* transcription unit, suggesting that nucleosomes are loosely positioned across this region. Surprisingly, conditions that reduce *SRG1* transcription result in dramatically reduced MNase protection at the *SER3* promoter, indicating a loss of nucleosome occupancy. By analyzing mutations in *SPT6* and *SPT16*, two genes that encode subunits of the Spt6/Spn1(Iws1) and FACT elongation complexes, respectively, we provide evidence that it is the nucleosomes assembled at the *SER3* promoter by intergenic *SRG1* transcription, not RNA pol II itself, that interfere with the binding of transcription factors to the *SER3* promoter. Our data are consistent with a general model in which transcription of ncDNA can assemble nucleosomes that occlude DNA from binding by sequence specific DNA-binding proteins.

2.2 MATERIALS AND METHODS

2.2.1 Yeast strains and media

All *S. cerevisiae* strains used in this study (Table 1) are isogenic with a *GAL2+* derivative of S288C (WINSTON *et al.* 1995). Strains were constructed using standard genetic crosses or by transformation (AUSUBEL 1991). The C-terminus of *RPB1* and *SPT16* were tagged with 13 copies of the c-Myc epitope by PCR-mediated transformation of diploid strains using pFA6a-

13myc-KanMX and pFA6a-13myc-HIS3MX, respectively (LONGTINE *et al.* 1998). The *spt16-22* and *spt16-23* alleles (FORMOSA *et al.* 2001) were integrated into a diploid strain by two-step gene replacement using *Sna*BI-digested pTF142-23 and pTF142-22 plasmids (kindly provided by T. Formosa, University of Utah, Salt Lake City, UT). The *ser3*Δ*UAS* mutation was constructed by replacing 37bp of *SER3* promoter sequence (from -228 to -198; *SER3* ATG=+1) with an *Avr*II restriction site by QuikChange mutagenesis (Agilent Technologies) to yield pRM08 plasmid. The *ser3*Δ*UAS* allele was then integrated into a diploid strain by two-step gene replacement using *Afe*I-digested pRM08. Several strains contain a *KanMX*-marked deletion of the *SER33* gene, which is a paralog of *SER3*. Based on previous studies (MARTENS *et al.* 2004; MARTENS and WINSTON 2002) and the results presented in this study, the deletion of *SER33* does not affect *SER3* regulation. Strains were grown in the following media as indicated in the figure legends: YPD (1% yeast extract, 2% peptone, 2% glucose), YPgal (1% yeast extract, 2% peptone, 2% galactose), YPraff (1% yeast extract, 2% peptone, 2% raffinose), and synthetic complete with 1mM serine (SC +serine) or without serine (SC-serine) (ROSE 1991).

2.2.2 Nucleosome scanning assay

Nucleosome scanning experiments were performed using a method adapted from those described previously (BRICKNER *et al.* 2007; LEE *et al.* 2007; WHITEHOUSE and TSUKIYAMA 2006). Cells were grown to 2×10^7 to 3×10^7 cells per milliliter and were treated with formaldehyde (2% final concentration) for 30 min at 30°C and then glycine (125mM final concentration) for 10 min at room temperature. Formaldehyde-treated cells (1.2×10^9) were harvested by centrifugation, washed with Tris-buffered saline, and then incubated in ZDB buffer (50 mM Tris Cl at pH 7.5, 1 M sorbitol, 10 mM β-mercaptoethanol) containing 1.5 mg of zymolase 20T for 30 min at 30°C

Table 1. *Saccharomyces cerevisiae* strains used in Chapter 2.

Name	Genotype	Reference or Source
FY4	*MAT***a**	(WINSTON *et al.* 1995)
FY5	*MAT*α	F. Winston
FY111	*MAT***a** *his4-914δ lys2-128δ trp1Δ63 ura3-52 spt6-140*	(HARTZOG *et al.* 1998)
FY346	*MAT***a** *leu2Δ1 lys2-128δ ura3-52 spt16-197*	(MALONE *et al.* 1991)
FY1221	*MAT*α *his4-914δ lys2-128δ trp1Δ63 ura3-52 spt6-14*	(HARTZOG *et al.* 1998)
FY1350	*MAT*α *leu2Δ0 lys2Δ0 ura3Δ0*	F. Winston
FY1411	*MAT***a** *his4-917δ leu2Δ1 lys2-173R2 trp1Δ63 ura3-52 nhp6aΔ::URA3 nhp6bΔ::URA3*	F. Winston
FY2097	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 ser33Δ::KanMX*	(MARTENS *et al.* 2004)
FY2099	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 ser33Δ::KanMX ser3-100*	(MARTENS and WINSTON 2002)
FY2134	*MAT***a** *his4-912δ leu2Δ1 lys2-128δ RPB3-HA1::LEU2 SPT6-FLAG CTR9-9MYC::KanMX*	(KAPLAN *et al.* 2003)
FY2180	*MAT***a** *his4-912δ leu2Δ1 lys2-128δ FLAG-spt6-1004*	(KAPLAN *et al.* 2003)
FY2250	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 ser33Δ::KanMX srg1-1*	(MARTENS *et al.* 2004)
FY2260	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 ser33Δ::KanMX srg1-1 ser3::GAL7UAS*	(MARTENS *et al.* 2004)
FY2425	*MAT*α *his3Δ200 leu2Δ1 lys2-128δ ura3-52 FLAG-spt6-1004*	F. Winston
FY2471	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 srg1-1*	(MARTENS *et al.* 2005)
GHY1199	*MAT*α *his4-914δ leu2Δ1 lys2-128δ trp1Δ63 ura3-52 iws1-7-MYC::TRP1*	(LINDSTROM *et al.* 2003)
GHY1200	*MAT*α *his4-914δ leu2Δ1 lys2-128δ trp1Δ63 ura3-52 iws1-13 MYC::TRP1*	(LINDSTROM *et al.* 2003)
KY719	*MAT***a** *ura3Δ0*	
KY912	*MAT***a** *his3Δ200 leu2Δ1 lys2-128δ ura3-52 set2Δ::HIS3*	K. Arndt
KY934	*MAT*α *his3Δ200 leu2Δ1 trp1Δ63 dot1Δ::HIS3*	K. Arndt
KY938	*MAT*α *his3Δ200 leu2Δ1 trp1Δ63 set1Δ::HIS3*	K. Arndt
KY1235	*MAT***a** *his3Δ200 lys2-128δ ura3-52 rco1Δ::HIS3*	K. Arndt
KY1806	*MAT***a** *set3Δ::KanMX*	K. Arndt
KY1822	*MAT***a** *leu2Δ0 set1Δ::KanMX set2Δ::KanMX*	K. Arndt
YJ275	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 ser33Δ::KanMX ser3-100*	This study
YJ582	*MAT***a** *srg1-1*	This study
YJ583	*MAT***a** *srg1-1*	This study
YJ585	*MAT*α *leu2Δ0 lys2Δ0 ura3Δ0 srg1-1*	This study
YJ586	*MAT*α *his3Δ200 leu2Δ0 ura3Δ0*	This study
YJ832	*MAT***a** *leu2Δ0 ura3Δ0 spt16-22*	This study
YJ833	*MAT***a** *ura3Δ0 spt16-23*	This study
YJ841	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2Δ0 ura3Δ0* or *ura3-52 RPB1-C13MYC::KanMX spt16-197*	This study
YJ842	*MAT*α *his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0* or *ura3-52*	This study

	RPB1-C13MYC::KanMX spt16-197	
YJ843	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0* or *ura3-52 RPB1-C13MYC::KanMX spt16-197*	This study
YJ844	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0 RPB3-HA::LEU2 spt16-197-C13MYC::HIS3MX*	This study
YJ845	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0 RPB3-HA::LEU2 spt16-197-C13MYC::HIS3MX*	This study
YJ846	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2Δ0 ura3Δ0 RPB3-HA::LEU2 spt16-197-C13MYC::HIS3MX*	This study
YJ847	*MATα leu2Δ1* or *leu2Δ0 lys2-128δ* or *lys2Δ0 FLAG-SPT6*	This study
YJ850	*MAT***a** *leu2Δ1* or *leu2Δ0 lys2-128δ ser3::GAL7UAS FLAG-spt6-1004*	This study
YJ855	*MATα leu2Δ1* or *leu2Δ0 lys2-128δ ser33Δ::KanMX FLAG-spt6-1004*	This study
YJ859	*MAT***a** *leu2Δ1* or *leu2Δ0 lys2-128δ ura3-52* or *ura3Δ0 spt16-197*	This study
YJ862	*MAT***a** *leu2Δ1* or *leu2Δ0 lys2-128δ FLAG-spt6-1004*	This study
YJ864	*MAT***a** *his3Δ200 his4-912δ leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0 ser33Δ::KanMX*	This study
YJ867	*MATα leu2Δ0* or *leu2Δ1 lys2Δ0 ura3Δ0* or *ura3-52 ser3::GAL7UAS spt16-197*	This study
YJ868	*MATα his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0* or *ura3-52 ser3::GALUAS spt16-197*	This study
YJ869	*MATα leu2Δ0* or *leu2Δ1 lys2Δ0 ura3Δ0* or *ura3-52 ser3::GAL7UAS spt16-197*	This study
YJ871	*MATα leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ ura3Δ0* or *ura3-52 ser3::GAL7UAS*	This study
YJ872	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ ura3Δ0* or *ura3-52 ser3::GAL7UAS*	This study
YJ873	*MAT***a** *his4-912δ leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ ura3Δ0 ser3::GAL7UAS SPT6-FLAG*	This study
YJ875	*MATα his4-912δ leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0 ser3::GAL7UAS FLAG-spt6-1004*	This study
YJ876	*MAT***a** *his4-912δ leu2Δ0* or *leu2Δ1 lys2-128δ ser3::GAL7UAS FLAG-spt6-1004*	This study
YJ877	*MAT***a** *leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ ura3Δ0* or *ura3-52 RPB1-C13MYC::KanMX FLAG-SPT6*	This study
YJ878	*MATα his3Δ200 leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ RPB1-C13MYC::KanMX FLAG-SPT6*	This study
YJ879	*MATα his3Δ200 leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ ura3Δ0* or *ura3-52 RPB1-C13MYC::KanMX FLAG-SPT6*	This study
YJ884	*MATα leu2Δ0* or *leu2Δ1 lys2Δ0* or *lys2-128δ RPB3-HA:LEU2 RPB1-C13MYC::KanMX FLAG-SPT6*	This study
YJ886	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ RPB1-C13MYC::KanMX FLAG-spt6-1004*	This study
YJ887	*MATα his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0* or *ura3-52 RPB1-C13MYC::KanMX FLAG-spt6-1004*	This study
YJ888	*MAT***a** *his3Δ200 leu2Δ0* or *leu2Δ1 lys2Δ0 ura3Δ0* or *ura3-52 RPB1-C13MYC::KanMX FLAG-spt6-1004*	This study
YJ892	*MATα his3Δ200 leu2Δ0* or *leu2Δ1 lys2-128δ ura3Δ0* or *ura3-52*	This study

	RPB3-HA::LEU2 RPB1-C13MYC::KanMX FLAG-spt6-1004	
YJ916	*MATα leu2Δ1* or *leu2Δ0 lys2-128δ ura3-52* or *ura3Δ0 ser33Δ::KanMX spt16-197*	This study
YJ947	*MAT***a** *ura3Δ0 ser3ΔUAS*	This study
YJ950	*MATα ura3Δ0 leu2Δ0* or *leu2Δ1*	This study
YJ954	*MATα ura3Δ0 lys2-128δ leu2Δ0* or *leu2Δ1 ser3ΔUAS*	This study
YJ955	*MAT***a** *ura3Δ0 ser3ΔUAS*	This study
YJ956	*MATα ura3Δ0 lys2-128δ ser3ΔUAS*	This study
YJ958	*MATα lys2-128δ* or *LYS2 leu2Δ0* or *leu2Δ1 FLAG-spt6-1004*	This study
YJ962	*MATα lys2-128δ* or *LYS2 leu2Δ0* or *leu2Δ1 FLAG-spt6-1004 ser3ΔUAS*	This study
YJ966	*MATα ura3Δ0* or *ura3-52 lys2-128δ* or *LYS2 leu2Δ0* or *leu2Δ1 spt16-197*	This study
YJ970	*MATα ura3Δ0* or *ura3-52 lys2-128δ* or *LYS2 leu2Δ0* or *leu2Δ1 spt16-197 ser3ΔUAS*	This study
TF7783-24	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 his4-912δ lys2-128δ spt16-24*	T. Formosa
TF8030-1	*MATα leu2Δ1 trp1Δ63 ura3-52 his4-912δ lys2-128δ spt16-11*	T. Formosa
TF8031-1	*MATα leu2Δ1 trp1Δ63 ura3-52 his4-912δ lys2-128δ pob3-7*	T. Formosa

on a rocker platform. Spheroplasts were pelleted by low-speed centrifugation, gently washed with NP buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris Cl at pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.075% NP-40, 1 mM β-mercaptoethanol, 500 mM spermidine), and resuspended in 1.8 mL of NP buffer. Samples were divided into six 300-mL aliquots that were then digested with 0, 1, 2.5, 5, 10, and 20 U of MNase (Nuclease S7 from Roche) for 45 min at 37°C. Digestions were stopped with 75 mL of Stop buffer (5% SDS, 50 mM EDTA) and were treated with 100 mg of proteinase K for 12–16 h at 65°C. DNA was extracted by phenol/chloroform using PLG-H tubes (5 Prime), and was incubated with 50 mg of RNase A for 1 h at 37°C. DNA was re-extracted with phenol/chloroform, precipitated with an equal volume of isopropanol, washed with 80% ethanol, and resuspended in 100 mL of TE. MNase digestions were evaluated by two methods. First, one-fifth of digested DNA was separated by gel electrophoresis. Second, previously characterized *GAL1* promoter sequences (BRICKNER *et al.* 2007; FLOER *et al.* 2010; LOHR 1984) - one within a positioned nucleosome (*GAL1* NB), and a second adjacent region (*GAL1* NUB) that is rapidly digested by MNase - were amplified by qPCR from MNase-treated and untreated samples. The MNase concentration that resulted in mostly mononucleosome-sized DNA (HAINER *et al.* 2011) with a *GAL1* NUB/NB ratio of <15% was subjected to further qPCR using tiled *SER3* primer pairs (*SER3*-1 to *SER3*-41) (Table 2). For each *SER3* primer set, the amount of protected template was calculated as a ratio between MNase-digested and undigested samples and then normalized to the amount of protected *GAL1* NB template. All nucleosome scanning assays were done in triplicate using at least two independent strains as indicated in the figure legends.

2.2.3 Northern analysis

Northern analysis was performed as described previously (AUSUBEL 1991) on 20 mg of total RNA isolated from cells grown to 1×10^7 to 2×10^7 cells per milliliter. DNA probes were generated by random prime-labeling PCR fragments for *SER3* (+111 to +1342), *SRG1* (-454 to -123 relative to *SER3* ATG), and *SCR1* (-163 to +284). *SCR1* serves as a loading control, since its RNA levels are unaffected by the mutations and growth conditions used in this study.

2.2.4 Chromatin Immunoprecipitation (ChIP) analysis

For histone H3, TBP, and Rpb1-C13myc ChIPs, cells were grown in YPD at 30°C to 1×10^7 to 2×10^7 cells per milliliter. For Gal4 ChIPs, cells were grown in YPraff at 30°C to 0.8×10^7 cells per milliliter, and then an additional 4 h at 30°C after addition of 2% galactose. Chromatin preparation and treatment were preformed as described previously (SHIRRA *et al.* 2005). Breifly, Gal4, histone H3, TBP, and Rpb1-13myc were immunoprecipitated by incubating sonicated chromatin overnight at 4°C with 1 mL of anti-GAL4 DBD antibody (sc-577, Santa Cruz Biotechnology), 5 mL of anti-histone H3 antibody (ab1791, Abcam), 2 mL of anti-TBP antibody (kind gift from G. Prelich, Albert Einstein College of Medicine), and 4 mL of anti-c-myc A-14 antibody (sc-789, Santa Cruz Biotechnology), respectively. Dilutions of input and immunoprecipitated DNA were subjected to qPCR. All ChIP signals were normalized to a control: either *GAL1* NB template (histone H3 ChIP), *TELVI* template located within a telomeric region on chromosome VI (Gal4 ChIP), or ‘‘No ORF’’ template located within a region of chromosome V that lacks ORFs (Rpb1-C13myc and TBP ChIPs). Details regarding the primers used for qPCR in each ChIP experiment are listed in Table 2.

Table 2. Oligonucleotides used in Chapter 2.

Name	Forward Primer	Reverse Primer	Position*	Length[$]	Midpoint[&]
GAL1 NB	CCCCACAAACCTTCAAATTAACG	CGCTTCGCTGATTAATTACCC	II:278751-278850	00	
GAL1 NUB	CGGATTAGAAGCCGCCGA	ATCTTTATTGTTCGGAGCAGTG	II:278568-278697	30	
SER3-1	CGGTACCAACCAAGTTGACTTAGAC	ATTTCAGCGATGACCAATTCTGCTAC	V:323074-323181	08	447
SER3-2	CGAAGAATCTGGTTTGTATTGGTTG	TTGGAGAAAGGCGAGTTGAAAAC	V:323040-323145	06	412
SER3-3	ACTAGATTAACTTCAAATGTCTTACAACATG	TGGTAGCGTAGTCTAAGTCAAC	V:323009-323108	00	378
SER3-4	GACGTTCATGCTATTGGTATCAGATC	TACCGATACAGAAACAACCAATACAA	V:322979-323078	00	348
SER3-5	TGCCCGAGGAAGAGTTGATC	CAAACCAGATTCTTCGCATGTTGTAA	V:322947-323055	09	320.5
SER3-6	GAAGAGCAAGGTTACCAAGTCGAAT	CTAGTCTTTGATCTGATACCAATAGCATGAAC	V:322907-323013	07	279.5
SER3-7	AAACGTTAATCAAACTGCTATTACAATCTT	GATCTTTTCGATCAACTCTTCCTCGG	V:322876-322975	00	245
SER3-8	GCCTTTCTCAACGGGTGATATG	CAATGAAGATTTATAGAATTCGACTTGGTAAC	V:322837-322948	12	212
SER3-9	ATGCTGTAAAGCACCCAAAAATTT	CGAAGATTGTAATAGCAGTTTGATTAACG	V:322809-322907	9	177.5
SER3-10	CATGAATACCGTTCCACAGCG	ACGTTTTCTAATAGTAAAATCTTCATATCACC	V:322783-322881	9	152
SER3-11	CCCAGGCGCTGTTTGTACTT	AAAGGCTTCAAAATTTTTGGGTG	V:322747-322842	6	114
SER3-12	ACCTTTCAACAAGCTATGAATATGAGC	ACAGCATTCAAGCGCTGTGGA	V:322715-322815	01	84.5
SER3-13	AATGACAAGCATTGACATTAACAACTTAC	CATGAAAGATTGCGTAGGTGAAGTAC	V:322681-322786	06	52
SER3-14	TACAGAACTCTATAAAGAACCACAGAAAAATC	AGCCGCTCATATTCATAGCTTGTTG	V:322643-322745	03	12.5
SER3-16	GGAAGAACCATTTCTAGTTATTTCACTTTT	CATTGCTGTCGATTTTTCTGTGGTTC	V:322585-322684	00	-47
SER3-17	GCAGAGGATAAGGAAATTCTTAAAACTG	GTTCTGTATTTTTACTAAGATAGTTGACAAG	V:322544-322650	07	-84.5
SER3-19	GGATGAAAAAATCAGACAAATATCCAA	CCTTTATATACATAACAGTTTTAAGAATTTCC	V:322485-322586	02	-145
SER3-20	TTAAGAAAATGCAACGCTGCC	GCTCCCTCCTTCCAACAAAG	V:322444-322545	02	-187
SER3-21	GTCCTTGACTTCTACCACGAGAAAA	TTACTCATAACTTGGATATTTGTCTGATTTTTTC	V:322416-322522	07	-212.5
SER3-22	ATTCTTCTCGTTCCCACCTAATTTC	TCAGAAAACCCTGCACGGG	V:322381-322481	01	-250.5
SER3-23	GGAACAACTTCGGTCTCAGCA	TTTCTTAATTTTTTTCTCGTGGTAGAAG	V:322352-322451	00	-280
SER3-24-2	CGATATTTACTCACAAATGGAATTCAAG	GAAGTCAAGGACAATAAATTGCGAA	V:322323-322427	05	-306.5
SER3-25-2	AAACCTAATTTTTTTTGTGGACCCA	AACGAGAAGAATAATTAAAGTGCTGAGAC	V:322294-322392	9	-338.5
SER3-26	TAAAAATTTGGTTAAGCAGTTAGGCTG	TCCCCTTGAATTCCATTTGTGAGTAAATAT	V:322250-322354	05	-379.5
SER3-27	GCCAAGCTATGTGCAAATATCACAAA	TGGGTCCACAAAAAAAATTAGGTT	V:322223-322318	6	-411
SER3-28	CATTGTTTTAGTTTTTTACTCACAATCGA	AGGTCCAGCCTAACTGCTTA	V:322179-322281	03	-451.5
SER3-29	AGAAATGCCATTGTTTAATCCTGATT	TTTAATTTGTGATATTTGCACATAGCTTGG	V:322147-322253	07	-481.5

SER3-30-2	TCCCCATTATCTTTGAATTTTCCTC	CTCGATTGTGAGTAAAAAACTAAAACAATG	V:322094-322207	14	-531
SER3-31	CATCTCCACCTTTCTCCCCAT	ACAATGTAGATAATCAGGATTAAACAATGGC	V:322080-322183	04	-550
SER3-32	GAACTTTCAAATTTACGATAGGTGGAG	TGGCATTTCTATGGATTTGTTGTTCTCTT	V:322050-322156	07	-578.5
SER3-33	GCGTGATGTTTGGGTGCAAT	GAGGAAAATTCAAAGATAATGGGGAGAAA	V:322017-322118	02	-614
SER3-34	TGCTGGATTGGATATATTGATAACGT	ATGTATCTCCACCTATCGTAAATTTGAAAG	V:321978-322082	05	-651
SER3-35	TCCATTTACTAATCAACTTAACAATGCTG	GTTCCGCTTTTCCGCCAAT	V:321954-322053	00	-678
SER3-36	TAAAACCCTTTTTTGTACACAATGGA	CAAACATCACGCAACGCTTTTT	V:321922-322028	07	-706.5
SER3-37-2	TATAACAAAATAATCAAGTTAAAACCCT	CGTTATCAATATATCCAATCCAGCATT	V:321903-322002	00	-724
SER3-38-2	TTCTTTACCTCATTCAACTGTATAGAACGT	GTTAAGTTGATTAGTAAATGGAAGAGATTCC	V:321873-321975	03	-762.5
SER3-40-2	GAGACTACACCGTGAAGCAACCT	AACGTTCTATACAGTTGAATGAGGTAAAGA	V:321805-321903	9	-827.5
SER3-41	TGATCAACTATTAAATTCCGGCAGTA	TTTAGTATAGATTATTTGGTAGCTTCAGG	V:321761-321853	3	-874.5
SER3-3'	TGCAATCGATTCTCATACTGTCAAC	TGCCTCAAGCATTCTTCTATCCA	V:324094-324197	04	+1465
GAL7/*SER3*	GAAAGGGTCCAAAAAGCGCTCGGA	CCTTTATATACATAACAGTTTTAAGAATTTCC	Details upon request	05	
TELVI	GCGTAACAAAGCCATAATGCCTCG	CTCGTTAGGATCACGTTCGAATCC	VI:269487-269624	38	
NO ORF	GTGTTTGACCCGAGGGTATG	TAAGGTCCACACCGTCATCA	V:9797-10013	17	

*location of amplified product (chromosome: bp-bp) from the *Saccharomyces* Genome Database (http://yeastgenome.org/) $length (bp) of amplified product &midpoint of amplified product relative to *SER3* ATG plotted in nucleosome scanning graphs

2.2.5 Quantitative PCR (qPCR)

All qPCR data were obtained using an ABI 7300 or StepOnePlus Real-Time PCR system, SYBR green reagents (Fermentas), and the primer sets listed in Table 2. All calculations were performed using Pfaffl methodology for relative quantitation of real-time PCR (PFAFFL 2001). Pfaffl methodology takes into account the efficiency of the reaction and the reference standard used in the reaction in order to determine the ΔCt value.

2.3 RESULTS

2.3.1 Evidence that nucleosomes occupy the *SER3* promoter in repressing conditions

Previously, we showed that transcription of intergenic *SRG1* DNA is required for *SER3* repression (MARTENS *et al.* 2004). Several pieces of data suggest that chromatin structure also plays an important role in *SER3* repression. First, we identified histones and two activators of histone gene expression, Spt10 and Spt21 (DOLLARD *et al.* 1994; ERIKSSON *et al.* 2005; HESS *et al.* 2004), in a genetic screen for repressors of *SER3* expression (PRUNESKI 2011). Second, DNA microarray experiments revealed that depletion of histone H4 resulted in strong *SER3* derepression (WYRICK *et al.* 1999). Third, a mutation in *SPT6*, a gene that encodes a protein required to maintain proper chromatin structure over genes during transcription (CHEUNG *et al.* 2008; KAPLAN *et al.* 2003), also results in *SER3* derepression (KAPLAN *et al.* 2003).

To investigate a possible role for chromatin structure in *SER3* repression, we first determined the positions of nucleosomes across the *SER3* locus in wild-type cells grown in *SER3*

repressing conditions (YPD) by a nucleosome scanning assay (LEE *et al.* 2007; SEKINGER *et al.* 2005). Briefly, cells are treated with formaldehyde, spheroplasted, and then incubated with increasing amounts of MNase to digest nonnucleosomal DNA (see the Materials and Methods for details). As described previously (BRICKNER *et al.* 2007), we monitored MNase digestion of two sequences located in the *GAL1–10* promoter - one within a well-positioned nucleosome (*GAL1* NB), and one within an adjacent MNase-sensitive region (*GAL1* NUB) - by quantitative PCR (qPCR) (J. Martens, data not shown). DNA isolated from the MNase concentration where we observed significant protection of *GAL1* NB relative to *GAL1* NUB was then used to assess MNase protection across *SRG1*/*SER3*. We performed qPCR with 38 uni que primer pairs to amplify overlapping *SRG1*/*SER3* sequences (Figure 10A) from both MNase-digested and undigested DNA. MNase protection for each of these sequences was quantified as the ratio of template present in MNase-digested DNA over undigested DNA that was then normalized to the amount of MNase-protected *GAL1* NB template. Using this method, we identified peaks of MNase protection, indicating the presence of a positioned nucleosome at the 3' end of *AIM9* (the gene adjacent to *SRG1*) and two at the 5' end of the *SER3* ORF (Figure 10B). We also found a 200bp MNase sensitive region (from -750 to -550 with respect to the *SER3* ATG) corresponding to the *SRG1* promoter, indicating a nucleosome-depleted region that is a hallmark of many yeast promoters (ALBERT *et al.* 2007; LEE *et al.* 2007; YUAN *et al.* 2005). In addition, we identified a broad region of MNase protection that begins at the *SRG1* transcription start site (-475) and extends across the *SER3* promoter to the *SER3* translational start site, a region that defines the *SRG1* transcription unit. This pattern of strong MNase protection implies the presence of nucleosomes that are positioned randomly across the *SRG1* transcription unit.

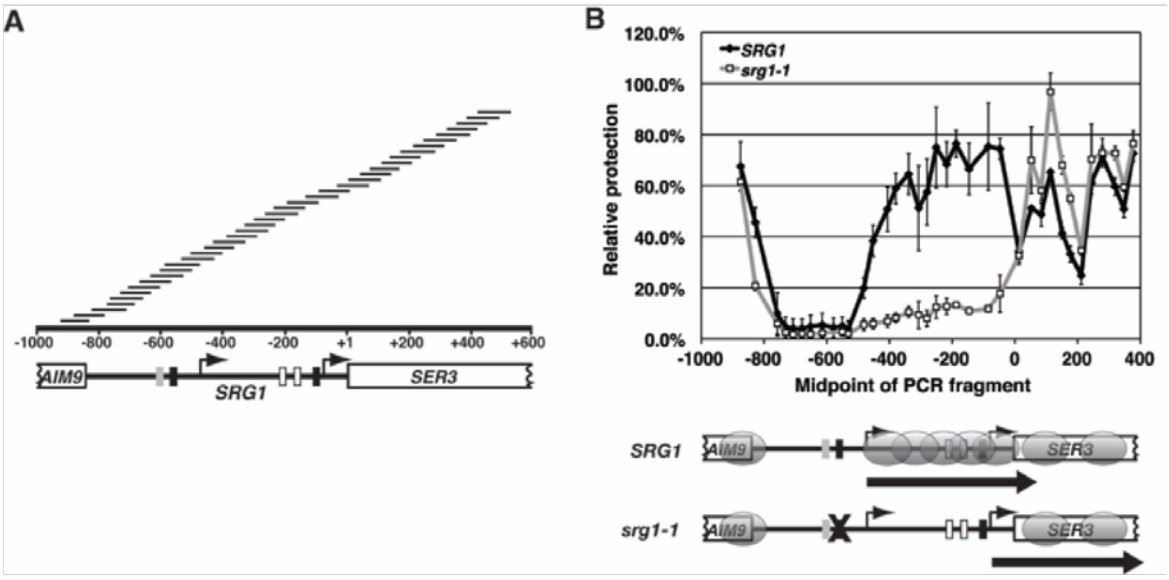


Figure 10. Nucleosome positions and relative occupancy at *SER3* in the presence and absence of *SRG1* transcription.

A) Schematic of *SER3* locus. The arrows at -475 and -75 indicate the TTS of *SRG1* and *SER3,* respectively. Blocks of intergenic sequence identity between *S. cerevisiae* and related yeast strains are marked, including the *SRG1* and *SER3* TATAs (black boxes), sequences required for *SER3* activation (white boxes), and a Cha4 binding site (grey box). The scale represents the distance from the *SER3* translation start (+1). The tiled black bars indicate the DNA fragments amplified by qPCR to quantify nucleosome position and relative occupancy (see Table 2). B) Nucleosome scanning assay was performed on wild-type (FY4, FY2097, FY1350) and *srg1-1* (YJ582, FY2250, YJ585) cells that were grown in YPD medium at 30°C. The relative MNase protection of each *SER3* template was calculated as a ratio to the control *GAL1* NB template. Each point on the graph shows the mean +/- SEM from three independent experiments that are plotted at the midpoint of each PCR product. Below the graph, a diagram of the *SER3* locus indicates the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data. The block arrows indicate the transcription activity of *SRG1* and *SER3* respectively. *srg1-1* strains have a m utated TATA sequence (marked by an **X**) that inhibits *SRG1* transcription causing *SER3* derepression.

Therefore, the *SER3* promoter lacks the typical nucleosome depleted region (ALBERT *et al.* 2007; LEE *et al.* 2007; YUAN *et al.* 2005). These results are consistent with our previously reported indirect-labeling experiments (MARTENS and WINSTON 2002) and with genome-wide nucleosome positioning experiments (LEE *et al.* 2007). To determine if *SRG1* transcription affects the chromatin structure at *SER3*, we repeated the nucleosome scanning assay using *srg1-1* strains, which carry a mutation of the *SRG1* TATA sequence. This mutation severely reduces *SRG1* transcription, resulting in strong derepression of *SER3* (MARTENS *et al.* 2004). In the *srg1-1* cells, MNase protection was reduced specifically over the *SRG1* transcription unit as compared with wild-type cells, indicating a dramatic loss of nucleosome occupancy (Figure 10B). Our results reveal a positive correlation between *SRG1* transcription and nucleosome occupancy across *SRG1*, an unexpected finding given the negative correlation between transcription and nucleosome occupancy generally observed for protein-coding genes (LEE *et al.* 2004; SCHWABISH and STRUHL 2004).

2.3.2 Serine-dependent transcription of *SRG1* intergenic DNA controls nucleosome occupancy over the *SER3* promoter

We showed previously that *SER3* expression is tightly controlled by the serine-dependent regulation of *SRG1* transcription (MARTENS *et al.* 2005). Therefore, we also measured MNase accessibility at *SER3* in wild-type strains that were grown in synthetic complete (SC) + serine (*SRG1* induced; *SER3* repressed) and then shifted to SC - serine (*SRG1* repressed, *SER3* induced) for 25 min. Since the extent of the MNase digestion of the *GAL1* NB region was identical in these different growth conditions (J. Martens, data not shown), we again normalized all *SER3* data to this region. As expected for cells grown in serine-rich media, the relative MNase

protection across *SRG1*/*SER3* is nearly identical to that observed for cells grown in YPD (compare wild-type strains in Figures 10B, 11A). When cells were shifted to media lacking serine, we measured a significant decrease in MNase protection over the *SRG1* transcribed region. However, rather than extending across the entire *SRG1* transcription unit, as was observed for *srg1-1*, the reduced MNase protection was restricted to a 200bp region that included sequences that had been determined previously to be required for *SER3* activation (MARTENS *et al.* 2004). An MNase-protected region of ~350bp, consistent with two closely associated nucleosomes or possibly one nucleosome that adopts multiple positions, remains near the 59 end of *SRG1*. This MNase-protected region begins at a more 5' position, including the *SRG1* transcription start site and possibly the *SRG1* TATA, as compared with the beginning of the broad peak of MNase protection that was measured for cells grown in serine rich media. Thus, in contrast to the complete loss of nucleosomes across *SRG1* that occurs in the *srg1-1* strains, serine starvation depletes nucleosomes specifically over sequences required for *SER3* activation. Therefore, in response to serine starvation, the *SER3* promoter adopts the typical promoter architecture, with +1 and -1 nucleosomes flanking a nucleosome depleted UAS (ALBERT *et al.* 2007; LEE *et al.* 2007).

To determine if the loss of nucleosome occupancy at the *SER3* promoter is caused by a loss of *SRG1* transcription and is not simply an effect of the resulting increase in *SER3* transcription, we repeated the nucleosome scanning assay using strains that contain a mutation in the *SER3* TATA sequence (*ser3-100*). Although the *ser3-100* mutation strongly inhibits *SER3* activation when cells are shifted from serine-rich to serine starvation media (10-fold decrease in *SER3* mRNA levels) (Figure 11B), the changes in MNase protection between these growth conditions were identical to those observed for a wild type (Figure 11, compare A and C).

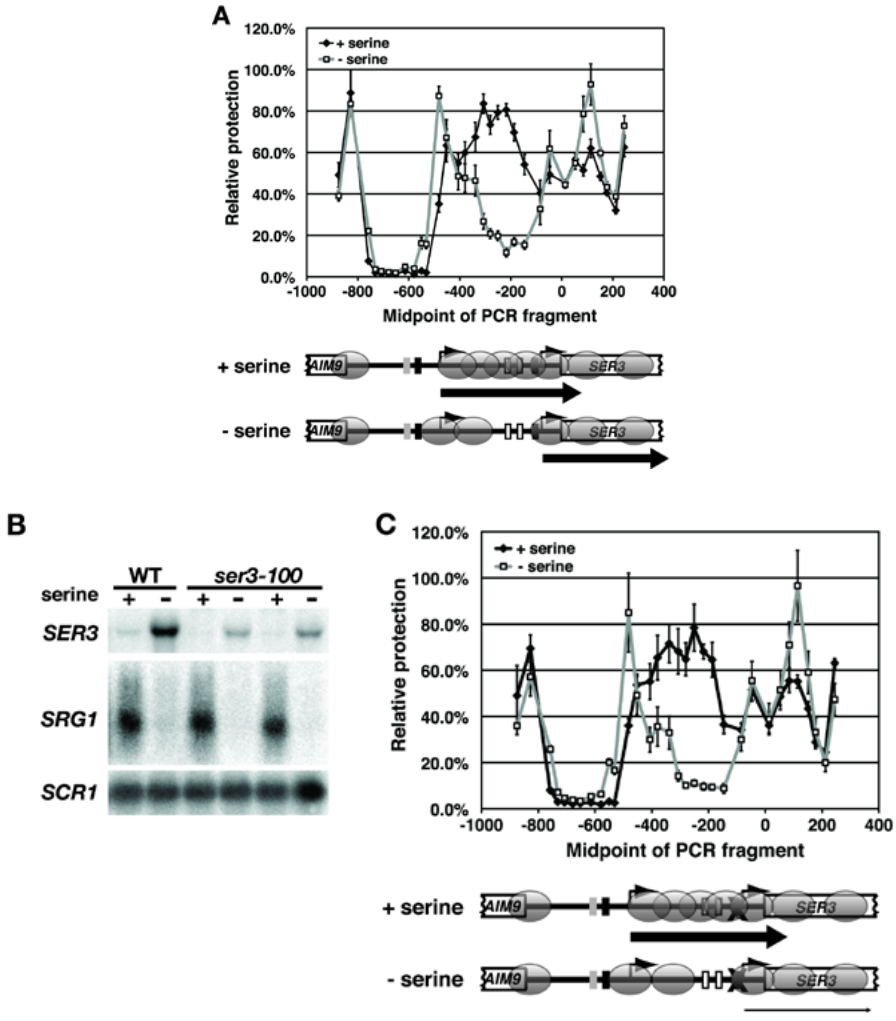


Figure 11. Effect of serine on nucleosome positions and relative occupancy at *SER3*.

A) Nucleosome scanning assay was performed on wild-type cells (FY2097 and FY4) that were grown at 30°C in SC+serine media (+ serine) and then shifted to SC-serine media for 25 minutes (- serine) as described in Figure 10. Each point on the graph shows the mean relative MNase protection +/-SEM from four independent experiments (two for each strain) plotted at the midpoint of each PCR product. Results for amplicons *SER3*-7 to *SER3*-41 are shown. B) Northern analysis of *SER3* and *SRG1* was performed on a wild type (FY2097) and two *ser3-100* strains (YJ275 and FY2099) that have a mutated *SER3* TATA. Cells were grown at 30°C in SC+serine (+ serine) and then shifted to SC-serine media for 25 minutes (- serine). *SCR1* serves as a loading control. C) Nucleosome scanning assay was performed on *ser3-100* strains (YJ275 and FY2099) as described in (A).

Therefore, reduced nucleosome occupancy over the *SER3* promoter is not a consequence of increased *SER3* expression.

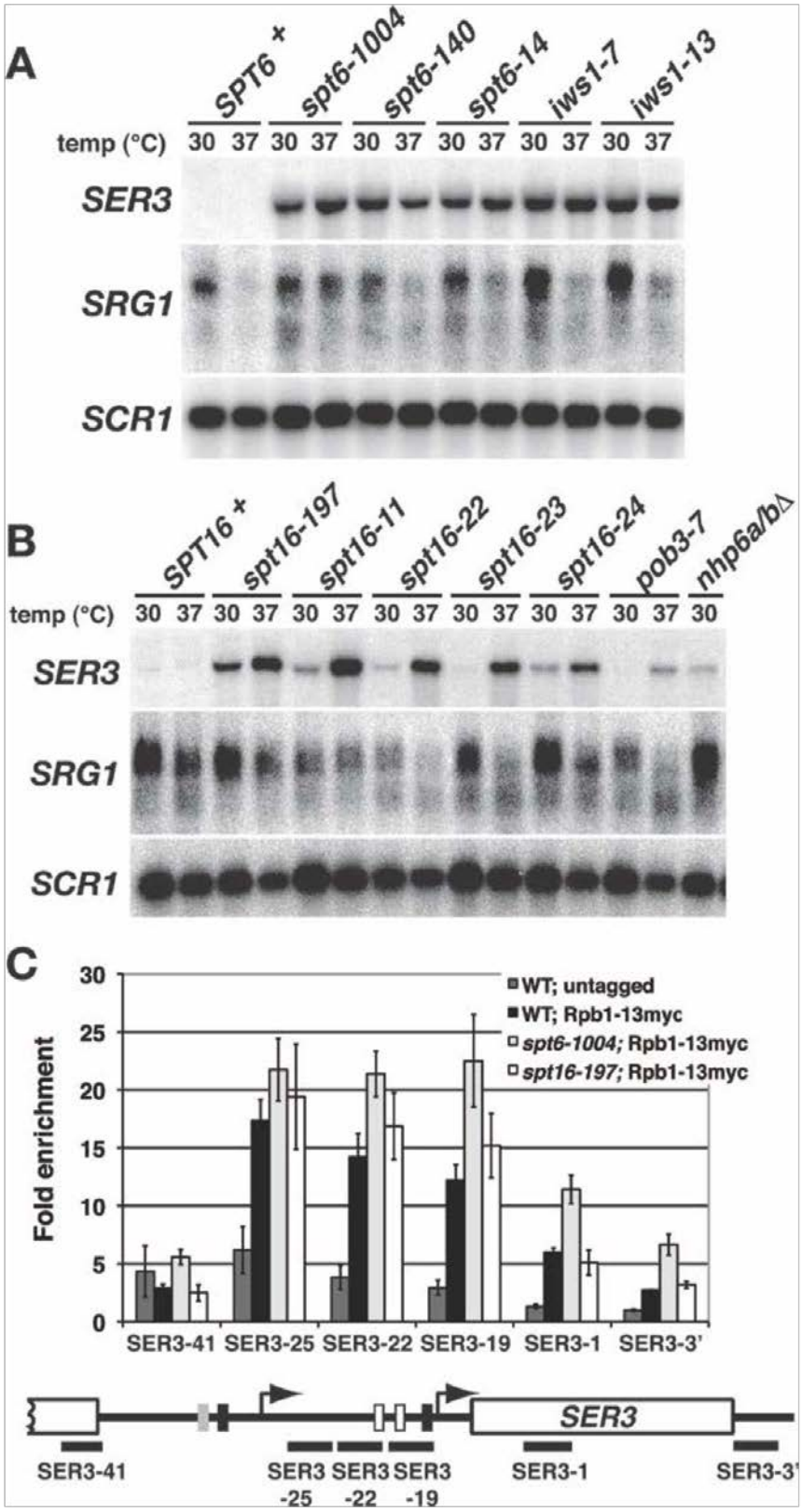
2.3.3 FACT and Spt6/Spn1(Iws1) are required to repress *SER3*

Our results thus far are consistent with two possible mechanisms for transcription interference at *SER3*. In the first possibility, similar to the conventional transcription interference mechanism (GREGER *et al.* 2000), RNA pol II elongating across *SRG1* competes with transcription factors for binding to the *SER3* promoter. In the second possibility, the nucleosomes maintained over the *SER3* promoter by *SRG1* transcription compete with transcription factor access to the *SER3* promoter. If the latter possibility is true, we reasoned that disrupting nucleosome reassembly during transcription might cause *SER3* derepression. Several studies have implicated the essential, highly conserved FACT and Spt6/Spn1(Iws1) transcription elongation complexes in transcription-dependent chromatin reassembly (BELOTSERKOVSKAYA *et al.* 2003; CHEUNG *et al.* 2008; JAMAI *et al.* 2009; KAPLAN *et al.* 2003; MASON and STRUHL 2003). I performed Northern analyses on several temperature-sensitive mutants of the Spt6/Spn1(Iws1) and FACT complexes that were grown in YPD at permissive (30°C) and nonpermissive (37°C) temperatures. Large increases in *SER3* mRNA levels were detected in multiple *spt6* and *spn1*(*iws1*) mutants at both 30°C and 37°C (Figure 12A). While increases were more modest and variable in the FACT mutants (*spt16*, *pob3*, and *nhp6*), I did find that, in at least one mutant, *spt16-197*, a significant increase in *SER3* mRNA levels occurred at 30°C (Figure 12B). Importantly, *SRG1* RNA levels were not significantly reduced in most of the mutant strains as compared with a wild type at 30°C.

We also performed chromatin immunoprecipitation (ChIP) experiments to measure RNA

pol II occupancy across the *SRG1*/*SER3* locus in a wild-type strain and two of these mutants (*spt6-1004* and *spt16-197*) that express either untagged Rpb1 (control) or a myc-tagged version of Rpb1 (Rpb1-13myc). The *spt6-1004* and *spt16-197* mutants have both been well characterized and share similar phenotypes characteristic of transcription defects, including sensitivity to the nucleotide analog 6-azauracil, suppression of Ty insertions, and cryptic intragenic transcription (KAPLAN *et al.* 2003; MASON and STRUHL 2003). Consistent with my Northern data, RNA pol II strongly associates with the *SRG1* transcription unit (Figure 12C) to similar levels in wild-type, *spt6-1004*, and *spt16-197* cells. Taken together, these results show that *SER3* repression is strongly dependent on both Spt6/Spn1(Iws1) and FACT. When these factors are mutated, *SER3* is derepressed without affecting RNA pol II levels at *SRG1*. This result argues against a model in which it is the level of active transcription that confers transcription interference.

Beyond the primary sites of *SRG1* transcription termination, we found a two-fold increase in RNA pol II occupancy in the *spt6-1004* cells as compared with wild type cells, which is consistent with our Northern data (Figure 12C). However, we did not detect an increase in RNA pol II in the *spt16-197* cells. Although surprising given the increase in *SER3* mRNA levels in this mutant, this result may be reconciled if we consider that *SRG1* transcription does not always terminate properly, resulting in the production of a minor readthrough that extends to the end of *SER3* (MARTENS *et al.* 2004; THOMPSON and PARKER 2007). Importantly, I found that the level of *SRG1–SER3* readthrough product is reduced in both *spt6-1004* and *spt16-197* mutants (data not shown), which is likely due to increased initiation at the *SER3* promoter. Therefore, increased RNA pol II occupancy in these mutant strains that would better reflect the observed increases in *SER3* transcription are likely masked by the RNA pol II that occupies *SER3* as a result of the synthesis of an *SRG1–SER3* readthrough product.


A
SPT6 +
spt6-1004
spt6-140
spt6-14
iws1-7
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temp (°C)
30 37 30 37 30 37 30 37 30 37 30 37
SER3
SRG1
SCR1
B
SPT16 +
spt16-197
spt16-11
spt16-22
spt16-23
spt16-24
pob3-7
nhp6a/bΔ
temp (°C)
30 37 30 37 30 37 30 37 30 37 30 37 30 37 30
SER3
SRG1
SCR1
C
Fold enrichment
30
25
20
15
10
5
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WT; untagged
WT; Rpb1-13myc
spt6-1004; Rpb1-13myc
spt16-197; Rpb1-13myc
SER3-41 SER3-25 SER3-22 SER3-19 SER3-1 SER3-3'
SER3
SER3-41
SER3 -25
SER3 -22
SER3 -19
SER3-1
SER3-3'

Figure 12. Repression of *SER3* is dependent on Spt6/Spn1(Iws1) and the FACT complex.

A) Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (FY4), *spt6-1004* (FY2425), *spt6-140* (FY111), *spt6-14* (FY1221), *iws1-7* (GHY1199), *iws1-13* (GHY1200) strains. Cells were grown in YPD at 30°C to mid-log and then shifted to 37°C for 60 minutes. B) Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (FY4), *spt16-197* (FY346), *spt16-11* (TF8030-1), *spt16-22* (YJ832), *spt16-23* (YJ833), *spt16-24* (TF7783-24), *pob3-7* (TF8031-1), *nhp6a*Δ*::URA3 nhp6b*Δ*::URA3* (FY1411) strains that were grown in YPD. C) ChIP analysis was performed on chromatin isolated from wild-type (YJ877, YJ878, YJ879, YJ884), *spt6-1004* (YJ886, YJ887, YJ888, YJ892), and *spt16-197* (YJ841, YJ842, YJ843) strains expressing Rpb1-C13myc and untagged control strains (FY4, FY5, YJ586). Rpb1-C13myc was immunoprecipitated with α-myc A14 antibody from chromatin prepared from cells that were grown in YPD at 30°C. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material and expressed as the fold enrichment over a control region of chromosome V that lacks open reading frames (No ORF). Each bar represents the mean +/- SEM from at least three independent experiments. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

2.3.4 Nucleosome occupancy over the *SER3* promoter is reduced in *spt6-1004* and *spt16-197* mutants at permissive temperature

To test whether the level of nucleosomes over *SRG1* affects *SER3* repression, we next performed nucleosome scanning assays to compare MNase accessibility across *SRG1* in wild-type, *spt6-1004*, and *spt16-197* cells that were grown in YPD at 30°C. We again normalized MNase protection of each *SRG1*/*SER3* region to the *GAL1* NB region, as the MNase accessibility of the *GAL1* control regions was indistinguishable between these strains (data not shown). Compared with wild-type cells, we measured a significant reduction of MNase protection specifically across the *SRG1* transcribed unit in *spt6-1004* cells (four-fold decrease) and to a slightly lesser extent in *spt16-197* cells (three-fold decrease) (Figure 13A), indicating nucleosome depletion across *SRG1*. These results are strikingly similar to the nucleosome scanning results we obtained for the *srg1-1* mutant (Figure 10B). However, while *SRG1* transcription was greatly reduced in *srg1-1* strains, it remained at wild-type levels in the *spt6-1004* and *spt16-197* mutants.

To complement our MNase experiments, we performed histone H3 ChIP assays in these same strains grown under the same conditions (Figure 13B). In wild-type cells, we detected significant histone H3 occupancy over the *SER3* promoter as compared with the *SRG1* promoter, which is consistent with nucleosomes occupying the *SER3* promoter. Moreover, at least for *spt6-1004* cells, there is a two-fold to three-fold decrease in histone H3 occupancy specifically over the *SER3* promoter that parallels the increase in MNase sensitivity over this region. Curiously, we did not observe a similar decrease in histone H3 occupancy over the *SER3* promoter in *spt16-197* cells. Since the loss of MNase protection is less pronounced in the *spt16-197* mutants as compared with the *spt6-1004* mutants, it is possible that histone H3 ChIP is not sensitive enough to detect a change in histone occupancy between wild-type and *spt16-197* strains. Alternatively,

nucleosomes may only partially reassemble in the *spt16-197* mutant in a manner that makes them more accessible to MNase without altering histone H3 occupancy. Based on previous studies (BELOTSERKOVSKAYA *et al.* 2003; XIN *et al.* 2009), an intriguing possibility is that reassembly of the H2A/H2B dimers at the *SER3* promoter may be specifically reduced by the *spt16-197* mutation. Taken together, these data support a model whereby FACT and Spt6/Spn1(Iws1) are required for *SRG1* transcription-dependent assembly of nucleosomes that repress *SER3*.

2.3.5 *spt6-1004* and *spt16-197* mutants are defective for transcription interference at *SER3*

To test whether *SRG1* transcription-dependent nucleosomes interfere with transcription factor binding to the *SER3* promoter, we performed ChIP experiments in *spt6-1004* and *spt16-197* mutants. Because sequence specific activators of *SER3* remain unknown, we first used a previously described *ser3*::*GAL7UAS* allele in which the putative *SER3* UAS is replaced with two binding sites for the Gal4 transcription activator (MARTENS *et al.* 2004). We then measured Gal4 occupancy by ChIP in wild-type, *srg1-1*, *spt6-1004*, and *spt16-197* strains that all contain the *ser3::GAL7UAS* allele and were grown in YPgal (Figure 14A). Consistent with our previous data (MARTENS *et al.* 2004), Gal4 occupancy at the *SER3* promoter increases eight-fold in the *srg1-1* control strain where *SRG1* is no longer transcribed and the *SER3* promoter is depleted of nucleosomes. In the *spt6-1004* and *spt16-197* strains where *SRG1* is transcribed at wild-type levels but nucleosome occupancy at the *SER3* promoter is reduced, Gal4 occupancy at the *SER3* promoter was also increased two-fold and four-fold, respectively (Figure 14A, left panel). Based on our *SER3* expression and nucleosome occupancy data (Figures 12A, 13A), the twofold increase in Gal4 occupancy at the *SER3* promoter in the *spt6-1004* strains was lower than

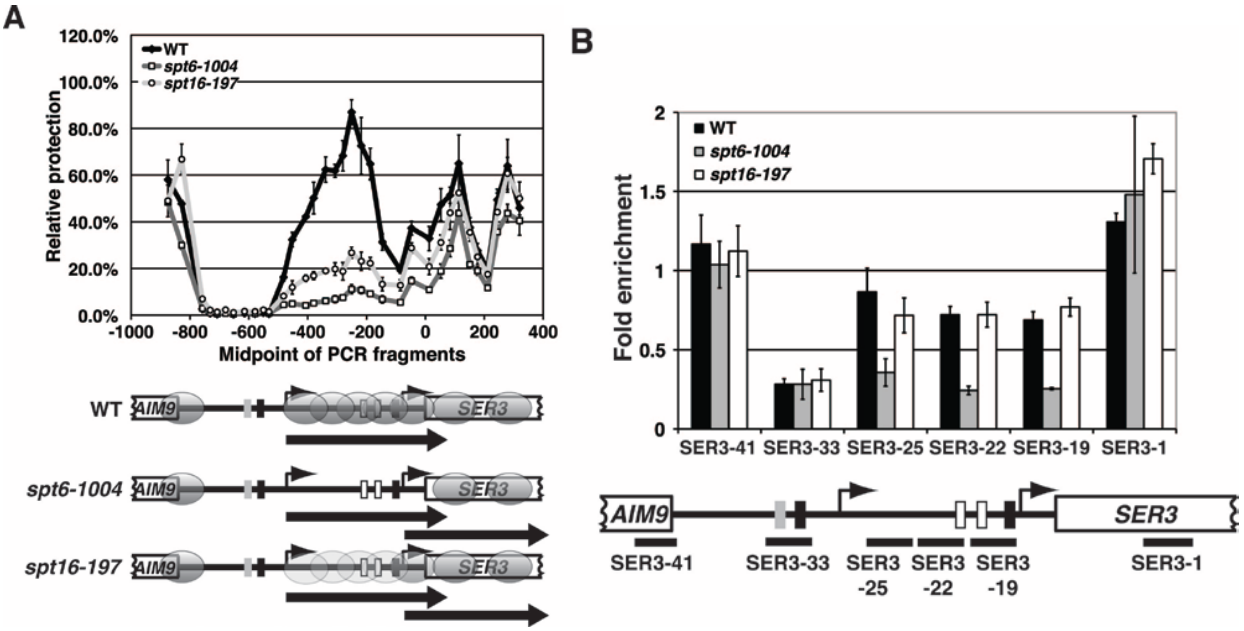

Figure 13. Nucleosome positions and relative occupancy at *SER3* in *spt6-1004* and *spt16-197* mutants.

A) Nucleosome scanning assay was performed on wild-type (FY2134, YJ864, YJ847), *spt6-1004* (FY2180, YJ855, YJ862), and *spt16-197* (FY346, YJ859, YJ916) strains that were grown in YPD at 30°C as described in Figure 10. The light gray ovals over the *SRG1* transcription unit in the *spt16-197* strain reflect that this region is slightly more protected from MNase digestion as compared to the *spt6-1004* strain. B) Histone H3 ChIP was performed on chromatin isolated from wild-type (FY4, FY5, YJ586), *spt6-1004* (YJ886, YJ887, YJ888), and *spt16-197* (YJ844, YJ845, YJ846) cells that were grown in YPD. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material and expressed as the fold enrichment over *GAL1* NB. Each bar represents the mean +/- SEM of at least three independent experiments. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

expected. However, this result is likely related to the fact that we also found reduced Gal4 occupancy at the control *GAL1* promoter in *spt6-1004* cells as compared with wild-type, *srg1-1*, and *spt16-197* cells (Figure 14A, right panel).

We also compared TBP occupancy by ChIP at the *SRG1* and *SER3* TATA sequences in wild-type, *srg1-1*, *spt6-1004*, and *spt16-197* strains that contain the endogenous *SRG1*/*SER3* locus (Figure 14B). The *SRG1* and *SER3* TATA sequences are both conserved among related yeast strains, bind TBP, and are required for *SRG1* and *SER3* transcription, respectively (MARTENS *et al.* 2004; MARTENS and WINSTON 2002). At the *SRG1* TATA, there is little difference in TBP occupancy in the *spt6-1004* and *spt16-197* mutants as compared with the wild-type strains, which agrees with our Northern and RNA pol II ChIP data (Figure 12). At the *SER3* TATA, TBP occupancy increased two-fold in *spt6-1004* cells as compared with a four-fold increase in *srg1-1* control cells, suggesting that the loss of nucleosomes over the *SER3* promoter in the *spt6-1004* mutants either increases TBP binding directly or possibly indirectly by allowing an unknown *SER3* activator protein better access to the *SER3* promoter. Interestingly, we did not observe a significant difference in TBP occupancy in the *spt16-197* mutant. This result may not be surprising, considering the increase in *SER3* expression is significantly lower in this mutant as compared with the *spt6-1004* mutant. Therefore, this assay may lack the sensitivity to detect a significant difference in TBP occupancy between wild-type and *spt16-197* cells.

From these data, we conclude that transcription interference at *SER3* is dependent, at least in part, on Spt6 and Spt16. Taken together with results described earlier, our findings suggest that transcription interference of *SER3* is partially mediated by nucleosomes that occupy the *SER3* promoter as a consequence of *SRG1* transcription from intergenic DNA.

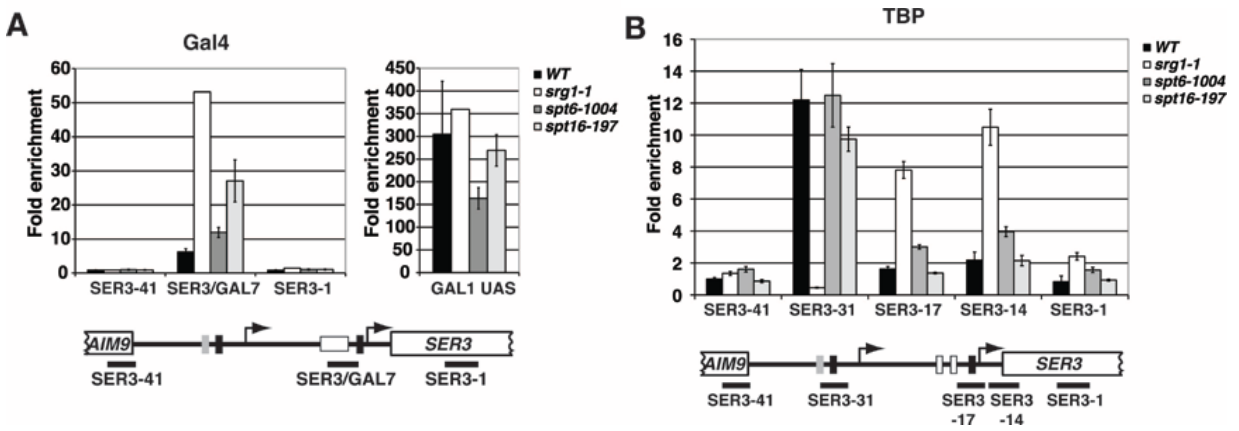

Figure 14. *spt6-1004* and *spt16-197* mutants are defective for transcription interference at *SER3*.

A) Gal4 ChIP was performed on wild-type (YJ871, YJ872, YJ873), *spt6-1004* (YJ875, YJ876, YJ850), *spt16-197* (YJ867, YJ868, YJ869), and positive control *srg1-1* (FY2260) cells that all contain the *ser3::GAL7UAS* allele. Chromatin was prepared from cells grown at 30°C in YPraf to 0.8 × 10^7 cells/ml and then for an additional four hours at 30°C after the addition of 2% galactose. Gal4 ChIP signals were determined by qPCR at the three *SER3* locations (left histogram) and at *GAL1* as a positive control (right histogram). All values were normalized to a control region located near the telomere of chromosome VI (TELVI) and represent the mean +/- SEM. Below the graph is a diagram of the *ser3::GAL7UAS* allele in which the putative *SER3* UAS region was replaced with the *GAL7* UAS region containing two Gal4-binding sites (white box). The black bars indicate the regions of *SER3* amplified by qPCR. B) TBP ChIP was performed on chromatin isolated from wild-type (FY4, FY5, YJ586, KY719), *spt6-1004* (YJ886, YJ887, YJ888, YJ892), *spt16-197* (YJ841, YJ842, YJ843, and YJ844), and positive control *srg1-1* (FY2471, YJ582, YJ583, YJ585) strains that were grown in YPD at 30°C as described in Figure 12C.

2.3.6 Histone modifications that suppress cryptic intragenic transcription are not required for *SER3* regulation

Spt6 and Spt16 have been shown previously to suppress transcription initiation from cryptic promoters that are located within protein-coding regions (KAPLAN *et al.* 2003; MASON and STRUHL 2003). Cryptic intragenic transcription is also suppressed by a cascade of transcription dependent post-translational histone modifications (LEE and SHILATIFARD 2007; LI *et al.* 2007a). During transcription, Set2 methylates Lys 36 of histone H3, thereby marking nucleosomes associated with recently transcribed DNA (POKHOLOK *et al.* 2005; RAO *et al.* 2005). Dimethylated H3 K36 acts as a binding site for the Rpd3S histone deacetylase complex (YOUDELL *et al.* 2008). Upon recruitment, Rpd3S deacetylates the reassembled nucleosomes on the N-terminal tails of histones H3 and H4, which suppresses cryptic intragenic transcription, presumably by occluding transcription factor access (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). Recently, Set1-dependent methylation of H3 K4 has also been implicated as a signal for transcription-dependent histone deacetylation by Rpd3S (PINSKAYA *et al.* 2009) and the Set3 complex (KIM and BURATOWSKI 2009). Because of these observations, a likely hypothesis is that Set1 and Set2 may contribute to *SER3* repression by regulating similar histone modifications over the *SER3* promoter in response to *SRG1* transcription. To test this possibility, I performed a Northern analysis to measure the effect of deleting the genes encoding the Set1, Set2, and Dot1 histone methyltransferases; the Rco1 subunit of Rpd3S; and the Set3 subunit of the Set3 complex on *SER3* and *SRG1* expression. Deletions of any one of these genes or a *set1*Δ*set2*Δ double deletion has no effect on *SER3* or *SRG1* mRNA levels (Figure 15). Moreover, mutations of histone H3 K4 (methylated by Set1), K36 (methylated by Set2), or K79 (methylated by Dot1) also has little to no effect on *SER3* repression (see Chapter 4). Therefore,

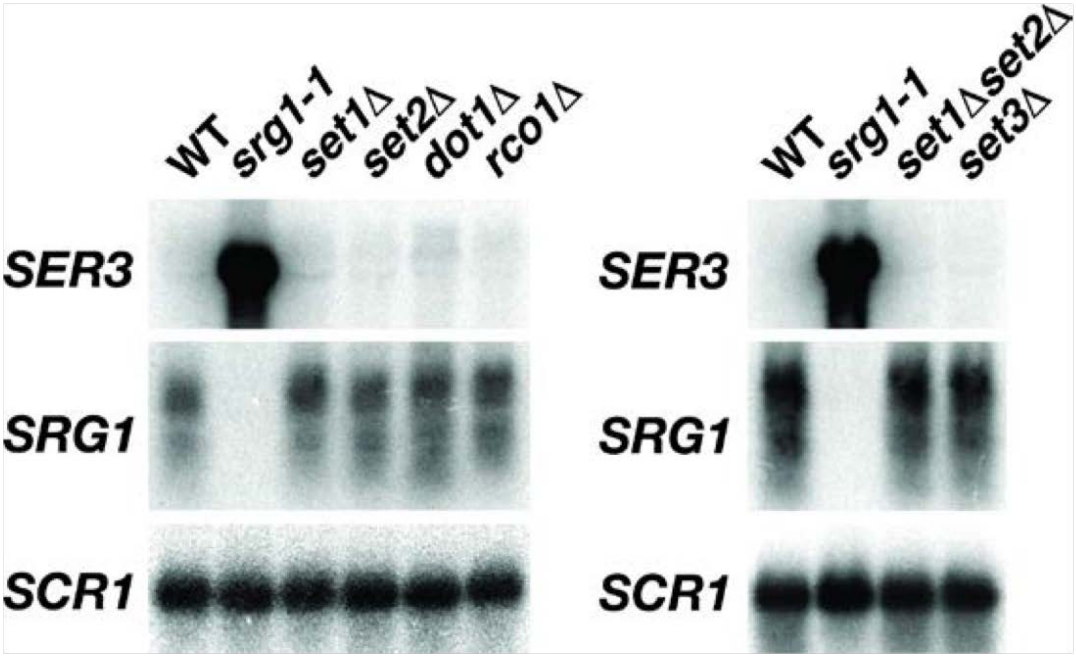

Figure 15. Repression of *SER3* does not require histone methyltransferases or the Rpd3S and Set3C histone deacetylase complexes.

Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (YJ586), *srg1-1* (FY2471), *set1*Δ (KY938), *set2*Δ (KY912), *dot1*Δ (KY934), *rco1*Δ (KY1235), *set1*Δ *set2*Δ (KY1822) and *set3*Δ (KY1806) strains that were grown in YPD at 30°C.

our results suggest that the relative contribution of these histone reassembly mechanisms may vary at different loci throughout the genome.

2.4 DISCUSSION

In this Chapter, we provide evidence that intergenic transcription represses adjacent gene transcription by assembling a repressive chromatin structure, rather than by the act of transcription. First, we showed that *SRG1* intergenic transcription is not only required for repression of the adjacent *SER3* gene, but is also required to maintain MNase protection of the *SER3* promoter. Second, we determined that changes in the MNase protection of the *SER3* promoter are caused by changes in *SRG1* transcription and not an effect of the changes to *SER3* transcription. Third, we found that cells expressing mutant versions of the Spt6 and Spt16 elongation factors derepress *SER3* and reduce MNase protection across the *SER3* promoter without altering *SRG1* RNA levels or RNA pol II occupancy across *SRG1*. These results clearly implicate the nucleosomes assembled on the *SER3* promoter as the key factor in *SER3* repression. Finally, we found that Spt6 and Spt16 are required to inhibit transcription factor binding to the *SER3* promoter, which suggests that the nucleosomes assembled at the *SER3* promoter by these factors interfere with the binding of transcription factors to their sites on DNA.

Taken together with previous studies (MARTENS *et al.* 2004; MARTENS and WINSTON 2002; MARTENS *et al.* 2005) we propose the following model for *SER3* regulation. When cells are grown in serine-rich medium, the Cha4 DNA binding protein recruits the Swi/Snf and SAGA complexes resulting in the induction of *SRG1* transcription. RNA pol II transcribes *SRG1* across

the *SER3* promoter, disassembling nucleosomes in its path and then reassembling them in its wake by a mechanism that involves both Spt6 and Spt16. *SRG1* transcription is thus required to maintain nucleosomes across the *SER3* promoter, interfering with transcription factor binding. When cells are then transferred to serine starvation conditions, Cha4 no longer recruits Swi/Snf and SAGA, resulting in decreased *SRG1* transcription. Without intergenic transcription to maintain them, nucleosomes are depleted over the *SER3* UAS allowing transcription factors, either an as yet unknown site-specific DNA binding activator or possibly TBP and RNA pol II, to bind and activate *SER3*. Two positioned nucleosomes remain at the 5' end of *SRG1* where they are likely to inhibit *SRG1* transcription.

In addition to its role in nucleosome assembly during transcription, Spt6 has also been reported to reassemble nucleosomes at the promoters of *PHO5* and several other yeast genes during repression (ADKINS and TYLER 2006). Therefore, an alternative model for *SER3* repression is that Spt6, and possibly Spt16, reassemble nucleosomes over the *SER3* promoter independently of *SRG1* transcription. Thus, mutations in these factors may bypass the normal role for *SRG1* transcription, which is to interfere with the recruitment of chromatin remodeling factors needed to displace the repressive nucleosomes at the *SER3* promoter. A prediction of this model is that the increased levels of *SER3* expression observed in the *spt6-1004* and *spt16-197* mutants would no l onger be dependent on s equence-specific activators to recruit chromatin remodeling factors, analogous to what has been observed for *PHO5* (ADKINS and TYLER 2006). To test this alternative model, we first identified a 37bp sequence within the *SER3* promoter (from -192 to -228; *SER3* ATG=+1) that when deleted, is required for *SER3* activation in response to serine starvation (Figure 17A). When this sequence was deleted in the *spt6-1004* and *spt16-197* strains, *SER3* mRNA levels were reduced as compared to similar strains expressing

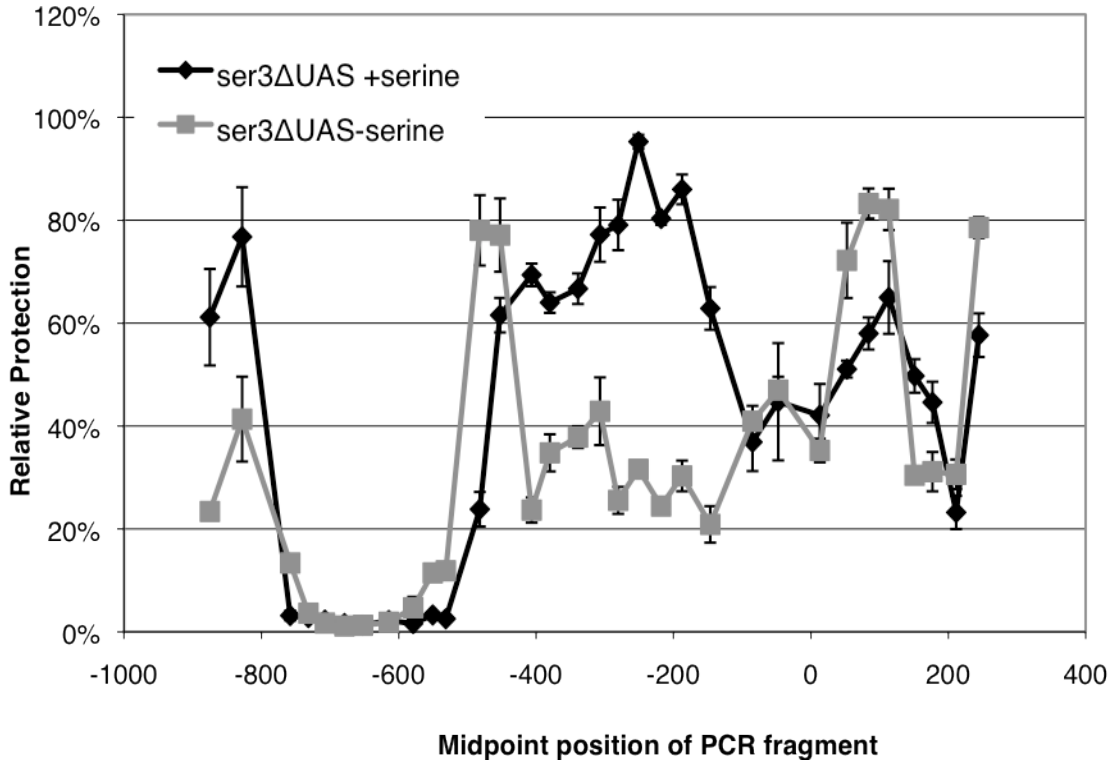

Figure 16. Deleting the *SER3* UAS does not alter nucleosome positions over the promoter.

Nucleosome scanning assay was performed on *ser3ΔUAS* (YJ954, YJ955, YJ956) strains that were grown in SC+serine media (+ serine) and then shifted to SC-serine media for 25 minutes (- serine) at 30°C as described in Figure 10.

wild-type *SER3* (Figure 17B). Also, nucleosomes were still lost in the *ser3*Δ*UAS* mutant after shifting to low serine (Figure 16). Therefore, *spt6-1004* and *spt16-197* mutations do not bypass the requirement of the *SER3* UAS for *SER3* activation, which argues against this alternative model.

Although MNase accessibility has been extensively used to predict nucleosome occupancy in eukaryotic organisms (for examples see (LEE *et al.* 2007; YUAN *et al.* 2005)), we cannot rule out the possibility that DNA-binding proteins may contribute to the protection of the *SER3* promoter from MNase digestion in serine-rich conditions. However, our observation that MNase protection over the *SER3* promoter was reduced in *spt6-1004* and *spt16-197* mutants without affecting RNA pol II occupancy suggests that at least RNA pol II and its associated factors do not affect MNase digestion.

If *SRG1* transcription from intergenic DNA is required to maintain nucleosomes over the *SER3* UAS, then from where might these nucleosomes originate? An intriguing source of these nucleosomes would be those positioned over the *SRG1* transcription start site and TATA (Figure 22), which likely inhibit *SRG1* transcription in the absence of serine. Based on this study and our previous work (MARTENS *et al.* 2004; MARTENS and WINSTON 2002; MARTENS *et al.* 2005), Swi/Snf, when recruited to the *SRG1* promoter in response to serine, may slide these nucleosomes toward *SER3*, to facilitate preinitiation complex assembly and *SRG1* transcription. Once RNA pol II begins to transcribe *SRG1*, the nucleosomes originally moved by Swi/Snf are disassembled to allow passage of RNA pol II and then reassembled behind RNA pol II by Spt6 and Spt16. Therefore, the activities of Swi/Snf, Spt6/Spn1, and FACT may combine to establish and maintain nucleosomes over the *SER3* promoter, which interfere with transcription factor binding to this region. This scenario would also explain the difference in nucleosome occupancy

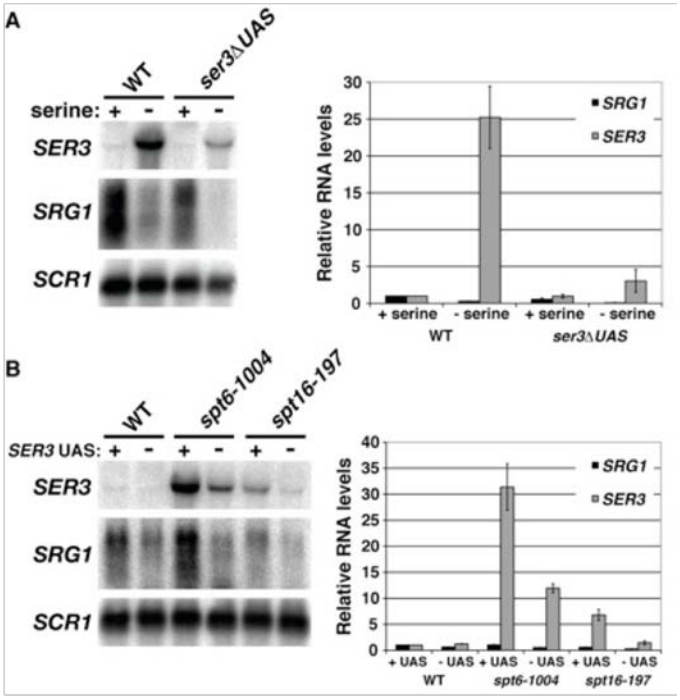

Figure 17. Identification of promoter sequence required for *SER3* activation.

A) Northern analysis (left panel) of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (KY719) and *ser3*Δ*UAS* (YJ947) strains that were grown at 30°C in SC+serine media (+ serine) and then shifted to SC-serine for 25 minutes (- serine). The *ser3*Δ*UAS* allele replaces a 37bp of the *SER3* promoter (–228 to -198; *SER3* ATG=+1) with an *Avr*II restriction enzyme site. The bar graph (right panel) summarizes *SER3* and *SRG1* RNA levels (normalized to *SCR1*) of three independent experiments. Each bar represents the mean +/- SEM of either *SRG1* or *SER3* RNA levels relative to wild-type cells that were grown in serine-rich media, which was arbitrarily set to 1. B) *SER3* activation in *spt6-1004* and *spt16-197* is impaired in the absence of *SER3* UAS. Northern analysis (left panel) of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type, *spt6-1004*, and *spt16-197* strains expressing either wild-type *SER3* (YJ950, YJ958, YJ966) or the *ser3*Δ*UAS* allele (YJ954, YJ962, YJ970) that were grown in YPD at 30°C. The bar graph (right panel) summarizes *SER3* and *SRG1* RNA levels (normalized to *SCR1*) of four independent experiments. Each bar represents the mean +/- SEM of either *SRG1* or *SER3* RNA levels relative to wild type cells that were grown in serine-rich media, which was arbitrarily set to 1.

at the 5' end of *SRG1* observed for wild-type cells grown in the serine starvation media as compared to *srg1-1* cells grown in serine-rich media, two conditions in which *SER3* is strongly derepressed (Figures 10 and 11A). In contrast to wild-type cells grown in serine starvation medium where it is no longer recruited, Swi/Snf is presumably still recruited by Cha4 in the *srg1-1* (*SRG1* TATA mutant) cells that are grown in serine-rich media. Thus, Swi/Snf can remodel the nucleosomes at the 5' end of *SRG1*; however, these nucleosomes cannot be maintained in the absence of *SRG1* transcription.

In addition to the nucleosome reassembly activity of Spt6/Spn1 and FACT, it has been well documented that a cascade of transcription-dependent post-translational modifications of histones found within nucleosomes over protein-coding genes contribute to the repression of intragenic transcription initiation (LEE and SHILATIFARD 2007; LI *et al.* 2007a). However, our studies show that *SER3* repression appears to be independent of at least some of these marks, including Set1-mediated methylation of histone H3 K4, Set2-mediated methylation of K36, and the removal of histone H3 and H4 acetylation by the Rpd3S and Set3C histone deacetylase complexes. Although we cannot rule out the possibility that other post-translational histone modifications may be involved, our results indicate a difference in the requirement of transcription-dependent post-translational histone modifications between *SER3* repression by *SRG1* transcription and repression of cryptic intragenic transcription. This difference may be related to the fact that *SRG1* is a relatively short transcription unit (~400bp) that is highly transcribed. It has been recently reported that cryptic intragenic transcription preferentially occurs at lowly transcribed genes (CHEUNG *et al.* 2008; LI *et al.* 2007b; LICKWAR *et al.* 2009). Therefore, it is possible that highly transcribed *SRG1* may not be dependent on H3 K36 methylation and subsequent histone deacetylation for protection from intragenic transcription

because of the frequent passage of RNA pol II. Alternatively, short, highly transcribed genes may never establish this histone mark since histone H3 K36 methylation predominates towards the 3' ends of transcribed genes (POKHOLOK *et al.* 2005). In support of this possibility, genome-wide analyses of K36 methylation and K79 methylation indicate little K36 trimethylation and K79 di- and trimethylation at *SRG1* (POKHOLOK *et al.* 2005; SCHULZE *et al.* 2011). Conversely, H3 K4 has been shown to be trimethylated, but not acetylated, and H2B K123 has been shown to be ubiquitylated over *SRG1* (GUILLEMETTE *et al.* 2011; SCHULZE *et al.* 2011).

In contrast to the characteristic transcription-dependent depletion of nucleosomes seen at protein-coding genes (LEE *et al.* 2007; YUAN *et al.* 2005), we show transcription-dependent assembly of nucleosomes across intergenic *SRG1*. How does one account for this apparent contradiction between nucleosome occupancy and transcription? Several recent studies have indicated that DNA sequence can either favor or refract nucleosome formation thereby influencing genome-wide nucleosome positioning (FIELD *et al.* 2008; IOSHIKHES *et al.* 2006; KAPLAN *et al.* 2009; PECKHAM *et al.* 2007; SEGAL *et al.* 2006; YUAN *et al.* 2005). As has been proposed for yeast genes containing nucleosome depleted promoter regions (SEGAL and WIDOM 2009b), one possibility is that the underlying DNA sequence of the *SER3* promoter may normally disfavor nucleosome formation to facilitate transcription factor binding. Therefore, by reassembling nucleosomes after each passage of RNA pol II, *SRG1* transcription effectively maintains nucleosomes over DNA that is normally refractory to nucleosomes. Several observations support this possibility. First, the *SER3* UAS region that is nucleosome-depleted in the absence of *SRG1* transcription contains poly(dA:dT) tracts; a sequence motif that resists bending and thus disfavors nucleosome formation (SEGAL and WIDOM 2009a; SEGAL and WIDOM 2009b). Second, the *SER3* UAS sequence is predicted to have a low nucleosome-

forming potential by an algorithm developed using comparative genomics (IOSHIKHES *et al.* 2006). Finally, the *SER3* UAS sequence failed to form a stable nucleosome in a genome-wide *in vitro* nucleosome reconstitution assay (KAPLAN *et al.* 2009).

In *Saccharomyces cerevisiae*, cells respond to changes in serine availability by rapidly inducing or repressing transcription of *SER3*. This response involves a dynamic competition between nucleosomes and transcription factors that is controlled by the transcription of *SRG1* from intergenic ncDNA. Our findings raise the intriguing possibility that widespread transcription of ncDNA may impact genome-wide chromatin architecture. In doing so, transcription of ncDNA may influence not only gene expression, but also other cellular processes that are dependent on protein-DNA interactions.

3.0 IDENTIFICATION OF MUTANT VERSIONS OF THE SPT16 HISTONE CHAPERONE THAT ARE DEFECTIVE FOR TRANSCRIPTION-COUPLED NUCLEOSOME OCCUPANCY

The work discussed in this Chapter has been adapted from published material (HAINER *et al.* 2012) and is reprinted, with alterations, by permission from the Genetics Society of America, copyright 2012. This work was a collaborative project in the Martens lab. Erin Walker and I piloted the genetic screen described below and the 2009 University of Pittsburgh Summer Gene Team led by Justin Pruneski, Alison Slinskey-Legg, and Lewis Jacobson performed the large-scare screen for *spt16* mutants. Brittany Charsar and Shayna Cohen verified, subcloned, and sequenced the 25 mutants described in this study. Brittany Charsar and I performed the Northern analysis and Western analysis shown in Figure 21. Shayna Cohen and I performed the dilution analysis shown in Figures 20 and 23. I preformed the rest of the experiments presented in this Chapter.

3.1 INTRODUCTION

The highly conserved heterodimer FACT (Facilitates Chromatin Transactions) is a prominent member of the histone chaperone family with reported functions in multiple nuclear processes including DNA replication, DNA repair, transcription initiation, and transcription elongation

(reviewed in (DUINA 2011; FORMOSA 2008; FORMOSA 2011; WINKLER and LUGER 2011). Its role in transcription elongation has been particularly well supported by both genetic and biochemical experiments involving yeast and mammalian systems (FORMOSA 2011). These include the sensitivity of yeast FACT mutants to the transcription elongation inhibitor 6-azauracil, the genetic interaction of these mutants with other known elongation factors, the co-localization of FACT with RNA pol II across transcribed regions of eukaryotic genomes, the physical association of FACT with other transcription elongation factors, and the requirement of human FACT to allow RNA pol II to transcribe a nucleosomal DNA template *in vitro* (BELOTSERKOVSKAYA *et al.* 2003; FORMOSA *et al.* 2001; FORMOSA *et al.* 2002; KROGAN *et al.* 2002; ORPHANIDES *et al.* 1998; SIMIC *et al.* 2003; SQUAZZO *et al.* 2002). Although the precise molecular functions of FACT in transcription elongation remain under investigation, several studies have strongly implicated FACT in facilitating the nucleosome dynamics that occur during transcription elongation. These studies suggest that FACT associates with a nucleosome in front of RNA pol II resulting in the reorganization of histones that eventually lead to the displacement of H2A-H2B dimers and the passage of RNA pol II (BELOTSERKOVSKAYA *et al.* 2003; MCCULLOUGH *et al.* 2011; ORPHANIDES *et al.* 1998). Once RNA pol II has passed, FACT is also required to assist in the reassembly of nucleosomes to protect recently transcribed DNA from spurious transcription from cryptic intragenic promoters (BELOTSERKOVSKAYA *et al.* 2003; FORMOSA *et al.* 2002; JAMAI *et al.* 2009; ORPHANIDES *et al.* 1999; SCHWABISH and STRUHL 2004; STUWE *et al.* 2008; VANDEMARK *et al.* 2008).

While a role for FACT in facilitating transcription-dependent nucleosome dynamics has been well documented, less is known concerning the precise contribution of the individual FACT subunits. Yeast FACT is composed of two proteins, Spt16 and Pob3, that are essential for

viability and can bind nucleosome *in vitro* when aided by a third protein, the HMG box-containing protein Nhp6 (FORMOSA *et al.* 2001; WITTMEYER and FORMOSA 1997). Pob3 consists of three separate domains defined by limited proteolysis: an N-terminal (NT/D) domain that is thought to be involved in dimerization with Spt16, a middle (M) domain that contains a double pleckstrin homology motif, and an acidic C-terminal (C) domain (LIU *et al.* 2010; VANDEMARK *et al.* 2006). The pleckstrin homology motif has been implicated in assisting the interactions between FACT and RPA, an essential protein involved in DNA replication and repair (VANDEMARK *et al.* 2006). Spt16 has been characterized as having four distinct domains referred to as the N-terminal (NTD), dimerization (D), middle (M), and C-terminal (C) domains (VANDEMARK *et al.* 2006; VANDEMARK *et al.* 2008). Structures of Spt16-NTD, the one domain that is dispensable for viability, from both *S. cerevisiae* and *S. pombe* have been solved by X-ray crystallography, revealing a motif that is structurally similar to bacterial aminopeptidases (STUWE *et al.* 2008; VANDEMARK *et al.* 2008). Although interactions between the Spt16-NTD and histones H2A, H3, and H4 have been reported, the fact that this domain is expendable for Spt16 functions *in vivo* suggests that there are likely to be other regions of Spt16 that functionally and physically interact with histones (O'DONNELL *et al.* 2004; VANDEMARK *et al.* 2008). The Spt16-D domain is thought to interface with the NT/D domain of Pob3 to form the FACT dimer (VANDEMARK *et al.* 2006). Although molecular functions of the Spt16-M domain are not known, mutations altering residues within this domain have resulted in phenotypes indicative of transcription initiation and elongation defects, defects in replication, and defects in cell wall integrity, indicating the functional significance of this domain (MYERS *et al.* 2011; O'DONNELL *et al.* 2009; STEVENS *et al.* 2011). Spt16-C is an acidic domain that is essential for viability whose most 3' end has been shown to functionally interact with histone H3

(BELOTSERKOVSKAYA *et al.* 2003; EVANS *et al.* 1998). Recent *in vitro* analysis of the human Spt16-C domain have implicated this domain in the active displacement of nucleosomal DNA during nucleosome reorganization (WINKLER *et al.* 2011).

We have provided evidence that FACT contributes to a new mechanism of gene regulation operating at the *S. cerevisiae SER3* gene based on its ability to promote transcription-coupled nucleosome dynamics (described in Chapter 2) (HAINER *et al.* 2011; MARTENS *et al.* 2004). In the presence of serine, transcription of intergenic *SRG1* DNA initiates 5' of the adjacent *SER3* gene, which encodes an enzyme for serine biosynthesis (MARTENS *et al.* 2004; MARTENS *et al.* 2005). As a consequence of *SRG1* transcription across the *SER3* promoter, FACT assists in the assembly and maintenance of nucleosomes over this region that is normally depleted of nucleosomes (HAINER *et al.* 2011). The presence of these nucleosomes at the *SER3* promoter inhibits the binding of transcription factors required to induce *SER3* transcription. In this Chapter, I present the results of an unbiased genetic screen to identify mutations of *SPT16* that derepress *SER3* transcription. Our analyses of these mutants indicate that the integrity of both the Spt16-D and Spt16-M domains are not only required for *SRG1* transcription-dependent nucleosome assembly and *SER3* repression, but are more broadly required for transcription-coupled nucleosome occupancy at highly transcribed genes. I provide evidence suggesting a possible role for the Spt16-D and Spt16-M domains in promoting the association of FACT to genes being actively transcribed.

3.2 MATERIALS AND METHODS

3.2.1 Strains and Media

All *S. cerevisiae* strains used in this study (Table 3) are isogenic to a *GAL2*$^{+}$ derivative of S288C (WINSTON *et al.* 1995). All strains were constructed by transformation or by genetic crosses (AUSUBEL 1991). YJ920 and YADP50 have been previously described (HAINER *et al.* 2011; MYERS *et al.* 2011). Strains YJ1089-YJ1092 were derived from YJ920. The *spt16*Δ::*KanMX* and *lys2-128*δ alleles have been previously described (CLARK-ADAMS *et al.* 1988; MYERS *et al.* 2011). The *lyp1*Δ::*SER3*pr-*HIS3* allele was generated by replacing the *URA3* open reading frame in *lyp1*Δ::*SER3*pr-*URA3* (HAINER and MARTENS 2011a) with a PCR product containing the *HIS3* open reading frame that was amplified from pRS403 (SIKORSKI and HIETER 1989). pAO01 and p*SPT16-URA3* are centromeric plasmids marked with *LEU2* and *URA3*, respectively, that contain wild-type *SPT16* (kindly provided by A. Duina) (MYERS *et al.* 2011). Derivatives of pAO01 containing *spt16-G132D* and *spt16-T828I/P859S* alleles were generated by standard cloning methods and verified by sequencing. All other *spt16* mutants characterized in this study are expressed from plasmids derived from pAO01. Yeast extract-peptone-dextrose (YPD), synthetic complete (SC), omission (SC-), 5-fluoroorotic acid (5-FOA), and galactose media have been previously described (ROSE 1991). YPD was supplemented with 5ug/mL cyclohexamide (CHX) or 200mM hydroxyurea (HU) as indicated. 3-amino-1,2,4-triazole (3-AT; Sigma) was added to SC medium lacking leucine and histidine at the indicated concentrations.

Table 3. *Saccharomyces cerevisiae* strains used in Chapter 3.

Strain	Genotype	Reference or Source
YJ920	*MATα ura3Δ0 leu2Δ0 his3Δ200 lyp1Δ::SER3pr-URA3*	(HAINER and MARTENS 2011a)
YJ1089	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ200 lyp1Δ::SER3pr-HIS3 spt16Δ::KanMX <pSPT16-URA3>*	This study
YJ1090	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ200 lyp1Δ::SER3pr-HIS3*	This study
YJ1091	*MATα ura3Δ0 leu2Δ0 lys2-128δ trp1Δ63 spt16Δ::KanMX <pSPT16-URA3>*	This study
YJ1092	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ200 spt16Δ::KanMX KanMX-GAL1pr-FLO8-HIS3 <pSPT16-URA3>*	This study
YADP50	*MATα his3Δ200 leu2Δ1 ura3-52 lys2-128δ (hht1-hhf1)Δ::HIS3 hht2-11 spt16Δ::KanMX pSPT16*	(MYERS *et al.* 2011)
YJ586	*MATα ura3Δ0 leu2Δ0 his3Δ200*	(HAINER *et al.* 2011)
YS117	*MAT***a** *ura3-52 leu2Δ1 lys2-128δ spt16-ΔN(*469-1035*)*	This study

3.2.2 Screen for *spt16* mutants that derepress *SER3*

Using a previously described strategy (MYERS *et al.* 2011), two regions of *SPT16,* from +764 to +2044 (region B) and from +1430 to +3521 (region C), were amplified from pAO01 plasmid (gift from A. Duina) using GoTaq polymerase (Invitrogen) and standard PCR conditions. Amplified DNA was co-transformed into YJ1089 with pAO01 plasmid that had been digested with either *Eag*1 and *Sna*B1 (region B) or *Sna*B1 and *Xba*I (region C). Transformants containing gap-repaired plasmids were selected on SC medium lacking leucine and then replica-plated onto medium containing 5-FOA to select for cells that lost the *URA3*-marked plasmid carrying a wild-type copy of *SPT16* (p*SPT16-URA3*). The resulting colonies were then replica-plated to SC medium lacking histidine and leucine that was supplemented with 5mM 3-AT. Candidate plasmids were recovered from strains resistant to 5mM 3-AT, re-transformed into YJ1089 and retested for their ability to confer 3-AT resistance. The region of *SPT16* that was subjected to PCR mutagenesis was first subcloned into a new copy of pAO01 before retransformation. For each plasmid that retested for 3-AT resistance, both strands of the entire *SPT16* gene were sequenced and compared to the wild type gene.

3.2.3 Northern analysis

Cells were grown to approximately 2×10^7 cells/ml in YPD at 30°C. Total RNA isolation and Northern analysis was performed as previously described (COLLART and OLIVIERO 2001). Radiolabeled DNA probes to *SRG1* (-454 to -123 relative to *SER3* ATG), *SER3* (+111 to +1342), and *SCR1* (-163 to +284) were generated by random-primed labeling of PCR fragments

amplified from genomic DNA. RNA levels were quantified using a PhosphorImager (FLA-5000) and ImageJ software.

3.2.4 Western analysis

Whole cell extracts (WCE) were prepared from cells grown in YPD at 30°C to approximately 3 × 10^7 cells/ml using trichloroacetic acid as previously described (COX *et al.* 1997; ZHENG *et al.* 2010). Equal amounts of WCE were separated by 10% acrylamide SDS-PAGE, transferred to Protean nitrocellulose (Whatman), and assayed by immunoblotting. The antibodies used to detect Spt16, Pob3, and G6PDH were as follows: anti-Spt16 (1:500; gift from Tim Formosa), anti-Pob3 (1:2000; gift from Tim Formosa), anti-G6PDH (1:50,000; Sigma). After incubation with HRP-conjugated IgG secondary antibody (1:5000; GE Healthcare), the immunoreactive proteins were visualized by enhanced chemiluminescence detection (Perkin-Elmer) using a Kodak image station 440CF. Spt16 and Pob3 protein levels were calculated by measuring their signal intensities in these western blots using Kodak ID 3.6 software and normalizing these values to those obtained for the G6PDH control.

3.2.5 Dilution growth assays

Cells were grown at 30°C overnight to saturation then washed twice with water. Starting at 1 × 10^8 cells/ml, cultures were serially diluted 10-fold. 3 µl of each dilution was spotted onto indicated media and incubated at 30°C for the indicated number of days.

3.2.6 Nucleosome scanning assays

Cells were grown at 30°C to approximately 2 × 10^7 cells/ml in YPD and subjected to a nucleosome scanning assay, as previously described in Chapter 2 (HAINER *et al.* 2011). For each of the 38 *SER3* primer pairs, the amount of template protected from digestion by micrococcal nuclease (MNase) was calculated as a ratio between MNase-digested and undigested samples and then normalized to the amount of MNase-protected control template (*GAL1* NB) that is located within a well-positioned nucleosome in the *GAL1* promoter (BRICKNER *et al.* 2007; FLOER *et al.* ; FLOER *et al.* 2010).

3.2.7 Chromatin Immunoprecipitation (ChIP) assays

Cells were grown in YPD at 30°C to approximately 2 × 10^7 cells/ml. Chromatin was prepared as previously described (SHIRRA *et al.* 2005). Histone H3, Spt16, or Rpb3 were immunoprecipitated by incubating sonicated chromatin overnight at 4°C with 1µl anti-histone H3 antisera (previously described in (TOMSON *et al.* 2011)), 1µl anti-Spt16 antisera (kindly provided by Tim Formosa), or 2.5µl anti-Rpb3 antisera (W0012, Neoclone) followed by the addition of IgG-Sepharose beads (GE Healthcare) for 2 hours at 4°C. Dilutions of input DNA and immunoprecipitated DNA were analyzed by qPCR reactions. Primer sets that amplify the following regions were used for qPCR: *SER3*-41 (-921 to -828, relative to +1 ATG of *SER3*), *SER3*-25 (-338 to -289, relative to +1 ATG of *SER3*), *SER3*-22 (-300 to -200, relative to +1 ATG of *SER3*), *SER3*-7 (+195 to +295), *PYK1* (5': +62 to +164, 3': +1173 to +1279), *PMA1* (5': +691 to +794, 3': +1689 to +1791), *ADH1* (+845 to +943), *CYC1* (+122 to +217), *TUB2* (5': +105 to +202, 3': +1083 to +1189) and *GAL1* (5': +79 to +175, 3': +1366 to + 1487). Histone H3, Spt16, and Rpb3 ChIP signals for each gene

were normalized to a No ORF control template, which is located within a region of chromosome V that lacks open reading frames (KOMARNITSKY *et al.* 2000).

3.2.8 Quantitative PCR (qPCR)

All qPCR data for the nucleosome scanning and ChIP assays were obtained by using an ABI StepOne Plus Real-time system using SYBR green reagents (Fermentas) and the indicated primers (HAINER *et al.* 2011). Calculations were performed using Pfaffl methodology (PFAFFL 2001).

3.3 RESULTS

3.3.1 Identification of *spt16* mutants that derepress *SER3*

In Chapter 2, I described a new mechanism of gene regulation in *S. cerevisiae* whereby transcription of *SRG1* ncDNA assembles nucleosomes over the promoter of the adjacent *SER3* gene to maintain *SER3* repression (HAINER *et al.* 2011). Furthermore, we provided evidence that the histone chaperones, Spt6 and Spt16, are required to maintain this nucleosome occupancy, and repress *SER3*, likely through their ability to disassemble and reassemble nucleosomes during active transcription (BELOTSERKOVSKAYA *et al.* 2003; HAINER *et al.* 2011). To investigate the role of Spt16 in this mechanism, we performed an unbiased genetic screen to identify novel mutations in *SPT16* that derepress *SER3* during *SRG1* transcription. A PCR-based strategy that has been previously described (see Materials and Methods) (MYERS *et al.* 2011) was used to

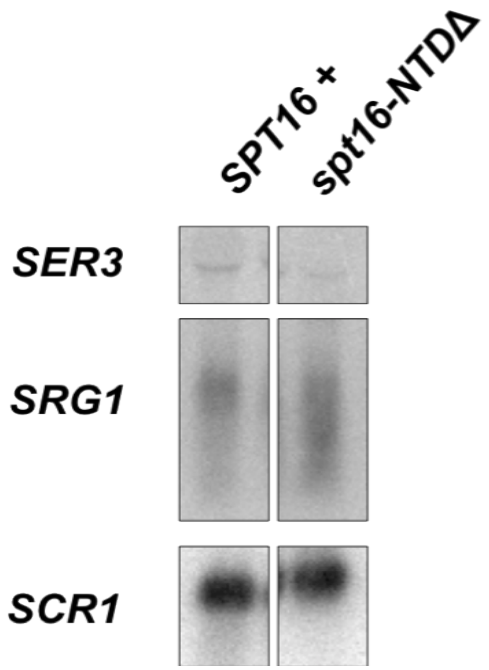

Figure 18. The N-terminal domain of Spt16 is not required for *SER3* regulation.

Northern blot analysis examining the effect of *spt16*-NTDΔ mutant on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from cells expressing either wild-type (YJ586) or *spt16*-NTDΔ (YS117) alleles that were grown to a density of ~2 × 10^7 cells/ml in YPD at 30°C.

target mutagenesis of the 3' half of *SPT16* that excludes most of the N-terminal domain (NTD), which is dispensable for *SER3* repression (Figure 18). These PCR fragments were co-transformed with a gapped *LEU2*-marked plasmid that contained homology to the PCR fragments into an *spt16*Δ *his3*Δ strain containing an integrated *SER3*pr-*HIS3* reporter (Figure 19A) and expressing a wild type copy of *SPT16* from a *URA3*-marked plasmid (YJ1089). Following gap-repair and loss of the *URA3*-marked plasmid expressing *SPT16*, we screened for *spt16* mutants that derepress the *SER3*pr-*HIS3* reporter by their ability to confer growth in the presence of 3-AT, a competitive inhibitor of the *HIS3* gene product (Figure 19).

With this screen, we initially identified 522 mutants that permit growth on medium containing 5mM 3-AT. *SPT16*-containing plasmids were then recovered from a subset of these strains that conferred resistance up to 40mM 3-AT to enrich for mutations that most strongly derepress *SER3*. After retesting for their ability to derepress the *SER3*pr-*HIS3* reporter, the entire *SPT16* gene contained on each of these plasmids was sequenced. Sequencing of 38 plasmids identified 25 unique *spt16* mutants harboring nucleotide changes that result in either single (12), double (11), or triple (two) amino acid substitutions. For the 12 single amino acid substitution mutants, the location of the altered amino acids varies - three are located at the very 3' end of the Spt16-NTD, three are found in Spt16-D, and the remaining six are found in Spt16-M, including four residues that are within 13 amino acids of each other (Figure 19B). Interestingly, nine of the 13 double or triple mutants contain one of the single amino acid substitutions, indicating that the effect on the *SER3* reporter from these mutation combinations is likely through the isolated single substitution. Interestingly, only one of these mutations, *spt16-E857K*, has been previously reported (O'DONNELL *et al.* 2009; STEVENS *et al.* 2011). In these studies, *spt16-E857K* was isolated as a dominant suppressor of a transcription defect caused by the insertion of a δ element

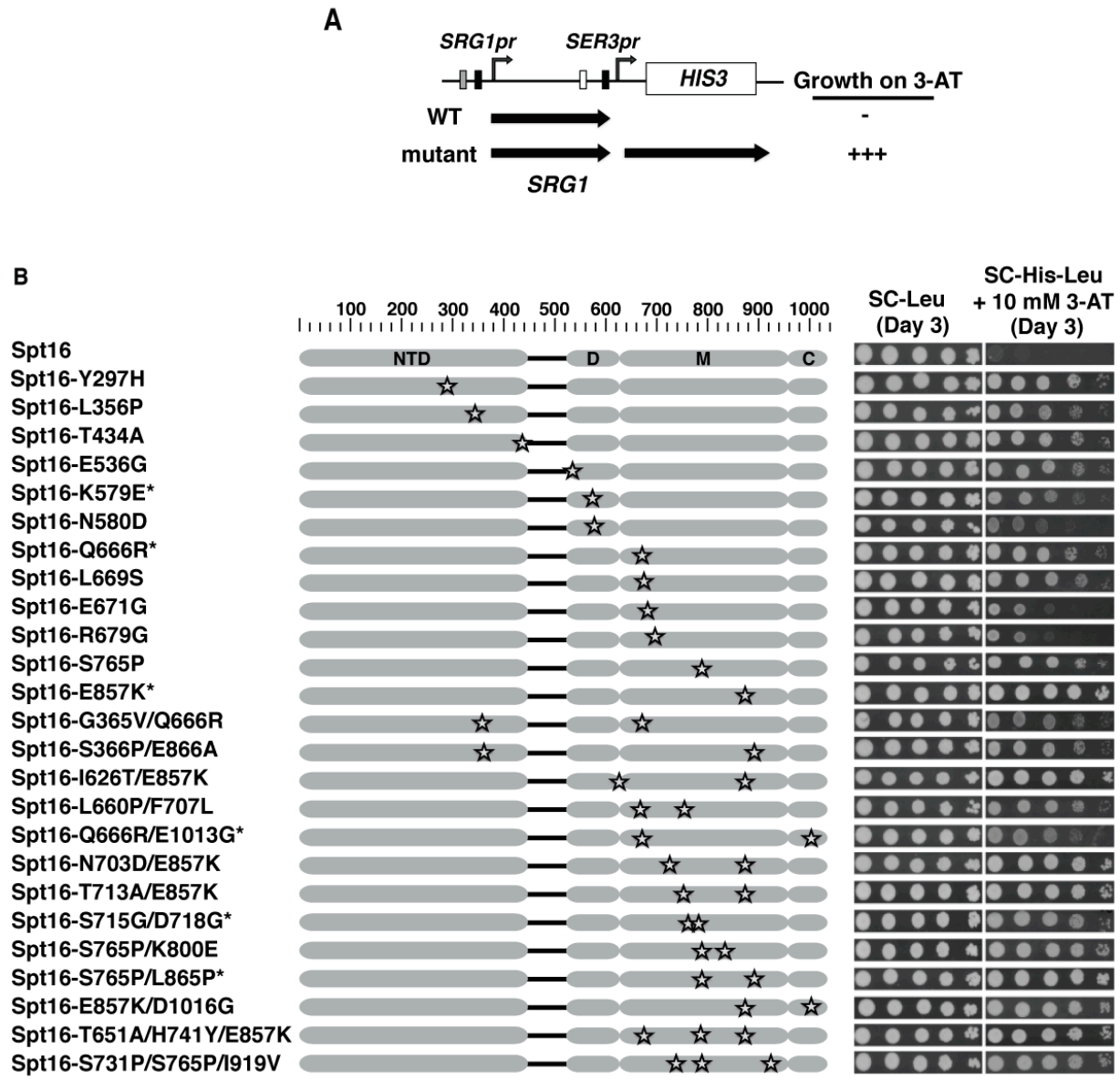

Figure 19. Identification of *spt16* mutants that derepress an ectopically expressed *SER3*pr-*HIS3* reporter gene.

A) Diagram of *SER3*pr-*HIS3* reporter. The *LYP1* ORF was replaced by *SER3* intergenic sequence from -713 to -1, including *SRG1* and its promoter, fused to the *HIS3* ORF. Block arrows beneath the diagram indicate the expected *SRG1* and *SER3-HIS3* transcripts in wild-type and mutant strains grown in serine rich media (YPD). The expected growth of these strains on SC-His-Leu plates containing 3-AT is indicated on the right. B) Growth assays indicating that newly isolated *spt16* mutants derepress *SER3*pr-

HIS3 reporter. *spt16*Δ cells (YJ1089) containing *LEU2*-marked plasmids expressing either wild-type or mutant Spt16 protein, as indicated, were grown to saturation in YPD, diluted to 10^8 and then spotted in a 10-fold serial dilution series on SC-His-Leu (control) and SC-His-Leu+10mM 3-AT plates. Plates were incubated at 30°C for 3 days. Results were obtained for two independent growth assays where each plate contained control strains and five to six mutants. Shown are representative dilutions for the control strains and each *spt16* mutant strain. Each Spt16 mutant protein is named to describe the location and nature of the amino acid substitution. The location of the amino acid substitutions in each of these mutants are also indicated (marked by stars) in diagrams of Spt16 where gray ovals the N-terminal (NTD), dimerization (D), and middle (M) domains and the C-terminal acidic tail region (C). Note that the five mutants marked by an asterisk each have an additional silent mutation.

5' of the *LYS2* and *HIS4* genes (Spt$^-$ phenotype) and was found to genetically interact with mutations in other transcription elongation factors.

3.3.2 Phenotypic analysis of the *spt16* mutants

To further characterize these mutants, we tested these strains for temperature sensitivities and growth defects on YPD medium supplemented with cycloheximide (CHX), hydroxyurea (HU), mycophenolic acid (MPA), and caffeine. Surprisingly, we found that not one of the *spt16* mutants that we isolated confer a growth defect at elevated temperatures (39°C) or in the presence of HU (Figure 20A), phenotypes that have been previously described for other *spt16* alleles, including *spt16-G132D* (Figure 20A, row 2) and *spt16-T828I/P859S* (Figure 20A, row 3) (FORMOSA *et al.* 2001). Interestingly, one mutant, *spt16-S715G/D718G*, confers cold sensitivity at 15°C, and a number of the mutants cause varying sensitivities to CHX (Figure 20A). No detectable growth defects were observed when strains expressing any one of the isolated *spt16* mutants were exposed to MPA or caffeine (S.B.C, data not shown).

We also tested whether the *spt16* mutants that we isolated are dominant for repression of the *SER3*pr-*HIS3* reporter. YJ1090 cells containing wild-type *SPT16* at its genomic location, *SER3*pr-*HIS3*, and a plasmid expressing either wild-type or mutant versions of Spt16, were spotted onto media containing 3-AT to test for expression of the *SER3*pr-*HIS3* reporter. One mutant allele, *spt16-E857K,* is dominant for derepression of the *SER3* reporter gene, suggesting that it may be a gain-of-function mutation (Figure 20B). Interestingly, our analysis of the more complex mutants identified amino acid substitutions at *I626T* and one or both of *T651A* and *H471Y* as intragenic suppressors of the dominant effect of this *E857K* substitution. Additionally, our analysis revealed that whereas the mutant *spt16-S765P* allele alone does not confer

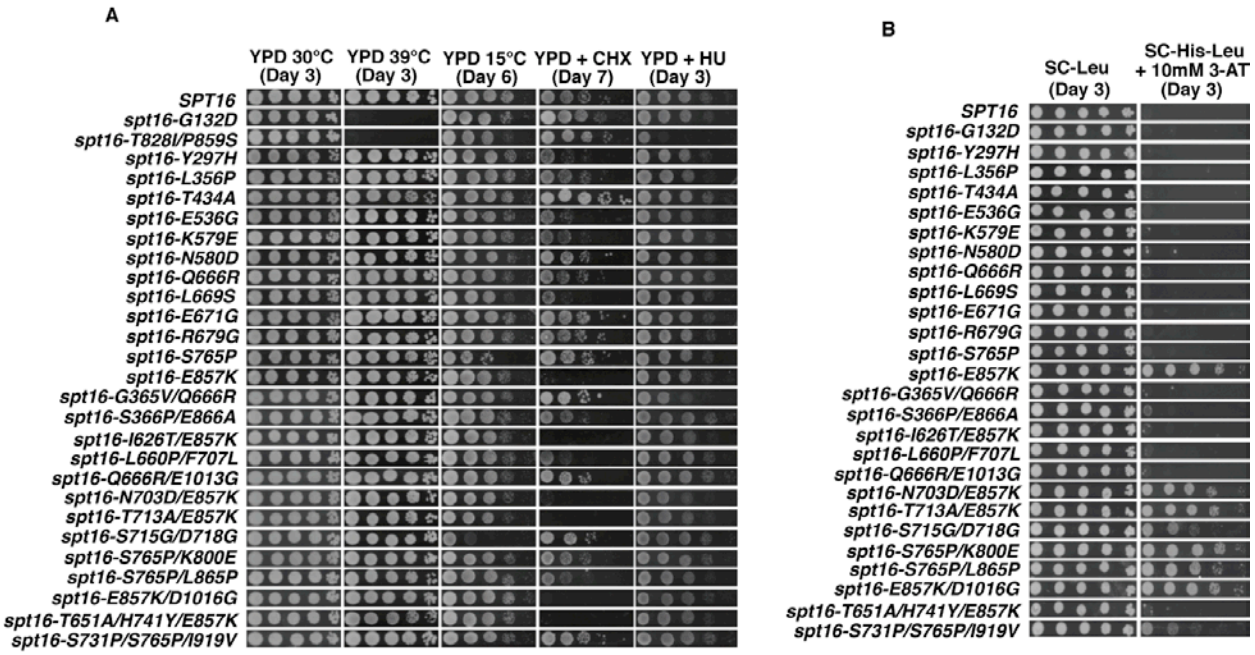

Figure 20. Phenotypic characterization of newly isolated *spt16* mutants.

A) Temperature, cycloheximide (CHX), and hydroxyurea (HU) sensitivity of *spt16* mutants. *spt16*Δ cells (YJ1091) expressing either wild-type or the indicated mutant alleles of *SPT16* from a *LEU2*-marked plasmid were growth to saturation in YPD at 30°C, diluted to 10^8, spotted in 10-fold serial dilutions and incubated for the indicated number of days on solid media. Cells spotted on YPD were incubated at 30°C, 39°C, or 15°C, while cells spotted on YPD+CHX or YPD+HU were grown at 30°C. These results are a representative of two biological replicates. B) Dominance test. Cells expressing wild-type *SPT16* from its genomic location and the *SER3*pr-*HIS3* reporter (YJ1090) were transformed with plasmids containing either wild-type or the indicated mutant *SPT16* alleles. Transformants were grown to saturation in YPD at 30°C and spotted on SC-His-Leu (control) and SC-His-Leu+10mM solid medium, which were then incubated at 30°C for three days. These results are representative of two biological replicates.

dominance, it is synthetically dominant for *SER3* derepression with either a *K800E* or *L865P* substitution mutation.

3.3.3 *spt16* mutants derepress endogenous *SER3*

We next determined the effect of these *spt16* mutants on endogenous *SER3* and *SRG1* RNA levels. We transformed plasmids containing either wild-type *SPT16*, a previously characterized *spt16-G132D* mutant (MALONE *et al.* 1991) or one of our newly isolated *spt16* mutants into YJ1091 and YJ1092 strains and performed Northern assays on these strains (Figure 21A and 21B). For these and subsequent experiments, we limited our analysis to the 12 *spt16* mutants having single amino acid substitutions. All of the *spt16* mutants tested derepress *SER3* with effects ranging from very strong (30-fold increase for *spt16-E857K*) to more mild effects (two-fold for *spt16-Y297H, spt16-N580D, spt16-E671G,* and *spt16-S765P*) that are similar to what we had previously observed for *spt16-G132D* (HAINER *et al.* 2011). Although we did observe some variability in *SRG1* RNA levels between experiments, average results from four independent experiments indicate that these *spt16* mutants do not significantly alter *SRG1* RNA levels. Consistent with these Northern data, we find equivalent levels of RNA pol II localized across the *SRG1* transcription unit in strains expressing either wild-type or mutant versions of Spt16 (Figure 24B). Moreover, Western analyses show that these newly isolated *spt16* mutants do not alter the levels of Spt16 or its' interacting partner, Pob3 (Figure 21C and 21D). Taken together, these data identify amino acids in Spt16 that are critical for *SER3* repression.

3.3.4 Effect of *spt16* mutants on nucleosome occupancy over the *SER3* promoter

To examine the effect of a subset of the *spt16* mutants on nucleosome occupancy at *SER3*, I performed nucleosome scanning assays on seven of the single amino acid substitutions that most strongly derepress *SER3* (Figure 22). As previously described (HAINER *et al.* 2011), micrococcal nuclease (MNase) protection across *SER3* was normalized to the protection of a well-studied, nucleosome-bound region of the *GAL1* promoter whose digestion by MNase is unaffected by these *spt16* mutants (see Materials and Methods for details). Compared to strains containing wild-type control plasmids, protection from MNase digestion was reduced across the *SRG1* transcribed region in all the *spt16* mutants examined to degrees approximately equal to or exceeding that of *spt16-G132D* (Figure 22), which we had previously shown to decrease nucleosome occupancy across the *SER3* locus (Chapter 2) (HAINER *et al.* 2011). MNase protection across the *SER3* promoter region was most dramatically reduced in the *spt16-E857K* mutant (Figure 22H), which is consistent with the strong derepression of *SER3* that is observed in this mutant. The other six mutants that display more modest defects in *SER3* repression also have more modest reductions in the MNase protection across the *SER3* promoter. However, I did observe subtle differences in the MNase protection patterns between these mutants. Two of the *spt16* mutants resulted in greater sensitivity to MNase towards the 5' of *SRG1* relative to the 3' of *SRG1* (*spt16-K579E* and *spt16-L669S*), compared to the other mutants that had increases in MNase sensitivity that were more evenly distributed across the *SRG1* transcription unit (Figure 22, compare -400 and -200 regions in panels D and F to panels E and G).

To confirm that the changes in MNase protection across the *SRG1* transcription unit caused by these *spt16* mutants reflect changes in nucleosome occupancy, I measured histone occupancy across this region by ChIP. For the most part, histone H3 occupancy across the *SRG1*

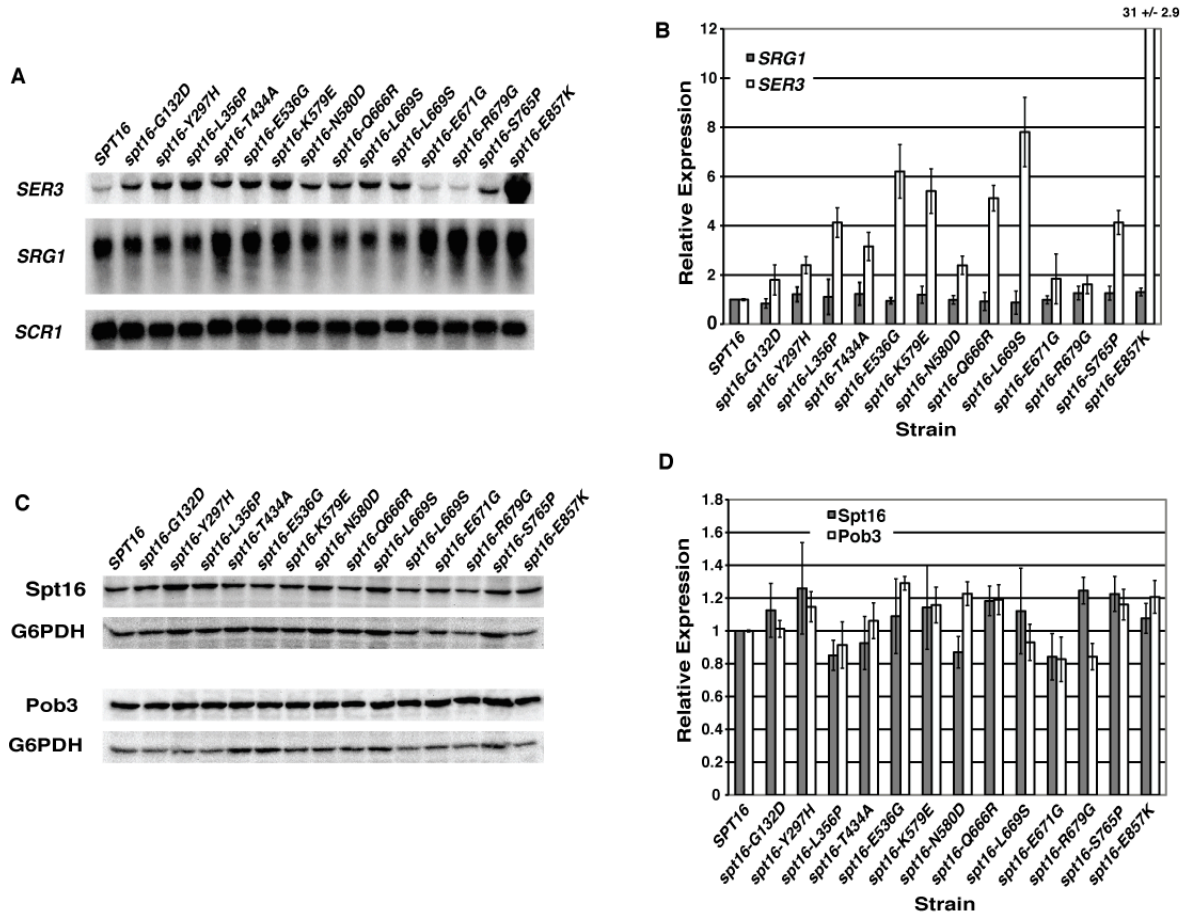

Figure 21. Single amino acid substitutions in Spt16 strongly derepress endogenous *SER3*.

A) Northern blot analysis examining the effect of *spt16* mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from *spt16*Δ cells (YJ1091) carrying plasmid-borne wild-type or mutant *SPT16* alleles that were grown to a density of ~2 × 10^7 cells/ml in YPD at 30°C. B) Quantitation of Northern data. *SRG1* (gray bars) and *SER3* (white bars) RNA levels for the *spt16* mutants are normalized to the level of the *SCR1* loading control and are relative to strains expressing wild-type *SPT16* (arbitrarily set to 1). Each bar indicates the mean RNA level +/- SEM from four independent experiments using two transformations each of YJ1091 and YJ1092. C) Western analysis examining the effect of *spt16* mutant alleles on mutant Spt16 and Pob3 protein levels. Whole cell extracts were prepared from the same set of strains described in Panel A grown to ~3 × 10^7 cells/ml in YPD at 30°C and subjected to Western analysis using anti-Spt6 and anti-Pob3 antibodies (kindly provided by T. Formosa). Blots were re-probed with

anti-G6PDH antibody as a loading control. D) Quantitation of Western data. Spt16 (gray bars) and Pob3 (white bars) protein levels are normalized to the G6PDH loading control and are relative to strains expressing wild-type *SPT16* (arbitrarily set to 1). Each bar indicates the mean protein level +/- SEM from three independent experiments using the same set of strains as in Panel B.

transcription unit was reduced in the *spt16* mutants to degrees that correlate with the results of our MNase experiments (Figure 22I). Taken together, these data identify Spt16 residues whose integrity are required to maintain *SER3* repression by facilitating *SRG1* transcription-dependent nucleosome occupancy across the *SER3* promoter.

3.3.5 Effect of *spt16* mutations on phenotypes associated with defects in transcription and chromatin structure

Having shown a role for at least seven of the *spt16* single mutants in regulating chromatin structure at *SER3*, we tested whether all 12 single mutants confer other phenotypes indicative of chromatin-related transcriptional defects. We first determined if these *spt16* mutants can confer an Spt$^-$ phenotype (suppressor of Ty δ element insertion), which is caused by defects in chromatin and aberrant transcription initiation (CLARK-ADAMS *et al.* 1988). *spt16*Δ strains containing the *lys2-128*δ allele were transformed with plasmids containing either wild-type *SPT16* or mutant *spt16* alleles and assayed for their ability to grow on medium lacking lysine (Figure 23A). As a control, I also introduced a plasmid expressing the *spt16-G132D* allele, which has been previously shown to have an Spt$^-$ phenotype (EVANS *et al.* 1998). Compared to the cells expressing wild-type *SPT16,* most of the *spt16* mutants grow robustly in the absence of lysine, similar to what is observed for the *spt16-G132D* control, indicating that these mutants confer a strong Spt$^-$ phenotype (Figure 23A). In contrast, the two *spt16* mutants that most weakly derepress *SER3*, *spt16-E671G* and *spt16-E679G*, had no detectable Spt$^-$ phenotype.

Next, we tested whether these *spt16* mutants permit the production of aberrant intragenic transcripts, a phenotype that has been associated with defects in transcription-coupled nucleosome reassembly (CARROZZA *et al.* 2005; KAPLAN *et al.* 2003). For these experiments, we

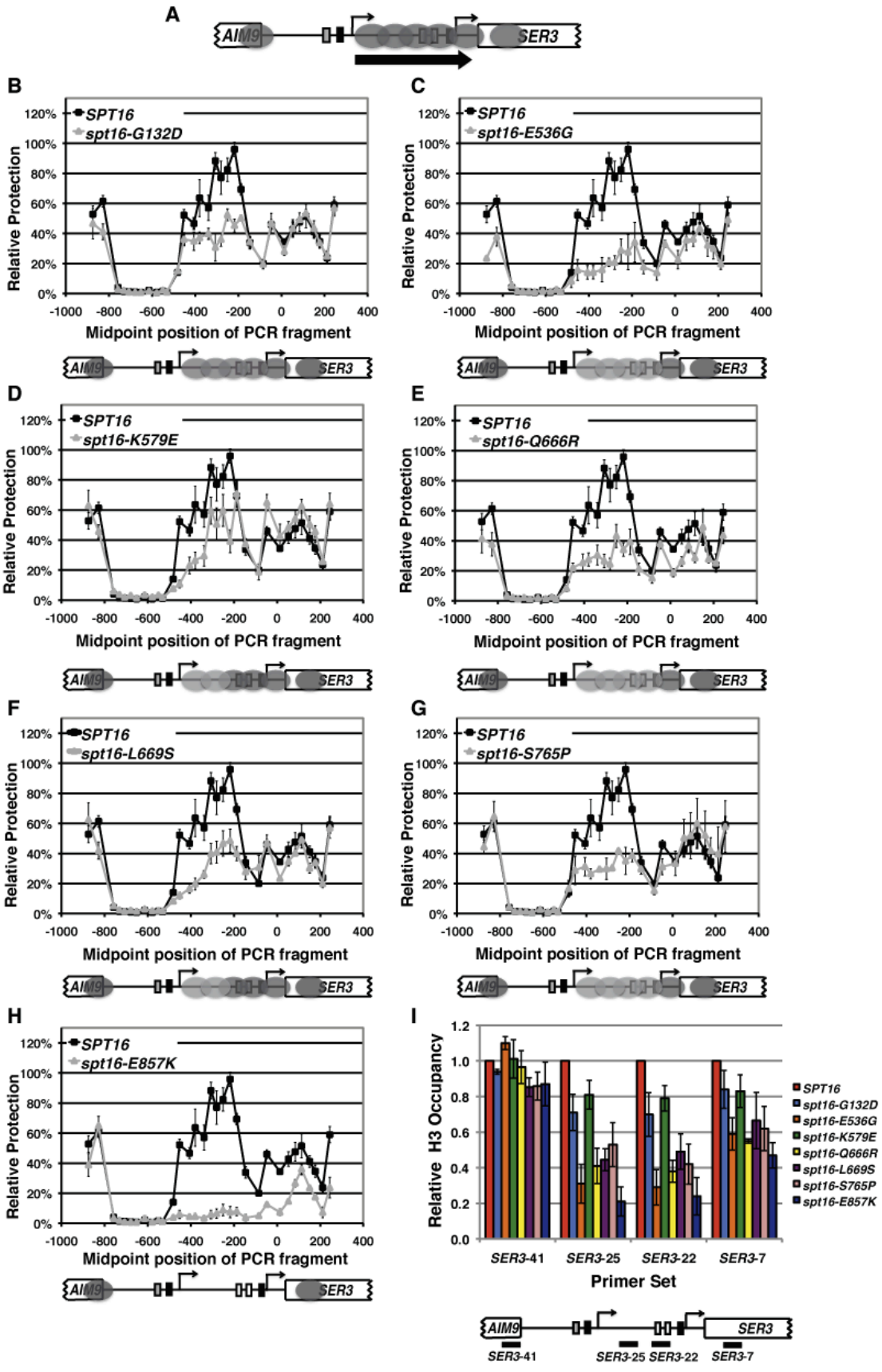
A
AIM9
SER3
B
SPT16
spt16-G132D
Relative Protection
Midpoint position of PCR fragment
C
SPT16
spt16-E536G
Relative Protection
Midpoint position of PCR fragment
D
SPT16
spt16-K579E
Relative Protection
Midpoint position of PCR fragment
E
SPT16
spt16-Q666R
Relative Protection
Midpoint position of PCR fragment
F
SPT16
spt16-L669S
Relative Protection
Midpoint position of PCR fragment
G
SPT16
spt16-S765P
Relative Protection
Midpoint position of PCR fragment
H
SPT16
spt16-E857K
Relative Protection
Midpoint position of PCR fragment
I
Relative H3 Occupancy
SER3-41
SER3-25
SER3-22
SER3-7
Primer Set
SPT16
spt16-G132D
spt16-E536G
spt16-K579E
spt16-Q666R
spt16-L669S
spt16-S765P
spt16-E857K

Figure 22. Effect of *spt16* mutants on chromatin structure at *SER3*.

