The *Saccharomyces cerevisiae* Paf1 transcription elongation complex is connected to chromatin modification through the multifunctional Rtf1 subunit and the inositol polyphosphate signaling pathway

by

Marcie Helene Warner

B.S., Duquesne University, 2000

Submitted to the Graduate Faculty of

Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

SCHOOL OF ARTS AND SCIENCES

This dissertation was presented

by

Marcie Helene Warner

It was defended on

October 10, 2008

and approved by

Deborah Chapman, PhD, Associate Professor, Dept. of Biological Sciences

Sanford Leuba, PhD, Assistant Professor, Dept. of Cell Biology and Physiology

William Saunders, PhD, Associate Professor, Dept. of Biological Sciences

Anthony Schwacha, PhD, Assistant Professor, Dept. of Biological Sciences

Dissertation Advisor: Karen Arndt, PhD,

Associate Professor, Dept. of Biological Sciences

The *Saccharomyces cerevisiae* Paf1 transcription elongation complex is connected to chromatin modification through the multifunctional Rtf1 subunit and the inositol polyphosphate signaling pathway

Marcie Helene Warner, PhD

University of Pittsburgh, 2008

Transcription in eukaryotes takes place in the context of a repressive chromatin template. Access to the DNA is facilitated by histone modifying enzymes and ATP-dependent chromatin remodeling complexes, which modify chromatin structure. The activities of chromatin modifying proteins are often coordinated by nonenzymatic accessory factors that interact with actively transcribing RNA Polymerase II (Pol II). One such factor is the *Saccharomyces cerevisiae* Paf1 transcription elongation complex. This complex, which is minimally composed of Paf1, Ctr9, Rtf1, Cdc73, and Leo1, physically interacts with Pol II and localizes to the coding regions of active genes.

The Rtf1 subunit of the Paf1 complex performs several cotranscriptional functions: it facilitates recruitment of the chromatin remodeling enzyme Chd1, promotes covalent modification of specific lysine residues in histones H2B and H3, and mediates association of other Paf1 complex subunits. Using a collection of internal deletion mutations that remove 20 to 50 amino acid segments across the length of Rtf1, I demonstrated that Rtf1's known functions are mediated by nonoverlapping regions, implying that the multiple functions of this protein are not completely interrelated. Deletion of the regions of Rtf1 that are required for promoting histone modification or its association with active genes resulted in the strongest transcription-related phenotypes, which suggested that promoting cotranscriptional histone modification is a critical means by which Rtf1 exerts its effects on transcription. Detailed analysis of the region of Rtf1

required for histone modification determined that it is sufficient to promote Rtf1-dependent histone modifications and that this function is dependent on several highly conserved residues.

Additionally, a screen for factors that become essential in the absence of Rtf1 uncovered mutations in the first two enzymes of the inositol polyphosphate (IP) signaling pathway: Plc1 and Arg82. The IP signaling pathway has been linked to the function of several chromatin remodeling complexes. I uncovered strong genetic interactions between Arg82, Paf1, and mutations in the SWI/SNF and INO80 chromatin remodeling complexes and demonstrated that the expression of several target genes was strongly impaired by mutations in these factors. Together, these data suggest that transcription elongation, IP signaling, and chromatin remodeling cooperate to coordinate proper gene expression.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

My experience as a graduate student in the Department of Biological Sciences at the University of Pittsburgh has been a fantastic experience that would not have been possible without the support and assistance of so many wonderful people. Most importantly, I must acknowledge Karen Arndt; thank you, Karen, for welcoming me into your lab and sharing your knowledge while teaching me to think critically and independently. I also need to thank past and present members of the Arndt lab. It has been a privilege to work with so many talented and intelligent people.

I also must thank the members of my thesis committee: Debbie Chapman, Sanford Leuba, Bill Saunders, and Tony Schwacha. I appreciate the helpful suggestions and advice as I progressed through my graduate career. Thanks also to the Martens, VanDemark, Winston, Prelich, Tansey, and Wente labs for their assistance and collaboration. The main and fiscal offices also deserve acknowledgement for their assistance in helping me meet my requirements for graduation. I also need to thank the community of graduate students in this department. I've been fortunate to meet so many wonderful people and develop many lasting friendships.

Finally, I must acknowledge my family who have always supported me and encouraged my pursuit of whatever made me happy. I'm thankful to have always been surrounded by unconditional love and know that I never would've made it this far without you.

1.0 INTRODUCTION

The genomes of eukaryotes are intricately packaged into a nucleoprotein material known as chromatin by association with the histone proteins; this arrangement facilitates their efficient storage and organization within the comparatively small cell nucleus. However, this storage system imposes a significant impediment to processes that require access to the genetic material, such as DNA repair and replication, recombination, and transcription. Eukaryotic cells have, therefore, developed several methods to counter the repressive nature of chromatin structure, including covalent modification of the histone proteins and disassembly or remodeling of nucleosomes, which represent the basic unit of chromatin.

The studies described in this dissertation were performed using the yeast *Saccharomyces cerevisiae* as a model system to explore the mechanisms underlying chromatin modification during the transcription of messenger RNA. Therefore, the information provided in this chapter is specifically tailored toward detailing the current understanding of these processes in *S. cerevisiae.*

Chromatin modification during transcription appears to be an ongoing process that is tightly regulated throughout the transcription cycle. This careful control is necessary to ensure proper levels of gene expression and to prevent aberrant initiation from cryptic promoter sequences present in active open reading frames (ORFs) that become exposed when chromatin structure has been perturbed by the passage of RNA polymerase II (Pol II). The regulation of

chromatin modification during transcription depends, in part, upon accessory factors that interact with actively transcribing Pol II and coordinate the recruitment of chromatin-modifying factors to active genes and, in some cases, stimulate their enzymatic activity.

1.1 THE EUKARYOTIC GENOME IS PACKAGED INTO CHROMATIN

Eukaryotic cells condense and organize large genomes within the relatively small confines of their nuclei. The genome of *S. cerevisiae*, which represents one of the simplest eukaryotic genomes, totals nearly 13,000,000 base pairs (bp) in length and contains nearly 6,000 genes (GOFFEAU *et al.* 1996). The *S. cerevisiae* haploid genome consists of 16 linear chromosomes of varying size which must be packaged into a nuclear space that measures approximately $3\mu m^3$ (JORGENSEN *et al.* 2007).

Organization and compaction of eukaryotic DNA molecules is achieved through their association with the histone proteins. The histones are a class of small, highly conserved proteins that interact with each other to form an octamer containing two copies each of histones H2A, H2B, H3, and H4 (LUGER *et al.* 1997). The histones are positively charged, leading to a tight interaction between the histone octamer and the negatively-charged phosphodiester backbone of a DNA molecule.

Approximately every 200 bp along the length of a eukaryotic chromosome, a 146 bp length of DNA coils nearly two revolutions around a histone octamer to form a structure known as a nucleosome (Figure 1) (LUGER *et al.* 1997). The resulting nucleoprotein material, known as chromatin, resembles “beads on a string” and represents the first order of chromosome organization (KORNBERG and THOMAS 1974). The genome can be further compacted through

the formation of higher-order chromatin structures which require, in part, a fifth histone protein (histone H1) which contacts both the proteins in the histone octamer and DNA present in the linker region between adjacent nucleosomes (ISHIMI *et al.* 1981). Highly compact chromatin domains, known as heterochromatin, often contain important structural elements such as telomeres or centromeres. The remainder of the genome is referred to as euchromatin, which is largely composed of coding regions for both active and inactive genes.

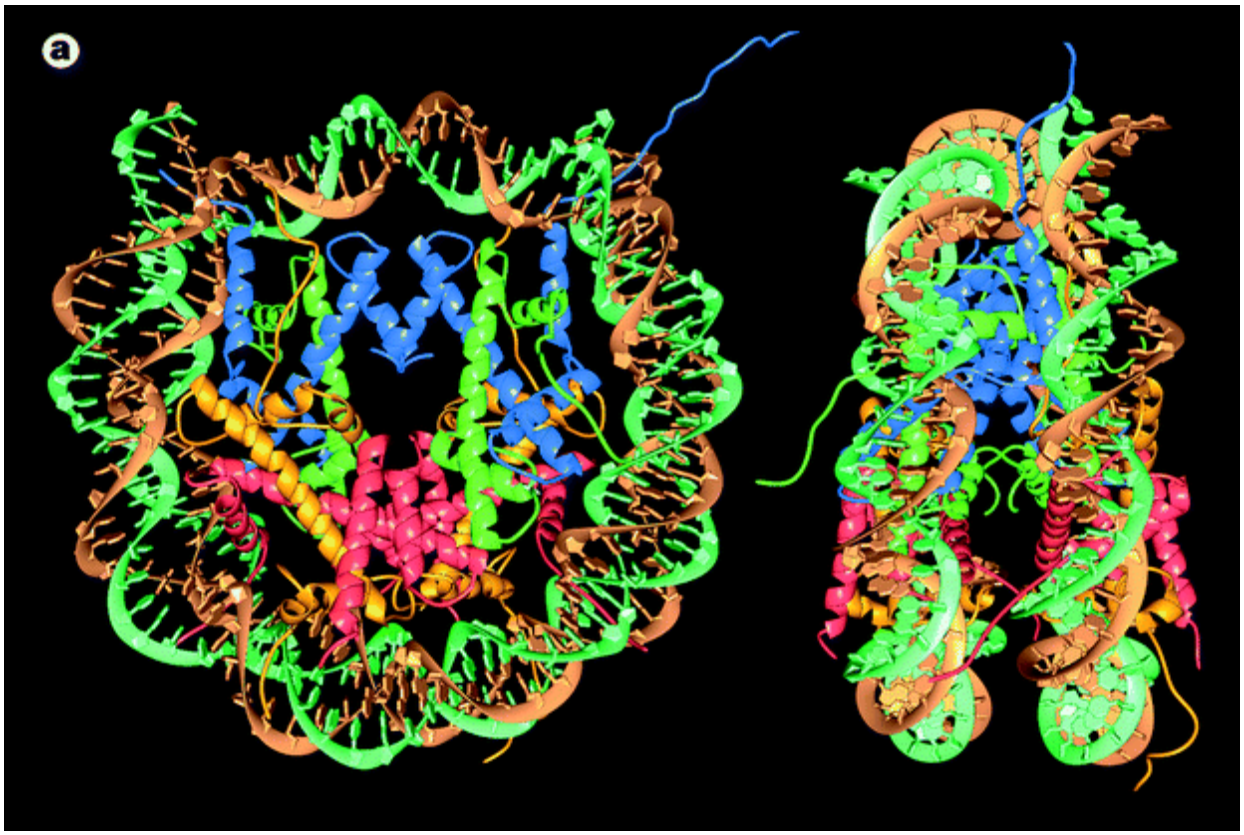

Figure 1. The crystal structure of the nucleosome.

The crystal structure of the nucleosome was solved at 2.8A resolution. Models for the structure of 146 bp of DNA wrapped around a histone octamer are shown (Reprinted by permission from Macmillan Publishers Ltd: [Nature] LUGER *et al.* 1997, copyright 1997). Two copies each of H2A (orange), H2B (pink), H3 (blue), and H4 (green) can be seen in the model on the left. In addition to the alpha-helical histone core domains, several histone "tails" can be seen protruding from the nucleosome core in both models. Two coils of DNA around the histone octamer can be seen clearly in the model on the right.

1.2 CHROMATIN STRUCTURE IS DYNAMICALLY REGULATED BY SEVERAL MECHANISMS

The arrangement of eukaryotic chromosomes into chromatin is an efficient means of storing the genetic material in a highly organized fashion. However, organization of the genome into chromatin impacts DNA-based processes such as DNA repair, DNA replication, recombination, and transcription, by introducing structural constraints and occluding the recognition sites of DNA-binding proteins. Specifically, nucleosomes are known to impede Pol II processivity along ORFs of active genes, often resulting in stalling or arrest of the polymerase (KIREEVA *et al.* 2005). Eukaryotic cells have, therefore, developed a number of mechanisms to modify chromatin structure in order to facilitate the proper execution of DNA-based processes. The most common mechanisms of chromatin modification in yeast, including chromatin disassembly, incorporation of histone variants, covalent histone modification, and ATP-dependent chromatin remodeling, are described in more detail in the following sections. Higher eukaryotes also regulate chromatin modification through heritable methylation of the DNA, although this mechanism appears to be largely absent from the *S. cerevisiae* genome.

1.2.1 Nucleosome disassembly and the incorporation of histone variants

Chromatin assembly is closely linked to genome replication. Expression of the histone genes takes place specifically during the S phase of the cell cycle and histones are incorporated into newly synthesized DNA immediately following the passage of DNA polymerase (TABANCAY

and FORSBURG 2006). Histone octamers are assembled from free H2A/H2B and H3/H4 dimers. Incorporation of new nucleosomes into DNA takes place in a stepwise fashion in which two H3/H4 dimers are incorporated first, followed by the addition of two H2A/H2B dimers (SCHULTZ *et al.* 1997).

Nucleosome positioning is not entirely random; nearly 70% of the *S. cerevisiae* genome is incorporated into well-positioned nucleosomes that consistently occupy a 140 bp length of DNA (YUAN *et al.* 2005). Additionally, chromatin structure assumes a characteristic pattern over the bodies of yeast genes. Many yeast gene promoters contain an approximately 200 bp length of DNA that is devoid of nucleosomes. This nucleosome free region (NFR) is succeeded into the ORF by several well-positioned nucleosomes (MAVRICH *et al.* 2008; YUAN *et al.* 2005). In many cases, the precise location of nucleosomes becomes less well-defined as distance from the promoter increases, suggesting the NFR may function as a nucleosome positioning signal.

The NFR is known to be flanked by two well-positioned nucleosomes that contain the histone variant H2A.Z in place of canonical H2A. Histone variants are differentiated from canonical histones largely by their continued expression outside of S phase and their primarily replication-independent incorporation into chromatin (LI *et al.* 2007a). H2A.Z, encoded by the *HTZ1* gene, shares only about 60% sequence similarity with canonical H2A, which implies an early evolutionary divergence between these two proteins (ZLATANOVA and THAKAR 2008). H2A.Z containing nucleosomes appear to be destabilized more easily than their canonical H2A containing counterparts (ZHANG *et al.* 2005). The presence of H2A.Z in nucleosomes flanking promoters may, therefore, facilitate transcription initiation by promoting efficient eviction of nucleosomes that occlude transcription factor binding sites or inhibit the ability of the transcription machinery to assemble on gene promoters.

The presence of nucleosomes in the bodies of genes impedes the processivity of Pol II (KIREEVA *et al.* 2005). Therefore, chromatin must be disassembled during active transcription to ensure efficient gene expression. Removal of a single H2A/H2B dimer from each nucleosome appears to be sufficient to allow the passage of Pol II in vitro (KIREEVA *et al.* 2002). Translocating Pol II is believed to induce positive supercoiling of the DNA template ahead of its position, which may be adequate to dislodge an H2A/H2B dimer (LEVCHENKO *et al.* 2005). However, chromatin is also believed to be actively disassembled by the activity of numerous histone chaperones including the CAF-I complex, Nap1, Spt6, and the yeast FACT complex; these histone chaperone complexes also play important roles in reassembling chromatin following the passage of Pol II (ARMSTRONG 2007). Chromatin reassembly is critical to prevent aberrant initiation from cryptic promoters present within ORFs (KAPLAN *et al.* 2003).

1.2.2 Histone modification

Changes in chromatin structure can also be elicited through modification of the histone proteins. The crystal structure of the nucleosome demonstrates that each histone protein contains an alpha-helical core domain, which interacts with other histones and the DNA backbone; however, unstructured amino- and carboxy-terminal tails are also apparent (Figure 1) (LUGER *et al.* 1997). Histone modifications primarily occur on these histone tails, although modifications in the core domains have also been identified.

Histones can be posttranslationally modified by the phosphorylation of serines, the acetylation, ubiquitylation, or sumoylation of lysines, and the methylation of lysines and arginines (Figure 2); additional modifications, such as isomerization, ADP-ribosylation and deimination, have also been identified (KOUZARIDES 2007; KREBS 2007). Many histone

modifications are conserved throughout eukaryotes, which emphasizes the importance of modifying chromatin structure for normal cellular function.

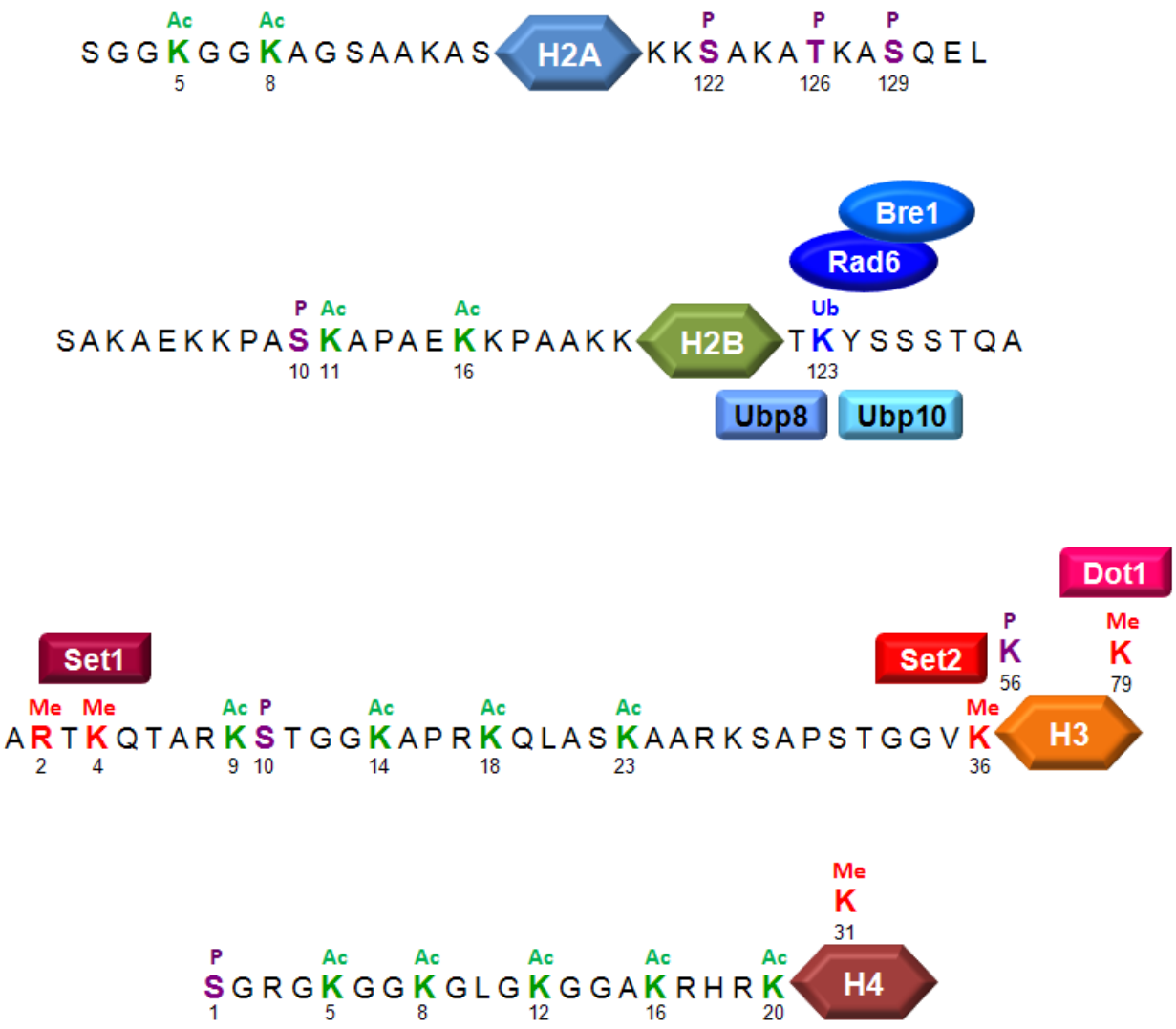
Ac Ac
S G G K G G K A G S A A K A S
5 8
H2A
P P P
K K S A K A T K A S Q E L
122 126 129
Bre1
Rad6
P Ac Ac
S A K A E K K P A S K A P A E K K P A A K K
10 11 16
H2B
Ub
T K Y S S S T Q A
123
Ubp8
Ubp10
Dot1
Set1
Set2
P
K
56
Me
K
79
Me Me Ac P Ac Ac Ac Ac Me
A R T K Q T A R K S T G G K A P R K Q L A S K A A R K S A P S T G G V K
2 4 9 10 14 18 23 36
H3
Me
K
31
P Ac Ac Ac Ac Ac
S G R G K G G K G L G K G G A K R H R K
1 5 8 12 16 20
H4

Figure 2. Posttranslational histone modifications in yeast.

The four core histones are represented; hexagons symbolize core domains and the sequences of amino- and/or carboxy-terminal tails are provided. By convention, histone H2A is numbered from the methionine at position 1, which is cleaved posttranslationally; all other histones are numbered according to the first amino acid present in the mature protein. Modifications on the C-terminal tails of histones H3 and H4 have not been identified; the C-terminal tails have, therefore, been omitted for simplicity. Known covalent histone modifications in yeast are indicated; acetylation (Ac) is shown in green; phosphorylation (P) is shown in purple; monoubiquitylation (Ub) is shown in blue; and methylation (Me) is shown in red. In several instances, the same residue is known to be modified by both acetylation and methylation; these modifications are mutually exclusive. For simplicity, only the most common modification or the modification most relevant to the data contained in this document is shown. Select histone modifying enzymes that are of particular relevance to this thesis are illustrated in proximity to their sites of action. Rad6 and Bre1 cooperate to monoubiquitylate histone H2B at K123; Ubp8 and Ubp10 are ubiquitin proteases that remove this modification. Set1, Set2, and Dot1 are histone lysine methyltransferases that catalyze the methylation of histone H3 at lysines 4, 36, and 79, respectively. The same enzyme catalyzes mono-, di-, and trimethylation of the particular lysine that it modifies. The four core histones are also known to be sumoylated, but specific sites of sumoylation have not been identified. This illustration is an adaptation of Figure 1 from KREBS 2007.

Specific enzymes that mediate each type of histone modification have been identified. In many cases, enzymes have also been identified that reverse or remove these modifications. The existence of these classes of enzymes underscores the extreme flexibility of this mode of chromatin modification. Furthermore, the reversibility and complexity of histone modification suggests that chromatin, which is generally believed to repress DNA-based processes, is also utilized by eukaryotic cells in a regulatory capacity.

The placement and removal of histone modifications is tightly regulated, both spatially and temporally. This results in the establishment of distinct patterns and combinations of histone modifications at specific chromosomal landmarks, such as genes, telomeres, or areas of DNA damage. These carefully crafted domains of histone modification patterns have, therefore, been postulated to represent a "histone code" in which specific histone modification patterns elicit distinct effects on different DNA-dependent processes (STRAHL and ALLIS 2000).

Histone modifications are believed to impact chromatin structure in at least three ways: the attachment of functional groups to the histones inherently alters their structure, acetylation of lysines is believed to weaken histone-DNA interactions by neutralizing the positive charge of this residue, and the presence of histone modifications can introduce binding sites for additional factors that further modify chromatin structure. The establishment and consequences of three of the most well-studied classes of histone modifications, (acetylation, ubiquitylation, and methylation) are described in more detail in the following sections.

1.2.2.1 Histone Acetylation

Histone acetylation was the first covalent histone modification to be identified and it remains the most common. The four core histone proteins, H2A, H2B, H3, and H4, are acetylated at multiple residues in vivo (Figure 2) and the histone variant H2A.Z is also known to

be acetylated. Histones are acetylated almost exclusively on their amino-terminal tails; although, modifications within the histone core domain have also been identified.

Acetylation of histones is carried out by a group of enzymes known as histone acetyltransferases (HATs); individual HATs often possess the ability to modify multiple histone lysine residues. In vitro experiments have demonstrated that HATs often require association with additional factors to facilitate acetylation of histones that are incorporated into a nucleosomal template. At least four major HAT-containing complexes have been identified in yeast: SAGA, ADA, NuA3, and NuA4. The activities of these complexes are carried out by the Gcn5 (SAGA and ADA), Esa1, and Sas3 HATs, respectively (ALLARD *et al.* 1999; GRANT *et al.* 1997; JOHN *et al.* 2000). Gcn5 specifically acetylates histone H2B at lysines 11 and 16 and histone H4 at lysines 9, 14, 18, and 23; Esa1 primarily acetylates histone H4 at lysines 5, 8, 12, and 16, but also displays some activity toward lysine 7 of histone H2A; and Sas3 exclusively acetylates histone H3 at lysines 14 and 23 (MILLAR and GRUNSTEIN 2006; STERNER and BERGER 2000). Several additional HATs, including Hpa3, Hat1, Elp3, Sas2, and Rtt109, have also been identified in yeast (KLEFF *et al.* 1995; OSADA *et al.* 2001; TSUBOTA *et al.* 2007; WITTSCHIEBEN *et al.* 1999; YOW *et al.* 2004).

HAT-containing complexes are frequently transcriptional coactivators, which are specifically recruited to gene promoters to facilitate gene expression. It is, therefore, not surprising that histone acetylation, primarily of histones H3 and H4, is predominantly associated with promoter regions and is almost exclusively linked to transcription activation (KOUZARIDES 2007; LI *et al.* 2007a; STERNER and BERGER 2000). Acetylation has been proposed to weaken histone-DNA contacts due to its ability to neutralize the positive charge of the lysine residue to which it is attached (HONG *et al.* 1993). This idea is supported by the observation that acetylated

histones can be evicted from chromatin more easily than their unmodified counterparts (CHANDY *et al.* 2006; ERKINA and ERKINE 2006; GOVIND *et al.* 2007; WILLIAMS *et al.* 2008). Because histones are acetylated at multiple residues, it is likely that charge neutralization has a cumulative effect whereby histone-DNA contacts are affected more significantly as multiple modifications accumulate on a single histone tail. Histone acetylation has also been postulated to promote transcription activation by hindering the compaction of chromatin into higher-order structures (KOUZARIDES 2007; SHOGREN-KNAAK *et al.* 2006). Many HATs have also been implicated in telomeric silencing, chromatin assembly, and DNA repair (COUTURE and TRIEVEL 2006; MILLAR and GRUNSTEIN 2006).

Another function that has been identified for histone acetylation is the recruitment of additional chromatin modifying factors. Acetylated histones are the binding sites for proteins that contain bromodomains, which are typically found in ATP-dependent chromatin remodeling factors (described in more detail in Section 1.2.3) and other proteins that impact chromatin structure (ZENG and ZHOU 2002). Histone acetylation, therefore, represents a means to target additional enzymatic activities to specific locations in the genome.

Histone acetylation is actively reversed by enzymes known as histone deacetylases (HDACs). These proteins generally function as gene repressors (KOUZARIDES 2007). Additionally, low levels of histone acetylation have been identified within gene coding regions. Accumulation of histone acetylation within ORFs can allow aberrant initiation from cryptic promoters that become exposed when chromatin structure has been modified to allow the passage of Pol II (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; LI *et al.* 2007b). Histone acetylation within coding regions is removed by the Rpd3 HDAC by a mechanism described in detail in Section 1.2.2.3.

1.2.2.2 Histone Ubiquitylation

Histones in *S. cerevisiae* are known to be ubiquitylated on lysine 123 in the carboxy-terminal tail of histone H2B (H2B K123 Ub) (Figure 2) (ROBZYK *et al.* 2000). This modification is conserved in higher eukaryotes, which also possess the ability to ubiquitylate histone H2A at K119 (NICKEL and DAVIE 1989). These modifications are primarily limited to monoubiquitylation, which is reversible and is believed to signal for involvement in non-degradation associated functions. A recent study indicates that histone H2B may also be polyubiquitylated, which likely signals for its degradation (GENG and TANSEY 2008).

Ubiquitylation of H2B K123 in *S. cerevisiae* is performed by the Rad6 ubiquitin conjugating-enzyme and the Bre1 ubiquitin ligase (Figure 2) (HWANG *et al.* 2003; ROBZYK *et al.* 2000; WOOD *et al.* 2003a). An additional factor, Lge1, is found in a complex with Rad6 and Bre1 and is also required for H2B K123 Ub, but its role is not clearly defined (HWANG *et al.* 2003). H2B K123 Ub is found along the promoters and coding regions of active genes (Figure 3), where the enzymes involved in this modification are known to localize; Bre1 is recruited to gene promoters by interaction with transcriptional activator proteins, which leads to the subsequent recruitment of Rad6 (HWANG *et al.* 2003; WOOD *et al.* 2003a; WOOD *et al.* 2003b; XIAO *et al.* 2005). However, recruitment alone is not sufficient for Bre1 and Rad6 activation. Rad6 associates with Pol II when it transitions to an elongation-competent form and remains associated along the entirety of the ORF; this interaction and the presence of a Pol II-associated transcription accessory complex, known as the Paf1 complex (described in greater detail in Section 1.4.2.1), are required for Rad6 to become catalytically active (XIAO *et al.* 2005). Additionally, in vitro experiments demonstrated that, in addition to Pol II and the Paf1 complex,

Rad6 requires the presence of NTPs to transition to an active form, suggesting that transcription itself is also required for H2B K123 Ub (PAVRI *et al.* 2006).

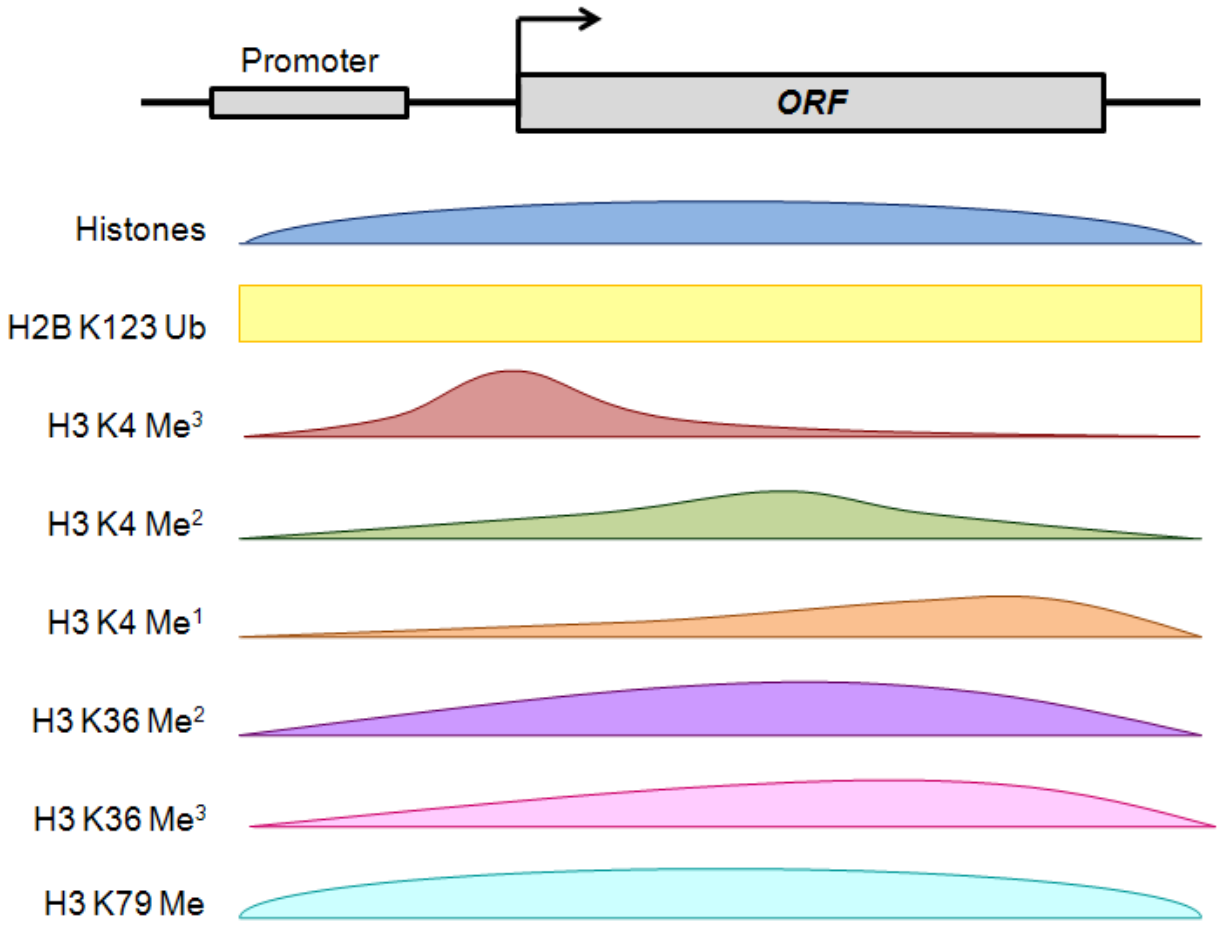

Figure 3. Distribution of H2B K123 ubiquitylation and H3 K4, K36, and K79 methylation at a typical active yeast gene.

The open reading frame (ORF) and promoter of a model yeast gene are illustrated at the top. A typical yeast gene contains higher total histone levels in the ORF than at the promoter or immediately downstream of the ORF. The relative abundance of total histones or each of the indicated histone modifications are indicated by the height of the curved lines. Me^3 = trimethylation; Me^2 = dimethylation; Me^1 = monomethylation. H3 K79 Me refers to all methyl-lysine states for this residue. This figure is adapted from Figure 1 in Li *et al.* 2007a.

H2B K123 Ub is rapidly reversed by deubiquitylating enzymes. In yeast, this activity is mediated by the ubiquitin specific proteases Ubp8 and Ubp10 (DANIEL *et al.* 2004; EMRE *et al.* 2005; GARDNER *et al.* 2005; HENRY *et al.* 2003). While the activities of Ubp8 and Ubp10 are partially redundant (GARDNER *et al.* 2005), unique functions have been identified for each of these enzymes. Ubp8 is a component of the SAGA transcriptional coactivator complex, which is recruited to gene promoters and also contains HAT activity; integrity of the SAGA complex is required for Ubp8 activity (DANIEL *et al.* 2004; HENRY *et al.* 2003). Ubp10 functions independently of SAGA and has instead been shown to associate with Sir4, a protein involved in establishing transcriptional silencing at the telomeres and rDNA loci (described in more detail in Section 1.3). Accordingly, Ubp10 is required for normal telomeric and rDNA silencing (KAHANA and GOTTSCHLING 1999; OSLEY 2006).

The exact function of H2B K123 Ub remains unknown, although several models have been proposed. Ubiquitin, a 76 amino acid protein, likely represents a bulky attachment to histone H2B, which itself is only 132 amino acids in length. The presence of a monoubiquitin moiety on H2B has, therefore, been postulated to function as a "wedge" that might loosen the interaction of the histone octamer with DNA or prevent tight packing of adjacent chromatin fibers, thereby making the underlying DNA more accessible to the transcription machinery (MURATANI and TANSEY 2003). It has also been reported that deubiquitylation of H2B K123 Ub is required for association of the Ctk1 kinase with transcribing Pol II (WYCE *et al.* 2007). Ctk1 phosphorylates Pol II during transcription elongation at specific sites on an unstructured domain at the C-terminus of the largest subunit (JONES *et al.* 2004). Ctk1-mediated phosphorylation of Pol II is known to stimulate the association of numerous factors involved in chromatin modification and mRNA maturation with active Pol II (AHN *et al.* 2004; LI *et al.* 2003;

LICATALOSI *et al.* 2002). Because the association of Ctk1 with Pol II is inhibited until H2B K123 Ub is removed, it has been suggested that this modification may function to impose a checkpoint during transcription elongation (WEAKE and WORKMAN 2008). Additionally, H2B K123 Ub is known to be a prerequisite for the subsequent methylation of histone H3 at lysines 4 and 79 (BRIGGS *et al.* 2002; SUN and ALLIS 2002). These histone methylation events (described in more detail in the following section) are believed to prevent aberrant silencing of transcriptionally active regions (NG *et al.* 2003c); H2B K123 Ub is, therefore, also implicated in this process.

1.2.2.3 Histone Methylation

Histones can also be modified by the enzymatic addition of methyl groups to arginine and lysine residues on histones H3 and H4. Histone arginine residues can be mono- or dimethylated; dimethylation may occur in symmetric or asymmetric configurations (Figure 4; bottom). The arginine residue at position 2 of histone H3 is known to be methylated in *S. cerevisiae*; this modification is typically associated with inactive genes (KIRMIZIS *et al.* 2007). To date, enzymes that remove histone arginine methylation have not been identified.

Histone lysine methylation occurs in mono-, di-, or trimethylated forms (Figure 4; top); all three methyl-lysine states are mediated by the same enzyme. Nucleosomes present in the bodies of active genes in *S. cerevisiae* are frequently methylated on histone H3 at lysines 4, 36, and 79 (K4, K36, and K79); these modifications are mediated by the Set1, Set2, and Dot1 methyltransferases, respectively (Figure 2) (BERNSTEIN *et al.* 2002; LI *et al.* 2007a; SANTOS-ROSA *et al.* 2002; XIAO *et al.* 2005). The activity of Set1 and Set2 is carried out by a conserved catalytic motif known as a SET domain. This domain is named for the *Drosophila melanogaster* gene regulators Su(var) 3-9, Enhancer of zeste, and Trithorax, which represent the first proteins

in which this domain was identified (JONES and GELBART 1993; TSCHIERSCH *et al.* 1994). SET domains, which have since been documented in numerous histone lysine methyltransferases throughout eukaryotes, were originally characterized by their involvement in heterochromatin formation, a process that has subsequently been demonstrated to be highly dependently on global patterns of histone modifications (RUSCHE *et al.* 2003). Dot1 represents the only known histone methyltransferase that lacks a SET domain (FENG *et al.* 2002). K4 and K36 are located in the H3 amino-terminal tail; while K79 is present on the outer surface of the H3 core domain. The different localization of their substrates may account for the unique catalytic motifs present in the SET-domain containing histone methyltransferases and Dot1.

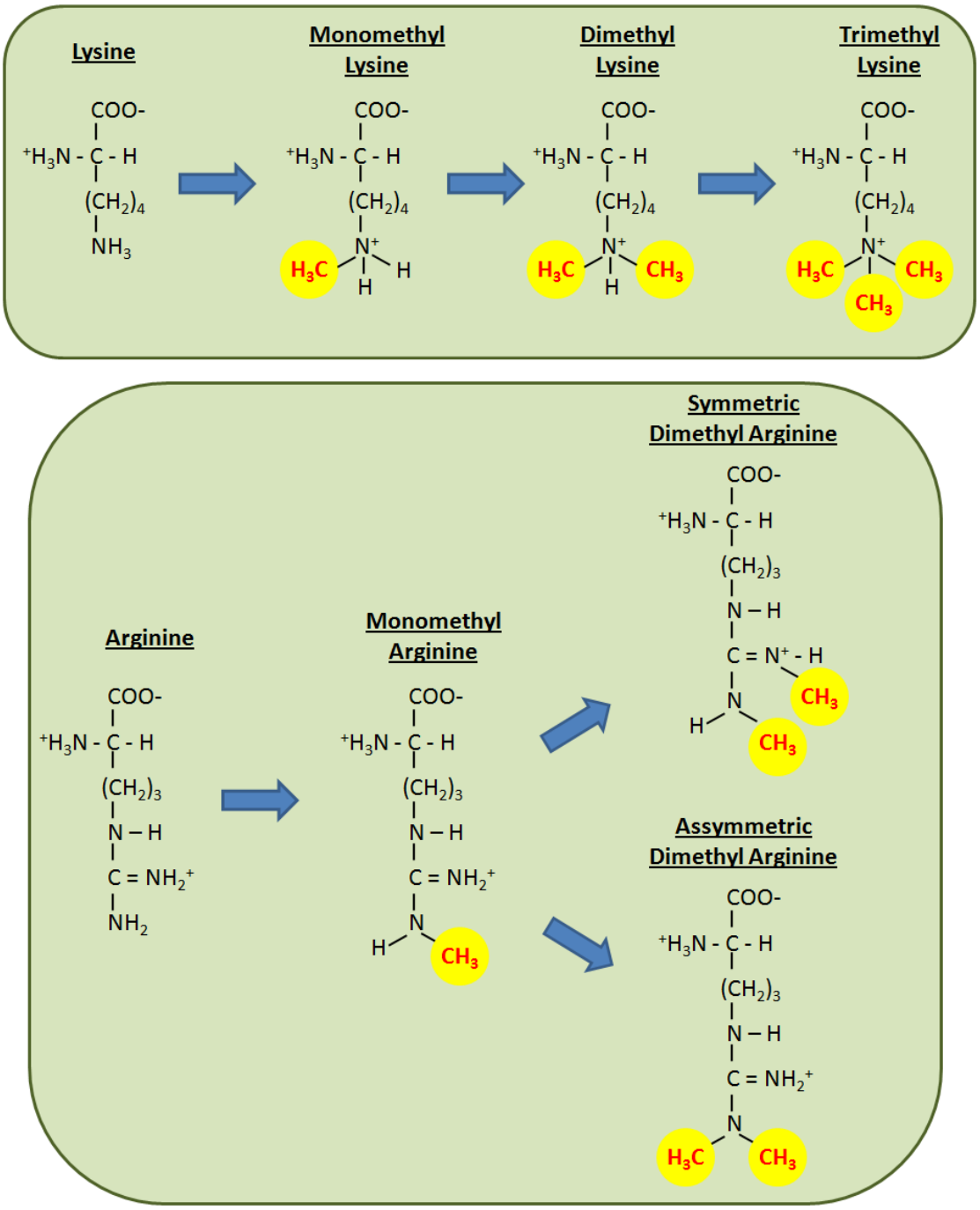

Figure 4. The states of methylation on histone lysine and arginine residues.

Top; Histones can be mono-, di-, or trimethylated on lysine residues. Bottom; Histones can be mono- or dimethylated on arginine residues. Arginine dimethylation can occur in symmetric or asymmetric configurations. This figure is an adaptation of Figure 1 from SHILATIFARD 2006.

While Set2 and Dot1 appear to function independently of binding partners, Set1 acts in the context of COMPASS (complex of proteins associated with Set1), which is conserved throughout eukaryotes. In addition to Set1, COMPASS contains Cps60, Cps50, Cps40, Cps35, Cps30, Cps25, and Cps15 (MILLER *et al.* 2001; NAGY *et al.* 2002; ROGUEV *et al.* 2001). Association of Set1 with other COMPASS subunits is required for its enzymatic activity and unique subunits have distinct effects on the fate of COMPASS and the methyl-lysine states that can be achieved at H3 K4 (DEHE and GELI 2006; MUELLER *et al.* 2006; ROGUEV *et al.* 2001; SCHNEIDER *et al.* 2005): Cps50 and Cps30 are required for Set1 stability and COMPASS integrity and are, therefore, required for all H3 K4 methyl states; loss of Cps25 and/or Cps60 decreases H3 K4 dimethylation and eliminates H3 K4 trimethylation; and Cps40 specifically affects H3 K4 trimethylation, potentially by influencing Set1 stability. Cps35, which has also been found to associate with the RNA 3' end formation machinery, is essential for cell viability (DEHE and GELI 2006; ROGUEV *et al.* 2001).

Histone H3 lysine methylation occurs in distinct patterns across the bodies of active genes. H3 K4 methylation displays a graded pattern across ORFs in which a peak of trimethylation is present at the 5' end of a gene, dimethylation is enriched in the center of a gene, and the 3' end is marked by monomethylation (Figure 3) (LI *et al.* 2007a; POKHOLOK *et al.* 2005). H3 K36 di- and tri-methylation are distributed across the coding regions of active genes with a slight bias toward the 3' end (Figure 3); no distinct pattern has been identified for monomethylation of H3 K36. H3 K79 methylation marks nearly all histone H3 in euchromatin, which accounts for 90% of the yeast genome (VAN LEEUWEN *et al.* 2002). This modification is found in the coding regions of active genes, although no particular distribution of individual methyl-lysine states is apparent (LI *et al.* 2007a; POKHOLOK *et al.* 2005).

These distinct patterns of individual histone H3 lysine methylation marks are established through several mechanisms. Set1 and Set2 are specifically recruited to the coding regions of active genes through a physical interaction with Pol II itself. Rpb1, the largest subunit of Pol II, features an unstructured "tail" at its carboxy-terminus, which is composed of 26 repeats of a tyrosine-serine-proline-threonine-serine-proline-serine (YSPTSPS) consensus heptapeptide repeat (ALLISON *et al.* 1985). Pol II is phosphorylated on this unstructured domain, known as the carboxy-terminal domain (CTD), in a distinct pattern as it transcribes an mRNA. The serine at position 5 (Ser5) of the heptapeptide repeat is phosphorylated during early transcription elongation. Ser5 phosphorylation is erased as Pol II approaches the 3' end of a gene, while phosphorylation of the serine at position 2 (Ser2) concomitantly accumulates (PALANCADE and BENSAUDE 2003). Set1 is known to interact with the Ser5 phosphorylated form of Pol II (KROGAN *et al.* 2003) and Set2 contains a motif that binds directly to the CTD when it is phosphorylated at Ser2 (KIZER *et al.* 2005). Dot1 is not known to physically interact with Pol II, but instead is likely recruited to chromatin through an interaction with a basic patch on the surface of histone H4 (FINGERMAN *et al.* 2007).

In addition to targeted recruitment of their corresponding histone methyltransferases, H3 K4 and K79 di- and tri-methylation are also confined to discrete spatial domains through a trans-histone modification pathway; H2B K123 Ub is a prerequisite for the subsequent di- and trimethylation of H3 at these sites (BRIGGS *et al.* 2002; NG *et al.* 2002; SUN and ALLIS 2002; WOOD *et al.* 2003b). Monomethylation of H3 at K4 and K79 does not require H2B K123 Ub (DEHE *et al.* 2005; SHAHBAZIAN *et al.* 2005). In vitro studies have demonstrated that monoubiquitylation of K120 of human H2B, which corresponds to H2B K123 of *S. cerevisiae*,

leads to methylation of H3 K79 by the human homolog of Dot1 within the same nucleosome (MCGINTY *et al.* 2008).

Several models have been proposed to explain the mechanism by which H2B K123 Ub controls di- and trimethylation of histone H3 at K4 and 79. In the simplest model, the addition of ubiquitin to H2B at K123 decondenses chromatin structure to allow better access of the Set1 and Dot1 methyltransferases to their corresponding substrates. Additionally, a recent study demonstrated that H2B K123 Ub is necessary for the recruitment of the COMPASS subunit Cps35 to active genes and that this interaction is required for normal di- and trimethylation of H3 K4 (LEE *et al.* 2007). Interestingly, the same study identified a physical interaction between Cps35 and Dot1, suggesting that a similar mechanism may also control Dot1's ability to catalyze di- and trimethylation. The proteasomal ATPases Rpt4 and Rpt6 and the Ccr4-Not mRNA production and processing complex are also involved in the steps linking H2B K123 Ub to H3 K4 and K79 di- and trimethylation, but the mechanism by which these factors participate in this process is unclear (EZHKOVA and TANSEY 2004; LARIBEE *et al.* 2007).

Histone lysine methylation is a relatively stable mark; it endures for several hours at recently transcribed yeast genes despite dissociation of the histone lysine methyltransferases from coding regions coincident with cessation of transcription (NG *et al.* 2003c). It was, therefore, believed that histone lysine methylation was not actively removed and was instead turned over slowly as a passive result of the incorporation of unmodified histones during subsequent rounds of genome replication. However, multiple enzymes have recently been discovered that possess the ability to enzymatically remove specific methyl marks from histones. Two classes of histone lysine demethylases, which utilize unique catalytic mechanisms, have been identified: the first class, represented by LSD1 (lysine specific demethylase 1; originally

identified in humans) can remove mono- and dimethylation of H3 K4 (SHI *et al.* 2004); the second class is composed of multiple proteins containing a Jumonji domain. Histone lysine demethylases containing Jumonji domains have been shown to catalyze the removal of di- and trimethylation from specific histone lysine residues (KIM and BURATOWSKI 2007; TSUKADA *et al.* 2006). In spite of the discovery of histone demethylases, histone methylation remains a persistent mark. This suggests that histone lysine demethylases function to fine tune histone lysine methylation domains and do not catalyze their complete erasure. While the effects of histone demethylases on global histone methylation levels and transcription are relatively subtle, they have been demonstrated to have roles in development in higher eukaryotes (AGGER *et al.* 2008).

Histone lysine methylation is conserved throughout evolution, suggesting that it serves an important purpose and experimental evidence implies that these modifications are linked to several processes. H3 K4 and K79 methylation have been proposed to function as a transcriptional memory mechanism (NG *et al.* 2003c). Because these modifications are present on the bodies of active genes and persist for an extended period of time in the absence of transcription, histone lysine methylation may represent a means of maintaining recently transcribed genes in a state that is poised for reactivation. The presence of histone lysine methylation in recently transcribed coding genes is also known to prevent the spread of transcriptional silencing into areas of active gene expression (described in detail in Section 1.3.)

Additionally, histone lysine methylation is known to introduce binding sites for several classes of enzymes that contain chromo-, Tudor, or PHD domains (KREBS 2007). These histone methyl-lysine binding proteins can sometimes discriminate among different methylation states, implying that unique stages of methylation can lead to distinct downstream effects. Proteins

containing histone methyl-lysine binding motifs are often found in multisubunit complexes that contain enzymatic activity, thus functioning as a mechanism to tether catalytic activity to specific sites in the genome. For example, H3 K36 di- and trimethylation are known to function as binding sites for the chromodomain containing Eaf3 protein, a subunit of the Rpd3(S) complex (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; LI *et al.* 2007b). Rpd3 is an HDAC and its targeted recruitment to domains where H3 K36 di- and trimethylation are present leads to the removal of acetylation from histones present in the bodies of active genes. Deacetylation of histones in coding regions by this mechanism prevents aberrant initiation from cryptic promoters present within open reading frames.

1.2.3 ATP-Dependent Chromatin Remodeling

An additional method of modifying chromatin is to reposition or restructure nucleosomes to facilitate or restrict access to the underlying DNA. This is achieved through the activity of several families of multisubunit complexes, called chromatin remodeling complexes, that contain a catalytic subunit with homology to the super family 2 (SF2) of DEAD/H-box nucleic acid-stimulated ATPases (EISEN *et al.* 1995). Although the SF2 family of enzymes includes DNA helicases, chromatin remodeling enzymes do not possess helicase activity.

Chromatin remodeling enzymes are likely recruited to chromatin through interactions that are mediated by regulatory subunits present within their respective complexes; the ATPase subunits themselves do not appear to possess any sequence specific DNA-binding activity and are instead believed to interact with DNA nonspecifically through the phosphodiester backbone. Chromatin remodeling enzymes utilize the energy derived from ATP hydrolysis to disrupt the 14 points of contact between a histone octamer and the DNA backbone (LUGER *et al.* 1997). In

some cases, disrupting the interactions between histones and DNA is sufficient to permit access of DNA-binding proteins to the underlying DNA or to allow the passage of RNA or DNA polymerase.

However, chromatin remodeling complexes do not typically cause only transient disruptions in histone-DNA contacts, but rather result in the repositioning, eviction, or restructuring of entire nucleosomes. The current model for how chromatin remodeling enzymes facilitate these effects on nucleosome positioning is believed to involve a mechanism of intranucleosomal looping (GANGARAJU and BARTHOLOMEW 2007; VAN VUGT *et al.* 2007). Chromatin remodeling enzymes are thought to contact DNA at two points and spool the DNA from one contact point through its active site, while holding the other point of contact stationary, resulting in the formation of an intranucleosomal loop (Figure 5). The force of translocating the DNA is likely sufficient to disrupt histone-DNA contacts.

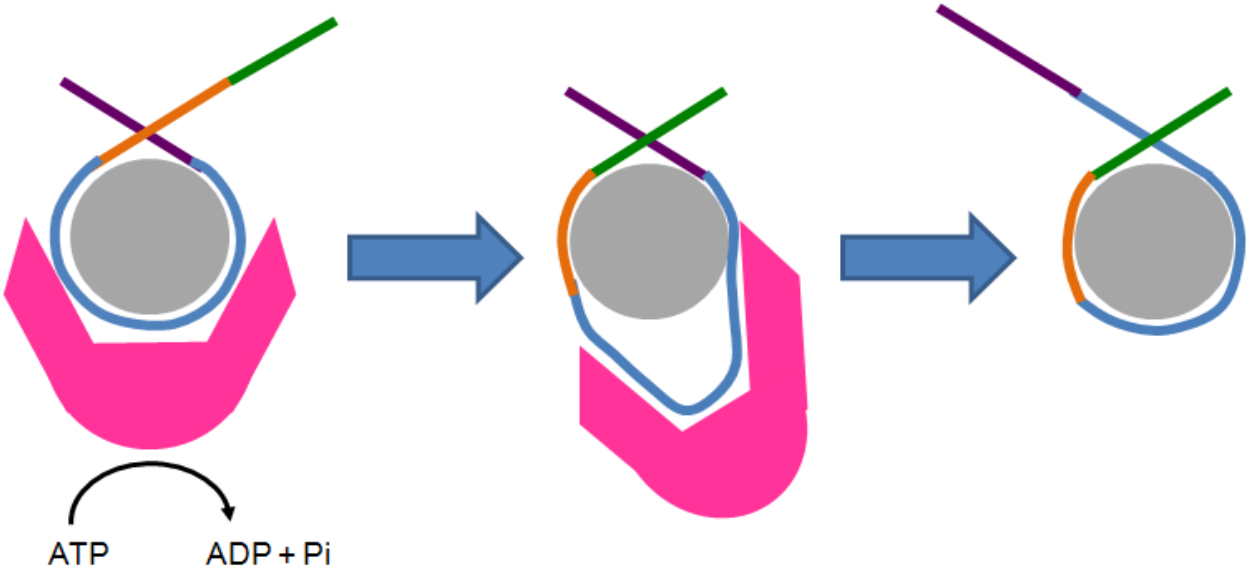

Figure 5. Potential mechanism of intranucleosomal looping and nucleosome repositioning induced by chromatin remodeling enzymes.

A chromatin remodeling enzyme (pink) may contact nucleosomal DNA nonspecifically through the phosphodiester backbone at two points. The energy derived from ATP hydrolysis might lead to a shifting of one DNA contact point while the second point of contact is held stationary. This would result in an intranucleosomal loop, as depicted in the center illustration. A nucleosome could be repositioned if the histone octamer forms stable contact with the DNA that was translocated into proximity with the histones during formation of the intranucleosomal loop (shown in orange). Propagation of the intranucleosomal loop could then result in shifting of the DNA at the other end of the nucleosome (shown in purple). For simplicity, only one revolution of DNA around the histone octamer is shown. This figure is adapted from Figure 5 in GANGARAJU and BARTHOLOMEW 2007.

Individual complexes create different lengths of intranucleosomal loops. It is likely that the size of intranucleosomal loop created by a particular complex is responsible for the characteristic style of activity by which that complex functions, such as the incorporation of histone variants or the formation of regularly spaced nucleosome arrays. The mechanism by which a particular chromatin remodeling complex functions is generally thought to determine whether it facilitates or restricts access to the underlying DNA.

S. cerevisiae contains 8 known chromatin remodeling complexes, which range in complexity from 2 to 17 subunits. Homologs of *S. cerevisiae* chromatin remodeling complexes have been identified in all eukaryotes. These complexes are split into 4 families, which are classified by similarities in their ATPase subunits (GANGARAJU and BARTHOLOMEW 2007). SWI2/SNF2-type complexes are characterized by bromodomains, which bind acetylated lysines, in their catalytic subunit; ISWI-type complexes contain catalytic subunits that contain SANT and SLIDE domains, which facilitate histone tail and DNA binding, respectively, CHD-type chromatin remodeling proteins harbor chromodomains that are believed to interact with methylated lysines on histones, and INO80-type complexes contain DNA-binding DBINO domains in their catalytic subunits.

In yeast, the SWI2/SNF2 family is represented by the SWI/SNF and RSC complexes; the ISWI family is represented by the ISW1a, ISW1b, and ISW2 complexes, the INO80 family is represented by the INO80 and SWR1 complexes, and Chd1 is the sole CHD-type chromatin remodeling enzyme. With the exception of the essential RSC complex, different chromatin remodeling complexes appear to be somewhat redundant, although each complex also appears to fulfill its own unique roles. Each of these complexes is described in more detail in the following subsections.

1.2.3.1 SWI/SNF

SWI/SNF was the first chromatin remodeling complex discovered in yeast. Mutations in genes encoding the members of this complex were identified in independent genetic screens for factors that could not activate mating type switching (SWI = mating type switching defect) or use sucrose as a carbon source (SNF = sucrose non-fermenting) (WINSTON and CARLSON 1992). SWI and SNF mutations were discovered to affect overlapping sets of factors. The likely cause of the defects in SWI and SNF mutants was the inability to induce expression of the gene encoding the mating type switching HO endonuclease or *SUC2*, which encodes a sucrose hydrolyzing enzyme; these observations suggested SWI and SNF mutants resulted in a general defect in transcription activation. Suppressors of SWI and SNF mutations were generally found to relieve the repressive nature of chromatin (LAURENT *et al.* 1991; PETERSON and HERSKOWITZ 1992), suggesting a role for factors encoded by SWI and SNF genes in gene activation through effects on chromatin structure.

The product of the *SNF2* gene was found to encode a protein with similarity to the SF2 family of DEAD/H-box nucleic acid-stimulated ATPases (CAIRNS *et al.* 1994; VAN VUGT *et al.* 2007). Snf2 was purified as a member of a 12 subunit complex that also contains the products of many other SWI and SNF genes (CAIRNS *et al.* 1994; PETERSON *et al.* 1994). This complex was, therefore, named SWI/SNF and was found to be present at about 100-200 copies per cell (GHAEMMAGHAMI *et al.* 2003). In addition to its ATPase domain, Snf2 also contains bromodomains and other subunits of SWI/SNF contain ARID, SWIRM, and SANT domains which participate in interactions with DNA and histones (GANGARAJU and BARTHOLOMEW 2007). SWI/SNF has subsequently been shown to disrupt nucleosome structure and increase DNA accessibility to activators in an ATP-dependent manner (COTE *et al.* 1994); additional

biochemical analysis indicates that SWI/SNF functions at individual nucleosomes to create large intranucleosomal loops that are approximately 50 bp in length (ZOFALL *et al.* 2006). SWI/SNF is strongly required for expression of only about 5% of genes in rich medium (SUDARSANAM *et al.* 2000). However, it is likely that there is a requirement for this complex during activation of many genes; this idea is supported by the initial identification of SWI/SNF subunits as participating in *HO* and *SUC2* activation. In addition to its known role in transcription activation, less well-defined roles for SWI/SNF have also been identified in rDNA and telomeric silencing, nucleotide excision repair, and double strand break repair (VAN VUGT *et al.* 2007).

1.2.3.2 RSC

RSC, which stands for Remodels the Structure of Chromatin, is an additional member of the SWI/SNF family of chromatin remodeling complexes in *S. cerevisiae*. This complex is present in the cell at a 10-fold greater abundance than SWI/SNF (GHAEMMAGHAMI *et al.* 2003). The catalytic component of RSC, Sth1, was identified based on sequence homology to Snf2 (LAURENT *et al.* 1992). Sth1 contains a similar domain composition as Snf2 and creates similar-sized intranucleosomal loops (VAN VUGT *et al.* 2007). RSC is a 17-member complex that exists in two isoforms that are defined by the presence or absence of the Rsc3 and Rsc30 subunits; 10 of the 17 subunits of RSC, including Sth1, are essential for cell viability (CAIRNS *et al.* 1999). This is likely due to a general requirement for RSC in global transcription; 40% of all yeast genes are strongly affected by depletion of Sth1 (VAN VUGT *et al.* 2007).

RSC likely functions in concert with histone acetylation to maintain global transcriptional activity. Multiple bromodomains are found in the Rsc1, Rsc2, and Rsc4 subunits of RSC, in addition to those in the Sth1 ATPase. Together, the bromodomains present in RSC account for 7 of the 15 known bromodomains in yeast (VAN VUGT *et al.* 2007). The abundance of

bromodomains contained in the RSC complex may suggest that RSC is recruited to genes through an interaction with acetylated histones. This idea is supported by the observation that RSC stimulates the passage of Pol II through nucleosomes most efficiently in the presence of the SAGA and NuA4 histone acetyltransferase complexes (CAREY *et al.* 2006). In addition to its role in transcription regulation, RSC has also been implicated in sister chromatin cohesion, chromosome stability, and DNA repair (VAN VUGT *et al.* 2007).

1.2.3.3 ISW1

The Imitation SWItch, or ISWI, family of chromatin remodeling complexes in yeast is represented by ISW1a, ISW1b, and ISW2; these ATPases are present in the cell at approximately 1500 copies each (GHAEMMAGHAMI *et al.* 2003). The ISWI ATPases, Isw1 and Isw2, are very similar in structure to SWI/SNF-type ATPases except that they lack bromodomains. The SANT domains of ISWI-type ATPases, known as SLIDE domains (SANT-like ISWI domain) mediate the DNA-binding activity of ISWI-family members and are necessary for their full catalytic activity (GANGARAJU and BARTHOLOMEW 2007). The full activity of these enzymes also requires interaction with the same basic patch on histone H4 that interacts with the histone lysine methyltransferase Dot1 (CLAPIER *et al.* 2002).

ISWI-containing complexes are the smallest chromatin remodeling complexes identified; the 4 known ISWI-containing complexes in yeast contain only 2 to 4 subunits. ISW1a is composed of Isw1 and Ioc3 and functions primarily at promoters to repress gene expression; ISW1b contains Isw1, Ioc2, and Ioc4 and is believed to restore nucleosomes within ORFs, and is, therefore, also typically associated with inhibition of transcription (VAN VUGT *et al.* 2007). However, the repositioning of nucleosomes into ORFs by ISW1b may also function to assist in

promoter clearance of Pol II during transcription initiation, which suggests that this complex may also have a positive impact on transcription (MORILLON *et al.* 2003).

ISW1a and ISW1b move nucleosomes in opposite directions in vitro, suggesting that regulatory subunits impart directionality to the catalytic subunit (GANGARAJU and BARTHOLOMEW 2007; VAN VUGT *et al.* 2007). ISW1a creates regularly spaced nucleosome arrays (SIF 2004), which are believed to inhibit transcription by restricting access to the underlying DNA. ISWI-type ATPases result in the formation of considerably smaller intranucleosomal DNA loops than SWI/SNF family members; the loops created by ISWI-type ATPases appear to measure only about 10 bp in length (ZOFALL *et al.* 2006).

1.2.3.4 ISW2

The Isw2 ATPase forms a complex with Itc1, Dbp4, and Dls1, which is believed to have a role in maintenance of telomeric silencing and in preventing inappropriate antisense transcription (WHITEHOUSE *et al.* 2007) (IIDA and ARAKI 2004). A complex consisting of only Isw2 and Itc1 has also been identified; this two subunit complex appears to be recruited to gene promoters through interaction with the transcriptional repressor Ume6 and functions in parallel with the Rpd3(L) histone deacetylase complex to repress gene expression (VAN VUGT *et al.* 2007).

Isw2-containing complexes are thought to function in a primarily repressive capacity, similar to their Isw1-containing counterparts. Isw2 also functions to create regularly spaced nucleosome arrays, although the activity of this enzyme does not appear to be as tightly regulated as that of Isw1. Isw2 results in the spacing of nucleosomes approximately every 200 bp, while Isw1-positioned nucleosomes are about 175 bp apart (GANGARAJU and BARTHOLOMEW 2007).

1.2.3.5 CHD1

Chd1 is the sole representative of the CHD (chromodomain helicase DNA-binding)-type ATPase in yeast cells. It shares significant domain similarity to Snf2, but also contains two chromodomains, which recognize methyl-lysine residues on histones (WOODAGE *et al.* 1997). The presence of chromodomains suggests that Chd1 might be targeted to active chromatin through a physical interaction with methylated H3 K4, but conflicting data regarding this interaction have been reported in yeast (OKUDA *et al.* 2007; PRAY-GRANT *et al.* 2005). The normal expression of about 2-4% of all yeast genes requires Chd1, which is present at about 1600 copies per cell (GHAEMMAGHAMI *et al.* 2003; TRAN *et al.* 2000). This chromatin remodeling enzyme demonstrates the ability to reposition nucleosomes, but does not create large stretches of free DNA like those created by SWI/SNF (TRAN *et al.* 2000).

Chd1 interacts with chromatin throughout the yeast genome (SIMIC *et al.* 2003; TRAN *et al.* 2000). It is known to associate with the SAGA transcriptional coactivator/histone acetyltransferase complex, implying a potential role in transcription activation (PRAY-GRANT *et al.* 2005). However, yeast Chd1 also physically interacts with the Paf1 and Spt4/Spt5 transcription elongation complexes and human CHD1 co-immunoprecipitates the FACT histone chaperone/transcription elongation complex (SIMIC *et al.* 2003; SIMS *et al.* 2007); these interactions strongly suggest a role for Chd1 in transcription elongation.

1.2.3.6 INO80

The INO80 family of chromatin remodeling proteins is the most recently identified class of these factors in yeast. The catalytic components (Ino80 and Swr1) of these complexes (INO80 and SWR1) are characterized by a split ATPase domain that contains a large spacer region (GANGARAJU and BARTHOLOMEW 2007). The INO80 complex, which is composed of 15

subunits, is present at about 7000 copies per cell and is necessary for normal transcription of about 150 genes (GHAEMMAGHAMI *et al.* 2003; JONSSON *et al.* 2001).

Ino80 contains Rvb1 and Rvb2; these proteins share homology with the bacterial Holliday junction helicase RuvB and impart 3'-5' helicase activity to the INO80 complex (SHEN *et al.* 2000). Additionally, the INO80 complex contains actin and three actin-related proteins (ARPs), Arp4, Arp5, and Arp8. While the exact mechanism by which ARPs participate in chromatin remodeling is not understood, they have been hypothesized to interact with histones or components of the nuclear matrix (SIF 2004; VAN VUGT *et al.* 2007).

In addition to its known role in regulating a subset of yeast genes, the INO80 complex has also been implicated in DNA repair. Mutations in INO80 complex members result in an increased sensitivity to DNA-damaging agents (MORRISON *et al.* 2004; VAN ATTIKUM *et al.* 2004). INO80 is targeted to DNA double stranded breaks through an interaction of the Arp4 subunit with H2A that is phosphorylated on serine 129 in response to DNA damage (DOWNS *et al.* 2004). However, the exact mechanism by which INO80 participates in the repair of DNA double strand breaks is not well understood.

1.2.3.7 SWR1

The SWR1 (SWI-related) complex, which is composed of 15 subunits, is present at about 700 copies per cell (GHAEMMAGHAMI *et al.* 2003; KOBOR *et al.* 2004; VAN VUGT *et al.* 2007). The catalytic component of this complex, Swr1, participates in the only known example of ATP-dependent chromatin remodeling that results in the incorporation of a histone variant. Swr1 physically interacts with H2A.Z and catalyzes the replacement of canonical H2A/H2B dimers with H2A.Z/H2B dimers (KOBOR *et al.* 2004; MIZUGUCHI *et al.* 2004). This exchange happens primarily at gene promoters (ZHANG *et al.* 2005). H2A.Z/H2B dimers are more easily displaced

from chromatin than their canonical counterparts and are, therefore, believed to poise genes for activation (ZHANG *et al.* 2005). Although the exact mechanism by which the SWR1 complex is targeted to promoters is not entirely understood, its recruitment may be facilitated by interaction of two bromodomains within the Bdf1 subunit with acetylated histones (VAN VUGT *et al.* 2007). In addition to its effects on transcription through H2A.Z incorporation, the SWR1 complex is also known to have roles in DNA repair and preventing the spread of telomeric silencing (GANGARAJU and BARTHOLOMEW 2007).

1.3 TELOMERIC SILENCING IS CONTROLLED BY GLOBAL PATTERNS OF HISTONE MODIFICATION

Structural segments of linear chromosomes, such as telomeres and centromeres, are assembled into compact, transcriptionally silent heterochromatin domains. Compaction of these areas of the genome is believed to protect their structural integrity, in part, by excluding the transcription machinery and other DNA-based enzymes from accessing the genomic material in these regions. In *S. cerevisiae*, rDNA repeats and the mating type loci are also actively silenced to properly control their expression. Because transcriptional silencing of the telomeres is the category of silencing that is most relevant to the data presented in this document, the remainder of this section focuses specifically on establishment and maintenance of this type of silencing. However, silencing of rDNA repeats and the mating type loci are carried out by similar, though not identical, mechanisms (RUSCHE *et al.* 2003).

Telomeric DNA is composed of repetitive sequences that contain binding sites for the sequence specific DNA binding protein Rap1 (CONRAD *et al.* 1990). The silent information

regulator (Sir) proteins Sir4 and Sir2 form a complex that is recruited to the telomeres by a physical interaction between Rap1 and Sir4 (MORETTI *et al.* 1994). Sir2 is an HDAC that functions to deacetylate the amino-terminal tails of histones H3 and H4 in nearby telomeric nucleosomes (LANDRY *et al.* 2000; SMITH *et al.* 2000). Hypoacetylated histone H3 and H4 tails are bound tightly by Sir4 and also recruit a third Sir family member, Sir3 (HECHT *et al.* 1995). Recruitment of Sir4 to hypoacetylated H3 and H4 tails results in the arrival of additional molecules of Sir2 as part of the Sir4-Sir2 complex. Multiple cycles of Sir2-dependent histone deacetylation followed by recruitment of Sir3 and the Sir4-Sir2 complex leads to the spreading of these factors over several kilobases of DNA.

Telomere-associated Sir proteins bind tightly to each other, leading to compaction of the underlying DNA. This creates a dense and highly ordered arrangement of nucleosomes in telomeres. The tightly packed Sir proteins and closely spaced nucleosomes create a barrier that results in inaccessibility of the underlying genomic DNA to DNA-binding proteins.

Transcriptional silencing of the telomeres is highly dependent on global histone modification patterns. In addition to the absence of histone H3 and H4 acetylation, telomeric nucleosomes also lack H2B K123 Ub and H3 K4 and K79 methylation (BERNSTEIN *et al.* 2002; NG *et al.* 2003a; SANTOS-ROSA *et al.* 2004; VAN LEEUWEN *et al.* 2002). H2B K123 Ub is likely excluded from histones in the telomeres due to the known physical interaction between the Ubp10 deubiquitylating enzyme and Sir4 (GARDNER *et al.* 2005; KAHANA and GOTTSCHLING 1999). Because H2B K123 Ub is a prerequisite for H3 K4 and K79 methylation, the active exclusion of H2B K123 Ub from telomere-associated histones likely prevents H3 K4 and K79 methylation in these nucleosomes. H3 K4 and K79 methylation that was present when silencing was established is likely eliminated by histone replacement during subsequent rounds of genome

replication. However, it is also possible that histone demethylases are involved in the removal of H3 K4 and K79 methylation from telomeric nucleosomes.

The enzymes required for H2B K123 Ub and H3 K4 and K79 methylation are necessary for normal telomeric silencing (HUANG *et al.* 1997; KROGAN *et al.* 2002a; VAN LEEUWEN *et al.* 2002). The absence of these enzymes leads to an abundance of hypomodified histone tails throughout the genome, which results in titration of the Sir proteins away from the telomere (NG *et al.* 2003b). If the Sir proteins are not present at a high enough concentration in telomeric regions, DNA compaction is not achieved thereby allowing the transcription machinery and other DNA-binding proteins to access the underlying DNA. Because H2B K123 Ub and H3 K4 and K79 methylation inhibit the formation of silenced chromatin, it is likely that a major function of the specific targeting of these modifications to the bodies of actively transcribed genes is to protect areas of ongoing or recent gene activity from being silenced incorrectly.

1.4 TRANSCRIPTION BY RNA POLYMERASE II IS HIGHLY REGULATED BY ACCESSORY PROTEINS

The genetic information contained in DNA is copied into RNA by the activity of RNA polymerases. Three major RNA polymerases exist in eukaryotic cells: Pol I, Pol II, and Pol III. Pol I transcribes ribosomal RNA (rRNA); Pol III primarily transcribes transfer RNA, but also transcribes 5S rRNA. Transcription of protein coding genes into messenger RNA (mRNA) is carried out by Pol II; this enzyme also catalyzes the expression of several species of noncoding RNAs, including small nuclear RNAs and small nucleolar RNAs.

Pol II is a highly conserved 550 kDa complex that is composed of 12 subunits (CRAMER 2004). Structural studies of Pol II indicate that it adopts a "crab claw"-like configuration. The upper and lower "jaws" are composed primarily of the two largest subunits of Pol II: Rpb1 and Rpb2 (Figure 6) (CRAMER *et al.* 2000; KETTENBERGER *et al.* 2004). Template DNA is funneled through this cleft, where the Watson and Crick strands are separated and brought into contact with the active site of the polymerase. Individual nucleotides enter the active site through a channel (CRAMER *et al.* 2000) and base pair with the template strand where Pol II catalyzes the formation of a phosphodiester bond between the arriving nucleotides. The "bubble" introduced between the Watson and Crick strands of the DNA can accommodate an RNA-DNA hybrid that measures approximately 8 nucleotides in length (KETTENBERGER *et al.* 2004).

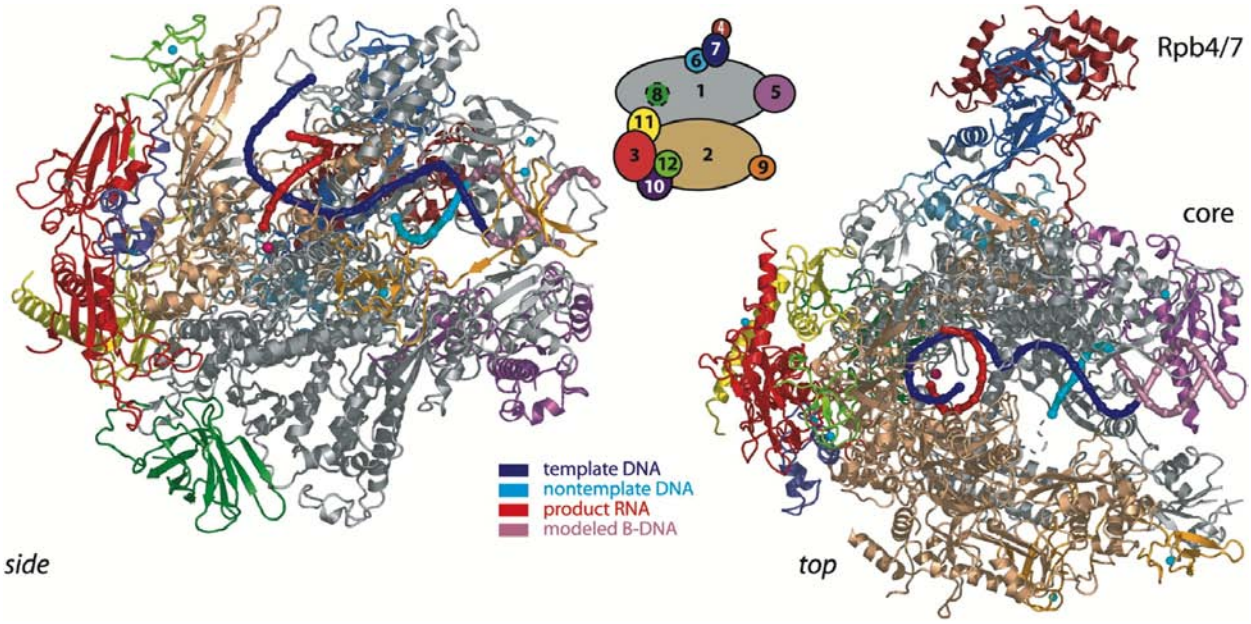

Figure 6. The crystal structure of RNA Polymerase II.

The crystal structure of Pol II was solved in complex with template DNA and a short RNA product (Reprinted from KETTENBERGER *et al.* 2004, Copyright 2004, with permission from Elsevier). Two views (left = side view; right = top view) are shown. Pol II subunits 1-12 (Rpb1-Rpb12) are colored according to the key shown between the two structural representations. Template DNA, nontemplate DNA, and product RNA are shown in blue, cyan, and red, respectively; downstream DNA is shown in light pink.

As the length of the RNA chain increases, it exits the polymerase through a channel that emerges near the carboxy-terminus of Rpb1 (KETTENBERGER *et al.* 2004). This places the growing RNA in close proximity to the Rpb1 carboxy-terminal domain (CTD) (KETTENBERGER *et al.* 2004). In yeast, the CTD is composed of 26 repeats of the heptapeptide consensus sequence YSPTSPS, which are unstructured in crystallographic studies (ALLISON *et al.* 1985). The CTD is highly conserved throughout eukaryotes, although the number of repeats increases with the complexity of the organism. The CTD is a unique feature of Pol II that is not present on the largest subunits of Pols I and III.

Removal or substantial truncation of the Rpb1 CTD results in lethality in yeast (WEST and CORDEN 1995), which likely reflects its critical role in coordinating the cotranscriptional processing of nascent RNAs. Transcription of an mRNA consists of distinct initiation, elongation, and termination stages. The CTD of Rpb1 is phosphorylated in a characteristic pattern as Pol II progresses through each transcriptional stage (Figure 7), which influences the association of unique classes of RNA processing factors with the CTD; at a minimum, these factors participate in adding a 5' cap and 3' polyadenylated (poly(A)) tail to an emerging pre-mRNA to produce a mature mRNA. The importance of the CTD in coordinating cotranscriptional pre-mRNA processing is further supported by the identification of over 100 yeast proteins that interact with the CTD (PHATNANI *et al.* 2004). The emergence of the nascent RNA from a channel near the CTD places it in close proximity to processing factors that bind to the polymerase through this domain; this likely facilitates the coupling of transcription to pre-mRNA processing.

The association of pre-mRNA processing factors is influenced by the phosphorylation state of the CTD, which changes dynamically during the transcription cycle (Figure 7) (BENTLEY

2005; KOMARNITSKY *et al.* 2000; PALANCADE and BENSAUDE 2003; PRELICH 2002). During transcription initiation, when Pol II is recruited to a gene promoter, the CTD is un- or hypophosphorylated. Coincident with the release of Pol II into the ORF at the onset of transcription elongation, CTD repeats become phosphorylated specifically on the serine at position 5 (Ser5) by the protein kinase Kin 28. Ser5-phosphorylated CTD repeats facilitate the binding of enzymes that participate in processing of the pre-mRNA 5' end, including the 7-methyltransferase Abf1, the guanylyltransferase Ceg1, and the RNA triphosphatase Cet1 which catalyze the addition of a methylguanosine cap to the pre-mRNA at the 5' end. Capping of the pre-mRNA appears to positively influence transcription elongation, suggesting that checkpoints exist throughout the transcription cycle to ensure an emerging RNA is properly maturing.

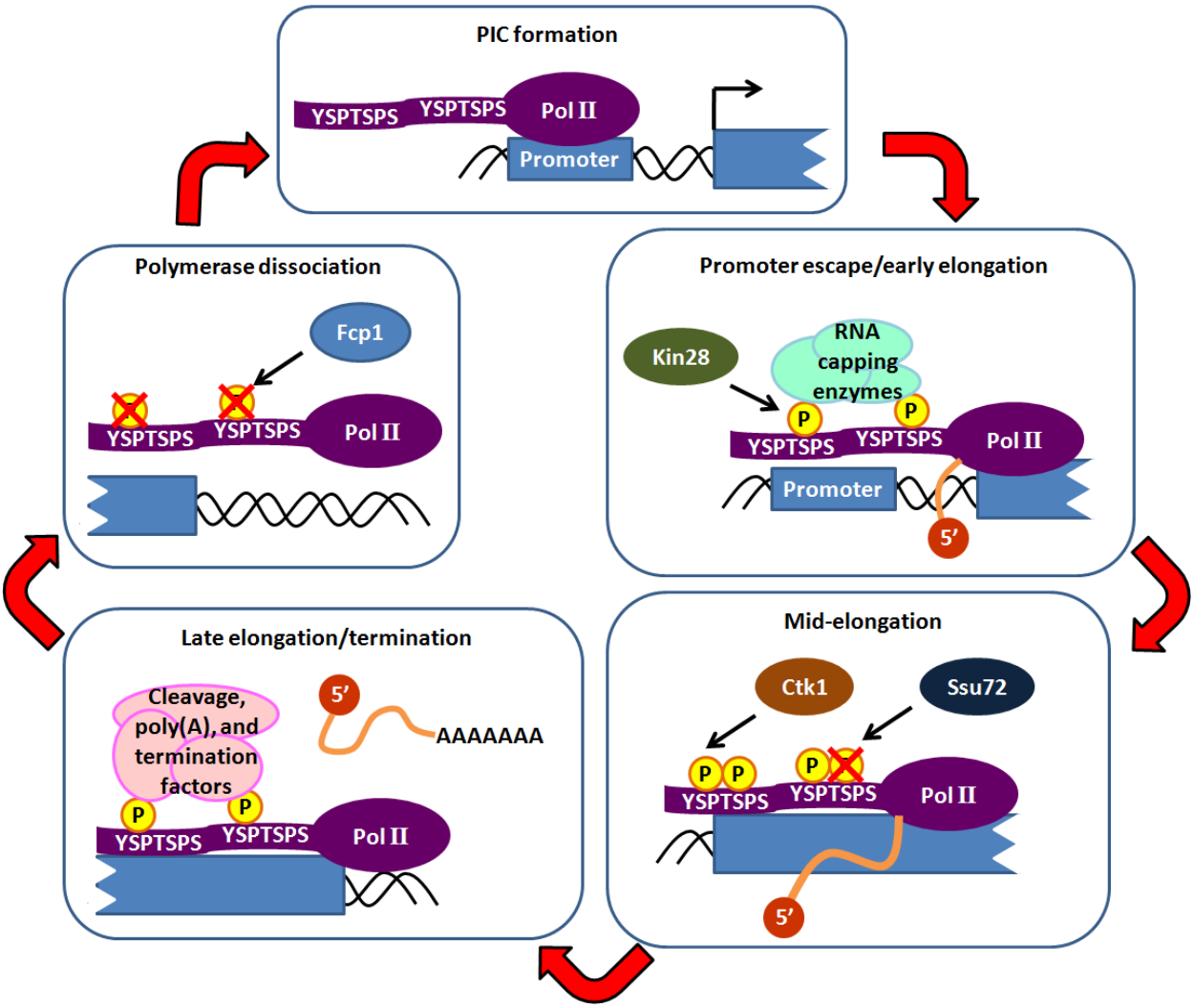
PIC formation
YSPTSPS
YSPTSPS
Pol II
Promoter
Promoter escape/early elongation
Kin28
RNA capping enzymes
P
P
YSPTSPS
YSPTSPS
Pol II
Promoter
5'
Mid-elongation
Ctk1
Ssu72
P
P
P
YSPTSPS
YSPTSPS
Pol II
5'
Late elongation/termination
Cleavage, poly(A), and termination factors
5'
AAAAAAA
P
P
YSPTSPS
YSPTSPS
Pol II
Polymerase dissociation
Fcp1
YSPTSPS
YSPTSPS
Pol II

Figure 7. Phosphorylation of the CTD is dynamically regulated throughout the transcription cycle.