A) Diagram of the *SER3* locus. The gray ovals mark the position of nucleosomes when wild-type cells are grown in *SER3* repressing conditions (YPD). The block arrow indicates *SRG1* transcription. B-J) Nucleosome scanning assays were performed on *spt16*Δ cells (YJ1091 and YJ1092) carrying plasmids expressing either wild-type *SPT16* or mutant *spt16* alleles as indicated. Mononucleosome-sized DNA fragments were generated by micrococcal nuclease (MNase) digestion of formaldeyde-treated chromatin that was isolated from cells grown to ~2×10^7 cells/mL in YPD media at 30°C . MNase protection across the *SER3* locus relative to a positioned nucleosome within the *GAL1* promoter was determined by qPCR. For each PCR amplicon, the mean MNase protection +/- SEM from three independent experiments is plotted at its midpoint. Shown below each graph is a diagram of the *SER3* locus indicating the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data for each *spt16* mutant. The light gray ovals are indicative of less dramatic reductions in MNase protections as compared to the wild-type control shown in Panel A. I) Histone H3 ChIP was performed on chromatin isolated from the same strains used in panel B-H). The amount of immunoprecipitated DNA was determined by qPCR as a fraction of the input that was then normalized to a control region in chromosome V and made relative to strains expressing wild-type *SPT16* (arbitrarily set to 1). Each bar represents the mean +/- SEM of three independent experiments. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

employed a previously described *GAL*pr-*FLO8-HIS3* reporter gene whereby *HIS3* gene expression is dependent on transcription initiation from a cryptic promoter within the *FLO8* coding sequence (CHEUNG *et al.* 2008). Therefore, cryptic intragenic transcription can be measured by the growth of *his3*Δ cells containing this reporter construct on medium lacking histidine. For this assay, strains expressing plasmid-borne *SPT16* or the indicated *spt16* mutant alleles were monitored for growth on medium lacking histidine. When grown in galactose-containing medium, all but two of the *spt16* mutants allowed cells to grow in the absence of histidine, indicative of robust transcription initiation from the cryptic promoter within the *FLO8* coding sequence (Figure 23B). For the most part, these data correlate well with the Spt$^-$ phenotypic data, suggesting that the molecular defects resulting in these two phenotypes are likely related. Interestingly, only those mutations within the N-terminal domain of Spt16 allow cells to grow in glucose-containing medium lacking histidine, suggesting that these mutants permit cryptic transcription initiation even in the absence of significant levels of transcription across this region.

Finally, we tested the *spt16* mutants for their ability to suppress a cold sensitive (cs) phenotype of a histone mutant, H3 L61W, a phenotype that has been previously described for a distinct class of mutations located within the Spt16 M-domain (MYERS *et al.* 2011). For this assay, *spt16*Δ cells containing the H3 L61W mutant as the sole source of histone H3 (YADP50) and plasmid-borne copies of wild type or mutant versions of *SPT16* were monitored for growth on YPD at 15°C (Figure 23C). When compared with the *spt16-E735G* control (kindly provided by Andrea Duina), none of our identified *spt16* mutants suppress the cold sensitivity of H3 L61W. Therefore, the *spt16* mutants we isolated as being defective for *SER3* repression represent

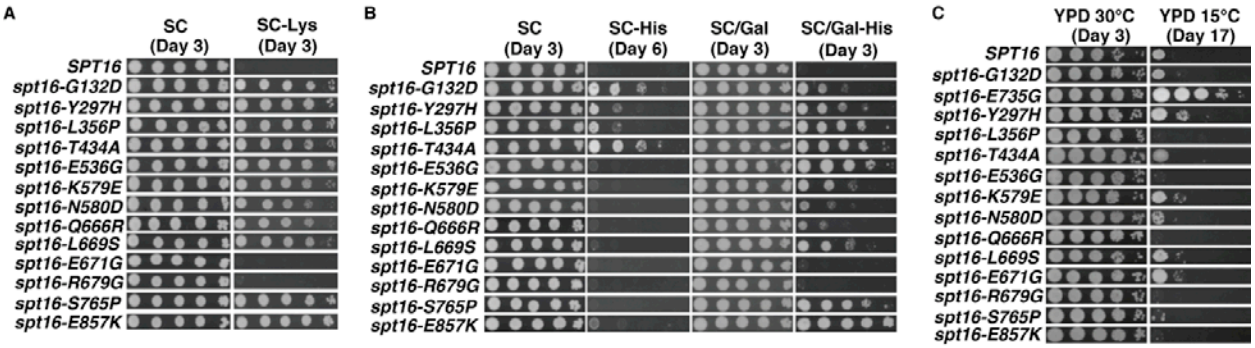

Figure 23. Analysis of *spt16* mutants for phenotypes associated with defects in transcription and chromatin.

A) Assay for Spt$^-$ phenotype. *spt16*Δ cells containing the *lys2-128*δ allele and plasmids that express either wild-type or mutant Spt16, as indicated, were grown to saturation in YPD at 30°C, diluted to 10^8, and spotted in 10-fold serial dilutions onto solid synthetic complete medium (SC) and synthetic complete medium lacking lysine (SC-Lys). Plates were incubated at 30°C for 3 days. These results are representative of two independent assays using transformations of YJ1091. B) Assay for cryptic transcription initiation. *spt16*Δ cells containing the *FLO8-HIS3* reporter and plasmids that express either wild-type or mutant Spt16, as indicated, were grown to saturation in YPD at 30°C. Serial diluted cells were spotted onto solid synthetic complete medium with or without histidine containing either glucose (SC and SC-His) or galactose (SC/Gal and SC/Gal-His) as a carbon source. Plates were incubated at 30°C for either 3 days (SC, SC/Gal, and SC/Gal-His) or 6 days (SC-His). These results are representative of two independent assays using transformants of YJ1092. C) Assay for suppression of histone H3 L61W mutant. *spt16*Δ cells expressing the H3-L61W mutant as its sole source of histone H3 and plasmids expressing either wild-type or mutant Spt16, as indicated, were grown to saturation in YPD at 30°C. Serial diluted cells were spotted onto solid YPD medium as described in panel A and grown at 30°C (3 days) or 15°C (17 days). These results were generated with strains derived from YADP50 (kindly provided by A. Duina) and are representative of two independent assays.

a distinct class of mutants from those that suppress the cs phenotype of the H3 L61W mutant and may define functionally distinct regions of the Spt16-D and Spt16-M domains.

3.3.6 Occupancy of mutant versions of Spt16 is reduced across *SRG1* and the *SER3* promoter region

I next considered the possibility that these mutant versions of Spt16 fail to be recruited normally to transcribed regions, which may account for their multiple phenotypes related to defects in transcription-coupled nucleosome occupancy. Therefore, I performed ChIP experiments to assess the binding of selected Spt16 mutant proteins across the *SRG1* transcription unit (Figure 24A). In general, I detected reduced binding of most of the mutant versions of Spt16 that parallel the loss of histone H3 occupancy across this region that we observed in these mutant versions (compare Figures 24A to 22I). The lone exception is the *spt16-K579E* mutant where we detect a stronger decrease in the occupancy of the mutant protein expressed from this allele then expected based on a relatively modest decrease in histone H3 occupancy. Because Spt16 strongly co-localizes with RNA pol II across transcribed genes, I tested whether the decrease in the occupancy of the mutant versions of Spt16 might be indirect due to a decrease in RNA pol II occupancy at *SER3*. To this end, I performed ChIP analysis of Rpb3, a subunit of RNA pol II, over *SRG1* (Figure 24B). Consistent with our Northern analysis (Figure 21), I found that all but one of these *spt16* mutants did not cause a decrease in RNA pol II occupancy as compared to cells expressing wild type *SPT16*. Interestingly, the *spt16-L669P* and *spt16-L669S* mutants did cause a slight, but significant decrease ($P<0.05$) in Rpb3 binding across *SRG1*. However, by normalizing the binding of these mutant versions of Spt16 to Rpb3 binding, it is clear that these minor decreases in Rpb3 binding alone cannot account for the reduced binding of these two mutant versions of

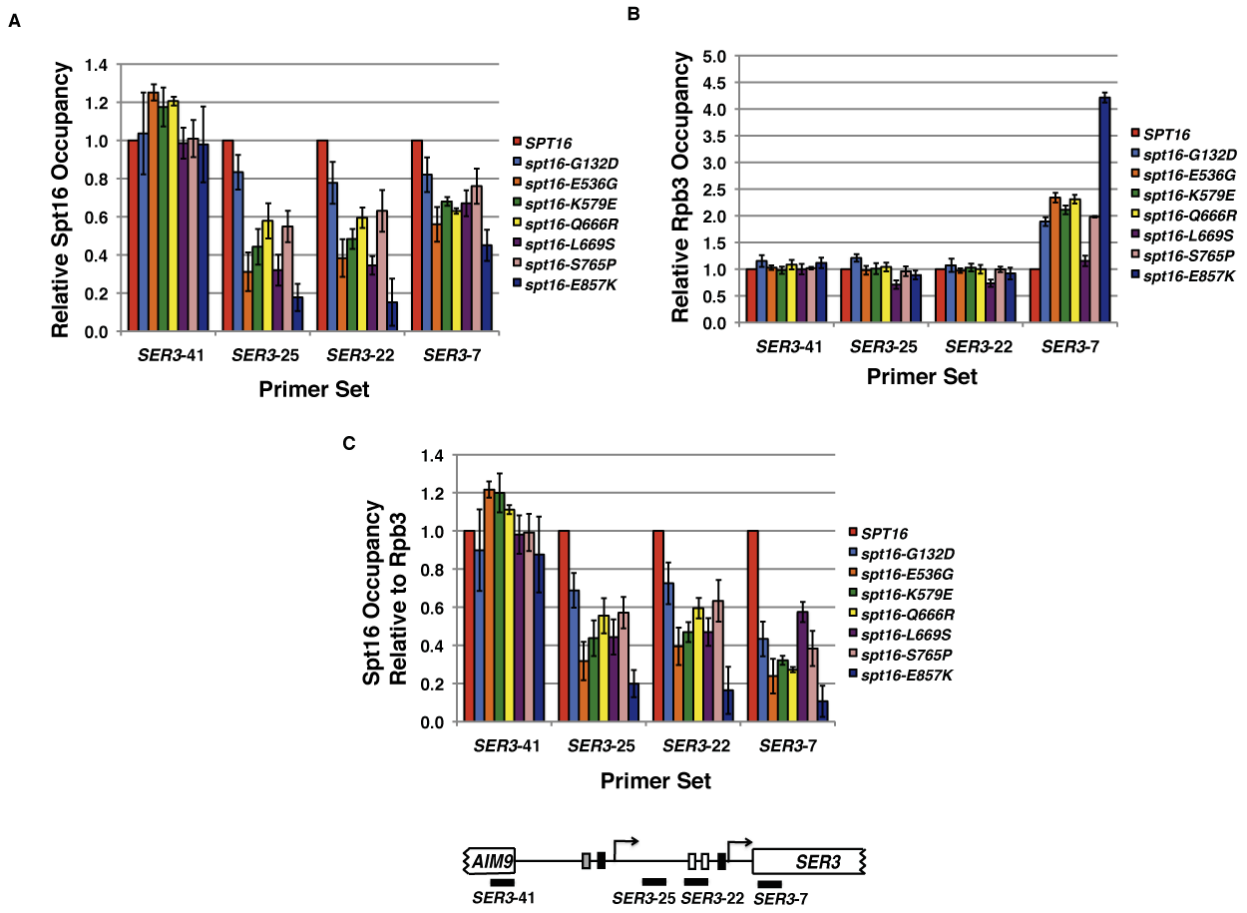

Figure 24. Relative occupancy of Spt16 and RNA pol II across *SER3* in *spt16* mutants.

Spt16 (A) and Rpb3 (B) ChIP experiments were performed on chromatin prepared from *spt16*Δ strains expressing either wild-type or mutant Spt16, as indicated, that were grown in YPD at 30°C. The amount of immunoprecipitated DNA at four locations across *SER3* (indicated by black bars in the diagram of *SER3* below the graphs) was determined by qPCR as a fraction of the input material and normalized to a control region in chromosome V. Each bar represents the mean +/- SEM of three independent experiments using strains derived from YJ1091 and YJ1092. Occupancy of these factors in the strains expressing wild-type Spt16 was arbitrarily set to 1 at each *SER3* location. C) Occupancy of Spt16 across *SER3* was recalculated relative to Rpb3 occupancy.

Spt16 across *SRG1* (Figure 24C). Taken together, these data indicate that the amino acids defined by these mutants are required to maintain Spt16 co-localization with RNA pol II across *SRG1*.

3.3.7 Effect of *spt16* mutants on histone H3, Spt16, and RNA pol II occupancy at other genes

To investigate whether the *spt16* mutants that reduce nucleosome occupancy across *SRG1* have a general defect in transcription-coupled nucleosome occupancy, I measured histone H3 occupancy across the coding sequences of a subset of yeast genes by ChIP (Figure 25A). At three highly transcribed genes, *PMA1* (100 mRNA/hr), *PYK1* (95 mRNA/hr), and *ADH1* (125 mRNA/hr) (HOLSTEGE *et al.* 1998), histone H3 levels were reduced in all of the mutants to a similar extent as I observed across *SRG1*. Conversely, histone H3 occupancy at three lowly transcribed genes, *GAL1* (repressed), *TUB2* (12 mRNA/hr), and *CYC1* (10 mRNA/hr) (HOLSTEGE *et al.* 1998), was unaffected in the mutants.

I next examined the occupancy of these mutant derivatives of Spt16 across the coding sequence of this subset of yeast genes (Figure 25B). Consistent with the results found at *SRG1*, I found that at the highly transcribed genes, *PMA1*, *PYK1,* and *ADH1,* the binding of the mutant Spt16 proteins were generally reduced in accordance with the decrease in histone H3 levels across these regions. Interestingly, the decrease in the occupancy of these mutant Spt16 proteins is greater at the 5' end of these genes as compared to regions towards the 3' end. For the most part, these changes in Spt16 binding occur in the absence of any change in RNA pol II binding to these regions (Figure 25C). Interestingly, a small but significant decrease in Rpb3 levels in the *spt16-L669S* mutant ($P<0.05$) was detected at these highly transcribed genes comparable to what

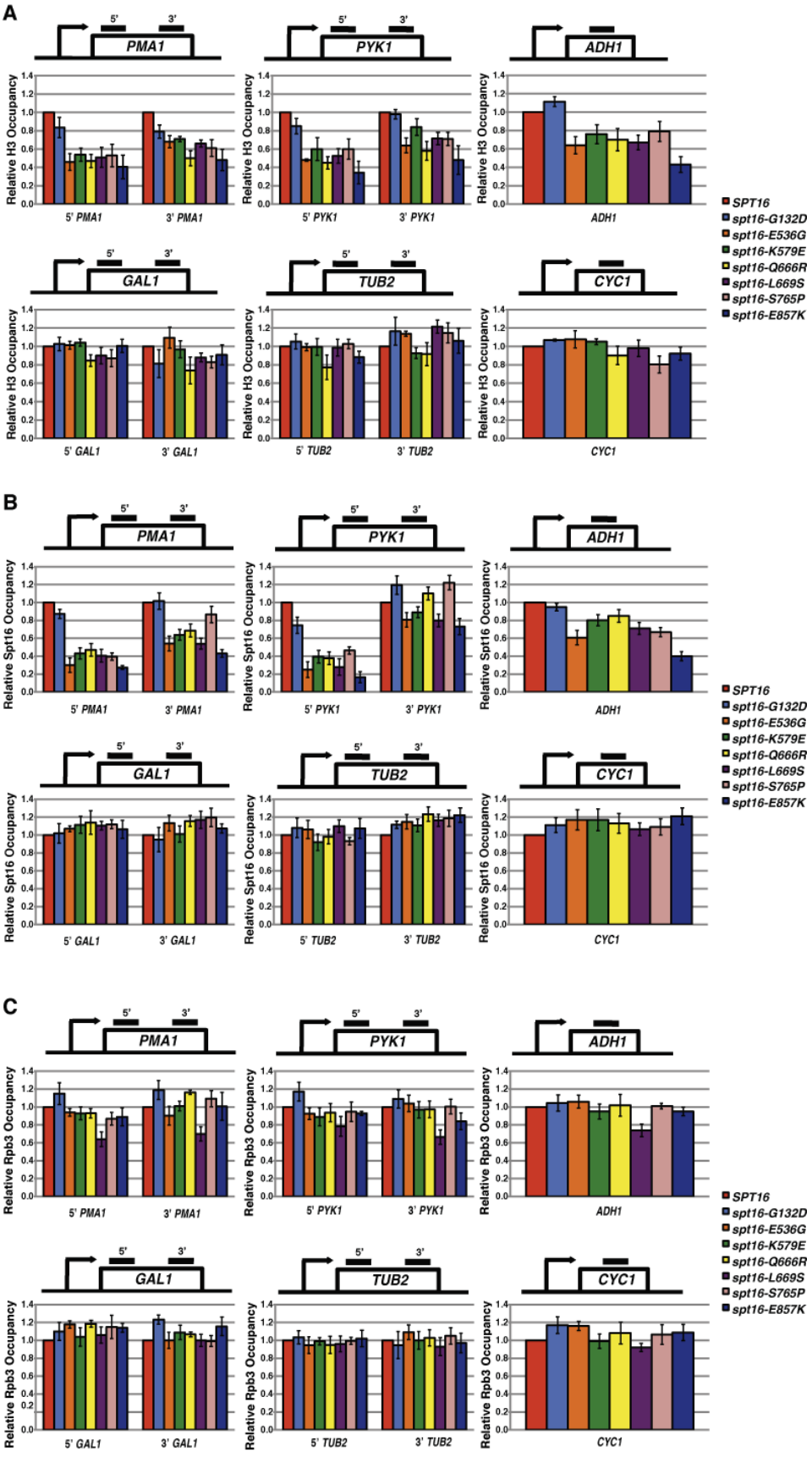
A
B
C
PMA1
PYK1
ADH1
GAL1
TUB2
CYC1
5'
3'
Relative H3 Occupancy
Relative Spt16 Occupancy
Relative Rpb3 Occupancy
5' PMA1
3' PMA1
5' PYK1
3' PYK1
ADH1
5' GAL1
3' GAL1
5' TUB2
3' TUB2
CYC1
SPT16
spt16-G132D
spt16-E536G
spt16-K579E
spt16-Q666R
spt16-L669S
spt16-S765P
spt16-E857K

Figure 25. Relative occupancy of histone H3, Spt16, and Rpb3 across the coding regions of a subset of yeast genes.

Histone H3 (A), Spt16 (B) and Rpb3 (C) was measured by ChIP within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1* (top panel in A, B, and C) and over three lowly transcribed genes: *GAL1*, *TUB2*, and *CYC1* (bottom panel in A, B, and C) as described in Figure 24. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values represent the mean +/- SEM of three independent experiments.

we observed at *SRG1*. In contrast to what I observed at highly transcribed genes, occupancy of the mutant Spt16 proteins and Rpb3 at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1*, were largely unaffected. Importantly, I found that when these mutants are grown in galactose-containing medium to induce high levels of *GAL1* expression, I now detect reduced occupancy of both H3 and the mutant Spt16 proteins to the *GAL1* coding sequence similar to what we observed for *SRG1* and other highly transcribed genes (Figure 26). Thus, we have identified mutant *spt16* alleles that cause reduced occupancy of both the mutant version of Spt16 encoded by these alleles and histones specifically over highly transcribed regions of the genome. Taken together, our studies suggest that the integrity of the Spt16-D and Spt16-M domains is generally required to maintain nucleosome occupancy at highly transcribed genes, possibly by facilitating Spt16 recruitment to those genes.

3.4 DISCUSSION

Spt16 is an essential, highly conserved component of the FACT elongation complex with a dual role in transcription elongation – the disassembly of nucleosomes to allow the passage of RNA pol II and their reassembly in the wake of RNA pol II (reviewed in (DUINA 2011; FORMOSA 2011; REINBERG and SIMS 2006; WINKLER and LUGER 2011). In this Chapter, I provide evidence indicating that the integrity of both the Spt16-D and Spt16-M domains are required to support the histone chaperone activities of Spt16 during transcription elongation. We utilized a previously characterized system where this activity of Spt16 is required for *SRG1* transcription-dependent repression of the *S. cerevisiae SER3* gene (HAINER *et al.* 2011) to identify a largely novel class of mutations in *SPT16* that derepress *SER3*. Seven mutations that most strongly

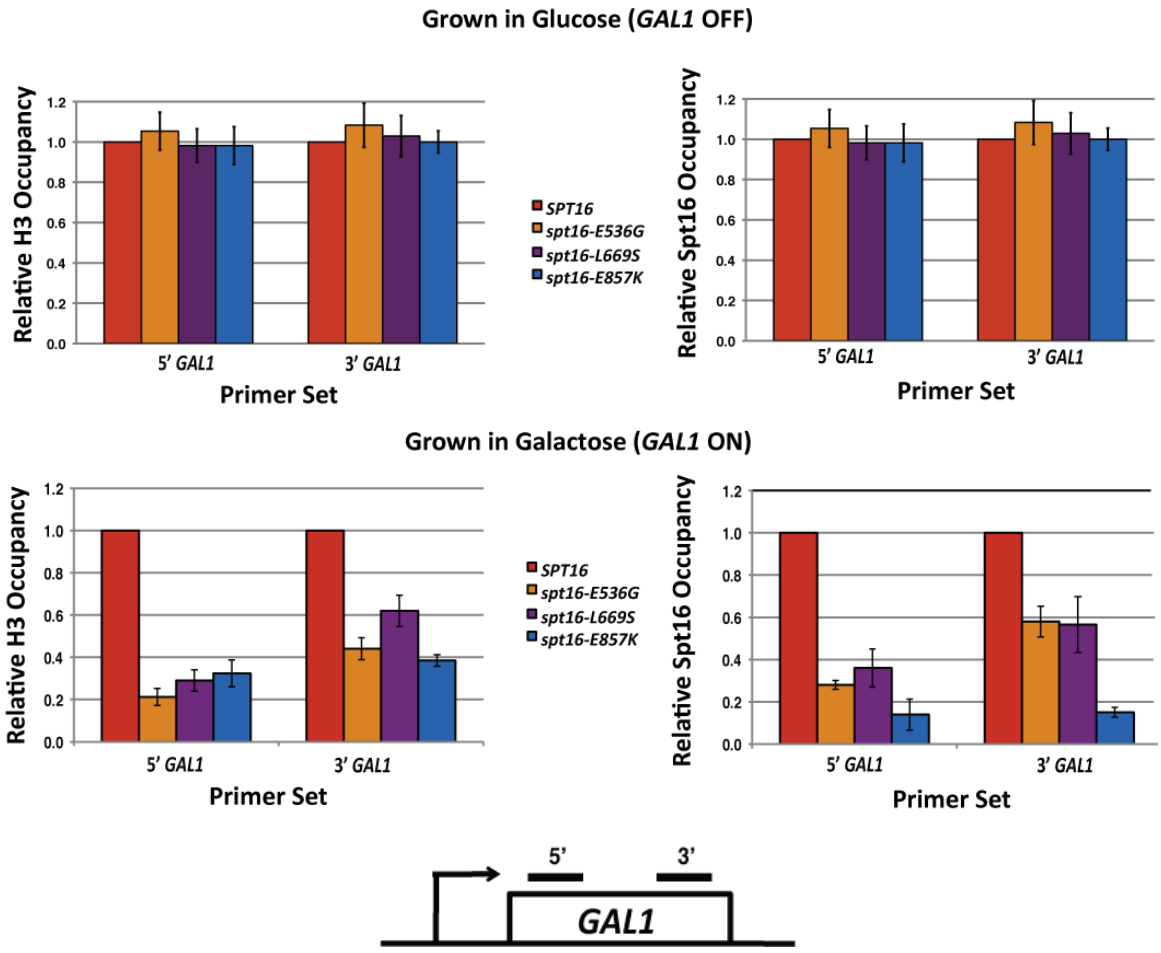

Figure 26. Relative occupancy of histone H3, Spt16 and RNA pol II in *spt16* mutants over *GAL1*.

Histone H3 (A), Spt16 (B), and Rpb3 (C) ChIP experiments were performed on chromatin prepared from *spt16*Δ strains containing either plasmid-borne copies of either wild-type *SPT16* or the indicated *spt16* mutant alleles, which were grown in YPRaff at 30°C and shifted to YPGal for 1 hr. The amount of immunoprecipitated DNA at 5' and 3' locations within the *GAL1* open reading frame (indicated by black bars in the diagram of *GAL1* below the graphs) was determined by qPCR as a fraction of the input material and normalized to a control region in chromosome V. Each bar represents the mean +/- SEM of three independent experiments using strains derived from YJ1091 or YJ1092. Occupancy of these factors in the strains expressing wild-type Spt16 was arbitrarily set to 1 at each *GAL1* location. D) Occupancy of Spt16 over *GAL1* was recalculated relative to Rpb3 occupancy.

derepress *SER3* contain single amino acid substitutions in either the Spt16-D or Spt16-M domains. For this subset of mutants, *SRG1* transcription-coupled nucleosome occupancy over the *SER3* promoter is reduced to degrees that generally correlate with *SER3* derepression. Moreover, we provide evidence that these mutations broadly disrupt transcription-coupled nucleosome occupancy at highly transcribed regions of the yeast genome. Finally, we show that while these mutant versions of Spt16 are expressed at wild type levels, their association with highly transcribed genes is significantly reduced. These data suggest that the integrity of the Spt16-D and Spt16-M domains is required for transcription-coupled nucleosome occupancy, possibly by promoting or maintaining FACT association with transcribed regions of the genome.

With one exception (*spt16-E857K*), the *spt16* mutants that we identified in this work are distinct from those that have been previously identified by other genetic approaches (FORMOSA *et al.* 2002; MALONE *et al.* 1991; MYERS *et al.* 2011; O'DONNELL *et al.* 2009; STEVENS *et al.* 2011). Although most members of this new class of *spt16* mutants confers an Spt$^-$ phenotype similar to many previously characterized *spt16* mutants, additional phenotypic studies indicate that there are important functional differences between these mutants. First, these mutants do not confer lethality at elevated temperature as is common for many previously characterized *spt16* mutant alleles (FORMOSA *et al.* 2002; MYERS *et al.* 2011; O'DONNELL *et al.* 2009). This result suggests that the amino acid substitutions caused by these mutations are not likely to affect the general stability of the Spt16 protein. Furthermore, these results indicate that the ability of Spt16 to promote nucleosome assembly during transcription is not essential for viability. Second, these mutants do not confer a growth defect in the presence of hydroxyurea – a phenotype conferred by other *spt16* mutants (FORMOSA *et al.* 2002; MYERS *et al.* 2011; O'DONNELL *et al.* 2009) that is indicative of a defect in DNA replication and/or DNA repair (HAMPSEY 1997). Therefore, this

new group of *spt16* mutants may define an activity for Spt16 that is specific to its role in transcription elongation rather than a histone chaperone activity that may be generally required for all of Spt16 functions. Third, these *spt16* mutants do not suppress a cold sensitive growth defect conferred by a histone H3 L61W as has been recently described for a distinct set of *spt16* mutant alleles (MYERS *et al.* 2011). This is somewhat surprising given that both groups of *spt16* mutants have amino acid substitutions within the Spt16-M domain. Moreover, one of the *spt16* mutants isolated as a suppressor of the cold sensitivity of the histone H3 L61W mutant contains a glutamine substitution of glutamic acid residue at position 847, the same residue that, when substituted for a lysine, confers strong *SER3* derepression and transcription-coupled nucleosome assembly defects. However, the lysine substitution did not suppress the cold sensitivity of the H3 L61W mutation. Taken together, these data show that we have identified a new class of *spt16* mutants that interfere with an Spt16 activity that is specific to its role in transcription-coupled nucleosome assembly rather than one that is generally required Spt16 functions in transcription, cell viability and/or DNA replication/DNA repair.

During our phenotypic analyses, we found that most of the *spt16* mutants that were isolated based on their ability to derepress *SER3* also confer sensitivity to cycloheximide – a phenotype that has not been previously described for *spt16* mutant alleles. Cycloheximide is a potent inhibitor of eukaryotic protein synthesis that is normally toxic to yeast cells (MCCUSKER and HABER 1988). However, at low doses, a sensitivity to this drug has been shown to reveal mutations that reduce protein synthesis or impair cell cycle progression (HAMPSEY 1997). Therefore, while the identification of this phenotype may be interesting, the interpretation of the data is unclear. I hypothesize that the subset of *spt16* mutants causing cycloheximide sensitivity do so as a result of the misregulation of one or more genes encoding proteins that are either

essential for viability, regulate protein synthesis, or regulate intracellular levels of cycloheximide.

Interestingly, the *spt16-E857K* allele, which we found to confer a dominant negative effect on *SER3* repression, was previously isolated as a dominant suppressor of the transcription defects of δ element insertions just 5' of both the *LYS2* and *HIS4* genes (O'DONNELL *et al.* 2009; STEVENS *et al.* 2011). This is not surprising given the striking similarities between *SER3* repression by *SRG1* transcription and *LYS2* and *HIS4* repression by the δ element insertions (CLARK-ADAMS and WINSTON 1987; MARTENS *et al.* 2004; WINSTON *et al.* 1984). Both *SRG1* and the δ element insertion are promoting transcription across the promoters of their adjacent genes, *SER3* and either *LYS2* or *HIS4*, respectively. Our finding that *SER3* derepression in the *spt16-E857K* mutant is the result of reduced *SRG1* transcription-dependent nucleosome assembly at the *SER3* promoter suggests that a similar transcription-defect in nucleosome occupancy may play a role in alleviating repression of *LYS2* and *HIS4* caused by these δ element insertions. Interestingly, we found that while three of the five double mutants containing the *E857K* substitution also act in a dominant manner, two of these combinations, *spt16-I626T/E857K* and *spt16-T651A/H741Y/E857K* do not. Moreover, we found that the level of *SER3* derepression in these two mutant alleles to be significantly lower to that caused by the *E857K* substitution alone (B. Charsar, unpublished). Therefore, *I626T* and one or both of *T651A* and *H741Y* substitutions appear to suppress the negative effects of the *E857K* substitution.

Our analysis of the single amino acid substitutions in the Spt16-D and Spt16-M domains revealed a strong correlation between defective transcription-dependent nucleosome assembly and reduced association of these mutant versions of Spt16 at highly transcribed regions of the yeast genome. Several possible models could account for these observations. First, these mutant

versions of Spt16 may interfere with the normal recruitment of FACT to transcribed DNA. In this model, the reduced recruitment of FACT would be the cause of the defect in transcription-coupled nucleosome assembly. Although several studies have determined that FACT physically associates with DNA that is being transcribed (DUINA *et al.* 2007; KIM *et al.* 2004; MASON and STRUHL 2003; MAYER *et al.* 2010), the molecular mechanism of this association is not known. Previous studies have implicated a number of factors that may facilitate Spt16 association with transcribed DNA including the Chd1 chromatin remodeling factor, the Paf1 elongation complex, RNA pol II, and histone proteins (ADELMAN *et al.* 2006; BISWAS *et al.* 2007; FORMOSA *et al.* 2001; MASON and STRUHL 2003; PRUNESKI *et al.* 2011; SIMIC *et al.* 2003; WINKLER *et al.* 2011). It is conceivable that the amino acid substitutions within the Spt16-D and Spt16-M domains that interfere with transcription-coupled nucleosome assembly do so by altering FACT interactions with one or more of these factors. Second, the reduction in Spt16 association with transcribed regions may be a consequence of the reduced nucleosome occupancy due to a defect in transcription-coupled nucleosome assembly. In this model, the amino acid substitutions in the Spt16-D and Spt16-M domain would not alter initial Spt16 recruitment to transcribed DNA or its ability to associate with nucleosomal DNA but rather interfere with its nucleosome remodeling activity that leads to disassembly and/or reassembly of nucleosomes during transcription. Additional molecular and biochemical experiments to investigate the affect of these mutants on FACT interactions with other proteins and the nucleosome remodeling activity of Spt16 will be necessary to distinguish between these models.

Although the possibility that the Spt16-D and Spt16-M domains may directly mediate protein-protein interactions or FACT nucleosome remodeling activity is intriguing, we cannot rule out a more indirect role for these domains. For example, it is possible that the three

mutations in the Spt16-D domain may simply disrupt the Spt16-Pob3 interface (VANDEMARK *et al.* 2006; VANDEMARK *et al.* 2008). However, if this were the case we would expect any changes in the Spt16-Pob3 dimer interface to be subtle - specifically affecting the activity of the FACT in transcription-dependent nucleosome assembly rather than a more general histone chaperone role for FACT. Large perturbations in the Spt6-Pob3 interactions would most likely lead to more broad defects in cell growth and DNA replication/repair, which were not detected in these mutants by our phenotypic assays.

In summary, we have identified a novel class of *spt16* mutants that specifically impair transcription-coupled nucleosome occupancy across highly transcribed regions of the *S. cerevisiae* genome and result in reduced association of the mutant Spt16 proteins to these regions.

4.0 IDENTIFICATION OF HISTONE MUTANTS THAT ARE DEFECTIVE FOR TRANSCRIPTION-COUPLED NUCLEOSOME OCCUPANCY

The majority of the work discussed in this Chapter has been adapted from published material (HAINER and MARTENS 2011a) and is reprinted, with alterations, by permission from the American Society for Microbiology, copyright 2011.

4.1 INTRODUCTION

Chromatin is a dynamic participant in regulating the function of both large genomic regions and individual genes (reviewed in (BERGER 2007; CAIRNS 2009; LEE *et al.* 2010; LI *et al.* 2007a). Nucleosomes are the fundamental unit of chromatin, consisting of 147bp of DNA wrapped around an octamer of histones, including two H2A/H2B heterodimers and one H3/H4 heterotetramer (KORNBERG 1974; LUGER *et al.* 1997). Not surprisingly, nucleosomes have a major impact on the regulation of transcription in several ways. At promoters, nucleosomes interfere with the binding of sequence-specific transcription factors. Over transcribed sequences, nucleosomes act both negatively as a barrier to elongating RNA polymerases and positively by inhibiting transcription factor access to cryptic intragenic promoters to prevent aberrant transcription. Therefore, a major strategy for gene regulation that is shared among eukaryotes is

to control nucleosome architecture (reviewed in (BAI and MOROZOV 2010; CAIRNS 2009; NARLIKAR *et al.* 2002; SMITH and SHILATIFARD 2010).

Eukaryotic cells have three major classes of proteins that contribute to transcription regulation by altering chromatin: chromatin remodelers, post-translational histone modifiers, and histone chaperones. Chromatin remodelers, such as the yeast Swi/Snf complex, use the energy from ATP hydrolysis to reposition or remove nucleosomes primarily at promoter regions thus allowing sequence-specific proteins to bind DNA (CAIRNS 2005; CLAPIER and CAIRNS 2009; FLOER *et al.*). Post-translational histone modifiers catalyze the covalent addition of methyl, acetyl, phosphoryl, and ubiquityl groups to the side chains of specific amino acids encoded by the histone genes (CAMPOS and REINBERG 2009; SHILATIFARD 2006; SUGANUMA and WORKMAN 2008). These modifications have been shown to impact gene regulation by facilitating the activity of chromatin remodelers and by providing a binding platform for additional regulatory proteins. Histone chaperones, including Asf1, Spt6, and Spt16, interact with histones and contribute to the disassembly and reassembly of nucleosomes at promoters and over coding sequences during transcription (EITOKU *et al.* 2008; KIM *et al.* 2007c; WILLIAMS and TYLER 2007).

In Chapter 2, I described a new mechanism for controlling chromatin at promoters involving the transcription of ncDNA (HAINER *et al.* 2011). In the presence of serine, transcription of *SRG1* ncDNA is initiated upstream of the adjacent *SER3* gene and extends across the *SER3* promoter (MARTENS *et al.* 2004; MARTENS *et al.* 2005). We provided evidence that during *SRG1* transcription, Spt6 and Spt16 histone chaperones reassemble nucleosomes over the *SER3* promoter after the passage of RNA pol II, which then interfere with transcription factor binding resulting in *SER3* repression (HAINER *et al.* 2011). In response to serine starvation, *SRG1*

transcription is reduced causing nucleosome depletion over the *SER3* promoter, which in turn allows transcription factors to bind the *SER3* promoter and activate *SER3* transcription.

Although histone chaperones, including Spt6/Spn1, FACT, and Asf1, have been implicated in mediating transcription-coupled nucleosome assembly, less is known about how histone proteins contribute to this mechanism (BELOTSERKOVSKAYA *et al.* 2003; BORTVIN and WINSTON 1996; CHEUNG *et al.* 2008; EITOKU *et al.* 2008; ENGLISH *et al.* 2006; IVANOVSKA *et al.* 2010; JAMAI *et al.* 2009; KAPLAN *et al.* 2003; KIM *et al.* 2007c; MASON and STRUHL 2003). Several studies have begun to identify specific histone residues that may be involved in this process (CHEUNG *et al.* 2008; DU and BRIGGS 2010; DU *et al.* 2008; ZHENG *et al.*). Among these residues, lysine 36 on h istone H3 and several other lysines within the amino terminal tail of histone H4 are sites of post-translational modifications that are required to protect recently transcribed DNA from aberrant transcription (DROUIN *et al.* 2010; DU *et al.* 2008; LI *et al.* 2007b; PSATHAS *et al.* 2009; RAO *et al.* 2005; YOUDELL *et al.* 2008). However, in Chapter 2, I provided evidence that *SER3* repression by intragenic *SRG1* transcription is independent of these histone modifications (HAINER *et al.* 2011).

In this Chapter, I will discuss the results of a modified synthetic genetic array (SGA) screen using a comprehensive library of histone H3 and H4 mutants (DAI *et al.* 2008) to identify histone residues required for *SER3* repression. Mutations altering five histone H3 (K122, Q120, V117, R49, V46) and three histone H4 (S47, I46, R36) residues that most strongly derepress *SER3* show reduced nucleosome occupancy over the *SER3* promoter. Chromatin immunoprecipitation (ChIP) assays at a subset of yeast genes suggest that these residues, in particular H3 K122, H3 Q120, H3 V117, H4 I46, and H4 R36, are generally required for transcription-dependent nucleosome occupancy at highly transcribed genes. In addition, I

provide evidence that two of these residues, histone H3 R49 and V46, have a distinct role in repressing cryptic intragenic transcription by promoting Set2-dependent methylation of lysine 36 of histone H3. Overall, my results have identified a subset of histone H3 and H4 residues that are required for normal transcription-dependent nucleosome occupancy.

4.2 MATERIALS AND METHODS

4.2.1 Strains and Media

All *S. cerevisiae* strains used (Table 4) are isogenic to a *GAL2*$^{+}$ derivative of S288C (WINSTON *et al.* 1995). All strains were constructed by transformation or by genetic crosses (AUSUBEL 1991). The *lyp1*Δ::*SER3*pr-*lacZ* allele was generated by two successive PCR-mediated integrations. First, the *LYP1* open reading frame was replaced with a 1523bp PCR product containing *SRG1* and *SER3* sequences (-713 to -1 relative to the *SER3* ATG (+1)) and the *URA3* open reading frame. The *URA3* open reading frame at *lyp1* was then replaced with a 3046bp PCR product containing the *lacZ* open reading frame that was amplified from p180 plasmid (HINNEBUSCH 1985; MUELLER *et al.* 1987). Transformants were selected by growth on plates containing 5FOA, screened for *β*-galactosidase activity, and confirmed by PCR. The *snf2*Δ::*KanMX, snf2*Δ::*LEU2, spt6-1004* and *spt16-197* alleles have been previously described (CAIRNS *et al.* 1996; KAPLAN *et al.* 2003; MALONE *et al.* 1991; MARTENS *et al.* 2004). All strains comprising the comprehensive histone mutant library are derivatives of JDY86 and were kindly provided by J. Boeke (DAI *et al.* 2008). YJ1082 is a derivative of JDY86 generated by gene replacement of the *URA3* gene 3' of *HHTS-HHFS* with *KanMX*, which was PCR-amplified from pRS400 (BRACHMANN *et al.* 1998).

Table 4. *Saccharomyces cerevisiae* strains used in Chapter 4.

Strain	Genotype	Reference or Source
FY4	*MAT***a**	(WINSTON *et al.* 1995)
YJ586	*MATα ura3Δ0 leu2Δ0 his3Δ200*	(HAINER *et al.* 2011)
FY2425	*MATα lys2-128δ his3Δ leu2Δ1 ura3-52 FLAG-spt6-1004*	F. Winston
KY1907	*MATα dot1Δ::KanMX*	K. Arndt
KY1755	*MATα set1Δ::KanMX*	K. Arndt
KY1716	*MAT***a** *set2Δ::KanMX*	K. Arndt
YAAD828	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128*δ (*hht2-hhf2*)Δ::*HIS3*	A. Duina
YAAD958	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128*δ (*hht2-hhf2*)Δ::KanMX	A. Duina
YAAD959	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128*δ (*hht2-hhf2*)Δ::KanMX	A. Duina
YJ112	*MATα ura3Δ0 lys2Δ0 leu2Δ0 snf2::LEU2*	This study
JDY86*	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/HHFS-URA3 can1Δ::MFApr-HIS3*	(DAI *et al.* 2008)
YJ920	*MATα ura3Δ0 leu2Δ0 his3Δ200 lyp1Δ::SER3pr-URA3*	This study
YJ921	*MATα ura3Δ0 leu2Δ0 his3Δ200 lyp1Δ::SER3pr-LacZ*	This study
YJ922	*MATα ura3Δ0 leu2Δ0 his3Δ200 trp1Δ63 lyp1Δ::SER3pr-LacZ*	This study
YJ923	*MATα his3Δ200 leu2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 lyp1Δ::SER3pr-LacZ can1Δ::MFApr-HIS3*	This study
YJ924	*MATα ura3Δ0 snf2Δ::KanMX lyp1::SER3pr-LacZ*	This study
YJ925	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhfs-K122A/HHFS-URA3*	This study
YJ926	*MATα his3Δ200 leu2Δ0 trp1Δ63 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-K122A/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ927	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ928	*MAT***a** *leu2Δ0 lys2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/HHFS-URA3*	This study
YJ929	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-K122R/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ930	*MATα his3Δ200 leu2Δ0 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-K122R/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ931	*MATα his3Δ200 leu2Δ0 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-K122Q/HHFS-URA3*	This study
YJ932	*MAT***a** *his3Δ200 leu2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-K122Q/HHFS-URA3*	This study

YJ933	*MATα his3Δ200 leu2Δ0 ura3Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-Q120A/HHFS-URA3*	This study
YJ934	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-Q120A/HHFS-URA3*	This study
YJ935	*MATα his3Δ200 leu2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-V117A/HHFS-URA3*	This study
YJ936	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-V117A/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ937	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-R49A/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ938	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-R49A/HHFS-URA3*	This study
YJ939	*MATα his3Δ200 leu2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/hhfs-S47D-URA3*	This study
YJ940	*MAT**a** his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/hhfs-S47D-URA3*	This study
YJ941	*MAT**a** his3Δ200 leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/hhfs-I46A-URA3*	This study
YJ942	*MATα his3Δ200 leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/hhfs-I46A-URA3*	This study
YJ943	*MAT**a** his3Δ200 leu2Δ0 ura3Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/hhfs-R36A-URA3*	This study
YJ944	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/hhfs-R36A-URA3*	This study
YJ945	*MATα his3Δ200 leu2Δ0 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-V46A/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ946	*MAT**a** his3Δ200 leu2Δ0 ura3Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-V46A/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ974	*MAT**a** spt16-197 ura3(Δ0* or *52) leu2Δ(0* or *1) his3Δ200 trp1Δ63 met15Δ0 lyp1Δ::SER3pr-LacZ*	This study
YJ975	*MAT**a** spt16-197 ura3(Δ0* or *52) leu2Δ(0* or *1) trp1Δ63 lyp1Δ::SER3pr-LacZ*	This study
YJ976	*MATα spt16-197 ura3(Δ0* or *52) leu2Δ(0* or *1) his3Δ200 trp1Δ63 met15Δ0 lyp1Δ::SER3pr-LacZ*	This study
YJ977	*MATα FLAG:spt6-1004 ura3(Δ0* or *52) leu2Δ(0* or *1) his3Δ200 met15Δ0 lyp1Δ::SER3pr-LacZ*	This study
YJ978	*MATα FLAG:spt6-1004 ura3(Δ0* or *52) leu2Δ(0* or *1) his3Δ200 met15Δ0 (hht1-hhf1)Δ::NatMX lyp1Δ::SER3pr-LacZ*	This study
YJ979	*MATα FLAG:spt6-1004 ura3(Δ0* or *52) leu2Δ(0* or *1) his3Δ200 lyp1Δ::SER3pr-LacZ*	This study
YJ980	*MAT**a** his3Δ200 leu2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX lyp1Δ::SER3pr-LacZ can1Δ::MFApr-HIS3*	This study
YJ981	*MATα ura3Δ0 leu2Δ0 his3Δ200 lyp1Δ::SER3pr-LacZ*	This study
YJ982	*MATα snf2Δ::KanMX lyp1Δ::SER3pr-LacZ*	This study
YJ983	*MAT**a** ura3Δ0 his3Δ200 snf2Δ::KanMX lyp1Δ::SER3pr-LacZ*	This study

YJ1047	*MAT***a** *leu2Δ0 ura3Δ0 lys2Δ0 snf2Δ::LEU2 hht1-hhf1Δ::NatMX (hht2-hhf2)Δ::HHTS/HHFS-URA3*	This study
YJ1049	*MATα leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 his3Δ200 snf2Δ::LEU2 (hht2-hhf2)Δ::HHTS/HHFS-URA3 can1Δ::MFApr-HIS3*	This study
YJ1051	*MATα ura3Δ0 leu2Δ0 lys2Δ0 met15Δ0 can1Δ::MFApr-HIS3 snf2Δ::LEU2 (hht2-hhf2)Δ::hhts-K122A/HHFS-URA3*	This study
YJ1054	*MAT***a** *ura3Δ0 leu2Δ0 lys2Δ0 can1Δ::MFApr-HIS3 snf2Δ::LEU2 (hht2-hhf2)Δ::hhts-K122R/HHFS-URA3*	This study
YJ1057	*MAT***a** *ura3Δ0 leu2Δ0 lys2Δ0 snf2Δ::LEU2 (hht2-hhf2)Δ::hhts-K122Q/HHFS-URA3*	This study
YJ1060	*MAT***a** *ura3Δ0 leu2Δ0 snf2Δ::LEU2 (hht2-hhf2)Δ::hhts-Q120A/HHFS-URA3*	This study
YS284	*MAT***a** *ura3-52 leu2Δ1 lys2-128*δ *his4-912*δ or *his3Δ200* (*hht1*(*T118I*)-*HHF1*)Δ16'::*LEU2* (*hht2-hhf2*)Δ::KanMX	This study
YS285	*MAT***a** *ura3-52 leu2Δ1 lys2-128*δ *his4-912*δ or *his3Δ200* (*hht1*(*T118I*)-*HHF1*)Δ16'::*LEU2* (*hht2-hhf2*)Δ::KanMX	This study
YS286	*MAT***a** *ura3-52 leu2Δ1 lys2-128*δ *his4-912*δ or *his3Δ200* (*hht1*(*T118I*)-*HHF1*)Δ16'::*LEU2* (*hht2-hhf2*)Δ::KanMX	This study

* Indicates strains from the histone H3/H4 library. All strains in the library have identical auxotrophies with the *(hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/HHFS-URA3 can1Δ::MFApr-HIS3* alleles as described (Dai et al., 2008).

The *hhts-T118I* allele was generated by PCR-based site-directed mutagenesis followed by one-step gene replacement. First, a portion of the *HHTS-HHFS::KanMX* cassette beginning 50bp 5' of the T118 codon of *HHFS* and extending to the 3' end of *KanMX* was PCR-amplified from YJ1082 genomic DNA using a forward primer that contains a C to T base change converting the threonine codon at 118 to isoleucine. A second DNA fragment consisting of the 400bp 5' of the T118 codon was PCR-amplified from YJ1082 genomic DNA. These two fragments were mixed together and subjected to PCR amplification to generate one long DNA fragment containing the mutation that converts the T118 codon to isoleucine, which was then used to transform YJ112. Transformants were selected by growth on plates containing G418 and confirmed by PCR and sequencing. Solid media used for the modified SGA screen, were as follows: synthetic complete lacking uracil and lysine (SC-Ura-Lys), synthetic complete lacking histidine, lysine, and uracil that was supplemented with 50 mg/L thialysine (SC-His-Lys-Ura+thialysine) and sporulation media supplemented with histidine, lysine, tryptophan, methionine and cysteine (TONG and BOONE 2006). Thialysine is an analogue of lysine that is toxic to yeast cells expressing a functional Lyp1 lysine permease. Therefore, strains containing the *lyp1*Δ::*SER3pr-lacZ* reporter allele can be selected by their growth in the presence of thialysine (SYCHROVA and CHEVALLIER 1993). All other media was prepared as previously described (ROSE 1991). YPD, YPraff and YPgal media contained 2% glucose, 2% raffinose, and 2% galactose, respectively, as the sole carbon source. Solid YPraff and YPgal media also contained 1 mg/L antimycin A.

4.2.2 SGA screen of histone H3/H4 library

A previously described manual synthetic genetic array (SGA) screen (TONG and BOONE 2006) was modified to utilize a comprehensive library of histone H3 or H4 mutants (DAI *et al.* 2008).

First, I systematically mated 422 histone mutant strains to YJ923 for 1 day at room temperature. I then pinned the mated cells to SC-Ura-Lys plates and incubated at 30°C for 2 days to select for diploids. Next, I pinned diploid cells to sporulation plates and incubated at 22°C for 5 days. After two successive rounds of selection on S C-His-Lys-Ura+thialysine plates, I replica printed haploid cells containing both the histone substitution and the *lyp1*Δ::*SER3pr-lacZ* reporter to YPD plates and subjected the resulting patches to an X-gal overlay as previously described (DUTTWEILER 1996). Briefly, yeast cells that were grown as small patches on YPD plates at 30°C for 2 days were permeabilized by covering the patches with 5-10 ml of chloroform for 5 min. The chloroform was decanted and excess chloroform was allowed to evaporate. A warm agarose solution containing 1% low melting-point agarose, 0.1 M $NaPO_4$ and 25 mg/ml X-gal was poured over the cells and allowed to set. After 35 min, each strain was scored for the appearance of blue color as compared to the YJ980 control strain expressing one wild-type copy of histone H3 and H4.

4.2.3 Western analysis

Whole cell extracts (WCE) were prepared from cells grown in YPD at 30°C to 3-4 × 10^7 cells/ml using trichloroacetic acid as previously described (COX *et al.* 1997; ZHENG *et al.*). Equal amounts of WCE were separated by 15% acrylamide SDS-PAGE, transferred to Protean nitrocellulose (Whatman) and assayed by immunoblotting. The antibodies used to detect histone H3 and H4 levels and specific post-translational modifications of these histones were as follows: anti-H3 (1:20,000) (gift from LeAnn Howe), anti-H4 (1:2500) (ab10158; Abcam), anti-H3K4me2 (1:3000) (39255; Active Motif) anti-H3K4me3 (1:2500) (ab8580; Abcam), anti-H3K36me2 (1:2500) (39255; Active Motif), anti-H3K36me3 (1:500) (ab9050; Abcam), and anti-

H3K79me2/3 (1:1000) (ab2621; Abcam). After incubation with HRP-conjugated IgG secondary antibody (1:5000, GE Healthcare), the immunoreactive proteins were visualized by enhanced chemiluminescence detection (Perkin-Elmer).

4.2.4 Northern analysis

Cells were grown to 1-2 × 10^7 cells/ml in YPD at 30°C. Total RNA isolation and Northern analysis was performed as previously described (COLLART and OLIVIERO 2001). Radiolabeled DNA probes were generated by random-primed labeling of PCR fragments for *SRG1* (-454 to -123 relative to *SER3* ATG), *SER3* (+111 to +1342), *FLO8* (+1515 to +2326), *STE11* (+1868 to +2110), *SYF1* (+2032 to +2525) *PMA1* (+903 to +1246), *PYK1* (+333 to +654), *GAL10* (+212 to +509) and *SCR1* (-163 to +284) that were amplified from genomic DNA. RNA levels were quantified using a PhosphorImager (Instant Imager, Packard Instrument Co.) and normalized to the *SCR1* loading control.

4.2.5 Dilution growth assays

Cells were grown at 30°C to saturation then washed twice with water. Starting at 1 × 10^8 cells/ml, cultures were serially diluted ten-fold. 3 µl of each dilution was spotted onto YPD, YPraff, and YPgal media and incubated at 30°C for the indicated number of days.

4.2.6 Nucleosome scanning assay

Nucleosome scanning assays were performed as described in Chapter 2 (HAINER *et al.* 2011) on cells grown at 30°C to 2 × 10^7 cells/ml in YPD. For each of the 38 *SER3* primer pairs, the amount of template protected from digestion by micrococcal nuclease was calculated as a ratio between MNase-digested and undigested samples and then normalized to the amount of MNase-protected control template (*GAL1* NB) that is located within a well-positioned nucleosome in the *GAL1* promoter (BRICKNER *et al.* 2007; FLOER *et al.* ; FLOER *et al.* 2010).

4.2.7 Chromatin Immunoprecipitation (ChIP) assay

For H3 ChIP over galactose-induced *GAL1*, cells were grown in YPraff to approximately 1.5 × 10^7 cells/mL and then 2% galactose was added for 1 hr. For all other ChIP experiments, cells were grown in YPD at 30°C to 1-2 × 10^7 cells/ml. Chromatin was prepared as previously described (SHIRRA *et al.* 2005). Histone H3 was immunoprecipitated by incubating sonicated chromatin overnight at 4°C with 5 µl anti-histone H3 antibody (ab1791; Abcam) and followed by the addition of IgG-Sepharose beads (GE Healthcare) for 2 hr at 4°C. Dilutions of input DNA and immunoprecipitated DNA were analyzed by qPCR reactions. Primer sets that amplify the following regions were used to measure H3 occupancy by qPCR: *PYK1* (5': +62 to +164, 3': +1173 to +1279), *PMA1* (5': +691 to +794, 3': +1689 to +1791), *ADH1* (+845 to +943), *CYC1* (+122 to +217), *TUB2* (5': +105 to +202, 3': +1083 to +1189) and *GAL1* (5': +79 to +175, 3': +1366 to + 1487). Histone H3 ChIP signals for each gene were normalized to a No ORF control template, which is located within a region of chromosome V that lacks open reading frames (KOMARNITSKY *et al.* 2000).

4.2.8 Quantitative PCR (qPCR)

All qPCR data for the nucleosome scanning assays were obtained by using an ABI 7300 Real-time PCR system, SYBR green reagents (Fermentas) and primer sets tiling *SER3* (HAINER *et al.* 2011). All qPCR data for the ChIP assays were obtained using a StepOnePlus Real-time PCR system, SYBR green reagent (Fermentas) and the indicated primers. Calculations were performed using Pfaffl methodology (PFAFFL 2001).

4.3 RESULTS

4.3.1 Identification of histone mutations that derepress *SER3*

We have previously shown that transcription of *SRG1* ncDNA represses *SER3* transcription by assembling nucleosomes across the overlapping *SER3* promoter (HAINER *et al.* 2011). Although several studies have identified factors that contribute to transcription-coupled nucleosome reassembly, including the Spt6/Spn1(Iws1) and FACT transcription elongation complexes and the HMG-like Spt2 protein (HAINER *et al.* 2011; THEBAULT *et al.* 2011), less is known about how histone proteins themselves may contribute to this mechanism. To investigate the role of histones in *SER3* repression, I performed a comprehensive genetic screen to identify mutations in the genes encoding histones H3 and H4 that derepress *SER3* in normal repressing conditions. First, I constructed a *SER3*pr-*lacZ* reporter gene to monitor *SER3* repression using a standard β-galactosidase assay by replacing the *SER3* coding sequence with the coding sequence for the *E.coli lacZ* gene (Figure 27A; see Materials and Methods). Since deletion of the *SER3* gene leads

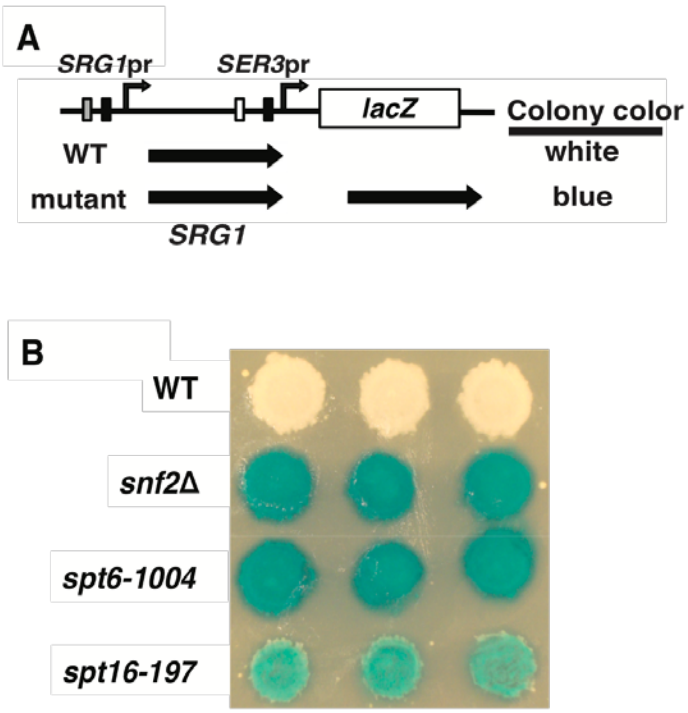

Figure 27. Detection of *SER3* derepression from an ectopically expressed *SER3*pr-*lacZ* reporter.

A) Diagram of *SER3*pr-*lacZ* reporter. The *LYP1* ORF was replaced by *SER3* 5' UTR sequence from -713 to -1, including *SRG1* and its promoter, fused to the *lacZ* ORF. Block arrows beneath the diagram indicate the expected *SRG1* and *SER3-lacZ* transcripts in wild-type and mutant strains grown in serine-rich media (YPD). The table on the right indicates the expected results for an X-gal overlay assay for wild-type and mutant strains. B) X-gal overlay detects *SER3*pr-*lacZ* derepression in *snf2*Δ (YJ924, YJ982, and YJ983), *spt6-1004* (YJ977, YJ978, and YJ979), and *spt16-197* (YJ974, YJ975, and YJ976) strains as compared to wild-type strains (YJ921, YJ980, and YJ981). Cells were grown on YPD media and incubated with X-gal for 32 minutes.

to increased expression from the *SER3* promoter (J. Martens, unpublished data), the *SER3*pr-*lacZ* reporter is integrated at *LYP1*. *LYP1* encodes a lysine permease and its depletion has no effect on *SER3* regulation (J. Martens, unpublished data). A β-galactosidase overlay assay demonstrates the *SER3*pr-*lacZ* reporter effectively detects mutations that are known to derepress endogenous *SER3* (Figure 27B).

Using this *SER3*pr-*lacZ* reporter strain in a modified SGA strategy, I systematically screened a library of histone H3 and H4 mutants (kindly provided by J. Boeke) for those that display increased β-galactosidase activity as compared to a control strain expressing wild-type histones. The histone H3 and H4 mutant library consists of 422 a lleles including alanine substitutions of all non-alanine residues, serine substitutions of all alanines, a number of additional substitutions that exploit the physical characteristics of several side chains (for example, lysine to arginine mutations maintain charge) or that mimic different post-translational modifications (for example, lysine to glutamine to mimic acetylation), and a series of histone tail deletions (DAI *et al.* 2008). In these strains, one copy of the histone genes, *HHT1-HHF1*, is deleted while the second copy, *HHT2-HHF2*, has been replaced with a synthetic version of these genes (*HHTS-HHFS*) that has been mutated (DAI *et al.* 2008). In the initial phase of the screen, I identified 139 histone H3 and H4 mutants that increase β-galactosidase activity. I then performed Northern analyses, in duplicate, on all 139 mutants to assay changes to the endogenous *SER3* and *SRG1* expression levels (see Table 5). Note that strains expressing a single copy of the synthetic histone (*HHTS-HHFS*) module increase *SER3* levels 3-fold as compared to a strain with two wild type copies of the histone H3 and H4 genes. This result is consistent with previous results indicating that *SER3* expression is sensitive to histone gene dosage (WYRICK *et al.* 1999). Therefore, the changes in RNA levels in the histone mutants were normalized to a control strain

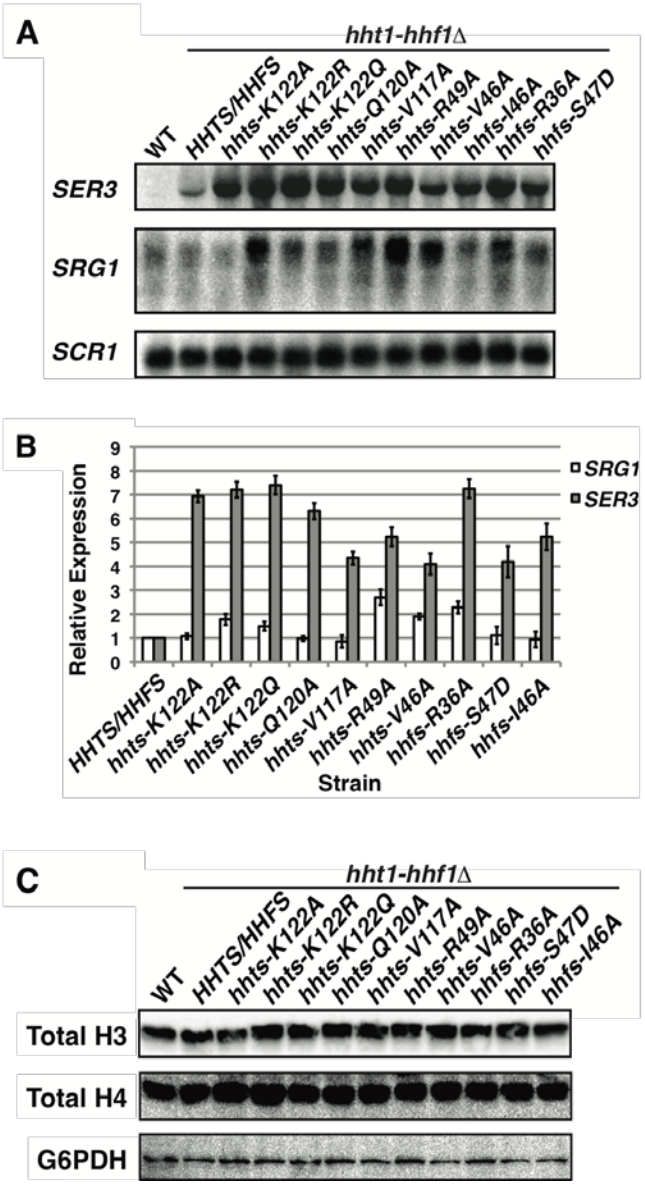

Figure 28. Single amino acid substitutions in histones H3 and H4 strongly derepress *SER3*.

A) Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from a wild-type strain (FY4) and derivatives of JDY86 expressing either synthetic, wild-type copies of histone H3 and H4 (*HHTS/HHFS)* or mutants *hhts-K122A*, *hhts-K122R*, *hhts-K122Q*, *hhts-Q120A*, *hhts-V117A*, *hhts-R49A*, *hhts-V46A*, *hhfs-I46A*, *hhfs-R36A*, and *hhfs-S47D* that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C. B) Quantitation of Northern

analyses. *SRG1* (white bars) and *SER3* (grey bars) RNA levels for the histone mutants are normalized to the *SCR1* loading control and are relative to the *SRG1* and *SER3* RNA levels measured in control *HHTS-HHFS* strains (arbitrarily set to 1). Each bar represents the mean +/- SEM from three independent experiments involving JDY86 derivatives (Panel A) and related strains generated by genetic crosses (YJ925-YJ946). C) Western analysis examining the effect of histone mutants on total histone H3 and histone H4 protein levels. Strains expressing the indicated histone alleles were grown to ~3×10^7 cells/ml in YPD at 30°C. Proteins were extracted with trichloroacetic acid and subjected to Western analysis using anti-H3, anti-H4, and anti-G6PDH (loading control). Similar results were obtained for three independent experiments using the strains listed in Panel B.

expressing a single copy of the synthetic histone genes (*HHTS-HHFS*). Of the initial 139 mutants, 12 mutants resulted in at least a four-fold increase in *SER3* mRNA levels as compared to the *HHTS-HHFS* control, while another 54 mutants resulted in more modest increases in *SER3* mRNA levels (1.5- to 4-fold). These data further emphasize the important role of chromatin in *SER3* regulation.

Of the 12 histone mutants that strongly derepress *SER3*, only alanine substitutions of histone H3 H39 and R72 significantly decrease *SRG1* RNA levels (Table 5). Therefore, histone H3 H39 and R72 may contribute to *SER3* repression indirectly by impairing *SRG1* transcription. In contrast, *SRG1* RNA levels are either unaffected or slightly elevated by the other ten mutants, which include: histone H3 K122A, K122R, K122Q, Q120A, V117A, R49A, V46A and histone H4 R36A, S47D, and I46A (Figure 28A and 28B). Since previous studies have shown that *SER3* is derepressed when histone H4 is depleted (WYRICK *et al.* 1999), I also tested the effect of these mutants on histone H3 and H4 protein levels by Western analysis (Figure 28C). All ten histone mutant strains express levels of histone H3 and H4 indistinguishable from a wild type *HHTS-HHFS* strain. Taken together, these data identify eight amino acids, five in histone H3 and three in histone H4, that are strongly required to repress *SER3* by a mechanism that is independent of the regulation *SRG1* transcription.

Recent large-scale phenotypic analyses have reported a range of phenotypes for mutations that alter these eight residues (summarized in Table 6) (ENGLISH *et al.* 2006; HUANG *et al.* 2009; MATSUBARA *et al.* 2007; SAKAMOTO *et al.* 2009; SEOL *et al.* 2008). Most notably, substitutions of some of these residues confer phenotypes linked to defects in chromatin structure, including telomeric silencing defects and suppression of a *LYS2* transcriptional defect caused by a Ty retrotransposon insertion (*SPT*$^-$ phenotype of the *lys2-128δ* allele)

Table 5. Results from Northern analysis on candidates identified through reporter screen

Substitution	Fold change in *SER3* expression*	Fold change in *SRG1* expression*
H3 K122R	+7	+1.5
H3 K122Q	+7	+1.5
H3 K122A	+7	+2
H3 Q120A	+6	+1
H3 R49A	+5	+3
H3 H39A	+5	-3.5
H3 V46A	+4	+2
H3 V117A	+4	+1
H3 R72A	+4	-3
H3 Δ4-35	+3	+2
H3 Δ13-32	+3	-2
H3 V71A	+3	+5
H3 Q85A	+3	+2
H3 L65A	+3	+4
H3 A110S	+3	+2
H3 Δ17-32	+2.5	+4
H3 Δ1-24	+2.5	+2
H3 K37R	+2.5	+2
H3 D106N	+2.5	+1.5
H3 Δ4-30	+2	+3
H3 Δ21-32	+2	+3
H3 Δ1-28	+2	-1.5
H3 Δ1-20	+2	+3
H3 Y99D	+2	+1
H3 Y99A	+2	+2
H3 V101A	+2	+1.5
H3 S87D	+2	+1
H3 S86A	+2	+1.5
H3 R63A	+2	+2
H3 K14A	+2	+1.5
H3 I89A	+2	+2
H3 D81N	+2	+1.5
H3 R134A	+2	+1.5
H3 R131A	+2	+1
H3 G132A	+2	+1
H3 E105A	+2	+2
H3 A111S	+2	+1.5
H3 Δ1-28	+1.5	+1
H3 Δ1-20	+1.5	+2
H3 K4R	+1.5	+2
H3 K42R	+1.5	+1
H3 K36R	+1.5	+1
H3 K125R	+1.5	+1.5
H3 A88S	+1.5	+2
H3 A114S	+1.5	+1
H3 Δ9-28	+1	+1
H3 Δ9-24	+1	+2
H3 Δ9-20	+1	+2
H3 Δ9-16	+1	+1.5
H3 Δ5-28	+1	+1
H3 Δ5-24	+1	+1.5
H3 Δ5-20	+1	+2
H3 Δ5-16	+1	+1
H3 Δ4-20	+1	+3
H3 Δ33-36	+1	+1
H3 Δ21-36	+1	+2
H3 Δ17-28	+1	+1.5
H3 Δ17-20	+1	-1
H3 Δ13-28	+1	+1
H3 Δ13-24	+1	+2
H3 Δ1-12	+1	+1
H3 T58A	+1	+5
H3 T107A	+1	+1.5
H3 S86D	+1	+1.5
H3 S57A	+1	+4
H3 S135A	+1	+2
H3 R69K	+1	+2
H3 R40A	+1	+1
H3 R17A	+1	+2
H3 Q93E	+1	-1
H3 Q68A	+1	+1
H3 P66A	+1	+1
H3 N108A	+1	+1.5
H3 L60A	+1	+1
H3 L100A	+1	+1.5
H3 K79A	+1	-1
H3 K64Q	+1	+1
H3 K4Q	+1	+2
H3 K42Q	+1	+1
H3 K42A	+1	+1.5
H3 K37Q	+1	+1.5
H3 K18Q	+1	+2
H3 I74A	+1	+3
H3 F104A	+1	+1
H3 E73A	+1	+3

H3 Δ1-16	+1	+1	H4 I29A	+1	+1
H3 A98S	+1	+2	H4 Δ1-8	+1	+1.5
H4 R36A	+7	+2	H4 G28A	+1	+3
H4 I46A	+5	+1	H4 T82A	+1	+3
H4 S47D	+4	+1	H4 I50A	+1	-1
H4 T30D	+3	+2	H4 R55A	+1	+1.5
H4 E52Q	+3	+1.5	H4 K77A	+1	+2
H4 K44A	+2.5	+1	H4 D85A	+1	+1
H4 G42A	+2	+1.5	H4 R19K	+1	+1.5
H4 I66A	+2	+2	H4 R40K	+1	+3
H4 V43A	+2	+3	H4 R67K	+1	+1
H4 E74A	+2	+1.5	H4 D68N	+1	+2
H4 R39K	+2	+1	H4 Q93E	+1	+2
H4 L49A	+2	+2	H4 Δ21-24	+1	+1.5
H4 K77R	+2	+3	H4 R78K	+1	+2
H4 K31Q	+2	+1.5	H4 V57A	+1	+2.5
H4 A38S	+2	+2	H4 L58A	+1	+1.5
H4 Y88F	+1.5	+1	H4 T80A	+1	+1.5
H4 Δ1-4	+1.5	+1	H4 R92K	+1	+1
H4 V87A	+1.5	+1	H4 R95K	+1	+1
H4 V54A	+1.5	+3	H4 Δ17-24	+1	+1
H4 H18A	+1.5	+1	H4 R3K	+1	+1
H4 F100A	+1.5	+2	H4 R55K	+1	-1
H4 L37A	+1	+1	H4 K44R	+1	+1.5
H4 K77Q	+1	+3	H4 G101A	+1	+2
H4 V86A	+1	+1	H4 Δ13-24	+1	-1
H4 L97A	+1	+1	H4 Δ17-20	+1	-2

* data from Northern analysis performed in duplicate on JDY86* strains expressing the indicated histone mutant

(SIMCHEN *et al.* 1984). Of particular note is the *SPT⁻* phenotype; a phenotype that arises due to transcription across the promoter of *LYS2*. Mutations in several genes encoding transcription-related factors that cause *SPT⁻* phenotypes, including the *SPT6* and *SPT16* histone chaperones, also derepress *SER3* (CLARK-ADAMS and WINSTON 1987; HAINER *et al.* 2011; MALONE *et al.* 1991). The lack of correlation between *SER3* derepression and *SPT⁻* phenotype for these histone mutants suggests that the transcription interference mechanisms that regulate *SER3* and *lys2-128δ* may be distinct.

Examination of the X-ray crystal structure indicates that all eight of these histone residues track DNA on the lateral surface of the nucleosome (Figure 29) and are therefore unlikely to be involved in the general stability of the histone octamer. Five of the eight amino acids, histone H3 K122, Q120, V117, and histone H4 S47 and I46, cluster at the nucleosome dyad. These residues are part of the L1L2 loop region of the $(H3\text{-}H4)_2$ tetramer that contacts DNA on either side of the nucleosome dyad (LUGER *et al.* 1997). Interestingly, two of these residues, histone H3 K122 and H4 S47, are highly conserved among eukaryotes and are targets for post-translational modifications, although the functional significance of these modifications is not known. H3 K122 has been shown to be methylated in mice and humans (PETERS *et al.* 2003; SU *et al.* 2007) and acetylated in bovine (ZHANG *et al.* 2003), however, similar modifications in yeast have not yet been detected (FREITAS *et al.* 2004; POKHOLOK *et al.* 2005). Phosphorylation of H4 S47 has been detected in yeast (FREITAS *et al.* 2004; HYLAND *et al.* 2005; ZHANG *et al.* 2003) and a phenotype associated with this residue is dependent on the substitution: S47A and S47E both confer sensitivity to HU, while S47D is not sensitive (HYLAND *et al.* 2005). Interestingly, I found that *SER3* expression is also affected differentially depending on the substitution at H4 S47. In this case, an H4 S47A mutant more weakly derepresses *SER3* than an

Table 6. Known histone mutant phenotypes

Amino acid change	Known phenotypes*
H3 K122A	Telomeric silencing, K56 hyperacetylation, zeocin^{s}, CHX^{s}
H3 K122R	Telomeric silencing
H3 K122Q	Telomeric silencing, K56 hyperacetylation, zeocin^{s}
H3 Q120A	Telomeric silencing, *SPT*$^{-}$, CHX^{s}
H3 V117A	Telomeric silencing, *SPT*$^{-}$
H3 R49A	Ribosomal and telomeric silencing, HU^{s}, ts, MMS, 6AU^{s}, *SPT*$^{-}$, CHX^{s}
H3 V46A	MMS
H4 R36A	Ribosomal and telomeric silencing, cs, ts, HU^{s}, MMS, *SPT*$^{-}$
H4 S47D	
H4 I46A	Ribosomal and telomeric silencing, ts

* Phenotypes were obtained from http://www.histonehits.org (HUANG *et al.* 2009), English et al. (ENGLISH *et al.* 2006), Matsubara et al. (MATSUBARA *et al.* 2007), and Sakamoto et al. (SAKAMOTO *et al.* 2009). HU^{s} = sensitivity to hydroxyurea; cs = cold sensitivity; ts = temperature sensitivity; 6AU^{s} = sensitivity to 6-azauracil; MMS = sensitivity to methyl methanesulfonate; *SPT*$^{-}$ = suppressor of Ty insertion phenotype; CHX^{s} = sensitivity to cycloheximide. (CHX data is from S. Hainer, unpublished)

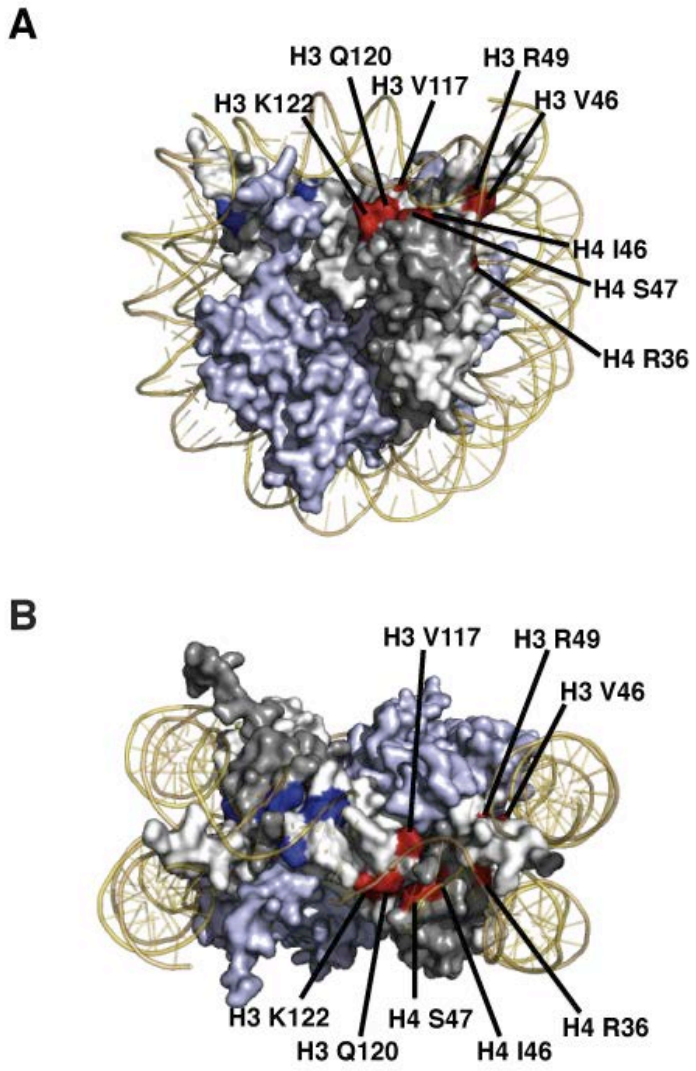

Figure 29. Mapping of the eight H3/H4 histone residues that strongly derepress *SER3* onto the yeast nucleosome crystal structure.

A) A surface representation of the yeast nucleosome core particle viewed down the DNA superhelical axis. Histone proteins are color coded as follows: H3 in white, H4 in grey, and H2A/H2B in blue. The DNA helix is shown in yellow. The five histone H3 and three histone H4 residues required for *SER3* repression are highlighted in red (one H3-H4 dimer) and blue (second H3-H4 dimer). B) Rotation of the view in Panel A by 90° around the horizontal axis revealing the lateral surface surrounding the nucleosome dyad. These images were generated by Pymol (PDB#1ID3).

S47D mutant (Figure 30). The other three residues, R49 and V46 on the N-terminal α-helix of histone H3 and R36 on α-helix 1 of histone H4 are located near the DNA entry/exit sites on the nucleosome (WHITE *et al.* 2001). Although these eight histone residues share similar locations on the nucleosome and are all required to repress *SER3*, it is unclear whether they function together in a common mechanism.

4.3.2 Nine of ten histone mutants that strongly derepress *SER3* do not confer a *sin* phenotype

Previous studies have genetically identified mutations in several genes, including the histone genes, that suppress transcriptional defects caused by the loss of a component of the Swi/Snf chromatin remodeling complex (HIRSCHHORN *et al.* 1992; HSIEH *et al.* 2010; KRUGER *et al.* 1995; KURUMIZAKA and WOLFFE 1997). The *sin* mutations (Swi/Snf independent) identified within the histone H3 and H4 genes had the following amino acid substitutions: H3 T118I, H3 R116H, H3 D123, H3 E105K, H4 R45H/C, and H4 V43I. Three of these residues, H3 T118, H3 R116, and H4 R45, lie within the L1L2 loop at the nucleosome dyad along with six of the eight H3 and H4 residues that derepress *SER3* (MUTHURAJAN *et al.* 2004). Moreover, mutations that change these amino acids to alanines confer lethality in *S. cerevisiae*, resulting in their absence from the library of histone mutations that I used for my screen (DAI *et al.* 2008). Therefore, I tested each of the histone mutants that confer strong *SER3* derepression for a *sin* phenotype using a previously described growth assay (HIRSCHHORN *et al.* 1992). Wild-type, *snf2*Δ, and *snf2*Δ strains expressing one wild-type copy of histone H3 and H4 genes (*HHT1-HHF1)* and having the second copy replaced by a synthetic copy of these genes that is either wild-type (*HHTS-HHFS*),

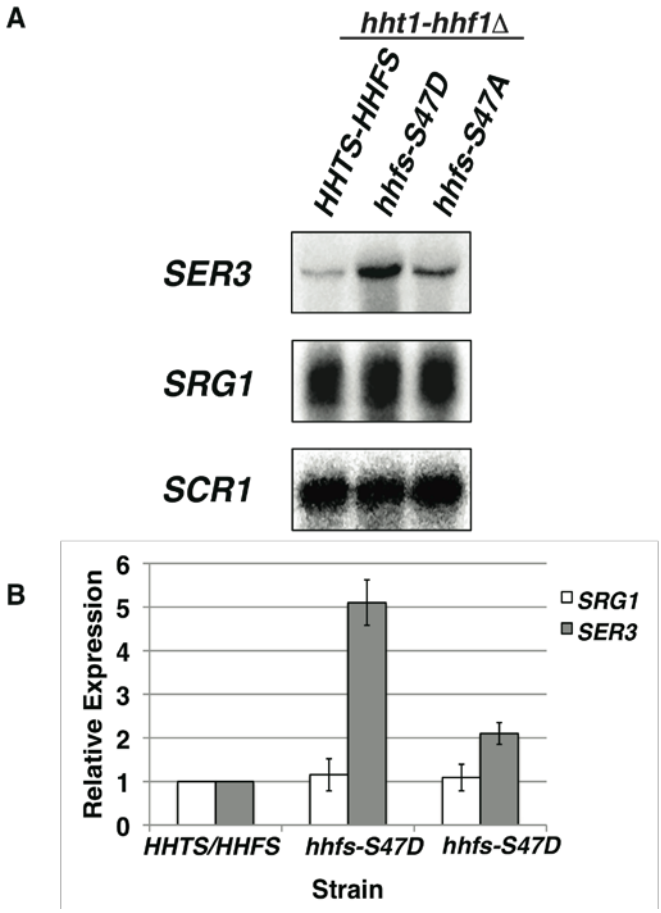

Figure 30. Derepression of *SER3* in H4 S47D and S47A.

A) Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from derivatives of JDY86 expressing synthetic wild type copies of histone H3 and H4 (*HHTS/HHFS*) or mutants *hhfs-S47D* and *hhts-S47A* that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C. B) Quantitation of Northern analyses. *SRG1* (white bars) and *SER3* (grey bars) RNA levels for the histone mutants are normalized to the *SCR1* loading control and are relative to the *SRG1* and *SER3* RNA levels measured in control *HHTS-HHFS* strains (arbitrarily set to 1). Each bar represents the mean +/- SEM from three independent experiments involving JDY86 derivatives.

or contains one of the ten mutations (for example, *hhts-K122A-HHFS*) were spotted on YPD, YPraff, and YPgal (Figure 31). As expected, a *snf2*Δ strain fails to grow on YPraff and YPgal media and this growth defect is suppressed by the *sin* mutant allele, *hhts-T118I*. Of the ten mutant histone alleles that strongly derepress *SER3*, only one, *hhts-V117A*, confers a *sin* phenotype similar to the T118I mutant. V117 lies between T118 and a second residue that confers a strong *sin* phenotype suggesting that these three amino acids are, at least in part, functionally related. Importantly, these data indicate that, with the exception of V117A, the histone mutations that confer strong *SER3* repression appear to be distinct from those that confer a *sin* phenotype.

4.3.3 Role of histone H3 T118I, a known *sin* mutation, in *SER3* regulation

The histone residues described above surround histone H3 T118, which when mutated to alanine is lethal and when mutated to isoleucine results in a *sin* (Swi/Snf independent) phenotype (BORTVIN and WINSTON 1996; KRUGER *et al.* 1995). A *sin* phenotype indicates the mutant is able to bypass the requirement of Swi/Snf in transcriptional activation (KRUGER *et al.* 1995). Therefore, an alternative hypothesis for the function of the described residues is they may facilitate the function if T118, which has been shown to allow more efficient passage of RNA pol II and decrease the stability of nucleosomes (HYLAND *et al.* 2005; KURUMIZAKA and WOLFFE 1997). Therefore, I examined the effect of T118 on *SER3* by performing Northern and nucleosome scanning analyses using a T118I mutant, which was not available in the original collection of histone mutants screened. I obtained an overexpression construct of T118I, where the substitution is in copy one of the histone genes, whereas the other histone mutants were all contained within copy two of the histone genes. With the caution that this construct is not

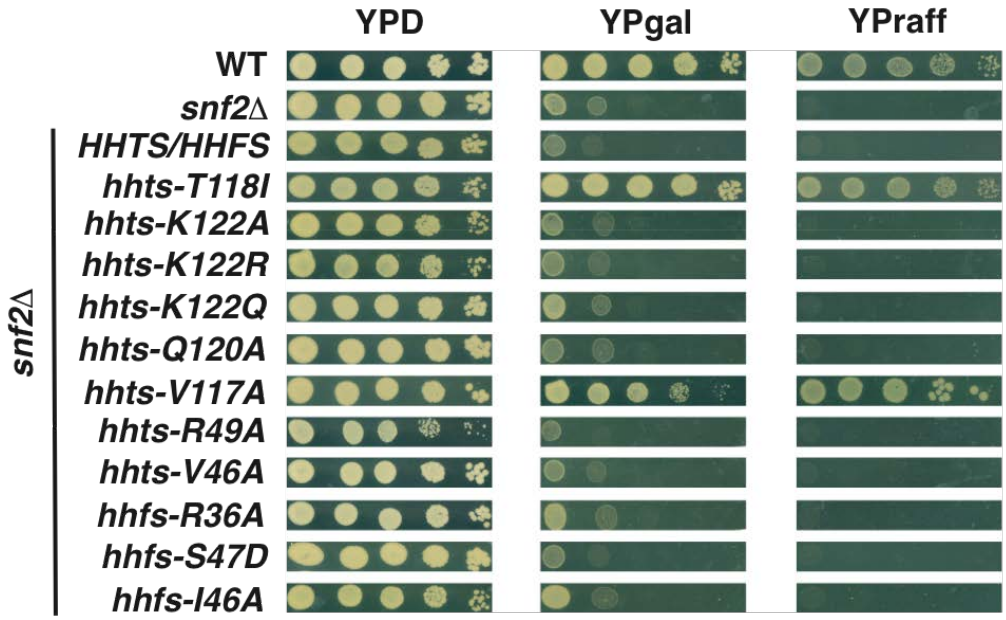

Figure 31. Analysis of histone mutants for *sin* phenotype.

Wild-type (FY4), *snf2*Δ (YJ112), *HHTS-HHFS snf2*Δ (YJ1049), *hhts-T1181 snf2*Δ (YJ1081), *hhts-K122A snf2*Δ (YJ1051), *hhts-K122R snf2*Δ (YJ1054), *hhts-K122Q snf2*Δ (YJ1057), *hhts-Q120A snf2*Δ (YJ1060), *hhts-V117A snf2*Δ (YJ1063), *hhts-R49A snf2*Δ (YJ1066), *hhts-V46A snf2*Δ (YJ1069), *hhfs-R36A snf2*Δ (YJ1072), *hhfs-S47D snf2*Δ (YJ1075), and *hhfs-I46A snf2*Δ (YJ1078) were grown to saturation in YPD at 30°C. 3 µl of 10-fold serial dilutions were spotted onto solid YPD (left panels), YPgal (middle panels), and YPraff (right panels) media and incubated for 3 days. A representative growth assay of three biological replicates that produced equivalent results is shown.

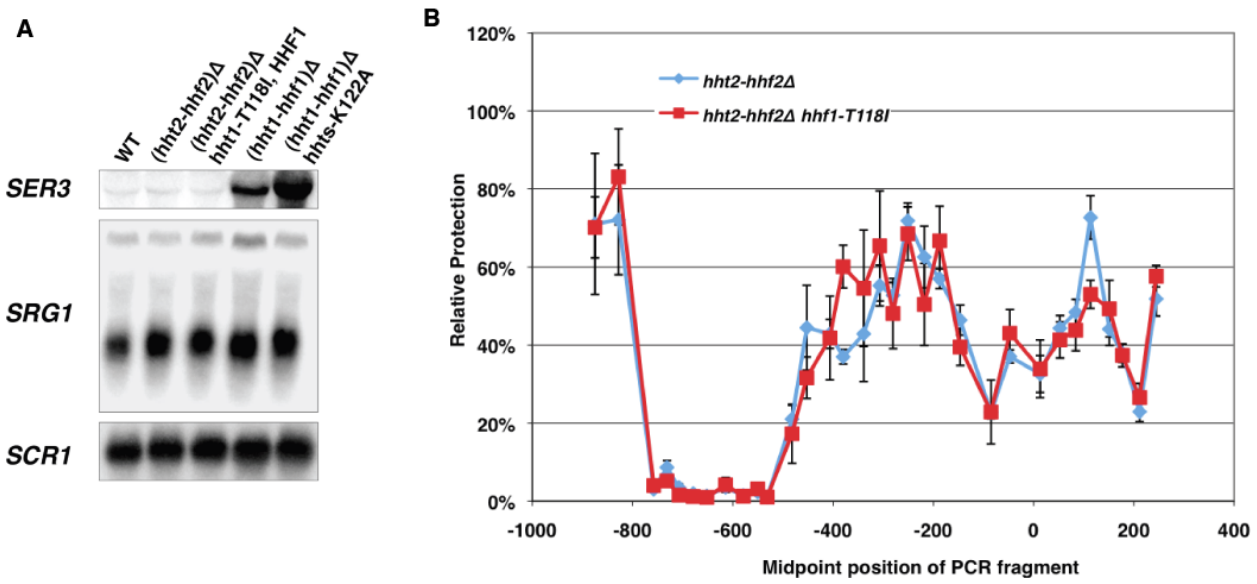

Figure 32. H3 T118I does not alter nucleosome positions over the *SER3* locus or effect *SER3* expression.

A) Northern blot analysis examining the effect of H3 T118I on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from a wild-type strain (FY4), a strain expressing only *HHT1-HHF1* (control for T118I) (YAAD828), the *hht1-T118I* mutant (YS284), and strains expressing either synthetic wild type copies of histone H3 and H4 (*HHTS/HHFS)* (control for K122A) (YJ927) or mutant *hhts-K122A* (for comparison) (YJ926), that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C. B) Nucleosome scanning assays were performed on (*hht2-hhf2*)Δ strains expressing either wild-type copies of histone H3 and H4 (*HHT1*/*HHF1;* YAAD828, YAAD958, and YAAD959) or the *hht1-T118I* mutant allele (YS284-YS286). Cells were grown in YPD media at 30°C. Each experiment was done in triplicate and the mean +/- SEM for the three replicates is plotted at the midpoint for each PCR product. Shown below each graph is a diagram of the *SER3* locus indicating the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data.

equivalent to the other mutants, I performed Northern analysis using a control proper to this mutation (*hht1-hhf1*)Δ and found this mutant had no effect on *SER3* expression (Figure 32A). Furthermore, nucleosome scanning assays revealed no loss in histone occupancy over the *SER3* locus in this mutant (Figure 32B). From these data, I conclude the H3/H4 mutants I have been characterizing are not functioning through the T118 essential residue.

4.3.4 Effect of histone mutants on nucleosome occupancy over the *SER3* promoter

To examine the effect of these ten histone mutants on nucleosome occupancy at *SER3*, I performed nucleosome scanning assays (Figure 34) as described in Chapter 2 (HAINER *et al.* 2011). Micrococcal nuclease (MNase) protection across *SER3* was normalized to the protection of a well-studied, nucleosome-bound region of the *GAL1* promoter whose digestion by MNase is unaffected by these histone mutants (Figure 33; see Materials and Methods for details). Compared to control *HHTS-HHFS* strains, protection from MNase digestion was reduced across the *SRG1* transcribed region in all ten histone mutants. MNase protection was more dramatically reduced in the H3 K122 (R/Q), H3 Q120A, H3 V117A, H4 R36A, and H4 I46A mutants than the H3 K122A, H3 R49A, H3 V46A, and H4 S47D mutants. When plotted against my Northern data, MNase protection across the *SRG1* transcription unit correlates well with *SER3* repression (Figure 35). As a control, I found that an H3 K115A mutant, which has no effect on *SER3* expression (data not shown), does not alter the nucleosome architecture across the *SER3* locus (Figure 34J). I performed histone H3 ChIP assays in these strains to complement my MNase experiments (Figure 36). In strains showing a dramatic reduction in MNase protection, I detected a significant loss of histone H3 occupancy over the *SER3* promoter, as compared with the adjacent *AIM9* ORF. For the mutants having a more modest loss of MNase protection, I was

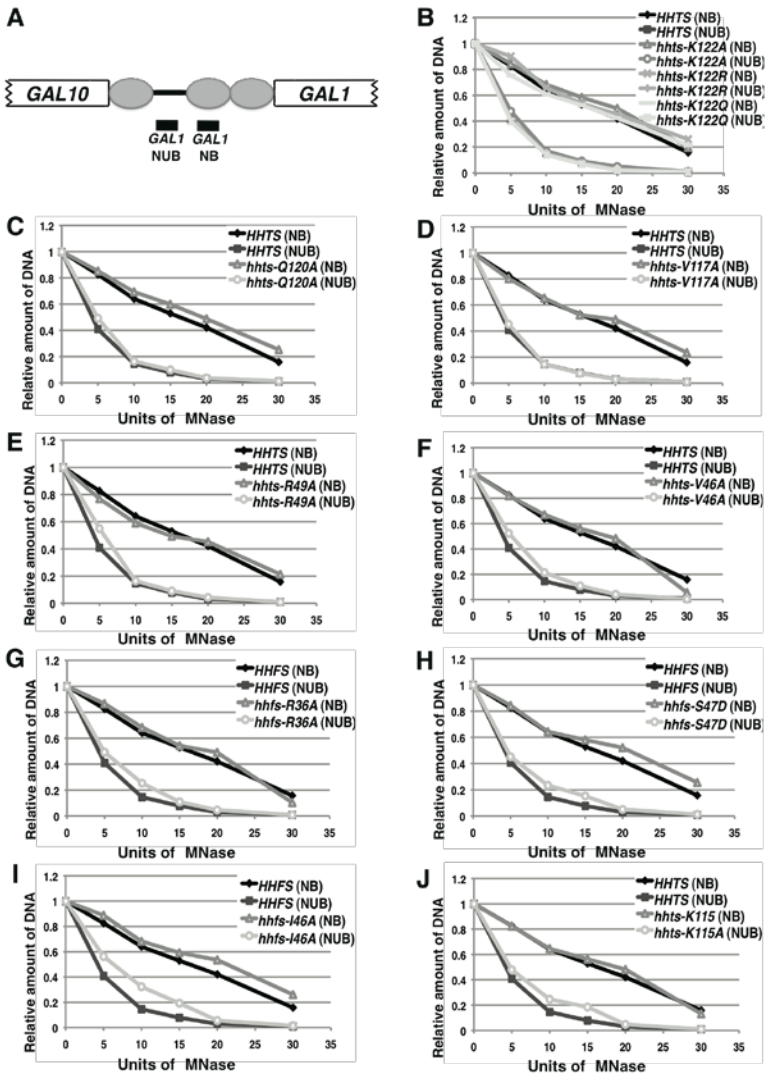

Figure 33. Histone mutations do not effect MNase digestion of *GAL1* promoter regions.

A) Schematic of the *GAL1-10* locus. Grey ovals represent well-characterized positions of three nucleosomes. Below are locations of DNA fragments amplified by qPCR to measure the amount of MNase protection of a region in the locus within a nucleosome (*GAL1* NB) and a nucleosome free region (*GAL1* NUB). B-J) The relative amount of *GAL1* NB and *GAL1* NUB amplified DNA was determined by qPCR and shown plotted against the MNase concentration used to digest the DNA. DNA isolated with a NUB/NB ratio <15% was subjected to qPCR with *SER3* primer sets. Shown is a digestion profile for one of the three strains used for each wild-type and mutant genotype, with similar results obtained for all three strains.

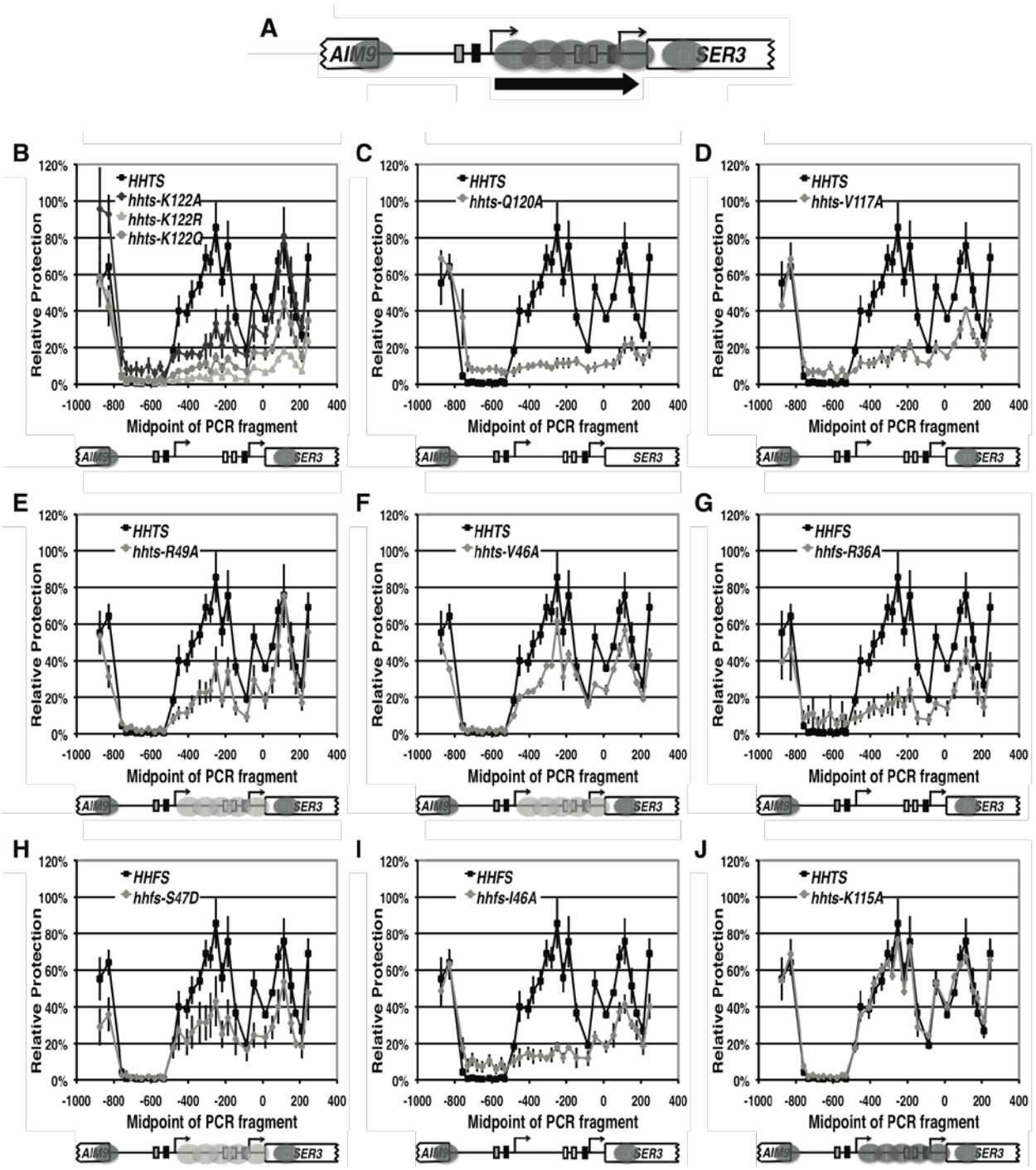
A
AIM9
SER3
B
HHTS
hhts-K122A
hhts-K122R
hhts-K122Q
C
HHTS
hhts-Q120A
D
HHTS
hhts-V117A
E
HHTS
hhts-R49A
F
HHTS
hhts-V46A
G
HHFS
hhfs-R36A
H
HHFS
hhfs-S47D
I
HHFS
hhfs-I46A
J
HHTS
hhts-K115A
Relative Protection
Midpoint of PCR fragment

Figure 34. Effect of histone mutants on nucleosome positions at *SER3*.

A) Diagram of the *SER3* locus. The gray ovals mark the position of nucleosomes when wild-type cells are grown in *SER3* repressing conditions (YPD). The block arrow indicates *SRG1* transcription. B-J) Nucleosome scanning assays were performed on *(hht1-hhf1)*Δ strains expressing either synthetic wild-type copies of histone H3 and H4 (*HHTS* and *HHFS*) or the indicated histone mutant alleles. Cells were grown in YPD media at 30°C. Each experiment was done in triplicate using one set of strains from the original histone mutation library (JDY86 derivatives) and two additional sets of strains generated by genetic crosses (YJ925-YJ946). MNase protection across the *SER3* locus relative to a positioned nucleosome within the *GAL1* promoter was determined by qPCR and the mean +/- SEM for the three replicates is plotted at the midpoint for each PCR product. Shown below each graph is a diagram of the *SER3* locus indicating the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data for each histone mutant. The light gray ovals are indicative of less dramatic reductions in MNase protections as compared to the wild-type control shown in Panel A.

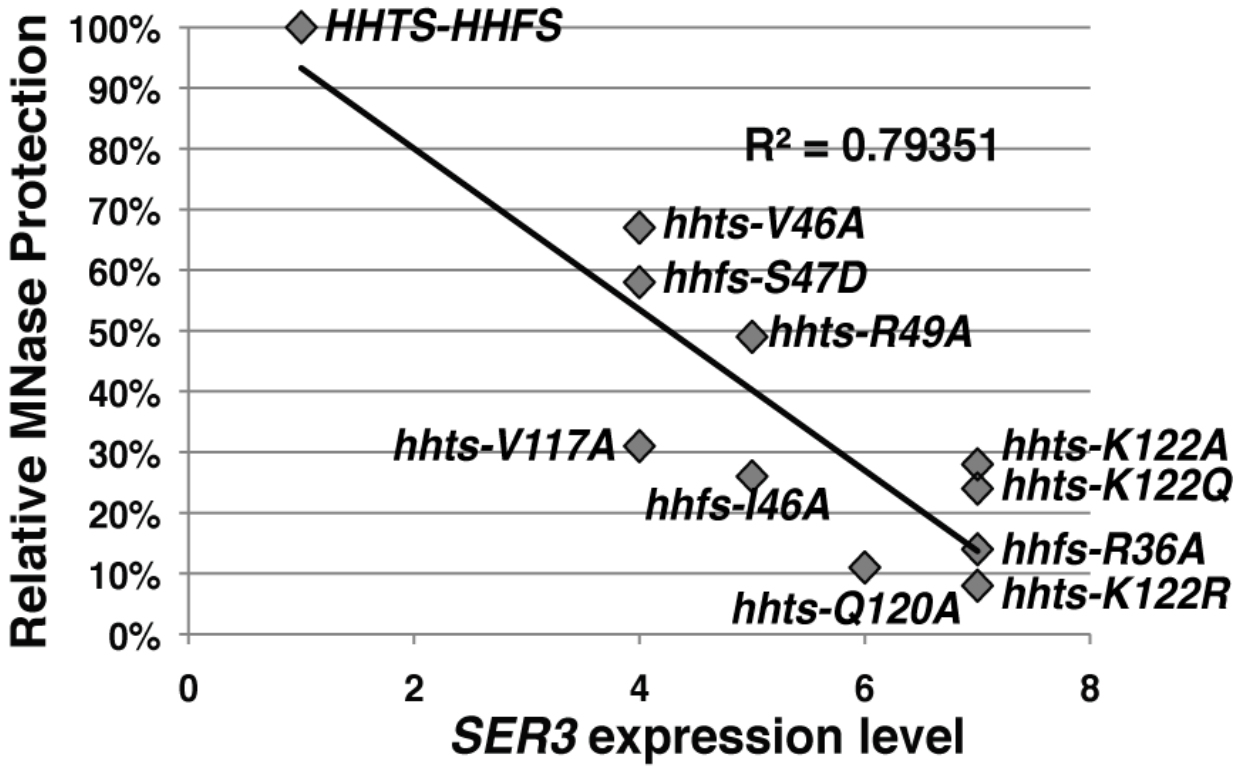

Figure 35. Correlation between MNase protection of *SRG1* and *SER3* expression.

The extent of MNase protection across the *SRG1* transcribed unit for wild-type and histone mutant strains (Figure 33) was plotted against the relative level of *SER3* expression in these strains as determined by Northern analysis (Figure 28). Change in MNase protection was calculated by taking the area under the curve over the *SRG1* transcription unit in the histone mutant strain and subtracting this from the area under the curve over the *SRG1* transcription unit in the wild-type control. All values were normalized to strains expressing synthetic copies of wild type histone H3 and H4 genes (*HHTS-HHFS*) where the MNase protection across *SRG1* was set to 100% and *SER3* expression was set to 1. Line of best fit and R^2 value were determined by linear regression.

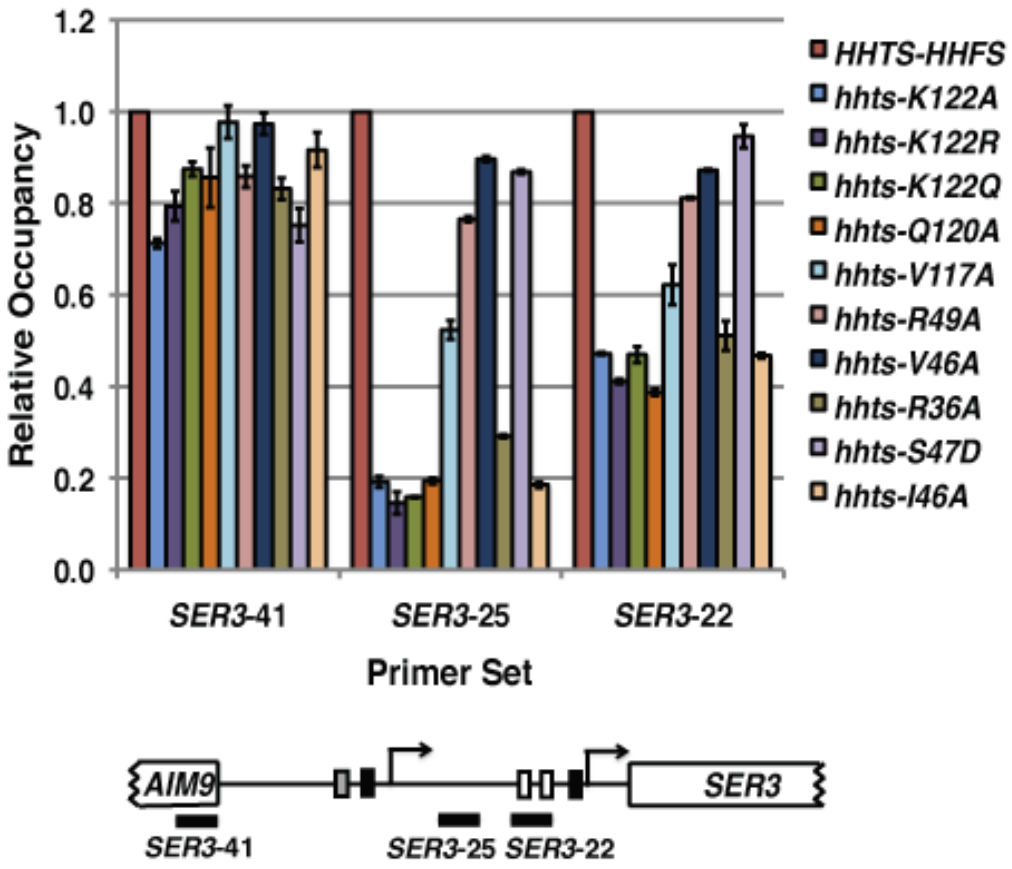

Figure 36. Relative occupancy of histone H3 in histone mutants over *SER3*.

Histone H3 ChIP was performed on chromatin isolated from (*hht1-hhf1*)Δ strains expressing *HHTS-HHFS* alleles (JDY86, YJ927, and YJ928) or the indicated histone mutant alleles (JDY86 derivative, YJ925, YJ926, YJ930-YJ946) that were grown in YPD at 30°C. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and represents the mean +/- SEM of three experiments. Histone H3 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

unable to detect a significant decrease in histone H3 occupancy over the *SER3* promoter. Taken together, my data identify eight histone residues that contribute to *SER3* repression by facilitating *SRG1* transcription-dependent nucleosome occupancy across the *SER3* promoter.

4.3.5 Histone H3 V46 and R49 are required to repress cryptic intragenic transcription

A previous study has shown that transcription from cryptic promoters located within protein-coding genes is repressed by maintaining normal chromatin structure across these regions during transcription (KAPLAN *et al.* 2003). Mutations that impair a number of factors that alter either nucleosome assembly or post-translational modifications to histone proteins, such as the Spt6 and Spt16 transcription factors and the Set2 histone methyltransferase, have been shown to allow cryptic transcription (CARROZZA *et al.* 2005; CHEUNG *et al.* 2008; JOSHI and STRUHL 2005; KAPLAN *et al.* 2003; POKHOLOK *et al.* 2005; RAO *et al.* 2005). In addition, several amino acid substitutions in histone H3 were identified in a genetic selection for mutations that promote cryptic transcription (CHEUNG *et al.* 2008; DU and BRIGGS 2010; DU *et al.* 2008; DUINA *et al.* 2007; PSATHAS *et al.* 2009; ZHENG *et al.*). To test whether the ten histone mutants that reduce nucleosome occupancy over the *SER3* promoter have a more general defect in transcription-dependent nucleosome assembly, I determined whether these mutants permit cryptic intragenic transcription by Northern analysis (Figure 37A). I examined the production of previously observed aberrant transcripts that initiate within the protein coding sequences of three genes, *FLO8*, *STE11*, and *SYF1* (CHEUNG *et al.* 2008; KAPLAN *et al.* 2003). Two of the ten histone mutants, H3 R49A and H3 V46A, produce aberrant transcripts similar to those previously described for an *spt6-1004* mutant (KAPLAN *et al.* 2003). Therefore, histone H3 R49 and V46 may have a more general role in regulating chromatin structure during transcription.

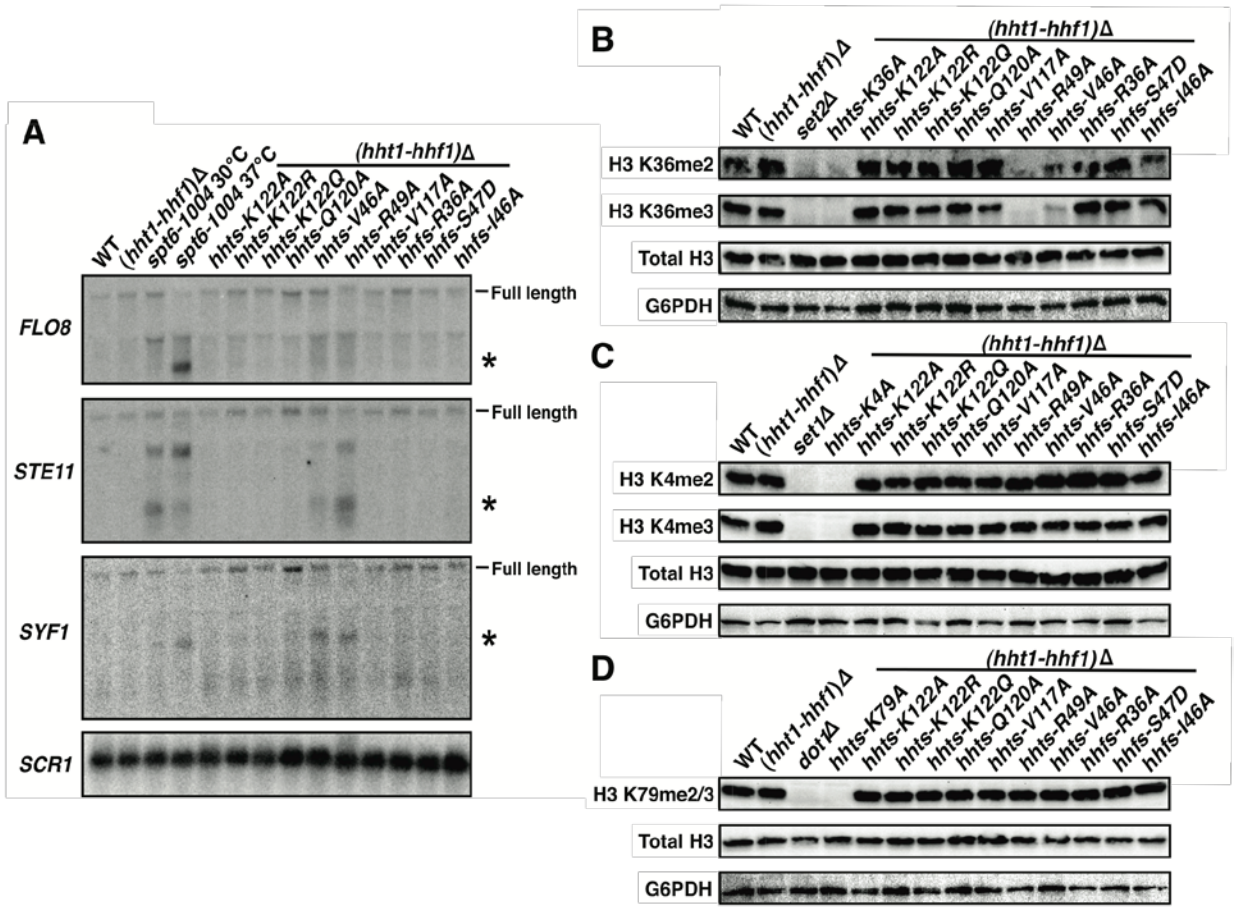

Figure 37. Effect of histone mutants on cryptic intragenic transcription and post-translational histone modifications.

A) Northern analysis of *FLO8, STE11,* and *SYF1* for cryptic intragenic transcription. Total RNA was isolated from *(hht1-hhf1)*Δ strains that express either synthetic wild-type copies of histone H3 and H4 or the indicated histone mutant alleles (JDY86 derivatives). Strains wild type for both copies of histone H3 and H4 (WT) and expressing either a normal copy of *SPT6* (WT) or the *spt6-1004* mutant allele were included as negative and positive controls for cryptic transcription. All strains were grown in YPD at 30°C except for the *spt6-1004* mutant which was also shifted to 37°C for 60 minutes as indicated. Cryptic

transcripts for each gene are marked with an asterisk. *SCR1* serves as a loading control. B-D) Western analyses of post-translational histone modifications. Whole cell extracts were prepared from wild-type (FY4), *set2*Δ (KY1716), *set1*Δ (KY1755), and *dot1*Δ (KY1907) strains and *(hht1-hhf1)*Δ strains expressing either synthetic wild-type copies of histone H3 and H4 or the indicated histone mutant alleles (JDY86 derivatives) that were grown in YPD at 30°C. Immunoblots of WCEs were probed with H3 K36 (Panel B), H3 K4 (Panel C), or H3 K79 (Panel D) methyl-specific antibodies. Immunoblots of total H3 and G6PDH are provided as loading controls. Similar results were observed for two distinct sets of strains (YJ925-YJ946).

Set2-dependent methylation of histone H3 K36 has been shown to play a role in the repression of cryptic transcription by recruiting and/or directing activity of the Rpd3S histone deacetylase complex to remove acetylation marks from the amino terminal tails of histone H4 after passage of RNA pol II (CARROZZA *et al.* 2005; CHEUNG *et al.* 2008; DROUIN *et al.* 2010; JOSHI and STRUHL 2005; KEOGH *et al.* 2005; POKHOLOK *et al.* 2005; RAO *et al.* 2005; YOUDELL *et al.* 2008). The resulting hypoacetylated nucleosomes are thought to protect recently transcribed DNA from the binding of transcription factors and intragenic transcription. Therefore, I tested whether the histone H3 R49A and V46A mutants were defective in H3 K36 methylation by Western analysis (Figure 37B). In agreement with my cryptic intragenic transcription data, the H3 R49A and H3 V46A substitutions dramatically reduced global levels of histone H3 K36 di- and tri-methylation. These defects were specific to histone H3 K36 as the methylation at two other sites on histone H3, K4 and K79, were unaffected (Figure 37C and 37D). In contrast, the remaining eight histone mutants had little to no effect on the methylation state at all three of these histone H3 lysines. These data suggest that histone H3 R49 and V46 repress cryptic intragenic transcription by maintaining normal Set2-dependent methylation of histone H3 K36. However, since I have previously shown that *SER3* repression is independent of the methylation states of histone H3 K4, K36, and K79 (Chapter 2) (HAINER *et al.* 2011), histone H3 R49 and V46 are likely to repress *SER3* by a mechanism independent from their involvement in histone H3 K36 methylation. I also used a previously described reporter growth assay to examine the effect of the histone mutants on cryptic intragenic transcription (as described in Chapter 3), and found that most of the histone mutants increased growth of the reporter strain on media containing galactose as the sole carbon source, whereas only H3 R49A had a strong increased growth of the reporter strain on media containing glucose as the sole carbon source

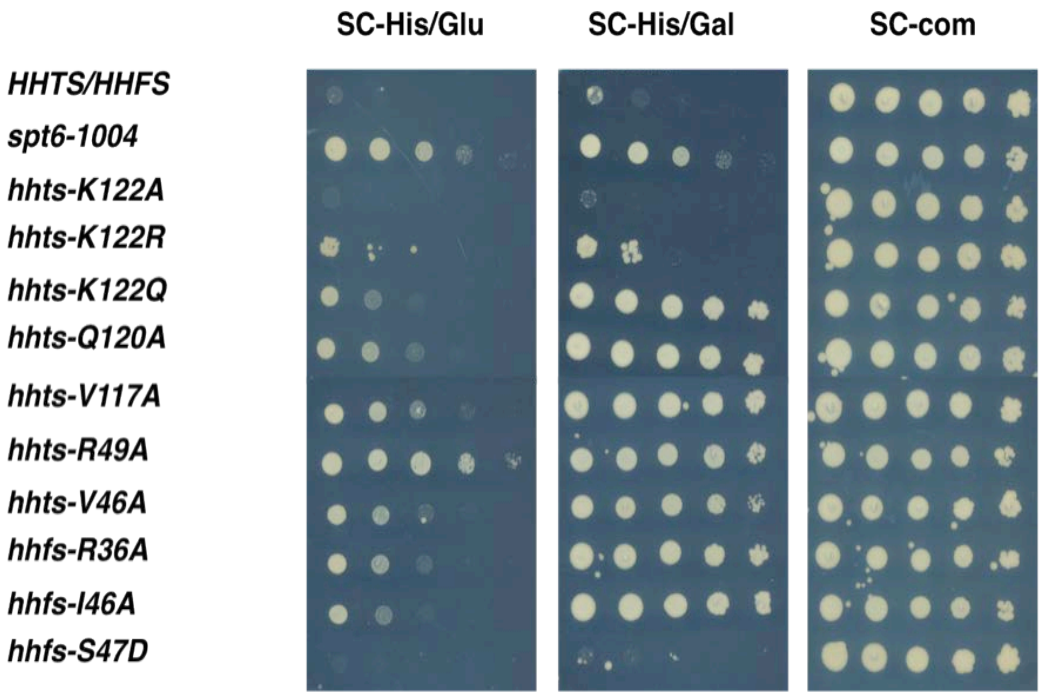

Figure 38. Most histone mutants result in cryptic initiation using an inducible reporter. Strains were created in which the *FLO8* gene was driven under the inducible *GAL1*pr containing the histone mutations. Cells were serially diluted and plated onto media containing glucose (*GAL1*pr off) and on media containing galactose (*GAL1*pr on).

(Figure 38). Therefore, with the exception of R49A, the glucose results suggest that the histone mutations play only a minor role in disrupting chromatin in the absence of transcription.

4.3.6 Effect of histone mutants on histone H3 occupancy at other genes

Several studies have indicated that cryptic intragenic transcription is more common within genes that are lowly transcribed (CHEUNG *et al.* 2008; LI *et al.* 2007b). In contrast, transcription run-on and RNA pol II ChIP experiments have indicated that *SRG1* is a highly transcribed region of the yeast genome (MARTENS *et al.* 2004; MARTENS *et al.* 2005). Therefore, I performed histone H3 ChIP assays to test whether these ten histone mutants cause a more general defect in nucleosome occupancy at other highly transcribed yeast genes (Figure 39). At three highly transcribed genes, *PMA1* (100 mRNA/hr), *PYK1* (95 mRNA/hr), and *ADH1* (125 mRNA/hr) (HOLSTEGE *et al.* 1998), histone H3 levels were reduced in seven of the ten mutants corresponding to those that show the strongest effects on nucleosome occupancy at the *SER3* promoter (H3 K122(A/R/Q), H3 Q120A, H3 V117A, H4 R36A, and H4 I46A). The only exception is the H3 V117A mutant, which results in reduced histone occupancy at *PMA1* and *PYK1* but not over *ADH1*. Conversely, histone H3 occupancy at three lowly transcribed genes, *GAL1* (repressed), *TUB2* (12 mRNA/hr), and *CYC1* (10 mRNA/hr) (HOLSTEGE *et al.* 1998), was either unaffected or slightly increased in nine of the ten histone mutants. The only exception was the H4 S47D mutant where we found a surprising 2-fold increase in histone H3 levels towards the 3' end of all three lowly transcribed genes. Furthermore, inducing high levels of *GAL1* expression by growing cells in the presence of galactose, uncovered histone H3 occupancy defects in the histone mutants similar to those detected at the other highly transcribed genes (Figure 40). While an effect on gene expression of these genes might not be expected, I performed preliminary Northern blot analysis using all ten

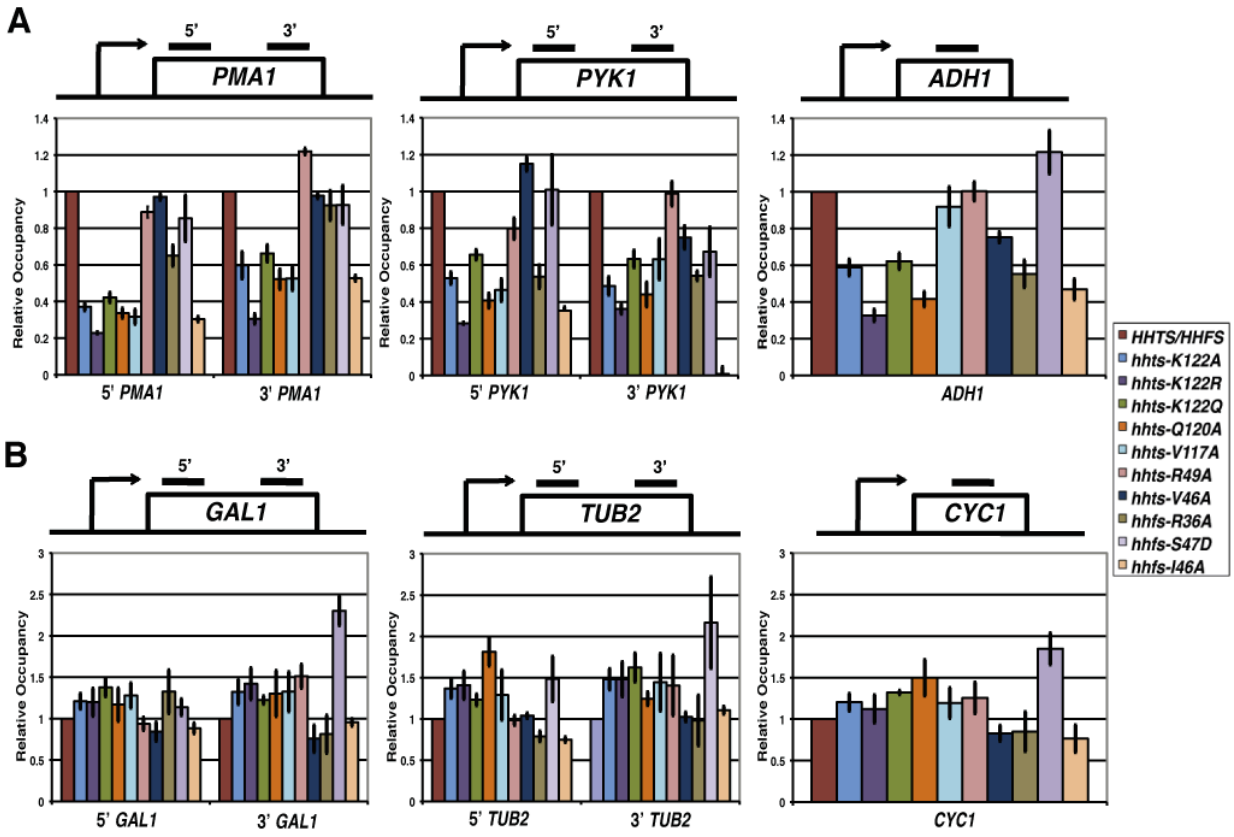

Figure 39. Effect of histone mutants on histone H3 occupancy over the coding regions of a subset of yeast genes.