The predominant form of Pol II recruited to the promoter during PIC formation is un- or hypomodified on the Rpb1 CTD. Coincident with promoter escape and the transition to early elongation, CTD repeats become phosphorylated on Ser5 by Kin28; this facilitates the recruitment of the pre-mRNA capping enzymes, which catalyze the addition of a methylguanosine cap to the 5' end of the emerging RNA (shown in orange). As transcription continues, Ser5 phosphorylation is removed from the CTD repeats by Ssu72, and the CTD is concomitantly phosphorylated on Ser2 by the Ctk1 kinase. Ser2 phosphorylated CTD repeats are essential for the recruitment of many cleavage, polyadenylation, and termination factors; the association of these factors peak during late elongation and as Pol II traverses the poly(A) site. Downstream of the poly(A) site, CTD phosphorylation at Ser2 is removed by the Fcp1 phosphatase and the polymerase dissociates from the template. Dephosphorylation of Pol II renders it competent to initiate another round of transcription. This figure is an adaptation of Figure 1 in PRELICH 2002.

As transcription progresses, Ser5 phosphorylation is removed by the Ssu72 phosphatase, resulting in a graded pattern of Ser5 phosphorylation across the ORF in which it is highest at the 5' end. Conversely, CTD repeats are phosphorylated on the serine at position 2 (Ser2) by the Ctk1 kinase beginning in early elongation and intensifying as the polymerase reaches the 3' end of the ORF. The activity of Ctk1, therefore, results in a Ser2 phosphorylation pattern that is inverse to that of Ser5 phosphorylation. Phosphorylation of CTD repeats at Ser2 facilitates the recruitment of factors involved in late transcriptional events including 3' end processing, transcript termination, and mRNA export (described in more detail in Sections 1.4.3 and 1.4.4). As Pol II encounters the polyadenylation site, Ser2 phosphorylation is removed by the Fcp1 phosphatase. This restores the CTD to its unmodified form, thus rendering the polymerase competent to reinitiate transcription of additional RNAs once it dissociates from the template.

In addition to the CTD-interacting factors described here, a plethora of additional proteins participate in transcription. These factors facilitate recruitment of Pol II to gene promoters, modification of the chromatin template during transcription, reactivation of stalled polymerases, 3' end formation of the RNA, transcript termination, and export of mRNA to the cytoplasm for translation. Factors that play prominent roles in these processes are detailed in the following sections.

1.4.1 Promoter Binding and Transcription Initiation

Genes are divided into coding regions and control regions. Coding regions contain the DNA sequence information necessary to instruct production of the gene's RNA or protein product. Control regions, or promoters, are composed of various DNA sequence elements that direct the binding and positioning of transcriptional activators, coactivators, and the basal

transcription machinery. Approximately 20% of eukaryotic promoters contain an element known as a TATA box, which generally conforms to the consensus sequence TATA(A/T)A(A/T)N and is found approximately 40 to 120 bases upstream of the transcription start site in *S. cerevisiae* (BASEHOAR *et al.* 2004; BUCHER 1990; STRUHL 1989). The TATA box is involved in assembly and positioning of the preinitiation complex (PIC), which is composed of Pol II and several general transcription factors (GTFs).

Eukaryotic promoters contain several additional conserved sequence elements that contribute to transcription initiation. The initiator (Inr) element defines the transcription start site and generally contains an adenosine at the transcription start site, a cytosine at the -1 position, and several neighboring pyrimidines (SMALE and KADONAGA 2003). Many TATA-less promoters in higher eukaryotes also contain a downstream promoter element (DPE). This element contains sequences that interact with the GTFs TFIID and TFIIB to define the site of PIC assembly in the absence of a TATA box (KUTACH and KADONAGA 2000; SMALE and KADONAGA 2003). Many gene promoters also contain upstream activating sequences (UASs) or upstream repressing sequences (URSs); these sequence elements frequently contain binding sites for sequence-specific transcriptional activators or repressors.

1.4.1.1 Assembly of the Preinitiation Complex

Pol II is insufficient to initiate transcription on its own and requires interaction with the GTFs to stably associate with gene promoters (HA *et al.* 1993). In vitro experiments demonstrated that the GTFs assemble sequentially into the PIC during transcription activation (Figure 8) (BURATOWSKI *et al.* 1989; FANG and BURTON 1996; HA *et al.* 1993). The first step in PIC assembly is binding of the TATA binding protein (TBP) to the TATA box. TBP, which is encoded by the *SPT15* gene in *S. cerevisiae*, is a component of the GTF TFIID. In addition to

TBP, TFIID also contains at least 12 TBP-associated factors, or TAFs, which interact with promoter sequences other than the TATA box, as well as activators and coactivators, to enhance or restrict interaction of TBP with the TATA box (WOYCHIK and HAMPSEY 2002). At TATA-less promoters, the TAFs and the GTF TFIIB are critical for positioning the PIC through association with other promoter elements. Binding of TFIID to the TATA box is enhanced by the subsequent association of TFIIA (ORPHANIDES *et al.* 1996). PIC formation continues with the recruitment of TFIIB, which interacts asymmetrically with the DNA upstream and downstream of TATA-associated TFIID. Transcription generally initiates from a PIC in only one direction, and the asymmetrical binding of TFIIB is thought to impart this directionality (NOGALES 2000). TFIIB physically interacts with a complex of TFIIF and Pol II, which is unmodified on its CTD, and recruits it to the promoter (FLORES *et al.* 1989). The association of Pol II-TFIIF stabilizes the interaction of TFIIB and TFIID with promoter DNA and facilitates the recruitment of TFIIE (BURATOWSKI *et al.* 1989; FLORES *et al.* 1989). TFIIE appears to assist closure of Pol II around the DNA template and to stimulate TFIIH's catalytic activities (NOGALES 2000; WOYCHIK and HAMPSEY 2002). TFIIH, the final GTF recruited to the PIC, contains at least two known catalytic functions. It contains two helicases of opposite polarity, Rad3 and Rad25, which are believed to unwind the DNA template to facilitate Pol II binding (GUZMAN and LIS 1999; WOYCHIK and HAMPSEY 2002).

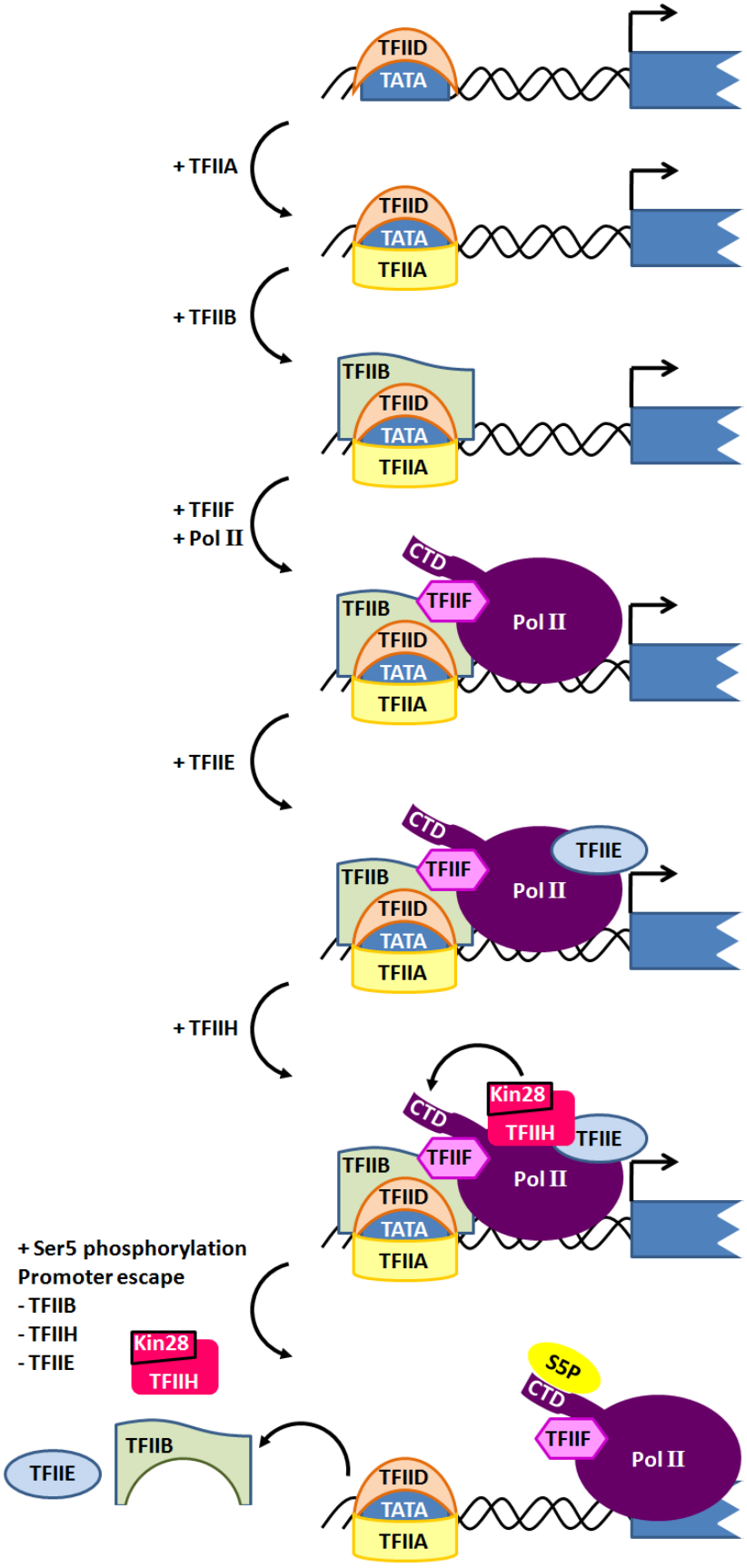
TFIID
TATA
+ TFIIA
TFIID
TATA
TFIIA
+ TFIIB
TFIIB
TFIID
TATA
TFIIA
+ TFIIF
+ Pol II
CTD
TFIIB
TFIIF
Pol II
TFIID
TATA
TFIIA
+ TFIIE
CTD
TFIIE
TFIIB
TFIIF
Pol II
TFIID
TATA
TFIIA
+ TFIIH
Kin28
CTD
TFIIH
TFIIE
TFIIB
TFIIF
Pol II
TFIID
TATA
TFIIA
+ Ser5 phosphorylation
Promoter escape
- TFIIB
- TFIIH
- TFIIE
Kin28
TFIIH
S5P
CTD
TFIIF
Pol II
TFIIB
TFIIE
TFIID
TATA
TFIIA

Figure 8. Assembly of the preinitiation complex.

The preinitiation complex (PIC) is composed of Pol II and several general transcription factors (GTFs). In vitro, the GTFs and Pol II are recruited in a stepwise fashion, as described in the text; the order of GTF and Pol II recruitment is illustrated. Following promoter escape, a subset of GTFs remains at the promoter to facilitate additional rounds of transcription. This figure is an adaptation of Figure 1 in MARTINEZ 2002.

Although PIC assembly is complete upon the association of TFIIH, in vitro experiments have demonstrated that Pol II does not immediately transition to an elongation-competent form. Initially, Pol II remains associated with the promoter as it generates short 8-10 bp transcripts; a phenomenon known as abortive initiation. The exact trigger that releases Pol II from the promoter is not entirely understood, but it appears to involve a stable association with the template DNA and the availability of necessary cofactors and nucleotides (DVIR 2002). Additionally, Pol II becomes phosphorylated at Ser5 coincident with promoter escape; this activity is mediated by the Kin28 kinase, which is a subunit of TFIIH (MARTINEZ 2002; PRELICH 2002). Whether Ser5 phosphorylation precedes or immediately follows promoter escape is unknown. Upon promoter clearance, a subset of the GTFs remain assembled at the promoter, often in contact with activator and coactivator proteins, which facilitates reinitiation during subsequent rounds of transcription of the same gene (MARTINEZ 2002; YUDKOVSKY *et al.* 2000).

The stepwise model of PIC assembly was determined through in vitro experiments. It has also been proposed that the PIC may be recruited to gene promoters as a "holoenzyme" in which Pol II is preassembled with the GTFs and coactivator proteins (BURATOWSKI 2000). In vivo PIC formation likely proceeds by a mechanism that is intermediate to the stepwise and holoenzyme models and that the precise pathway of PIC formation is likely to differ at individual promoters.

1.4.1.2 Transcriptional activators and coactivators

The PIC is sufficient to support basal transcription from a promoter, but the level of gene expression must often be enhanced or reduced to meet the physiological needs of the cell. The intensity and timing of gene expression is generally controlled through the regulated binding of sequence-specific DNA binding proteins, known as transcriptional activators or repressors, to

sites within the gene promoter. Transcriptional activators, such as Gal4, Gcn4, and Swi5 in *S. cerevisiae*, are typically composed of a DNA binding domain and an activation domain. The DNA binding domain of a transcriptional activator interacts with a binding site in the promoters of its target genes. Activation domains enhance transcription by physically interacting with the basal transcription machinery and facilitating its association with the promoter or by recruiting coactivators (discussed in more detail in the following paragraph). Transcriptional repressors generally function to antagonize assembly of the PIC, block the access of transcriptional activators to their binding sites, or interfere with the downstream functions of activators by physically interacting with activation domains.

Coactivators, which are generally thought to lack sequence-specific DNA-binding activity, are typically recruited to promoters through physical interactions with transcriptional activators. Coactivators usually function either to establish a physical link between the transcriptional activator and Pol II or to modify chromatin structure at the promoter; both of these modes of functionality are believed to assist PIC formation. Coactivator proteins that are known to influence the structure of promoter chromatin include the SWI/SNF chromatin remodeling complex and the SAGA HAT complex. These proteins appear to stimulate transcription by creating a more accessible chromatin structure at the promoter that facilitates binding of the GTFs and Pol II. Mediator, which is composed of 20 subunits, is the most recognizable representative of coactivators that function to facilitate and stabilize the binding of Pol II at gene promoters. This complex, which is believed to function at the majority of yeast promoters, physically interacts with many transcriptional activators and with Pol II (KORNBERG 2005). Mediator has also been demonstrated to interact with several transcriptional repressors to inhibit transcription initiation. Several of the TAFs associated with TFIID also function as co-

activators that appear to stabilize TBP binding through interaction with transcriptional activators and promoter elements (FEATHERSTONE 2002; WOYCHIK and HAMPSEY 2002).

1.4.2 Transcription Elongation

Once Pol II clears the promoter, it enters the ORF and begins incorporating free nucleotides into a growing RNA chain during the elongation phase of the transcription cycle. Transcription elongation is a highly regulated process which requires numerous accessory factors. Crystallographic studies have demonstrated that the structure of Pol II is modified in several ways following the initiation to elongation transition (GNATT 2002); these structural modifications may generate new protein-protein interaction surfaces which function to recruit transcription elongation factors to the transcribing polymerase. Transcription elongation factors have been identified that physically interact with Pol II to coordinate cotranscriptional modification of the nascent mRNA or chromatin and to reactivate Pol II that has stalled or arrested within the ORF. Additional factors are known to associate directly with the chromatin template to modulate its structure during transcription. Four yeast transcription elongation factors with particular relevance to the data presented in this document, the Paf1 complex, the Spt4/Spt5 complex, TFIIS, and yFACT, are described in more detail in the following sections.

1.4.2.1 Paf1 complex

The *S. cerevisiae* Paf1 complex, minimally composed of Paf1, Ctr9, Rtf1, Cdc73, and Leo1, colocalizes with Pol II from the transcription start site to the poly(A) site of actively transcribed genes (KAPLAN *et al.* 2005; KROGAN *et al.* 2002b; MUELLER and JAEHNING 2002; POKHOLOK *et al.* 2002; SIMIC *et al.* 2003; SQUAZZO *et al.* 2002). In the absence of Rtf1 or

Cdc73, the remaining Paf1 complex subunits remain physically associated with each other, but are not recruited to genes and no longer interact with Pol II (MUELLER *et al.* 2004). Paf1, Cdc73, and Rtf1 are present in the cell at about 25,000 copies each, which is roughly equivalent to the expression level of Rpb3 (the 3rd largest subunit of Pol II) (MUELLER *et al.* 2004; PORTER *et al.* 2005); this suggests that there is a Paf1 complex available for interaction with every Pol II in an individual cell. Consistent with this idea, the Paf1 complex has been found to associate with every active ORF that has been tested.

Despite their ubiquitous association with active genes, the subunits of the Paf1 complex are not essential for cell viability. However, deletion of genes encoding the Paf1 complex results in numerous phenotypes. Deletion of *CTR9* and *PAF1* result in a severe growth defect, deletion of *CDC73* causes an intermediate growth phenotype, and deletion of *RTF1* or *LEO1* cause only minor or undetectable effects on cell growth (BETZ *et al.* 2002). This pattern of mutational severity (*paf1Δ = ctr9Δ > cdc73Δ > rtf1Δ = leo1Δ*) is reflected in several other phenotypes caused by deletion of the genes encoding the Paf1 complex. These phenotypes include genetic interaction with CTD truncations or deletion of the gene encoding the H3 K36 methyltransferase Set2 and sensitivity to the base analog 6-azauracil (6-AU) (CHU *et al.* 2007; SQUAZZO *et al.* 2002). These observations suggest that individual subunits of the Paf1 complex are necessary for distinct functions.

Microarray and other gene expression analyses have determined that the Paf1 complex affects the expression of a subset of genes. Most genes whose expression is affected by the Paf1 complex are involved in cell wall biosynthesis, rRNA maturation, and cell cycle progression (KOCH *et al.* 1999; PENHEITER *et al.* 2005; PORTER *et al.* 2005). Accordingly, deletion of *PAF1* causes phenotypes associated with cell wall defects and a decreased tolerance to cell stress (BETZ

et al. 2002). These phenotypes include sensitivity to high temperature, caffeine, and SDS. Interestingly, 68% of genes who expression is downregulated in a *paf1Δ* strain are essential, and many of these genes are also downregulated in the absence of Ctr9 (PENHEITER *et al.* 2005). These observations likely account for the severe growth defects observed in *paf1Δ* and *ctr9Δ* cells.

The severity of phenotypes caused by loss of Paf1 or Ctr9 may also be accounted for by the observation that these subunits are required for normal levels of Rtf1, Cdc73, and Leo1, while the reverse effect is not observed (MUELLER *et al.* 2004; PORTER *et al.* 2005). Intriguingly, Paf1 and Ctr9 appear to retain functionality even when they are not associated with active genes or Pol II (MUELLER *et al.* 2004); this observation is supported by the less severe growth defect observed in *rtf1Δ* or *cdc73Δ* strains, in which the remaining Paf1 complex subunits are no longer associated with chromatin, as compared to the extreme slow growth of *paf1Δ* and *ctr9Δ* strains.

The Paf1 complex has been linked to the initiation, elongation, and termination stages of transcription. Database analyses indicate that the subunits of the Paf1 complex contain no recognizable functional motifs or domains suggestive of catalytic activity in their primary amino acid sequences. Instead, the Paf1 complex may represent a recruitment platform for other factors that positively stimulate transcription.

Paf1 and Cdc73 were initially purified in association with a component of the GTF TFIIH and Rpb1 that is unphosphorylated on its CTD (the predominant form of Pol II found in the PIC) (SHI *et al.* 1997). However, subsequent studies have demonstrated that a significantly greater amount of Paf1 complex interacts with Rpb1 that is phosphorylated at Ser5 or Ser2 of the CTD (MUELLER *et al.* 2004; QIU *et al.* 2006). This observation suggests that the Paf1 complex predominantly associates with elongating polymerase. Additionally, expression of luciferase

was unaffected by the deletion of Paf1 in strains where the gene encoding luciferase was placed under the control of promoters from genes that depend on the Paf1 complex for normal expression (PENHEITER *et al.* 2005). These observations suggest that the Paf1 complex does not play a major role in transcription initiation.

The presence of the Paf1 complex across the entirety of active ORFs implies that this complex functions during transcription elongation. An initial link between the Paf1 complex and transcription elongation was suggested by the results of a screen for factors that become essential in the absence of the Paf1 complex subunit Rtf1. This screen identified mutations in the CTD Ser2 kinase Ctk1, the CTD Ser2 phosphatase Fcp1, and the Pob3 component of the yFACT histone chaperone/transcription elongation complex (COSTA and ARNDT 2000); these factors all have established roles in transcription elongation. The Rtf1 subunit of the Paf1 complex is also known to physically interact with the Chd1 chromatin remodeling enzyme, which has been linked to transcription elongation, and this interaction is necessary for normal recruitment of Chd1 to active genes (SIMIC *et al.* 2003).

Additional subunits of the Paf1 complex have subsequently been implicated in transcription elongation. Deletion of the genes encoding subunits of the Paf1 complex results in genetic interaction with mutations in known elongation factors (Spt4, Spt5, and TFIIS) and sensitivity to the base analog 6-AU (SQUAZZO *et al.* 2002). Exposure to 6-AU results in reduction of intracellular nucleotide pools (EXINGER and LACROUTE 1992); this is believed to increase polymerase stalling and arrest and result in a greater dependence on elongation factors for normal transcription. Therefore, sensitivity to 6-AU is a hallmark of defects in transcription elongation. Physical interactions have been identified between the Paf1 complex and other protein complexes which are known to affect transcription elongation, including the Spt4/Spt5

and yFACT complexes (Figure 9) (SQUAZZO *et al.* 2002). Additionally, the Spt4/Spt5 and Bur1/Bur2 transcription elongation complexes are necessary for normal recruitment of the Paf1 complex to active genes (LARIBEE *et al.* 2005; QIU *et al.* 2006).

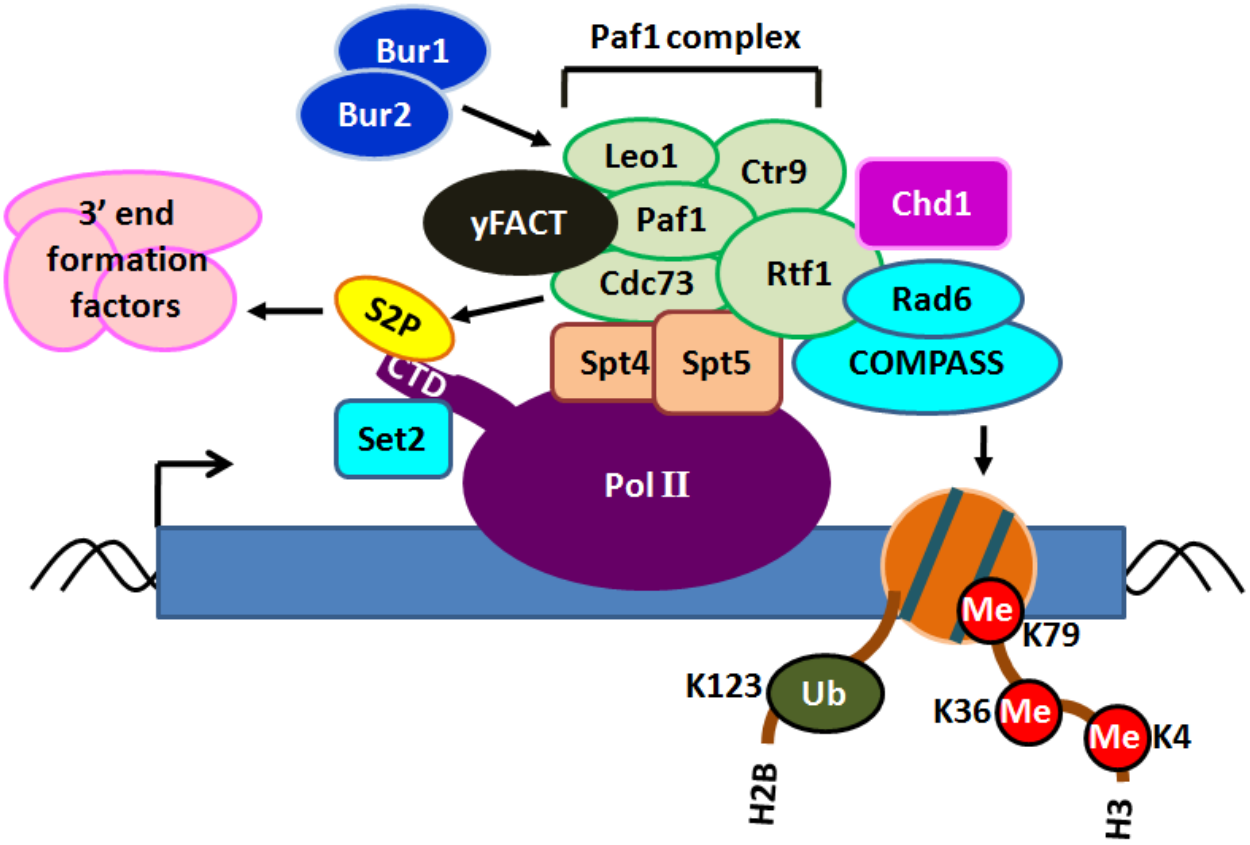

Figure 9. Physical interactions and functions of the Paf1 complex.

The Paf1 complex physically interacts with the Spt4/Spt5 and yFACT transcription elongation complexes. The Spt4/Spt5 and Bur1/Bur2 complexes are required for association of the Paf1 complex with active genes; a physical interaction between the Paf1 and Bur1/Bur2 complexes has not been demonstrated. The Paf1 complex is required for normal levels of Ser2 CTD phosphorylation, which is necessary for normal recruitment of the H3 K36 methyltransferase Set2 and several factors involved in RNA 3' end formation. Rtf1 is known to physically interact with Chd1 and mediate its full recruitment to active ORFs. Subunits of the Paf1 complex are also required for normal association of the Rad6 ubiquitin conjugating enzyme and the COMPASS complex (which contains the Set1 H3 K4 methyltransferase) with active genes. In addition to its role in the recruitment of histone modifying enzymes, the Paf1 complex affects multiple covalent histone modifications: H2B K123 Ub and methylation of H3 at K4, K36, and K79. The Paf1 complex affects only trimethylation of H3 K36. S2P = Ser2 phosphorylation

A known Paf1 complex-dependent function associated with transcription elongation is the covalent modification of specific lysine residues on histones H2B and H3 (Figure 9). Monoubiquitylation of histone H2B at lysine 123 (K123) by the ubiquitin-conjugating enzyme Rad6 and the ubiquitin-protein ligase Bre1 is eliminated in strains lacking Rtf1 or Paf1 (NG *et al.* 2003b; WOOD *et al.* 2003b). This modification is a prerequisite for the methylation of histone H3 on K4 and K79 by the methyltransferases Set1 and Dot1, respectively (BRIGGS *et al.* 2002; SUN and ALLIS 2002). Therefore, Rtf1 and Paf1 are also required for histone H3 K4 and K79 methylation (KROGAN *et al.* 2003; NG *et al.* 2003b; NG *et al.* 2003c). Interestingly, low levels of monomethylation at H3 K4 are still detected in *rtf1Δ* and *paf1Δ* cells, suggesting that the Paf1 complex controls the processivity of Set1 (DEHE *et al.* 2005). Rtf1 is likely the primary component of the Paf1 complex that regulates these modifications because Rtf1 levels are significantly reduced in strains lacking Paf1, while Paf1 levels remain unchanged in the absence of Rtf1 (MUELLER *et al.* 2004; PORTER *et al.* 2005).

H2B K123 ubiquitylation and H3 K4 methylation are enriched in the coding regions of active genes (BERNSTEIN *et al.* 2002; SANTOS-ROSA *et al.* 2002; XIAO *et al.* 2005). Rad6 and Set1 are recruited to ORFs coincident with gene activation and modify histones during transcription. Rtf1 colocalizes with these histone modifying enzymes on active genes and is required for their optimal recruitment and activation (KROGAN *et al.* 2003; NG *et al.* 2003c; XIAO *et al.* 2005). Rtf1-dependent histone modifications are required for normal Sir-mediated silencing (HUANG *et al.* 1997; KROGAN *et al.* 2002a; VAN LEEUWEN *et al.* 2002). Consistent with this observation, mutations in the Paf1 complex lead to defects in telomeric and rDNA silencing (KROGAN *et al.* 2003; MUELLER *et al.* 2006; NG *et al.* 2003b).

The Paf1 complex also has additional roles in histone modification. Histone H3 is also methylated on lysine 36 (K36) by the Set2 methyltransferase (STRAHL *et al.* 2002). The trimethyl state of this modification is severely reduced by the deletion of *PAF1* or *CTR9* and is decreased in the absence of *CDC73*; mono- and dimethylation of this residue are unaffected (CHU *et al.* 2007). Interestingly, deletion of *RTF1*, which has severe effects on H3 K4 and K79 methylation, has no effect on methylation of K36. Chromatin immunoprecipitation analysis demonstrated that H3 K36 trimethylation was severely reduced in the coding regions of several genes and that this reduction was greater at the 5' end of the ORF than at the 3' end of the ORF.

The decrease in H3 K36 trimethylation caused by loss of Paf1 correlates with an increase in acetylation of histones H3 and H4 (CHU *et al.* 2007). Histone acetylation and H3 K36 methylation have previously been linked; Set2-mediated H3 K36 methylation results in recruitment of the Rpd3(S) histone deacetylase complex, which removes histone acetylation from coding regions to prevent aberrant initiation from cryptic promoters (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; LI *et al.* 2007b). Interestingly, deletion of *SET2* does not result in increased acetylation at the 5' ends of coding regions, as is seen in *paf1Δ* strains (CHU *et al.* 2007). Additionally, a *paf1Δ set2Δ* strain results in a significant increase in cryptic initiation from the *FLO8* ORF than is observed in either single mutant. These observations suggest the Paf1 complex functions to control cryptic initiation and histone acetylation in the bodies of active genes by a mechanism that is redundant with the function of Set2.

The Paf1 complex is also connected to the termination stage of transcription through several recognized roles in RNA 3' end formation. Components of the Paf1 complex are necessary for normal Ser2 phosphorylation of the Rpb1 CTD (MUELLER *et al.* 2004). This modification is necessary for the cotranscriptional recruitment of numerous cleavage,

polyadenylation, and termination factors to transcribing Pol II. Specifically, the Paf1 complex is known to be required for association of the cleavage factors Cft1 and Pcf11 with active genes (MUELLER *et al.* 2004; NORDICK *et al.* 2008). A physical interaction has also been identified between the Paf1 complex and Hpr1, a subunit of the TREX transcription elongation and mRNA export complex (CHANG *et al.* 1999). Consistent with a role in pre-mRNA cleavage and polyadenylation, mutations in the Paf1 complex are known to affect poly(A) site selection and result in the shortening of poly(A) tails from the normal length of 75 nucleotides to 65 nucleotides (MUELLER *et al.* 2004; PENHEITER *et al.* 2005). These effects on RNA 3' end formation can lead to nonsense mediated decay of the resulting transcripts and it has been demonstrated that the decreased mRNA levels observed for some Paf1 complex-dependent genes are a result of mRNA instability and not reduced gene expression (PENHEITER *et al.* 2005).

The Paf1 complex also affects 3' end formation of several nonpolyadenylated transcripts. Deletion of genes encoding subunits of the Paf1 complex leads to transcriptional readthrough of the *SNR13* and *SNR47* snoRNA genes, resulting in the production of hybrid transcripts that extend into the neighboring gene (SHELDON *et al.* 2005). snoRNAs are not polyadenylated; instead, their 3' ends are generated through the function of the Nab3 and Nrd1 heterologous nuclear ribonucleoproteins and the nuclear exosome (STEINMETZ *et al.* 2001). The role of the Paf1 complex in 3' end formation of nonpolyadenylated RNAs can, therefore, likely be attributed to its involvement in the recruitment of Nab3 and Nrd1 to snoRNA genes (SHELDON *et al.* 2005).

Despite the evidence linking it to transcription termination through its roles in 3' end formation, the Paf1 complex is primarily considered an elongation factor. While a direct role for the Paf1 complex in transcription termination cannot be ruled out, it is known to dissociate from

Pol II at the poly(A) site and has not been found to associate with RNA (KAPLAN *et al.* 2005); these observations make it unlikely that the Paf1 complex participates directly in 3' end formation or transcription termination. The known roles for the Paf1 complex in 3' end formation or transcription termination can largely be attributed to the role of this complex in facilitating phosphorylation of Rpb1 CTD repeats on Ser2, which occurs during transcription elongation.

Although there is considerable genetic and biochemical evidence linking the Paf1 complex to transcription elongation, the absence of Paf1 complex subunits appears to have relatively minor effects on the distribution of Pol II throughout the genome. However, Pol II levels were assessed at constitutively active genes in many of these studies. Recent reports have demonstrated that Pol II levels are reduced at the 5' end of the galactose-inducible *GAL1* gene shortly after activation in strains lacking Paf1 complex subunits (MARTON and DESIDERIO 2008; XIAO *et al.* 2005). Additionally, the sensitivity of strains lacking Paf1 complex subunits to heat and caffeine suggests that this complex may be involved in the cellular stress response, which is largely mediated by rapid gene induction. These results may imply that the Paf1 complex is necessary to efficiently promote optimal gene expression during the pioneering rounds of transcription and that it may become less important during the maintenance of steady-state gene expression. This hypothesis is consistent with the known roles for the Paf1 complex in creating a more permissible chromatin structure in ORFs through it roles in histone modification.

Further supporting the idea that the Paf1 complex contributes to a more accessible chromatin structure, several studies have demonstrated that histone H3 levels are elevated in ORFs in the absence of Paf1 complex subunits (DEHE *et al.* 2005; MARTON and DESIDERIO 2008). Interestingly, deletion of genes encoding the subunits of the Paf1 complex often cause

more severe phenotypes than mutation of the histone modifying enzymes that mediate Paf1 complex-dependent histone modifications. This suggests that the function of the Paf1 complex is not solely mediated through its effects on histone modification. The role of Rtf1 in recruiting Chd1 to ORFs, or an as-yet-unidentified function for the Paf1 complex, may also contribute to modifying chromatin structure upon gene activation.

The subunits of the Paf1 complex are conserved throughout eukaryotes and they have been demonstrated to carry out many of the same functions as their *S. cerevisiae* counterparts. Deletion or multiplication of the genes encoding the human homologs of Paf1 complex subunits have been implicated in the etiology of numerous cancers, and the homologs of *Drosophila melanogaster* (fly) Paf1 complex subunits are involved in Wnt and Notch signaling (AKANUMA *et al.* 2007; CHAUDHARY *et al.* 2007; TENNEY *et al.* 2006). Interestingly, while Rtf1 is conserved throughout eukaryotes, it is absent from the human and fly Paf1 complex equivalents (CHAUDHARY *et al.* 2007; YART *et al.* 2005; ZHU *et al.* 2005). Additionally, the human Paf1 complex has incorporated the Ski8 component of the human SKI complex, which cooperates with the exosome to mediate 3'–5' mRNA decay (ZHU *et al.* 2005). This observation implies that the human Paf1 complex has incorporated additional functions.

1.4.2.2 Spt4/Spt5 complex

Spt4 physically associates with the essential Spt5 protein in vivo to form the Spt4/Spt5 transcription elongation complex in *S. cerevisiae* (HARTZOG *et al.* 1998). This complex is conserved in higher eukaryotes, where it is known as DSIF (DRB sensitivity-inducing factor). Human DSIF was identified as a factor that induced Pol II pausing in conjunction with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (WADA *et al.* 1998). The Spt4/Spt5 complex is recruited to the ORFs of active genes and is known to

physically interact with Pol II and multiple elongation factors including TFIIS, yFACT, the Paf1 complex, and Spt6 (KROGAN *et al.* 2002b; LINDSTROM *et al.* 2003; MUELLER and JAEHNING 2002; SQUAZZO *et al.* 2002). Spt5 shares a region of homology with the bacterial elongation factor NusG (WADA *et al.* 1998). These observations suggested a role for the Spt4/Spt5 complex in transcription elongation and it has been demonstrated to affect this process both positively and negatively (HARTZOG *et al.* 1998; WADA *et al.* 1998). Mutations in *SPT4* and *SPT5* cause growth defects when combined with CTD truncations or mutations in CTD kinases, suggesting that the Spt4/Spt5 complex affects transcription through a pathway that functions in parallel to the CTD of Rpb1 (LINDSTROM and HARTZOG 2001). Despite the experimental evidence linking the Spt4/Spt5 complex to transcription elongation, the mechanism by which it participates in this process remains unknown and the subunits of this complex are not predicted to function enzymatically.

The Spt4/Spt5 complex is also known to interact with transcription initiation factors, RNA processing factors, and mRNA capping enzymes and to affect polyadenylation of mRNA (CUI and DENIS 2003; LINDSTROM *et al.* 2003). Additionally, Spt4 and Spt5 associate with Pol I and are necessary for normal expression of rRNA (SCHNEIDER *et al.* 2006). These observations suggest that the function of the Spt4/Spt5 complex is not restricted specifically to transcription elongation by Pol II.

1.4.2.3 TFIIS

Pol II is known to stall or arrest as it translocates across an ORF during transcription (KIREEVA *et al.* 2005). Pausing or arrest of Pol II can be induced by chromatin structure or by interaction with unfavorable nucleotide sequences. Arrested Pol II can be reactivated by the conserved transcription elongation factor TFIIS, which is encoded by the *DST1* gene

(KETTENBERGER *et al.* 2003; RUDD *et al.* 1994; WEILBAECHER *et al.* 2003). Deletion of *DST1* results in growth defects when strains harboring this deletion are grown in the presence of 6-AU or are combined with deletion in genes encoding other elongation factors, including components of the Paf1, Spt4/Spt5, and the yFACT complexes (COSTA and ARNDT 2000; HARTZOG *et al.* 1998; ORPHANIDES *et al.* 1999). These observations implicate TFIIS in transcription elongation.

TFIIS is known to physically interact with Pol II and the structure of this interaction has been solved (KETTENBERGER *et al.* 2004). TFIIS binds to the surface of Pol II near the pore through which nucleotides enter the active site. A hairpin loop extending from TFIIS inserts into the pore where it stimulates an intrinsic cleavage activity of Pol II. This activity repositions the new RNA 3' end properly in the Pol II active site and stimulates the resumption of transcription.

1.4.2.4 yFACT

The FACT (FAcilitates Chromatin Transcription) complex was first identified in HeLa cell nuclear extracts as a factor that stimulates transcription on a chromatin template in vitro (ORPHANIDES *et al.* 1998). This complex was found to be conserved in yeast (yFACT), where it is composed of the Spt16, Pob3, and Nhp6 subunits. ChIP analyses have demonstrated that yFACT associates with the coding regions of active genes and mutations in subunits of yFACT genetically interact with mutations in the transcription elongation factors TFIIS and Spt4 (MASON and STRUHL 2003; ORPHANIDES *et al.* 1998). Furthermore, yFACT is known to physically interact with the Chd1 chromatin remodeling enzyme, which has been linked to transcription elongation, and the Paf1 complex (KROGAN *et al.* 2002b; SQUAZZO *et al.* 2002). Together, these observations implicated yFACT in transcription elongation.

yFACT is also known to physically interact with histones and has been demonstrated to possess histone chaperone activity (ORPHANIDES *et al.* 1998). yFACT can stimulate the

displacement of an H2A/H2B dimer from a nucleosome, which is sufficient to permit the passage of Pol II (BELOTSERKOVSKAYA *et al.* 2003). These activities of yFACT are strongly enhanced in vitro by the addition of the Paf1 complex and H2B K123 Ub (PAVRI *et al.* 2006). The histone chaperone activity of yFACT has also been linked to the reassembly of chromatin following cessation of transcription (BELOTSERKOVSKAYA *et al.* 2003). These observations suggest that yFACT facilitates transcription elongation by creating a chromatin environment that allows Pol II to navigate the template more easily; yFACT then functions to reassemble chromatin once transcription has ceased.

1.4.3 3' End Formation and Transcription Termination at Protein Coding Genes

The final steps in production of a functional mRNA are cleavage and polyadenylation of the 3' end of the transcript. These processes are tightly coupled to dissociation of Pol II from the DNA template. The factors involved in these processes are frequently essential and many associate with Pol II on active genes through an interaction with Ser2 phosphorylated CTD repeats. Cleavage and polyadenylation take place in yeast at a site downstream of the coding region that contains 3 characteristic DNA sequence elements (Figure 10) (ZHAO *et al.* 1999). The most 5' component of the cleavage and polyadenylation signal is a UA-rich efficiency element, which functions to activate a nearby A-rich positioning element. Cleavage of the pre-mRNA typically takes place about 20 nucleotides downstream of the positioning element at a PyA_n (Py = pyrimidine) consensus site.

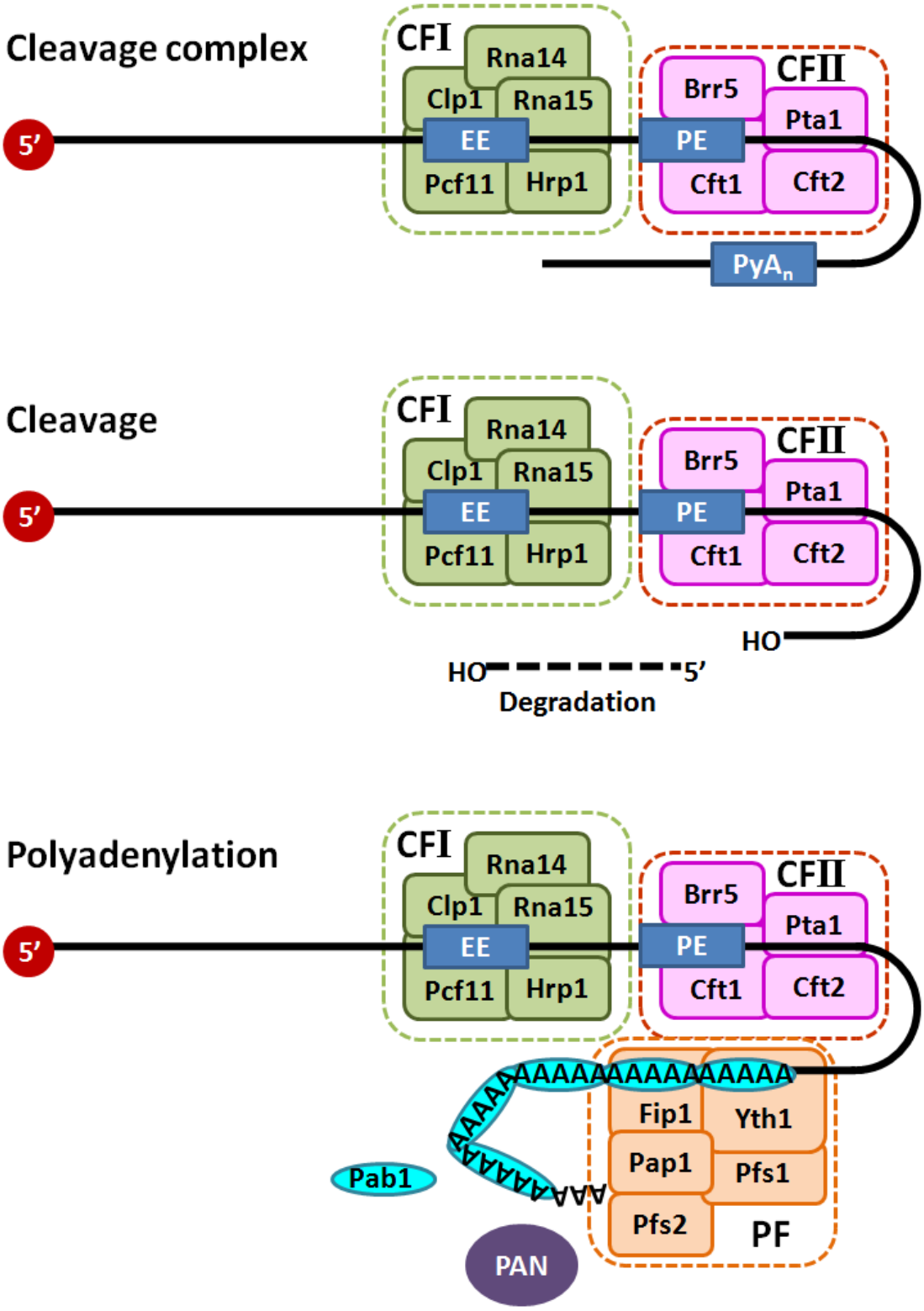
Cleavage complex
CFI
Rna14
Clp1
Rna15
EE
Pcf11
Hrp1
CFII
Brr5
Pta1
PE
Cft1
Cft2
5'
PyA$_n$
Cleavage
CFI
Rna14
Clp1
Rna15
EE
Pcf11
Hrp1
CFII
Brr5
Pta1
PE
Cft1
Cft2
5'
HO
HO
5'
Degradation
Polyadenylation
CFI
Rna14
Clp1
Rna15
EE
Pcf11
Hrp1
CFII
Brr5
Pta1
PE
Cft1
Cft2
5'
AAAAAAAAAAAAAAAAAA
AAAAAA
AAAAAA
Fip1
Yth1
Pap1
Pfs1
Pfs2
PF
Pab1
PAN

Figure 10. Factors involved in RNA 3' end formation.

Cleavage of a pre-mRNA requires two multisubunit complexes, CFI and CFII, which bind to the RNA. Two sequence elements, the efficiency element (EE) and the positioning element (PE), are involved in positioning of the cleavage complex. Cleavage takes place at a PyA_n (Py = pyrimidine) element that is usually found about 20 nucleotides downstream of the PE. Association of a third multisubunit complex (polyadenylation factor = PF), results in the addition of approximately 75 adenines to the new 3' OH on the pre-mRNA that was generated by the cleavage reaction. The addition of the poly(A) tail is catalyzed by poly(A) polymerase (Pap1). The activity of Pap1 is opposed by poly(A) nuclease (PAN). poly(A) binding protein (Pab1) coats the poly(A) tail to help stabilize the transcript. This figure is an adaptation of Figure 4 in ZHAO *et al.* 1999.

Transcript cleavage is carried out by cleavage factor I (CFI; composed of Rna14, Rna15, Pcf11, Clp1, and Hrp1) and cleavage factor II (CFII; composed of Cft1, Cft2, Brr5, and Pta1) (ZHAO *et al.* 1999). The exact sites at which CFI and CFII bind RNA are unknown, but these complexes are known to crosslink to RNA (MINVIELLE-SEBASTIA *et al.* 1998). The efficiency element and positioning element are believed to interact with CFI and CFII to direct cleavage activity to the PyA_n site. Although the identity of the endonuclease that carries out the cleavage reaction remains unknown, recent evidence suggests the Cft2 subunit of CFII may perform this activity (RYAN *et al.* 2004).

Following cleavage, polyadenylation factor (PF; composed of Fip1, Pap1, Yth1, Pfs1, and Pfs2), is recruited to the RNA through physical interaction with CFII and directs the addition of a poly(A) tail to the free 3' OH of the RNA at the cleavage site through the catalytic activity of Pap1 (poly(A) polymerase) (PREKER *et al.* 1997; ZHAO *et al.* 1999). The action of Pap1 is opposed by poly(A) nuclease (PAN) (LOWELL *et al.* 1992); together, the antagonism between these enzymes results in the formation of a poly(A) tail that typically measures 75 nucleotides in length (BLOCH *et al.* 1978). The poly(A) tail is coated by poly(A) binding protein (Pab1), which protects the mature mRNA transcript from degradation in both the nucleus and cytoplasm (ZHAO *et al.* 1999).

Transcription termination at protein coding genes in yeast does not appear to take place at a consensus sequence, but instead occurs stochastically downstream of the poly(A) site. Eukaryotic transcription has been proposed to take place by two mechanisms: the "anti-terminator model" and the "torpedo model". Experimental evidence supports both hypotheses, suggesting that distinct mechanisms may operate downstream of specific genes. In the anti-terminator model, the addition of the poly(A) tail to the transcript may lead to the displacement

of a positive elongation factor or the association of a negative elongation factor (BURATOWSKI 2005; LOGAN *et al.* 1987). This may lead to increased stalling and pausing of the polymerase, making it more prone to dissociate from the DNA template. Interestingly, the Paf1 complex is known to dissociate from Pol II near the poly(A) site and deletion of genes encoding Paf1 complex subunits lead to shortened poly(A) tails (KAPLAN *et al.* 2005; MUELLER *et al.* 2004).

Alternatively, the torpedo model of transcription termination proposes that cleavage of the transcript at the poly(A) site introduces a new 5' RNA end that is not capped; this may create a loading point for an exonuclease or helicase that tracks along the transcript until it reaches the polymerase and facilitates its dissociation from the DNA template (BURATOWSKI 2005). This model is supported by the physical interaction of the Rat1 5' to 3' exonuclease with Rtt103, a phospho-Ser2 CTD binding protein that is thought to play a role in mRNA 3' end formation and transcription termination (KIM *et al.* 2004b).

1.4.4 mRNA export

Before a mature mRNA can be translated into protein, it must be exported to the cytoplasm. mRNA is exported to the cytoplasm in association with proteins as a messenger ribonucleoprotein particle (mRNP) (Figure 11). Formation of mRNPs takes place cotranscriptionally. The mRNA export adaptor proteins Npl3, Yra1, and Sub2 physically interact with Pol II along active ORFs and are transferred to the growing pre-mRNA chain during synthesis (KOHLER and HURT 2007; ROUGEMAILLE *et al.* 2008). Yra1 and Sub2 mediate association of the assembling mRNP with the Mex67-Mtr2 export receptor complex. Mex67-Mtr2 physically interacts with the nuclear pore to facilitate movement of the mRNP through the channel (SANTOS-ROSA *et al.* 1998; SEGREF *et al.* 1997).

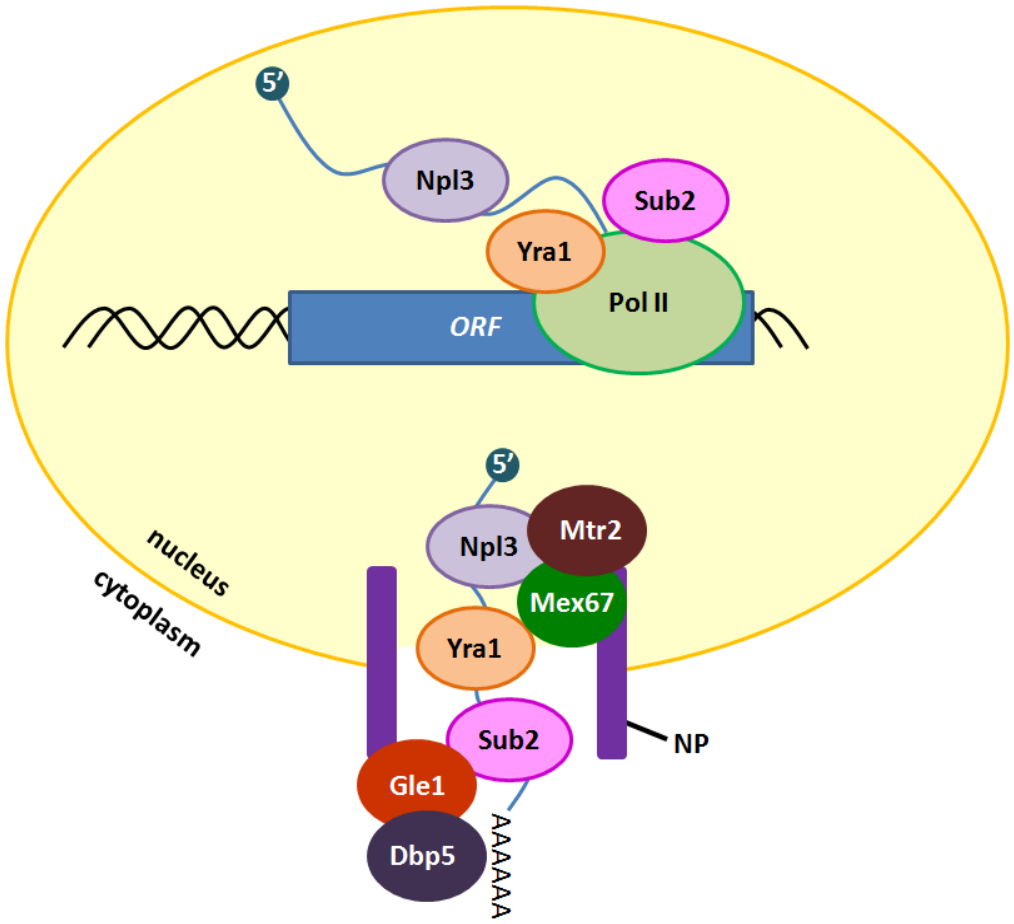

Figure 11. The mRNA export pathway.

mRNA is exported to the cytoplasm for transcription in complex with several proteins as a messenger ribonucleoprotein particle (mRNP). mRNPs are generated cotranscriptionally; the Yra3, Sub2, and Npl3 mRNA export adaptor proteins associate with Pol II and the bodies of active genes. These adaptor proteins associate with the pre-mRNA as it is being transcribed. Following transcription termination, the mRNP is released from the polymerase. Passage of the mRNP into the cytoplasm is facilitated by the Mex67-Mtr2 export receptor complex, which associates with the nuclear pore (NP). Backtracking of mRNPs into the nucleus is inhibited by the helicase activity of Dbp5, which is believed to unravel mRNPs as they enter the cytoplasm. The activity of Dbp5 is specifically stimulated in the cytoplasm by physical interaction with Gle1. This figure is an adaptation of Figure 1 in ROUGEMAILLE *et al.* 2008.

To prevent backtracking of the mRNP through the NPC, mRNPs are remodeled as they emerge into the cytoplasm by the helicase activity of Dbp5 (LUND and GUTHRIE 2005). Although Dbp5 can shuttle between the nucleus and cytoplasm, its helicase activity is only activated in the cytoplasm. This is because activation of Dbp5 requires interaction with Gle1, which associates with filaments on the cytoplasmic side of the nuclear pore (ALCAZAR-ROMAN *et al.* 2006; WEIRICH *et al.* 2006). Association with Gle1 tethers Dbp5 activity in a position to unravel mRNPs immediately upon entry into the cytoplasm to prevent backtracking of these particles into the nucleus.

1.5 THE INOSITOL POLYPHOSPHATE SIGNALING PATHWAY AFFECTS TRANSCRIPTION

Signaling pathways represent a primary means by which cells sense and respond to changing environmental stimuli. A major signaling pathway utilized by eukaryotic organisms is the inositol polyphosphate signaling pathway. Inositol, a 6-carbon ring that bears a hydroxyl group on each carbon, is imported into cells or is synthesized from glucose-6-phosphate through the enzymatic activity of inositol-1-phosphate synthase (Ino1 in *S. cerevisiae*) (ALCAZAR-ROMAN and WENTE 2008). Inositol, which is essential for cell viability, is incorporated into membrane-anchored lipids resulting in the formation of phosphatidylinositols (PIPs). PIPs, which have recognized functions in vesicular trafficking and actin cytoskeleton rearrangements, can be phosphorylated in various arrangements.

PIP_2 (phosphatidylinositol 4,5 bisphosphate) is cleaved by phospholipase C (Plc1 in *S. cerevisiae*) to generate membrane-bound diacylglycerol and soluble inositol trisphosphate (IP_3)

(Figure 12) (FLICK and THORNER 1993). IP_3 is phosphorylated by the Arg82 kinase to inositol tetrakisphosphate (IP_4) and inositol pentakisphosphate (IP_5) (SAIARDI *et al.* 1999). IP_5 is further phosphorylated to inositol hexakisphosphate (IP_6) by the Ipk1 kinase (YORK *et al.* 1999). Although Arg82 and Ipk1 are localized to the nucleus, IPs are found throughout the cell (ALCAZAR-ROMAN and WENTE 2008). Plc1 is necessary for a variety of cellular functions including survival in nutrient-limited conditions, transition from budding to pseudohyphal growth, sporulation, heat-tolerance, and response to osmotic shock; the IP signaling pathway has also been implicated in DNA repair and telomere homeostasis (ALCAZAR-ROMAN and WENTE 2008; MICHELL *et al.* 2003). These observations suggest that the inositol polyphosphate signaling pathway is involved in the cellular stress response.

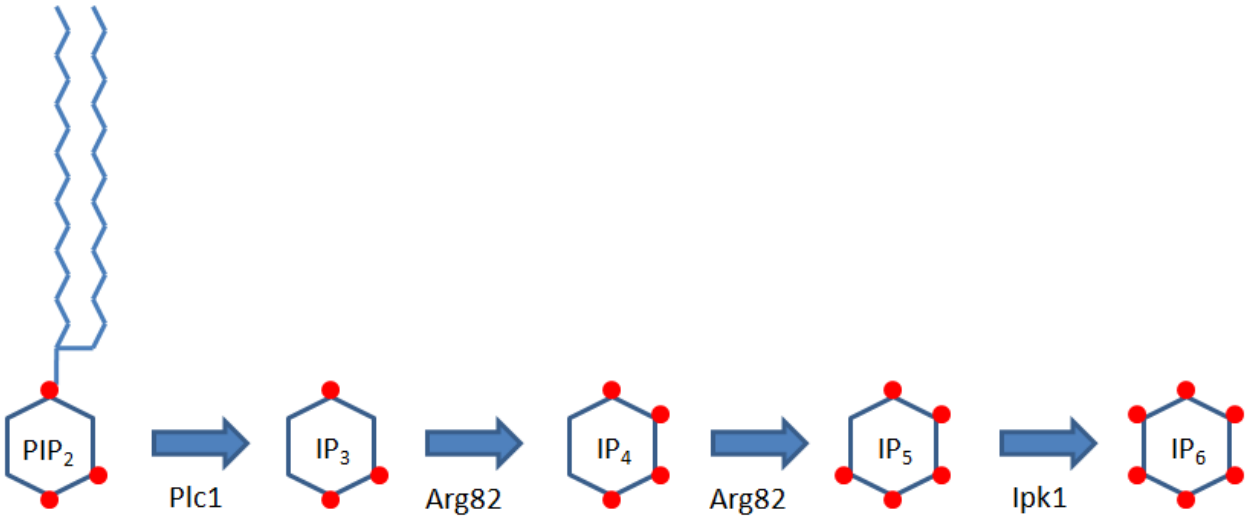

Figure 12. The inositol polyphosphate signaling pathway.

Inositol is a 6-carbon ring (represented by the blue hexagons) that bears a hydroxyl group on each carbon (hydroxyl groups are not shown. Plc1 cleaves the membrane-associated phospholipid PIP_2 into IP_3 and diacylglycerol (DAG; not shown). Arg82 is a bifunctional kinase that phosphorylates IP_3 to IP_4 and IP_5. IP_5 is further phosphorylated to IP_6 by Ipk1. Phosphate groups are represented by red dots. This figure is an adaptation of Figure 2 in ALCAZAR-ROMAN and WENTE 2008.

IPs are known to bind to various proteins, although no consensus sequence for an IP binding pocket has been identified (ALCAZAR-ROMAN and WENTE 2008). The association of IPs with proteins has been proposed to modulate protein function in several ways: IPs may function as essential structural cofactors, induce allosteric changes, or inhibit the binding of other ligands.

Many signaling pathways are known to impact gene expression, and the IP signaling pathway has several recognized connections to this activity. The products of Arg82 and Ipk1 catalytic activity have roles in chromatin remodeling and mRNA export and Arg82 is a subunit of the ArgR/Mcm1 transcription regulatory complex. These roles for the IP signaling pathway are described in more detail in the following sections.

1.5.1 Arg82 is required for normal expression of arginine metabolic genes

Arg82 is known to associate with and stabilize Mcm1, a member of the MADS box transcription factor family (EL BAKKOURY *et al.* 2000). Arg82 is, therefore, necessary for normal expression of Mcm1 targets, which include genes involved in the pheromone response. Additionally, Arg82 and Mcm1 associate with Arg80 and Arg81 to form the ArgR/Mcm1 transcription regulatory complex (EL BAKKOURY *et al.* 2000; MESSENGUY *et al.* 1991) (SHEARS 2000). This complex binds to the promoters of 8 genes involved in arginine metabolism and regulates their expression. Five genes involved in arginine synthesis (*ARG8*, *ARG3*, *ARG1*, and the bifunctional *ARG5,6*) are repressed by ArgR/Mcm1 binding, while two genes involved in arginine catabolism (*CAR1* and *CAR2*) are induced in response to association of this complex. Interestingly, Arg82 kinase activity does not appear to required for its role in expression of arginine metabolic genes (DUBOIS *et al.* 2000; EL ALAMI *et al.* 2003). This observation suggests that Arg82 may possess functionality that is not related to the IP signaling pathway.

1.5.2 IP_6 is necessary for mRNA export

Mutations in *PLC1*, *ARG82*, and *IPK1* were identified in a screen for factors that became essential in combination with a *gle1-1* mutation (YORK *et al.* 1999). As discussed in Section 1.4.4, Gle1 is a cytoplasmic factor that interacts with Dbp5 and stimulates its helicase activity to facilitate the forward translocation of mRNP particles from the nucleus to the cytoplasm. IP6 is required for Gle1-dependent stimulation of Dbp5 activity (ALCAZAR-ROMAN *et al.* 2006; KOHLER and HURT 2007; WEIRICH *et al.* 2006). The role of IP_6 in mRNA export suggests that this process can be modulated in response to physiological stress.

1.5.3 Chromatin remodeling is affected by inositol polyphosphates

The IP signaling pathway has been implicated in the control of chromatin remodeling in several ways. A genetic screen for mutations that impaired induction of the *PHO5* gene, which is highly regulated by chromatin structure, identified a mutation in *ARG82* (STEGER *et al.* 2003). Further analysis demonstrated that nucleosomes, which are displaced from the *PHO5* promoter upon activation, were not efficiently remodeled in the absence of Arg82 kinase activity. The requirement for the kinase function of Arg82 suggests that effects on *PHO5* chromatin remodeling are mediated through Arg82's role in IP signaling and are likely not due to its function as a transcriptional regulator as a subunit of the ArgR/Mcm1 complex.

This study further demonstrated that remodeling of chromatin structure at several phosphate responsive promoters is impaired by mutations in the SWI/SNF and INO80 chromatin remodeling complexes and that mutations in these factors also decreases expression of *PHO5* and *PHO84*. In the absence of Arg82, SWI/SNF and INO80 fail to localize to the *PHO84*

promoter upon gene induction and INO80 is not recruited to the *PHO5* promoter. Interestingly, the Pho4 transcriptional activator is still efficiently recruited to *PHO5* and *PHO84* in the absence of Arg82, suggesting that regulation of SWI/SNF and INO80 recruitment by the IP signaling pathway takes place independent of transcription factor binding.

An additional study demonstrated that IPs could modulate the activity of chromatin remodeling complexes in vitro. IP_4 and IP_5, the products of Arg82 catalytic function, stimulated nucleosome sliding by SWI/SNF while IP_6, the product of Ipk1 kinase activity, inhibited nucleosome repositioning by the INO80, ISW2, and the fly ISWI-containing NURF complexes (SHEN *et al.* 2003). SWI/SNF, ISW2, and INO80 are required for normal expression of the *INO1* gene in *S. cerevisiae* (GOLDMARK *et al.* 2000; PETERSON *et al.* 1991; SHEN *et al.* 2000). Deletion of *PLC1* or *ARG82*, or mutation of the Arg82 catalytic domain, impaired *INO1* induction (SHEN *et al.* 2003). This observation suggests that the effects of IP_4, IP_5, and IP_6 on chromatin remodeling complexes in vitro may have physiological relevance.

The different effects of individual IPs on chromatin remodeling complexes implies that activity of IP signaling enzymes can be modulated to elicit distinct effects on gene expression. Interestingly, *INO1* and the *PHO* genes are induced under conditions of nutrient limitations; therefore, the effects of mutations in IP signaling enzymes on expression of these genes further implicates the IP signaling pathway in the cellular stress response. Genes involved in the cellular stress response are often poised for rapid activation and in several cases, chromatin remodeling complexes are associated with the promoters of these genes under repressing conditions (RANDO *et al.* 2003). These genes may then be rapidly activated or inhibited in response to environmental stimuli by modulating the activity of enzymes in the IP signaling pathway.

1.6 THESIS AIMS

The *S. cerevisiae* Paf1 transcription elongation complex contributes to normal transcription in multiple ways. However, the mechanisms underlying Paf1 complex function remain largely unknown. A major focus of my thesis research was to investigate the means by which the Rtf1 subunit of the Paf1 complex carries out its known transcription-related functions. Rtf1 physically interacts with the ATP-dependent chromatin remodeling protein Chd1 and recruits it to active genes, promotes posttranslational modification of specific lysine residues in histones H2B and H3 along active ORFs, and mediates association of other Paf1 complex subunits with transcriptionally active regions. Using a collection of sequential Rtf1 internal deletion mutants, I identified nonoverlapping regions of Rtf1 that were necessary for each of these processes. These observations suggest that the known activities of Rtf1 are not completely interrelated. I performed a more detailed analysis of the region of Rtf1 required for histone modification and found that this region of the protein was sufficient to promote Rtf1-dependent histone modifications when expressed in isolation and that the function of this region depended on several highly conserved residues.

Furthermore, a screen for factors that become essential when *RTF1* is deleted identified mutations in the IP signaling enzymes Plc1 and Arg82. I sought to better understand the nature of the interaction between these factors. I performed genetic analyses which demonstrated that deletion of *ARG82* impaired cell growth in combination with loss of four subunits of the Paf1 complex (Paf1, Ctr9, Rtf1, and Cdc73). Additional genetic analyses established that deletion of *ARG82* or *PAF1* genetically interacted with disruptions of the same chromatin remodeling complexes. These results established a connection between IP signaling, transcription elongation, and chromatin remodeling.

2.0 RTF1 IS COMPOSED OF DISCRETE FUNCTIONAL REGIONS

2.1 INTRODUCTION

The Paf1 complex colocalizes with actively transcribing Pol II (KROGAN *et al.* 2002b; POKHOLOK *et al.* 2002; PORTER *et al.* 2005; SIMIC *et al.* 2003) and orchestrates modifications to the chromatin template during transcription elongation. In particular, the Rtf1 subunit of the Paf1 complex contributes to chromatin modification in several ways. Rtf1 is necessary for the cotranscriptional monoubiquitylation of histone H2B at lysine 123 (H2B K123 Ub) along active ORFs by the Rad6 ubiquitin conjugating enzyme and the Bre1 ubiquitin ligase (NG *et al.* 2003b; WOOD *et al.* 2003b; XIAO *et al.* 2005). H2B K123 Ub is a prerequisite for the subsequent methylation of histone H3 at lysine 4 (H3 K4 Me) and lysine 79 (H3 K79 Me) within active genes by the Set1 and Dot1 methyltransferases, respectively (BERNSTEIN *et al.* 2002; BRIGGS *et al.* 2001; SANTOS-ROSA *et al.* 2002; SUN and ALLIS 2002). Therefore, these histone H3 methylation marks are also Rtf1-dependent (KROGAN *et al.* 2003; NG *et al.* 2003b; NG *et al.* 2003c). Additionally, Rtf1 impacts chromatin structure through its physical interaction with the ATP-dependent chromatin remodeling protein Chd1. Our lab has demonstrated that this physical interaction is necessary for the normal recruitment of Chd1 to active genes (SIMIC *et al.* 2003).

Although multiple transcription-related roles have been identified for Rtf1, the mechanism by which it functions in these processes remains unknown. Rtf1's primary amino

acid sequence contains no recognizable motifs or domains that provide insight into the mechanism by which it functions. Therefore, a series of sequential deletions along the length of the protein was created in the Arndt lab to identify the functional regions of the protein. I performed genetic and biochemical assays on these mutants to identify residues required for the various activities of Rtf1. My results establish that discrete non-overlapping segments of Rtf1 are necessary for recruitment of the ATP-dependent chromatin remodeling protein Chd1 to genes, promoting covalent modification of histones H2B and H3, recruitment to active ORFs, and association with other Paf1 complex subunits. Transcription-related defects were observed when regions of Rtf1 that mediate histone modification or association with active genes were deleted, but disruption of the physical association between Rtf1 and other Paf1 complex subunits caused only subtle mutant phenotypes. Together, our results indicate that Rtf1 influences transcription and chromatin structure through several independent functional domains and that Rtf1 may function independently of its association with other members of the Paf1 complex.

2.2 MATERIALS AND METHODS

2.2.1 Media and yeast strains

Rich (YPD), synthetic complete (SC), synthetic minimal (SD), and 5-fluoroorotic acid (5-FOA) media were prepared as described previously (ROSE *et al.* 1990). Where indicated, 6-azauracil was added to SC-Ura media at a final concentration of 50 μg/ml.

S. cerevisiae strains used in these studies are listed in Table 1. All strains, with the exceptions of OKA93 and PJ69-4A, are *GAL2*$^{+}$ derivatives of S288C (WINSTON *et al.* 1995).

Transformation, mating, sporulation, and tetrad dissection were performed according to standard methods (ROSE *et al.* 1990). Complete disruptions of *RTF1* and *CHD1* were created by a PCR-based gene replacement method (AUSUBEL *et al.* 1988). Constructs expressing HA-Rtf1Δ3 or HA-Rtf1Δ4 were integrated into the yeast chromosome to replace the endogenous *RTF1* locus by a two-step gene replacement method (ROTHSTEIN 1991). Tagging of Ctr9 at the carboxy-terminus with six copies of the Myc epitope and Chd1 at the amino-terminus with three copies of the HA epitope was previously described (SIMIC *et al.* 2003; SQUAZZO *et al.* 2002).

2.2.2 Plasmid construction

Standard cloning techniques were used to construct all plasmids (AUSUBEL *et al.* 1988). pLS20 and pLS21-5 are derivatives of pRS314 (SIKORSKI and HIETER 1989) that express Rtf1 or triple HA-tagged Rtf1, respectively (STOLINSKI *et al.* 1997). Site-directed mutagenesis (KUNKEL *et al.* 1987) or PCR-based approaches were used to remove segments of the *RTF1* coding region from pLS21-5 to create a series of sequential *RTF1* internal deletion mutations, which are detailed in Figure 13A. Each *rtf1* deletion mutation was confirmed by DNA sequencing. pKR37, which expresses HA-Rtf1Δ1, was digested with Nde1 to remove the triple HA-tag and religated to generate pPC61. High-copy vectors used to overexpress HA-Rtf1 (pMW6) and HA-Rtf1Δ7 (pMW7) were constructed by subcloning a SalI-SpeI fragment from pLS21-5 or pKR14 (the pLS21-5 derivative carrying *HA-rtf1Δ7*) into SalI-SpeI digested pRS426 (SIKORSKI and HIETER 1989). To create glutathione S-transferase (GST)-Rtf1 fusions, plasmid pJS2 was generated by introducing a BamHI site immediately upstream of *RTF1* by site directed-mutagenesis of pKA61, which carries *RTF1* on a 3.6 kb insert (STOLINSKI *et al.* 1997). A plasmid expressing a fusion of GST to full-length Rtf1 (pJS4) was constructed by subcloning a 2.4 kb *RTF1*-

containing BamHI/EcoRI fragment from pJS2 into BamHI/EcoRI digested pGEX-3X (SMITH and JOHNSON 1988). pJS3, which expresses a GST fusion to the most amino-terminal 261 amino acids of Rtf1, was created by digesting pJS2 at the introduced BamHI and at a natural SmaI site in the *RTF1* coding region and subcloning this fragment into BamHI/SmaI digested pGEX-3X. A plasmid expressing a GST fusion to the most carboxy-terminal 297 amino acids of Rtf1 (pJS1) was constructed by digesting pKA61 at the internal *RTF1* SmaI site and at a downstream EcoRI site and cloning this 1.6 kb fragment into SmaI/EcoRI digested pGEX-3X. pMW4, which expresses a GST fusion to Rtf1 segments 11 through 13, was created by amplifying the 3' end of the *RTF1* coding region from pJS1 to introduce a SmaI site adjacent to nucleotide 1306 of *RTF1* (corresponding to the start of amino acid 436). PCR product was digested with SmaI and EcoRI and the resulting 1.1 kb fragment was cloned into SmaI/EcoRI digested pGEX-3X.

2.2.3 Yeast growth assays

Strains were grown to saturation at 30^{o}C in appropriate media. Cells were washed twice and serially diluted in sterile water. Three microliters of each dilution were spotted on indicated media and incubated at 30^{o}C.

2.2.4 Sequence Alignment

A BLAST search was conducted using *S. cerevisiae* Rtf1 protein sequence (GenBank accession #: NP_011270.1) as the query. Sequences corresponding to Rtf1 homologs in *Schizosaccharomyces pombe* (NP_595507.1), *Caenorhabditis elegans* (NP_505473.1), and *Homo sapiens* (NP_055953.1) were downloaded from the NCBI database. A complete sequence

alignment was performed using Clustal X (JEANMOUGIN *et al.* 1998) and the resulting alignment was exported into JalView (http://www.jalview.org) to apply grayscale shading at a threshold value of 20% identity. To search for proteins containing a similar sequence, amino acids 63-152 of *S. cerevisiae* Rtf1 were used to query the nonredundant NCBI protein database in four iterations of a Psi-BLAST search.

2.2.5 Immunoblotting analyses

Transformants of KY404 containing pRS314, pRS424, pMW6, pMW7, pLS20, pLS21-5, or derivatives of pLS21-5 expressing each Rtf1 internal deletion mutant were grown to approximately 4 x 10^7 cells/mL in SC-Trp medium. Whole cell extracts were made by glass bead lysis essentially as described previously (SHIRRA *et al.* 2005) except that radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES, pH 7.9, 2 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, and protease inhibitors) was used. To examine expression of Rtf1 internal deletion mutants, 20 µg of extract was run on a 15% SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were probed with anti-HA (Boehringer Mannheim) or anti-L3 (VILARDELL and WARNER 1997) primary antibodies at a final concentration of 1:3000. Sheep anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) were used at a 1:5000 dilution and the presence of immunoreactive proteins was visualized by enhanced chemiluminescence detection (Perkin Elmer). Bulk levels of histone modifications were assayed similarly except that 30 µg of protein was analyzed using primary antibodies specific for H3 K4 Me^3 (Abcam; 1:2000 dilution), H3 K79 Me^2 (Upstate; 1:2500 dilution), or total H3 (Upstate; 1:2000 dilution). Donkey anti-rabbit HRP-conjugated secondary antibodies (GE Healthcare) were used at a 1:5000 dilution.

2.2.6 Analysis of histone H2B K123 monoubiquitylation

Histone H2B K123 monoubiquitylation was measured using a protocol provided by William Tansey. FY406, KY982, KY1216, and KY1217 were transformed with *CEN*/*ARS*/*HIS3* plasmids carrying *HTA1/HTB1* or *HTA1/FLAG-HTB1* (NG *et al.* 2002). *URA3*-marked *HTA1/HTB1* plasmids were eliminated by counterselection on 5-FOA medium. Resulting strains were transformed with 2-micron, *URA3*-marked plasmids expressing untagged or HIS-tagged ubiquitin under control of the *CUP1* promoter (pUb175 and pUb221, respectively; gift of Daniel Finley). Transformants were grown to approximately 1 x 10^7 cells/ml in SC-His-Ura medium and expression of ubiquitin was then induced with a final concentration of 0.5 mM $CuSO_4$ for 4.5 h. Whole cell extracts were made by glass bead lysis in Buffer A, pH 8.0 (6M guanidine-HCl, 0.1 M sodium phosphate). Extract (2 mg) was incubated with 250 µl 50% Ni-NTA agarose (Qiagen) at room temperature for 2 h. Affinity precipitated proteins were washed, separated on a 15% SDS-PAGE gel, and analyzed by immunoblotting using anti-FLAG (Sigma) or anti-HIS tag (Invitrogen) antibodies at a final concentration of 1:5000.

2.2.7 Chromatin immunoprecipitation assays

Transformants of KY452 or KY995 containing pRS314, pRS424, pLS20, pMW6, pMW7, pLS21-5, or derivatives of pLS21-5 and transformants of KY623 containing pRS314, pLS20, or pPC61, were grown in SC-Trp medium to approximately 1 x 10^7 cells/mL. Chromatin preparation and treatment were performed essentially as described previously (SHIRRA *et al.* 2005). Sonicated chromatin was incubated overnight with anti-HA or anti-Myc conjugated agarose (Santa Cruz Biotechnology) to immunoprecipitate HA-Rtf1, HA-Chd1 or Ctr9-Myc.

PCR was performed using primers that amplify segments in the 5' ORFs of *PYK1* (+195/381; ATG = +1), *CLN2* (+126/373; ATG = +1), or *TEF2* (+40/291; ATG = +1). Reactions were multiplexed with control primers that amplify an intergenic region of chromosome VIII (coordinates: 535129/535268). Two dilutions of input and immunoprecipitated DNA were amplified in the presence of [α-^{32}P]dATP (Perkin Elmer) and Platinum Taq DNA polymerase (Invitrogen). PCR products were separated on 6% native polyacrylamide gels and signals were visualized and quantitated with a Fujifilm FLA-5100 phosphorimager and MultiGauge software. Signals from input and immunoprecipitated DNA were normalized to the chr VIII control signal and relative association of Rtf1, Chd1, or Ctr9 at each locus was determined by dividing the average of the two immunoprecipitated samples by the average of the two input samples.

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

Strain[a]	Genotype
FY406	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6= *HTA1-HTB1/CEN/ARS/URA3*]
OKA93	*MATα rtf1Δ::kanMX4 TEL-VR::URA3 ura3-52 trp1*$^{-}$
KY100	*MAT***a** *spt15-122 his4-917δ lys2-173R2 ura3-52 ade8*
KY284	*MATα his4-917δ ura3-52 trp1Δ63*
KY343	*MAT***a** *rtf1Δ::URA3 his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
KY386	*MATα spt15-122 rtf1Δ::URA3 his4-917δ lys2-173R2 ura3-52 leu2Δ1 ade8*
KY404	*MAT***a** *rtf1Δ::LEU2 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY432	*MAT***a** *rtf1-1 his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
KY440	*MATα spt15-122 rtf1-1 his4-917δ lys2-173R2 leu2Δ1 ade8*
KY452	*MAT***a** *rtf1Δ::URA3 his3Δ200 lys2-173R2 ura3-52 trp1Δ63*
KY457	*MAT***a** *rtf1Δ::URA3 leu2Δ1 ura3-52 trp1Δ63*
KY619	*MAT***a** *rtf1Δ::ARG4 his4-912δ lys2-173R2 leu2Δ1 trp1Δ63 arg4-12*
KY623	*MATα rtf1Δ ::LEU2 3XHA-CHD1 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY638	*MATα spt15-122 his4-917δ lys2-173R2 ura3-52*
KY639	*MAT***a** *spt15-122 chd1Δ::URA3 his4-917δ leu2Δ1 ura3-52 trp1Δ63*
KY640	*MAT***a** *his4-917δ ura3-52*
KY641	*MATα chd1Δ::URA3 his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
KY680	*MATα 3XHA-rtf1Δ1 his4-917δ lys2-173R2 leu2Δ1 ura3-52*
KY982	*MAT***a** *rtf1Δ::kanMX4 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200*

	lys2-128δ leu2Δ1 ura3-52 trp1Δ63 [pSAB6= *HTA1-HTB1/CEN/ARS/URA3*]
KY995	*MAT*α *rtf1Δ::URA3 CTR9-6XMYC::LEU2 his3Δ200 leu2Δ(0 or 1) ura3(Δ0 or -52) trp1Δ63*
KY1216	*MAT***a** *rtf1Δ3 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 arg4-12* [pSAB6= *HTA1-HTB1/CEN/ARS/URA3*]
KY1217	*MAT***a** *rtf1Δ4 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 arg4-12* [pSAB6= *HTA1-HTB1/CEN/ARS/URA3*]
KY1265	*MAT***a** *spt15-122 3XHA-rtf1Δ1 his4-917δ lys2-173R2 ura3-52 ade8*

[a] FY406 was generated in the laboratory of Fred Winston.

2.3 RESULTS

2.3.1 Conserved regions of Rtf1 direct normal transcription

Although Rtf1 homologs are found in many eukaryotes, the primary amino acid sequences of these proteins contain no recognizable functional domains or motifs and the mechanism by which this protein affects transcription remains unknown. To begin dissecting the functional regions of Rtf1, a series of sequential internal deletions across the *RTF1* coding region was constructed in the Arndt lab. These internal deletion mutations express HA-epitope tagged mutant versions of Rtf1 that lack between 23 and 52 amino acids (Figure 13A) from *CEN/ARS* plasmids. I performed immunoblot analysis to demonstrate that all Rtf1 internal deletion mutants were expressed (Figure 13B) and verified by serial dilution analysis that cell growth was not impaired by any of the mutations on control medium (Figure 14, right panel).