A) Histone H3 ChIP analysis was performed on chromatin prepared from *(hht1-hhf1)*Δ strains expressing *HHTS-HHFS* alleles (JDY86, YJ927, and YJ928) or the indicated histone mutant alleles (JDY86 derivative, YJ925, YJ926, YJ930-YJ946) that were grown in YPD at 30°C. Histone H3 occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Histone H3 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Histone H3 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

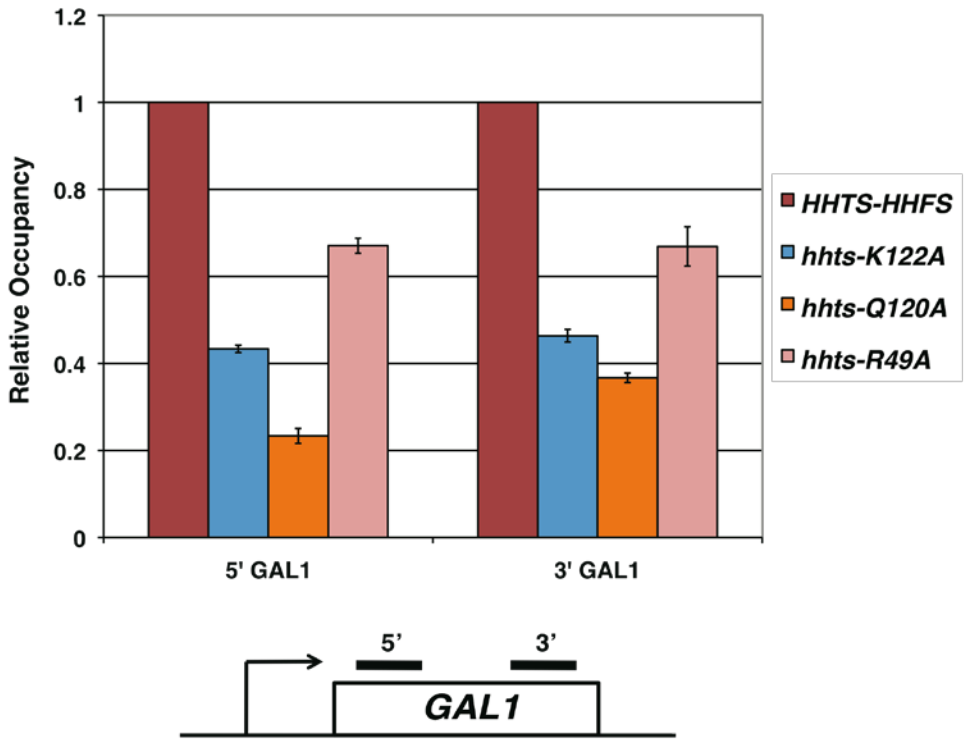

Figure 40. Relative occupancy of histone H3 in histone mutants over *GAL1*.

Histone H3 ChIP was performed on c hromatin isolated from (*hht1-hhf1*)Δ strains expressing *HHTS-HHFS* alleles (JDY86, YJ927, and YJ928) or the indicated histone mutant alleles (JDY86 derivative,YJ926, YJ927, YJ933, YJ934, YJ937, and YJ938) that were grown in YPraff at 30°C and shifted to YPgal for 1 h r. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and represents the mean +/- SEM of three experiments. Histone H3 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. Below the graph is a schematic of *GAL1* with black bars corresponding to the regions amplifed by qPCR.

histone mutants, examining their effect on two of the highly transcribed genes, *PYK1* and *PMA1* (Figure 41). As predicted, the histone mutants did not dramatically alter expression of these genes, with the exception of *hhfs-I46A*, which resulted in an increase in *PMA1* expression, and a subset of the mutants slightly increasing *PYK1* expression. Additionally, I preliminarily examined the effect of the various K122 point mutations on *GAL10* gene expression, to which the mutants did not alter expression levels in either repressing (YPD), non-inducing (YPRaff), or inducing conditions (YPGal) (Figure 42).

Taken together with my analysis at *SRG1*, I identified a new set of histone mutants that are defective for transcription-coupled nucleosome occupancy specifically at highly transcribed genes. These data support recent studies which suggest that high levels of transcription result in nucleosome displacement whereas regions with low levels of transcription maintain nucleosome occupancy (DION *et al.* 2007; IVANOVSKA *et al.* 2010; JAMAI *et al.* 2007; JIN *et al.* 2010; KIMURA and COOK 2001; KRISTJUHAN and SVEJSTRUP 2004; KULAEVA *et al.* 2009; KULAEVA *et al.* 2010; RUFIANGE *et al.* 2007; SCHWABISH and STRUHL 2004; THIRIET and HAYES 2005).

4.4 DISCUSSION

In this Chapter, I systematically tested the contribution of all non-essential amino acids in histones H3 and H4 to *SER3* repression. I identified changes of 52 histone H3 and H4 residues that derepress *SER3* where the increase in *SER3* RNA levels ranged from modest (1.5-fold) to strong (7-fold), further emphasizing the prominent role of nucleosomes in *SER3* repression by transcription of *SRG1* ncDNA. Of particular interest were substitutions of eight histone residues that most strongly derepress *SER3* (>4-fold) without reducing *SRG1* transcription. All eight

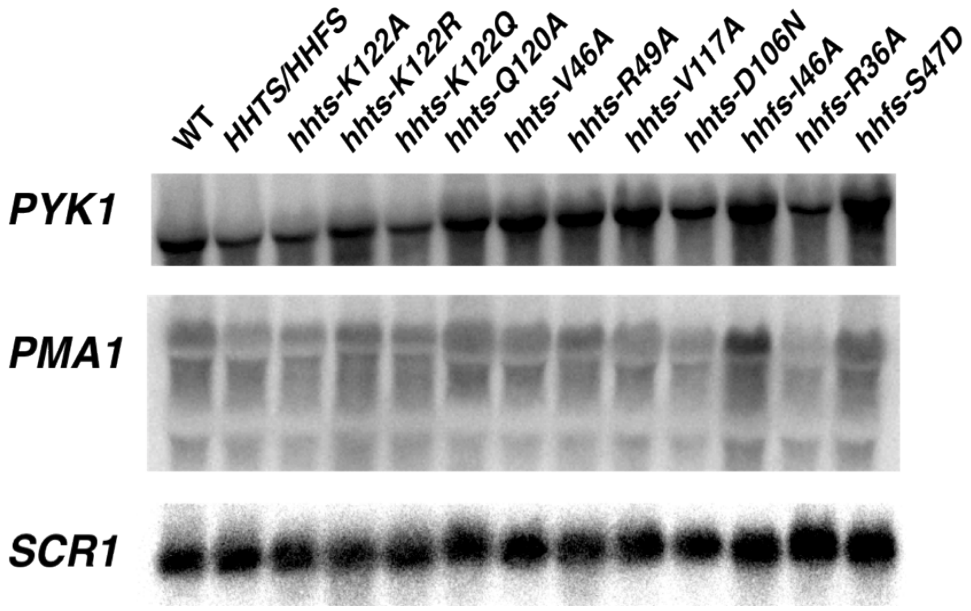

Figure 41. Effect of histone mutants on expression of other ORFS.

Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from a wild-type strain (FY4) and derivatives of JDY86 expressing either synthetic, wild-type copies of histone H3 and H4 (*HHTS/HHFS)* or mutants *hhts-K122A*, *hhts-K122R*, *hhts-K122Q*, *hhts-Q120A*, *hhts-V117A*, *hhts-R49A*, *hhts-V46A*, *hhfs-I46A*, *hhfs-R36A*, and *hhfs-S47D* that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C. Analysis was only performed once.

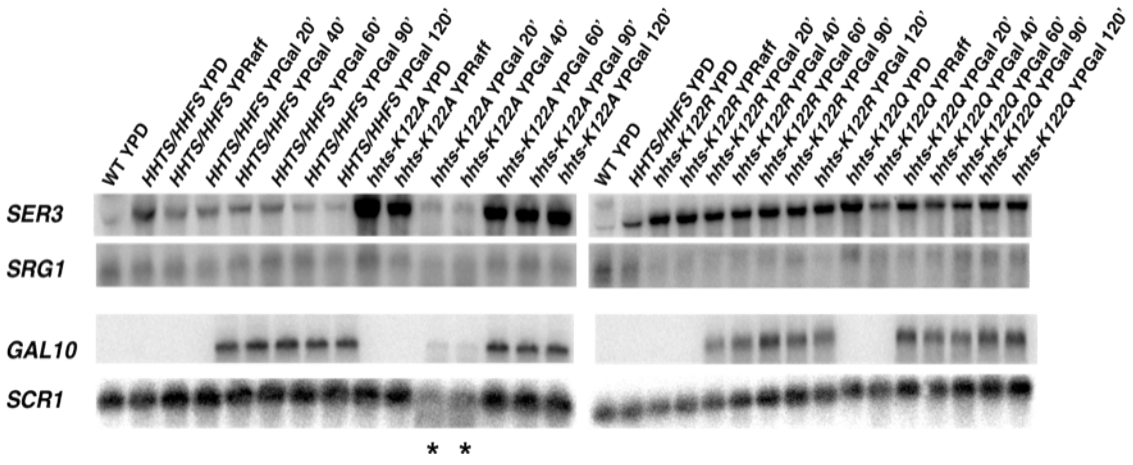

Figure 42. K122 mutations do not affect the expression of *GAL10*.

Northern analysis was performed on RNA isolated from cells grown to early log in YPraff and then for an additional 20 to 120 minutes in YPgal. Blots were probed with *SER3, SRG1, GAL10* and *SCR1* serves as a loading control. Mutations in K122 cause a derepression of *SER3*, no effect on *SRG1* mRNA levels, and no effect over wild-type on *GAL10*. Lanes designated with an asterisk (*) illustrate samples that were degraded during RNA preparation. Northern analysis was only performed once.

residues are located on the lateral surface of the nucleosome; five residues, H3 K122, H3 Q120, H3 V117, H4 I46, and H4 S47, that track the DNA binding surface near the nucleosome dyad, and three residues, H3 R49, H3 V46, and H4 R36, near the DNA entry/exit point (DAI *et al.* 2008; WHITE *et al.* 2001). My nucleosome scanning experiments show that substitutions of these non-essential residues impair nucleosome occupancy at the *SER3* promoter, thus resulting in *SER3* derepression. Moreover, substitutions of all but three of these residues reduce histone H3 occupancy over the open reading frames of highly transcribed, but not lowly transcribed genes. Of note, the three exceptions, H3 R49A, H3 V46A, and H4 S47D mutations more modestly reduce MNase protection at *SER3* compared to the other mutants, which was not evident by histone H3 ChIP. Therefore, these three mutants are also likely to cause modest effects on nucleosome occupancy at other highly transcribed genes that may only be detected by the more sensitive nucleosome scanning assay. Taken together, my data reveal a class of histone residues that are required for nucleosome occupancy specifically at locations of high transcription activity with a greater dependence on H3 K122, Q120, V117, and H4 R36 and I46 than H3 R49, V46, and H4 S47.

Results from this study and others suggest that the histone mutants that strongly derepress *SER3* are defective for *SRG1* transcription-dependent deposition of nucleosomes over the *SER3* promoter. First, we had previously established that nucleosome occupancy of the *SER3* promoter is dependent on *SRG1* transcription and the Spt6 and Spt16 histone chaperones, which are required to restore nucleosome occupancy after passage of RNA pol II (HAINER *et al.* 2011). Second, nucleosome occupancy over other highly transcribed genes, but not lowly transcribed genes, is reduced by mutations that alter these eight histone residues. Finally at *GAL1*, I showed that these histone residues are required to maintain nucleosome occupancy across its open

reading frame in cells that were grown in galactose (*GAL1* on), but not glucose (*GAL1* off), directly demonstrating that the effect of these mutants is transcription dependent.

An alternative possibility is that the rate or magnitude of chromatin remodeling at the *SER3* promoter that may be required for *SER3* activation is enhanced in these histone mutants. If this were the case, nucleosome occupancy at the *SER3* promoter should be restored in the absence of the chromatin remodeling factor and the sequence-specific activator that is responsible for its recruitment. However, I have found that deletion of the *SER3* UAS, which severely impairs *SER3* expression in the absence of *SRG1* transcription, does not restore nucleosome occupancy to the *SER3* promoter (HAINER *et al.* 2011). Although I cannot completely discount this alternative possibility, these results are more consistent with the histone mutations being defective for *SRG1* transcription-dependent deposition of nucleosomes over the *SER3* promoter.

Interestingly, changes of six of the eight histone residues required for *SER3* repression do not permit cryptic intragenic transcription, which is also controlled by transcription-dependent chromatin architecture (CARROZZA *et al.* 2005; KAPLAN *et al.* 2003; LI *et al.* 2007b). Based on my ChIP studies at other lowly transcribed genes, the absence of cryptic intragenic transcription is likely attributable to the low transcription frequency of the *FLO8, STE11,* and *SYF1* genes that were used in this assay (HOLSTEGE *et al.* 1998). The fact that histone H3 V46A and R49A mutants permit cryptic initiation is likely a consequence of the reduced di- and tri-methylation of H3 K36 that we observe in these mutants. Set2-dependent methylation of histone H3 K36 has been shown previously to play an important role in preventing cryptic intragenic transcription (DROUIN *et al.* 2010; DU and BRIGGS 2010; DU *et al.* 2008; PSATHAS *et al.* 2009). However, evidence from this work and others strongly suggests that the role of H3 V46 and R49 in

promoting K36 methylation is distinct from their role in *SER3* repression. First, alteration of these two residues does not alter nucleosome occupancy over lowly transcribed genes. Second, deletion of the Set2 methyltransferase that is responsible for histone H3 K36 methylation has no effect on *SER3* gene expression (Figure 37). Third, substitutions of H3 K36 have little to no effect on *SER3* gene expression (Table 5). Therefore, H3 V46 and R49 may have multiple roles in maintaining nucleosome integrity during transcription depending on the target gene; an indirect role by facilitating Set2-dependent methylation of lysine 36 on hi stone H3 or a more direct role in transcription-coupled histone deposition. It will be interesting to determine how histone H3 R49 and V46 functionally relate to other histone residues needed for proper H3 K36 methylation, including the N-terminal tail, histone H4 K44, three H2A residues, I112, L116, and L117, and two H3 residues, R52 and N108 (DROUIN *et al.* 2010; DU and BRIGGS 2010; DU *et al.* 2008; PSATHAS *et al.* 2009).

Several of the histone residues that strongly derepress *SER3* when substituted, cluster at the nucleosome dyad. Histone residues H3 K122, Q120, and V117 are located within the L2 loop of histone H3, which is juxtaposed to the L1 loop of histone H4 that includes I46 and S47, to form the L1L2 region of the $(H3\text{-}H4)_2$ tetramer that organizes the central two turns of nucleosomal DNA (LUGER *et al.* 1997). Recent single molecule studies have indicated this region makes the largest contribution to the DNA-histone interactions within a nucleosome (HALL *et al.* 2009). Several structural features are likely to contribute to the strength of this binding (LUGER and RICHMOND 1998). First, the overall structure of the L1L2 loop region at the nucleosome dyad puts it in close proximity to DNA allowing an extensive series of hydrogen bonds between the main peptide chain amides and the phosphate backbone of DNA. Second, histone H4 R45 extends into the minor groove, where it makes contact with the DNA backbone

and also stabilizes the position of H3 T118, which also contacts the DNA backbone. Third, histone H3 R116 forms a salt bridge with H3 D123 and a hydrogen bond with the backbone carbonyl of T118, further stabilizing the L1L2 loop. Interactions between Q120, K121, and K122 protect this salt bridge from solvent and also coordinate a chloride ion. Genetic studies have also indicated the functional significance of this region. In creating the histone mutant library, Dai *et al*. noted that of the surprisingly small number of alanine substitutions in histone H3 and H4 that cause lethality, many cluster within this L1L2 loop region, including H4 R45, H3 T118, and H3 R116 (DAI *et al.* 2008). As noted earlier, other amino acid substitutions of these three residues were also identified as *sin* mutants (HIRSCHHORN *et al.* 1992; HSIEH *et al.* 2010; KRUGER *et al.* 1995; KURUMIZAKA and WOLFFE 1997).

How might this class of histone residues affect transcription-coupled nucleosome occupancy of highly transcribed genes? One possibility is that mutations that alter any one of these eight residues may reduce the affinity of the histone octamer for DNA. In one scenario, these mutations may increase the mobility of nucleosomes similar to what has been shown for *sin* mutations located at the nucleosome dyad (FLAUS *et al.* 2004). Nucleosomes containing these histone mutants would be properly reassembled during *SRG1* transcription, but then are mobilized away from the *SER3* promoter. However, this is not likely to be the case as only one of the histone mutants, H3 V117A, confers a *sin* phenotype. Moreover, our nucleosome scanning experiments gave no i ndication of nucleosome mobility at other locations including more positioned nucleosomes over the open reading frames of *AIM9* and *SER3* that flank *SRG1* (Figure 34) and the repressed *GAL1* promoter (Figure 33). A more likely scenario is that a reduction in DNA affinity may slow nucleosome reassembly after passage of RNA pol II. This could account for my contrasting observations between lowly and highly transcribed regions of

the genome. At lowly transcribed genes, a nucleosome will have sufficient time to reassemble prior to the passage of the next RNA pol II so the density of nucleosomes will not be affected. However, at highly transcribed genes, nucleosomes will only be partially assembled before being disassembled by the next RNA pol II molecule, resulting in reduced nucleosome occupancy at these genes.

These eight histone residues may contribute to the overall histone-DNA affinity by distinct mechanisms. Alteration of the residues within the L1L2 region at the nucleosome dyad (H3 K122, Q120, and V117, and H4 I46 and S47) may disrupt this structure resulting in a reduced number of contacts between histone and DNA backbone in this region. Alternatively, these residues may be affecting a critical function of the essential residues in this region, such as the threonine at position 118 in histone H3 and the arginine at position 45 of histone H4. Interestingly, several *in vitro* studies investigating *sin* mutations, in particular those involving H3 T118 and H4 R45, have provided evidence to support their role in histone-DNA affinity (HSIEH *et al.* 2010; KURUMIZAKA and WOLFFE 1997; MUTHURAJAN *et al.* 2004). However, with the exception of V117A, substitutions of H3 K122, Q120, H4 I46A and H4 S47 do not confer a similar *sin* phenotype, suggesting that any role these residues may play in DNA affinity is either more moderate or distinct from those of H3 T118 and H4 R45. The other three mutations, histone H3 R49, V46 and H4 R36 are unlikely to affect the L1L2 loop region at the nucleosome dyad but rather may disrupt histone-DNA interactions at the DNA entry/exit within the nucleosome, where H3 R49 is also positioned within the minor groove of DNA.

In summary, I have provided evidence that at least eight histone residues, five in histone H3 and three in histone H4, are required for normal transcription-coupled nucleosome occupancy specifically at highly transcribed genes. I have also shown a distinct role for two of these

residues, histone H3 R49 and V46, in promoting Set2-dependent methylation of histone H3 K36. Further analysis of this new class of histone mutants is likely to provide answers to questions and will enhance our understanding of transcription-coupled nucleosome dynamics.

5.0 HISTONE RESIDUES REQUIRED FOR PROPER RECRUITMENT, ACTIVITY, AND BINDING OF HISTONE CHAPERONES

The work discussed in this Chapter is unpublished data of which I performed all the experiments described.

5.1 INTRODUCTION

Based on work presented in Chapter 4, I have identified eight amino acids in histones H3 and H4 that are required for nucleosome occupancy specifically over highly transcribed regions of the genome (HAINER and MARTENS 2011a). While I have revealed general phenotypes of the histone mutants, the mechanism through which these residues are required for maintenance of nucleosome occupancy has not been elucidated. Several hypotheses which could explain the requirement for these histone residue mutations are that they: 1) destabilize nucleosomes; 2) result in decreased histone chaperone occupancy/activity; and/or 3) cause defective nucleosome disassembly/reassembly.

The work I present in this Chapter has been aimed at determining the mechanism through which these histone residues regulate nucleosome architecture. While this work is still in progress, it suggests that the histone residues are required for Spt2, Spt6, and Spt16 occupancy

and therefore, when the residues are substituted, these proteins no l onger occupy highly transcribed regions of the genome, resulting in a decreased reassembly rate of the nucleosomes.

5.2 MATERIALS AND METHODS

5.2.1 Strains and Media

All *S. cerevisiae* strains used in this study (Table 7) are isogenic with a *GAL2+* derivative of S288C (WINSTON *et al.* 1995). Strains were constructed using standard genetic crosses or by transformation (AUSUBEL 1991). Tagged versions of Spt2 and Paf1 have been previously described (CRISUCCI and ARNDT 2011a; NOURANI *et al.* 2006). Asf1-TAP, Spt2-TAP, Spt6-TAP, and Spt16-TAP strains were validated from the TAP-tag collection (Open Biosystems) and amplified from this strain to tag Asf1, Spt2, Spt6, or Spt16, respectively, in our strain background. Synthetic histone strains were created by a one-step integration of plasmids expressing synthetic histone genes targeted for *HHT1/HHF1* tagged with a hygromycin resistant cassette (kind gift from J. Dai, Tsinghua University) into JDY86 strains expressing the same synthetic histone gene sequence at *HHF2/HHF2*. Briefly, plasmids were linearized with *Bci*VI and transformed into the correct JDY86 strain. Transformants were selected on YPD media containing 200ug/mL of hygromycin, and confirmed through PCR and sequencing. Strains were grown in the following media as indicated in the figure legends: YPD (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose), YPRaff (1% yeast extract, 2% peptone, 2% raffinose), and synthetic complete with 1mM serine (SC+serine) or without serine (SC-serine) (ROSE 1991).

5.2.2 Northern Analysis

Cells were grown to 1-2 × 10^7 cells/ml in YPD at 30°C. Total RNA isolation and Northern analysis was performed as previously described (COLLART and OLIVIERO 2001). Radiolabeled DNA probes were generated by random-primed labeling of PCR fragments for *SRG1* (-454 to -123 relative to *SER3* ATG), *SER3* (+111 to +1342), *FMP27* (+1982 to +2296), and *SCR1* (-163 to +284) that were amplified from genomic DNA. RNA levels were quantified using a PhosphorImager (Instant Imager, Packard Co.) and normalized to the *SCR1* loading control.

5.2.3 Western Analysis

Whole cell extracts (WCE) were prepared from cells grown in YPD at 30°C to approximately 3 × 10^7 cells/ml using trichloroacetic acid as previously described (COX *et al.* 1997; ZHENG *et al.* 2010). Equal amounts of WCE were separated by 12.5% acrylamide SDS-PAGE, transferred to Protean nitrocellulose (Whatman), and assayed by immunoblotting. The antibodies used to detect H3, H2B, Spt6, Spt16, Pob3, PAP, HA, Myc, and G6PDH were as follows: anti-H3 (1:30,000, described in (TOMSON *et al.* 2011)) anti-H2B (1:2,500, Active Motif), anti-Spt6 (1:1000, gift from Tim Formosa), anti-Spt16 (1:500; gift from Tim Formosa), anti-Pob3 (1:2000; gift from Tim Formosa), anti-TAP (1:2,000, Sigma), anti-HA (1:2,000, Santa Cruz), anti-Myc (1:1,000, Santa Cruz), and anti-G6PDH (1:50,000; Sigma). After incubation with HRP-conjugated IgG or secondary antibody (1:5000; GE Healthcare), the immunoreactive proteins were visualized by enhanced chemiluminescence detection (Perkin-Elmer) using a Kodak image station 440CF. Protein levels were calculated by measuring their signal intensities in these western blots using Kodak ID 3.6 software and normalizing these values to those obtained for the G6PDH control.

Table 7. *Saccharomyces cerevisiae* strains used in Chapter 5.

Strain	Genotype	Reference or Source
FY4	*MAT***a**	(WINSTON *et al.* 1995)
FY346	*MAT***a** *leu2Δ1 lys2-128δ ura3-52 spt16-197*	(MALONE *et al.* 1991)
FY2180	*MAT***a** *his4-912δ leu2Δ1 lys2-128δ FLAG-spt6-1004*	(KAPLAN *et al.* 2003)
YJ1	*MAT***a** *ura3Δ0 trp1Δ63 lys2Δ0 met15Δ0*	
YJ112	*MATα ura3Δ0 lys2Δ0 leu2Δ0 snf2Δ::LEU2*	
YJ584	*MAT***a** *ura3Δ0 leu2Δ0 lys2Δ0 his3Δ200 srg1-1*	(MARTENS *et al.* 2004)
YJ717	*MAT***a** *snf2*Δ::KanMX	
YJ718	*MAT***a** *snf2*Δ::KanMX	
YJ780	*MAT***a** *his3Δ200*, *lys2-128*δ, *leu2Δ1*, *spt2Δ0*::KanMX	
YJ926	*MATα his3Δ200 leu2Δ0 trp1Δ63 ura3Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-K122A/HHFS-URA3 can1Δ::MFApr-HIS3*	(HAINER and MARTENS 2011a)
YJ927	*MATα his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/HHFS-URA3 can1Δ::MFApr-HIS3*	(HAINER and MARTENS 2011)
YJ933	*MATα his3Δ200 leu2Δ0 ura3Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-Q120A/HHFS-URA3*	(HAINER and MARTENS 2011)
YJ938	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ63 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::hhts-R49A/HHFS-URA3*	(HAINER and MARTENS 2011)
YTM194	*MAT***a** *ura3Δ0 hht1Δ::HHTS-URA3* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	T. Mavrich
YTM202	*MAT***a** *ura3Δ0 hht1Δ::hhts-K122A-URA3* (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*	T. Mavrich
YS288	*MAT***a** *his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128*δ *met15Δ0 can1Δ::MFA1*pr-*HIS3 snf2Δ::LEU2 hht1Δ::URA3-hhts*(*K122A*), (*hht2-hhf2*)Δ::*URA3-hhts*(*K122A*)*-HHFS*	This study
YS289	*MATα his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128*δ *met15Δ0 can1Δ::MFA1*pr-*HIS3 snf2Δ::LEU2 hht1Δ::URA3-hhts*(*K122A*), (*hht2-hhf2*)Δ::*URA3-hhts*(*K122A*)*-HHFS*	This study
YS333	*MATα his3Δ200*, *leu2Δ0 ura3Δ0* (or *-52*) *lys2-128*δ *snf2Δ::LEU2 srg1-1 hht1Δ::URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts*(*K122A*)*-HHFS*	This study
YS337	*MAT***a** *his3Δ200* (or *4-912*δ) *leu2Δ0* (or *1*) *ura3Δ0* (or *-52*) *trp1Δ63 hht1Δ::URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts*(*K122A*)*-HHFS SNF2*-C18MYC::*TRP1 RPB3*-3HA::*LEU2*	This study
YS366	*MATα ura3Δ0 lys2*Δ0 *leu2Δ0 trp1Δ63 his3Δ200 SNF2*-Myc::*TRP1 RPB3*-3HA::*LEU2* (*hht1-hhf1*)Δ::*HHTS*/*HHFS-URA3* (*hht2-hhf2*)Δ::*HHTS*/*HHFS-URA3*	This study
YS367	*MAT***a** *ura3Δ0 lys2Δ0 leu2Δ0 trp1Δ63 his3Δ200 SNF2*-Myc::*TRP1 RPB3*-3HA::*LEU2* (*hht1-hhf1*)Δ::*HHTS*/*HHFS-URA3* (*hht2-hhf2*)Δ::*HHTS*/*HHFS-URA3*	This study
YS368	*MAT***a** *ura3Δ0 lys2Δ0 leu2Δ0 trp1Δ63 his3Δ200 SNF2*-Myc::*TRP1*	This study

	RPB3-3HA::*LEU2* (*hht1-hhf1*)Δ::*HHTS/HHFS-URA3* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	
YS370	*MAT***a** *ura3Δ0 lys2Δ0 leu2Δ0 trp1Δ63 his3Δ200 SNF2*-Myc::*TRP1* (*hht1-hhf1*)Δ::*hhts-K122A/HHFS-URA3* (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*	This study
YS371	*MATα ura3Δ0 lys2Δ0 leu2Δ0 trp1Δ63 his3Δ200 met15Δ0 SNF2*-Myc::*TRP1 RPB3*-HA::*LEU2* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS-URA3* (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3*	This study
YS380	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ200 lys2Δ0 met15Δ0 snf2*Δ::*LEU2* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS-URA3* (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3*	This study
YS381	*MAT***a** *ura3Δ0 leu2Δ0 lys2Δ0 met15Δ0 snf2*Δ::*LEU2 can1*Δ::*MFA1*pr-*HIS3* (*hht1-hhf1*)Δ::*HHTS/HHFS-URA3* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	This study
YS384	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ200 RPB3*-HA::*LEU2 GAL1*pr-*FMP27*::KanMX (*hht1-hhf1*)Δ::*HHTS/HHFS-URA3* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	This study
YS385	*MAT***a** *ura3Δ0 leu2Δ0 lys2Δ0 trp1Δ63 RPB3*-HA::*LEU2 GAL1*pr-*FMP27*::KanMX (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS-URA3* (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3*	This study
YS393	*MATα his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ snf2*Δ::*LEU2 srg1-1 hht1*Δ::*URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts*(*K122A*)-*HHFS*	This study
YS395	*MATα his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ snf2*Δ::*LEU2 srg1-1 hht1*Δ::*URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts*(*K122A*)-*HHFS*	This study
YS396	*MAT***a** *his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ srg1-1 hht1*Δ::*URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts-K122A*/*HHFS*	This study
YS397	*MATα his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ srg1-1 hht1*Δ::*URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts-K122A*/*HHFS*	This study
YS398	*MAT***a** *his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ srg1-1 hht1*Δ::*URA3-hhts*(*K122A*) (*hht2-hhf2*)Δ::*URA3-hhts-K122A*/*HHFS*	This study
YS399	*MAT***a** *his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ snf2*Δ::*LEU2 srg1-1 hht1*Δ::*URA3-HHTS* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	This study
YS400	*MATα his3Δ200 leu2Δ0 ura3Δ0* (or *-52*) *lys2-128δ srg1-1 hht1*Δ::*URA3-HHTS* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	This study
YS404	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS405	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-K122R*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122R*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS407	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-K122Q*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122Q*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS409	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS411	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-*	This study

	hhf1)Δ::*hhts-V117A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-V117A*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	
YS413	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-V46A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-V46A*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS415	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*HHTS*/*hhfs-I46A*-Hygro (*hht2-hhf2*)Δ::*HHTS*/*hhfs-I46A-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS417	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*HHTS*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS428	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	This study
YS454	*MAT*α *his3Δ200 ura3Δ0 lys2Δ0 met15Δ0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS455	*MAT*α *his3Δ200 ura3Δ0 lys2Δ0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS456	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2Δ0 met15Δ0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS458	*MAT*α *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS459	*MAT*α *his3Δ200 ura3Δ0 lys2Δ0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS460	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS462	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2Δ0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS463	*MAT*α *his3Δ200 ura3Δ0 trp1Δ63 lys2Δ0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS465	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS471	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2Δ0 trp1Δ63* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS472	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2Δ0 trp1Δ63 met15Δ0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-13Myc::KanMX HA-*PAF1*	This study
YS474	*MAT*α *his3Δ200 ura3Δ0 leu2Δ0 lys2Δ0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A-URA3 SPT2*-	This study

	13Myc::KanMX HA-*PAF1*	
YS482	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 lys2*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-TAP::*HIS3* HA-*PAF1*	This study
YS485	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 lys2*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13myc::KanMX HA-*PAF1 SPT6*-TAP::*HIS3*	This study
YS490	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 lys2*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13myc::KanMX HA-*PAF1 SPT16*-TAP::*HIS3*	This study
YS493	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3*	This study
YS494	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS495	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS497	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 trp1*Δ*63 leu2*Δ0 (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-TAP::*HIS3* HA-*PAF1*	This study
YS501	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 trp1*Δ*63 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13myc::KanMX *SPT16*-TAP::*HIS3*	This study
YS504	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 trp1*Δ*63 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS505	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS506	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 trp1*Δ*63 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS508	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0 trp1*Δ*63 leu2*Δ*0 lys2*Δ*0* (*hht1-hhf1*)Δ::*hhts-Q120A/HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-TAP::*HIS3* HA-*PAF1*	This study
YS511	MAT**a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 trp1*Δ*63 lys2*Δ*0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13myc::KanMX *SPT6*-TAP::*HIS3*	This study
YS514	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0 lys2*Δ*0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13myc::KanMX *SPT16*-TAP::*HIS3* HA-*PAF1*	This study
YS518	*MAT***a** *his3*Δ*200 ura3*Δ*0 lys2*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS519	*MAT*α *his3*Δ*200 ura3*Δ*0 met15*Δ*0 trp1*Δ*63 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study

YS521	*MAT***a** *his3*Δ*200 ura3*Δ*0 met15*Δ*0 trp1*Δ*63 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3*	This study
YS522	*MAT***a** *his3*Δ*200 ura3*Δ*0 met15*Δ*0 lys2*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-13myc::KanMX *SPT16*-TAP::*HIS3*	This study
YS525	*MAT*α *his3*Δ*200 ura3*Δ*0 met15*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3* HA-*PAF1*	This study
YS526	*MAT*α *his3*Δ*200 ura3*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3*	This study
YS527	*MAT*α *his3*Δ*200 ura3*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-13myc::KanMX *ASF1*-TAP::*HIS3*	This study
YS538	*MAT*α *ura3*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-13myc::KanMX *SPT6*-TAP::*HIS3* HA-*PAF1*	This study
YS541	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 lys2*Δ*0 leu2*Δ*0 HHT1-HHF1* (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3 SPT2*-TAP::*HIS3*	This study
YS544	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0* (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro *HHT2-HHF2 SPT2*-TAP::*HIS3*	This study
YS547	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 leu2*Δ*0 HHT1-HHF1* (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 SPT2*-TAP::*HIS3*	This study
YS550	*MAT*α *his3*Δ*200 met15*Δ*0 ura3*Δ*0 leu2*Δ*0 trp1*Δ*63* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro *HHT2-HHF2 SPT2*-TAP::*HIS3*	This study
YS553	*MAT***a** *his3*Δ*200 met15*Δ*0 lys2*Δ*0 trp1*Δ*63 ura3*Δ*0 leu2*Δ*0 HHT1-HHF1* (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3 SPT2*-myc::KanMX	This study
YS558	*MAT***a** *his3*Δ*200 met15*Δ*0 ura3*Δ*0 lys2*Δ*0 trp1*Δ*63* (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro *HHT2-HHF2 SPT2*-TAP::*HIS3*	This study
YS560	*MAT***a** *his3*Δ*200 met15*Δ*0 leu2*Δ*0 ura3*Δ*0 HHT1-HHF1* (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT16*-TAP::*HIS3 SPT2*-myc::KanMX	This study
YS563	*MAT***a** *his3*Δ*200 leu2*Δ*0 ura3*Δ*0 met15*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A/HHFS*-Hygro *HHT2-HHF2*	This study
YS565	*MAT***a** *his3*Δ*200 ura3*Δ*0 met15*Δ*0 leu2*Δ*0 lys2*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3 SPT2*-TAP::*HIS3*	This study
YS570	*MAT***a** *his3*Δ*200 ura3*Δ*0 leu2*Δ*0* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhfs-R49A*/*HHFS-URA3 SPT2*-myc::KanMX *SPT6*-TAP::*HIS3*	This study
YS591	*MAT*α *ura3*Δ*0*, *leu2*Δ*0*, *lys2*Δ*0*, *his3*Δ*200*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, FLAG-*spt6-1004*	This study
YS592	*MAT***a** *ura3*Δ*0*, *leu2*Δ*0*, *his3*Δ*200*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, FLAG-*spt6-1004*	This study
YS593	*MAT*α *ura3*Δ*0*, *leu2*Δ*0*, *lys2*Δ*0*, *his3*Δ*200*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*,	This study

	FLAG-*spt6-1004*, *RPB1*-C13Myc::KanMX	
YS594	*MAT*α *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, *met15Δ0*, (*hht1-hhf1*)Δ::*hhts-K122A/HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*, FLAG-*spt6-1004*	This study
YS595	*MAT*α *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-K122A/HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*, FLAG-*spt6-1004*, *RPB1*-C13Myc::KanMX	This study
YS596	*MAT*α *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3*, FLAG-*spt6-1004*	This study
YS597	*MAT*α *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, FLAG-*spt6-1004*	This study
YS598	*MAT***a** *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, *trp1Δ63*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, FLAG-*spt6-1004*, *RPB1*-C13Myc::KanMX, *can1*Δ::*MFA*pr-*HIS3*	This study
YS599	*MAT***a** *leu2Δ0*, *lys2Δ0*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, FLAG-*spt6-1004*, *can1*Δ::*MFA*pr-*HIS3*	This study
YS600	*MAT*α *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, *met15Δ0*, *trp1Δ63* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, FLAG-*spt6-1004*	This study
YS601	*MAT*α *ura3Δ0*, *leu2Δ0*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, *spt16-197*, *SPT2*-13Myc::KanMX	This study
YS602	*MAT*α *ura3Δ0*, *leu2Δ0*, *his3Δ200*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, *spt16-197*	This study
YS603	*MAT***a** *ura3Δ0*, *leu2Δ0*, *his3Δ200*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, *spt16-197*	This study
YS604	*MAT***a** *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *trp1Δ63*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A/HHFS-URA3*, *spt16-197*	This study
YS605	*MAT***a** *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, *spt16-197*, *can1*Δ::*MFA*pr-*HIS3*	This study
YS606	*MAT***a** *ura3Δ0*, *leu2Δ0*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS607	*MAT***a** *ura3Δ0*, *leu2Δ0*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS608	*MAT*α *ura3Δ0*, *leu2Δ0*, (*hht1-hhf1*)Δ::*HHTS/HHFS*-Hygro, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS609	*MAT*α *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS610	*MAT*α *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-Q120A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, *spt2Δ0*::KanMX, *can1*Δ::*MFA*pr-*HIS3*	This study
YS611	*MAT***a** *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-*	This study

	Q120A/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-Q120A*/*HHFS-URA3*, *spt2Δ0*::KanMX	
YS612	*MAT***a** *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, *his3Δ200*, *lys2Δ0*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS613	*MAT*α *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, *his3Δ200*, *lys2Δ0*, *met15Δ0*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS614	*MAT***a** *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, *met15Δ0*, *trp1Δ63* (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, FLAG-*spt6-1004*	This study
YS615	*MAT***a** *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *his3Δ200*, *met15Δ0*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, FLAG-*spt6-1004*, *RPB1*-C13Myc::KanMX	This study
YS616	*MAT*α *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, *met15Δ0*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, *spt16-197*	This study
YS617	*MAT***a** *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, *spt16-197*	This study
YS618	*MAT***a** *ura3Δ0*, *leu2Δ0*, *lys2Δ0*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A*/*HHFS-URA3*, *spt16*-197	This study
YS619	*MAT***a** *ura3Δ0*, *leu2Δ0*, *trp1Δ63*, *his3Δ200*, (*hht1-hhf1*)Δ::*hhts-R49A*/*HHFS*-Hygro, (*hht2-hhf2*)Δ::*hhts-R49A/HHFS-URA3*, *spt2Δ0*::KanMX	This study
YS640	*MAT*α *his3Δ200*, *lys2Δ0*, *leu2Δ0*, *ura3Δ0*, (*hht1-hhf1*)Δ::*hhts-K122A*-Hygro, (*hht2-hhf2*)Δ::*hhts-K122A*-KanMX	This study
YS641	*MAT***a** *his3Δ200*, *lys2Δ0*, *leu2Δ0*, *ura3Δ0*, (*hht1-hhf1*)Δ::*hhts-K122A*-Hygro, (*hht2-hhf2*)Δ::*hhts-K122A*-KanMX	This study
YS642	*MAT*α *ura3Δ0*, *leu2Δ0*, *his3Δ200*, *spt2Δ0*	This study

5.2.4 Chromatin Immunoprecipitation (ChIP)

For ChIP over galactose-induced *GAL1*pr-*FMP27*, cells were grown in YPraff to approximately 1×10^7 cells/mL and then 2% galactose was added at time zero. For ChIP over glucose-repressed *GAL1*pr-*FMP27*, cells were grown in YPgal to approximately 1×10^7 cells/mL and then 2% glucose was added at time zero. For all other ChIP experiments, cells were grown in YPD at 30°C to 1-2 $\times$ 10^7 cells/ml. Chromatin was prepared as previously described (SHIRRA *et al.* 2005). Histone H3, histone H2B, Spt6, Spt16, Rpb3, Spt2-Myc, or HA-Paf1 were immunoprecipitated by incubating sonicated chromatin overnight at 4°C with 1 µl anti-histone H3 (described previously (TOMSON *et al.* 2011)), 3µl anti-histone H2B (Active Motif), 1µl anti-Spt6 (gift from Tim Formosa), 1µl anti-Spt16 (gift from Tim Formosa), 2.5µl anti-Rpb3 (neoclone), 1µl anti-Myc (Santa Cruz), 1µl anti-HA (Santa Cruz), antibody and followed by the addition of IgG-Sepharose beads (GE Healthcare) for 2 hr at 4°C. Asf1-TAP was immunoprecipitated by incubating sonicated chromatin for 4 hr at 4°C with IgG-Sepharose beads (GE Healthcare). Dilutions of input DNA and immunoprecipitated DNA were analyzed by qPCR reactions. Primer sets that amplify the following regions were used to measure H3 occupancy by qPCR: *PYK1* (5': +62 to +164, 3': +1173 to +1279), *PMA1* (5': +691 to +794, 3': +1689 to +1791), *ADH1* (+845 to +943), *CYC1* (+122 to +217), *TUB2* (5': +105 to +202, 3': +1083 to +1189), *GAL1* (5': +79 to +175, 3': +1366 to + 1487), *FMP27* (pr: -194 to +35, 2kB: +1986 to +2199, 4kB: +4069 to +4268, 6kB: +5901 to +6074, 8kB: +7701 to +7850). ChIP signals for each gene were normalized to a No ORF control template, which is located within a region of chromosome V that lacks open reading frames (KOMARNITSKY *et al.* 2000).

5.2.5 Nucleosome Scanning Assay

Nucleosome scanning assays were performed as described in Chapter 2 (HAINER *et al.* 2011) on cells grown at 30°C to 2 × 10^7 cells/ml in YPD. For each of the 38 *SER3* primer pairs, the amount of template protected from digestion by micrococcal nuclease was calculated as a ratio between MNase-digested and undigested samples and then normalized to the amount of MNase-protected control template (*GAL1* NB) that is located within a well-positioned nucleosome in the *GAL1* promoter (BRICKNER *et al.* 2007; FLOER *et al.* ; FLOER *et al.* 2010).

5.2.6 Quantitative PCR (qPCR)

All qPCR data for the ChIP assays were obtained using a StepOnePlus Real-time PCR system, SYBR green reagent (Fermentas) and the indicated primers. Calculations were performed using Pfaffl methodology (PFAFFL 2001).

5.2.7 TAP-tag Pull Down Assay

To examine the interaction between Spt2, Spt6, or Spt16 with histone H3 or a subset of the histone mutants (H3 K122A, H3 Q120A or H3 R49A), strains expressing either wild-type synthetic histones or one of the mutations with TAP-tagged versions of either Spt2, Spt6 or Spt16 (YS482, YS485, YS490, YS497, YS501, YS508, YS511, YS514, YS522, YS538, YS565, YS570) were grown in YPD medium to approximately 3-4 × 10^7 cells/ml. Whole cell extracts were made by glass bead lysis in lysis buffer (20 mM HEPES, pH 7.4, 100mM sodium acetate, 2mM magnesium acetate, 100mM sodium acetate, 10mM EDTA, 10% glycerol, 1mM

dithiothreitol, and PMSF). Extract (5mg) was incubated at 4°C for 3.5 hours with 30µL IgG conjugated to sepharose beads (GE Healthcare). Bound complexes were washed twice with lysis buffer containing 400mM sodium chloride. Precipitates were resolved on a 12.5% SDS-PAGE and analyzed by immunoblotting with antibodies specific to histone H3 (1:30,000 dilution; (TOMSON *et al.* 2011)) or TAP (1:2,000; Sigma).

5.2.8 Protein Expression and Purification

Bacterial lysate was prepared from RIPL cells expressing wild-type or mutant versions (H3 K122A, H3 Q120A, or H3 R49A) of the yeast histone H3 gene (pET11A-H3) or wild-type H4 (pET11A-H4) created using the wild-type vector with site-directed mutagenesis. Briefly, cells were grown to an OD of 0.5, induced with 0.5mM IPTG for 3 hours, harvested by centrifugation and lysed via homogenization in 20mM Tris, pH 7.5, 500mM NaCl, 10% glycerol and 1 mM β-mercaptoethanol. Lysate was cleared by centrifugation and used for initial Far Western analysis or purified for further analysis. To purify the histone proteins, a previously described strategy was adopted (LUGER *et al.* 1999). After lysis and centrifugation, the pellet was washed in 50mM Tris, pH 7.5, 100m M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, 1% Triton X-100, and protease inhibitors and cleared by centrifugation. Histones were purified by ion exchange chromatography (HiTrap-Q and SP) after dialysis against a urea buffer (7M Urea, 1mM EDTA, 10mM Tris, pH 8.0, 100mM NaCl, 5 mM β-mercaptoethanol, and protease inhibitors). To elute the samples, a salt gradient was performed over the SP Sepharose column.

To purify Spt6, bacterial lysate was prepared from 6L of RIPL cells expressing Spt6 from a plasmid that was induced through autoinduction (STUDIER 2005). This procedure was adapted from a previously described method (CLOSE *et al.* 2011). Cells were lysed in 50mM Tris, pH 7.5,

500mM NaCl, 5% glycerol, and 15mM imidazole. After lysis and centrifugation, the supernatant was applied to a Nickel-NTA column that was prewashed with lysis buffer. After washing with lysis buffer, the resin was washed with 25mM HEPES, pH 7.0, 100mM NaCl, 5% glycerol, and 30mM imidazole, and then Spt6 was eluted from the resin with 25mM HEPES, pH 7.0, 100mM NaCl, 5% glycerol, and 300mM imidazole. The eluant was applied to a heparin column and to elute the samples, a salt gradient was performed over the heparin column. Protein was pooled and concentrated, and applied to a Superdex 200 sizing column.

5.2.9 Far Western Analysis

Far Western analysis was performed as previously described (WU *et al.* 2007). Briefly, 25 µg of bacterial lysate expressing either wild-type or mutant versions of the histone H3 protein were separated by 12.5% acrylamide SDS-PAGE and transferred to Protean nitrocellulose (Whatman). Transferred proteins are then denatured and renatured in freshly prepared AC buffer (100mM NaCl, 20mM Tris, pH 7.5, .5mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skim milk powder and 1mM DTT) by gradually reducing the concentration of guanidine hydrochloride (from 6M to to 0M). After blocking the renatured proteins with 5% milk in TBST buffer, the membrane is incubated with purified Spt16 (gift from T. Formosa) or Spt6 (prepared as described above) in freshly prepared protein binding buffer (100 mM NaCl, 20mM Tris, pH 7.5, 0.5mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skim milk powder, and 1mM DTT) overnight. After washing the membrane, interacting proteins are detected by incubating with an antibody against Spt16 (1:500, gift from T. Formosa) or Spt6 (1:2000, gift from T. Formosa), respectively. After incubation with HRP-conjugated IgG secondary antibody (1:5000; GE Healthcare), the immunoreactive proteins were visualized by enhanced chemiluminescence detection (Perkin-Elmer) using a

Kodak image station 440CF. As a control, membranes were also probed with an antibody against histone H3 (1:30,000).

5.3 RESULTS

5.3.1 Creating histone mutant strains where both copies of the histone genes express synthetic versions

To test the above hypotheses, I created double mutant versions of a subset of the residue substitutions, where both copies of the histone genes contain the substitution of the particular residue and utilized these strains for the following experiments. To do t his, I obtained intergrating plasmids of the ten histone mutants that I have been examining from Junbiao Dai (Tsinghua University, Beijing, China). These plasmids contain the synthetic versions of the histone mutants described in Chapter 4, but were targeted to *HHT1-HHF1* (see Materials and Methods). After successfully intergrating each mutation, I performed Northern blot analysis to examine the effect of the newly created strains which now contain the histone mutant allele at both histone loci (Figure 43). Compared to the original strains, which contained a deletion of the *HHT1-HHF1* locus and the mutation at the *HHT2-HHF2* locus (Chapter 4, Figure 28), the new strains, containing a substitution at both histone loci, upregulated *SER3* to approximately the same degree as the original histone substitutions. However, as the background of (*hht1-hhf1*)Δ no longer exists, there is no upregulation for the wild-type strain (Figure 43, compare lane one with lane three and five), and therefore the fold upregulation of *SER3* is more dramatic. For the rest of the analysis I performed in the histone mutants described throughout this Chapter, I

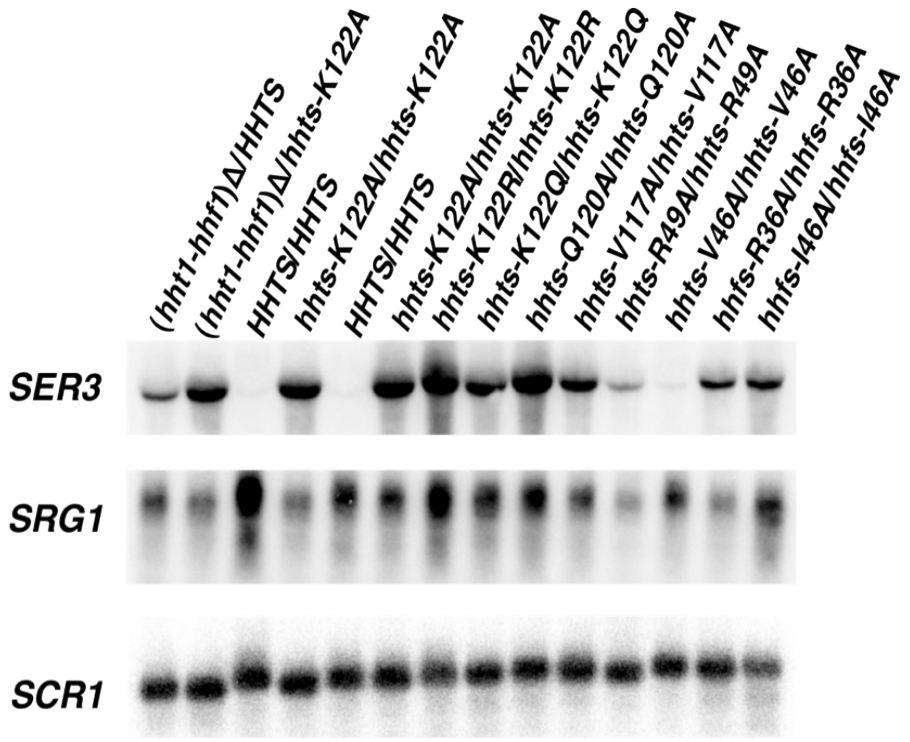

Figure 43. Single amino acid substitutions expressed at both *HHT1-HHF1* and *HHT2-HHF2* strongly derepress *SER3*.

Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from derivatives of JDY86 expressing either synthetic, wild-type copies of histone H3 and H4 (*HHTS/HHFS*) (YJ927) or mutants *hhts-K122A* (YJ926), and from strains expressing either synthetic wild-type copies at both genmoic locations (YTM194, YS417) or mutants *hhts-K122A* (YTM202, YS404)*, hhts-K122R* (YS405), *hhts-K122Q* (YS407), *hhts-Q120A* (YS409), *hhts-V117A* (YS411), *hhts-R49A* (YS428), *hhts-V46A* (YS413), *hhfs-I46A* (YS415), and *hhfs-R36A*, also expressed at both genomic locations, that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C.

concentrated on a subset of the histone residue substitutions: H3 K122A, H3 Q120A, and H3 R49A. I chose these three residues for the following reasons. K122 and Q120 are found over the dyad of the nucleosome, where the DNA makes a strong interaction with the histone proteins. K122 is especially interesting, as I isolated three individual substitutions for this amino acid (K122A, K122R, and K122Q) during my initial screen, and also, K122 has been previously described as a binding site for the histone chaperone, Asf1 (ENGLISH *et al.* 2006). I chose to continue examining H3 R49A as well, due to its alternate location compared to K122 and Q120 (it is found at the entry/exit point of the DNA wrapping around the histone octamer), as well as its additional phenotypes, described in Chapter 4.

5.3.2 A subset of the histone substitutions are dominant for upregulating *SER3* expression

I next examined whether the histone mutants are dominant in their effect at *SER3*. To determine this, I tested the dominant effect each histone mutant had on *SER3* in two ways. First, I transformed a wild-type strain (YJ1) with plasmids expressing a subset of the histone mutants on plasmids (Figure 44A), obtained from the Shilatifard lab at the Stowers Institute (NAKANISHI *et al.* 2008). For at least a subset of the mutants (H3 K122A, Q120A, and V117A), I observed a strong upregulation of *SER3*, which appears to mimic the effect of strains expressing only the histone mutations (Figure 43). As an alternative method to examine the histone mutants for dominance, I created strains expressing both wild-type and a subset of the histone mutant alleles from either genomic locus (Figure 44B). In these strains, either *HHT1/HHF1* is wild-type and *HHT2/HHF2* is replaced with either wild-type or mutant synthetic alleles, or *HHT2/HHF2* is wild-type and *HHF1/HHF2* is replaced with the synthetic alleles. Therefore, I expressed the synthetic wild-type or mutant from either locus and tested their effect on *SER3* expression by

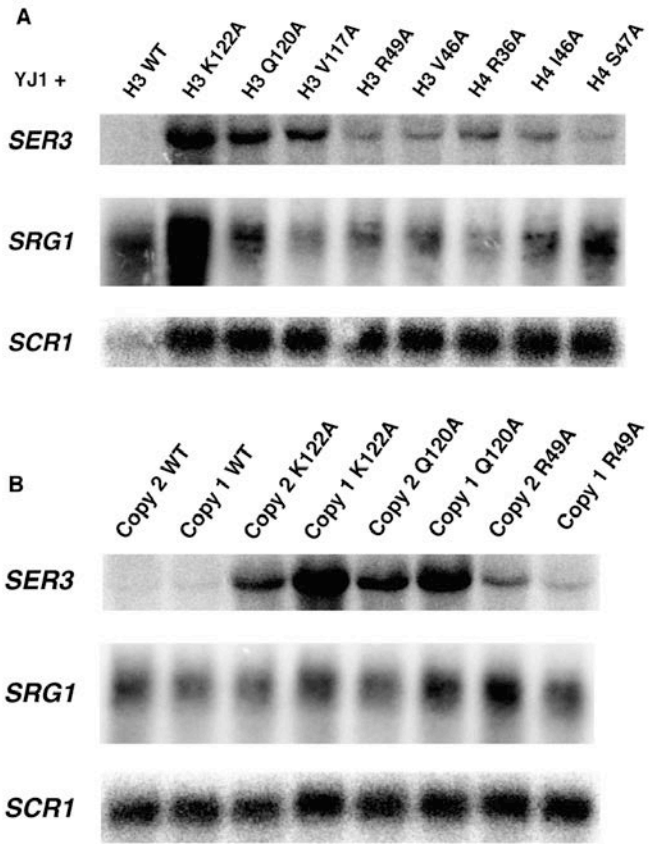

Figure 44. A subset of the histone mutants are dominant.

A) Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control) expressed from a plasmid in the presence of the wild-type histone genes. Total RNA was isolated from YJ1 expressing p*HHT1-HHF1*, p*hht1-K122A*, p*hht1-Q120A*, p*hht1-R49A,* p*hht1-V46A*, p*hhf1-R36A*, p*hhf1-I46A*, or p*hhf1-S47A* from a plasmid that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C. B) Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control) in the presence of a wild-type copy of the histone genes. Total RNA was isolated from derivatives of JDY86 expressing either a synthetic, wild-type copy of histone H3 and H4 (YS541,YS544) or mutants *hhts-K122A* (YS547, YS550), *hhts-Q120A* (YS553, YS558), and *hhts-R49A* (YS560, YS563) that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C. The copy number indicates which histone genomic location contains the synthetic version, whereas the un-named copy is the wild-type genomic locus.

Northern analysis. Interestingly, at least H3 K122A and Q120A appear to upregulate *SER3* in the presence of a wild-type locus, and the effect is strongest when the mutation is expressed at the *HHT1-HHF1* locus. This makes sense, as the histone loci have been previously shown to have imbalanced roles in processes, where the *HHT1-HHF1* locus can overcompensate compared to the *HHT2-HHF2* locus. While finding that a subset of these histone mutants are dominant for *SER3* regulation does not assist in determining through which mechanism the histone mutants are responsible for regulating transcription dynamics, it does provide an additional phenotype of these mutations, permitting a clearer interpretation of data.

5.3.3 Effect of histone mutants on *SER3* regulation in the absence of serine

It is possible that the histone residues are functioning to remove/destabilize the two nucleosomes, that are originally located at the 5' end of *SRG1* in the absence of serine. In serine rich conditions these 5' nucleosomes are subsequently remodeled over the entire *SRG1* transcript, likely through the action of the chromatin remodeler, Swi/Snf. In order to determine if the histone mutants result in decreased occupancy of these 5' nucleosomes in the absence of serine, which could then lead to decreased nucleosome occupancy over the entire *SRG1* transcription unit in the presence of serine, I examined the effect of the histone mutants on nucleosome occupancy in the absence of serine. In wild-type cells, as previously published (HAINER *et al.* 2011), I detected nucleosome occupancy over the entire *SRG1* transcript in the presence of serine and two peaks of MNase protection, indicating two primary nucleosomes, over the 5' end of *SRG1* in the absence of serine (Figure 45A). In order to begin examining whether the histone mutants disrupt the occupancy of these two nucleosomes, I examined the nucleosome architecture over the *SER3* locus in +/- serine conditions in three of the histone mutants (H3 K122A, Q120A, and R49A). From the

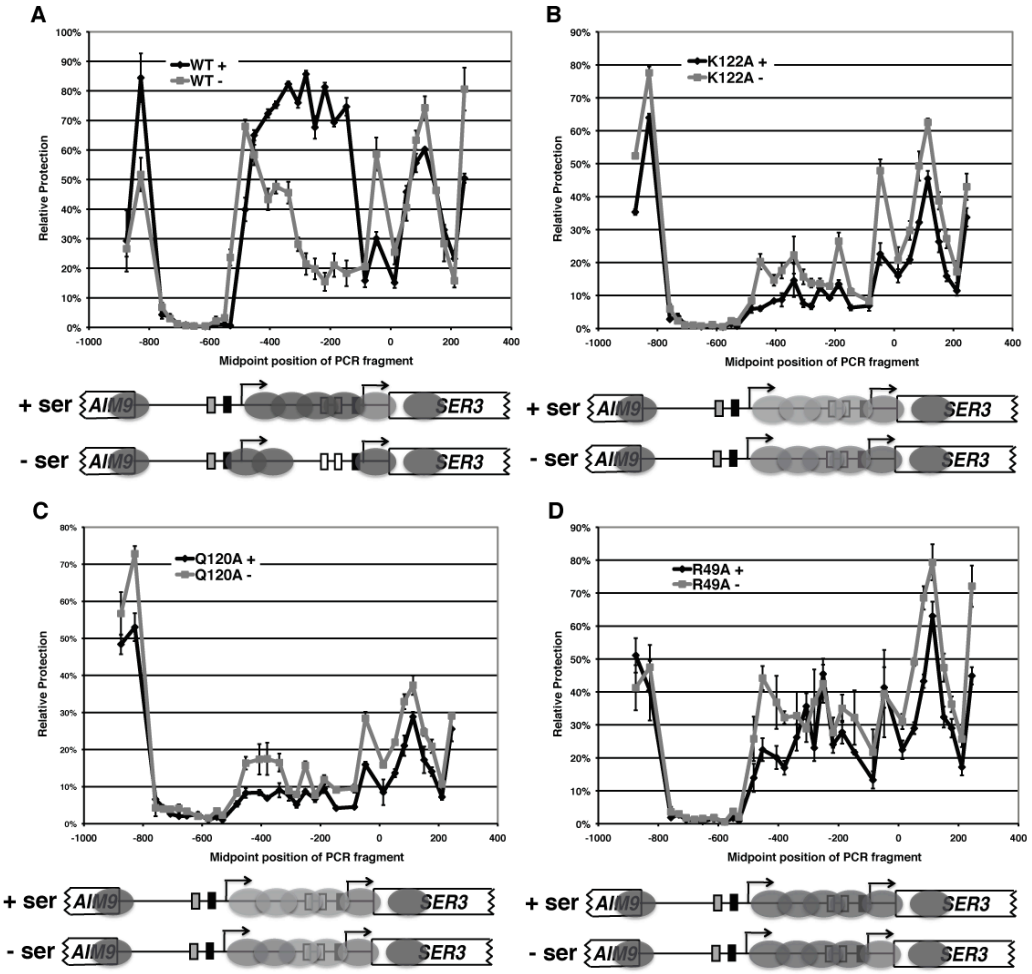

Figure 45. Effect of histone mutants on nucleosome occupancy over *SRG1*/*SER3* in media lacking serine.

A-D) Nucleosome scanning assays were performed on strains expressing either synthetic wild-type copies of histone H3 and H4 (A, YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474). Cells were grown at 30°C in SC+serine media (+) and then shifted to SC-serine media for 25 minutes (-). Each experiment was done in triplicate. MNase protection across the *SER3* locus relative to a positioned nucleosome within the *GAL1* promoter was determined by qPCR and the mean +/- SEM for the three replicates is plotted at the midpoint for each PCR product. Shown below each graph is a diagram of the *SER3* locus indicating the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data for each histone mutant. The light gray ovals are indicative of less dramatic reductions in MNase protections as compared to the wild-type control shown in Panel A.