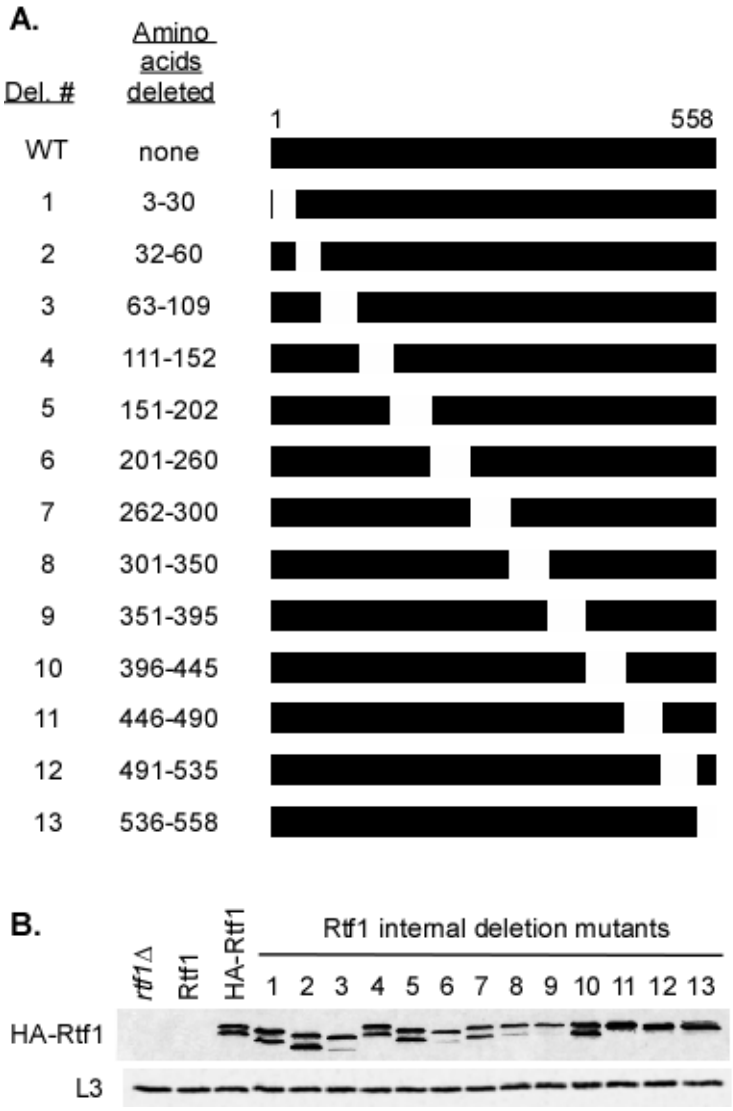

Figure 13. Rtf1 internal deletion mutants.

(A) Schematic representation of amino acids removed by *rtf1* internal deletion mutations. Each Rtf1 mutant protein is amino-terminally tagged with a triple HA epitope. (B) Immunoblot analysis of wild-type and mutant Rtf1 protein levels. Extracts were prepared from an *rtf1Δ* strain (KY404) transformed with *CEN/ARS* plasmids that express the indicated HA-tagged Rtf1 derivatives. Immunoblots were probed with anti-HA antibody and, as a loading control, anti-L3. The faster migrating band observed in most lanes in the anti-HA immunoblot is likely a product of proteolysis, which we frequently observe in extracts prepared by glass bead lysis, but is less pronounced in extracts prepared by a rapid boiling method (data not shown).

I first utilized the Rtf1 internal deletion mutants to explore which regions of the protein are responsible for the transcription defects observed when Rtf1 is absent from the cell. Deletion of *RTF1* causes the suppressor of Ty (Spt$^-$) phenotype (STOLINSKI *et al.* 1997); that is, it suppresses the transcriptional effects of promoter insertion mutations caused by a yeast retrotransposon Ty or its long terminal repeat (δ). In particular, I examined the Spt$^-$ phenotype of *rtf1* deletion strains that contain *his4-912δ*, a *HIS4* allele where a Ty δ element is positioned 37 bp upstream of the native TATA box (ROEDER and FINK 1982). Transcriptional effects at this locus were assayed by growth on medium lacking histidine; growth on this medium indicates an Spt$^-$ phenotype. Deletion of regions 2, 3, 4, or 13 of Rtf1 caused a weak Spt$^-$ phenotype, while deletion of region 5 caused a moderate Spt$^-$ phenotype (Figure 14, left panel). In contrast, a significantly stronger Spt$^-$ phenotype was observed when regions 6, 7, 8, or 9 were removed. To rule out the possibility that the severe phenotype caused by these deletions arose from mislocalization of the mutant proteins, indirect immunofluorescence assays were performed (K.L. Roinick and K.M. Arndt, data not shown). These experiments demonstrated that Rtf1 internal deletion mutants 6, 7, 8, and 9 localized to the nucleus, where wild-type Rtf1 is known to reside (STOLINSKI *et al.* 1997).

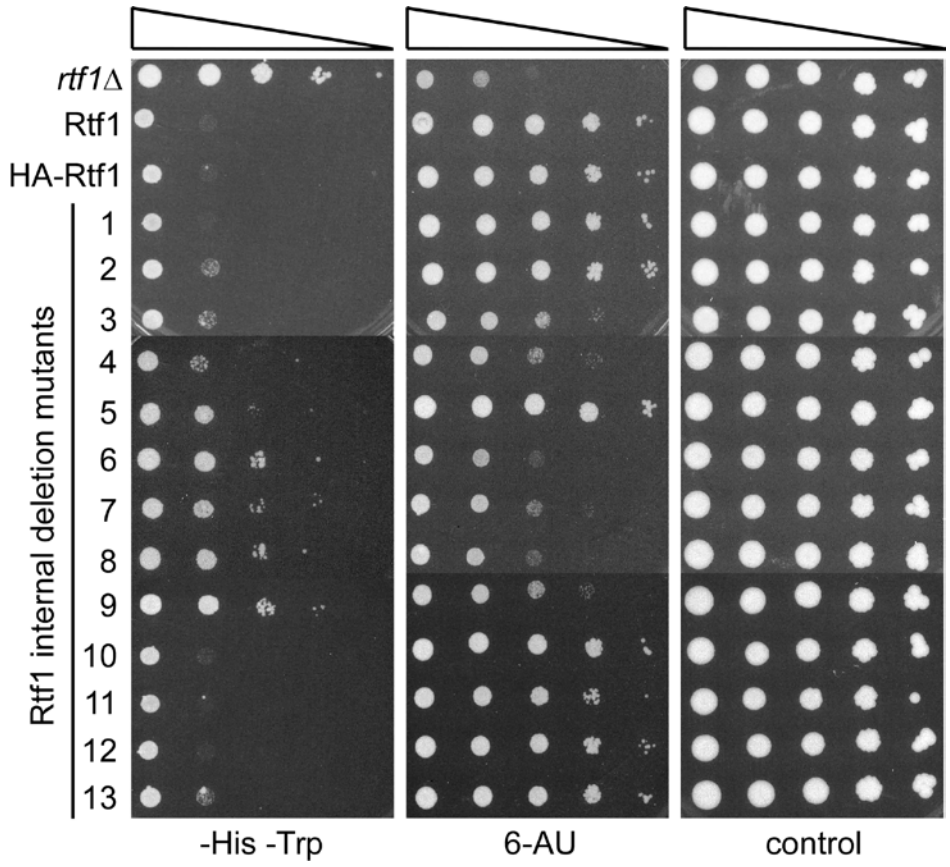

Figure 14. Deletions of discrete regions of Rtf1 cause phenotypes associated with transcriptional defects.

Ten-fold serial dilutions, ranging from 1 x 10^8 cells/mL to 1 x 10^4 cells/mL, of an *rtf1Δ* strain (KY619) expressing the indicated Rtf1 derivatives were spotted on SD-His-Trp medium to examine the Spt$^-$ phenotype, SC-Ura-Trp medium containing 50 μg/mL 6-AU to assess 6-AU sensitivity, or SC-Trp medium as a control for growth. Plates were incubated at 30^{o}C for 5 days.

Deletion of *RTF1* also causes sensitivity to the base analog 6-azauracil (6-AU) (COSTA and ARNDT 2000), a drug that lowers intracellular nucleotide pools by inhibiting enzymes in the ribonucleotide synthesis pathway (EXINGER and LACROUTE 1992). Sensitivity to 6-AU is considered an indicator of defects in transcription elongation because Pol II is believed to become more dependent on accessory factors to overcome elongation impediments under low nucleotide conditions. My analysis demonstrated that removal of regions 6, 7, or 8 from Rtf1 caused strong sensitivity to 6-AU, while deletion of regions 3, 4, and 9 caused moderate sensitivity (Figure 14, center panel).

Interestingly, loss of any single region of *RTF1* did not cause an effect equal to a complete *RTF1* disruption for either phenotype tested, suggesting that each internal deletion mutant retains some functionality. This raises the possibility that Rtf1 may be composed of distinct functional parts. I performed an alignment of Rtf1 homologs from four species, which revealed a total of 26 invariant residues. All of these invariant amino acids reside in either regions 3-4 (8 invariant residues) or 6-9 (18 invariant residues) (Figure 15). These conserved sections of the protein overlap with those that cause the most severe Spt^- and $6\text{-}AU^S$ phenotypes when deleted. Together, these observations suggest that Rtf1 requires two clusters of highly conserved amino acids to direct normal transcription *in vivo*.

Figure 15. Rtf1 homologs contain two clusters of highly conserved residues.

An alignment of Rtf1 protein sequences from four different species is shown. Conserved residues are highlighted in gray, with darker shades indicating a greater degree of conservation. Black and gray lines above the *S. cerevisiae* sequence denote Rtf1 regions 3-4 and 6-9, respectively.

2.3.2 A region near the amino-terminus of Rtf1 mediates physical interaction with Chd1

The observation that removal of discrete amino acid clusters from Rtf1 caused transcription defects led us to investigate whether these residues are responsible for any of Rtf1's known functions. The Arndt lab has previously demonstrated that Rtf1 physically associates with the ATP-dependent chromatin remodeling protein Chd1 and determined that this interaction is mediated by region 1 of Rtf1 (residues 3-30) (COSTA 2001). Rtf1 is required for normal recruitment of Chd1 to active ORFs (SIMIC *et al.* 2003) and I examined whether Rtf1 region 1 contributed to this association. I performed chromatin immunoprecipitation (ChIP) assays on wild type, *rtf1Δ1*, and *rtf1Δ* strains expressing HA-tagged Chd1. Association of Rtf1 was analyzed at the 5' ends of two ORFs: *PYK1*, a highly expressed gene where association of the Paf1 complex has been detected previously (QIU *et al.* 2006), and *TEF2*, where we have previously demonstrated a role for Rtf1 in the recruitment of Chd1 (SIMIC *et al.* 2003). My results show that deletion of region 1 of Rtf1 significantly reduced association of Chd1 at the 5' ORFs of *PYK1* and *TEF2*, although the effect was not as severe as that observed when *RTF1* was completely absent (Figure 16) suggesting that additional regions of Rtf1 may contribute to stabilizing the interaction of Chd1 with chromatin.

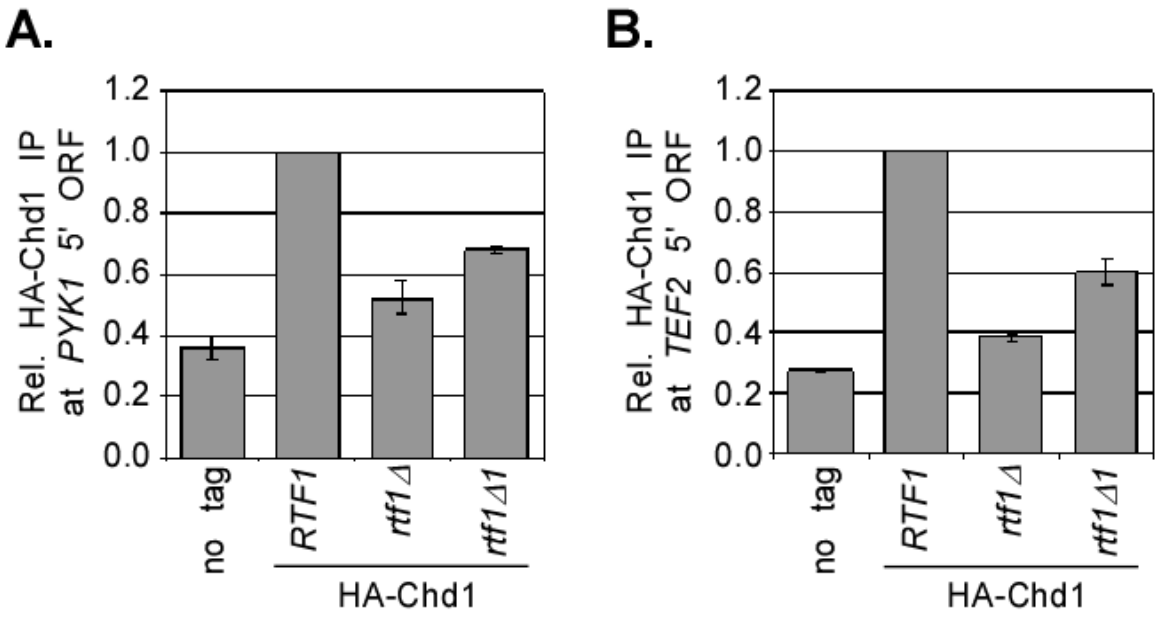

Figure 16. The amino-terminus of Rtf1 is required for recruitment of Chd1 to active genes.

ChIP analysis of Chd1 recruitment to active ORFs in *rtf1* mutants. HA-Chd1 and associated DNA were immunoprecipitated from extracts of a formaldehyde-treated *rtf1Δ* strain (KY623) that had been transformed with an empty vector or plasmids that express untagged wild-type Rtf1 or Rtf1Δ1. An untagged Chd1 strain (KY452) expressing untagged wild-type Rtf1 (pLS20) was used as a control. Association of Chd1 at the 5' ORFs of *PYK1* (A) or *TEF2* (B) was assessed by PCR. The mean of three independent experiments with standard error is shown. Signal from strains expressing full-length Rtf1 is set to 1.

A previous study in the Arndt lab identified a function for Rtf1 region 1 (STOLINSKI *et al.* 1997). An Rtf1 mutant with a substitution of phenylalanine for leucine at position 11 (Rtf1-1) was identified as a suppressor of the Spt$^-$ phenotype caused by *spt15-122*, a mutation in the gene encoding the TATA-binding protein (TBP). The TBP mutant encoded by *spt15-122*, TBP-L205F, exhibits altered DNA binding specificity *in vitro* and *in vivo* (ARNDT *et al.* 1992). Previous analysis in the lab demonstrated that Rtf1-1 also impairs the physical interaction between Rtf1 and Chd1 (COSTA 2001). The observation that the Rtf1-1 mutant protein interacted poorly with Chd1 suggested that suppression of *spt15-122* by certain *RTF1* mutations was due to a disruption of the Rtf1-Chd1 interaction. This possibility was tested by examining the effect of mutations that interfere with the Rtf1-Chd1 interaction on the Spt$^-$ phenotype of *spt15-122* in a strain carrying *his4-917δ*, a *HIS4* allele where a Ty δ element is inserted between the native TATA box and the transcription start site (ROEDER and FINK 1982). Previous work in the lab determined that *chd1Δ* and *rtf1-1* strongly suppressed the Spt$^-$ phenotype of the *spt15-122* mutation, similar to the effect of an *rtf1* null allele (COSTA 2001; STOLINSKI *et al.* 1997). I extended this analysis to demonstrate that *rtf1Δ1* also suppresses this phenotype (Figure 17). These observations suggest that while disrupting the Rtf1-Chd1 interaction does not cause noticeable transcription-related phenotypes in a wild-type strain (Figure 17), it may have effects on chromatin structure that become apparent in a TBP mutant strain.

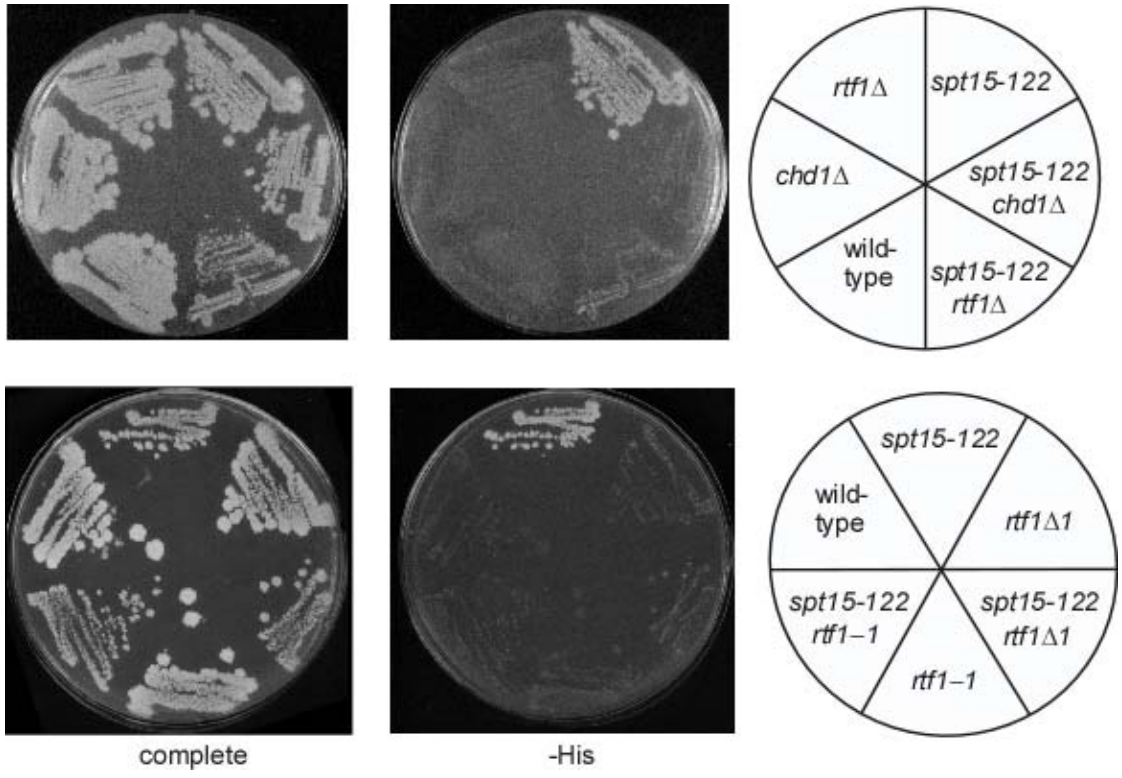

Figure 17. Mutations that disrupt the Rtf1-Chd1 interaction suppress a TBP mutant.

Mutations in *RTF1* or *CHD1* suppress the Spt$^-$ phenotype of an *spt15-122* strain. KY343, KY386, KY638, KY639, KY640, and KY641 (top row) or KY100, KY284, KY432, KY440, KY680, and KY1265 (bottom row) were grown on YPD medium and transferred by replica printing to SC complete as a control or SC-His medium to examine the Spt$^-$ phenotype. Plates were incubated at 30^{o}C for 3 days.

2.3.3 Conserved regions of Rtf1 are required for Rtf1-dependent histone modifications and telomeric silencing

I next sought to determine which residues are essential for Rtf1 to direct covalent modification of lysine residues in histones H2B and H3. I began by examining the effects of the Rtf1 internal deletion mutants on histone H3 methylation. Immunoblot analysis demonstrated that Rtf1 regions 3 and 4 are essential for dimethylation and trimethylation of histone H3 K4 and dimethylation of histone H3 K79 (Figure 18A and data not shown). I also observed a slight reduction of these modifications when regions 6, 7, 8, or 9 were eliminated from Rtf1. Interestingly, the regions of Rtf1 that are required for histone methylation correspond to the most highly conserved portions of the protein (Figure 15).

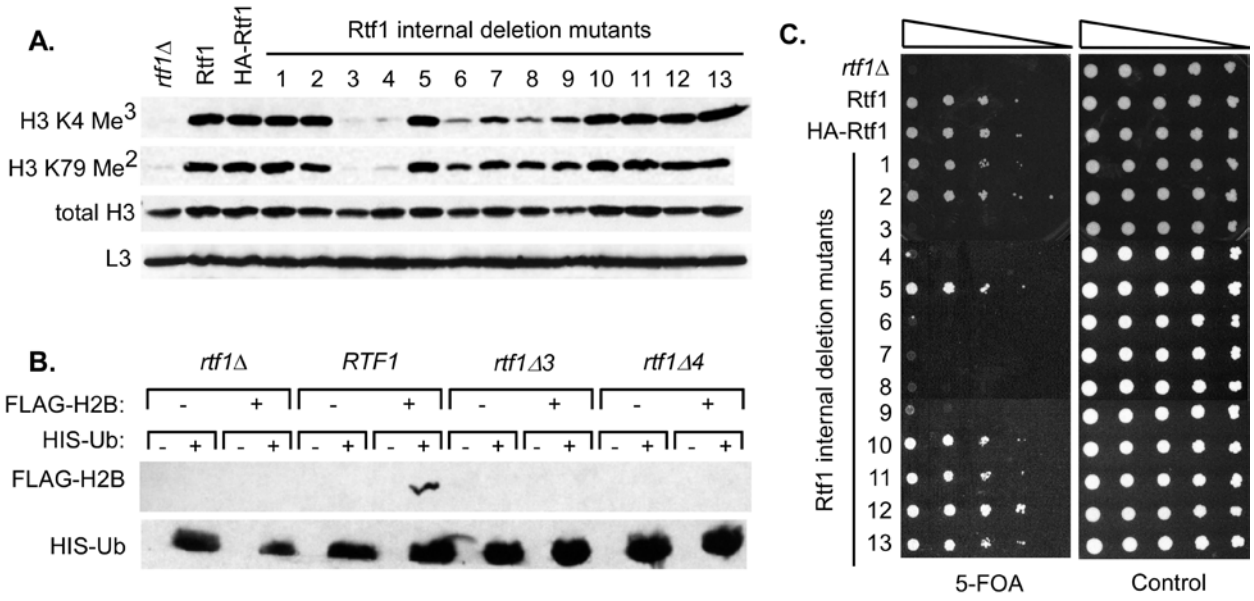

Figure 18. Conserved regions of Rtf1 are essential for Rtf1-dependent histone modifications and telomeric silencing.

(A) Immunoblot analysis of Rtf1-dependent histone methylation in strains expressing the Rtf1 internal deletion mutants. Extracts from an *rtf1Δ* strain (KY404) expressing the indicated Rtf1 derivatives were probed with antibodies specific for H3 K4 Me^3, H3 K79 Me^2, and total H3. An anti-L3 immunoblot was performed as a loading control. Me^3 = trimethylation; Me^2 = dimethylation. (B) Analysis of H2B K123 ubiquitylation levels in Rtf1 mutants defective for histone methylation. FY406, KY982, KY1216, and KY1217 were transformed with plasmids expressing untagged or HIS-tagged ubiquitin and a plasmid expressing wild type or FLAG-tagged histone H2B. The HIS-tagged protein fraction from each strain was isolated and analyzed by immunoblot using an anti-FLAG antibody to detect FLAG-H2B (top panel). An anti-HIS tag immunoblot was also performed (lower panel) to demonstrate expression and enrichment of HIS-ubiquitin (HIS-Ub) in the expected strains. (C) Analysis of telomeric silencing in strains expressing Rtf1 internal deletion mutants. Ten-fold serial dilutions, ranging from 1 x 10^8 cells/mL to 1 x 10^4 cells/mL, of an *rtf1Δ* strain (OKA93) expressing the indicated Rtf1 derivatives and containing an ectopic *URA3* gene integrated proximal to the telomere on the right arm of chromosome V were spotted on SC-Ura-Trp medium containing 5-FOA to assess telomeric silencing defects or SC-Trp medium as a control for growth. Plates were incubated at 30°C for 4 days.

Rtf1 is also required for monoubiquitylation of histone H2B K123 (NG *et al.* 2003b; WOOD *et al.* 2003b). Because removal of Rtf1 region 3 or 4 eliminates H3 K4 and K79 methylation, modifications that lie downstream of H2B K123 Ub, I examined the state of H2B K123 Ub in strains expressing these Rtf1 mutants. This modification was analyzed in strains expressing HIS-tagged ubiquitin (HIS-Ub) and FLAG-tagged histone H2B (FLAG-H2B). Expression of FLAG-H2B and HIS-Ub was confirmed by immunoblot analysis (data not shown and Figure 18B). Ubiquitylated proteins from yeast extracts were captured on Ni-NTA agarose, separated by SDS-PAGE, and analyzed by immunoblotting for the presence of FLAG-H2B. I detected ubiquitylated H2B in wild-type cells, but not in cells where *RTF1* had been deleted or replaced with *rtf1Δ3* or *rtf1Δ4* (Figure 18B). This observation demonstrates that Rtf1-dependent histone ubiquitylation is mediated by the same 90 amino acids (residues 63-152; regions 3 and 4) as Rtf1-dependent histone methylation.

Rtf1 and its downstream histone modifications are required for normal telomeric silencing (KROGAN *et al.* 2003; NG *et al.* 2003b). To assess which regions of Rtf1 affect telomeric silencing, I analyzed the effects of the Rtf1 internal deletion mutants on expression of an ectopic copy of *URA3* integrated proximal to the telomere on the right arm of chromosome V (*TEL-VR::URA3*). *URA3* expression causes toxicity on media containing 5-fluoroorotic acid (5-FOA). When telomeric silencing is active, transcription from *TEL-VR::URA3* is repressed and cell growth is largely unaffected by 5-FOA. However, mutations that compromise telomeric silencing derepress *TEL-VR::URA3*, resulting in growth inhibition or cell death on 5-FOA containing media. I found that deletion of Rtf1 regions 3, 4, 6, 7, 8, or 9 eliminated cell growth on media containing 5-FOA in a *TEL-VR::URA3* strain (Figure 18C, left panel). Therefore, the

regions of Rtf1 that affect telomeric silencing correspond to the same regions that eliminate or reduce Rtf1-dependent histone modifications.

2.3.4 Association of Rtf1 with active ORFs requires a conserved central region

Rtf1 occupies active ORFs coincident with Pol II. I performed ChIP assays on strains expressing the Rtf1 internal deletion mutants to investigate which regions of Rtf1 are required for its association with active ORFs. Association of Rtf1 was analyzed at the 5' ends of *PYK1* and *CLN2*, a gene that requires the Paf1 complex for normal expression (KOCH *et al.* 1999). My results show that deletion of segments 6, 7, or 8 reduced association of Rtf1 at the 5' ORFs of *PYK1* and *CLN2* to background levels observed in untagged or *rtf1Δ* strains (Figure 19A and B). A strong reduction in association of Rtf1 at these loci was also observed when Rtf1 region 9 was absent. Deletion of Rtf1 regions 3 and 4, which are required for Rtf1-dependent histone modifications, caused approximately a two-fold reduction of Rtf1 association with *PYK1*, but this effect was not observed for *CLN2*. These observations suggest that a large central region of Rtf1, spanning amino acids 201-350, is essential for recruitment of Rtf1 to the two genes tested and amino acids 351-395 contribute significantly to this interaction.

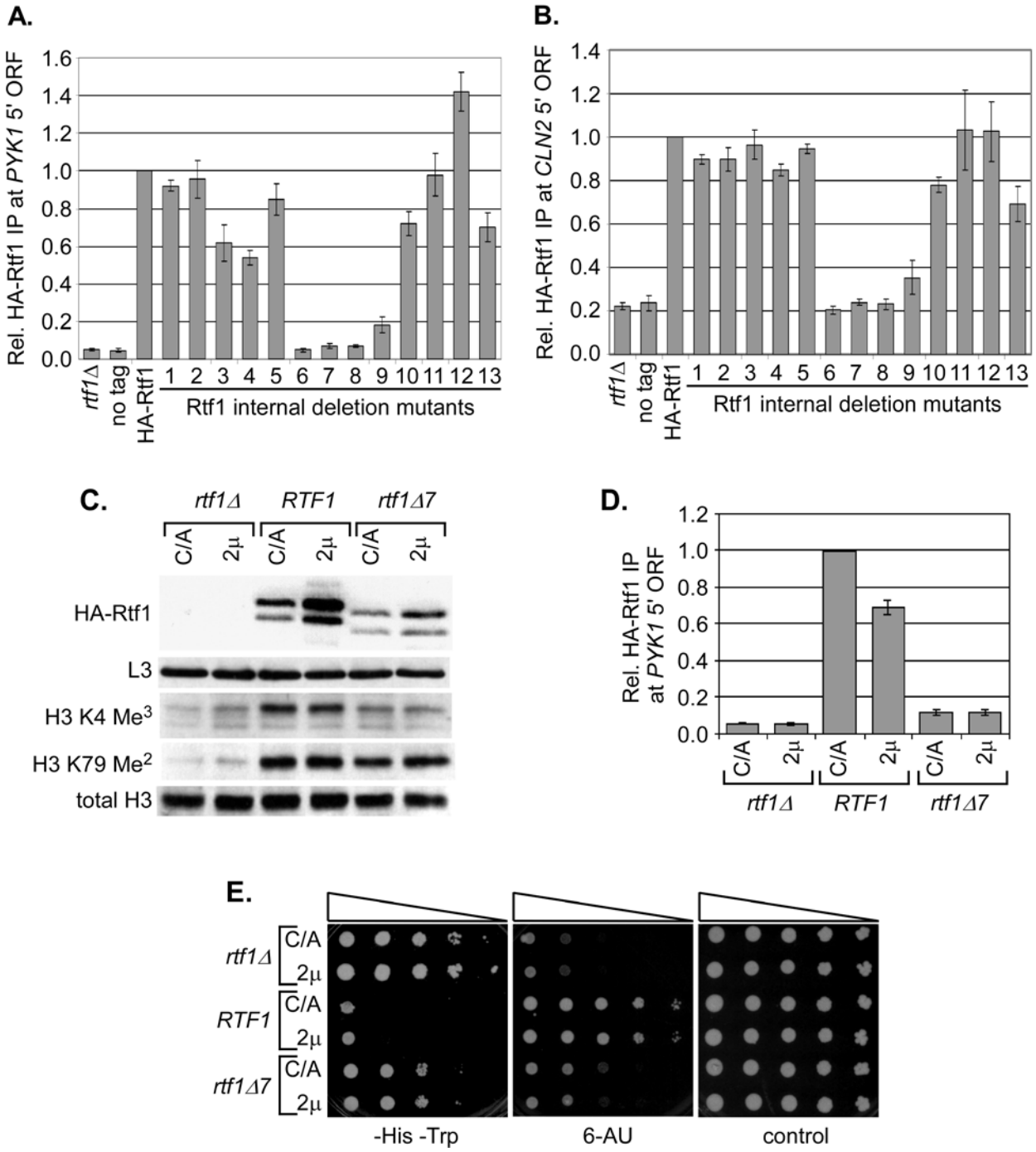
A.
Rel. HA-Rtf1 IP at PYK1 5' ORF
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
rtf1Δ
no tag
HA-Rtf1
1 2 3 4 5 6 7 8 9 10 11 12 13
Rtf1 internal deletion mutants
B.
Rel. HA-Rtf1 IP at CLN2 5' ORF
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
rtf1Δ
no tag
HA-Rtf1
1 2 3 4 5 6 7 8 9 10 11 12 13
Rtf1 internal deletion mutants
C.
rtf1Δ
RTF1
rtf1Δ7
C/A
2μ
HA-Rtf1
L3
H3 K4 Me3
H3 K79 Me2
total H3
D.
Rel. HA-Rtf1 IP at PYK1 5' ORF
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C/A
2μ
rtf1Δ
RTF1
rtf1Δ7
E.
rtf1Δ
RTF1
rtf1Δ7
C/A
2μ
-His -Trp
6-AU
control

Figure 19. A central conserved region mediates association of Rtf1 with active ORFs and influences protein stability.

(A) and (B) ChIP analysis of Rtf1 association with active ORFs. HA-tagged Rtf1 mutants and associated DNA were immunoprecipitated from extracts of a formaldehyde-treated *rtf1Δ* strain (KY452) that expressed the indicated Rtf1 derivatives. Association of wild-type or mutant Rtf1 proteins with the 5' ORFs of *PYK1* (A) or *CLN2* (B) was assessed by PCR. The mean of three independent experiments with standard error is shown. Signal from strains expressing HA-tagged full-length Rtf1 is set to 1. (C) Effects of Rtf1Δ7 overexpression on protein levels and histone modifications. 20 μg of extract from an *rtf1Δ* strain (KY404) expressing the indicated Rtf1 derivatives from low-copy (*CEN/ARS* = C/A) or high-copy (2μ) plasmids were subjected to immunoblot analysis with antibodies specific for the HA epitope, H3 K4 Me^3, H3 K79 Me^2 and total H3. An anti-L3 immunoblot was performed as a loading control. (D) ChIP analysis to assess effects of Rtf1Δ7 overexpression on association with active ORFs. Analysis was performed as described in (A) and (B) on extracts from KY452 transformed with plasmids expressing the indicated Rtf1 derivatives from *C/A* or 2μ plasmids. (E) Overexpression of Rtf1Δ7 does not suppress transcription-related phenotypes. Ten-fold serial dilutions, ranging from 1 x 10^8 cells/mL to 1 x 10^4 cells/mL, of an *rtf1Δ* strain (KY619) expressing the indicated Rtf1 derivatives from C/A or 2μ plasmids were spotted on SD-His-Trp medium to examine the Spt^- phenotype, SC-Ura-Trp medium containing 50 μg/mL 6-AU to assess 6-AU sensitivity, or SC-Trp medium as a control for growth. Plates were incubated at 30°C for 5 days.

Immunoblot analysis of the Rtf1 internal deletion mutants indicates that expression of Rtf1 deletions 6, 7, 8, and 9 is somewhat reduced in comparison to full-length Rtf1 (Figure 13B.), raising the possibility that the effects I observe when regions 6-9 are deleted may be a consequence of reduced protein levels. To address this possibility, I expressed Rtf1Δ7 from a high-copy yeast vector and asked whether increased levels of the mutant protein reduced the severity of its effects. I found that overexpression of Rtf1Δ7 increased its levels to those observed for wild-type Rtf1 that was expressed from a *CEN/ARS* (low copy) vector (Figure 19C), but did not increase association of Rtf1Δ7 with the 5' ORF of *PYK1* (Figure 19D) or suppress the transcription-related or histone modification defects observed in an *rtf1Δ7* strain (Figure 19C and E). These results support the idea that amino acids 201-395 are important for recruitment of Rtf1 to active genes and indicate that this association contributes to protein function and stability.

2.3.5 Rtf1 interacts with other Paf1 complex components through its carboxy-terminus

Rtf1 is a component of the five-member Paf1 transcription elongation complex. To identify the regions of Rtf1 that are necessary for its association with other Paf1 complex components, I immunoprecipitated the Rtf1 internal deletion mutants with an anti-HA antibody and co-immunoprecipitation of Paf1 or Myc-tagged Ctr9 was measured by immunoblot analysis (Figure 20A). I found that Paf1 and Ctr9 co-immunoprecipitated with full-length Rtf1 and Rtf1 internal deletion mutants 1 through 11. The slight decrease in the amounts of Paf1 and Ctr9 co-immunoprecipitated from strains expressing Rtf1 internal deletion mutants 6, 7, 8, and 9 is likely related to the reduced expression of these Rtf1 mutants (Figure 13B). However, when the most

carboxy-terminal segments of Rtf1, regions 12 or 13, were deleted, co-immunoprecipitation of Paf1 and Ctr9 was greatly reduced.

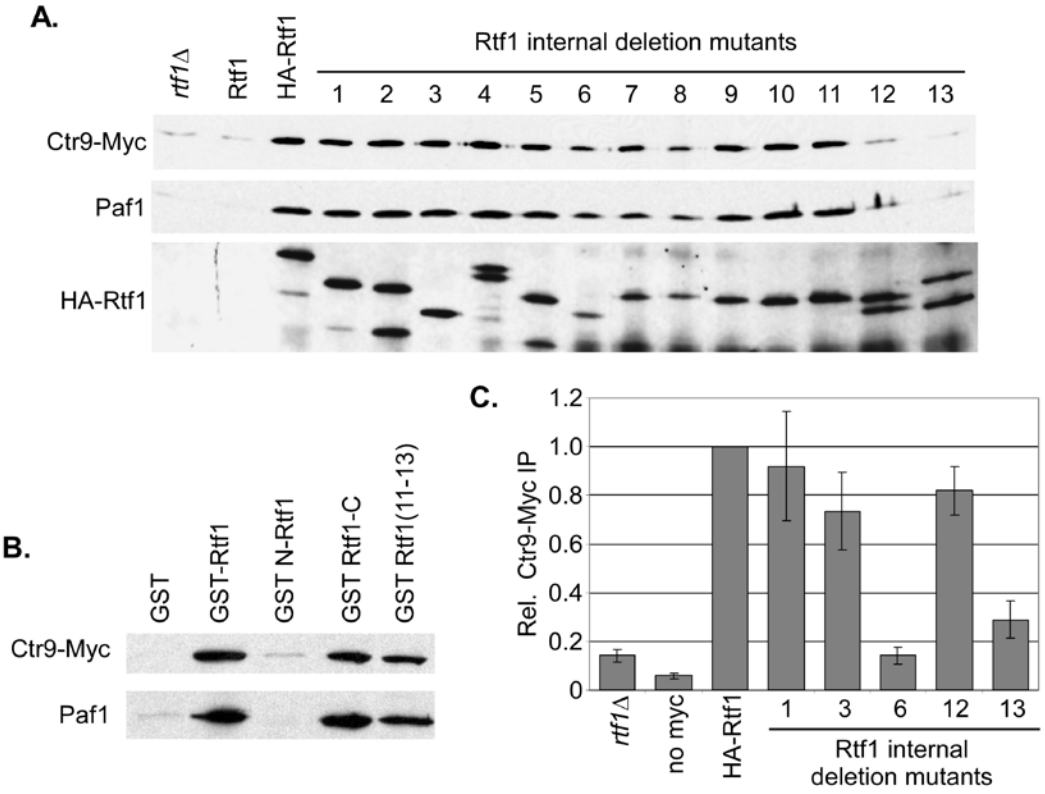
A.
rtf1Δ
Rtf1
HA-Rtf1
Rtf1 internal deletion mutants
1 2 3 4 5 6 7 8 9 10 11 12 13
Ctr9-Myc
Paf1
HA-Rtf1
B.
GST
GST-Rtf1
GST N-Rtf1
GST Rtf1-C
GST Rtf1(11-13)
Ctr9-Myc
Paf1
C.
Rel. Ctr9-Myc IP
1.2
1.0
0.8
0.6
0.4
0.2
0
rtf1Δ
no myc
HA-Rtf1
1 3 6 12 13
Rtf1 internal deletion mutants

Figure 20. The carboxy-terminus of Rtf1 mediates physical interaction with other Paf1 complex subunits.

(A) Co-immunoprecipitation analyses of Rtf1 internal deletion mutants and other Paf1 complex subunits. Extracts from an *rtf1Δ* strain (KY995) expressing Ctr9-Myc and the indicated Rtf1 derivatives were subjected to immunoprecipitation with an anti-HA antibody. The precipitated fraction was analyzed by immunoblotting using anti-Myc, anti-Paf1, or anti-HA antibodies. (B) Paf1 and Ctr9 bind to the carboxy-terminus of Rtf1. GST alone or GST fusions to full-length Rtf1 (GST-Rtf1), the amino-terminal half of Rtf1 (GST N-Rtf1), the carboxy-terminal half of Rtf1 (GST Rtf1-C), or Rtf1 segments 11 through 13 (GST Rtf1(11-13)) were purified from bacteria and incubated with yeast extract from an *rtf1Δ* strain (KY457) for GST pull-down assays. Bound proteins were detected by immunoblotting using anti-Myc and anti-Paf1 antibodies. (C) ChIP analysis of Ctr9-Myc in Rtf1 internal deletion mutant strains. Myc-tagged Ctr9 and associated DNA were immunoprecipitated from extracts of a formaldehyde-treated *rtf1Δ* strain (KY995) expressing Ctr9-Myc and the indicated Rtf1 derivatives. Association of Ctr9-Myc with the 5' ORF of *PYK1* was assessed by PCR. The mean of three independent experiments with standard error is shown. Signal from transformants expressing HA-tagged full-length Rtf1 is set to 1.

To further test the importance of the Rtf1 carboxy-terminus in Paf1 complex assembly, I purified bacterially-expressed GST fusions to full-length Rtf1, the amino-terminal half of Rtf1, the carboxy-terminal half of Rtf1, or the extreme carboxy-terminus of Rtf1 (regions 11 through 13) and examined the ability of these fusions to bind Paf1 or Myc-tagged Ctr9 in yeast lysates. I found that Paf1 and Ctr9 bound GST fusions to full-length Rtf1, the carboxy-terminal half of Rtf1, and the extreme carboxy-terminus of Rtf1 (Figure 20B). However, a GST fusion to the amino-terminal half of Rtf1 showed essentially no interaction with Paf1 or Ctr9. Together, these observations indicate that the extreme carboxy-terminus of Rtf1 is both necessary and sufficient to mediate interaction with other components of the Paf1 complex.

A previous report demonstrated that Paf1 complex components fail to associate with active ORFs in a strain completely lacking Rtf1 (MUELLER *et al.* 2004). I therefore decided to use our specific mutations to test whether disruption of the physical interaction between Rtf1 and the Paf1 complex is sufficient to eliminate recruitment of other Paf1 complex components to active ORFs. ChIP analyses were performed on a strain expressing Myc-tagged Ctr9 and a subset of the Rtf1 internal deletion mutants. I found that regions of Rtf1 required for the interaction with Chd1 (region 1) or histone modification (region 3) had only a modest effect on Ctr9 association at the *PYK1* gene (Figure 20C). However, removal of region 6, which is required for association of Rtf1 with active ORFs, or region 13, which is responsible for the interaction of Rtf1 with other Paf1 complex components, reduced Ctr9 association at *PYK1* essentially to the level observed in an *rtf1* null strain. Surprisingly, Ctr9 associates with the 5' ORF of *PYK1* at near wild-type levels in a strain expressing Rtf1Δ12. This observation suggests that a weak physical interaction between Rtf1Δ12 and Ctr9 exists *in vivo* and that this interaction can be detected once it has been stabilized by formaldehyde crosslinking. These results support

the conclusion that Rtf1 is required to tether the Paf1 complex to active ORFs. Additionally, I observed that deletion of region 13 from Rtf1 slightly reduces its occupancy on *PYK1* and *CLN2* (Figure 19), suggesting that other Paf1 components may play a reciprocal, though minor, role in stabilizing association of Rtf1 with active ORFs.

2.4 CONCLUSIONS

Using a collection of sequential *rtf1* deletion mutations, I demonstrated that discrete nonoverlapping segments of Rtf1 are required for recruitment of Chd1 to active genes, histone modification, association of Rtf1 with active ORFs, and interaction with other Paf1 complex subunits. These regions of the protein have been given designations which reflect their functionality: Chd1-interaction domain (CID), histone modification domain (HMD), ORF-association region (OAR), and the Paf1 complex interaction domain (PID), respectively. I observed that distinct transcriptional effects resulted from disruption of individual functions of Rtf1 (Figure 21). Combined with the observation that a complete deletion of *RTF1* causes more severe phenotypes than mutations that eliminate only one of its activities (Figure 14), these data suggest that the functions of Rtf1 are not entirely interdependent.

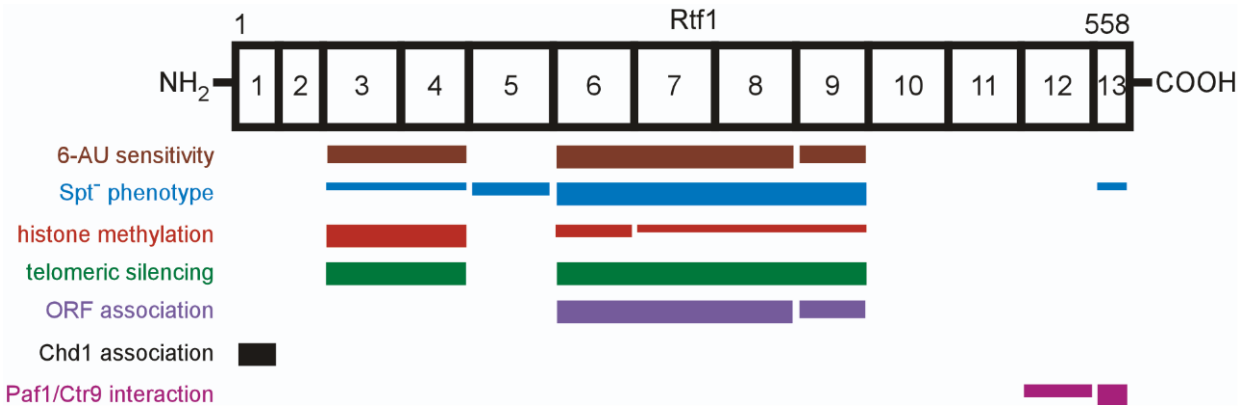

Figure 21. Rtf1 is composed of functionally distinct regions.

A schematic diagram of Rtf1 indicating the relative size of each Rtf1 internal deletion (numbered boxes) is shown. Regions of Rtf1 affecting each of the indicated processes or phenotypes are specified by colored lines. The width of the line represents the degree of phenotype or effect that was observed.

My analyses and other data from the Arndt lab indicate that amino acids 3-30 of Rtf1 (region 1) are required for physical interaction with Chd1 and for normal association of Chd1 with active genes. Although I observed no transcription-related defects when region 1 was deleted in an otherwise wild-type strain, previous work in the lab has demonstrated that *rtf1-1*, which results in substitution of phenylalanine for a leucine at position 11, and *chd1* and *rtf1* null alleles suppress the Spt$^-$ phenotype of the *spt15-122* mutation (COSTA 2001; STOLINSKI *et al.* 1997). I report here that this effect is also caused by *rtf1Δ1*. This result indicates that the interaction between Rtf1 and Chd1 is important for proper chromatin function particularly when the cell is sensitized to small changes in nucleosome positioning or stability, such as in a TBP mutant strain.

I also demonstrated that amino acids 63 through 152 of Rtf1, which correspond to regions 3 and 4, are required for monoubiquitylation of H2B K123 and methylation of histone H3 K4 and K79. Regions 3 and 4 of Rtf1 contain several highly conserved residues, but a Psi-BLAST search did not identify similar domains in other proteins. This suggests that regions 3 and 4 of Rtf1 stimulate the activity of the enzymes that promote Rtf1-dependent histone mechanisms (Rad6, Set1, and Dot1) by a unique conserved mechanism.

Rtf1-dependent histone modifications were also moderately reduced by deletion of regions 6-9, which span amino acids 201-395. My analyses show that residues in these regions are important for association of Rtf1 with active ORFs. In addition to its role in normal Rtf1-dependent histone modification levels, the association of Rtf1 with chromatin, and presumably transcribing Pol II, appears to be required for its full stability and for normal telomeric silencing.

All amino acids that are invariant across the four Rtf1 homologs examined in Figure 15 reside in either regions 3-4, which are required for histone modification, or in regions 6-9, which

regulate localization of Rtf1 to active ORFs. I also observe that the most severe transcription-related phenotypes result from deletion of these regions of the protein, indicating that directing cotranscriptional modification of histones is a critical means by which Rtf1 exerts its effects on transcription.

Finally, I demonstrated that the carboxy-terminus of Rtf1, defined by region 13, is necessary and sufficient for the interaction between Rtf1 and other Paf1 complex subunits. Consistent with a prior study, I confirmed that interaction with Rtf1 mediates recruitment of Paf1 and Ctr9 to active ORFs (MUELLER *et al.* 2004). Remarkably, disruption of the physical interaction between Rtf1 and the Paf1 complex causes only a mild Spt^- phenotype (Figure 14) and the known biochemical functions of Rtf1 remain largely intact. This observation suggests that Rtf1 retains function, independent of stable association with the Paf1 complex.

3.0 CONSERVED RESIDUES IN THE RTF1 HISTONE MODIFICATION DOMAIN DIRECT HISTONE H2B UBIQUITYLATION AND H3 METHYLATION

3.1 INTRODUCTION

In Section 2.3.3, I demonstrated that a 90 amino acid segment of Rtf1 mediates its roles in histone H2B monoubiquitylation, histone H3 K4 di- and trimethylation, and histone H3 K79 dimethylation. The region of Rtf1 required to promote these histone modifications spans amino acids 63 through 152 (regions 3 and 4) of the full-length protein. I have termed this protein region the histone modification domain (HMD) to reflect its functionality.

The Rtf1 HMD contains eight residues in a 33 amino acid span that are invariant across *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Homo sapiens,* and *Caenorhabditis elegans* (Figure 15). Although the HMD is conserved, its primary amino acid sequence does not resemble any recognized domains or motifs that imply the mechanism by which it functions; I, therefore, sought to explore this mechanism in several ways.

First, I isolated substitution mutations in conserved residues of the HMD and investigated their effects on the known functions for this region of the protein. Surprisingly, I found that different HMD substitution mutations had unique effects on Rtf1-dependent histone modifications. I also tested the effects of these mutations on transcription and chromatin-related phenotypes that result when *RTF1* is completely deleted. Interestingly, the results of these

analyses indicate that HMD substitution mutations often have similar effects on these phenotypes, despite their differential effects on Rtf1-dependent histone modifications. However, phenotypic effects of the HMD substitution mutations sometimes differ from those caused by an *rtf1* null mutation or partial deletions of the HMD. These results suggest that modifying the HMD and removing it are not functionally equivalent.

Additionally, I determined that the HMD alone was sufficient to promote Rtf1-dependent histone methylation in a strain where *RTF1* had been deleted. Intriguingly, the HMD did not need to be specifically targeted to DNA or to active genes to elicit this activity. Together, these results suggest that promoting histone modification is an intrinsic property of the HMD and that its function does not require a stable interaction with active genes.

3.2 MATERIALS AND METHODS

3.2.1 Media and yeast strains

Media were prepared and standard genetic methods were performed as described in Section 2.2.1. *S. cerevisiae* strains used in these studies are listed in Table 2. All strains are *GAL2*$^{+}$ derivatives of S288C (WINSTON *et al.* 1995). Constructs expressing HA-Rtf1 102-104A, HA-Rtf1 108-110A, or HA-Rtf1 F80V F123S, which are tagged at the amino-terminus with three copies of the HA epitope, were integrated into the yeast chromosome to replace endogenous *RTF1* by a two-step gene replacement method (ROTHSTEIN 1991).

3.2.2 Plasmid construction

Standard cloning techniques were used to construct all plasmids (AUSUBEL *et al.* 1988). pLS20 and pLS21-5 are derivatives of pRS314 (SIKORSKI and HIETER 1989) that express Rtf1 or triple HA-tagged Rtf1, respectively (STOLINSKI *et al.* 1997). pKR27 and pKR28 express HA-Rtf1Δ3 and HA-Rtf1Δ4, respectively, and were created as part of the Rtf1 internal deletion series as described in Section 2.2.2. Alanine substitution mutations in the Rtf1 HMD were created by site-directed mutagenesis using the QuikChange system from Stratagene. Using pLS21-5 as the template plasmid, three separate plasmids were created by this method: pJB1, which expresses HA-Rtf1 102-104A; pJB2, which expresses HA-Rtf1 108-110A, and pJB3, which expresses HA-Rtf1 120-121A. These mutations cause changes in the native amino acids at the corresponding numerical positions, i.e. 102-104, to alanines. The presence of the specified mutations in each plasmid was confirmed by DNA sequencing.

An amino-terminal Gal4 DNA binding domain fusion to full-length Rtf1 (GBD-Rtf1) is expressed from pLS28; construction of this plasmid is described in SIMIC *et al.* 2003. pLS28 is based on pGBT9 (Clontech), which expresses untagged GBD (corresponding to amino acids 1-147 of full-length Gal4.) pMW8 expresses an amino-terminal fusion of GBD to amino acids 63 through 152 of full-length Rtf1 (GBD-HMD); a single Myc tag is present between the GBD and HMD; this plasmid was created by amplifying the HMD coding sequence (nucleotides 187 through 456 of full-length *RTF1*) from pLS21-5 using primers that add an NdeI site to the 5' end and two stop codons and an EcoRI site to the 3' end. The resulting PCR product was digested with the appropriate enzymes and ligated into NdeI/EcoRI digested pGBKT7. pGBKT7 (Clontech) expresses the GBD tagged with a single Myc epitope at the carboxy-terminus (GBD-

Myc). pMW16 and pMW17 express GBD-myc fusions to amino acids 63 through 152 of Rtf1 F123S (GBD-F123S-HMD) and Rtf1-102-104A (GBD-102-104A-HMD), respectively. pMW16 was created by amplifying the HMD coding region from pDS1, which expresses full-length HA-Rtf1 F123S, and pMW17 was created by amplifying the HMD coding sequence from pJB1. A GBD-Myc fusion to the Rtf1 108-110A HMD (pMW15) was created, but no expression of this fusion protein could be detected in whole cell extracts (data not shown).

3.2.3 Error prone mutagenesis of the *RTF1* HMD coding sequence

To create random substitution mutations in the Rtf1 HMD, a PCR product encompassing nucleotides -23 through +1008 of *RTF1* was generated using Promega Taq DNA polymerase, a low fidelity DNA polymerase, under low nucleotide conditions (50μM each dNTP). The resulting PCR product was transformed into KY960 along with pKR27 that was digested with BglII and SmaI to remove nucleotides 190 through 783 (regions 3 through 6). Because the PCR product contains slightly greater than 200 bp overlaps upstream and downstream of the deleted region, homologous recombination should result in integration of the PCR product into the digested plasmid (MUHLRAD *et al.* 1992). Repaired plasmids are *TRP1* marked and can be selected on medium lacking tryptophan (SC-Trp). KY960 is deleted for the genomic copies of *RTF1* and *RKR1*, but is kept viable due to the presence of pKA69, which carries *RTF1* on a *URA3*-marked plasmid. Trp^+ transformants were exposed to medium containing 5FOA to counterselect for pKA69; transformants that were Trp^+, but sensitive to 5FOA, were further characterized. Null mutations were ruled out due to the degree of Spt^- phenotype they caused; an *rtf1* null mutation causes a strong Spt^- phenotype in a *his4-912δ* strain, while the effect of partial deletions of the HMD on this phenotype is relatively mild (Figure 14). Candidate plasmids that

caused a mild Spt$^-$ phenotype were isolated and sequenced. Two plasmids that were isolated in this screen, pKB1 (*HA-rtf1 F80V F123S*) and pKB2 (*HA-rtf1 E119K F123S*) were used in studies detailed in this chapter.

3.2.4 Yeast growth assays

Strains were grown to saturation at 30^{o}C in appropriate media. Cells were washed twice and serially diluted in sterile water. Three microliters of each dilution were spotted on indicated media and incubated at 30^{o}C.

3.2.5 Immunoblotting analyses

With the exception of the extracts analyzed in Figure 23C, all extracts were prepared by glass bead lysis in RIPA buffer and analyzed as described in Section 2.2.5. Extracts prepared from KY404 transformed with pLS21-5, pRS314, pKR27, pKR28, pJB1, pJB2, pJB3, pKB1, pKB2, and pLS20 that had been grown in SC-Trp medium were analyzed in Figure 22B; extracts prepared from KY279, KY1418, KY404, KY1193, KY1194, and KY1195 that had been grown in YPD were analyzed in Figure 23B; and extracts prepared from MHY119 transformed with pGBT9, pGBKT7, pLS20, pLS28, pMW8, pMW16, or pMW17 that had been grown in SC-Trp medium were analyzed in Figure 26 and Figure 29. 30 μg of extract from each strain was run on a 15% SDS-polyacrylamide gel and proteins were transferred to nitrocellulose for immunoblot analysis. An antibody specific for H3 K4 dimethylation (H3 K4 Me^2; Upstate) was used at 1:2500 dilution and an anti-Rtf1 antibody (SQUAZZO *et al.* 2002) was used at a 1:3000 dilution. Antibodies specific for glucose-6-phosphate dehydrogenase (G6PDH; 1:100,000; Sigma) or L3

(described in Section 2.2.5) were used to demonstrate that equivalent amounts of protein were loaded in each lane. All other antibodies were described in Section 2.2.5.

Extracts from KY1418, KY1194, and KY1195 were also prepared by a rapid boiling method to assess total H3 levels. Cells were grown in YPD to approximately 3 x 10^7 cells/mL and a volume equal to approximately 4.5 x 10^7 cells was centrifuged to remove growth media. The cell pellet was resuspended in 20 μL sample buffer (80mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol, and 1mM phenylmethylsulfonyl-fluoride) and boiled for 2 minutes. Cells were frozen in liquid nitrogen, and then thawed and vortexed at maximum speed in the presence of 0.12g acid-washed glass beads. 80 μL of additional sample buffer were added, extracts were boiled for an additional 3 minutes, and a volume equivalent to 1 x 10^7 cells was loaded on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and analyzed with the indicated antibodies.

3.2.6 Analysis of histone H2B K123 monoubiquitylation

Histone H2B K123 monoubiquitylation was measured using the protocol described in Section 2.2.6 and strains FY406 (*RTF1*), KY982 (*rtf1Δ*), KY1458 (*HA-RTF1*), MHY213 (*HA-rtf1 102-104A*), MHY215 (*HA-rtf1 108-110A*), and KY1462 (*HA-rtf1 F80V F123S*) expressing untagged or HIS-tagged ubiquitin and wild type or FLAG-tagged histone H2B. An anti-Ub (gift of Richard Gardner; 1:50 dilution) immunoblot was performed to demonstrate expression of HIS-Ub in the appropriate strains following enrichment with Ni-NTA agarose.

3.2.7 Chromatin immunoprecipitation assays

Chromatin was isolated and immunoprecipitated as described in Section 2.2.7. MHY119 transformed with pGBT9, pGBKT7, pLS20, pLS28, pMW8, pMW16, or pMW17 was analyzed in these experiments. Anti-Myc immunoprecipitation was performed as described in Section 2.2.7. Other immunoprecipitations were performed overnight at 4^{o}C with primary antibodies specific for H3 K4 trimethylation (H3 K4 Me^{3}; Abcam), H3 K79 dimethylation (H3 K79 Me^{2}; Upstate), total H3 (Upstate), or the largest subunit of Pol II (8WG16; Covance). Immune complexes were captured by incubating with Protein A-coupled sepharose for histone antibody immunoprecipitations (Amersham Biosciences) and Protein G-coupled sepharose (Amersham Biosciences) for 8WG16 immunoprecipitations. Association of these factors was analyzed at two loci: the *GAL7* UAS (-298/-92; ATG = +1) and the 5' end of *PYK1* (+195/381; ATG = +1). All PCR reactions were multiplexed with control primers that amplify a nontranscribed region in the right arm of telomere VI (*TEL-VIR*; coordinates: 269487/269624). IP/input was calculated as described in Section 2.2.7. IP/input values from the *GAL7* UAS or *PYK1* ORF are normalized to background signal observed with *TEL-VIR* primers; a ratio equal to "1" indicates no enrichment of the immunoprecipitated factors at the *GAL7* UAS or *PYK1* ORF over that observed at the *TEL-VIR*. Signals for H3 K4 Me^{3} and H3 K79 Me^{2} were not normalized to *TEL-VIR* control primers because histones in this region are modified at low levels in strains containing a WT HMD. TEL-VIR signal is, therefore, not equivalent across all strains used in this analysis and is unsuitable for normalization; specific signals are instead normalized to the amount of total H3 present at the locus being examined.

Table 2. *Saccharomyces cerevisiae* strains used in Chapter 3

Strain[a]	Genotype
FY406	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6= *HTA1-HTB1/CEN/ARS/URA3*]
KY279	*MAT***a** *leu2Δ1 ura3-52 trp1Δ63*
KY404	*MAT***a** *rtf1Δ::LEU2 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY619	*MAT***a** *rtf1Δ::ARG4 his4-912δ lys2-173R2 leu2Δ1 trp1Δ63 arg4-12*
KY960	*MAT***a** *rtf1Δ::LEU2 rkr1Δ::kanMX4 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [*pKA69 = RTF1/CEN/ARS/URA3*]
KY982	*MAT***a** *rtf1Δ::kanMX4 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6= *HTA1 HTB1/CEN/ARS/URA3*]
KY1010	*MATα his4-912δ lys2-128δ ade8*
KY1116	*MAT***a** *GAL1pr-FLO8-HIS3::kanMX his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY1193	*HA-rtf1 102-104A his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8*
KY1194	*HA-rtf1 108-110A his3Δ200 lys2-128δ leu2Δ1 ura3-52 ade8*
KY1195	*HA-rtf1 F80V-F123S his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8*
KY1207	*MAT***a** *rtf1Δ::ARG4 GAL1pr-FLO8-HIS3::kanMX his3Δ200* (*LYS2 or lys2-173R2*) *leu2Δ1 ura3-52 arg4-12*
KY1208	*MAT***a** *HA-rtf1Δ3 GAL1pr-FLO8-HIS3::kanMX his3Δ200 lys2-128δ leu2Δ1 ura3-52*

KY1209	*MAT***a** *HA-rtf1Δ4 GAL1pr-FLO8-HIS3::kanMX his3Δ200 lys2-128δ leu2Δ1 ura3-52 ade8*
KY1210	*MAT***a** *HA-rtf1 102–104A GAL1pr-FLO8-HIS3::kanMX his3Δ200 lys2-128δ leu2Δ1 ura3-52 arg4-12*
KY1211	*MATα HA-rtf1 108–110A GAL1pr-FLO8-HIS3::kanMX his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY1212	*MAT***a** *HA-rtf1 F80V F123S GAL1pr-FLO8-HIS3::kanMX his3Δ200 lys2-128δ leu2Δ1 ura3-52 ade8*
KY1226	*MATα TEL-VR::URA3 ura3-52*
KY1227	*MATα rtf1Δ::kanMX TEL-VR ::URA3 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY1418	*MATα HA-RTF1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY1458	*MATα HA-RTF1 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6= *HTA1 HTB1/CEN/ARS/URA3*]
KY1462	*MATα HA-rtf1 F80V F123S (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63 ade8* [pSAB6= *HTA1 HTB1/CEN/ARS/URA3*]
KY1506	*MATα HA-rtf1Δ3 his4-912δ lys2-128δ ade8*
KY1508	*MAT***a** *HA-rtf1Δ4 his4-912δ lys2-128δ leu2Δ1 trp1Δ63*
KY1510	*MATα HA-rtf1 102-104A his4-912δ lys2-128δ leu2Δ1 trp1Δ63*
KY1511	*MATα HA-rtf1 108-110A his4-912δ lys2-128δ trp1Δ63 ade8*
KY1512	*MAT***a** *HA-rtf1 F80V F123S his4-912δ lys2-128δ leu2Δ1 ade8*
MHY119	*MAT***a** *rtf1Δ::kanMX4 gal4Δ::LEU2 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*

MHY188	*MAT***a** *HA-rtf1Δ3 TEL-VR::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
MHY190	*MATα HA-rtf1Δ4 TEL-VR::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52*
MHY192	*MATα HA-rtf1 102–104A TEL-VR::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52*
MHY194	*MATα HA-rtf1 108–110A TEL-VR::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
MHY197	*MATα HA-rtf1 F80V F123S TEL-VR::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
MHY213	*MATα HA-rtf1 102-104A (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63* [pSAB6= *HTA1 HTB1/CEN/ARS/URA3*]
MHY215	*MATα HA-rtf1 108-110A (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63* [pSAB6= *HTA1 HTB1/CEN/ARS/URA3*]

[a] FY406 was generated in the laboratory of Fred Winston.

3.3 RESULTS

3.3.1 Point mutations were generated in the Rtf1 HMD by biased and unbiased methods

To explore the mechanism by which the Rtf1 HMD functions, I utilized two strategies to create mutations in this region. In a biased approach, site-directed mutagenesis was used to replace several highly conserved residues in the HMD with alanines. The HMD contains eight residues that are invariant across *S. cerevisiae*, *S. pombe*, *H. sapiens*, and *C. elegans* (Figure 15). These invariant residues reside in a 33 amino acid stretch spanning residues 88 through 120. I created three separate substitution mutations in this region: *rtf1 102-104A*, *rtf1 108-110A*, and *rtf1 120-121A*. These particular mutations were chosen between these residues flank the border between regions 3 and 4 of Rtf1. Each mutation changes at least one invariant residue and a neighboring residue to alanines, resulting in the neutralization of a charged patch (Figure 22A).

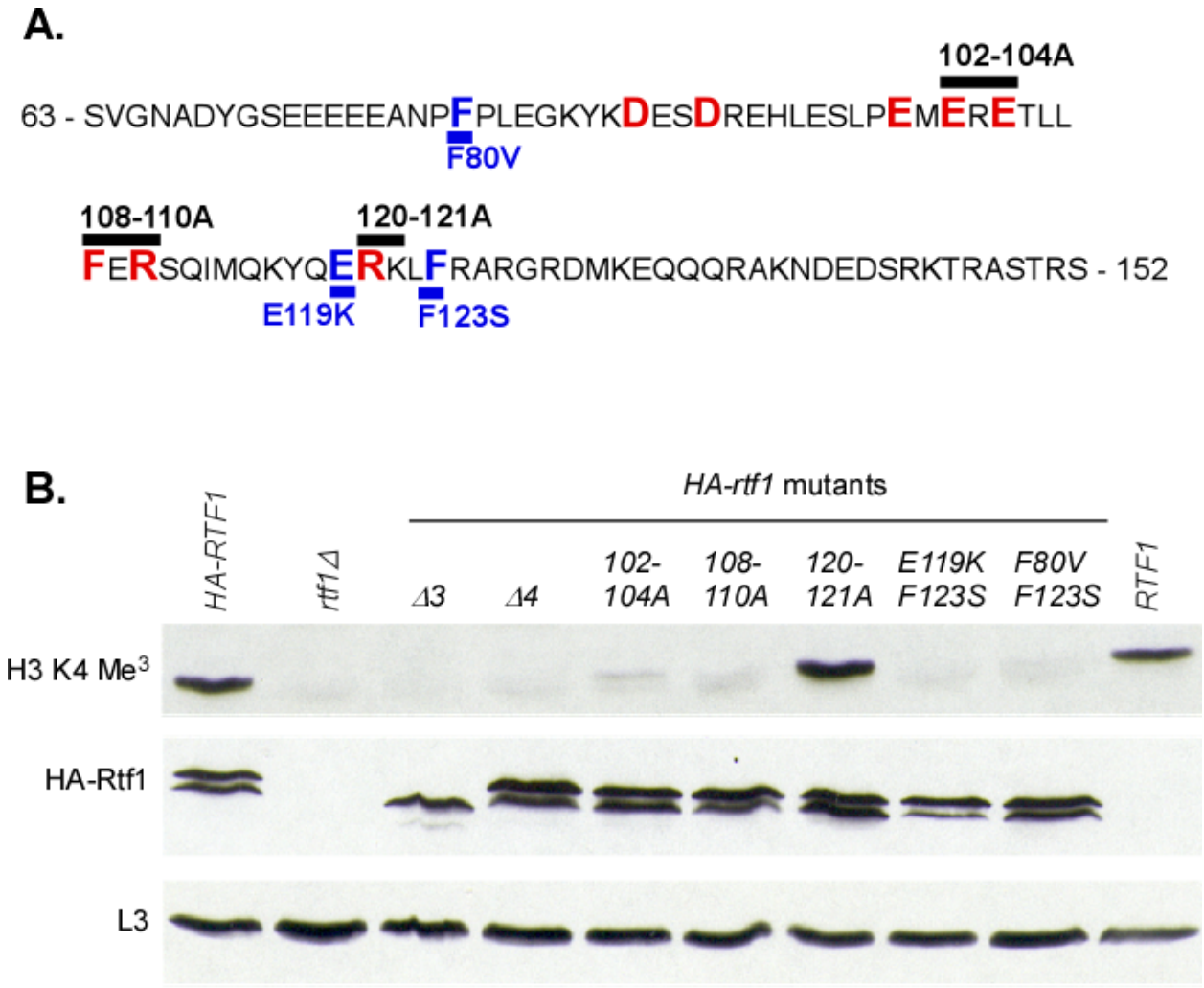
A.
102-104A
63 - SVGNADYGSEEEEEANPFPLEGKYKDESDREHLESLPEMERETLL
F80V
108-110A
120-121A
FERSQIMQKYQERKLFRARGRDMKEQQQRAKNDEDSRKTRASTRS - 152
E119K
F123S
B.
HA-rtf1 mutants
HA-RTF1
rtf1Δ
Δ3
Δ4
102-104A
108-110A
120-121A
E119K F123S
F80V F123S
RTF1
H3 K4 Me3
HA-Rtf1
L3

Figure 22. Substitution of conserved residues in the Rtf1 HMD affects H3 K4 trimethylation.

(A) Primary amino acid sequence of the Rtf1 HMD. The sequence of the *S. cerevisiae* Rtf1 HMD, corresponding to residues 63 through 152 of full-length Rtf1, is shown. Residues that are conserved across *S. cerevisiae*, *S. pombe*, *H. sapiens*, and *C. elegans* are shown in red. Black bars above the sequence denote clusters of charged residues that were mutated to alanines by site-directed mutagenesis; each mutation is referred to by its amino acid position followed by an "A", which signifies that the native residues have been replaced with alanines. Substitutions of the residues shown in blue were isolated in a genetic screen, as described in the text; the specific change that was isolated is indicated below the altered residue. (B) Immunoblot analysis of H3 K4 trimethylation levels in strains with substitution mutations in the Rtf1 HMD. Extracts from an *rtf1Δ* strain (KY404) expressing the indicated Rtf1 derivatives were probed with antibodies specific for H3 K4 Me^3 and total H3. An anti-L3 immunoblot was performed as a loading control and an anti-HA blot was performed to demonstrate approximately equal expression of HA-tagged wild type Rtf1 and mutant Rtf1 proteins harboring mutations in the HMD. The faster migrating band observed in most lanes in the anti-HA immunoblot is likely a product of proteolysis, which we frequently observe in extracts prepared by glass bead lysis, but is less pronounced in extracts prepared by a rapid boiling method (data not shown).

Furthermore, I performed an unbiased genetic screen to isolate additional mutations in the Rtf1 HMD. A deletion of *RTF1* is synthetically lethal in a cell that also lacks *RKR1*, which encodes a nuclear ubiquitin ligase (BRAUN *et al.* 2007). Previous work in the Arndt lab has demonstrated that this synthetic lethality results from the combined loss of Rtf1-dependent H2B ubiquitylation and an unidentified compensatory function that is mediated by Rkr1. I amplified a DNA fragment containing the HMD coding sequence using error-prone PCR to introduce random mutations. The resulting PCR products, along with a linearized *TRP1*-marked plasmid carrying triple HA-tagged *RTF1* that had been digested to remove the HMD coding sequence, were transformed into a strain where the genomic copies of *RTF1* and *RKR1* had been deleted. This strain was kept viable with a *URA3*-marked plasmid carrying wild type *RTF1*, which could be counterselected by growing these cells on media containing 5-FOA. Colonies that grew on media lacking tryptophan, but became inviable following exposure to 5-FOA, likely contained repaired plasmids harboring copies of *rtf1* with mutations in the HMD, since loss of Rtf1-dependent H2B ubiquitylation is lethal in the absence of Rkr1. *rtf1* null mutations were ruled out based on the strength of Spt$^-$ phenotype; a strong Spt- phenotype is associated with a null allele while HMD mutations cause a weak Spt$^-$ phenotype (Figure 14). Two HMD mutations were identified in this screen: *rtf1 F80V F123S* and *rtf1 E119K F123S* (Figure 22A). Although these residues are not invariant across the four Rtf1 homologs analyzed in Figure 15, the phenylalanine at position 123 in *S. cerevisiae* is conserved as a non-polar hydrophobic amino acid in the other three species examined.

3.3.2 Conserved residues in the Rtf1 HMD differentially affect H2B ubiquitylation and H3 methylation

I first tested the effects of the HMD point mutations on Rtf1-dependent histone modifications. Initially, I performed immunoblot analysis with an antibody specific for H3 K4 trimethylation on extracts prepared from an *rtf1Δ* strain that had been transformed with plasmids carrying each of the five *rtf1* HMD point mutations: *rtf1 102-104A*, *rtf1 108-110A*, *rtf1 120-121A*, *rtf1 F80V F123S*, and *rtf1 E119K F123S*. The resulting HMD mutant proteins are HA-tagged and I confirmed their expression is equivalent to wild type Rtf1 by anti-HA immunoblot analysis (Figure 22B). I also demonstrated that several of the HMD mutations do not impair cell growth on complete medium (Figure 24; right panel) and that the resulting mutant proteins were recruited normally to the 5' end of *CLN2* (data not shown). These results indicate that mutation of the HMD does not result in a gross misfolding of the resulting proteins. I found that four of the five HMD point mutations, with the exception of *rtf1 120-121A*, eliminated or greatly reduced H3 K4 trimethylation (Figure 22B).

To further characterize the consequences of these mutations, I integrated three of the alleles that affected H3 K4 trimethylation, *rtf1 102-104A*, *rtf1 108-110A*, and *rtf1 F80V F123S*, into the genome to replace wild type *RTF1*. Because I had observed that these mutations affected H3 K4 trimethylation, I next determined their effect on the upstream ubiquitylation of H2B at K123. I analyzed this modification using a strain that expressed HIS-Ub and FLAG-H2B, as described in Section 2.3.3. Interestingly, I found that *rtf1 108-110A* and *rtf1 F80V F123S* eliminated this modification, while it was essentially unaffected in an *rtf1 102-104A* strain (Figure 23A). I confirmed that the effect of *rtf1 108-110A* and *rtf1 F80V F123S* on H2B K123 ubiquitylation was not a consequence of decreased H2B levels by performing an anti-FLAG

immunoblot on whole cell extracts prepared from these strains and observed that equal or greater amounts of FLAG-H2B were extracted from HMD mutant strains when compared to a wild type strain (data not shown.)

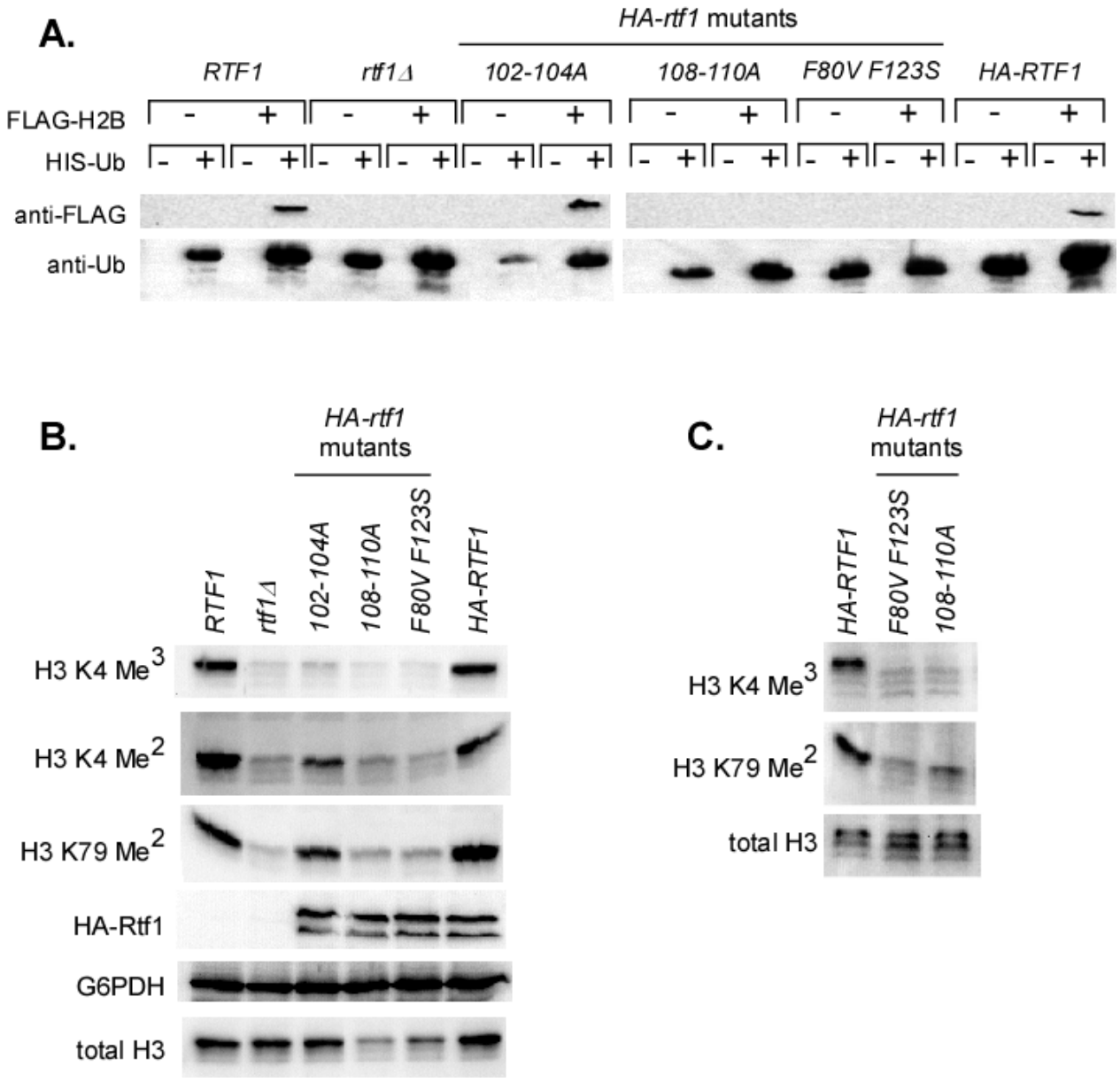
A.
HA-rtf1 mutants
RTF1
rtf1Δ
102-104A
108-110A
F80V F123S
HA-RTF1
FLAG-H2B
HIS-Ub
anti-FLAG
anti-Ub
B.
HA-rtf1 mutants
RTF1
rtf1Δ
102-104A
108-110A
F80V F123S
HA-RTF1
H3 K4 Me3
H3 K4 Me2
H3 K79 Me2
HA-Rtf1
G6PDH
total H3
C.
HA-rtf1 mutants
HA-RTF1
F80V F123S
108-110A
H3 K4 Me3
H3 K79 Me2
total H3

Figure 23. Conserved residues in the Rtf1 HMD differentially affect Rtf1-dependent histone modifications.

(A) Analysis of H2B K123 ubiquitylation levels in Rtf1 HMD substitution mutant strains. FY406 (*RTF1*), KY982 (*rtf1Δ*), KY1458 (*HA-RTF1*), MHY213 (*HA-rtf1 102-104A*), MHY215 (*HA-rtf1 108-110A*), and KY1462 (*HA-rtf1 F80V F123S*) were transformed with plasmids expressing untagged or HIS-tagged ubiquitin and plasmids expressing wild type or FLAG-tagged histone H2B as their only source of histone H2B. The HIS-tagged protein fraction from each strain was isolated and analyzed by immunoblot using an anti-FLAG antibody to detect FLAG-H2B (top panel). An anti-ubiquitin (anti-Ub) immunoblot was also performed (lower panel) to demonstrate expression and enrichment of HIS-ubiquitin (HIS-Ub) in the expected strains. (B) Immunoblot analysis of Rtf1-dependent histone methylation in Rtf1HMD substitution mutant strains. Extracts were prepared from KY279 (*RTF1*), KY1418 (*HA-RTF1*), KY404 (*rtf1Δ*), KY1193 (*HA-rtf1 102-104A*), KY1194 (*HA-rtf1 108-110A*), and KY1195 (*HA-rtf1 F80V F123S*) by glass bead lysis and probed with antibodies specific for H3 K4 Me^3, H3 K4 Me^2, H3 K79 Me^2, and total H3. An anti-G6PDH immunoblot was performed as a loading control and an anti-HA immunoblot was performed to demonstrate equivalent expression of HA-Rtf1 and Rtf1 HMD substitution mutant proteins. (C) Analysis of total H3 levels in extracts prepared from select Rtf1 HMD substitution mutant strains by a rapid boiling method. Rapid boiling extracts were prepared from KY1418 (*HA-RTF1*), KY1195 (*HA-rtf1 F80V F123*), and KY1194 (*HA-rtf1 108-110A*) and probed with antibodies specific for H3 K4 Me^3, H3 K79 Me^2, and total H3.

Due to the unexpected observation that unique HMD mutations had distinct effects on H2B ubiquitylation, I sought to perform a more thorough evaluation of the effects of these mutations on Rtf1-dependent H3 methylation. I prepared extracts from strains where *rtf1 102-104A*, *rtf1 108-110A*, or *rtf1 F80V F123S* had been integrated to replace the genomic copy of *RTF1* and performed immunoblot analysis using antibodies specific for di- or trimethylated H3 K4 or dimethylated H3 K79. As expected, the HMD mutations which eliminated H2B K123 Ub (*rtf1 108-110A* and *rtf1 F80V F123S*) also eliminated all three H3 methylation marks that were examined (Figure 23B). However, *rtf1 102-104A*, which did not affect H2B K123 ubiquitylation, specifically eliminates only H3 K4 trimethylation. Together, these results indicate that distinct conserved residues in the HMD differentially affect H2B ubiquitylation and H3 K4 trimethylation.

3.3.3 *rtf1 108-110A* and *rtf1 F80V F123S* may alter histone-DNA contacts

Another surprising observation made from the immunoblot analysis shown in Figure 23B is that *rtf1 108-110A* and *rtf1 F80V F123S* decrease the amount of total H3 present in whole cell extracts prepared by glass bead lysis (Figure 23B). However, I demonstrated that the effects of these mutations on Rtf1-dependent histone modifications are not simply a consequence of decreased H3 expression by repeating this immunoblot analysis on extracts prepared from *HA-RTF1*, *rtf1 108-110A*, and *rtf1 F80V F123S* strains by a rapid boiling method; when extracts are prepared in this manner, H3 K4 trimethylation and H3 K79 dimethylation are still dramatically decreased in *rtf1 108-110A* and *rtf1 F80V F123S* strains even though the amount of total H3 extracted under these conditions is equal to that observed in the *HA-RTF1* strain (Figure 23C).

A major difference between the two extract preparation methods is that the rapid boiling method lacks a centrifugation step that is performed during glass bead lysis. During this centrifugation step, intact genomic DNA is likely discarded along with any histones that are still associated. This suggests that the histone population being analyzed in extracts prepared by glass bead lysis represents free histones and histones which were dislodged from DNA during extract preparation. Because it appears that total histone H3 protein levels are unaffected by *rtf1 108-110A* and *rtf1 F80V F123S* mutations, my observations may instead suggest that these HMD mutations alter histone-DNA contacts or affect the balance of histones associated with DNA and in free pools. This effect is not likely due exclusively to the loss of H2B K123 ubiquitylation, because this modification is also absent from an *rtf1Δ* strain, where wild type levels of total H3 are extracted by glass bead lysis (Figure 23B). The different effects of *rtf1 108-110A* and *rtf1 F80V F123S* mutations on extraction of total H3 when compared to an *rtf1* null mutation may instead indicate that these HMD mutations represent gain-of-function alleles.