MNase studies, I found in the absence of serine, the mutants still result in a complete loss of nucleosomes over the *SRG1* transcript (Figure 45B-D). In comparison to the occupancy in the presence of serine, in all three cases, there is a slight, but reproducible, increase in this nucleosome occupancy. While this result would indicate the histone mutants are destabilizing the original two nucleosomes over the 5' of *SRG1*, Northern analysis demonstrates these mutants upregulate *SER3* in the presence and absence of serine (Figure 46A). Therefore, I hypothesized that the histone mutants result in constitutive expression of *SER3*, where *SER3* is never repressed in the mutants. This would explain the complete loss of nucleosomes over the entire *SRG1* transcribed unit, but still maintain the model for the role of these residues in nucleosome reassembly. To test whether the cells are, in fact, in an expression state of serine rich conditions when the cells are grown in the absence of serine, I performed ChIP analysis of the coactivator Swi/Snf over the *SRG1* promoter in the presence and absence of serine (Figure 46B). As observed previously, in the presence of serine, Snf2 ChIPs to the *SRG1* promoter and in the absence of serine, Snf2 no l onger occupies this region. However, in K122A strains, Snf2 occupies the *SRG1* promoter in both the presence and absence of serine. Therefore, at least for K122, and likely for the other histone mutants, I conclude that serine starvation cannot be achieved in these strains.

5.3.4 Stability of 5' nucleosomes over *SRG1* in H3 K122A

To test whether the histone mutants destabilize nucleosomes over the *SRG1* promoter, I examined the effect of H3 K122A on the nucleosomes in the context of a *SNF2* deletion. Snf2 is a subunit of the Swi/Snf complex and, when deleted, upregulates *SER3* in the presence of serine, while reducing *SRG1* levels by approximately four-fold (MARTENS *et al.* 2005). I reasoned that

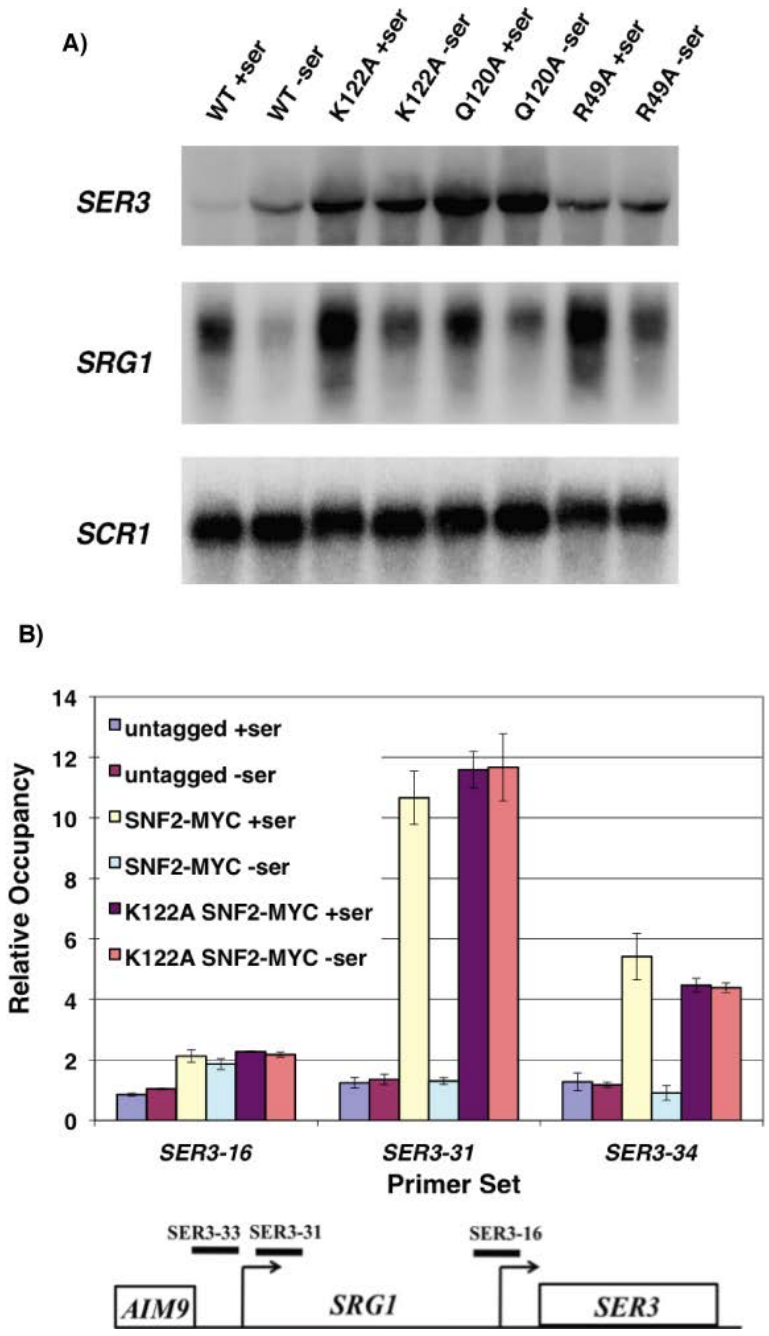

Figure 46. Histone mutants upregulate *SER3* in the absence of serine to a degree in which the cells are never starved for serine.

A) Northern blot analysis examining the effect of three histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from strains expressing either synthetic wild type copies of histone H3 and H4 (*HHTS/HHFS*; YJ927) or mutants *hhts-K122A* (YJ926), *hhts-Q120A* (YJ933), and

hhts-R49A (YJ938), that were grown to a density of 1×10^7 cells/ml in SC-ser +1mM serine at 30°C then shifted to either SC-ser +1mM serine (+) or SC-ser (-) for 25 minutes. Snf2 ChIP was performed on chromatin isolated from strains expressing *SNF2*-MYC alleles with *HHTS/HHFS* (SNF2-MYC; YS367-YS369) or *hhts-K122A/hhts-K122A* (K122A SNF2-MYC; YS337, YS370, YS371), or an untagged version of *SNF2* (FY4, YJ926, YJ927) grown in SC-ser +1mM serine at 30°C then shifted to either SC-ser +1mM serine (+) or SC-ser (-) for 25 minutes to a density of ~2×10^7. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and represents the mean +/- SEM of three experiments. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

Swi/Snf is, at least in part, responsible for providing the nucleosomes that reassemble over the *SRG1* transcription unit. In support of this hypothesis, the nucleosome architecture at the 5' end of *SRG1* in a *snf2*Δ strain resembles that of serine starvation conditions regardless of the serine conditions: two peaks of MNase protection at the 5' end of *SRG1* indicating two positioned nucleosomes. I examined the effect on nu cleosome architecture in the K122A mutant in combination with *snf2*Δ and found the two nucleosomes at the 5' of *SRG1* are still present in K122A, with a slight decrease in their occupancy levels (Figure 47). While there was a slight decrease in these strains, I hypothesized that the reason for this slight decrease in nucleosome occupancy could be due to the high rate of transcription occurring over this region, as *SRG1* is still being highly transcribed. Therefore, to examine the effect of K122A on the two nucleosomes at the 5' end of *SRG1* in the absence of *SRG1* transcription, I combined the K122A/*snf2*Δ with a mutation in the *SRG1* TATA box (*srg1-1*) which results in a loss of *SRG1* transcription (see Chapter 2). When I examined the effect of this strain on the nucleosome architecture over the *SRG1*/*SER3* locus, I observed an occupancy of the 5' source nucleosomes that almost completely mimics that of the occupancy in a *snf2*Δ strain. This indicates that at least for K122A, the loss of nucleosomes over *SRG1* cannot simply be explained by destabilization and subsequent loss of nucleosomes at the 5' end of *SRG1*.

5.3.5 Effect of histone mutants on Spt2, Spt6, Spt16, Paf1, Asf1 and RNA pol II occupancy at *SRG1/SER3*

Several studies have indicated that chromatin at highly transcribed genes is more dynamic than at lowly transcribed genes and is therefore more likely to be dependent on histone chaperones, such

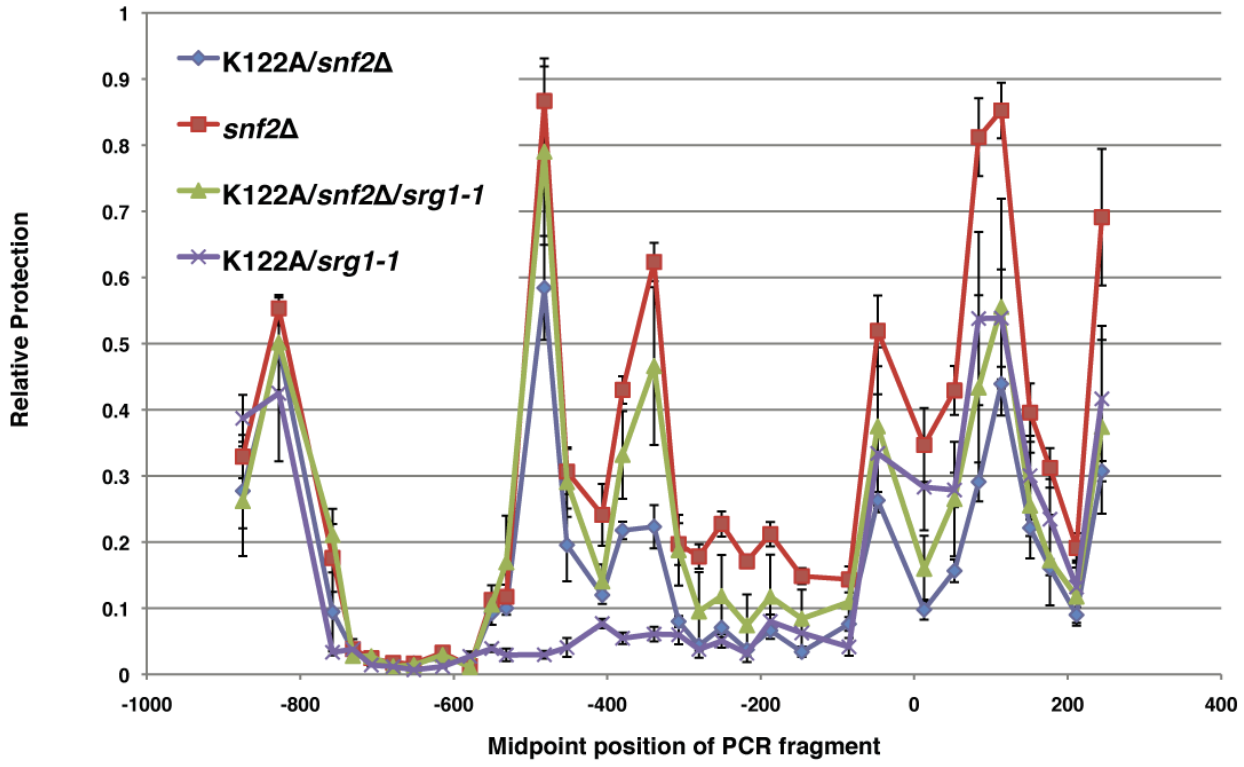

Figure 47. Combinatorial effect of *snf2*Δ, *srg1-1*, and K122A on nucleosome occupancy over the *SER3* promoter.

Nucleosome scanning assays were performed on strains expressing either *snf2*Δ (red) (YJ112, YJ717, and YJ718), K122A in combination with *snf2*Δ (blue) (YS288, YS289, and YS380), K122A in combination with *snf2*Δ and a TATA mutant in the *SRG1*pr (*srg1-1*; green) (YS333, YS393, and YS395), or K122A in combination with the *srg1-1* TATA mutant strain (purple) (YS396-YS398). Cells were grown in YPD media at 30°C. Each experiment was done in triplicate and the mean +/- SEM for the three replicates is plotted at the midpoint for each PCR product.

as Spt6 and FACT, for rapid nucleosome reassembly (DION *et al.* 2007; IVANOVSKA *et al.* 2010; JAMAI *et al.* 2007; JIN *et al.* 2010; KIMURA and COOK 2001; KRISTJUHAN and SVEJSTRUP 2004; KULAEVA *et al.* 2009; KULAEVA *et al.* 2010; RUFIANGE *et al.* 2007; SCHWABISH and STRUHL 2004; THIRIET and HAYES 2005). Because the eight histone residues are also required at highly transcribed genes, but not lowly transcribed genes, an attractive model is that at least a subset of these eight histone residues may disrupt the recruitment and/or function of histone chaperones that are directly involved in the transcription-dependent nucleosome assembly. Possible candidates include Spt6, its partner Spn1(Iws1), FACT, or Spt2(Sin1), which are all required for *SER3* repression. Spt6 and Spt16 (subunit of FACT) have been shown previously to interact with both histone H3 and with assembled nucleosomes (BELOTSERKOVSKAYA *et al.* 2003; BORTVIN and WINSTON 1996; FORMOSA *et al.* 2001; JAMAI *et al.* 2009; MCDONALD *et al.* 2010; ORPHANIDES *et al.* 1999). *In vitro* and *in vivo* experiments have provided evidence to support a histone chaperone role for these two factors in promoting assembly of nucleosomes (ADKINS and TYLER 2006; CHEUNG *et al.* 2008; IVANOVSKA *et al.* 2010; JENSEN *et al.* 2008; KAPLAN *et al.* 2003; MCDONALD *et al.* 2010). Spn1 has recently been shown to regulate the binding of Spt6 to nucleosomes *in vitro* (MCDONALD *et al.* 2010). Spt2 binds DNA non-specifically and is also required for transcription-coupled nucleosome assembly (NOURANI *et al.* 2006). In one possible scenario, the L1L2 loop may provide a binding surface for Spt6 or Spt16 to facilitate reassembly of histones after passage of RNA pol II. Recent structural studies have identified the L1L2 region as part of the binding interface between histone H3/H4 and another histone chaperone, Asf1 (AGEZ *et al.* 2007; ENGLISH *et al.* 2006). Regarding possible interactions with Spt2, histone H3 R49 and V46 are more likely candidates as this factor has shown affinity for four-way DNA junctions, a structure similar to what has been shown to occur at the DNA entry/exit points of the

nucleosome (KRUGER *et al.* 1995; LUGER and RICHMOND 1998). An alternative hypothesis is that Spt2 may be acting as a DNA binding factor for either Spt6 or Spt16, as it is a proposed HMG-box containing protein.

Based on these data, I considered the possibility that these mutant histones fail to recruit histone chaperones normally to transcribed regions, which may account for defects in transcription-coupled nucleosome occupancy. Therefore, I performed ChIP experiments to assess the binding of Spt6 and Spt16 across the *SRG1* transcription unit (Figure 48C,D). In general, I detected reduced binding of Spt6 and Spt16 that parallel the loss of histone H3 and H2B occupancy across this region that I observed in these mutant versions (compare Figure 48A,B with 48C,D). Interestingly, one of the histone mutants, H3 R49A, shows a more dramatic decrease in histone H2B occupancy compared to histone H3 occupancy. This may indicate a maintenance of H3/H4 tetramers, or even a hexasome where only one H2A/H2B dimer has been evicted.

Based on my hypothesis that Spt2 may facilitate Spt6 and/or Spt16 function in regulating nucleosome dynamics over highly transcribed regions, I tested whether these histone mutants also alter occupancy of Spt2 at *SER3*. I performed ChIP analysis of Spt2-Myc over *SRG1*/*SER3* (Figure 48E), and consistent with my hypothesis, I found that the histone mutants result in decreased Spt2 occupancy specifically over *SRG1*.

Previously, our lab has shown that the Paf1 transcription elongation complex is required for *SER3* regulation through maintaining nucleosome occupancy over the *SER3* promoter and possibly by regulating Spt6 and Spt16 recruitment to this region (PRUNESKI *et al.* 2011). Therefore, I tested whether the histone mutants alter Paf1 occupancy over the *SER3* promoter. To this end, I performed ChIP analysis of Paf1-HA (Figure 48F) and found that the histone mutants

result in only a slight decrease in Paf1 occupancy, but this decrease cannot account for the more dramatic decrease in Spt6 and Spt16 occupancy.

Because many of these factors strongly co-localize with RNA pol II across transcribed genes, I tested whether the decrease in the occupancy these factors might be indirect due to a decrease in RNA pol II occupancy at *SER3*. To this end, I performed ChIP analysis of Rpb3, a subunit of RNA pol II, over *SRG1* (Figure 48H). Consistent with my Northern analysis (Figure 43), I found that these histone mutants do not cause a decrease in RNA pol II occupancy as compared to cells expressing wild type histones.

Asf1 is another histone chaperone which has been shown previously to interact with histones, through binding which includes H3 K122 (ENGLISH *et al.* 2006). Previously, our lab determine that Asf1 plays only a minor role in regulating *SER3* expression and I found that deleting Asf1 does not alter nucleosome occupancy over the *SER3* promoter (data not shown). Therefore, while this histone chaperone may use K122 as a binding site, based on our previous data, I would hypothesize that Asf1 occupancy would not be altered in the histone mutants. To test this I performed ChIP analysis of Asf1-TAP over *SRG1/SER3* (Figure 48G) and found that Asf1 occupancy is not significantly altered over this region, supporting the idea that Asf1 is not responsible for regulating *SER3*.

Taken together, these data indicate that the amino acids defined by these mutants are required to specifically maintain occupancy of the Spt2, Spt6, and Spt16 histone chaperones, and not Asf1, across *SRG1*.

As a control, I tested the effect of these mutants on histone H3, H2B, Spt2, Spt6, Spt16, Pob3, Paf1, and Asf1 protein levels by Western analysis (Figure 49). All three histone mutant strains express levels of these proteins indistinguishable from a wild type *HHTS-HHFS* strain.

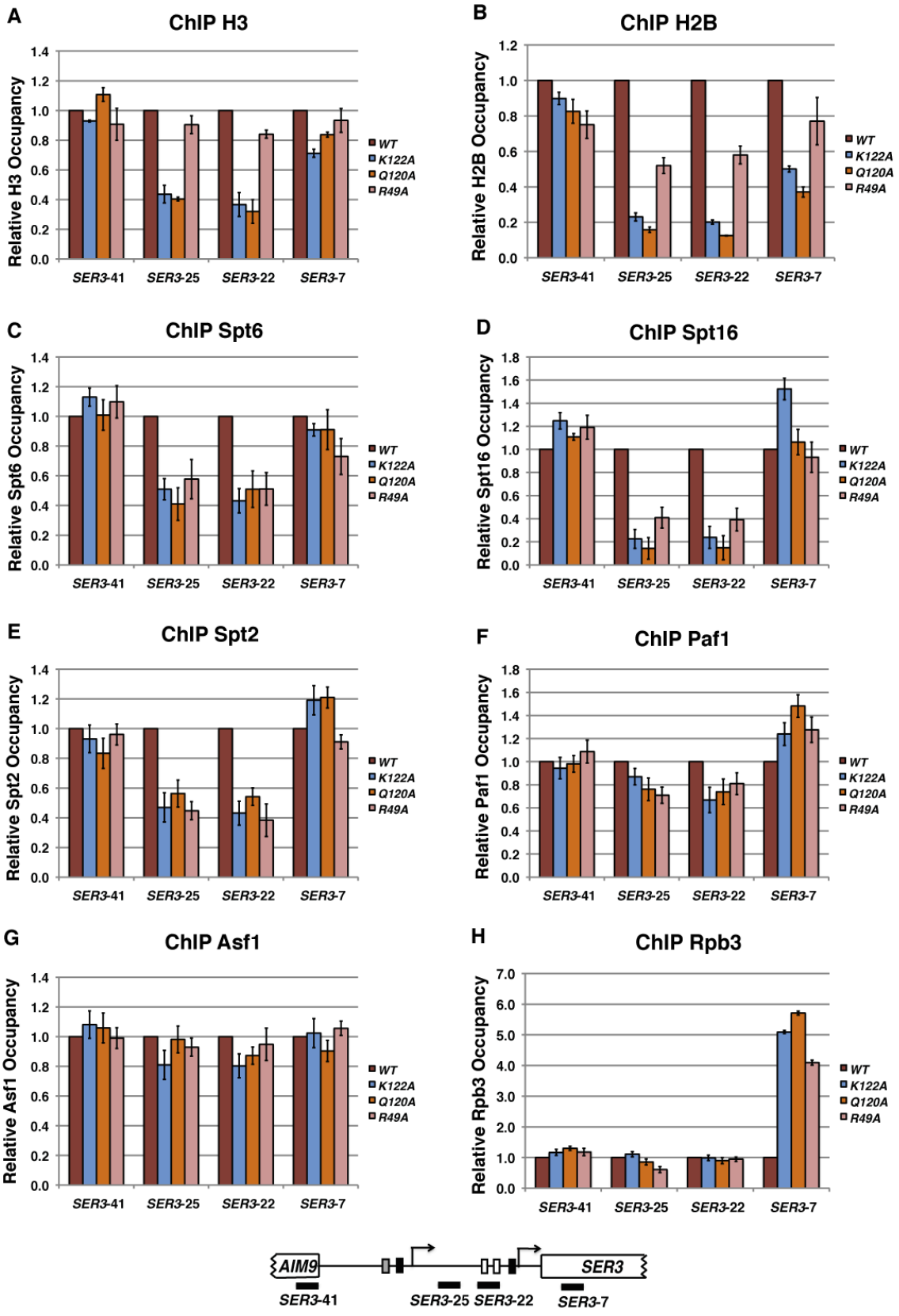
A
ChIP H3
Relative H3 Occupancy
B
ChIP H2B
Relative H2B Occupancy
C
ChIP Spt6
Relative Spt6 Occupancy
D
ChIP Spt16
Relative Spt16 Occupancy
E
ChIP Spt2
Relative Spt2 Occupancy
F
ChIP Paf1
Relative Paf1 Occupancy
G
ChIP Asf1
Relative Asf1 Occupancy
H
ChIP Rpb3
Relative Rpb3 Occupancy
WT
K122A
Q120A
R49A
SER3-41
SER3-25
SER3-22
SER3-7
AIM9
SER3

Figure 48. Occupancy of various factors over *SRG1/SER3*.

Histone H3 (A), H2B (B), Rpb3 (H), Spt6 (C), and Spt16 (D) ChIP was performed on chromatin isolated from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and represents the mean +/- SEM of three experiments. Spt2-13myc (E) and HA-Paf1 (F) ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Asf1-TAP (G) ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS493-YS495) or the indicated histone mutant alleles (YS504-YS506, YS518, YS519, YS521, YS525-YS527) that were grown in YPD at 30°C. Spt2-13myc, HA-Paf1, and Asf1-TAP occupancy values were subtracted with an untagged control (YS404, YS409, and YS417), normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

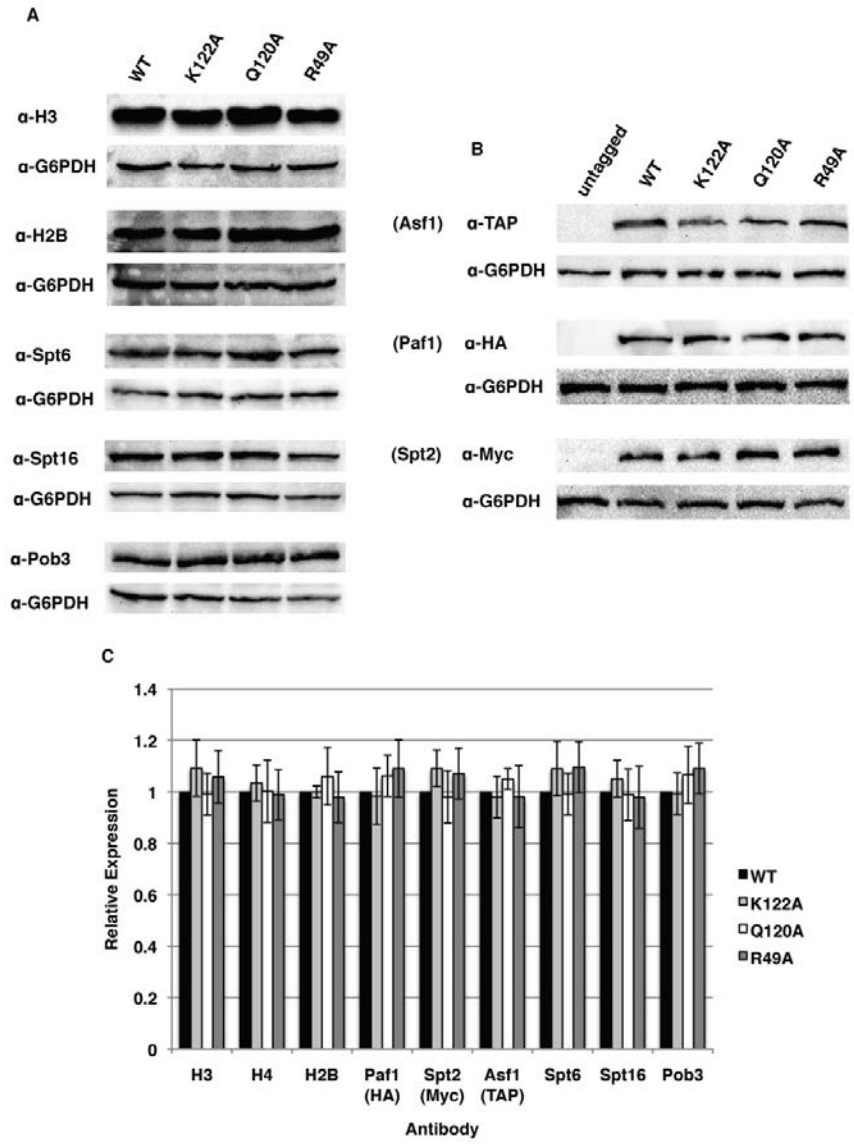

Figure 49. Histone mutants do not alter total protein levels.

A,B) Western analysis examining the effect of histone mutants on total histone H2B, Spt6, Spt16, Pob3, Asf1-TAP, HA-Paf1, and Spt2-Myc protein levels. Strains expressing the indicated histone alleles (YS417, YS404, YS409, YS428, YS454, YS458, YS462, YS471, YS493, YS504, YS518, YS525) were grown to ~3 × 10^7 cells/ml in YPD at 30°C. Proteins were extracted with trichloroacetic acid and subjected to Western analysis using anti-H2B, anti-Spt6, anti-Spt16, anti-Pob3, anti-PAP, anti-HA, anti-Myc, and anti-G6PDH (loading control). C) Quantitation of Western analysis where imilar results were obtained for three independent experiments and wild-type was arbitrarily set to one.

5.3.6 Effect of histone mutants on Spt2, Spt6, Spt16, Paf1, Asf1 and RNA pol II occupancy at other genes

To investigate whether the histone mutants that reduce histone chaperone occupancy across *SRG1* have a general defect in transcription-coupled occupancy, I measured H3, H2B, Spt2, Spt6, Spt16, Paf1, Asf1, and Rpb3 occupancy across the coding sequences of a subset of yeast genes by ChIP (Figures 50-57). At three highly transcribed genes, *PMA1* (100 mRNA/hr), *PYK1* (95 mRNA/hr), and *ADH1* (125 mRNA/hr) (HOLSTEGE *et al.* 1998), Spt2, Spt6, and Spt16 levels were reduced in all of the mutants to a similar extent as I observed across *SRG1* (Figures 53-55). Conversely, Spt2, Spt6, and Spt16 occupancy at three lowly transcribed genes, *GAL1* (repressed), *TUB2* (12 mRNA/hr), and *CYC1* (10 mRNA/hr) (HOLSTEGE *et al.* 1998), was unaffected in the mutants (Figures 42-44).

Similar to my analysis at *SRG1*/*SER3*, I examined the effect of the histone mutants on both Paf1 and Asf1 occupancy (Figures 56 and 57) and found that neither of these factors occupancy are significantly altered relative to wild-type.

For the most part, these changes in Spt2, Spt6, and Spt16 binding occur in the absence of any change in RNA pol II binding to these regions (Figure 52). Taken together, my studies suggest that these three residues are generally required to maintain nucleosome occupancy at highly transcribed genes, by facilitating Spt2, Spt6, and Spt16 recruitment to those genes.

5.3.7 Effect of histone mutants on *in vivo* interactions with Spt2, Spt6, and Spt16

I next examined whether the effect seen by ChIP on reduced Spt2, Spt6, and Spt16 in the three histone mutants (H3 K122A, Q120A, R49A), could be confirmed by performing pull down

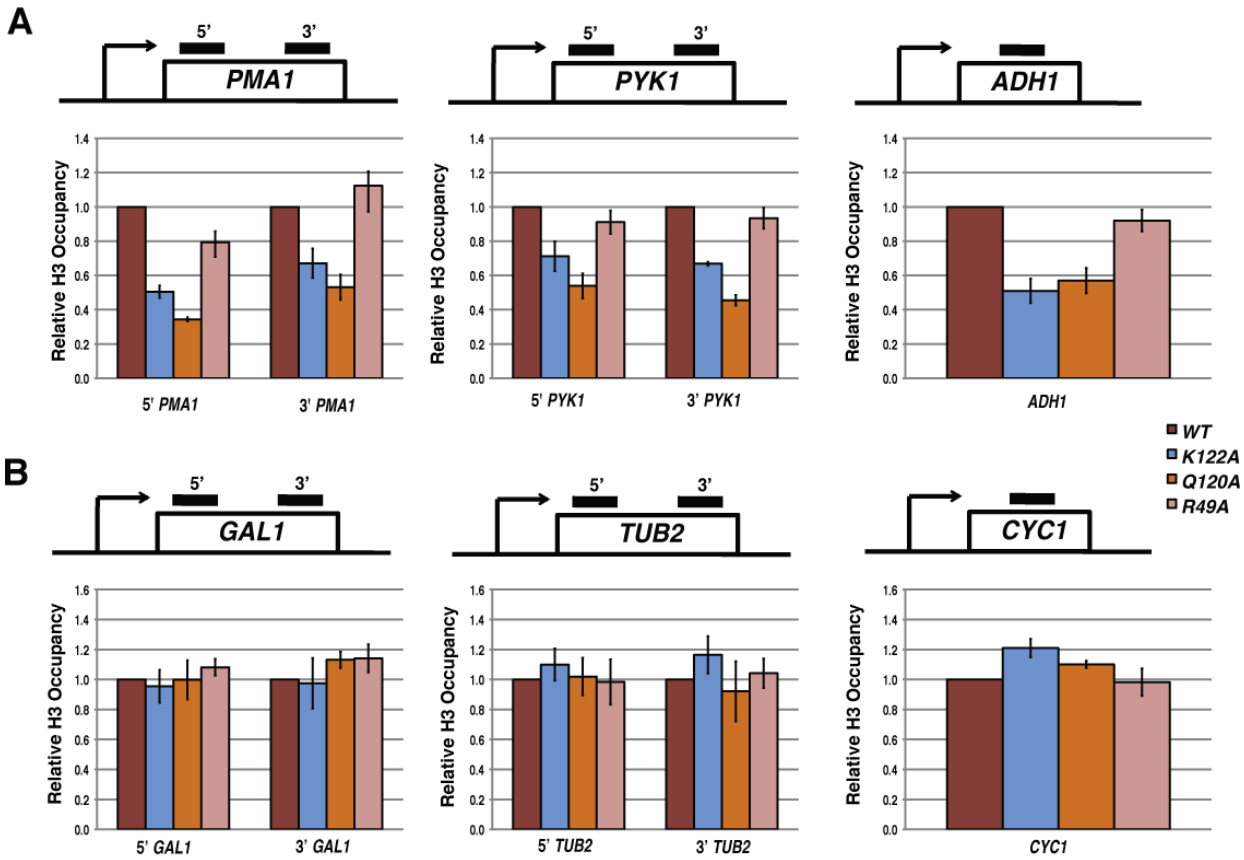

Figure 50. Effect of histone mutants on histone H3 occupancy over the coding regions of a subset of yeast genes.

A) Histone H3 ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Histone H3 occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Histone H3 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Histone H3 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

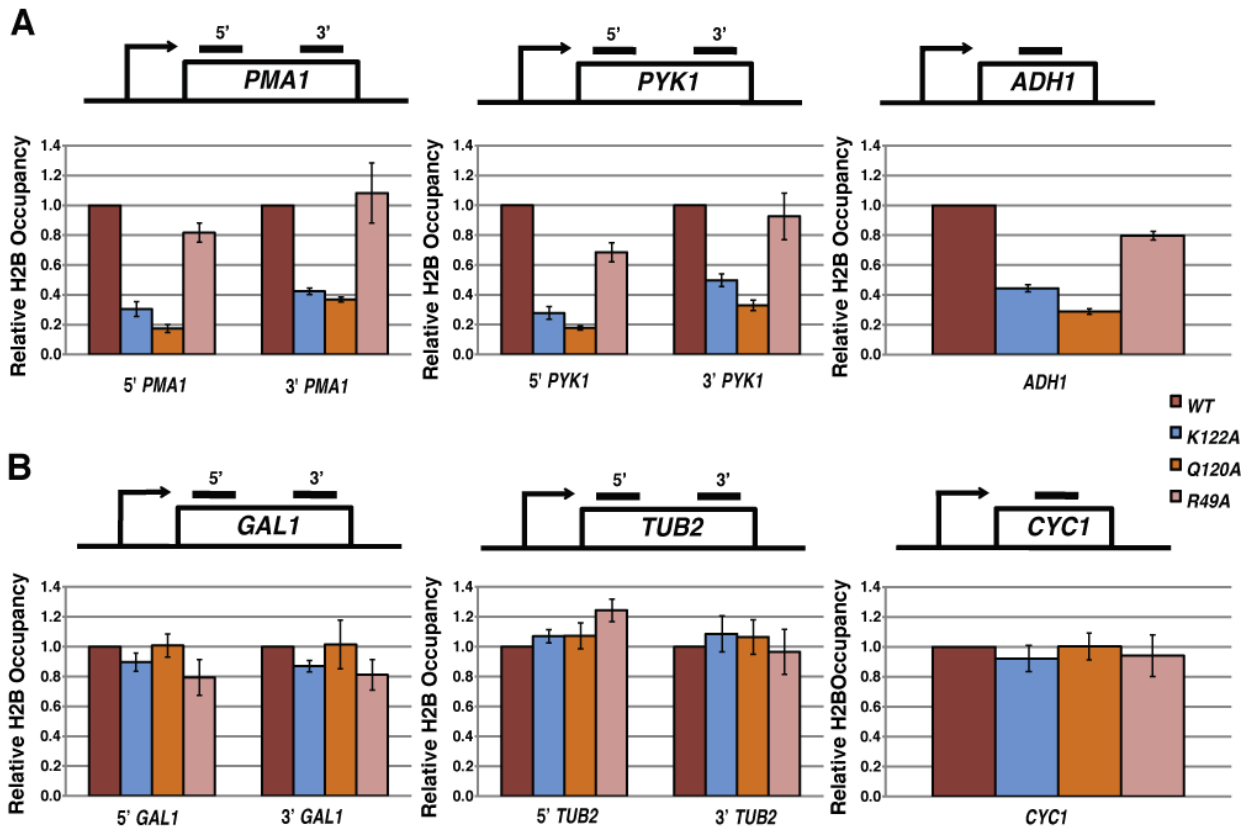

Figure 51. Effect of histone mutants on histone H2B occupancy over the coding regions of a subset of yeast genes.

A) Histone H2B ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Histone H2B occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Histone H2B occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Histone H2B occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

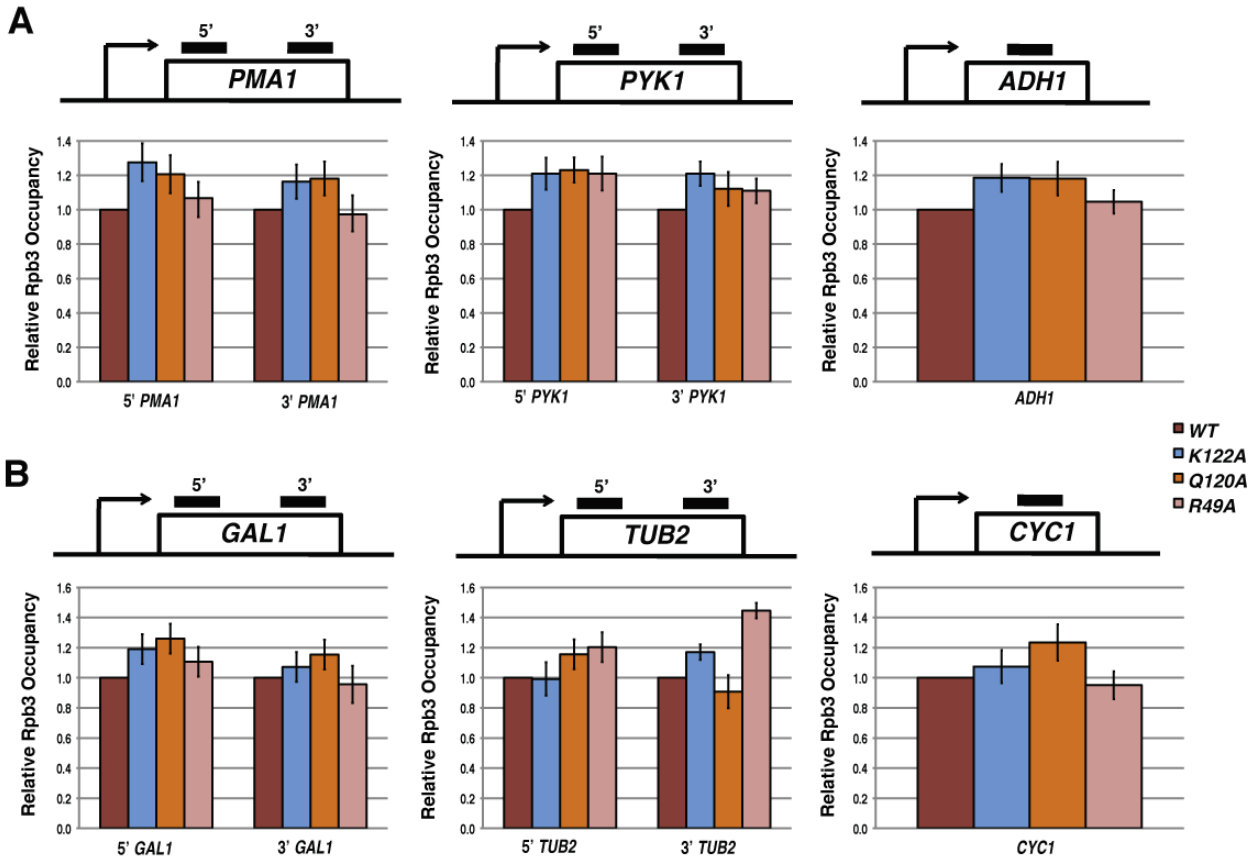

Figure 52. Effect of histone mutants on RNA pol II occupancy over the coding regions of a subset of yeast genes.

A) Rpb3 ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Rpb3 occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Rpb3 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Rpb3 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

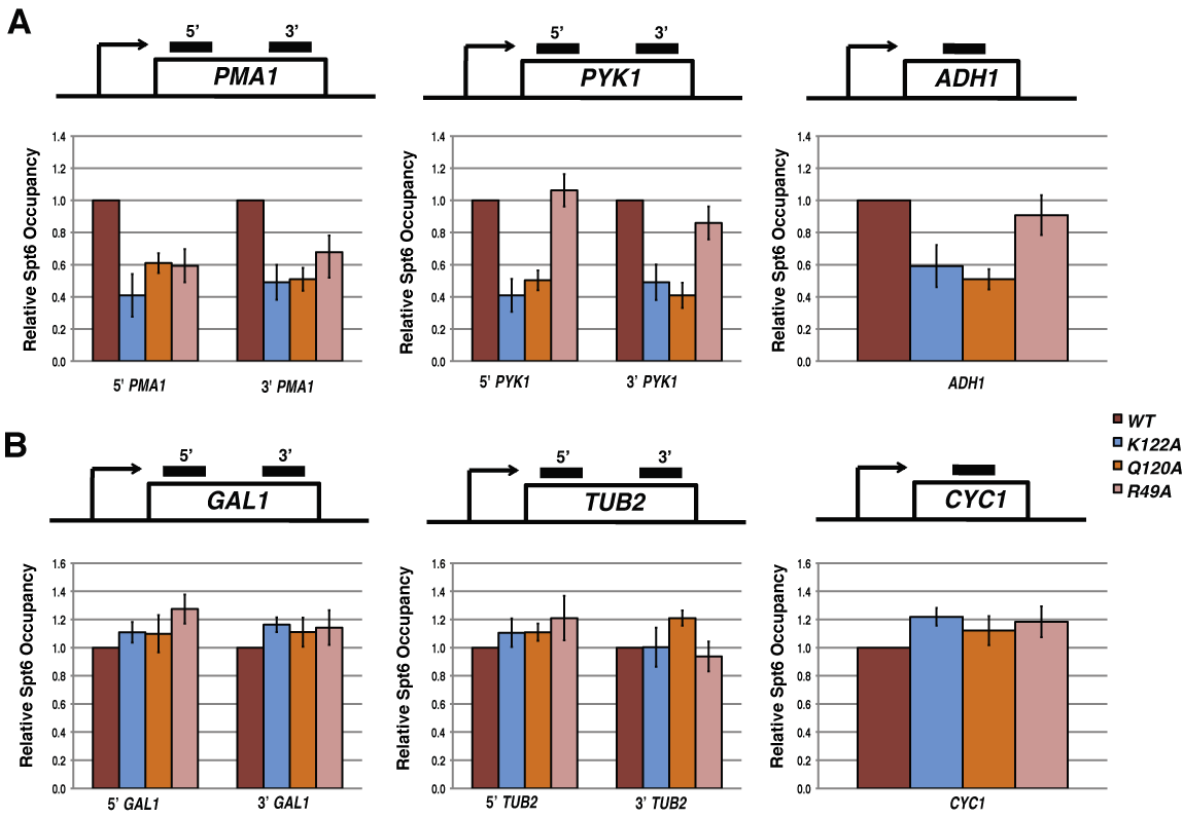

Figure 53. Effect of histone mutants on Spt6 occupancy over the coding regions of a subset of yeast genes.

A) Spt6 ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Spt6 occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Spt6 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Spt6 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

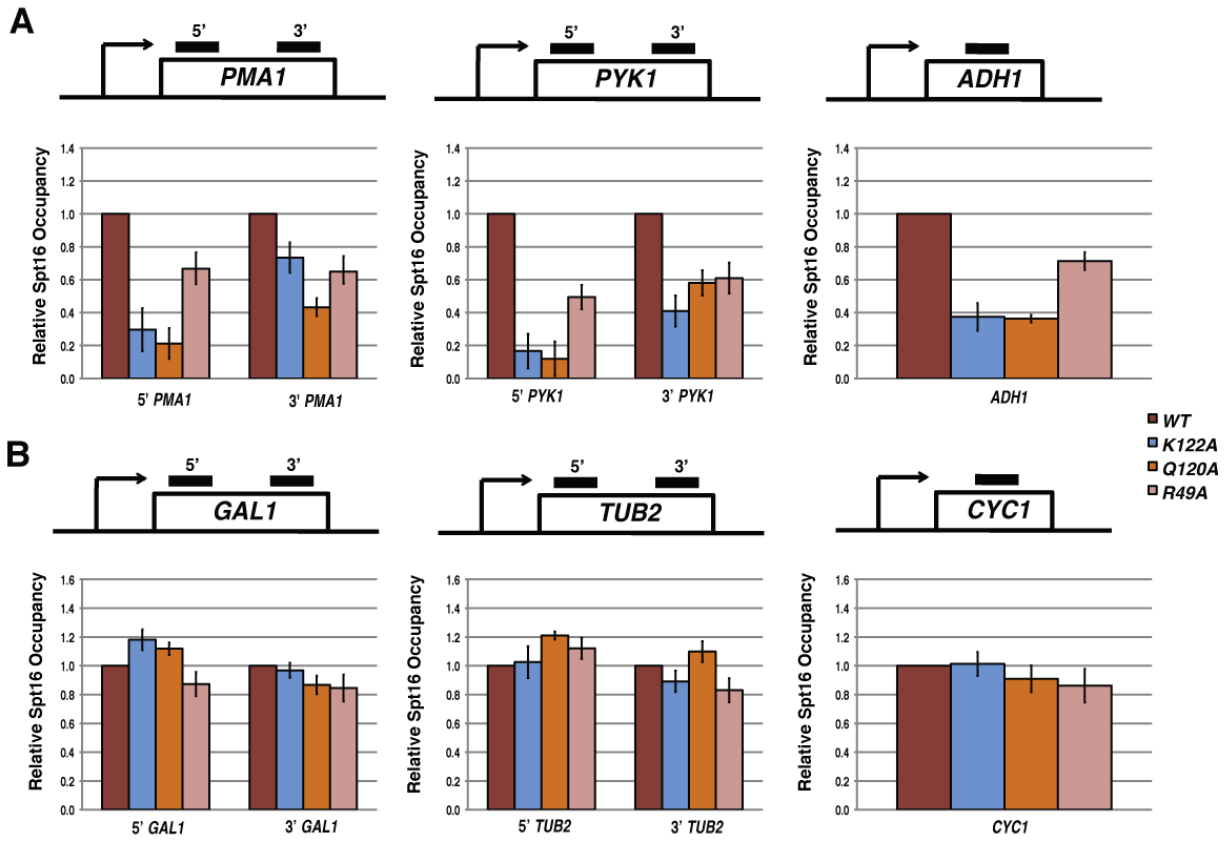

Figure 54. Effect of histone mutants on Spt16 occupancy over the coding regions of a subset of yeast genes.

A) Spt16 ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Spt16 occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Spt16 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Spt16 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

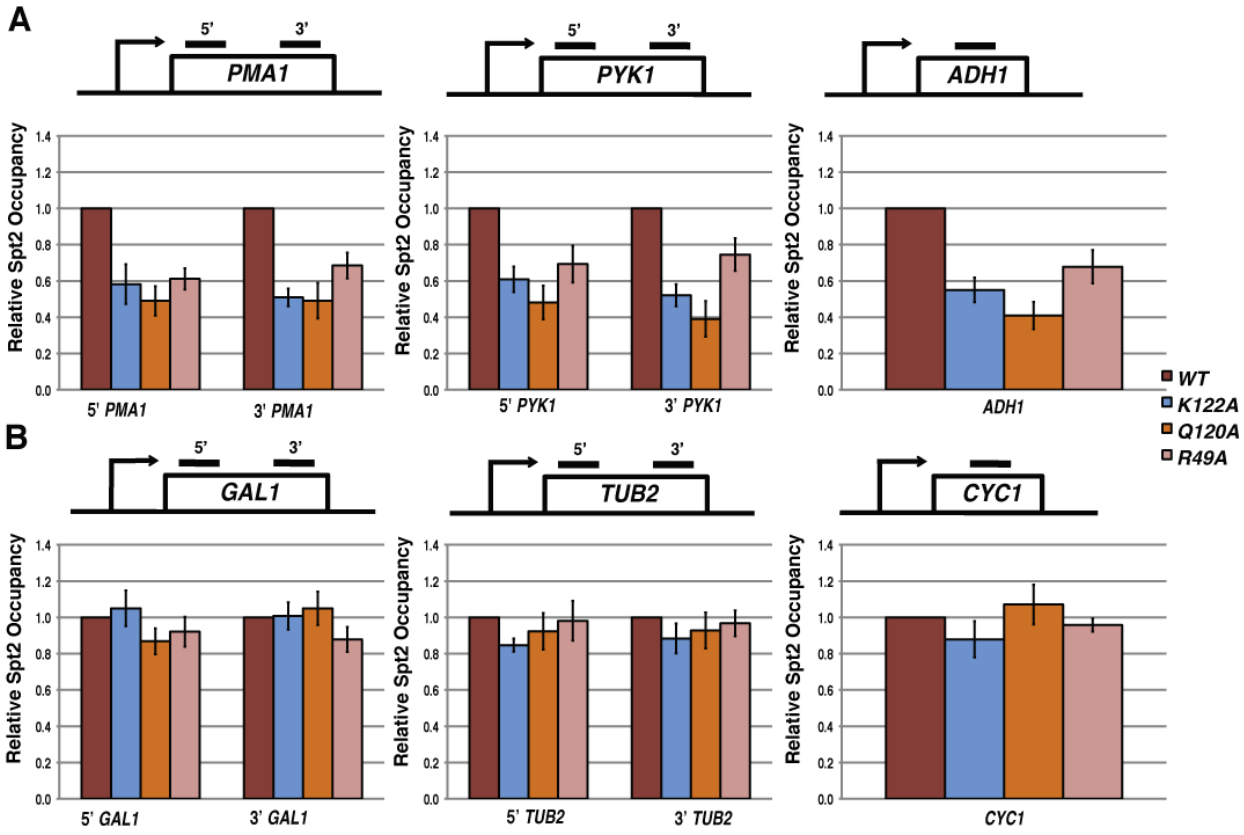

Figure 55. Effect of histone mutants on Spt2 occupancy over the coding regions of a subset of yeast genes.

A) Spt2-13myc ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. Spt2-13myc occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values were subtracted with an untagged control (YS404, YS409, and YS417), are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Spt2-13myc occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Spt2-13myc occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

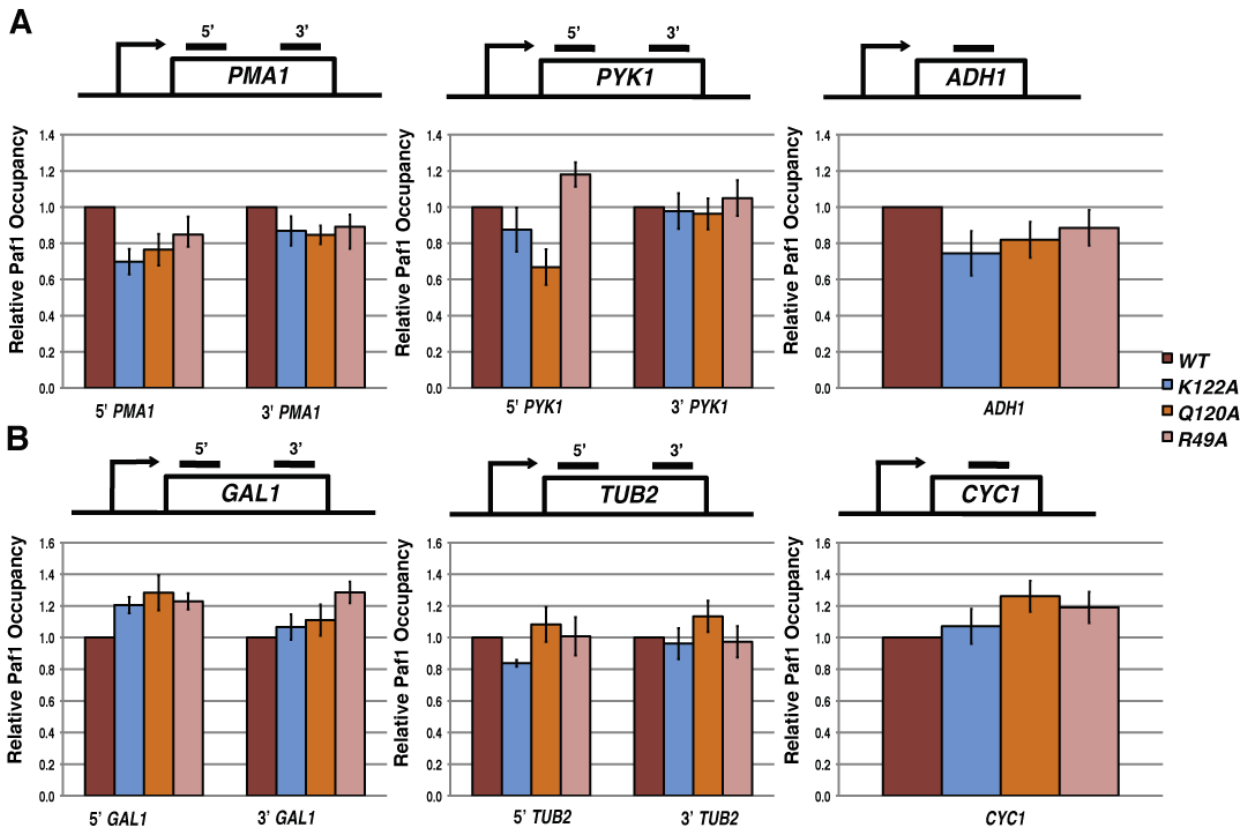

Figure 56. Effect of histone mutants on Paf1 occupancy over the coding regions of a subset of yeast genes.

A) HA-Paf1 ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS454-YS456) or the indicated histone mutant alleles (YS458-YS462, YS465, YS471, YS472, and YS474) that were grown in YPD at 30°C. HA-Paf1 occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values were subtracted with an untagged control (YS404, YS409, and YS417), are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. HA-Paf1 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) HA-Paf1 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

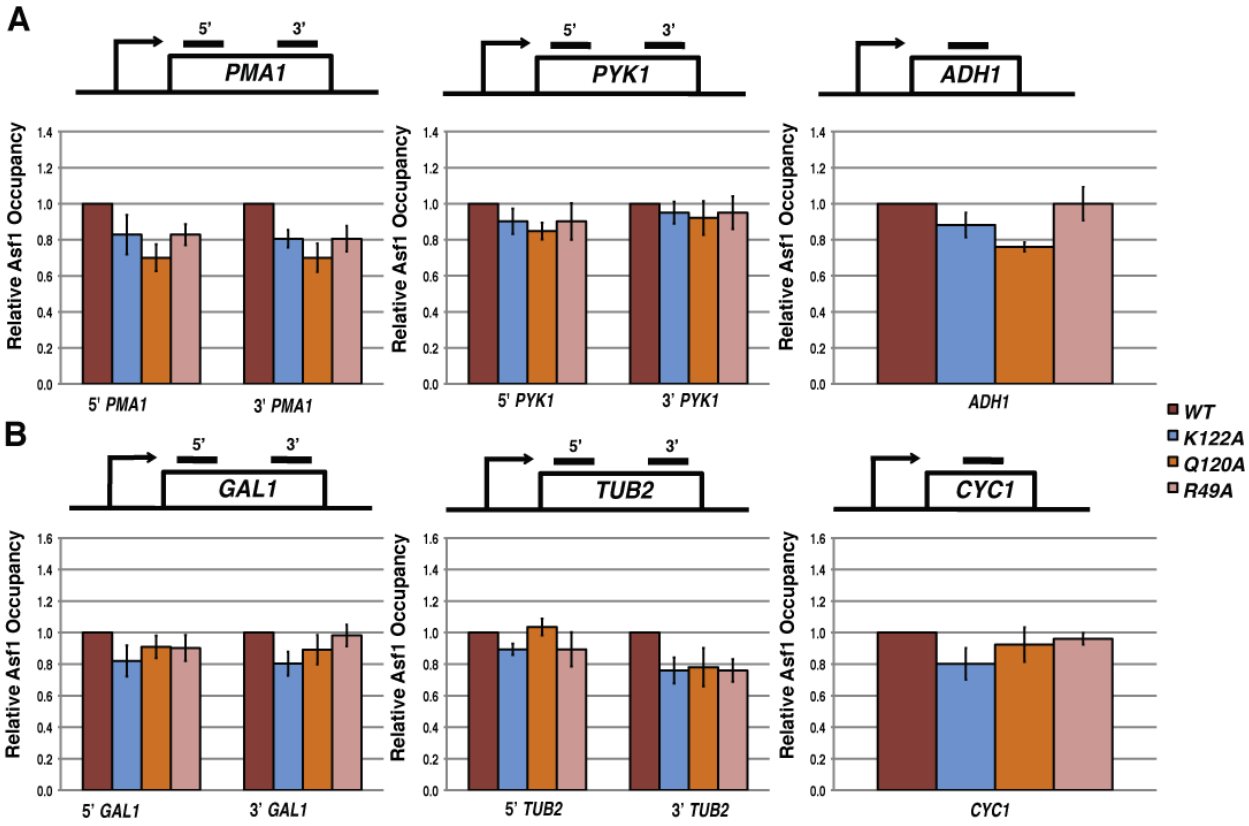

Figure 57. Effect of histone mutants on Asf1 occupancy over the coding regions of a subset of yeast genes.

A) Asf1-TAP ChIP analysis was performed on chromatin prepared from strains expressing *HHTS-HHFS* alleles (YS493-YS495) or the indicated histone mutant alleles (YS504-YS506, YS518, YS519, YS521, YS525-YS527) that were grown in YPD at 30°C. Asf1-TAP occupancy was measured within the coding region of three highly transcribed genes: *PMA1, PYK1,* and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values were subtracted with an untagged control (YS404, YS409, and YS417), are normalized to a control region in chromosome V and represent the mean +/- SEM of three experiments. Asf1-TAP occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. B) Asf1-TAP occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1* were determined as described in Panel A.

assays. To perform this experiment, I TAP-tagged either Spt2, Spt6, or Spt16 in strains expressing either wild-type histone alleles or one of the three histone mutations (see Materials and Methods). I next performed a TAP-pull down assay to test whether the histone amino acid substitutions reduced interactions with these factors (Figure 58). After immunoblotting for histone H3, I observed that Spt2, Spt6, and Spt16 all exhibited reduced interaction with the three histone mutants relative to wild-type. Taken together with the ChIP analysis (Figures 48, 50-57), my data indicate that these histone mutants result in a decreased interaction with Spt2, Spt6, and Spt16.

5.3.8 Effect of histone mutants on nucleosome disassembly and reassembly

Another likely scenario for how the histone mutants may be functioning is that a reduction in DNA affinity may slow nucleosome reassembly after passage of RNA pol II. This could account for my contrasting observations between lowly and highly transcribed regions of the genome. At lowly transcribed genes, a nucleosome will have sufficient time to reassemble prior to the passage of the next RNA pol II so the density of nucleosomes will not be affected. However, at highly transcribed genes, nucleosomes will only be partially assembled before being disassembled by the next RNA pol II molecule, resulting in reduced nucleosome occupancy at these genes. To test this hypothesis, I adopted a strategy that has been previously described (MASON and STRUHL 2003), where I put a very long gene, *FMP27* (over 8kB) under the control of the inducible *GAL1*pr (Figures 59 and 60). Using this construct, I can either turn off the promoter, in which case I can follow the last wave of RNA pol II, and subsequently nucleosome reassembly (Figure 59), or turn on the promoter, in which case I can follow the recruitment of RNA pol II, and subsequently nucleosome disassembly (Figure 60). Using this strategy, a pilot

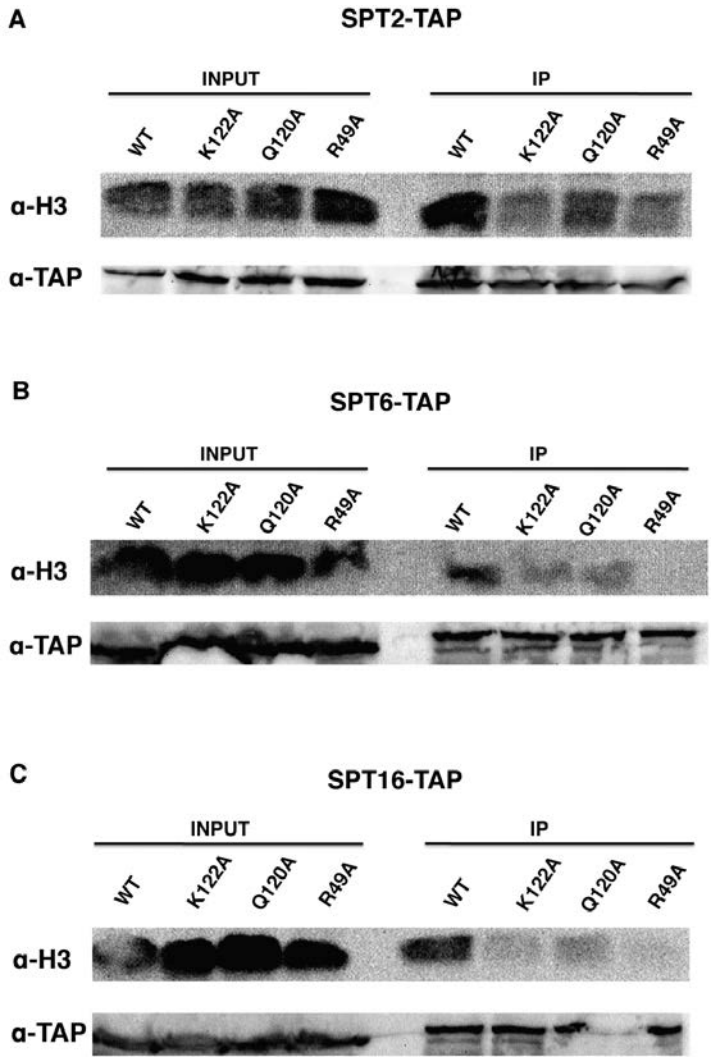

Figure 58. Histone mutants cause decreased interaction with Spt2, Spt6, and Spt16.

A-C) Pull down of Spt2-TAP, Spt6-TAP, or Spt16-TAP in strains expressing wild-type, H3 K122A, H3 Q120A, or H3 R49A histone alleles. Extracts from strains expressing wild-type (YS482, YS485, YS490), K122A (YS497, YS538, YS501), Q120A (YS508, YS511, YS514) or R49A (YS565, YS570, YS522) were incubated with IgG sepharose. Immunoblot analysis was performed to assess the presence of histone H3 and TAP-Spt2, Spt6, or Spt16 in the pulled down fractions (lanes 5-8). Lanes 1-4 in each blot represent 1% of the input.

experiment examining the difference between wild-type and K122A mutant strains, demonstrated that I could successfully follow the last wave of RNA pol II (turn off; GAL-> GLU) (Figure 59) and RNA Pol II recruitment (turn on; RAFF-> GAL) (Figure 60). In both cases, the expression of the construct (Figure 59B and Figure 60B), followed the predicted profile, given the carbon source provided.

When I examined the reassembly of nucleosomes by turning transcription off at this gene, compared to wild-type, the K122A mutant resulted in slowed reassembly of the nucleosomes, even though RNA pol II kinetics at this region were similar (Figure 59C-F). Similar to what has been previously found (MASON and STRUHL 2003), both Spt6 and Spt16 histone chaperones were found to follow the kinetics of RNA pol II in the wild-type strain (Figure 59G, 1). Interestingly, neither Spt6 nor Spt16 were found to occupy the gene in the K122A strain (Figure 59H, J).