3.3.4 Point mutations in the Rtf1 HMD cause defects in transcription

I demonstrated that defects in transcription, as measured by the Spt$^-$ phenotype and sensitivity to the base analog 6-azauracil (6-AU), are caused by partial deletions of the HMD coding sequence: *rtf1Δ3* and *rtf1Δ4* (Figure 14). I repeated this analysis to investigate whether Rtf1 HMD substitution mutations also cause transcription-related phenotypes. In contrast to an *rtf1* null mutation or partial HMD deletions, the HMD point mutations cause no discernible Spt$^-$ phenotype in a strain containing the *his4-912δ* allele (Figure 24; center panel). However, the HMD mutations caused the same degree of 6-AU sensitivity as partial HMD deletions (Figure

24; left panel). Because sensitivity to 6-AU is an indicator of a transcription elongation defect, this result supports a role for the Rtf1 HMD in proper transcription elongation.

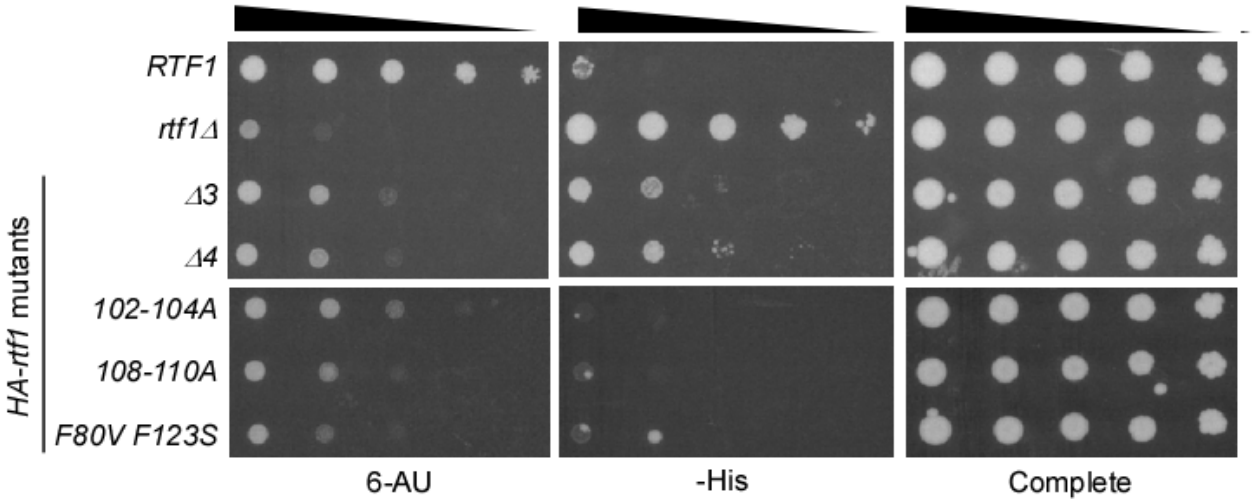

Figure 24. Rtf1 HMD substitution mutations cause phenotypes associated with transcription defects. Ten-fold serial dilutions, ranging from 1 x 10^8 cells/mL to 1 x 10^4 cells/mL, of KY1010 (*RTF1*), KY619 (*rtf1Δ*), KY1506 (*HA-rtf1Δ3*), KY1508 (*HA-rtf1Δ4*), KY1510 (*HA-rtf1 102-104A*), KY1511 (*HA-rtf1 108-110A*), and KY1512 (*HA-rtf1 F80V F123S*) were spotted on SC-Ura medium containing 50 μg/mL 6-AU to assess 6-AU sensitivity, SD-His medium to examine the Spt$^-$ phenotype, or SC Complete medium as a control for growth. Plates were incubated at 30^{o}C for 4 days.

3.3.5 Mutations in the Rtf1 HMD allow aberrant transcription initiation from cryptic promoters internal to coding regions and the telomeres

In addition to the transcription-related defects that occur in its absence, deletion of *RTF1* is also known to result in several phenotypes indicative of effects on normal chromatin function. As described in Section 2.3.3, loss of Rtf1 results in ineffective transcriptional silencing of the telomeres, a process which is highly sensitive to disruptions in global histone modification patterns. I analyzed the effectiveness of telomeric silencing in strains carrying the *TEL-VR::URA3* telomeric silencing reporter and integrated versions of *rtf1 102-104A*, *rtf1 108-110A*, or *rtf1 F80V F123S*. I demonstrated that these HMD mutations prevent growth on media containing 5-FOA, comparable to the effect observed when *RTF1* is completely deleted or when the HMD is partially removed (Figure 25A). Interestingly, *rtf1 120-121A*, the only *rtf1* HMD mutation that did not affect H3 K4 trimethylation in my initial analysis (Figure 22B), also did not affect telomeric silencing (data not shown.) This result very closely links Rtf1's roles in histone modification and telomeric silencing.

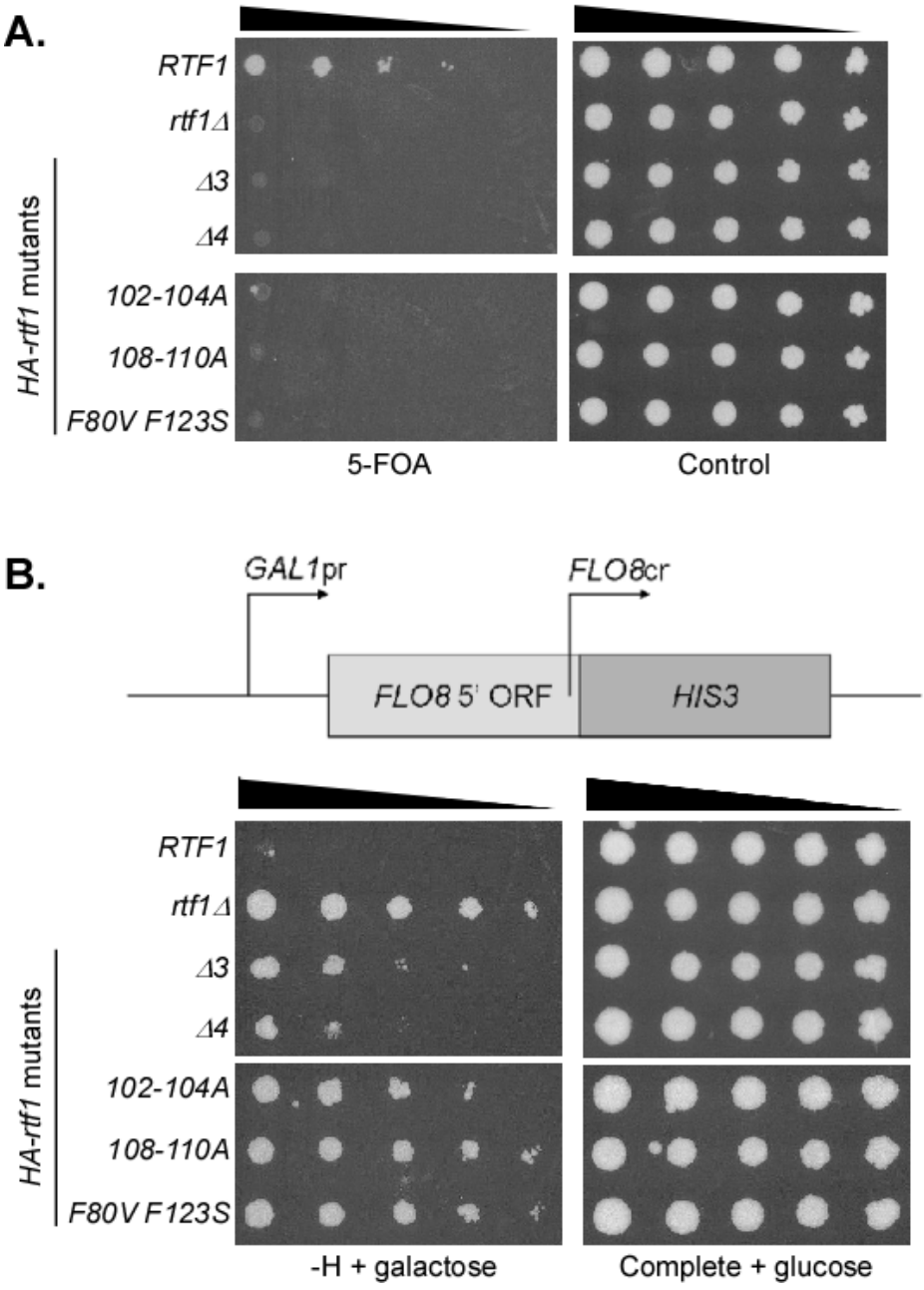
A.
RTF1
rtf1Δ
Δ3
Δ4
HA-rtf1 mutants
102-104A
108-110A
F80V F123S
5-FOA
Control
B.
GAL1pr
FLO8cr
FLO8 5' ORF
HIS3
RTF1
rtf1Δ
Δ3
Δ4
HA-rtf1 mutants
102-104A
108-110A
F80V F123S
-H + galactose
Complete + glucose

Figure 25. Substitution mutations in the Rtf1 HMD cause phenotypes associated with defects in chromatin structure.

(A) Analysis of telomeric silencing in strains expressing Rtf1 HMD substitution mutants. Ten-fold serial dilutions, ranging from 1 x 10^8 cells/mL to 1 x 10^4 cells/mL, of KY1226 (*RTF1*), KY1227 (*rtf1Δ*), MHY188 (*HA-rtf1Δ3*), MHY190 (*HA-rtf1Δ4*), MHY192 (*HA-rtf1 102-104A*), MHY194 (*HA-rtf1 108-110A*), and MHY197 (*HA-rtf1 F80V F123S*) were spotted on SC-Ura medium containing 5-FOA to assess telomeric silencing defects or SC Complete medium as a control for growth. Plates were incubated at 30°C for 3 days. (B) Substitution mutations in the Rtf1 HMD allow aberrant initiation from a cryptic promoter within the *FLO8* open reading frame. Top; Schematic of the *GAL1pr-FLO8-HIS3* reporter (PRATHER 2005). *GAL1pr* = *GAL1* promoter; *FLO8cr* = *FLO8* cryptic internal promoter. Bottom; Ten-fold serial dilutions (1 x 10^8 - 1 x 10^4 cells/mL) of KY1116 (*RTF1*), KY1207 (*rtf1Δ*), KY1208 (*HA-rtf1Δ3*), KY1209 (*HA-rtf1Δ4*), KY1210 (*HA-rtf1 102-104A*), KY1211 (*HA-rtf1 108-110A*), and KY1212 (*HA-rtf1 F80V F123S*) were spotted on SC-His + galactose medium to induce and monitor expression of the reporter and SC Complete medium as a control for growth. Plates were incubated at 30°C for 7 days. The *GAL1pr-FLO8-HIS3* reporter was kindly provided by Fred Winston.

Rtf1 is also required to suppress internal initiation from a cryptic promoter within the *FLO8* gene (WINSTON, F.; unpublished communication). This internal promoter is expressed when chromatin structure across the *FLO8* gene is disrupted, as is thought to occur in strains with mutations in certain transcription and chromatin-related factors. Effects on internal initiation from *FLO8* can be monitored using a reporter strain where the *HIS3* gene, which encodes an enzyme in the histidine biosynthesis pathway, is integrated into the *FLO8* reading frame downstream of the internal promoter; because *FLO8* is expressed at low levels, the native *FLO8* promoter was replaced with the highly inducible *GAL1* promoter (*GAL1 pr*; Figure 25B) (PRATHER 2005). Expression from the internal promoter can then be monitored by growing *GAL1pr-FLO8-HIS3* reporter strains on galactose-containing media that lacks histidine. I used this reporter to demonstrate that *rtf1 102-104A*, *rtf1 108-110A*, and *rtf1 F80V F123S* result in internal initiation at *FLO8* at a level equivalent to that observed when *RTF1* is completely deleted (Figure 25B). Interestingly, this effect is more severe than that caused by partial HMD deletions. This result indicates that substitution of conserved residues in the HMD is not functionally equivalent to completely removing these residues from the protein.

3.3.6 The Rtf1 HMD is sufficient to promote Rtf1-dependent histone modifications

I have established that the Rtf1 HMD is necessary for Rtf1-dependent histone modifications; I next sought to determine if it was also sufficient. To this end, I created a fusion of the Rtf1 HMD, encompassing residues 63 through 152 of the full-length protein, to the DNA binding domain of the Gal4 transcriptional activator (GBD-HMD). This construct also contains a single Myc epitope between the GBD and HMD. As controls, I also created a fusion of the Rtf1 102-104A HMD to GBD (GBD-102-104A-HMD) and a GBD fusion to an HMD containing a single

F123S substitution (GBD-F123S-HMD). I focused on the F123S substitution because it was isolated multiple times in the error-prone mutagenesis screen described in Section 3.2.3. I performed immunoblot analysis on extracts prepared from an *rtf1Δ gal4Δ* strain that had been transformed with plasmids expressing the GBD-HMD fusions. All three GBD-HMD fusions were expressed and recognized by an anti-Rtf1 antibody (Figure 26). I also analyzed expression of these fusions from the same extracts using an anti-Myc antibody; expression of GBD-Myc, GBD-HMD and GBD-F123S-HMD was detected in these extracts at nearly equivalent levels. However, GBD-102-104A-HMD could not be detected with an anti-Myc antibody in whole cell extracts (Figure 26). This is likely the result of the lower expression level of this protein when compared with expression of the other GBD fusions, as is observed in the anti-Rtf1 immunoblot analysis. Interestingly, all three GBD-HMD fusions impair growth of wild type, *rtf1Δ*, and *rtf1Δ gal4Δ* strains when compared to the same strains expressing GBD-Myc alone (data not shown). This result may suggest that the GBD-HMD fusions function as dominant-negatives, potentially by sequestering another factor involved in histone modification.

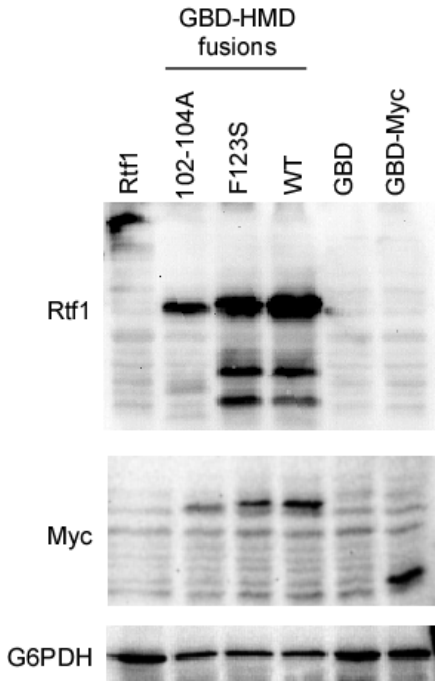

Figure 26. Expression of GBD-HMD fusion proteins.

Extracts were prepared from an *rtf1Δ gal4Δ* strain (MHY119) transformed with 2μ plasmids that express the indicated GBD constructs or pLS20, a *CEN/ARS* plasmid that expresses untagged full-length Rtf1. Immunoblots were performed on these extracts with anti-Rtf1 and anti-Myc antibodies to assess expression of the GBD-HMD fusions and, as a loading control, anti-G6PDH.

The motivation for creating the GBD-HMD fusions was to determine whether they were sufficient to induce Rtf1-dependent histone modifications when recruited to Gal4 binding sites present at a nontranscribed region of the genome. I performed ChIP assays on chromatin extracted from an *rtf1Δ gal4Δ* strain expressing GBD-Myc, untagged GBD, untagged Rtf1, full-length GBD-Rtf1, or each of the three GBD-HMD fusions. I determined whether H3 K4 trimethylation was present in these strains at the upstream activating sequence (UAS) of *GAL7*, which contains two Gal4 binding sites (Figure 27A) (LORCH and KORNBERG 1985). I determined that GBD-Myc and the three GBD-HMD fusions were recruited to this site, although wild type GBD-HMD is recruited at significantly higher levels than mutant GBD-HMD fusions or the GBD-myc control protein (Figure 27B). Chromatin utilized in these experiments was prepared from strains grown in glucose, a condition under which *GAL7* is transcriptionally inactive. I confirmed that Pol II was present at this locus at levels roughly equivalent to that observed at the telomeres, which are known to be transcriptionally silent (Figure 27C) (GOTTSCHLING *et al.* 1990). Expression of these fusion proteins also did not significantly affect levels of total histone H3 present at the *GAL7* UAS (Figure 27D).

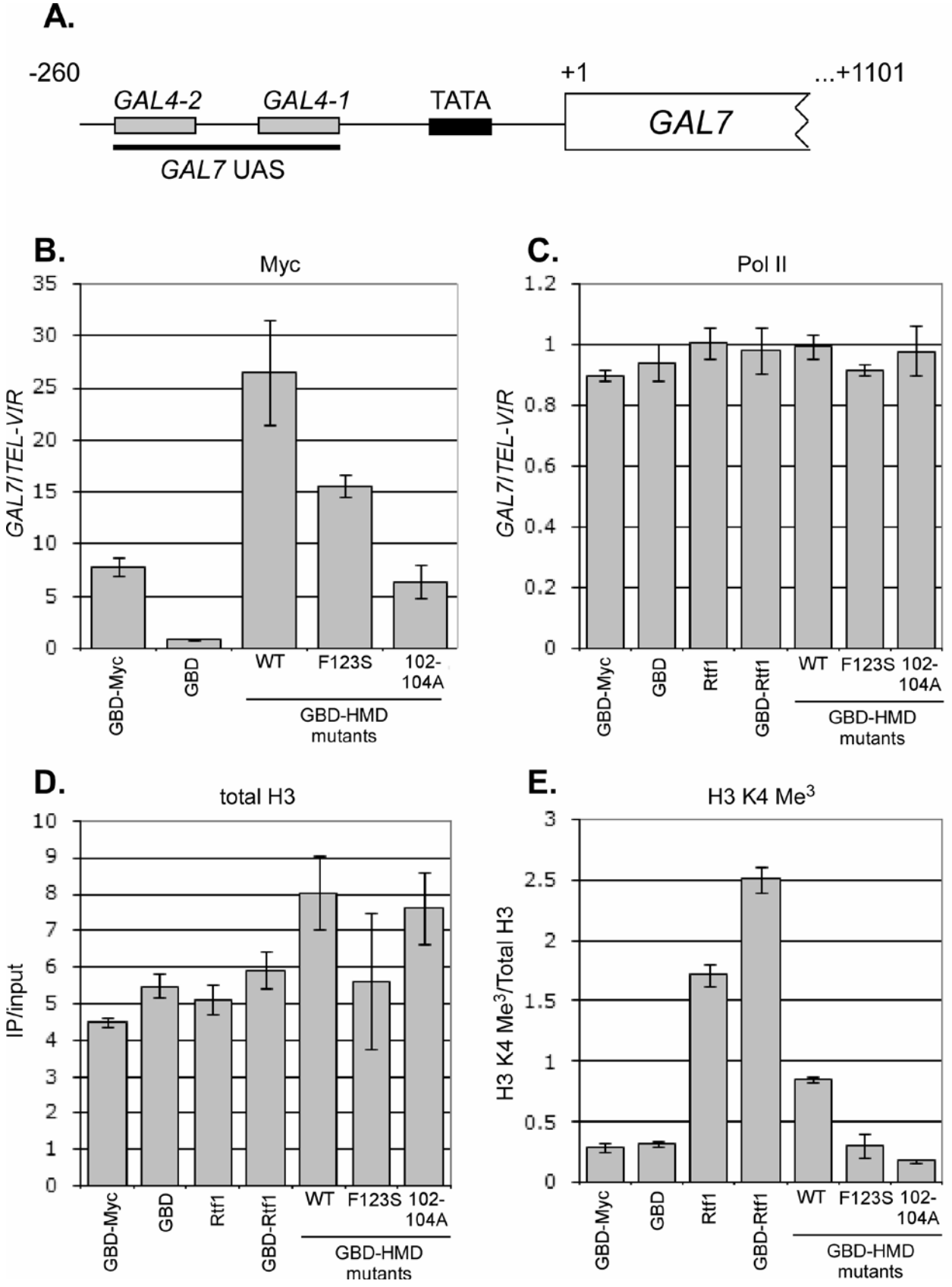
A.
-260
+1
...+1101
GAL4-2
GAL4-1
TATA
GAL7
GAL7 UAS
B.
Myc
GAL7/TEL-VIR
GBD-Myc
GBD
WT
F123S
102-104A
GBD-HMD mutants
C.
Pol II
GAL7/TEL-VIR
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants
D.
total H3
IP/input
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants
E.
H3 K4 Me3
H3 K4 Me3/Total H3
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants

Figure 27. The Rtf1 HMD is sufficient to induce H3 K4 trimethylation at the *GAL7* UAS.

(A) Schematic of the *GAL7* promoter region. The *GAL7* promoter contains a UAS with two 17 bp Gal4 binding sites, *GAL4-1* and *GAL4-2*, contained in a region spanning approximately 150 bp through 260 bp upstream of the translation start site. The region amplified in the *GAL7* UAS ChIP studies spans coordinates -298/-92 upstream of *GAL7*. (B) through (E) ChIP analyses of the effects of GBD-HMD fusions at the *GAL7* UAS. Antibodies specific for the Myc epitope (B), the largest subunit of Pol II (8WG16) (C), total H3 (D), and H3 K4 Me^3 (E) were used to immunoprecipitate these factors and associated DNA from a formaldehyde-treated *rtf1Δ gal4Δ* strain (MHY119) that had been transformed with 2μ plasmids expressing the indicated GBD or Rtf1 derivatives. Association of the indicated proteins or levels of histone modifications were assessed by PCR. Results in (B) and (C) are shown as a ratio of the signal observed at the *GAL7* promoter to the signal derived from a nontranscribed region in the right telomere of chromosome VI (*TEL-VIR*). Values in (D) and (E) are not normalized to *TEL-VIR*. *GAL7* UAS signal derived from the H3 K4 Me^3 samples (E) was normalized to total H3 levels (E) at the same position. The mean of three independent experiments with standard error is shown.

My analyses indicated that H3 K4 trimethylation at the *GAL7* UAS was increased three-fold in strains expressing GBD-HMD when compared to strains expressing GBD alone or GBD-Myc (Figure 27E). GBD-102-104A-HMD and GBD-F123S-HMD caused no detectable increase in H3 K4 trimethylation at this locus, consistent with the effect of these mutations in whole cell extracts. This result indicates that promoting Rtf1-dependent histone modifications is an intrinsic property of the HMD.

However, I also observed that full-length Rtf1 induced substantial levels of H3 K4 trimethylation at this locus, regardless of whether it was expressed as a GBD fusion or untagged (Figure 27E). The presence of this modification at the *GAL7* UAS in a strain expressing untagged Rtf1 is unexpected because *GAL7* should be inactive under these conditions and Rtf1-dependent histone modifications are typically found in transcriptionally active regions. This may indicate that transcription has recently occurred at this locus, or is currently occurring at a very low level, because H3 K4 trimethylation is known to be highly stable even several hours following the cessation of transcription (NG *et al.* 2003c).

Another explanation for the presence of H3 K4 trimethylation at this locus is that Rtf1 may not need to be recruited to DNA to induce Rtf1-dependent histone modifications. The *GAL7* UAS may be particularly poised to become modified in this way as long as a functional Rtf1 HMD is present in the cell. Precedence for the idea that Rtf1 does not require targeting to active genes to promote histone modifications can be found in my previous observation that when the region of Rtf1 that mediates its association with active genes (regions 6 through 9; Figure 19) is mutated, global levels of Rtf1-dependent histone methylation are relatively unaffected (Figure 18A).

To determine whether the HMD needed to be recruited to DNA to elicit Rtf1-dependent histone modifications, I examined the presence of these modifications at the 5' end of *PYK1*, a location which does not harbor Gal4 binding sites, in the same strains analyzed in the *GAL7* UAS studies. *PYK1* is constitutively expressed at high levels and I observed ten- to twenty-five-fold greater association of Pol II at the 5' end of *PYK1* than at the telomeres in all strains examined (Figure 28A). Due to the high expression level of *PYK1*, Rtf1-dependent histone modifications should be present within its 5' coding region in strains expressing full-length Rtf1. Accordingly, higher levels of H3 K4 trimethylation are observed at this locus in strains expressing untagged Rtf1 or GBD-Rtf1 than are seen in strains expressing GBD alone (Figure 28B). Full-length Rtf1 is known to associate with the 5' end of PYK1 (WARNER *et al.* 2007). However, the GBD-HMD fusions and control proteins should not associate with this locus due to the absence of the Rtf1 OAR and Gal4 binding sites; I verified that these proteins were not enriched in the 5' end of *PYK1* (Figure 28C). Total H3 levels at the 5' end of *PYK1* are also not dramatically impacted by expression of different Rtf1 constructs (Figure 28D); the most significant effect is a slightly greater than two-fold increase in total H3 levels at this locus in a strain expressing GBD-102-104A-HMD compared to a strain expressing wild type Rtf1.

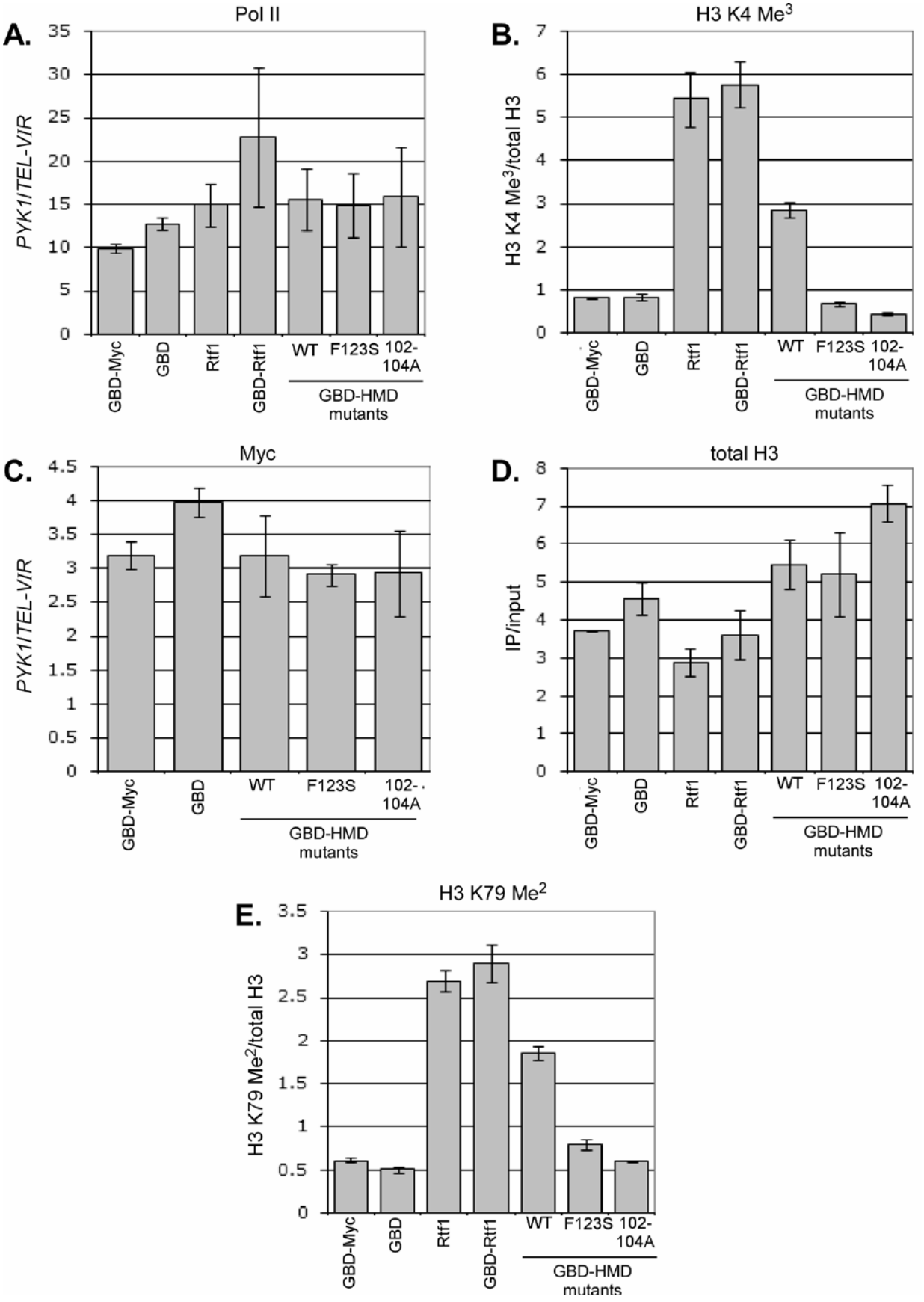
A.
Pol II
PYK1/TEL-VIR
35
30
25
20
15
10
5
0
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants
B.
H3 K4 Me3
H3 K4 Me3/total H3
7
6
5
4
3
2
1
0
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants
C.
Myc
PYK1/TEL-VIR
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
GBD-Myc
GBD
WT
F123S
102-104A
GBD-HMD mutants
D.
total H3
IP/input
8
7
6
5
4
3
2
1
0
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants
E.
H3 K79 Me2
H3 K79 Me2/total H3
3.5
3
2.5
2
1.5
1
0.5
0
GBD-Myc
GBD
Rtf1
GBD-Rtf1
WT
F123S
102-104A
GBD-HMD mutants

Figure 28. The Rtf1 HMD does not require targeting to DNA to induce Rtf1-dependent histone modifications.

ChIP analyses of the effects of GBD-HMD fusions at the 5' end of *PYK1*. Antibodies specific for the largest subunit of Pol II (8WG16) (A), H3 K4 Me^3 (B), the Myc epitope (C), total H3 (D), and H3 K79 Me^2 (E) were used to immunoprecipitate these factors and associated DNA from a formaldehyde-treated *rtf1Δ gal4Δ* strain (MHY119) that had been transformed with 2μ plasmids expressing the indicated GBD or Rtf1 derivatives. Association of the indicated proteins or levels of histone modifications are assessed by PCR. Results in (A) and (C) are shown as a ratio of the signal observed at the 5' end of *PYK1* to the signal derived from a nontranscribed region in the right telomere of chromosome VI (*TEL-VIR*). Values in (B), (D), and (E) are not normalized to *TEL-VIR*. *PYK1* signal derived from the H3 K4 Me^3 and H3 K79 samples (B and E) was normalized to total H3 levels at the same position. The mean of three independent experiments with standard error is shown.

Surprisingly, GBD-HMD induces H3 K4 trimethylation to the same extent at the 5' end of *PYK1* as was observed at the *GAL7* UAS (Figure 28B). I observed a similar induction of H3 K79 dimethylation at this locus in strains expressing untagged Rtf1, GBD-Rtf1, and GBD-HMD (Figure 28E). The increase in H3 K4 trimethylation and H3 K79 dimethylation observed at the 5' end of *PYK1* in strains expressing GBD-HMD was not seen in strains expressing GBD fusions to the 102-104A or F123S HMDs. *rtf1 102-104A* did not affect bulk H3 K79 dimethylation levels in whole cell extracts and the effect on this modification observed in this analysis is likely a consequence of the low expression level of the GBD-102-104A-HMD fusion protein.

To determine if the GBD-HMD fusions could affect Rtf1-dependent histone modifications on a global scale, I performed immunoblot analysis with antibodies specific for H3 K4 trimethylation and H3 K79 dimethylation on whole cell extracts from an *rtf1Δ gal4Δ* strain expressing GBD alone, GBD-Myc, full-length Rtf1, or the three GBD-HMD fusions. Interestingly, I observed nearly wild type levels of H3 K79 dimethylation and a modest restoration of H3 K4 trimethylation in the GBD-HMD strain, while these modifications were absent from GBD-102-104A-HMD and GBD-F123S-HMD strains (Figure 29). Together, the results of my *PYK1* ChIP analysis and the immunoblot analysis described here support the idea that the Rtf1 HMD possesses inherent activity and that it does not need to be specifically recruited to DNA to promote Rtf1-dependent histone modifications.

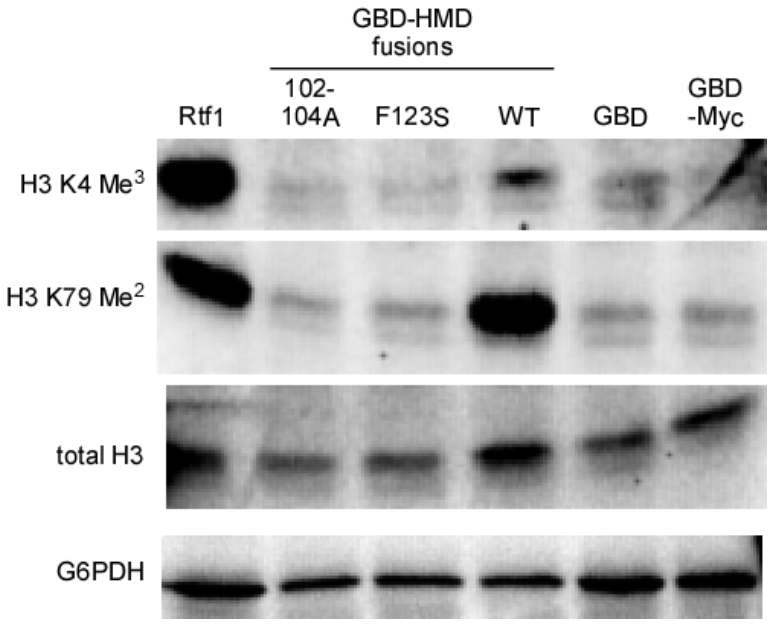

Figure 29. The Rtf1 HMD can partially restore global Rtf1-dependent histone modifications.

Extracts were prepared from an *rtf1Δ gal4Δ* strain (MHY119) transformed with 2μ plasmids that express the indicated GBD constructs or pLS20, a *CEN/ARS* plasmid that expresses untagged full-length Rtf1. Immunoblots were probed with antibodies specific for H3 K4 Me^3 and H3 K79 Me^2 to assess the levels of these modifications in the indicated extracts. A total H3 immunoblot was performed to demonstrate effects on these modifications are not due to a significant change in histone H3 expression and an anti-G6PDH immunoblot was performed as a loading control.

3.4 CONCLUSIONS

Rtf1 has been implicated in several transcription-related functions, but the mechanisms by which it participates in these processes remain undefined. My previous analyses identified a region of Rtf1, now termed the HMD, which is necessary for its role in the covalent modification of histones that are present in the bodies of transcriptionally active genes. The experiments described in this chapter were aimed at better characterizing the HMD to begin exploring the mechanism by which Rtf1 promotes histone modification.

I created substitution mutations in several conserved residues in the HMD and assessed the effects of these alterations on histone modification. Interestingly, I found that different substitution mutations in the HMD resulted in unique effects on Rtf1-dependent histone modifications. This result indicates that the Rtf1 HMD may promote histone H2B lysine 123 monoubiquitylation and histone H3 K4 trimethylation by unique mechanisms.

Because I had previously observed that partially deleting the HMD led to transcription-related phenotypes (Figure 14), I explored the effects of the HMD substitution mutations on these phenotypes. I found that all three mutations tested, *rtf1 102-104A*, *rtf1 108-110A*, and *rtf1 F80V F123S*, caused sensitivity to 6-AU. However, the HMD substitution mutations caused no obvious Spt$^-$ phenotype.

A complete deletion of *RTF1* also causes several phenotypes associated with defects in chromatin structure. I demonstrated that these phenotypes, loss of telomeric silencing and aberrant internal initiation from within the *FLO8* open reading frame, are also caused by *rtf1 102-104A*, *rtf1 108-110A*, and *rtf1 F80V F123S*. Effects on telomeric silencing reflect a global

disruption of histone modifications patterns, while internal initiation from a cryptic promoter within the *FLO8* open reading frame is a read-out of an effect on chromatin structure at this specific locus. These results therefore suggest that HMD substitution mutations lead to chromatin defects on both global and local scales.

Interestingly, aberrant initiation from the *FLO8* internal promoter and the Spt$^-$ phenotype both reflect the ability of the transcription machinery to gain access to abnormal transcriptional start sites. Rtf1-dependent histone modifications are found specifically within ORFs, where the *FLO8* internal promoter is located. The Spt$^-$ phenotype measures the ability to suppress insertions at specific promoters, which are devoid of Rtf1-dependent histone modifications. Because the Rtf1 HMD substitution mutations also display sensitivity to 6-AU, a phenotype associated with transcription elongation defects, these results may suggest that mutations in the Rtf1 HMD specifically affect chromatin structure within ORFs. Mutations in various elongation factors, including an *rtf1* null allele, are known to cause strong Spt$^-$ phenotypes. Therefore, it remains likely that the Spt$^-$ phenotype is influenced by effects on transcription elongation. However, the effect of Rtf1 on this phenotype appears to be mediated by a function other than its role in histone modification.

Despite their differential effects on Rtf1-dependent histone modifications, HMD substitution mutations always result in equivalent phenotypic effects. This may indicate that H3 K4 trimethylation is particularly important to prevent defects in transcription elongation and chromatin structure, since this is the only modification absent in all HMD substitution mutants tested in the phenotypic analysis. Interestingly, HMD substitution mutations sometimes result in phenotypes that are different from those caused by an *rtf1* null mutation or partial HMD deletions, suggesting that mutating the HMD and removing it are not functionally equivalent.

Finally, I found that expressing only the residues encompassing the HMD as a fusion to the Gal4 DNA binding domain (GBD-HMD) was sufficient to elicit a three-fold induction of H3 K4 trimethylation at the *GAL7* UAS, where this fusion protein is recruited by Gal4 binding sites, indicating that promoting histone modifications is an intrinsic property of the HMD. Surprisingly, I found that expression of the GBD-HMD also induced H3 K4 trimethylation and H3 K79 dimethylation at the 5' end of *PYK1*, where GBD-HMD binding is not detected, and at least partially restored both of these modifications in whole cell extracts. These observations indicate that the HMD does not need to be specifically recruited to DNA to elicit histone modifications. Overall, the analyses described in this chapter indicate that Rtf1's role in histone modification can be attributed to a number of highly conserved residues in the HMD and that these residues differentially contribute to H2B ubiquitylation and H3 K4 trimethylation. In addition to being necessary for Rtf1-dependent histone modifications, the HMD is also sufficient for this function and may not require targeting to active genes to elicit this activity.

4.0 THE PAF1 COMPLEX IS CONNECTED TO CHROMATIN REMODELING THROUGH GENETIC INTERACTIONS WITH THE INOSITOL POLYPHOSPHATE SIGNALING CASCADE

4.1 INTRODUCTION

A screen for factors that became essential for viability when *RTF1* was deleted identified several transcription-related factors and suggested a role for Rtf1 in transcription (COSTA and ARNDT 2000). The same screen also identified mutations in the genes that code for the first two enzymes in the inositol polyphosphate (IP) signaling cascade: *PLC1* and *ARG82* (Figure 12) (COSTA 2001). Plc1 catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol trisphosphate (IP_3) (FLICK and THORNER 1993). Arg82 converts nuclear IP_3 to tetrakis- and pentakisphosphorylated derivatives (IP_4 and IP_5) (SAIARDI *et al.* 1999). Because several factors that were identified as essential in the absence of *RTF1* were ultimately linked to transcription elongation, the function of Plc1 and Arg82 may also be connected to this process.

In addition to this possible connection to transcription elongation, disruption of the IP signaling cascade has been implicated in several other nuclear processes including chromatin remodeling and mRNA export. IP_4 and IP_5, the direct products of Arg82 activity, stimulate the SWI/SNF chromatin remodeling complex to mobilize nucleosomes *in vitro* (SHEN *et al.* 2003).

A separate study reported that the chromatin structure of the *PHO5* promoter was not efficiently remodeled and the SWI/SNF and INO80 complexes were poorly recruited to the promoter of *PHO84* in the absence of Arg82 (STEGER *et al.* 2003). Additionally, mutations in *PLC1* and *ARG82* were recovered in a screen for factors that become essential in combination with a temperature-sensitive version of Gle1, an mRNA export factor that associates with the nuclear pore complex (YORK *et al.* 1999). Further analysis demonstrated that mutations in *PLC1* and *ARG82* result in a striking accumulation of polyadenylated transcripts in the nucleus, suggesting that these mutations severely disrupt export of mRNA to the cytoplasm.

I investigated whether these known nuclear functions for the IP signaling cascade were responsible for the synthetic lethality observed when *PLC1* and *ARG82* are mutated in an *RTF1* deletion strain. Because a complete deletion of *PLC1* causes a severe growth defect, I performed these analyses using *arg82Δ* strains. I found that the absence of Arg82 strongly impaired the growth of several strains lacking known elongation factors, including four of the five subunits of the Paf1 complex. An *arg82Δ paf1Δ* strain was inviable; I determined that this lethality was not the result of an enhanced defect in mRNA export, but instead was likely due to interactions between Paf1, Arg82, and specific chromatin remodeling complexes. These results suggest that IP signaling, transcription elongation factors, and ATP-dependent chromatin remodeling cooperate to support normal cell growth.