When I next examined the disassembly of nucleosomes by inducing transcription, the RNA pol II kinetics were slightly affected in the K122A strain (Figure 60C,D). Interestingly, K122A resulted in slightly slowed occupancy of RNA pol II compared to the wild-type control. When I examined the occupancy of histones to this region upon induction, there was a slightly slowed decrease in histone occupancy in the K122A strain compared to wild-type (Figure 60E,F). However, while the nucleosome disassembly may seem slightly slowed in the K122A strain, the fact that RNA pol II occupancy was also slowed in the mutant strain indicates that any disassembly defect is slight, and the experiment would have to be repeated in order to ultimately determine if there is an effect. Similar to the analysis of nucleosome reassembly, I found that in the K122A mutant strain, neither Spt6 nor Spt16 is ever recruited to the region (Figure 60G-J). Taken together, these data suggest that K122 is required for the maintenance and/or recruitment

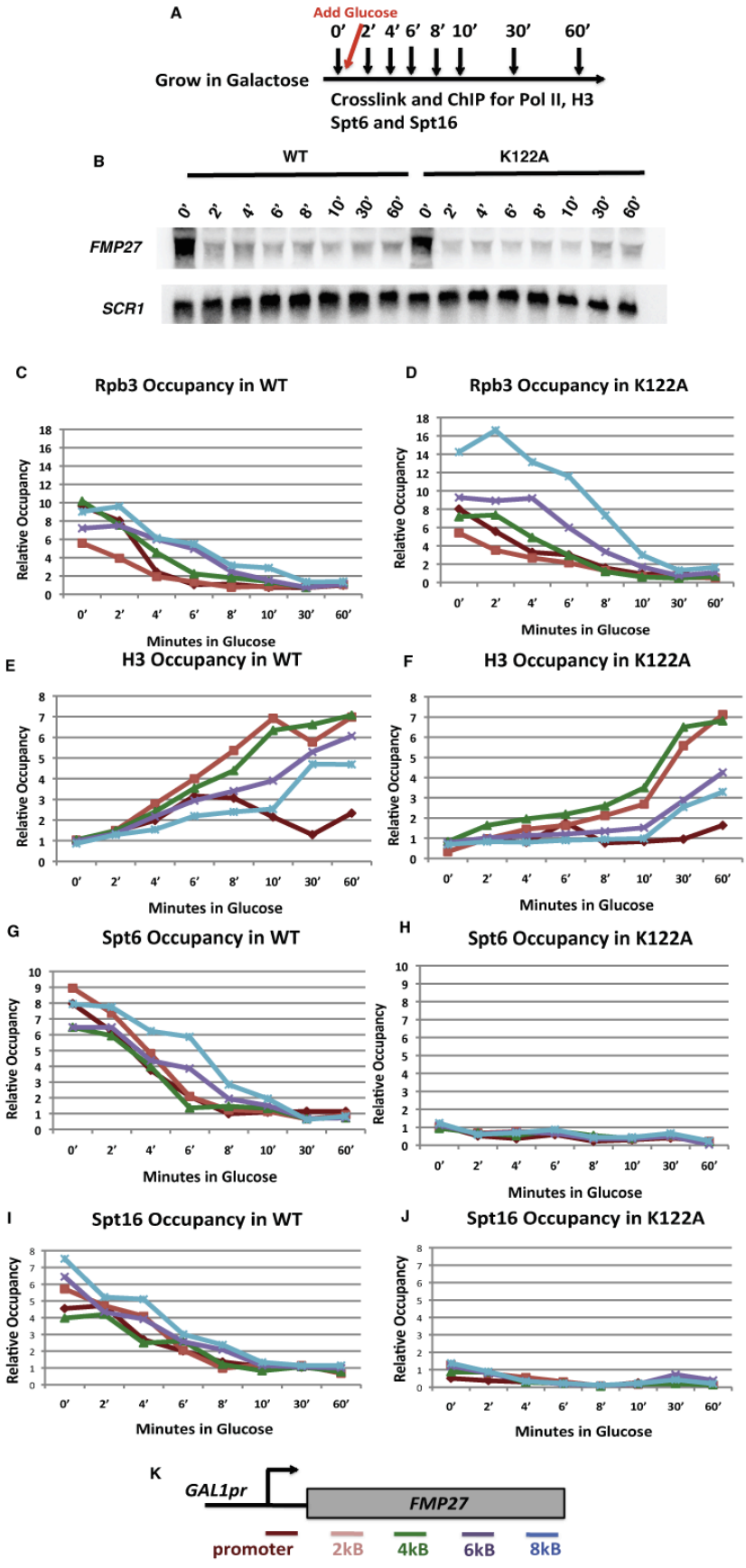
A
Add Glucose
0' 2' 4' 6' 8' 10' 30' 60'
Grow in Galactose
Crosslink and ChIP for Pol II, H3
Spt6 and Spt16
B
WT
K122A
0' 2' 4' 6' 8' 10' 30' 60' 0' 2' 4' 6' 8' 10' 30' 60'
FMP27
SCR1
C
Rpb3 Occupancy in WT
D
Rpb3 Occupancy in K122A
Relative Occupancy
Minutes in Glucose
E
H3 Occupancy in WT
F
H3 Occupancy in K122A
G
Spt6 Occupancy in WT
H
Spt6 Occupancy in K122A
I
Spt16 Occupancy in WT
J
Spt16 Occupancy in K122A
K
GAL1pr
FMP27
promoter
2kB
4kB
6kB
8kB

Figure 59. Time course ChIP analysis of Rpb3, H3, Spt6, Spt16 over *GAL1*pr-*FMP27* during transcription repression.

A) Diagram depicting the experimental procedure for determining RNA pol II loss, H3 recovery, and Spt6 and Spt16 occupancy upon repression of *GAL1*pr-*FMP27*. B) Northern blot analysis examining the effect of wild-type (YS384) and K122A (YS385) on *FMP27* expression during transcription repression. Rpb3 (C and D), H3 (E and F), Spt6 (G and H), and Spt16 (I and J) ChIP was performed on chromatin isolated from strains expressing *HHTS-HHFS* alleles (YS384) or *hhts-K122A* mutant alleles (YS385) expressing *GAL1*pr-*FMP27* that were grown in YPGal at 30°C to ~1×10^7 (0'), then repressed by adding glucose and time points were taken, as shown. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V.

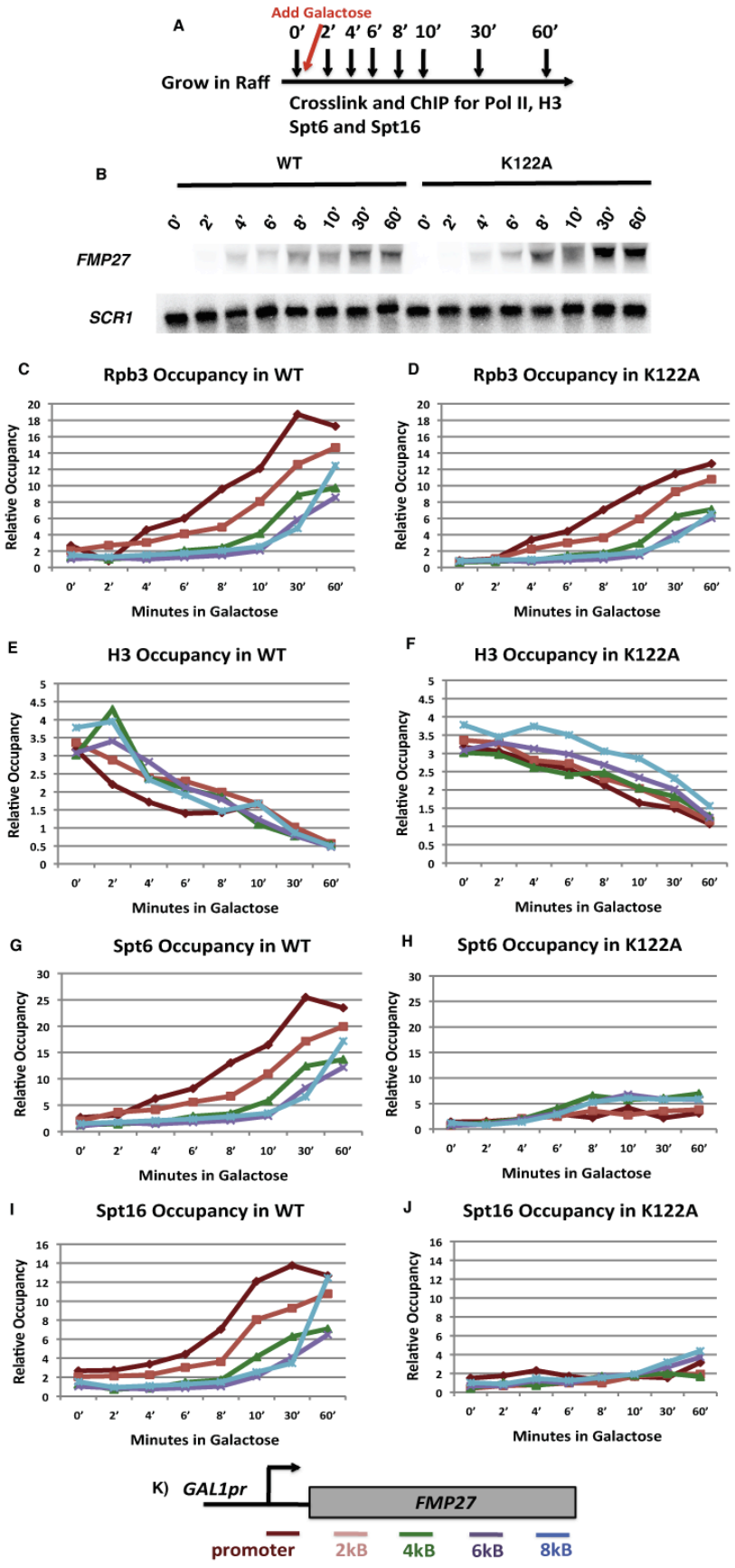
A
Add Galactose
0' 2' 4' 6' 8' 10' 30' 60'
Grow in Raff
Crosslink and ChIP for Pol II, H3
Spt6 and Spt16
B
WT
K122A
0' 2' 4' 6' 8' 10' 30' 60' 0' 2' 4' 6' 8' 10' 30' 60'
FMP27
SCR1
C
Rpb3 Occupancy in WT
D
Rpb3 Occupancy in K122A
E
H3 Occupancy in WT
F
H3 Occupancy in K122A
G
Spt6 Occupancy in WT
H
Spt6 Occupancy in K122A
I
Spt16 Occupancy in WT
J
Spt16 Occupancy in K122A
Relative Occupancy
Minutes in Galactose
K) GAL1pr
FMP27
promoter
2kB
4kB
6kB
8kB

Figure 60. Time course ChIP analysis of Rpb3, H3, Spt6, and Spt16 over *GAL1*pr-*FMP27* during transcription induction.

A) Diagram depicting the experimental procedure for determining RNA pol II recovery, H3 loss, and Spt6 and Spt16 occupancy upon induction of *GAL1*pr-*FMP27*. B) Northern blot analysis examining the effect of wild-type (YS384) and K122A (YS385) on *FMP27* expression during transcription induction. Rpb3 (C and D), H3 (E and F), Spt6 (G and H), and Spt16 (I and J) ChIP was performed on chromatin isolated from strains expressing *HHTS-HHFS* alleles (YS384) or *hhts-K122A* mutant alleles (YS385) expressing *GAL1*pr-*FMP27* that were grown in YPRaff at 30°C to ~1×10^7 (0'), then induced by adding galactose and time points were taken, as shown. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V.

of Spt6 and Spt16 to transcribed genes, and when these factors are not properly recruited, the rate of nucleosome reassembly is slowed.

5.3.9 Testing the effect of histone mutants on the direct interaction between histones and Spt16 or Spt6

Based on my strong *in vivo* evidence that the histone mutants cause decreased interaction with and occupancy of Spt6 and Spt16, I next wanted to determine if histone H3 directly interacts with these histone chaperones and whether the histone mutants then result in reduced direct interaction. In order to perform these analyses, I obtained bacterial plasmids expressing either wild-type or mutant versions of the histone proteins from Tim Formosa at the University of Utah, and also purified Spt16-Pob3. I transformed bacterial RIPL cells with each histone plasmid, induced their expression with IPTG, and confirmed their expression on a Coomassie stained denaturing gel (Figure 61A). After lysing the cells through homogenization, I separated the proteins by SDS-PAGE, transferred the protein to nitrocellulose membrane and took the protein through a series of washes to renature the proteins (see Materials and Methods). After incubating the membrane with purified Spt16-Pob3, I immunoblotted with antibody specific to Spt16 and found that while there was interaction between Spt16 and bacterial proteins, or potentially a cross reacting band with the antibody (higher molecular weight bands seen in Figure 61B, top panel), there is an interaction seen between Spt16 and histone H3, and this interaction appears to be decreased in the strains expressing mutant plasmids (Figure 61B, band corresponding to ~17kDa). To confirm this interaction was with histone H3, I stripped the membrane and immunoblotted with an antibody for total histone H3 and found that the same band Spt16 interacted with was histone H3 (Figure 61B, bottom panel). As this is preliminary analysis, in

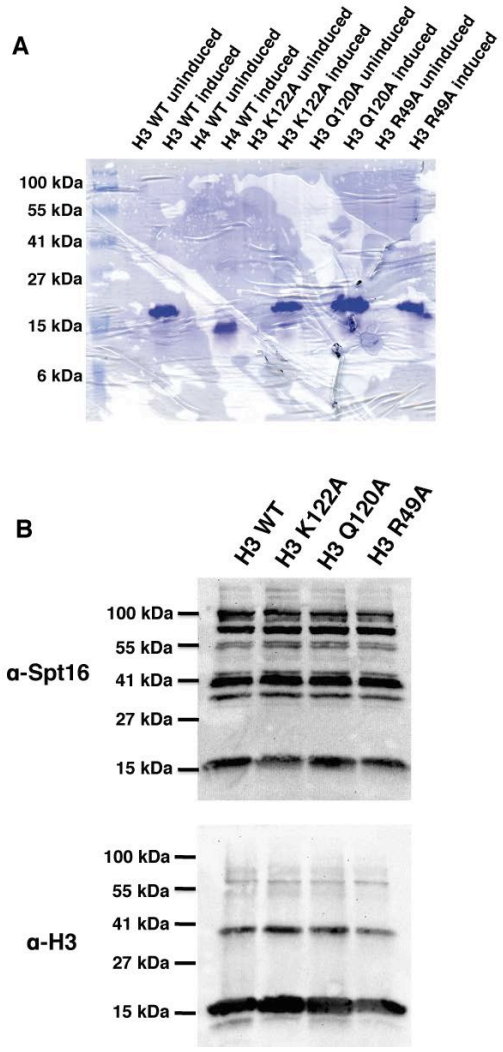

Figure 61. Effect of histone mutants on direct interaction with Spt16.

A) Coomassie stained denaturing gel of uninduced and IPTG induced bacterial RIPL cells expressing wild-type, K122A, Q120A, or R49A histone proteins. B) Far Western analysis between bacterial lysate expressing wild-type, K122A, Q120A, or R49A histones and Spt16. 25µg of bacterial lysate was separated on a 12.5% acrylamide SDS-PAGE, transferred to nitrocellulose, and taken through a series of washes to denature and renature proteins transferred to the membrane. After incubation with 5µg of purified Spt16-Pob3 (kindly provided by T. Formosa), the membrane was immunoblotted with anti-Spt16 (top panel) and anti-histone H3 (bottom panel).

order to confirm these studies I am currently performing this analysis using purified histone proteins, in order to get a clearer interpretation of the data, and I will use both histone H4 and histone H2A/H2B as controls for this assay.

Additionally, I purified Spt6 in order to perform similar analysis with this histone chaperone (Figure 62). In this Far Western analysis I separated the bacterial lysate by SDS-PAGE, transferred the protein to nitrocellulose membrane and took the protein through a series of washes to renature the proteins (see Materials and Methods). Then I either immunoblotted directly for Spt6 and histone H3 (Figure 62A) or incubated the membrane with purified Spt6 and then immunoblotted with antibody specific to Spt6 and histone H3. I found that there is an interaction able to be detected between Spt6 and histone H3, however this interaction does not seem to be altered in the histone mutants. The only mutant may show decreased interaction is H3 R49A, which may separate the functions of these histone mutants.

Based on this preliminary analysis, I have been able to show that Spt16 and Spt6 directly interact with histone H3. Interestingly, the interaction between Spt16 and histone H3 may be decreased in mutant histones, while the Spt6 interaction does not seem as effected.

5.3.10 Genetic relationship between histone residue substitutions and *SPT2*, *SPT6*, and *SPT16* mutations

To further understand the relationship between the histone amino acids and Spt2, Spt6, and Spt16, I performed crosses to generate strains expressing either wild-type, K122A, Q120A, or R49A histones with either *spt2*Δ, *spt6-1004*, or *spt16-197*. Interestingly, while I was able to create the majority of these strains, K122A in combination with *spt2*Δ or *spt16-197* is synthetically lethal (Figure 63A,B). I confirmed this tetrad analysis by covering the viable

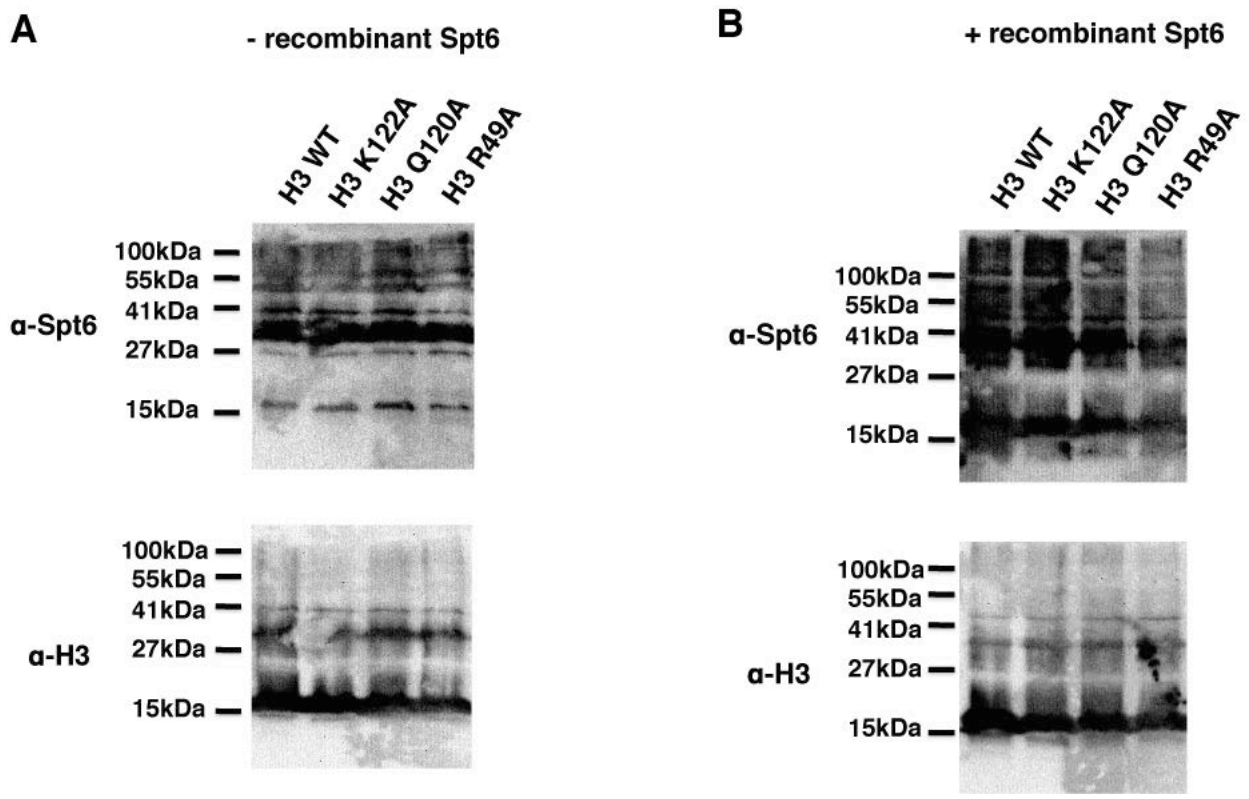

Figure 62. Effect of histone mutants on direct interaction with Spt6.

Far Western analysis between bacterial lysate expressing wild-type, K122A, Q120A, or R49A histones and Spt6. A,B) 25μg of bacterial lysate was separated on a 12.5% acrylamide SDS-PAGE, transferred to nitrocellulose, and taken through a series of washes to denature and renature proteins transferred to the membrane. A) Control blot. The membrane was immunoblotted with Spt6 (top panel) and histone H3 (bottom panel), with no incubation with recombinant Spt6. B) After incubation with 5μg of purified Spt6 (see Materials and Methods), the membrane was immunoblotted with anti-Spt6 (top panel) and anti-histone H3 (bottom panel).

diploid strain expressing both wild-type and mutant histones and wild-type or mutant *SPT2* or *SPT16* with a plasmid expressing either *HHT1/HHF1*, *SPT2,* or *SPT16* with the *URA3* gene for counter-selection on media containing 5FOA. Interestingly, I was unable to cover the lethality with the *HHT1*/*HHF1* plasmid, likely due to the dominant phenotype K122A displays over wild-type histones. However, I was able to cover the synthetic lethality with either the *SPT2* or *SPT16* plasmid: when I plated these spores on media containing 5FOA, I was unable to lose the plasmids in haploids containing K122A and *spt2*Δ or *spt16-197*.

With the double mutant strains I was able to generate, I performed Northern blot analysis to determine whether the histone mutants in combination with the *spt2*, *spt6*, or *spt16* mutants had an epastatic relationship (Figure 63C, D). Importantly, the wild-type synthetic histone strain did not alter the effect either *spt2*Δ*, spt6-1004*, or *spt16-197* has on their own, and none of the mutant strains examined significantly altered *SRG1* RNA levels. Interestingly, while K122A or Q120A did not have any combinatorial effects on *SER3* with *spt6-1004*, Q120A in combination with either *spt2*Δ or *spt16-197* did have an additive effect on *SER3* expression. Conversely, R49A in combination with all of the mutants resulted in an additive effect on *SER3* expression. Together these data reveal a genetic relationship between the histone residues and Spt2, Spt6, and Spt16.

5.4 DISCUSSION

In this Chapter, I tested multiple hypotheses as to how the histone mutants identified in Chapter 4 may be functioning to alter transcription-coupled nucleosome dynamics.

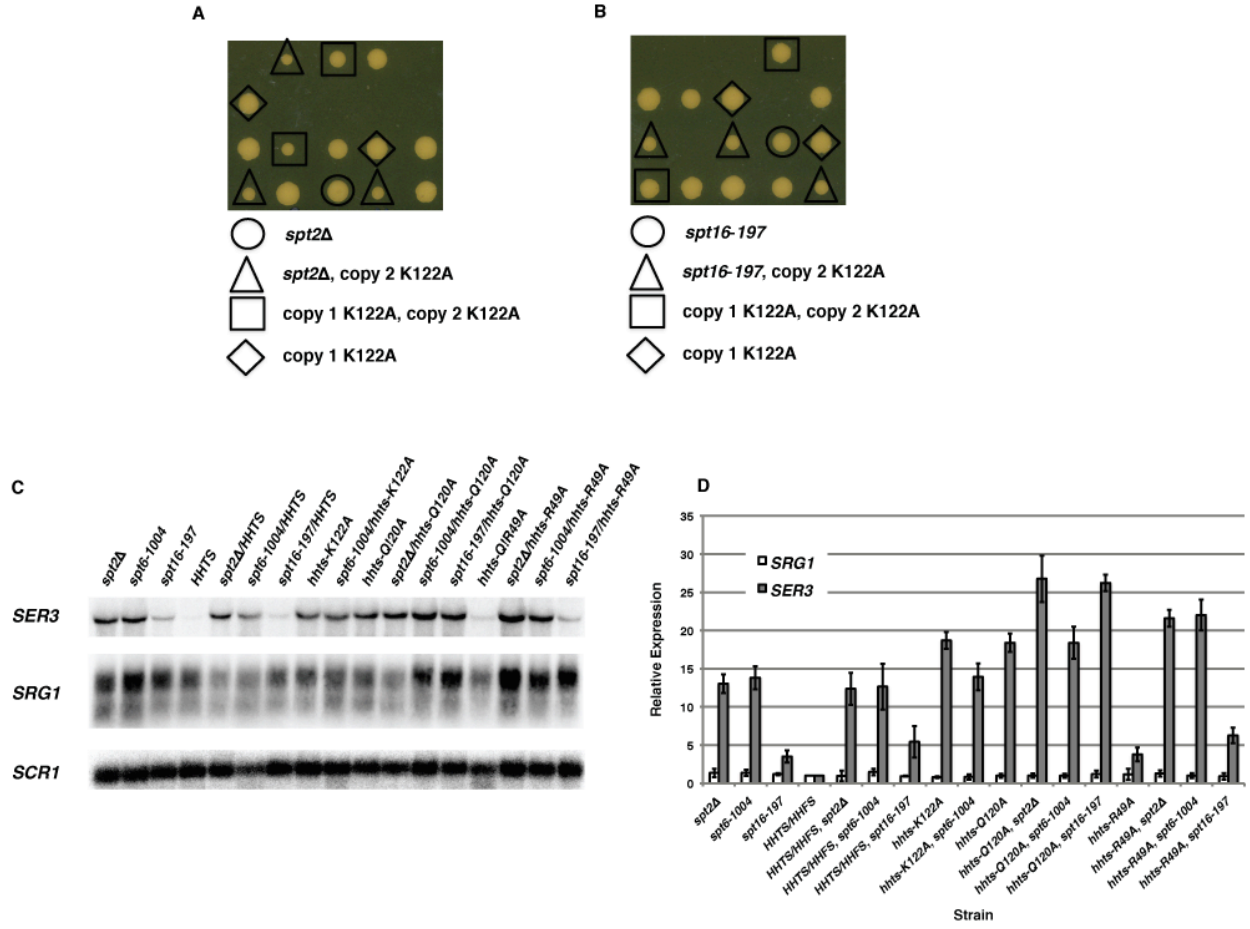

Figure 63. Effect of histone mutants on *SER3* expression in combination with either *spt2*Δ, *spt6-1004*, or *spt16-197*.

Tetrad analysis of *hhts-K122A/hhts-K122A* with *spt2*Δ (YS641XYS642; A) or *spt16-197* (YS640XYJ780; B). Tetrads are vertical, where those circled indicate either *spt2*Δ or *spt16-197* single alleles, those surrounded by a triangle express either *spt2*Δ or *spt16-197* in combination with *hhts-K122A* expressed at the *HHT2/HHF2* locus, those surrounded by a square express *hhts-K122A* at both histone loci, those surrounded by a diamond express *hhts-K122A* at the *HHT1/HHF1* locus, those which have a spore growing and are not indicated are wild-type for *SPT2* or *SPT16* and the histone loci, and those spores which do not grow are expressing either *spt2*Δ or *spt16-197* and either *hhts-K122A* at both histone loci, or only at the *HHT1/HHF1* locus. C) Northern blot analysis examining the effect of histone mutants

in combination with *spt2, spt6-1004* or *spt16-197* on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from FY346, FY2180, YJ804, YS404, YS409, YS417, YS428, YS591, YS594, YS597, YS600, YS601, YS604, YS606, YS609, YS612, and YS614 that were grown to a density of 1-2 x 10^7 cells/ml in YPD at 30°C. D) Quantitation of Northern analyses. *SRG1* (white bars) and *SER3* (grey bars) RNA levels for the histone mutants are normalized to the *SCR1* loading control and are relative to the *SRG1* and *SER3* RNA levels measured in control *HHTS-HHFS* strains (arbitrarily set to 1). Each bar represents the mean +/- SEM from three independent experiments strains generated by genetic crosses (YS591-YS619), except the *spt16-197/hhts-Q120A* strains, of which two independent strains were used, and the experiment was performed in triplicate (YS604, YS605).

To test whether the histone mutants are causing destabilization of nucleosomes, I performed multiple experiments, which suggest that this hypothesis is not correct. First, I found that the global protein levels of both histone H3 and H4 are equal to wild-type levels in all the histone mutants (Chapter 4) (HAINER and MARTENS 2011a). These mutations may increase the mobility of nucleosomes similar to what has been shown for *sin* mutations located at the nucleosome dyad (FLAUS *et al.* 2004). However, this is not likely to be the case as only one of the histone mutants, H3 V117A, confers a *sin* phenotype (Chapter 4) (HAINER and MARTENS 2011a). Moreover, my nucleosome scanning experiments gave no indication of nucleosome mobility at other locations including more positioned nucleosomes over the open reading frames of *AIM9* and *SER3* that flank *SRG1* and the repressed *GAL1* promoter (HAINER and MARTENS 2011a). Furthermore, I found that over the 5' end of *SRG1*, the positioned nucleosomes present in serine starvation conditions or when the catalytic subunit of the Swi/Snf chromatin remodeling complex is deleted, are not disrupted when there is no transcription of *SRG1*, supporting the idea that these mutants do not simply destabilize nucleosomes, like *sin* mutants.

These residues may also be affecting a critical function of the essential residues in this region, such as H3 T118. Interestingly, several *in vitro* studies investigating *sin* mutations, in particular those involving H3 T118, have provided evidence to support their role in histone-DNA affinity (HSIEH *et al.* 2010; KURUMIZAKA and WOLFFE 1997; MUTHURAJAN *et al.* 2004). However, with the exception of V117A, the other substitutions do not confer a similar *sin* phenotype, suggesting that any role these residues may play in DNA affinity is either more moderate or distinct from those of H3 T118. Furthermore, when I independently tested the effect of T118I on both *SER3* expression and nucleosome occupancy, I found no effect (Chapter 4).

A more likely scenario is that a reduction in DNA affinity may slow nucleosome reassembly after passage of RNA pol II. This could account for my contrasting observations between lowly and highly transcribed regions of the genome. At lowly transcribed genes, a nucleosome will have sufficient time to reassemble prior to the passage of the next RNA pol II so the density of nucleosomes will not be affected. However, at highly transcribed genes, nucleosomes will only be partially assembled before being disassembled by the next RNA pol II molecule, resulting in reduced nucleosome occupancy at these genes, and my data is consistent with this hypothesis. Expanding on this hypothesis, the reason disassembly and/or reassembly would be slowed in the histone mutants may be due to reduced occupancy and/or function of histone chaperones, such as Spt6 or Spt16. To test this hypothesis, I examined whether the histone mutations were altering histone chaperone occupancy and function. I found that both Spt6 and Spt16, but not Asf1, had reduced occupancy, specifically over highly transcribed regions, in three of the histone mutants (H3 K122A, Q120A, and R49A). Interestingly, Spt2 occupancy is also decreased in these histone mutants. Spt2 has been suggested to be an HMG-like protein that can interact with DNA, so it is possible that this protein may be required for interaction with the nucleosomal DNA, which is then loosened and provides a surface that histone chaperones such as Spt6 and Spt16 can interact. Therefore, an intriguing possibility is that the histone mutants result in reduced occupancy in all three of these factors because this mutated histone surface can no longer permit Spt2 to function, therefore resulting in decreased histone chaperone occupancy.

To continue the analysis of how the defect in histone chaperone occupancy is affecting nucleosome dynamics at highly transcribed regions of the genome, I created a previously reported allele, in which the expression of a long gene is inducible based on the sugar source

available in the cell. These data demonstrate that, while in a wild-type strain similar occupancy patterns to RNA pol II are observed for both Spt6 and Spt16, RNA pol II alone is not sufficient for the maintenance and/or recruitment of these histone chaperones. Due to the dramatic lack of occupancy of these histone chaperones in the K122A strain, I hypothesize that this amino acid is required for the maintenance of Spt6 and Spt16 occupancy over transcribed regions, once these factors are recruited to the chromatin. These data argue against a traditional view that RNA pol II is simply recruiting these factors, and rather supports the interplay between chromatin and transcription dynamics. Additionally, based on the nucleosome reassembly observed in K122A, and the lack of histone chaperone occupancy, this supports the hypothesis that the histone mutant causes slowed reassembly of nucleosomes due to the loss of histone chaperone binding. However, during induction of this gene, the histone chaperones are also absent, yet nucleosome disassembly is not dramatically affected. Together, these data indicate that while these histone chaperones are required for nucleosome reassembly, their role is not required during nucleosome disassembly, rather other factors, such as RNA pol II itself, are able to remove these barriers to promote RNA pol II traversal over the DNA, as has been demonstrated *in vitro* (HSIEH *et al.* 2010; KULAEVA *et al.* 2010; LUSE and STUDITSKY 2011).

While the actual affinity of the mutant nucleosomes to Spt2, Spt6, and Spt16 remains untested, I have provided preliminary data demonstrating a decreased interaction, that is direct, between Spt16 and the histone mutants. Taken together, these studies support a role in these histone residues in properly maintaining occupancy of Spt2, Spt6, and Spt16 in order for these factors to promote proper dynamics of transcribing nucleosomes. A possible model for these results is as follows. When the nucleosome contains one of the histone residue substitutions described, there is a weaker association of the DNA to the histone octamer. This loosened

interaction negates the requirement for Spt2 (or another DNA binding protein) to interact with the nucleosomal DNA, where this factor normally acts to loosen the DNA from histone contacts. Due to the loss of Spt2 interacting to the chromatin, subsequent histone chaperones, such as Spt6 and Spt16, are no longer promoted to these transcribed regions, and RNA pol II can successfully traverse the chromatinzed template, as the interaction is loosened, and the histone proteins can freely disassemble from the DNA. However, due to a decreased affinity for the mutant histone proteins to Spt6 and Spt16 histone chaperones, the reassembly rate of nucleosomes after RNA pol II has passed, is slowed. This model leaves many unanswered questions, including what factors are required for the recruitment of Spt6 and Spt16 histone chaperones; what is the affinity between the mutant histones and Spt6 and Spt16; what is the DNA binding protein responsible for initial loosening of the DNA before histone chaperones are able to bind; and how do nucleosomes properly reassemble onto transcribed regions without histone chaperones? This work has provided preliminary insight into the coordination of nucleosome dynamics during transcription, but it will be exciting to both confirm and expand on t his analysis to more completely understand this relationship.

Finally, I provided data showing a genetic relationship between the histone residue substitutions and Spt2, Spt6, and Spt16. These data show an additive role between some of the mutant combinations and also synthetic lethality between K122A and *spt2*Δ or *spt16-197*, which indicates that these factors are working through separate pathways. While this does not exactly support the hypothesis that there is a direct interaction between the histone chaperones and these histone amino acids, it does not rule out this claim. These factors have a variety of functions in the cell, and therefore exacerbating the effect that either one on its own causes to transcription-couple nucleosome reassembly could result in increased defects, as seen at *SER3*.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

The regulation of gene expression is critical for an organism to control cell development and this complex process involves many layers of control. My thesis has focused on the role of a relatively new layer of complexity involving the transcription of ncDNA, and, in particular, the effect of transcription, both coding and non-coding, on chromatin dynamics. First, I contributed to uncovering a novel mechanism of gene regulation in which transcription of a ncDNA, *SRG1*, promotes the maintenance of nucleosome occupancy in order to repress an adjacent coding gene, *SER3*. Second, I worked with a number of undergraduates and together we uncovered a number of unique amino acid substitutions in a histone chaperone, Spt16, which are required for the maintenance of Spt16 occupancy over *SRG1* and highly transcribed regions of the genome and therefore the maintenance of nucleosome occupancy. Finally, I identified a set of histone H3 and H4 residues which are required for maintaining both Spt6 and Spt16 histone chaperone occupancy, and therefore function, over highly transcribed regions of the genome. Thus, mutating these histone residues results in defective histone reassembly and leads to decreased nucleosome occupancy. My work has lead to a deeper understanding of the regulation of genes by transcription-coupled nucleosome dynamics and has provided a number of avenues of research for further studies in the field.

6.1.1 Identification of a novel mechanism of gene regulation

Previously a ncRNA, termed *SER3* Regulatory Gene 1 (*SRG1)*, was identified which initiates within the intergenic region 5' of the *SER3* gene and is transcribed across the promoter of the adjacent *SER3* gene (MARTENS *et al.* 2004). Our results have indicated that transcription of *SRG1* interferes with *SER3* expression by controlling nucleosome occupancy of the *SER3* promoter (Figure 64). In the absence of serine, *SRG1* is weakly transcribed leaving the *SER3* promoter depleted of nucleosomes. In response to serine, the Swi/Snf chromatin remodeling complex and SAGA histone acetyltransferase (HAT) are recruited to the *SRG1* promoter by the sequence specific Cha4 activator (MARTENS *et al.* 2005) where they function together to induce *SRG1* transcription. As a consequence of *SRG1* transcription, nucleosomes are assembled and continuously maintained over the *SER3* promoter that repress *SER3* expression by preventing transcription factors from binding to the *SER3* promoter. We found that both FACT and Spt6 histone chaperones are required to promote *SRG1* transcription dependent nucleosome assembly over the *SER3* promoter. Therefore, we have found that repression of *SER3* by intergenic *SRG1* transcription occurs through a novel mechanism, which can be used to study the dynamics of gene regulation.

6.1.2 Identification of unique Spt16 amino acids which are required for maintenance of Spt16 and histone occupancy over highly transcribed regions of the genome

Our finding that Spt16 controls *SER3* repression as a consequence of transcription across the promoter of this gene provided a unique model system to study nucleosome disassembly/reassembly during transcription and, more specifically, to genetically identify

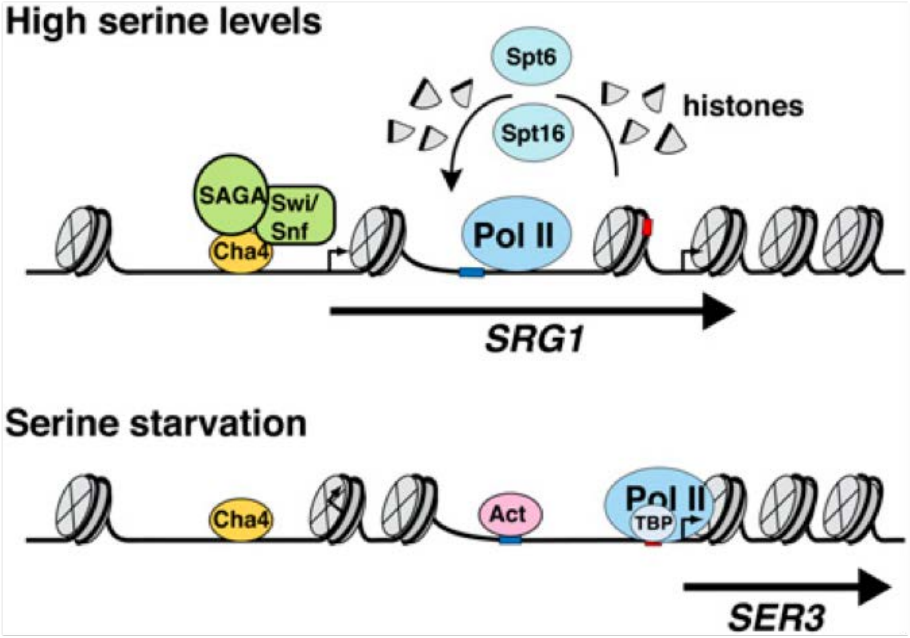

Figure 64. A model for *SER3* regulation by *SRG1* intergenic transcription.

When serine is available to cells, DNA-bound Cha4 recruits SAGA and Swi/Snf to initiate *SRG1* transcription, possibly by remodeling the two nucleosomes located at the 5' end of *SRG1* to expose the *SRG1* transcription start site. RNA pol II transcribes *SRG1* and, through Spt6 and Spt16, disassembles nucleosomes in its path and then reassembles them in its wake. As a result, nucleosomes continuously occupy the *SER3* UAS where they repress *SER3* by occluding the *SER3* promoter from transcription factor binding. In the absence of serine, *SRG1* transcription is repressed, possibly due to the presence of two nucleosomes at its 5' end that encompass its transcription start site. In the absence of *SRG1* transcription, the *SER3* UAS is depleted of nucleosomes, allowing an as yet unknown activator (Act) and/or TBP and RNA pol II to bind and activate *SER3* transcription. From (HAINER *et al.* 2011).

functional domains of Spt16 that may be critical for its role in transcription elongation. We identified 24 *spt16* mutant alleles that strongly derepress *SER3* by using a reporter gene to monitor *SER3* expression. Analysis of a subset of these mutants showed that they derepress *SER3* without reducing intergenic transcription or Spt16 protein levels, suggesting a direct effect on Spt16's ability to repress *SER3*. Phenotypic analyses indicated that these mutants are distinct from previously described *spt16* mutants and suggest that this new class of mutants are specific to Spt16's function during transcription rather than being required for replication/DNA repair and cell viability. Analysis of a subset of these mutants revealed a defect in intergenic transcription-dependent nucleosome assembly across the *SER3* promoter. Analysis examining nucleosome occupancy across selected protein-coding regions indicated that these mutants more broadly reduce transcription-coupled nucleosome assembly at highly transcribed genes. Finally, I found that association of these mutant Spt16 proteins with highly transcribed regions of the yeast genome were strongly reduced. The identification of individual amino acids that are specifically required for transcription dynamics of Spt16, rather than altering its contribution to other cellular processes, has provided a source of molecular tools to examine the contribution of Spt16 to transcription. Taken together, these results indicate that transcription-coupled nucleosome assembly at highly transcribed regions is dependent on the integrity of the Spt16-D and Spt16-M domains, likely by facilitating the association of Spt16 to these regions.

6.1.3 Identification of novel histone residue substitutions which are required for Spt6 and Spt16 interaction and function

Using a similar reporter system for *SER3*, I have also identified a unique collection of histone H3 and H4 mutants that regulate *SER3*. To investigate the role of histones in *SER3* regulation, I

screened a comprehensive library of histone H3 and H4 mutants for those that derepress *SER3*. I identified mutations altering eight histone residues (H3 V46, R49, V117, Q120, K122 and H4 R36, I46, S47) that strongly increase *SER3* expression without reducing transcription of the intergenic *SRG1* ncDNA. I detected reduced nucleosome occupancy across *SRG1* in these mutants to degrees that correlate well with the level of *SER3* derepression. Histone ChIP experiments at several other genes suggest that the loss of nucleosomes in these mutants may be specific to highly transcribed regions. Interestingly, two of these histone mutants, H3 R49A and H3 V46A, reduce Set2-dependent methylation of lysine 36 of histone H3 and allow transcription initiation from cryptic intragenic promoters. My analyses suggest that these histone residues are required to orchestrate transcription-coupled nucleosome assembly specifically at highly transcribed genes by providing a binding site for histone chaperones Spt6 and Spt16 to promote nucleosome reassembly.

6.2 FUTURE DIRECTIONS

While my thesis work has provided significant insight into the role of specific histone residues and histone chaperones in regulating transcription-coupled nucleosome dynamics, there are still many important questions that remain. For example, our model on *SER3* regulation demonstrates a number of factors that are involved in this process, however the exact molecular mechanisms by which these factors regulate chromatin dynamics is unknown. Which factors are initially recruited to the locus? Are the nucleosomes at the 5' end of *SRG1* source nucleosomes for those which occupy the *SER3*pr after *SRG1* induction? What is the rate of histone exchange occurring over the locus? What are the dynamics that occur between the identified factors to result in

nucleosome reassembly during *SRG1* transcription? I have been developing a method based on an inducible *SRG1* system to answer these questions (see Appendix E). Using this system, the above questions will be easily answered, leading to a deeper understanding of the regulation of *SER3* by *SRG1* transcription, and providing the field with novel insight into the dynamics which occur between many of this essential factors.

My work on the histone chaperone Spt16, described in Chapter 3, has lead to many interesting avenues of research as well. We have uncovered a set of unique amino acids which are responsible for maintaining Spt16 occupancy at highly transcribed regions, and therefore when they are mutated not only is Spt16 occupancy decreased, but also histone occupancy is decreased as a result. Therefore, initial analysis should focus on confirming this model by testing the effect these mutants have on the direct interaction between Spt16 and histone H3 through co-immunoprecipitation assays as well as *in vitro* binding assays. Additionally, the effect of these amino acid substitutions on FACT processes such as histone chaperone activities should be examined by performing analyses similar assays to those that have been previously described (XIN *et al.* 2009). While an defect between Spt16 and Pob3 interaction may expect to alter all processes FACT is involved in, and therefore likely is not the effect occurring in these mutants, it is important to test whether these *spt16* mutants alter Pob3 interactions. As additional controls for these residue substitutions, it is important to perform further analyses testing their effect on replication and DNA damage repair assays.

The effect of one dominant mutant in particular, *spt16-E857K*, would be interesting to analyze further. First, genome wide expression and nucleosome occupancy experiments would reveal whether this mutant (or any of the other *spt16* mutants) has a general defect, and what the overall impact this mutant has on transcription-coupled nucleosome occupancy. Furthermore,

this dominant mutant may represent a gain-of-function mutation whereby this form of Spt16 interacts with a target protein responsible for Spt16's association with DNA more tightly than wild-type Spt16, thus sequestering this protein away from transcribed DNA. Therefore, by performing affinity purification to identify proteins having altered affinity to the Spt16 mutant as compared to wild-type Spt16, the required protein(s) may be identified. Using this amino acid substitution in genetic analyses may also provide interesting insight into Spt16 function. For example, a genetic suppressor screen can be used as an unbiased method to identify proteins that physically or functionally interact with *spt16-E857K*, leading to mechanistic insight into this mutant.

Through the analysis described in Chapters 3 and 4, I identified intriguing phenotypic overlap between the *spt16* mutants and histone mutants. Together this work supports my further analysis on the histone mutants which demonstrated a region on the nucleosome (the dyad), and specific residues within this region, which are required for the maintenance and/or recruitment of Spt16. Due to the similar results, the data presented in Chapter 3 may indicate the specific region of Spt16 required for this interaction with nucleosomes. It will be interesting to examine what relationship these two collections of mutants have with each other.

Work discussed in Chapters 4 and 5 has lead to an exciting new avenue of research for the Martens' lab. I have discovered a novel class of histone mutants which leads to decreased occupancy of histone chaperones resulting in decreased reassembly of histone proteins and therefore reduced nucleosome occupancy over highly transcribed regions of the genome. Defining the precise molecular defect that these histone mutants promote is essential to understanding transcription-coupled nucleosome dynamics in general. My preliminary analysis on the effect of H3 K122A on histone reassembly and disassembly are the beginning of this

analysis. By utilizing this assay, or by using a novel *SRG1* induction system (see Appendix E), the exact effect of these histone mutants on transcription-coupled nucleosome reassembly can be revealed. Another testable hypothesis which my model has lead to, is what effect these histone mutants have on the histone chaperone activities of FACT and Spt6. By performing previously described Spt6 (BORTVIN and WINSTON 1996) and FACT (XIN *et al.* 2009) activity assays, it can be determined what the effect these histone mutants have on Spt6 or FACT ability to reassemble nucleosomes.

Another lingering question is how general is the repression mechanism that operates at *SER3*? Transcription over promoters is widespread, but how much of this is regulatory? Using my collection of histone mutants, genome-wide analysis can determine how transcription-coupled nucleosome assembly shapes overall chromatin and gene expression. I propose that these histone mutants will specifically reduce nucleosome occupancy over highly transcribed regions of the genome and, in cases where this transcription activity overlaps gene regulatory sequences, associated changes in transcription of those genes will be observed. These experiments will allow the construction of a comprehensive view of how these histone mutants alter chromatin to impact gene expression and ultimately lead to the identification of new cases of ncDNA transcription that control chromatin to regulate gene expression.

Cumulatively, my thesis work has contributed in a number of ways to the study of transcription-coupled nucleosome dynamics. First, we have revealed a novel mechanism in gene regulation in which a ncRNA promotes the maintenance of nucleosome occupancy to repress an adjacent coding gene. Second, we have uncovered a number of unique amino acid substitutions in Spt16 which are required for the maintenance of Spt16 occupancy over highly transcribed regions of the genome, and as a result, maintaining histone occupancy. Finally, I have identified

a set of novel histone H3/H4 residue substitutions which are defective in maintaining both Spt6 and Spt16 histone chaperone occupancy over highly transcribed regions of the genome, and therefore result in slowed reassembly of nucleosomes inevitably leading to reduced nucleosome occupancy. Beyond my progress, many interesting questions remain. Therefore, my work will serve as a foundation for future studies in the chromatin/transcription field.

APPENDIX A

ROLE OF H2B MONOUBIQUITLYATION PATHWAY IN *SER3* REGULATION

The work discussed in this Appendix has been adapted from my contribution to a published article (PRUNESKI *et al.* 2011) and is reprinted, with alterations, by permission from the American Society for Microbiology, copyright 2011.

Based on a genetic screen performed in the lab, we were interested in determining the role of the Paf1 complex in regulating *SER3* expression (PRUNESKI 2011). The Paf1 complex is a conserved, multi-subunit complex that plays a number of important roles in the transcription cycle (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). In higher eukaryotes, the Paf1 complex has important roles in embryonic development (AKANUMA *et al.* 2007; TENNEY *et al.* 2006), maintenance of stem cell fate (DING *et al.* 2009), and tumorigenesis (CHAUDHARY *et al.* 2007; LIN *et al.* 2008; MONIAUX *et al.* 2006). In *S. cerevisiae*, the Paf1 complex is comprised of five subunits, Paf1, Ctr9, Rtf1, Cdc73, and Leo1 (KROGAN *et al.* 2002; MUELLER and JAEHNING 2002; SHI *et al.* 1997; SQUAZZO *et al.* 2002) that co-localize with RNA pol II across transcribed genes exiting near the polyadenylation sites (KROGAN *et al.* 2002; MAYER *et al.* 2010; POKHOLOK *et al.* 2002).

One of the primary functions of the Paf1 complex is to promote histone modifications associated with active transcription (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). In yeast, the Paf1 complex promotes monoubiquitylation of histone H2B at lysine 123 (K123ub) by the ubiquitin conjugase Rad6 and ubiquitin ligase Bre1 (NG *et al.* 2003; WOOD *et al.* 2003). Ubiquitylation of H2B is required for subsequent methylation of histone H3 K4me and K79me by the Set1 and Dot1 methyltransferases, respectively (KROGAN *et al.* 2003; NG *et al.* 2003; NG *et al.* 2002; SHAHBAZIAN *et al.* 2005; SUN and ALLIS 2002; WOOD *et al.* 2003). These modifications are predominantly dependent on the Rtf1 subunit of the Paf1 complex, specifically involving a central region of the protein termed HMD for histone modification domain (PIRO *et al.* 2012b; TOMSON *et al.* 2011; WARNER *et al.* 2007). Furthermore, the Paf1 and Ctr9 subunits are required for trimethylation of histone H3 K36me3 by the Set2 methyltransferase (CHU *et al.* 2007; NG *et al.* 2002; SHAHBAZIAN *et al.* 2005; WOOD *et al.* 2003). Together, these modifications control histone acetylation across transcribed genes through the recruitment of histone deacetylases (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005; KIM and BURATOWSKI 2009; PINSKAYA *et al.* 2009; YOUDELL *et al.* 2008). Previously, I have shown by Northern analysis that either the deletion of the methyltransferases responsible for methylation of K4, K36, and K79 of histone H3 (discussed in Chapter 2) (HAINER *et al.* 2011) or the mutation of these lysine residues to alanines has little to no effect on *SER3* repression (discussed in Chapter 4) (HAINER and MARTENS 2011a). Despite there being no role for the downstream methylation marks, it is possible that the upstream H2B K123ub does regulate *SER3* repression. Therefore, I assayed the effect of histone H2B K123ub on *SER3* repression. Northern analyses revealed only modest increases in *SER3* expression in *rad6*Δ (two-fold) and *bre1*Δ (1.5-fold) mutants (Figure 65). Similarly, a conservative mutation that replaces H2B lysine 123 with

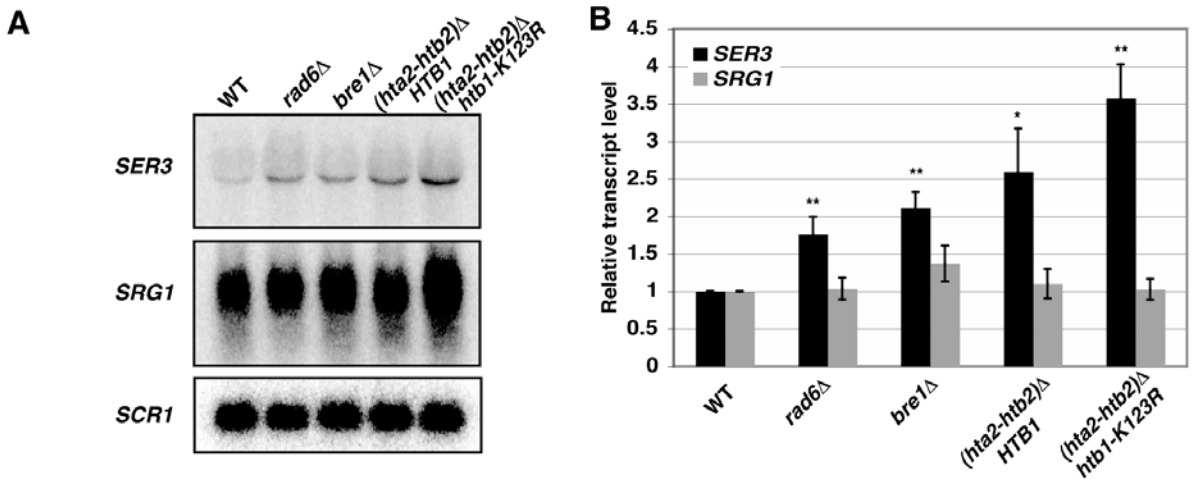

Figure 65. Role of H2B ubiquitylation pathway on *SER3* regulation.

A) Representative Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) RNA levels in wild-type (FY5) *rad6*Δ (KY1712), *bre1*Δ (KY1713), (*hta2-htb2*)Δ (KY2172), and (*hta2-htb2*)Δ *htb1-K123R* (KY2167) strains grown in YPD at 30°C. B) Quantitation of results from a minimum of three biological replicates. The values shown are the mean *SER3* (black) and *SRG1* (gray) transcript levels that have been normalized to the *SCR1* loading control and made relative to the wild-type strains. Error bars indicate SEM and asterisks indicate statistical significance compared to wild type (* $P < 0.05$, ** $P < 0.01$).

arginine also results in less than a two-fold increase in *SER3* levels when compared to the relevant control strain lacking one copy of the histone H2A and histone H2B genes (compare (*hta2-htb2*)Δ strains expressing *HTB1* or *htb1-K123R*). These results are consistent with the minor defect in *SER3* repression that was observed for cells lacking Rtf1, which has been previously shown to be the subunit primarily required for this modification (NG *et al.* 2003; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003). Taken together, *SER3* regulation seems to be largely independent of the H2B monoubiquitylation pathway.

Table 8. *Saccharomyces cerevisiae* strains used in Appendix A.

Strain	Genotype	Source
FY5	*MAT*α	(WINSTON *et al.* 1995)
KY1712	*MAT*α *rad6*Δ*::KanMX*	K. Arndt
KY1713	*MAT***a** *bre1*Δ*::KanMX*	K. Arndt
KY2167	*MAT*α *HTA1-htb1*$_{K123R}$ (*hta2-htb2*)Δ*::KanMX ura3*Δ*0*	K. Arndt

APPENDIX B

SCREEN FOR HISTONE H2A AND H2B RESIDUES REQUIRED FOR *SER3* REGULATION

Recent chromatin studies link nucleosomal assembly, disassembly, and histone dynamics to the control of transcription (reviewed in (LI *et al.* 2007a)). At gene promoters, histone dynamics modulate histone marks that play a critical role during activation or repression of genes (KREBS 2007). To uncover how nucleosomal assembly, disassembly, specific histone modifications and interacting regulators are important for regulation of *SER3* gene expression and to define the functional and physiological relevance histone residues play at the *SER3* locus, I screened a comprehensive library of histone mutants where every residue has been systematically mutated to an alanine (NAKANISHI *et al.* 2008). In order to perform this screen, I created a query strain which contains a *SER3*pr-LacZ reporter gene where the *SER3* coding sequence is replaced by the *E.coli lacZ* coding sequence, as described in Chapter 4 (see Figure 27) (HAINER and MARTENS 2011a).

From the Shilatifard lab (Stowers Institute), we obtained a scanning histone mutagenesis with alanine library (SHIMA) of histone H2A and H2B where every non-alanine residue has been systematically mutated to alanine (NAKANISHI *et al.* 2008). Each individual residue

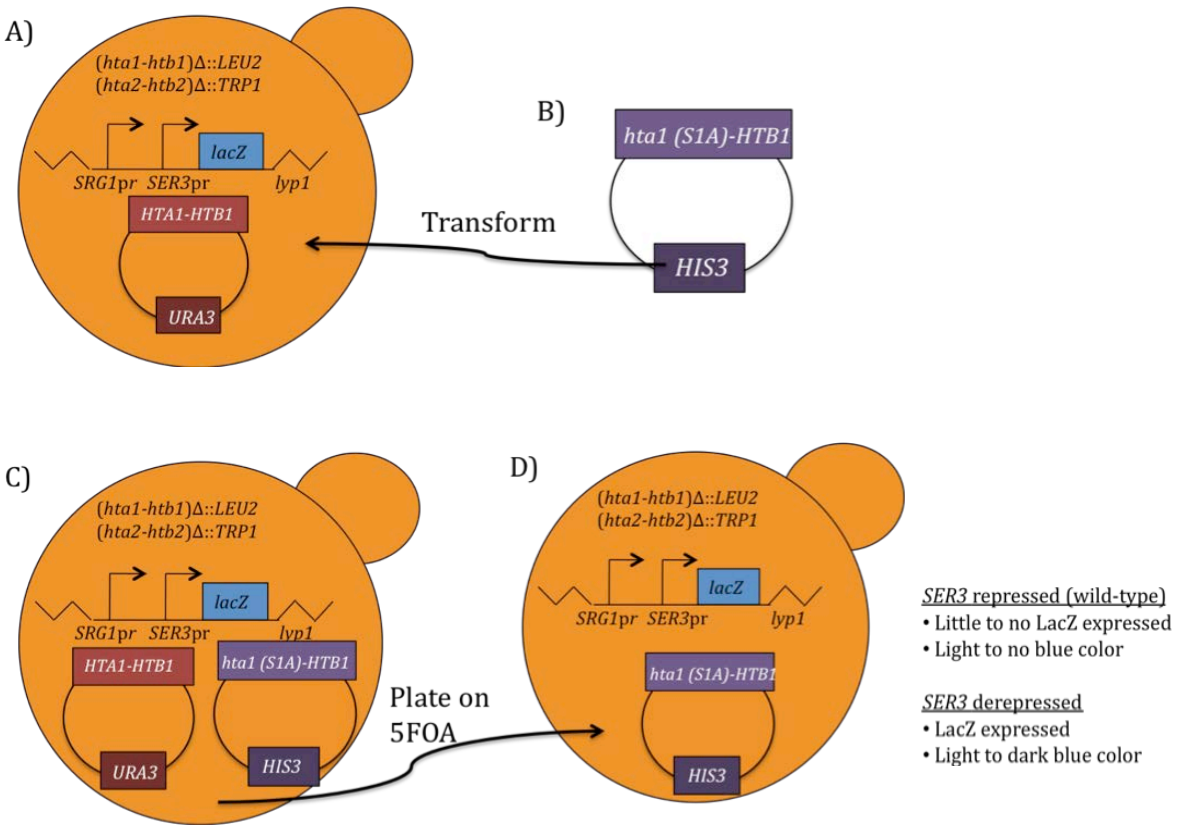

Figure 66. Screen of SHIMA H2A/H2B histone library.

A) Diagram of the *SER3*pr->LacZ query strain (YS093) created for histone library screens, where the *SER3* coding sequence is replaced by the *E.coli lacZ* coding sequence and integrated at the *LYP1* locus. B) Example of one of the 222 histone mutant plasmids kindly provided by the Shilatifard lab (Stowers Institute). In each plasmid there is a single residue substitution to alanine, comprehensively scanning across all non-alanine residues of the H2A/H2B histones. Here the first residue of the *HTA1* gene has been mutated from a serine to an alanine. All the plasmids have a *HIS3* marker for selection. Each plasmid was individually transformed into the query strain, and a plasmid shuffle was performed. C) After transforming the *HIS3*-marked substitution plasmid into my query strain, which contains a wild-type copy of histones H2A/H2B marked by the *URA3* gene, two plasmids were in the cells. In order to select for the loss of the wild-type plasmid, strains were plated on media containing 5-FOA (the *URA3* gene converts 5-FOA into a toxic byproduct, so cells containing the *URA3*-marked plasmid will be

inviable). Any colonies which did not retain either plasmid were inviable, as one copy of each histone gene is required for viability. D) Diagram of the resulting strain from our screen. Using a LacZ overlay assay I measured the effects these mutants had on the *SER3*pr->LacZ reporter. In a wild-type strain where *SER3* is repressed, LacZ expression is minimal so little blue color is produced. If *SER3* was derepressed by a histone mutation the cells turned blue and the intensity of blue varied depending on the amount of derepression: as the amount of derpression increased, LacZ cleaved more X-gal, more blue pigment will be produced, and the colony turned a darker blue color.

substitution is expressed on a plasmid, allowing for a plasmid shuffle screen (BOEKE *et al.* 1987). In the LacZ reporter for this screen (YS093), the H2A/H2B histone genes (*HTA1-HTB1, HTA2-HTB2*) have been knocked-out. Because histones are essential, the strain is covered by a *URA3* marked plasmid containing copy one of each histone gene (*HTA1-HTB1*). To screen the SHIMA library, I first transformed each histone substitution plasmid into our reporter strain, then selected for the removal of the *URA3* marked wild-type histone plasmid on plates containing 5-Fluoroorotic acid (5-FOA) which the *URA3* gene product converts to a toxic byproduct (Figure 66). Three 5-FOA resistant colonies from each transformation were patched onto YPD plates and the read-out of our reporter was a change in the intensity of blue color, from light to dark, as measured through a LacZ overlay assay over time (DUTTWEILER 1996).

The overlay assay, as described in Chapter 4, was adapted from a previously published strategy (DUTTWEILER 1996). This overlay assay was performed on the 222 strains transformed individually with each plasmid in triplicate and a representative plate is shown in Figure 67. Color change was recorded and the faster the cells turned blue, the greater expression of *SER3* would be assumed. 52 candidates were identified through this screen as derepressing *SER3* over wild-type expression levels (Table 9).

Strains identified to express higher levels of β-galactosidase compared to wild-type by the overlay assay were subjected to standard quantitation by liquid β-galactosidase assays. Displayed is a representative of some of the top and some of the lower candidates (Figure 68). The mutants ranged from two-fold to five-fold higher levels of β-galactosidase compared to wild-type, with an *snf2*Δ mutant having twelve-fold higher levels. These results indicate that my screen was successful at identifying mutations in histones H2A and H2B that altered the expression of *SER3* and helped to prioritize the mutants as to which to examine further.

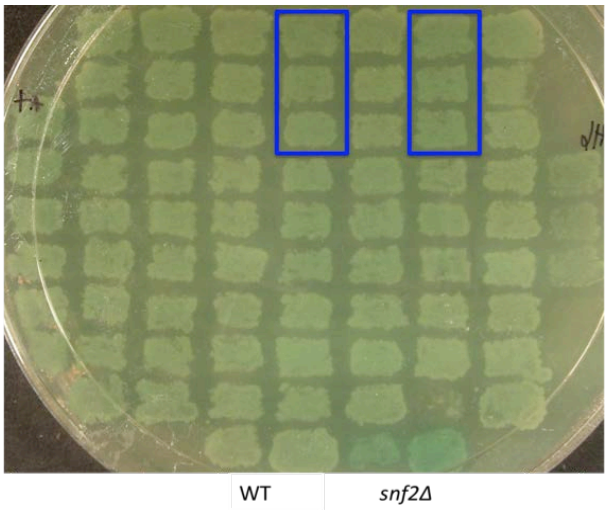

Figure 67. Overlay assay of a subset of H2A/H2B mutant candidates.

Shown is an example plate of strains taken through the plasmid screen (described in Figure 66). Each mutant plasmid transformation was plated in triplicate to prevent identification of any false positives/negatives. The overlay assay was adapted from Duttweiler *et al.* (DUTTWEILER 1996). In short, the cells were permeabilized by chloroform and then overlayed with a warm agarose containing 1% low melting agarose, .1M $NaHPO_4$ buffer and .25mg/mL X-Gal. Color change was observed by eye as the agarose hardened. On each plate WT and *snf2*Δ controls were included (bottom) and highlighted are two positive results of mutations derepressing *SER3*.

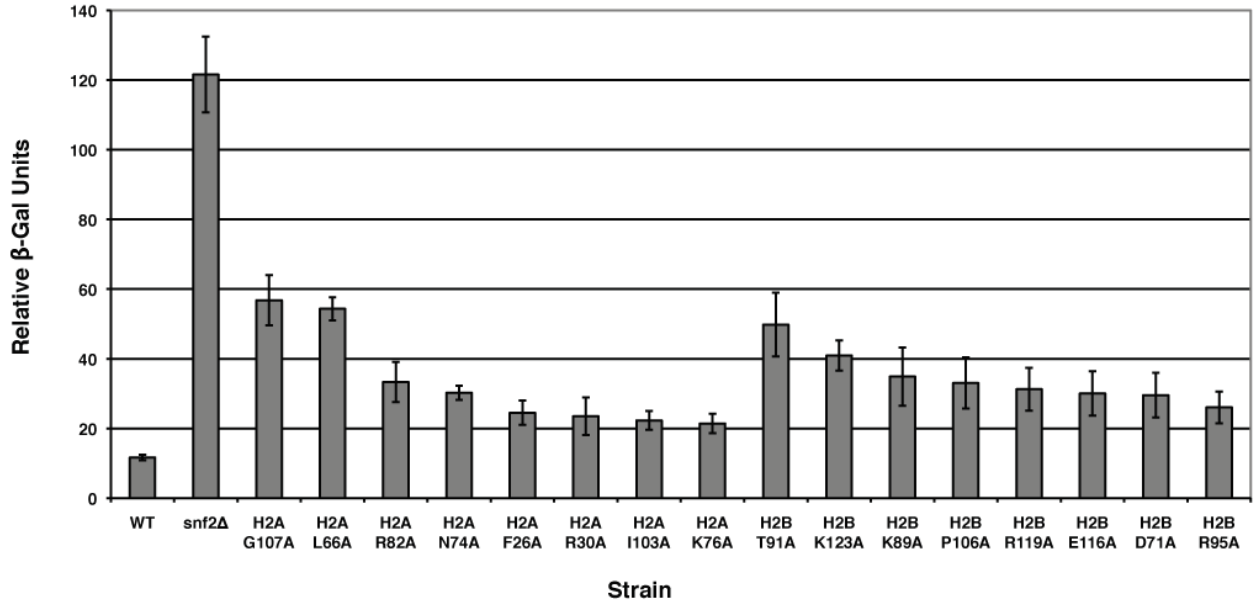

Figure 68. β-galactosidase assays for evaluating LacZ expression.

Due to the large number of candidates pulled out of the screen, I used β-galactosidase liquid assays on each candidate to quantitate the amount of LacZ expression. In short, the assay is performed by crudely isolating protein, adding this protein to a Z buffer and initiating the reaction by adding *ortho*-Nitrophenyl-β-galactoside (ONPG), a colorimetric and spectrophotometric substrate for detection of β-galactosidase activity. As the reaction proceeds a yellow color is reached and the reaction is stopped by adding Na_2CO_3. The absorbance is read with the amount of β-galactosidase units determined based on the absorbance over the time and volume during the reaction. Displayed is a representative of some of the top and some of the lower candidates. All of the mutants had at least a two-fold LacZ levels higher than wild-type, but even the strongest candidates were two-fold lower than *snf2*Δ strains. This is not too surprising since *snf2*Δ strains are one of the strongest mutants in derepressing *SER3* we have found, and also these histone mutants are all found on plasmids.

In order to confirm the results seen in β-galactosidase liquid assay for endogenous *SER3*, I performed Northern analysis on a number of the top and bottom candidates (Figure 69). This Northern shows that some mutants identified from the screen alter the expression of endogenous *SER3*. The levels of *SRG1* shown in this blot are two-fold higher than normal *SRG1* levels because the strains contain two copies of *SRG1*: the endogenous copy and the copy at the *LYP1* locus. For a number of the mutations, *SRG1* mRNA levels seem to be lower than wild-type, indicating the reason there may be some derepression of *SER3* is because of the loss of *SRG1* transcription. In order to examine the role of these histone residues further, however, the mutations should be integrated. A major reason I want to integrate these mutations is that there is evidence for the effects of the histone mutations to be masked when they are carried on a plasmid. For instance, I have used an H2B K123R integrated strain and found a seven-fold increase in *SER3* expression levels, whereas the H2B K123A plasmid increases *SER3* expression levels by only two-fold.

One additional test I performed, in collaboration with M. Shirra from the Arndt lab, was examining some plate phenotypes of the entire SHIMA library in the LacZ reporter strain, in order to further characterize the histone mutants. Phenotype sensitivity or growth tests included: mycophenolic acid (MPA), hydroxyurea (HU), caffeine, raffinose, galactose, sodium chloride, temperature, or cold sensitivity (Table 9). Although the results of this library screen are interesting, I decided to focus on the results of my H3/H4 histone screen for my thesis work (Chapter 4). Upon integration of these mutants, we may be able to determine a greater role for H2A/H2B in *SER3* regulation and transcription coupled nucleosome dynamics.

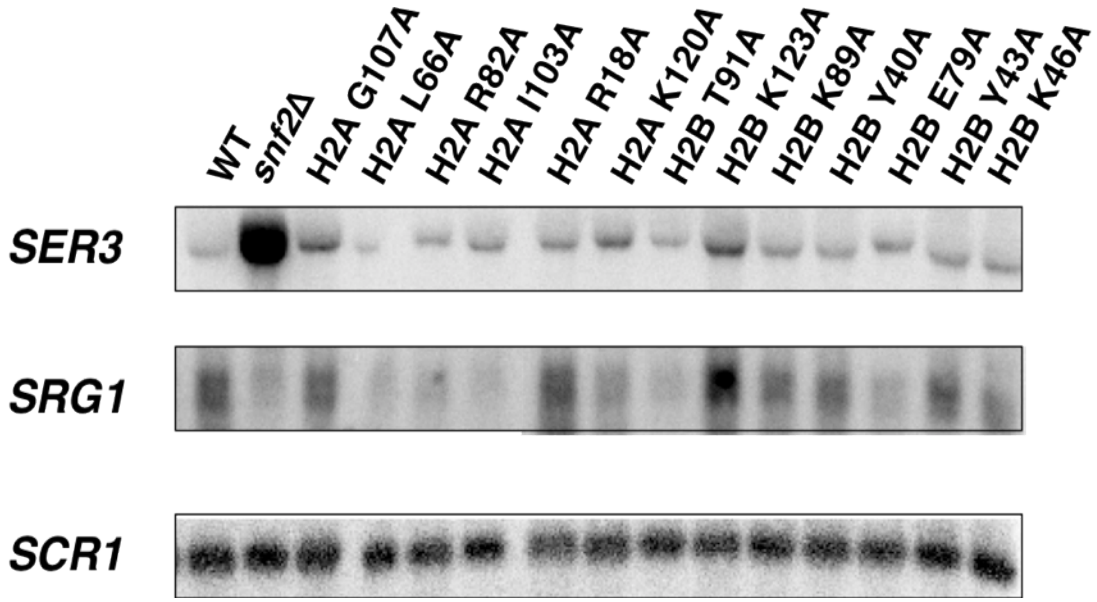

Figure 69. Effect of H2A/H2B mutations on endogenous *SER3* expression.

Northern analysis was performed to explore the effect H2A/H2B mutants had on endogenous *SER3*. The *SRG1* panel is misleading as these strains contain both the endogenous *SRG1* and the *SRG1* produced at the *LYP1* locus. *SCR1* serves as a loading control. RNA was isolated from cells grown to mid-log in YPD at 30°C.

Table 9. Summary of histone H2A/H2B screen data

Substitution	Time Overlay	B-gal Units	Phenotypes
T91A	8	50 +/- 13	
K123A	7	41+/- 4	caff, HU, MPA, raf, gal, NaCl
K89A	19	35+/- 12	
P106A	8	33+/- 7	
R119A	8	31 +/- 6	
E116A	25	30+/- 6	caff
D71A	8	30 +/- 6	
Y124A	13	28+/- 1	
R95A	12	26+/- 4	
G107A	20	26 +/- 1	caff, MPA
Y40A	30	24+/- 4	raf
F73A	15	24+/- .3	
E79A	9	23+/- 3	
T118A	13	23+/- 2	
T55A	15	23+/- 2	
S67A	13	22+/- 4	
R75A	13	21+/- 4	
H112A	20	21+/- 1	
V47A	15	20+/- 1	
F68A	25	19+/- 2	
L105A	25	19+/- 2	
R102A	12	19+/- 1	
S61A	25	18+/- 3	
Y43A	7	18 +/- 2	caff
Q50A	15	18 +/- 1	
K46A	11	17 +/- .5	

Table 10. *Saccharomyces cerevisiae* strains used in Appendix B.

Strain	Genotype	Source
YS093	*MAT***a** *ura3*Δ*0*, *leu2*Δ*0*, *his3*Δ*200*, *trp1*Δ*63*, *lyp1*::*SRG1*pr-lacZ, (*hta1-htb1*) Δ::*LEU2*, (*hta2-htb2*) Δ::*TRP1* pSAB6=*HTA1 HTB1 URA3* CEN	This study
YS107	*MAT***a** *ura3*Δ*0*, *his3*Δ*200*, *snf2*Δ::KanMX, lyp1::*SRG1*pr-LacZ	This study

APPENDIX C

DETERMINING WHETHER H3 K122 IS MODIFIED IN YEAST

As described in Chapter 4, I identified the amino acid K122 on histone H3 as being required for regulating *SER3* through maintenance of nucleosomes over the *SER3* promoter. Interestingly, histone H3 K122, a highly conserved residue, has been shown to be post-translationally modified in metazons, where it is methylated in mice and acetylated in humans (PETERS *et al.* 2003; SU *et al.* 2007; ZHANG *et al.* 2002). It is possible this residue is modified in yeast, and the modification is highly dynamic and therefore unable to be detected through previously performed genome-wide mass spectrometry experiments. To begin examining whether K122 is modified in yeast, I utilized an available antibody, specific to K122 acetylation, which was created against a human peptide sequence (Abcam). This initial work focused on acetylation only because the antibody was available, but further studies may consider including methylation specific antibodies. Western analysis on crude extracts of bulk histones support the dynamic nature of this modification, as I was unable to identify K122 acetylation through this procedure (Figure 70). While this blot may appear to show decreased K122-acetylation in the K122A and K122Q strains, I believe those strains are underloaded, and also, when repeated, I did not obtain the same results.

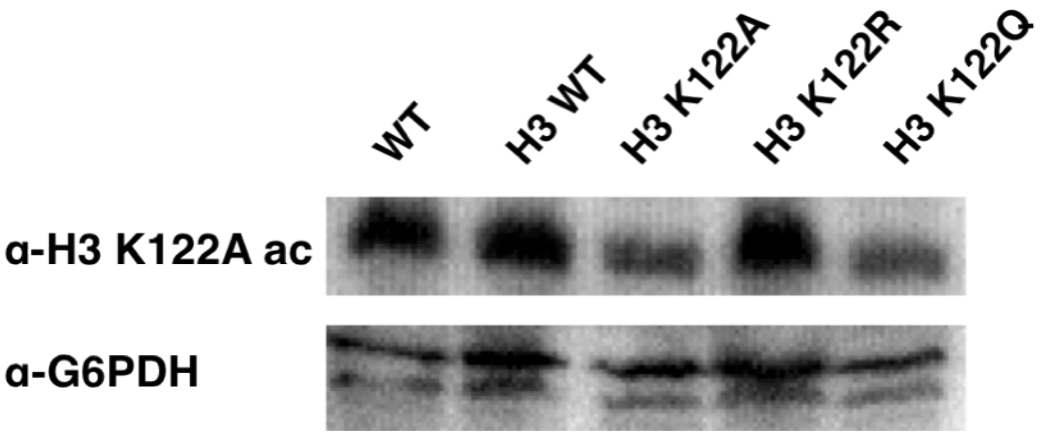

Figure 70. K122 global acetylation levels in wild-type vs mutant strains.

Whole cell extracts were prepared from *(hht1-hhf1)*Δ strains expressing either synthetic wild-type copies of histone H3 and H4, *hhts-K122A*, *hhts-K122R*, or *hhts-K122Q* that were grown in YPD at 30°C. Immunoblots of WCEs were probed with anti-acetylated K122 (Abcam – made to human peptide). Immunoblot of G6PDH is provided as a loading control.

One possible reason the global levels were not conclusive may be that this mark is transient and not found all over the genome. Therefore, I performed ChIP assays using total H3 and the modification specific antibody over the *SER3* locus, as it is possible that the modification state of K122 is responsible for the regulation of *SER3*, and by crosslinking the cells, I may be able to more readily identify the modification (Figure 71). These ChIP analyses did not reveal a decrease in H3 K122 acetylation in the K122A strain, as I would have predicted to see if K122 was acetylated. Together these data indicate that I am unable to detect acetylation of K122 in rich media conditions.

While I was unable to detect an effect on K122 acetylation in rich media, I hypothesized that the modification may occur in different growth conditions. To test this, I grew cells in rich media and shifted into minimal media for 30 minutes or two hours, and performed Northern analysis (Figure 72A) examining the effect of wild-type versus K122A strains on *SRG1* and *SER3* expression levels, Western analysis (Figure 72B) to examine the effect of wild-type and K122A strains on global total H3 and H3 K122 acetylation levels, and ChIP analysis (Figure 73) to examine the effect of wild-type and K122A strains on crosslinked chromatin, specifically at the *SRG1*/*SER3* locus. Together, these experiments were unable to reveal any defect in K122 acetylation in K122A compared to wild-type, indicating that I was unable to reveal a modification on K122 in these conditions.

As mentioned above, I choose to examine K122 acetylation only because an antibody was available for this analysis. However, K122 has been shown to be both acetylated and methylated in higher organisms (PETERS *et al.* 2003; SU *et al.* 2007; ZHANG *et al.* 2002). Therefore, to continue this analysis, I obtained strains which deleted various histone modifying

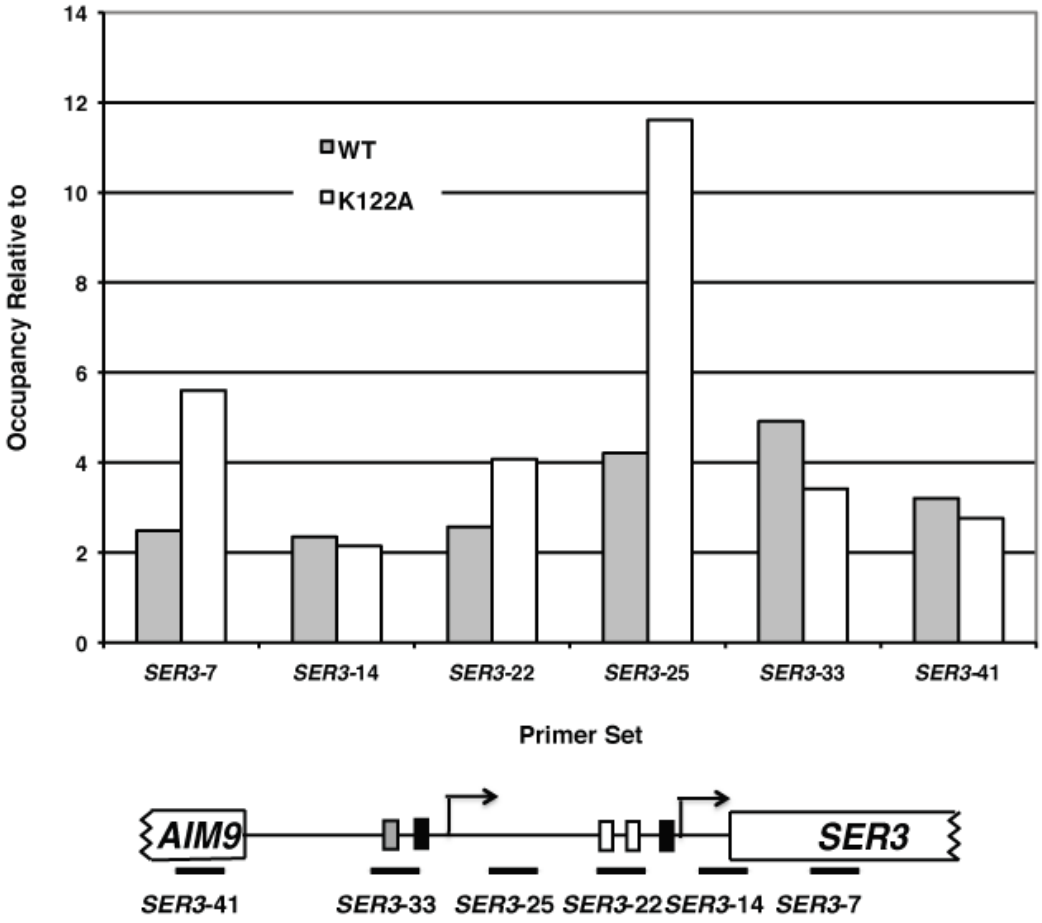

Figure 71. K122 acetylation levels in wild-type vs mutant strains over *SRG1*/*SER3*.

H3 and K122-acetyl ChIPs were performed on chromatin isolated from strains expressing either synthetic wild-type copies of histone H3 and H4 or *hhts-K122A* grown in YPD at 30°C to a density of ~2 × 10^7. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and signal for K122-acetyl was made relative to total histone H3. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

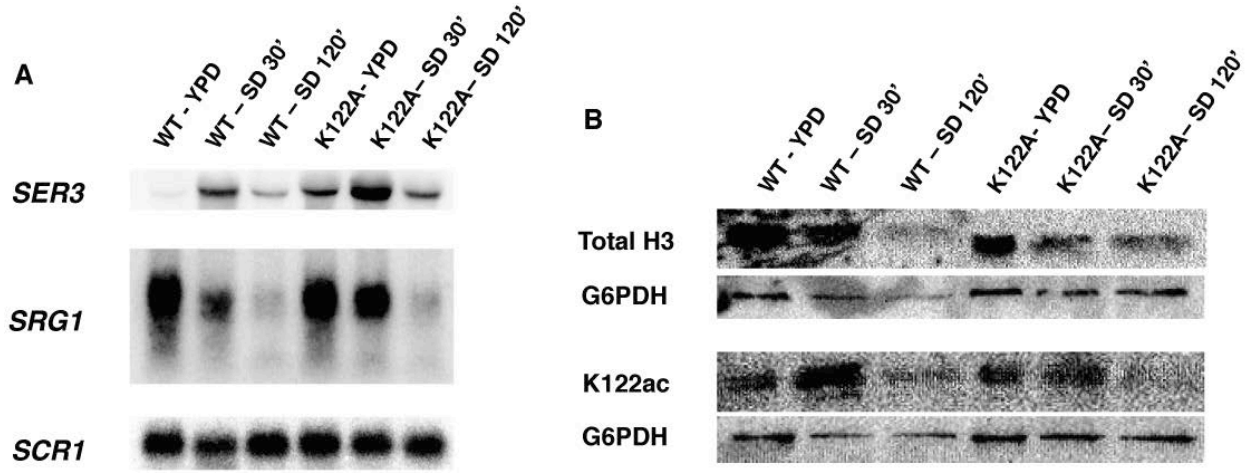

Figure 72. Global K122 acetylation levels in minimal media.

A) Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from derivatives of JDY86 expressing either synthetic, wild-type copies of histone H3 and H4 (*HHTS/HHFS)* or mutant *hhts-K122A* that were grown to a density of 1-2 × 10^7 cells/ml in YPD or SD at 30°C. B) Whole cell extracts were prepared from *(hht1-hhf1)*Δ strains expressing either synthetic wild-type copies of histone H3 and H4 or *hhts-K122A* that were grown in YPD or SD at 30°C. Immunoblots of WCEs were probed with either total H3 or anti-acetylated K122. Immunoblot of G6PDH is provided as a loading control.

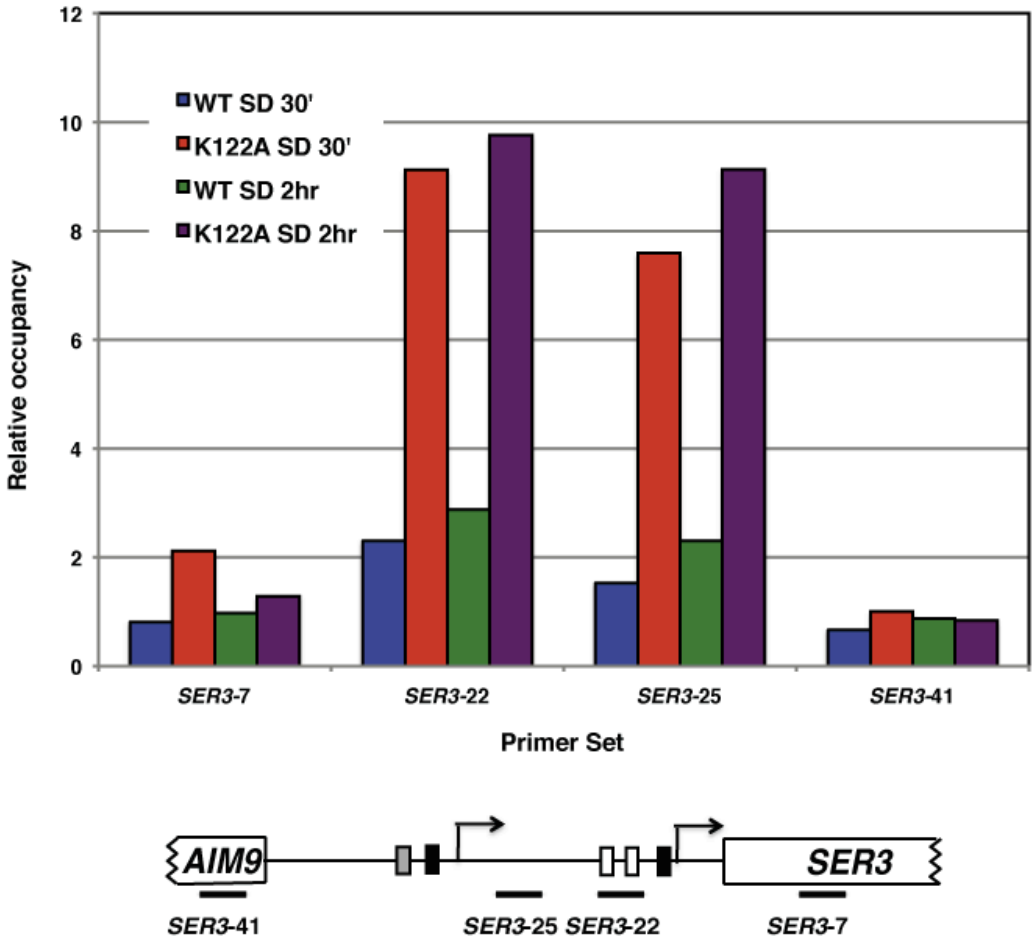

Figure 73. K122 acetylation levels in wild-type vs mutant strains over *SRG1*/*SER3* in minimal media.

H3 and K122-acetyl ChIPs were performed on chromatin isolated from strains expressing either synthetic wild-type copies of histone H3 and H4 or *hhts-K122A* grown in YPD at 30°C to a density of ~1 × 10^7 and shifted to SD for either 30 min or 2 hours. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and signal for K122-acetyl was made relative to total histone H3. Below the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR.

enzymes and performed Northern analysis to examine the effect of these enzymes on *SRG1*/*SER3* expression. I hypothesized that if K122 is modified, the enzyme required for this modification event would be important for regulating *SER3* expression levels. While I was able to confirm minor effects seen in strains lacking *RTT109* (PRUNESKI 2011), none of the deletion strains revealed an upregulation in *SER3* similar to what is seen in a K122A mutant (Figure 74). Deletion of *SAS3* did show a slight upregulation of *SER3*, indicating a possible role for this histone acetyltransferase in regulation *SER3* expression. However, the levels are *SRG1* are decreased in the *sas3*Δ strain, suggesting the reason *SER3* is slightly increased is due to decreased *SRG1* transcription. While the enzymes examined do not form a complete list, nor does this analysis examine the possible overlapping roles of the modifying proteins, this analysis was unsuccessful in revealing any potential modifying proteins for K122. However, it may be interesting to study the role of *SAS3*, and the NuA3 complex in general, in its role in regulating *SRG1* and *SER3*.

In summary, while I have identified that lysine 122 on histone H3 is required for regulation of *SER3*, I have not been successful in identifying whether this amino acid is modified in yeast, as seen in other eukaryotes. This may not be too surprising given the more open chromatin context which exists in yeast compared to other eukaryotes, and that genome-wide mass spectrometry analysis has been unable to reveal a modification on this residue. Further analysis may be undertaken to continue examining the possible modification state of K122 through modified mass spectrometry analyses, creating antibodies specific to a modified yeast peptide, or through screening more thoroughly, the role of histone modifying enzymes on *SER3* regulation.

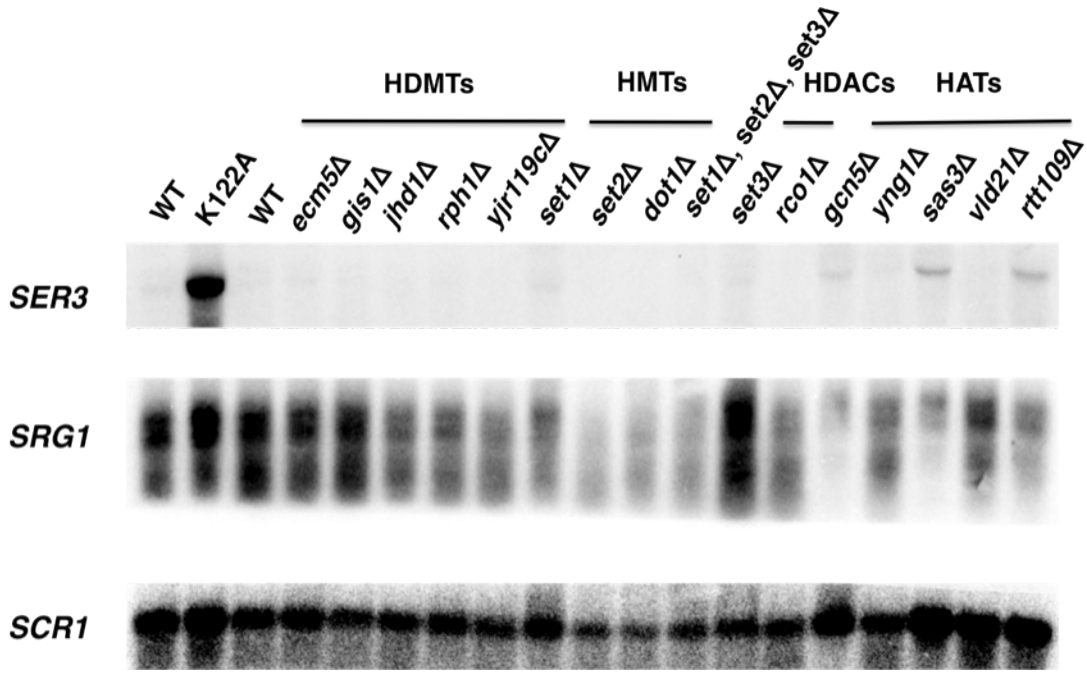

Figure 74. Northern analysis on strains deleted for histone modifying enzymes.

Northern analysis was performed to measure the affect of deleting histone demethylases, histone methyltransferases, histone deacetylases, and histone acetyltransferases on *SER3* and *SRG1* expression. *SCR1* serves as a loading control. Deletions of any one of these factors has no effect on *SER3* or *SRG1* mRNA levels. Wild-type and K122A strains are used as controls on this blot.

Table 11. *Saccharomyces cerevisiae* strains used in Appendix C.

Strain	Genotype	Source
JDY86	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 (hht1-hhf1)Δ::NatMX4 (hht2-hhf2)Δ::HHTS/HHFS* (or containing substitution)-*URA3 can1Δ::MFApr-HIS3*	(DAI *et al.* 2008)
YJ586	*MATα his3Δ200 leu2Δ0 ura3Δ0*	(HAINER and MARTENS 2011a)
KY912	*MAT***a** *his3Δ200 lys2-128 leu2Δ0 ura3-52 set2Δ::HIS3*	K. Arndt
KY934	*MATα his3Δ200 leu2Δ1 trp1Δ63 dot1Δ::HIS3*	K. Arndt
KY938	*MATα his3Δ200 leu2Δ1 trp1Δ63 set1Δ::HIS3*	K. Arndt
KY1235	*MAT***a** *his3Δ200 lys2-173 ura3-52 rco1Δ::HIS3*	K. Arndt
KY1304	*MAT***a** *vid21Δ::*KanMX *his3Δ200 lys2-173 ura3-52*	K. Arndt
KY1308	*MATα yng1Δ::*KanMX *his3Δ200 leu2Δ1 ura3-52*	K. Arndt
KY1313	*MATα sas3Δ*::KanMX *his3Δ200 leu2Δ1 ura3-52*	K. Arndt
KY1318	*MAT***a** *ecm3Δ*::KanMX *his3Δ200 ura3-52 trp1Δ63*	K. Arndt
KY1321	*MATα gis1Δ*::KanMX *his3Δ200 ura3-52*	K. Arndt
KY1326	*MAT***a** *yjr119cΔ*::KanMX *his3Δ200 ura3-52 arg4-12*	K. Arndt
KY1331	*MAT***a** *jhd1Δ*::KanMX *his3Δ200 ura3-52*	K. Arndt
KY1336	*MATα rhp1Δ*::KanMX *his3Δ200 leu2Δ1 ura3-52*	K. Arndt
KY1343	*MATα gcn5Δ::HIS3 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*	K. Arndt
KY1806	*MAT***a** *set3Δ*::KanMX	K. Arndt
KY1851	*MATα set1Δ*::KanMX *set2Δ*::KanMX *dot1Δ*::KanMX *leu2Δ0 ura3Δ0*	K. Arndt
YS404	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*hhts-K122A*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*hhts-K122A*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	Chapter 5
YS417	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0* (*hht1-hhf1*)Δ::*HHTS*/*HHFS*-Hygro (*hht2-hhf2*)Δ::*HHTS*/*HHFS-URA3 can1*Δ::*MFA*pr-*HIS3*	Chapter 5
YP043	*MAT***a** *rtt109Δ*::KanMX *ura3Δ0*	(PRUNESKI 2011)

APPENDIX D

IN VIVO GENOME-WIDE NUCLEOSOME OCCUPANCY OF H3 K122A

The work presented in this Appendix is the beginning of a collaboration performed with Megha Wal in Frank Pugh's laboratory at the Pennsylvania State University. For this analysis, Travis Mavrich created the strains, I performed the MNase digestion in Figure 75 on the samples used by the Pugh lab for the sequencing reaction and performed the Western blot analysis in Figure 76. Megha is performing the sequencing reaction and data analysis presented on the ChIP-seq samples.

Based on the results presented in Chapters 4 and 5, I was interested in determining the effect of the histone residue substitutions on nucleosome occupancy genome-wide. In order to perform this analysis, we formed a collaboration with Dr. Frank Pugh, a leading scientist in next-generation sequencing technology and nucleosome mapping. To begin the analysis, I selected one mutant, H3 K122A, to pilot the mapping, with the intention of continuing through more mutants if the data lead to an interesting result.

I grew strains generated by Travis Mavrich (YTM194, YTM197, YTM202, and YTM203), that express either two copies of *HHTS/HHFS* or two copies of *hhts-K122A* in YPD media at 30°C. I digested formaldehyde-treated chromatin to mononucleosomal size using

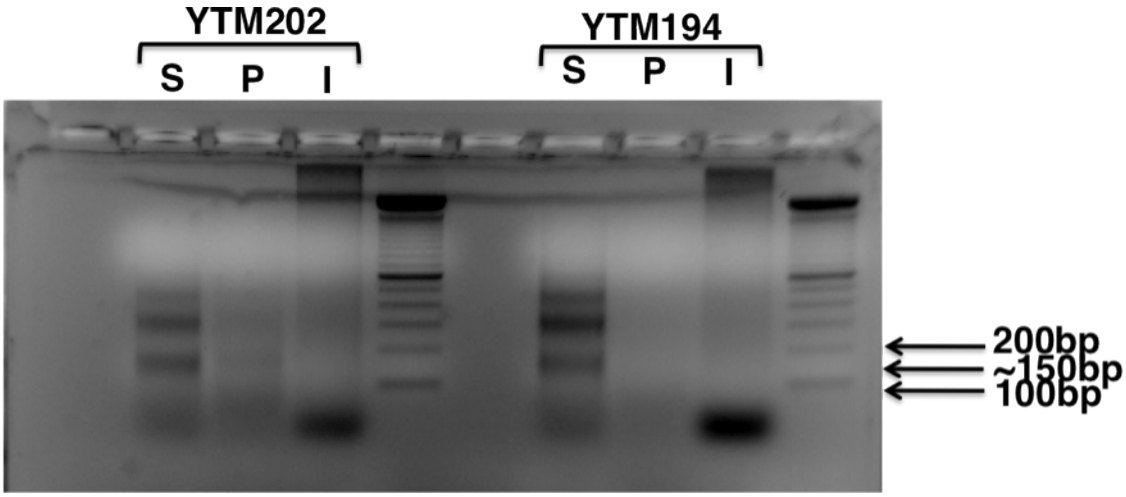

Figure 75. MNase digestion of samples for genome-wide nucleosome occupancy experiments.

This figure depicts the MNase digestion of two strains, YTM194 and YTM202, in preparation for histone H3 ChIP and sequencing analysis. The lanes represent the input (I) sample, the pellet (P), and the supernatant (S), from the MNase digestion. Supernatant fractions contain mostly mononucleosomal and dinucleosomal sizes of DNA.

micrococcal nuclease (MNase) (Figure 75). This was followed by a histone H3 ChIP to enrich for nucleosomal DNA and subjected to high-throughput next generation sequencing by Megha Wal in the Pugh lab. The resulting reads were mapped to the yeast genome to create a statistically derived probability map of nucleosome positions and occupancy.

As a control, I performed Western analysis on w ild-type and H3 K122A strains to examine the global histone H3, H2B, and H4 protein levels, to assure that in the strains we utilize in this analysis do not have any effect on these levels (Figure 76).

Now that the samples have been prepared and control experiments have been performed, the samples will undergo ChIP-seq reactions to provide insight into the genome-wide effect of H3 K122A on transcription-coupled nucleosome dynamics.

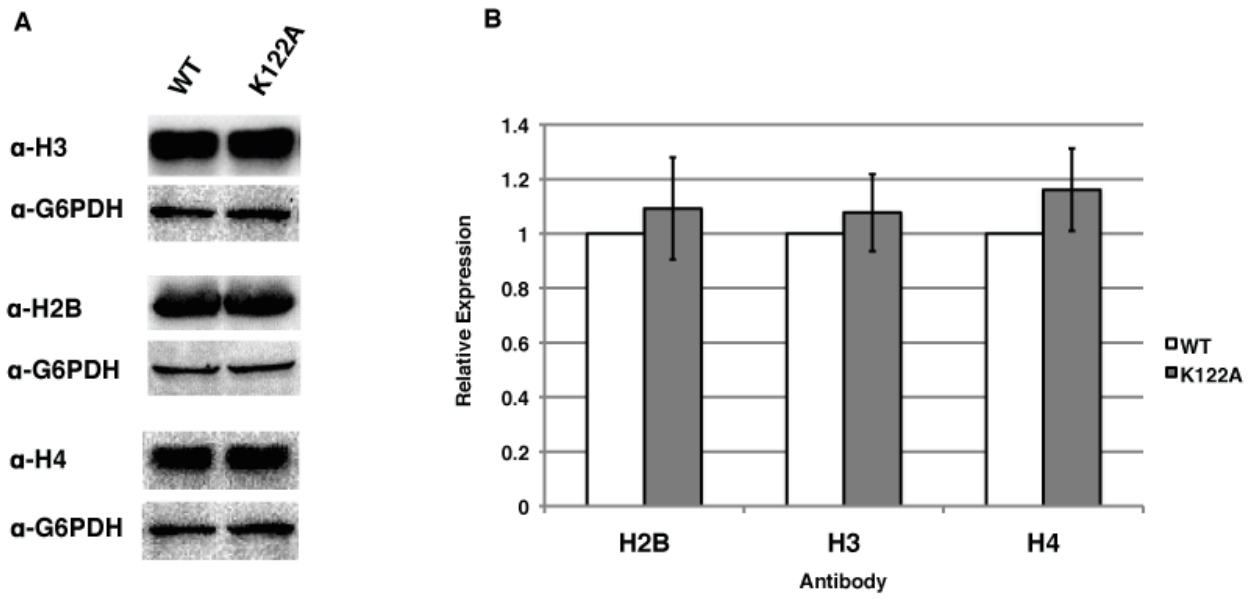

Figure 76. K122A does not alter global histone protein levels.

A) Western analysis examining the effect of histone mutants on total histone H3, H2B, and H4 protein levels. Strains expressing the indicated histone alleles (YTM194 and YTM202) were grown to ~3 × 10^7 cells/ml in YPD at 30°C. Proteins were extracted with trichloroacetic acid and subjected to Western analysis using anti-H3, anti-H2B, anti-H4, and anti-G6PDH (loading control). B) Quantitation of results from three biological replicates (YTM194, YTM197, YTM201, YTM202, YTM203, YTM210). The values shown are the wild-type (while) and K122A (gray) protein levels that have been made relative to the wild-type strains. Error bars indicate the average +/- SEM.

Table 12. *Saccharomyces cerevisiae* strains used in Appendix D.

Strain	Genotype	Source
YTM194	*MAT***a** *ura3Δ0, hht1Δ::HHTS-URA3*, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	T. Mavrich
YTM197	*MAT***a** *ura3Δ0, hht1Δ::HHTS-URA3*, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	T. Mavrich
YTM201	*MAT***a** *ura3Δ0, hht1Δ::hhts-K122A-URA3*, (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*	T. Mavrich
YTM202	*MAT***a** *ura3Δ0, hht1Δ::hhts-K122A-URA3*, (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*	T. Mavrich
YTM203	*MAT***a** *ura3Δ0, hht1Δ::hhts-K122A-URA3*, (*hht2-hhf2*)Δ::*hhts-K122A/HHFS-URA3*	T. Mavrich
YTM210	*MAT***a** *ura3Δ0, hht1Δ::HHTS-URA3*, (*hht2-hhf2*)Δ::*HHTS/HHFS-URA3*	T. Mavrich

APPENDIX E

AN INDUCIBLE SYSTEM FOR *SRG1* REGULATION

In this Appendix, I describe a novel system for *SRG1* regulation of *SER3* I piloted to determine the chromatin dynamics that *SRG1* utilizes to regulate *SER3*. As described in Chapter 2, we have identified a novel mechanism for gene regulation where a ncRNA, *SRG1*, is transcribed across the promoter of its downstream adjacent gene, *SER3,* in response to serine, resulting in the maintenance of nucleosomes over the *SER3* promoter and *SER3* repression (HAINER *et al.* 2011; MARTENS *et al.* 2004). Additionally, as described in Chapters 3, 4, and 5 and Appendix A, we have been able to utilize this system to identify a number of factors, including Spt2, Spt6, Spt16, Paf1, and specific histone residues that are required for the regulation of *SER3*, and, for a subset, are required for transcription dynamics in general (HAINER *et al.* 2012; HAINER and MARTENS 2011a; PRUNESKI *et al.* 2011; THEBAULT *et al.* 2011). Additionally, knowing that Swi/Snf is required to remodel nucleosomes positioned at the 5' end of *SRG1* (MARTENS *et al.* 2005), I hypothesize that in response to serine Swi/Snf slides nucleosomes over the *SER3* promoter and both Spt6 and Spt16 are recruited to this region and collaborate with *SRG1* transcription to maintain nucleosome occupancy through their recycling of nucleosomes. Other factors, such as Paf1 and Spt2, may facilitate this process. In order to test this hypothesis, I piloted the use of an

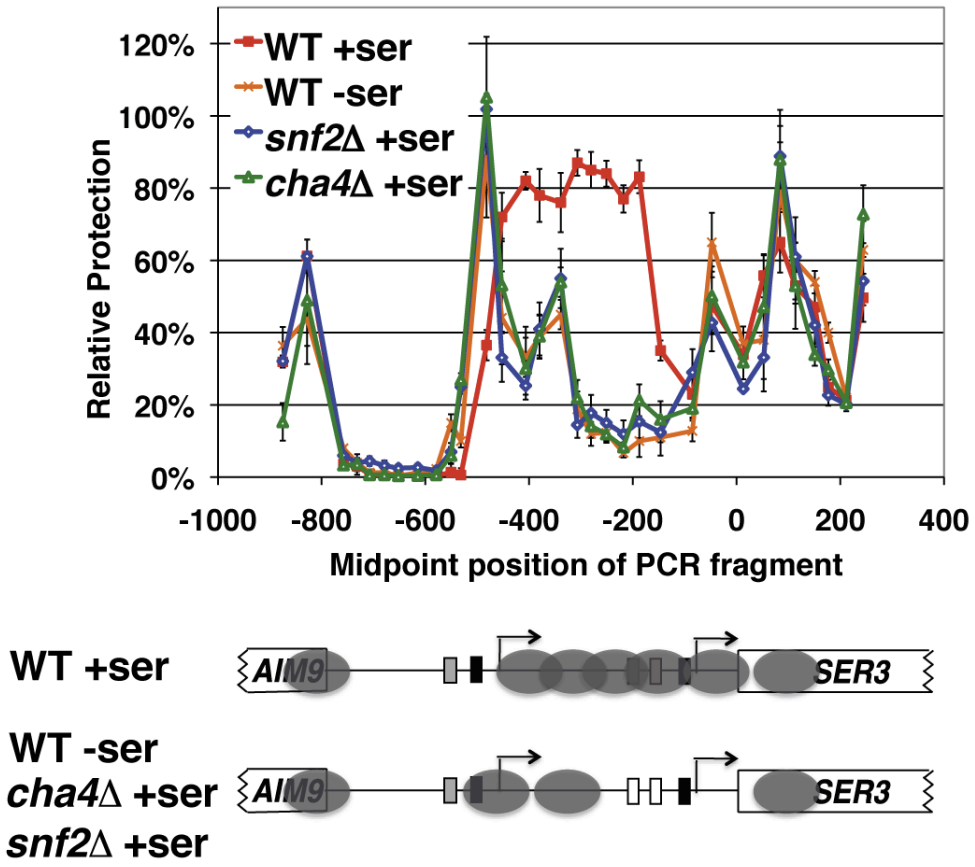

Figure 77. Deletion of *SNF2* or *CHA4* results in nucleosome occupancy patterns across *SRG1* similar to wild-type strains grown in media lacking serine.

Nucleosome scanning assay was performed on wild-type cells (FY4, YJ585, and YJ586) that were grown at 30°C in SC+serine media (+ serine, red) and then shifted to SC-serine media for 25 minutes (- serine, orange) and on strains expressing either *snf2*Δ (blue) (YJ112, YJ717, and YJ718), or *cha4*Δ (green; YJ580, YJ581, and YJ709) grown in YPD media at 30°C. Each experiment was done in triplicate and the mean +/- SEM for the three replicates is plotted at the midpoint for each PCR product.

inducible system for *SRG1* transcription which can be utilized to determine the precise molecular defects which occur in mutations of the factors that have been previously described.

In Chapter 2, we found that nucleosome occupancy over *SRG1* transitions from two positioned nucleosomes over the 5' end of *SRG1* in serine starvation conditions (*SRG1* repressed) to poorly positioned nucleosomes over the entire *SRG1* transcribed region, including the *SER3* promoter, in the presence of serine (*SRG1* expressed) (HAINER *et al.* 2011). Additionally, we know that both Cha4 and Swi/Snf are required for the expression of *SRG1* in the presence of serine (MARTENS *et al.* 2005). I found that when you delete either *CHA4* or *SNF2,* the catalytic subunit of the Swi/Snf chromatin remodeling complex, the nucleosome occupancy over *SRG1* in the presence of serine, mimics that of a wild-type strain grown in the absence of serine (Figure 77). This indicates that both Cha4 and Snf2 are required for the remodeling of nucleosomes over the *SER3* promoter through *SRG1* transcription.

In order to monitor the transition of *SER3* from an active to repressed state, a conditional allele for either *CHA4* or *SNF2* can be constructed and utilized. Initially, a *GAL1*pr-*CHA4* inducible construct was created (J. Martens), and the use of traditional carbon source availability as a method to induce *GAL1*pr driven constructs was attempted (C. Cucinotta, unpublished data). This method was unsuccessful in properly inducing and regulating *SER3*, likely due to the serine biosynthesis pathway, which begins with glucose conversion to 3-phosphoglycerate. Therefore, a new method for conditionally regulating *CHA4* or *SNF2* needed to be utilized.

Recently, a novel method for the inducible regulation of genes in yeast has been described (HICKMAN *et al.* 2011; MCISAAC *et al.* 2011). This system is based on the use of a chimeric transcriptional acitvator, Gal4dbd.ER.VP16 (GEV; the DNA binding domain of Gal4, with an estrogen receptor, and a strong, mammalian transcriptional activator VP16), which is put

under the highly expressed, constitutive yeast promoter of *ACT1*. To study the dynamic properties of a gene, its promoter is replaced with the *GAL1* promoter, to which the Gal4 DNA binding domain of GEV will bind once GEV has been induced by the addition of β-estradiol (McIsaac *et al.* 2011). In the GEV strain background, I can place either *CHA4* or *SNF2* under the *GAL1*pr and examine the dynamic role of chromatin transitions over *SRG1* in *SER3* regulation. In YPD, the strains containing *GAL1*pr-*CHA4* or *GAL1*pr-*SNF2* should mimic that of either *cha4*Δ or *snf2*Δ, respectively, and upon the addition of β-estradiol, expression of *CHA4* or *SNF2* will activate *SRG1* transcription and repress *SER3* so that changes in chromatin architecture across *SER3* can be examined.

I created the strains required to perform these assays, using both *CHA4* and *SNF2*. Interestingly, when I examined the effect of the *GAL1*pr-*CHA4* on *SER3* and *SRG1* expression, I found a surprising result (Figure 78). While *CHA4* was successfully induced over time, and resulted in the induction of *CHA1*, the system did not successfully induce *SRG1* expression, as *SRG1* was already expressed at the 0' time point. *CHA1* is another gene involved in the biosynthesis of serine in *S. cerevisiae*, which is also controlled by the serine responsive activator *CHA4* (see Chapter 1.5). Surprisingly, growth of the *GAL1*pr-*CHA4* strain in YPD without β-estradiol (0') did not mimic a *cha4*Δ strain. Not only was the 0' time point leaky, as *SER3* is not as active as in the *cha4*Δ strain, but the 0' time point also resulted in the presence of a larger band when probed with *CHA4*. Due to this result, I continued with my analysis using the *SNF2* inducible system.

When I performed Northern analysis examining the effect of the *GAL1*pr-*SNF2* inducible system on *SRG1*/*SER3* expression, I found a more straightforward result. *SNF2* was properly induced over time with the addition of β-estradiol, and no additional bands were observed. While

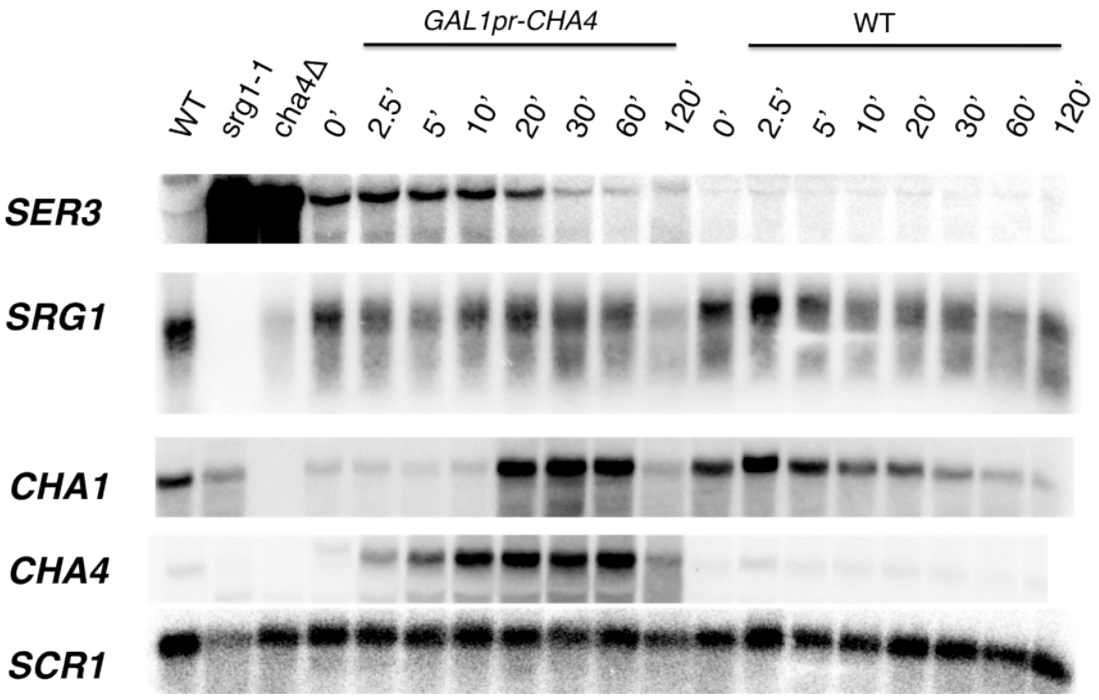

Figure 78. Estradiol induction system of *CHA4* successfully induces *CHA4* and *CHA1*, but does not affect *SRG1* expression.

Northern blot analysis examining the effect of inducing *CHA4* on *SER3*, *SRG1*, *CHA1*, *CHA4*, and *SCR1* (loading control). Total RNA was isolated from wild-type strains (FY4) or strains expressing *srg1-1* (FY2250), *cha4*Δ (YJ580) or expressing either the estradiol induction system with no gene under the control of the *GAL1*pr (WT; DBY12020) or the induction system where the *CHA4*pr is replaced with *GAL1*pr (*GAL1*pr-*CHA4*; YS468) that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C and induced with β-estradiol at 1μM concentration for the time course indicated above.

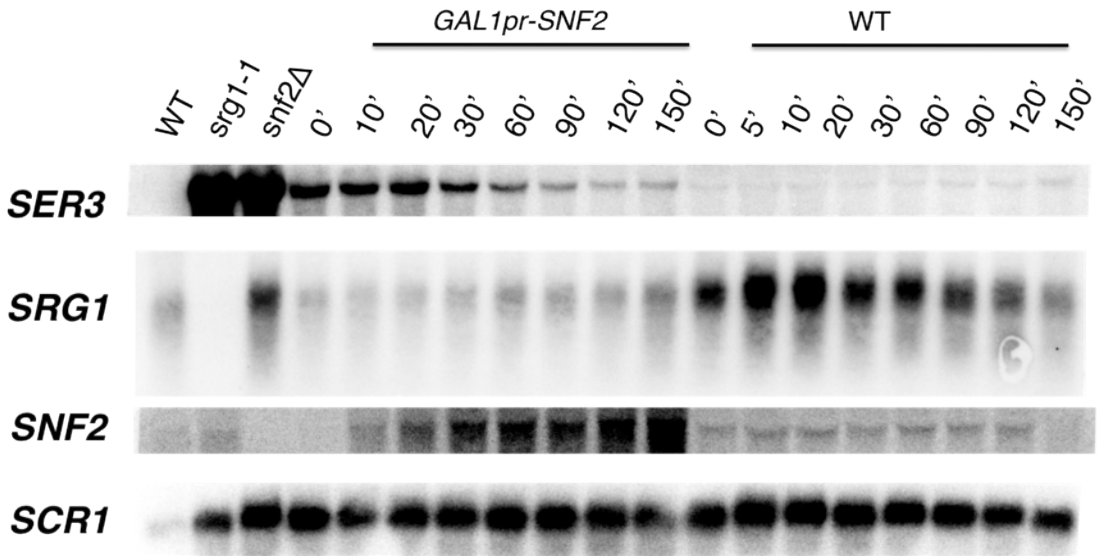

Figure 79. Estradiol induction system of *SNF2* successfully induces *SNF2* and *SRG1* while decreasing *SER3* expression.

Northern blot analysis examining the effect of inducing *SNF2* on *SER3*, *SRG1*, *SNF2*, and *SCR1* (loading control). Total RNA was isolated from wild-type strains (FY4) or strains expressing *srg1-1* (FY2250), *snf2*Δ (YJ112) or expressing either the estradiol induction system with no gene under the control of the *GAL1*pr (WT; DBY12020) or the induction system where the *SNF2*pr is replaced with *GAL1*pr (*GAL1*pr-*SNF2*; YS588) that were grown to a density of 1-2 × 10^7 cells/ml in YPD at 30°C and induced with β-estradiol at 1μM concentration for the time course indicated above.

the effect of the *GAL1*pr-*SNF2* without β-estradiol did not exactly mimic that of a *snf2*Δ, there was an observable increase in *SRG1* RNA and corresponding decrease in *SER3* expression, which better mimics the effect seen in *snf2*Δ than the *CHA4* inducible system 0' time point mimicked the *cha4*Δ effect (Figure 79).

Based on the results of the Northern blot analysis, I performed a preliminary nucleosome scanning assay using the *GAL1*pr-*SNF2* inducible system to examine whether this system could be utilized to examine the dynamic change in chromatin architecture over *SRG1*. Excitingly, the nucleosome profile of the *GAL1*pr-*SNF2* strain mimicked that of a *snf2*Δ strain before the addition of β-estradiol (Figure 80, 0') and upon induction of *SNF2* with β-estradiol, there was a transition from nucleosome occupancy of the 5' end of *SRG1* to over the entire *SRG1* transcribed region (Figure 80, compare 0' to 150'). Most interestingly, this loosely corresponds with the induction seen of *SNF2* (Figure 79), indicating that this system can be successfully used to examine the dynamic transition of chromatin occupancy over the *SER3* promoter.

Now that I have successfully piloted a system for inducible *SRG1* expression, the system can be utilized to explore the dynamic regulation of *SER3*. By combining the inducible system with a previously described system which employs yeast strains expressing two different versions of histone H3 to differentiate the reassembly of nucleosomes from previously those engaged on the DNA to those which are assembled from the pool of free histones. Also, the inducible system can be combined with the many mutants of histone chaperones, transcription elongation factors, and histones themselves, which I have previously discussed to determine the exact contribution of each factor to the regulation of *SER3* and its contribution to chromatin dynamics in general. By utilizing this system, the mechanism through which nucleosomes

employ to regulate *SER3* can be precisely defined as well as the precise role for the molecular contribution of each of the examined factors.

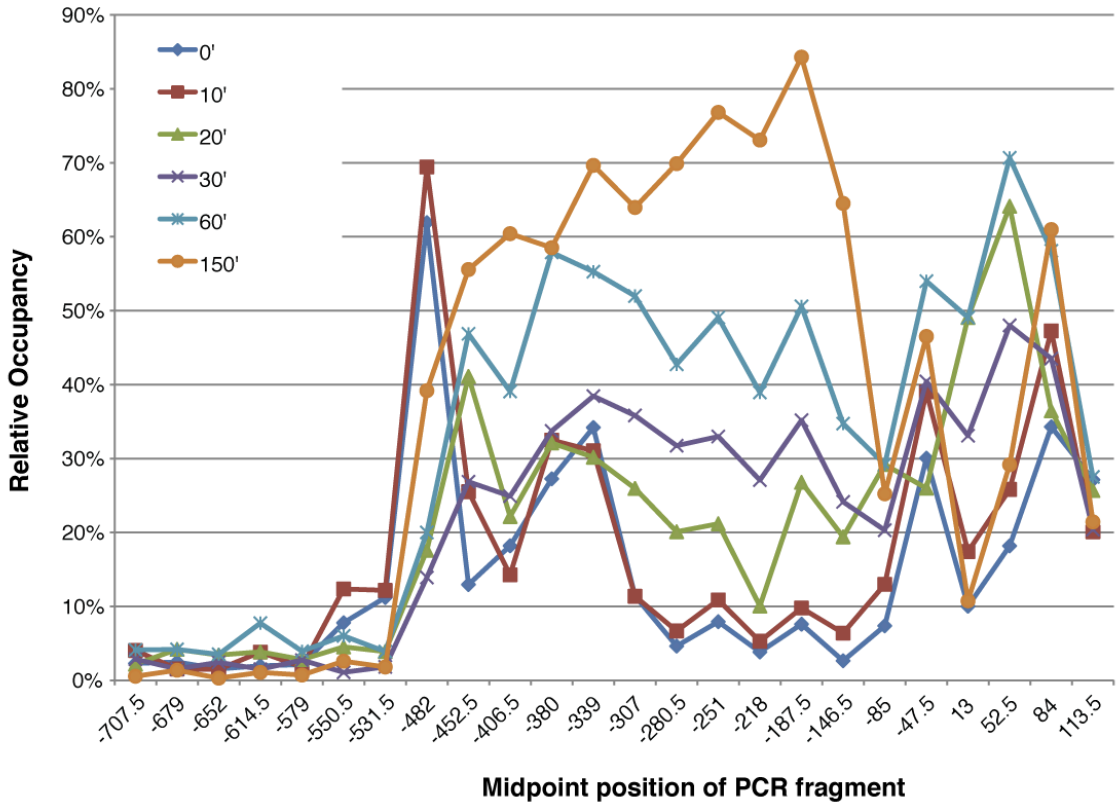

Figure 80. Inducing *SNF2* causes mobilization of nucleosomes to occupy the *SER3* promoter.

Nucleosome scanning assay was performed on estradiol inducible *GAL1*pr-*SNF2* (YS588) that were grown at 30°C in YPD (0'; dark blue) and then induced with 1 μM β-estradiol for a time course up to 150 min. The pilot experiment was performed one time.

Table 13. *Saccharomyces cerevisiae* strains used in Appendix E.

Strain	Genotype	Source
FY4	*MAT***a**	(WINSTON *et al.* 1995)
FY2250	*MAT***a** *his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 ser33Δ::KanMX srg1-1*	(MARTENS *et al.* 2004)
YJ112	*MATα ura3Δ0 lys2Δ0 leu2Δ0 snf2::LEU2*	(MARTENS *et al.* 2005)
YJ580	*MAT***a** *trp1Δ63 leu2Δ0 ura3Δ0 lys2Δ0 cha4Δ*::KanMX	(MARTENS *et al.* 2005)
YJ581	*MATα trp1Δ63 leu2Δ0 ura3Δ0 lys2Δ0 cha4Δ*::KanMX	(MARTENS *et al.* 2005)
YJ586	*MATα his3Δ200 leu2Δ0 ura3Δ0*	(HAINER and MARTENS 2011a)
YJ589	*MAT***a** *his3Δ200 leu2Δ0 ura3Δ0 lys2Δ0*	
YJ709	*MAT***a** *leu2Δ0 ura3Δ0 lys2Δ0 cha4Δ*::KanMX	(MARTENS *et al.* 2005)
YJ717	*MAT***a** *snf2Δ*::KanMX	(MARTENS *et al.* 2005)
YJ718	*MAT***a** *snf2Δ*::KanMX	(MARTENS *et al.* 2005)
DBY12020	*MAT***a** (*GAL10*pr+*gal1*)Δ::loxP, *leu2*Δ0::*ACT1*pr-GEV-NatMX, *gal4Δ*::*LEU2*, *HAP1*+,	(MCISAAC *et al.* 2011)
YS468	*MAT***a** (*GAL10*pr+*gal1*)Δ::loxP, *leu2*Δ0::*ACT1*pr-GEV-NatMX, *gal4Δ*::*LEU2*, *HAP1*+, KanMX::*GAL1*pr-*CHA4*	This study
YS588	*MAT***a** (*GAL10*pr+*gal1*)Δ::loxP, *leu2*Δ0::*ACT1*pr-GEV-NatMX, *gal4Δ*::*LEU2*, *HAP1*+, KanMX::*GAL1*pr-*SNF2*	This study

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