4.2 MATERIALS AND METHODS

4.2.1 Genetic methods

S. cerevisiae strains used in these studies are listed in Table 3. All strains are *GAL2*$^{+}$ derivatives of S288C (WINSTON *et al.* 1995). Complete disruptions of *RTF1, PAF1, CTR9, CDC73, LEO1, SPT4, DST1, SET1, BRE1, ARG82, ISW1, ISW2, CHD1, INO80, ARP8, SNF2,* and *SWR1* were created by a PCR-based gene replacement method (AUSUBEL *et al.* 1988). *spt5-194* is a temperature sensitive mutation in the essential *SPT5* gene.

Mating, sporulation, and tetrad dissection were performed according to standard methods (ROSE *et al.* 1990). Null mutations are marked by the incorporation of an auxotrophic marker in place of the native gene (Table 3). Following sporulation and dissection, sorting of marked deletions is assayed by replica printing to a series of media that lacks individual amino acids or nucleic acids; the auxotrophic profile of each spore can be determined by this method. Synthetic lethality of double mutants is inferred when spores containing marker genes for both knockouts sorting in a genetic cross are consistently not recovered. If both knockouts in a cross are marked by the same nutritional marker, the progeny of tetrads that sort 2:2 for growth on media lacking the corresponding nutrient (nonparental ditypes) are analyzed; the two spores capable of growth in the absence of this nutrient are surmised to contain both deletions. *spt5-194* is not marked by a nutritional marker; sorting of this mutation is followed by its known strong Spt- phenotype in a strain containing *his4-912δ*. *dst1Δ* was followed by sensitivity to mycophenolic acid.

4.2.2 Analysis of mRNA export

mRNA export analyses were conducted as described in (MURPHY *et al.* 1996) except that oligo-dT(30) prelabeled with digoxigenin (IDT) and Antidigoxigenin-rhodamine Fab fragments (Boehringer Mannheim) were used and strains were grown at 30°C. KY660 (wild type), KY804 (*paf1Δ*), MHY82 (*ipk1Δ paf1Δ*), KY875 (*ipk1Δ*), and KY743 (*arg82Δ*) were used in these analyses.

4.2.3 Northern analysis

KY292 (wild type), KY802 (*paf1Δ*), KY930 (*rtf1Δ*), MHY63 (*arg82Δ*), KY508 (*snf2Δ*), MHY88 (*arp8Δ*), MHY57 (*ino80Δ*), MHY75 (*isw1Δ*), KY884 (*isw2Δ*), KY632 (*chd1Δ*), KY907 (*set1Δ*), and KY968 (*bre1Δ*) were grown to approximately 1 x 10^7 cells/ml in YPD. RNA isolation and Northern analyses were performed as described previously (SWANSON *et al.* 1991). [α-^{32}P]dATP-labeled DNA hybridization probes were prepared by random prime labeling of *VTC3*, *SPL2*, or *SCR1* PCR products that had been amplified from yeast genomic DNA. *SCR1* encodes a cytoplasmic RNA that is transcribed by RNA Polymerase III (HANN and WALTER 1991) and is used as a loading control.

Table 3. *Saccharomyces cerevisiae* strains used in Chapter 4

Strain	Genotype
ECY57	*MATα paf1Δ::kanMX leu2Δ0 ura3Δ0*
KY292	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY404	*MAT***a** *rtf1Δ::LEU2 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY452	*MAT***a** *rtf1Δ::URA3 his3Δ200 lys2-173R2 ura3-52 trp1Δ63*
KY508	*MAT***a** *snf2Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY528	*MATα dst1Δ::HISG his4-912δ lys2-128δ leu2Δ1 ura3-52*
KY583	*MAT***a** *chd1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY632	*MATα chd1Δ::URA3 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY678	*MATα ctr9Δ::kanMX4 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY688	*MAT***a** *cdc73Δ::kanMX4 lys2Δ0 ura3Δ0 trp1Δ63*
KY715	*MAT***a** *spt5-194 his3Δ200 leu2Δ1 ura3-52*
KY740	*MATα arg82Δ::URA3 leu2Δ0 ura3Δ0 trp1Δ63 met15Δ0*
KY741	*MAT***a** *arg82Δ::URA3 his3Δ200 leu2Δ0 ura3Δ0 trp1Δ63 met15Δ0*
KY742	*MATα arg82Δ::URA3 leu2Δ0 ura3Δ0 met15Δ0*
KY743	*MAT***a** *arg82Δ::URA3 his3Δ200 leu2Δ0 ura3Δ0*
KY744	*MATα arg82Δ::URA3 his3Δ200 leu2Δ0 ura3Δ0 met15Δ0*
KY758	*MAT***a** *spt4Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY802	*MAT***a** *paf1Δ::URA3 his3Δ200 lys2-173R2 ura3(Δ0 or -52)*
KY804	*MATα paf1Δ::URA3 his3Δ200 leu2Δ(0 or 1) ura3(Δ0 or -52)*
KY806	*MAT***a** *leo1Δ::URA3 his3Δ200 lys2-173R2 ura3-52*

KY875	*MAT***a** *ipk1Δ::HIS3 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
KY879	*MATα arp8Δ::HIS3 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
KY884	*MAT***a** *isw1Δ::HIS3 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
KY885	*MATα isw2Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 ade8*
KY901	*MATα isw1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY907	*MAT***a** *set1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY930	*MATα rad6Δ::URA3 lys2-128δ ura3-52*
KY968	*MAT***a** *bre1Δ::kanMX4 his3Δ200 ura3-52*
KY972	*MATα swr1Δ::kanMX4 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
MHY8	*MAT***a** *arp8Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8*
MHY57	*MAT***a** *ino80Δ::HIS3 his3Δ200 his4-912δ lys2-173R2 leu2Δ1 ura3-52*
MHY63	*MATα arg82Δ::URA3 his3Δ200 leu2Δ(0 or 1) ura3(Δ0 or -52)*
MHY66	*MAT***a** *paf1Δ::URA3 his3Δ200 leu2Δ(0 or 1) ura3(Δ0 or -52) trp1Δ63*
MHY75	*MAT***a** *isw1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
MHY82	*MATα ipk1Δ::HIS3 paf1Δ::URA3 his3Δ200 ?lys2-173R2? leu2Δ(0 or 1) ura3(Δ0 or -52) trp1Δ63*
MHY86	*MAT***a** *paf1Δ::URA3 his3Δ200 leu2Δ(0 or 1) ura3(Δ0 or -52) ade8*
MHY88	*MAT***a** *arp8Δ::HIS3 his3Δ200 lys2-128δ leu2Δ(0 or 1) ura3(Δ0 or -52)*

4.3 RESULTS

4.3.1 *arg82Δ* genetically interacts with mutations in several known elongation factors

To determine whether the genetic interaction resulting from combination of mutations in IP signaling enzymes and deletion of *RTF1* was specific, I crossed an *arg82* null strain by strains harboring deletions in the genes encoding each subunit of the Paf1 complex: *PAF1*, *CTR9*, *RTF1*, *CDC73*, and *LEO1*. Deletion of *ARG82* results in synthetic lethality when combined with *paf1* or *ctr9* null mutations (Figure 30A and B and Table 4) and combination of an *ARG82* deletion with *rtf1* or *cdc73* null mutations resulted in considerable synthetic cell sickness (Figure 30C and D and Table 4). Although an *arg82Δ leo1Δ* strain displayed only a minor growth defect (Figure 30E and Table 4), the strong genetic interactions I observe between an *arg82* null mutation and deletion of four of the five genes encoding Paf1 complex subunits suggest that the connection between the IP signaling cascade and transcription elongation is not specifically mediated through Rtf1.

The severity of the genetic interaction between *rtf1* and *arg82* null mutations differs from the results of the original genetic screen where combination of an *RTF1* deletion with a point mutation in *ARG82* (*arg82-383*) was lethal (COSTA 2001). This disparity may reflect differences in the genetic background of the strains used in these analyses or may indicate that deletion of *ARG82* is not equivalent to *arg82-383*.

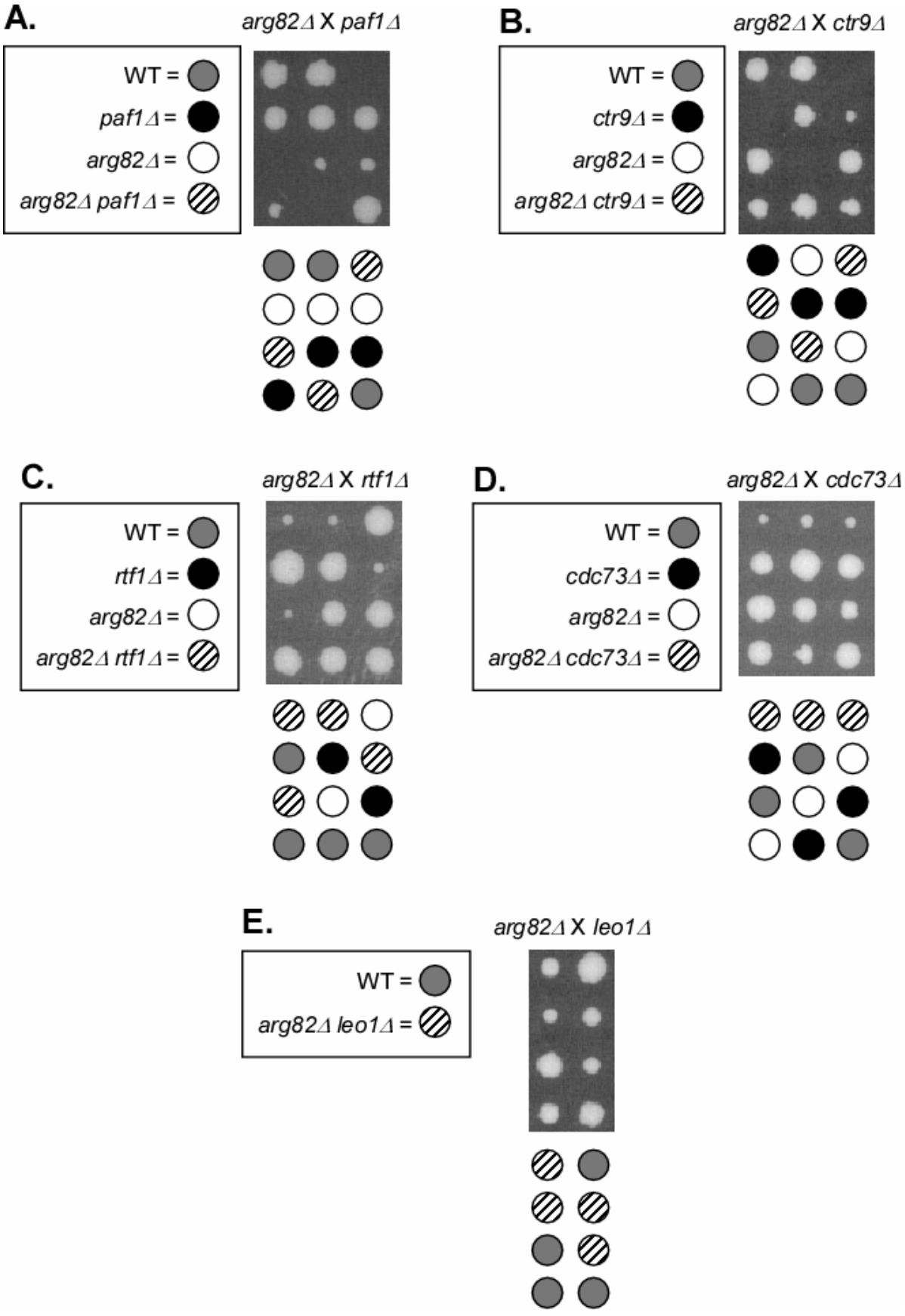
A.
arg82Δ X paf1Δ
WT =
paf1Δ =
arg82Δ =
arg82Δ paf1Δ =
B.
arg82Δ X ctr9Δ
WT =
ctr9Δ =
arg82Δ =
arg82Δ ctr9Δ =
C.
arg82Δ X rtf1Δ
WT =
rtf1Δ =
arg82Δ =
arg82Δ rtf1Δ =
D.
arg82Δ X cdc73Δ
WT =
cdc73Δ =
arg82Δ =
arg82Δ cdc73Δ =
E.
arg82Δ X leo1Δ
WT =
arg82Δ leo1Δ =

Figure 30. *arg82Δ* genetically interacts with deletions of the genes encoding Paf1 complex subunits.

Yeast strains of opposite mating type were mated, sporulated, and dissected on YPD. Strains used for each individual cross are indicated in the legend to Table 4. Each vertical row contains the four spores derived from meiosis of a single diploid yeast cell. Plates were incubated at 30^{o}C for 4 days. Because the deletions of *ARG82* and *LEO1* are both marked by *URA3*, only nonparental ditypes tetrads are shown (E).

Table 4. Genetic interactions between *arg82Δ* and mutations in known elongation factors

Strain	Relative growth rate
arg82Δ	+++++
dst1Δ arg82Δ	+++++
leo1Δ arg82Δ	++++
rtf1Δ arg82Δ	++
cdc73Δ arg82Δ	++
spt5-194 arg82Δ	+
spt4Δ arg82Δ	+
paf1Δ arg82Δ	-
ctr9Δ arg82Δ	-

The growth rate for an *arg82Δ* strain was arbitrarily set to "+++++"; an *arg82Δ* alone causes an intermediate growth defect when compared to a wild type strain, although this effect is no longer noticeable on dissection plates after 2 days growth at 30^{o}C. The growth rates of double mutants were determined by analyzing the progeny of the following crosses: *leo1Δ arg82Δ* = KY806 X KY744; *dst1Δ arg82Δ* = KY741 X KY678; *rtf1Δ arg82Δ* = KY404 X KY740; *cdc73Δ arg82Δ* = KY688 X KY740; *spt5-194 arg82Δ* = KY715 X KY742; *spt4Δ arg82Δ* = KY758 X KY744; *paf1Δ arg82Δ* =KY742 X ECY57; *ctr9Δ arg82Δ* = KY741 X KY528.

I next investigated whether mutations in IP signaling enzymes interacted specifically with mutations in the Paf1 complex or whether the growth effects caused by combination of mutations in these factors might be indicative of a functional relationship between IP signaling and transcription elongation. I crossed an *arg82Δ* strain by strains harboring mutations in *SPT4* and *SPT5*, which encode the subunits of the Spt4/Spt5 transcription elongation complex, or a deletion of *DST1*, which encodes a transcription elongation factor (TFIIS) that reactivates stalled Pol II by stimulating cleavage of nascent transcripts. I observed strong synthetic sickness in *arg82Δ spt4Δ* and *arg82Δ spt5-194* strains, but observed no obvious genetic interaction between *arg82Δ* and *dst1Δ* (Figure 31 and Table 4). This result suggests that while the connection between IP signaling and transcription elongation is not restricted specifically to mutation of the Paf1 complex, it is also not a general phenomenon that results from all disruptions of transcription elongation factors.

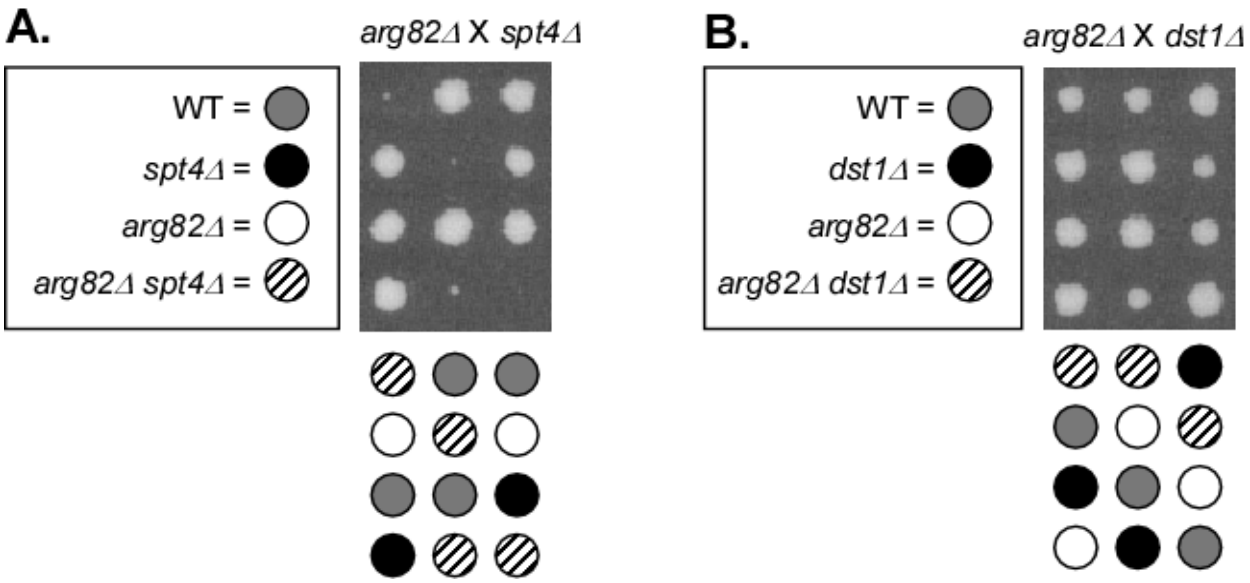

Figure 31. *arg82Δ* genetically interacts with mutations in the Spt4/5 elongation complex, but not TFIIS.

Yeast strains of opposite mating type were mated, sporulated, and dissected on YPD. Strains used for each individual cross are indicated in the legend to Table 4. Each vertical row contains the four spores derived from meiosis of a single diploid yeast cell. Plates were incubated at 30^{o}C for 4 days.

4.3.2 Enzymes that function downstream of Arg82 are not essential in the absence of Paf1 or Rtf1

The products of Arg82 kinase activity, IP_4 and IP_5, are further phosphorylated by two additional kinases, Ipk1 and Kcs1. Ipk1 phosphorylates IP_5 to IP_6 (YORK *et al.* 1999) and Kcs1 converts IP_4 and IP_6 to pyro-phosphorylated forms (SAIARDI *et al.* 1999). To determine if the Paf1 complex was required in all instances of disruptions in the IP signaling pathway, I created deletions of *IPK1* and *KCS1* and crossed the null strains by *paf1Δ* and *rtf1Δ* strains. I observed no major effects on growth of the resulting double mutant strains (data not shown). This result indicates that the connection between transcription elongation and IP signaling is specifically associated with Plc1 or Arg82 or the products of their enzymatic activity.

4.3.3 The synthetic lethality caused by the combined loss of *ARG82* and *PAF1* is not the result of an enhanced defect in mRNA export.

Mutations in *PLC1* and *ARG82* are known to result in a failure to properly export polyadenylated transcripts from the nucleus (YORK *et al.* 1999). To determine if the synthetic lethality observed in an *arg82Δ paf1Δ* strain might be the result of a combined effect on mRNA export, I examined the consequences of deleting *PAF1* on this process. The efficiency of mRNA export was assessed in fixed cells following in situ hybridization of a digoxigenin-labeled oligo-dT probe to the polyadenylated tails of mRNA. Hybridized probe was then detected with a rhodamine-labeled antidigoxigenin antibody and visualized on a fluorescence microscope. As expected, an *arg82Δ* strain displayed a striking accumulation of polyadenylated transcripts in the nucleus

(Figure 32). However, *paf1* null cells display diffuse cellular staining that is virtually indistinguishable from that observed in wild type cells, indicating that mRNA export was largely unaffected by this mutation (Figure 32). Deletion of *RTF1* or *CDC73* also does not result in a noticeable effect on mRNA export (data not shown). These results suggest that the inviability of an *arg82Δ paf1Δ* strain likely cannot be attributed to a combinatorial effect on mRNA export.

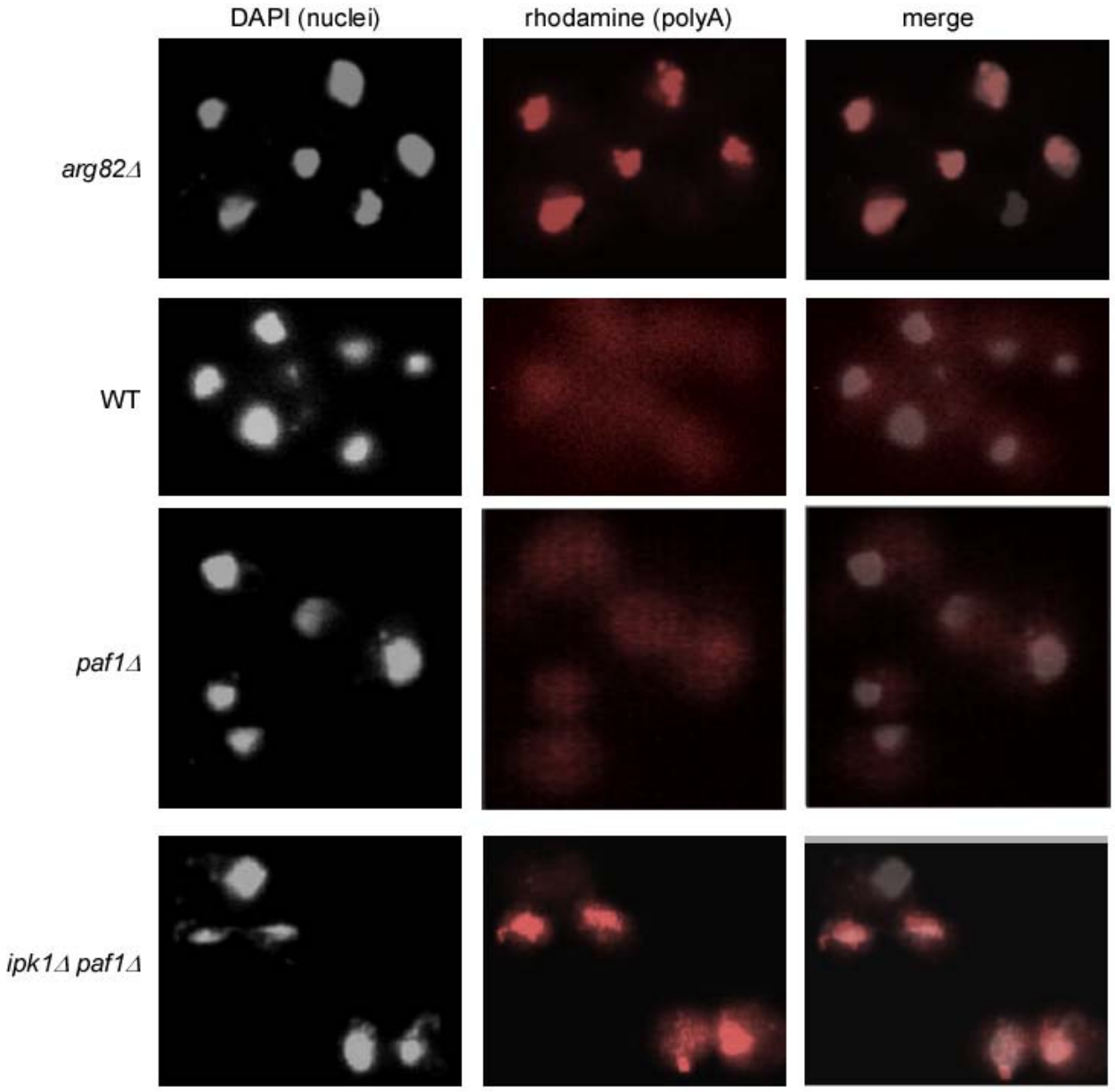

Figure 32. The inviability of an *arg82Δ paf1Δ* strain is not due to an enhanced defect in mRNA export.

The efficiency of mRNA export was assessed in fixed cells by in situ hybridization of a digoxigenin-labeled oligo-dT probe to polyadenylated (polyA) RNA followed by detection with a rhodamine-labeled antidigoxigenin antibody. DAPI was used to visualize the location of nuclei.

However, deletion of Paf1 complex subunits is known to result in a decrease in the average length of polyadenylated tails on RNA transcripts (MUELLER *et al.* 2004). This suggests that the absence of a strong nuclear signal in the mRNA export analysis of a *paf1Δ* strain may not reflect wild type levels of mRNA export, but instead be a simple consequence of the inability of the oligo-dT probe to hybridize as efficiently to abnormally short polyadenylated tails. To address this possibility, I performed mRNA export analysis on an *ipk1Δ paf1Δ* double mutant strain. Mutation of *IPK1* is known to cause a defect in mRNA export, similar to the effect observed in *arg82* mutant strains (YORK *et al.* 1999). A prominent signal is observed in the nuclei of *ipk1Δ paf1Δ* cells (Figure 32), suggesting that the reduction in polyadenylated tail length caused by deletion of *PAF1* does not affect the efficiency of this assay.

4.3.4 *paf1Δ* and *arg82Δ* genetically interact with mutations in the INO80, SWI/SNF, and SWR1 chromatin remodeling complexes

Inositol polyphosphates can modulate the activity of several ATP-dependent chromatin remodeling complexes in *S. cerevisiae,* including the INO80 and SWI/SNF complexes (SHEN *et al.* 2003). Additionally, the INO80 and SWI/SNF complexes are not efficiently recruited to the *PHO84* promoter and nucleosomes at the *PHO5* promoter are not efficiently remodeled in *arg82Δ* cells (STEGER *et al.* 2003). To investigate whether the inviability of an *arg82Δ paf1Δ* strain is the result of an enhanced impact on chromatin remodeling, *paf1Δ* cells were crossed by disruptions of all known nonessential chromatin remodeling complexes in *S. cerevisiae*: INO80, ISW1, ISW2, CHD1, SWI/SNF, and SWR1. Due to the requirement of the RSC complex for

viability, genetic interaction between deletion of *PAF1* and mutations in this complex were not analyzed.

The results of these crosses demonstrated that a *paf1* null mutation was synthetically lethal with deletions of *SWR1*, the catalytic subunit of the SWR1 complex, and *ARP8*, a component of the INO80 complex that is not found in other known chromatin remodeling complexes (Figure 33A and B and Table 5). An *ino80* null mutation causes severe sickness on its own (data not shown) and, therefore, was not used in my genetic analyses. Additionally, a deletion of *PAF1* resulted in severe sickness when combined with deletions of *ISW1* or *SNF2*, the catalytic subunits of the ISW1 and SWI/SNF chromatin remodeling complexes, respectively (Table 5). No obvious genetic interaction was observed in *paf1Δ chd1Δ* strains or *paf1Δ isw2Δ* strains; *CHD1* and *ISW2* encode the catalytic subunits of chromatin remodeling complexes by the same names (Table 5).

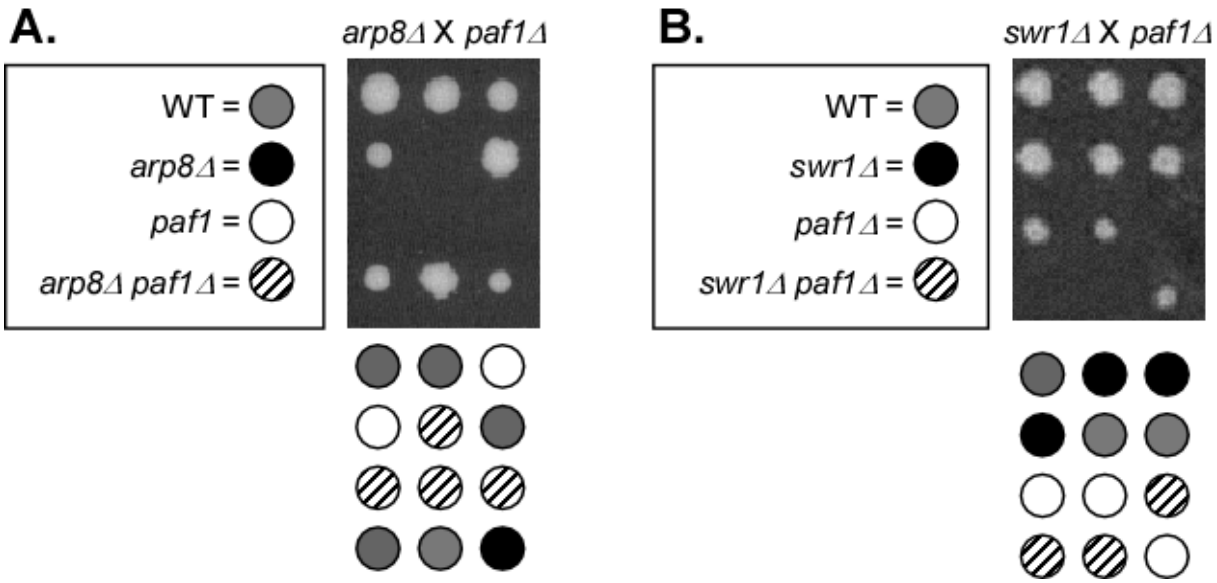

Figure 33. *paf1Δ* genetically interacts with disruptions in chromatin remodeling complexes.

Yeast strains of opposite mating type were mated, sporulated, and dissected on YPD. Strains used for each cross are indicated in the legend to Table 5. Each vertical row contains the four spores derived from meiosis of a single diploid yeast cell. Plates were incubated at 30^{o}C for 4 (A) or 3 (B) days.

Table 5. *paf1Δ* or *arg82Δ* genetically interact with disruptions of chromatin remodeling complexes

Chromatin remodeling complex	Subunit deleted	Growth defect when crossed by *paf1Δ*	Growth defect when crossed by *arg82Δ*
CHD1	*chd1Δ*	none	none
ISW1	*isw1Δ*	severe	none
ISW2	*isw2Δ*	none	none
SWI/SNF	*snf2Δ*	severe	lethal
INO80	*arp8Δ*	lethal	lethal
SWR1	*swr1Δ*	lethal	intermediate

paf1Δ and *arg82Δ* both cause growth defects on their own when compared to a wild type cell; the growth categories shown are relative to the normal growth of a *paf1Δ* or *arg82Δ* strain. The growth rates of double mutants were determined by analyzing the progeny of the following crosses: *chd1Δ paf1Δ* = KY583 X KY804; *chd1Δ arg82Δ* = KY583 X KY744; *isw1Δ paf1Δ* = MHY86 X KY901; *isw1Δ arg82Δ* = KY741 X KY901; *isw2Δ paf1Δ* = KY884 X KY804; *isw2Δ arg82Δ* = KY741 X KY885; *snf2Δ paf1Δ* = KY508 X KY804; *arp8Δ paf1Δ* = MHY8 X KY804; *arp8Δ arg82Δ* = KY741 X KY879; *swr1Δ paf1Δ* = MHY66 X KY972; *swr1Δ arg82Δ* = KY452 X KY972. *snf2Δ arg82Δ* double mutants were previously reported to be inviable (SHEN *et al.* 2003).

Deletion of *ARG82* is known to cause synthetic lethality in a cell lacking *SNF2* (SHEN *et al.* 2003). To more extensively analyze the connection between Arg82 and chromatin remodeling complexes, I introduced an *arg82* null mutation into strains harboring mutations in integral subunits of the other nonessential chromatin remodeling complexes. I found that *arg82Δ arp8Δ* double mutants were inviable (Figure 34A and Table 5). Loss of *ARG82* also resulted in synthetic sickness when combined with a deletion of *SWR1* (Figure 34B), while no discernible genetic interaction was obvious in crosses between an *arg82Δ* strain and strains lacking *CHD1*, *ISW1*, or *ISW2* (Table 5). Additionally, I performed genetic analyses which revealed that deletion of *ARG82* caused no growth defect in strains lacking *SET1*, which encodes the H3 K4 methyltransferase, or *BRE1*, the gene for the ubiquitin ligase required for H2B K123 ubiquitylation (data not shown). This suggests that Arg82 is not required for normal cell growth when chromatin structure is impacted by the loss of histone modifications, but instead appears to be specifically connected to chromatin remodeling.

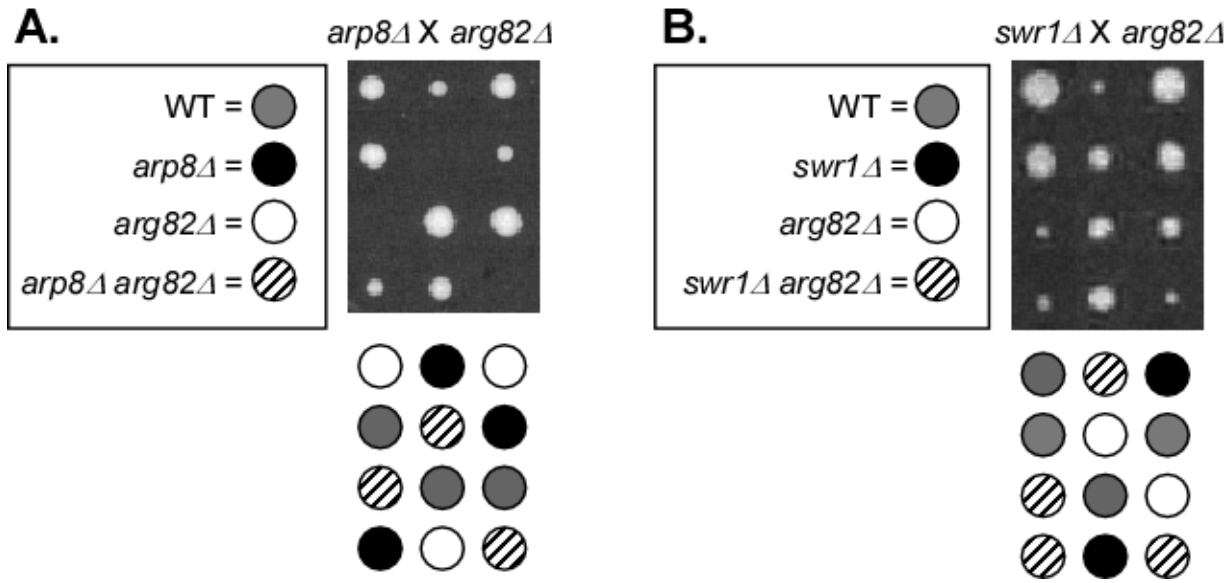

Figure 34. *arg82Δ* genetically interacts with disruptions in chromatin remodeling complexes.

Yeast strains of opposite mating type were mated, sporulated, and dissected on YPD. Strains used for each cross are indicated in the legend to Table 5. Each vertical row contains the four spores derived from meiosis of a single diploid yeast cell. Plates were incubated at 30^{o}C for 3 days.

Together, the results detailed in this section demonstrate that, when combined with disruptions of chromatin remodeling complexes, deletion of *ARG82* caused a similar pattern to that observed when *PAF1* is absent. Three of the four chromatin remodeling complexes (SWI/SNF, INO80, and SWR1) that were necessary for normal cell growth in the absence of *PAF1* were also required when *ARG82* was deleted. These genetic results imply that IP signaling and transcription elongation may work in parallel with a common set of chromatin remodeling complexes.

4.3.5 Expression of several genes is affected by loss of Paf1, Arg82, or mutations in the INO80 and SWI/SNF chromatin remodeling complexes

To further investigate the connection between IP signaling, transcription elongation, and ATP-dependent chromatin remodeling, I investigated whether factors involved in these processes were required for expression of common targets. Genes whose expression was known to be compromised in strains containing disruptions of chromatin remodelers were chosen as candidates. Expression of one such candidate gene, *SPL2* (OHDATE *et al.* 2003), is virtually eliminated in cells containing disruptions of the INO80 or SWI/SNF complexes or deletion of *PAF1*, and its expression is reduced in *arg82Δ* and *rtf1Δ* strains (Figure 35A). Transcription of a second candidate gene, *VTC3*, was essentially absent from *snf2Δ* and *ino80Δ* strains and was severely compromised by deletion of *PAF1*, *RTF1*, *ARG82*, and *ARP8* (Figure 35B). Chromatin remodelers that caused no growth defects when disrupted in both *paf1Δ* strains and *arg82Δ* strains (ISW1, ISW2, CHD1), or deletion of genes encoding histone modifying enzymes (*SET1* or *BRE1*), did not impact expression of these genes. These similar effects on expression of at

least two common genes further imply a specific relationship between the Paf1 complex, IP signaling, and the INO80 and SWI/SNF chromatin remodeling complexes.

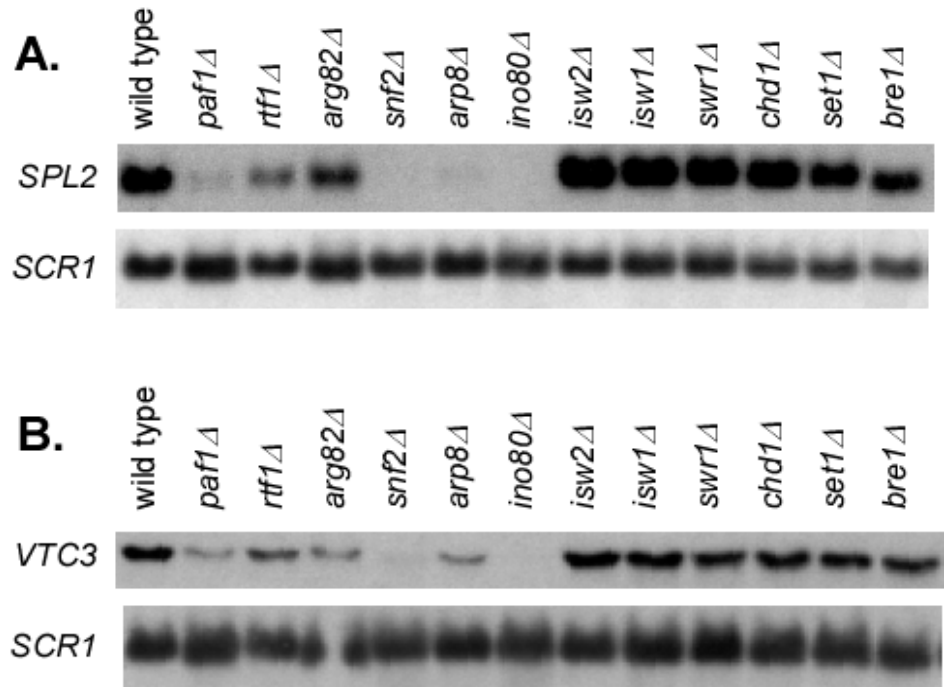

Figure 35. The Paf1 complex, Arg82, and the INO80 and SWI/SNF chromatin remodeling complexes are necessary for normal expression of *SPL2* and *VTC3*.

Expression of *SPL2* (A) and *VTC3* (B) was analyzed by Northern analysis. 10 μg of total RNA from strains harboring the indicated deletions was analyzed with probes specific for *SPL2* or *VTC3* and, after stripping, *SCR1* levels were assessed on the same filter as a loading control.

Surprisingly, deletion of *SWR1*, which was synthetically lethal in the absence of Paf1 or Arg82, does not impact expression of *VTC3* or *SPL2*. This may reflect the known function of the SWR1 complex as primarily required to facilitate the incorporation of the Htz1 histone variant in gene promoters whereas INO80 and SWI/SNF are primarily believed to reposition existing nucleosomes (MIZUGUCHI *et al.* 2004; SHEN *et al.* 2003). The genes examined in this analysis, *VTC3* and *SPL2*, may require nucleosome repositioning for expression, but not Htz1 incorporation. If I extended this analysis to explore effects on genes that are known to require Htz1 deposition for normal expression, I would likely uncover common target genes that are affected by *swr1Δ*, *paf1Δ*, and *arg82Δ*.

4.4 CONCLUSIONS

Previous work in the Arndt lab identified a synthetic lethal interaction between mutation of a transcription elongation factor, Rtf1, and two enzymes that function in the inositol polyphosphate signaling cascade, Plc1 and Arg82. While many connections between signaling pathways and transcription have been previously described, the effect is often manifested at the level of transcription initiation. The discovery of an interaction between the IP signaling cascade and the Paf1 transcription elongation complex implies that signaling pathways may also exert control during the elongation stage of transcription.

Because deletion of *PLC1* results in a severe growth defect, my studies were focused primarily on investigating the contribution of *ARG82* to this synthetic lethality. Arg82 is known to have at least three nuclear functions: transcriptional control of arginine-responsive genes as a subunit of the ArgR/Mcm1 transcriptional regulatory complex; involvement in the proper export

of mRNA from the nucleus, and effects on the activity of several chromatin remodeling complexes. I ruled out effects on Arg82's role as a transcriptional regulator as the likely cause of the synthetic lethality with *rtf1Δ* by demonstrating that deletion of *ARG80*, which encodes an additional subunit of the ArgR/Mcm1 complex, caused no growth defect in an *rtf1Δ* strain (data not shown). Furthermore, I demonstrated that deletion of multiple subunits of the Paf1 complex (*PAF1*, *CDC73*, or *RTF1*) did not affect mRNA export. This result indicates that the synthetic lethality caused by simultaneous mutation of *RTF1* and *ARG82* is not the result of a combined effect on mRNA export.

My genetic results suggest that the IP signaling cascade is connected to transcription elongation through its known interactions with several ATP-dependent chromatin remodeling complexes. Synthetic lethality is thought to result when two or more parallel processes that elicit a common essential function are simultaneously disrupted. Synthetic lethal interactions or strong effects on cell growth resulted from combinatorial deletions of factors involved in IP signaling (Arg82), transcription elongation (Paf1), and chromatin remodeling (Arp8, Snf2, and Swr1) (Figure 36). This result may imply that all three of these processes function in parallel; however, my genetic results demonstrate that a cell cannot tolerate the simultaneous loss of any two of these functions.

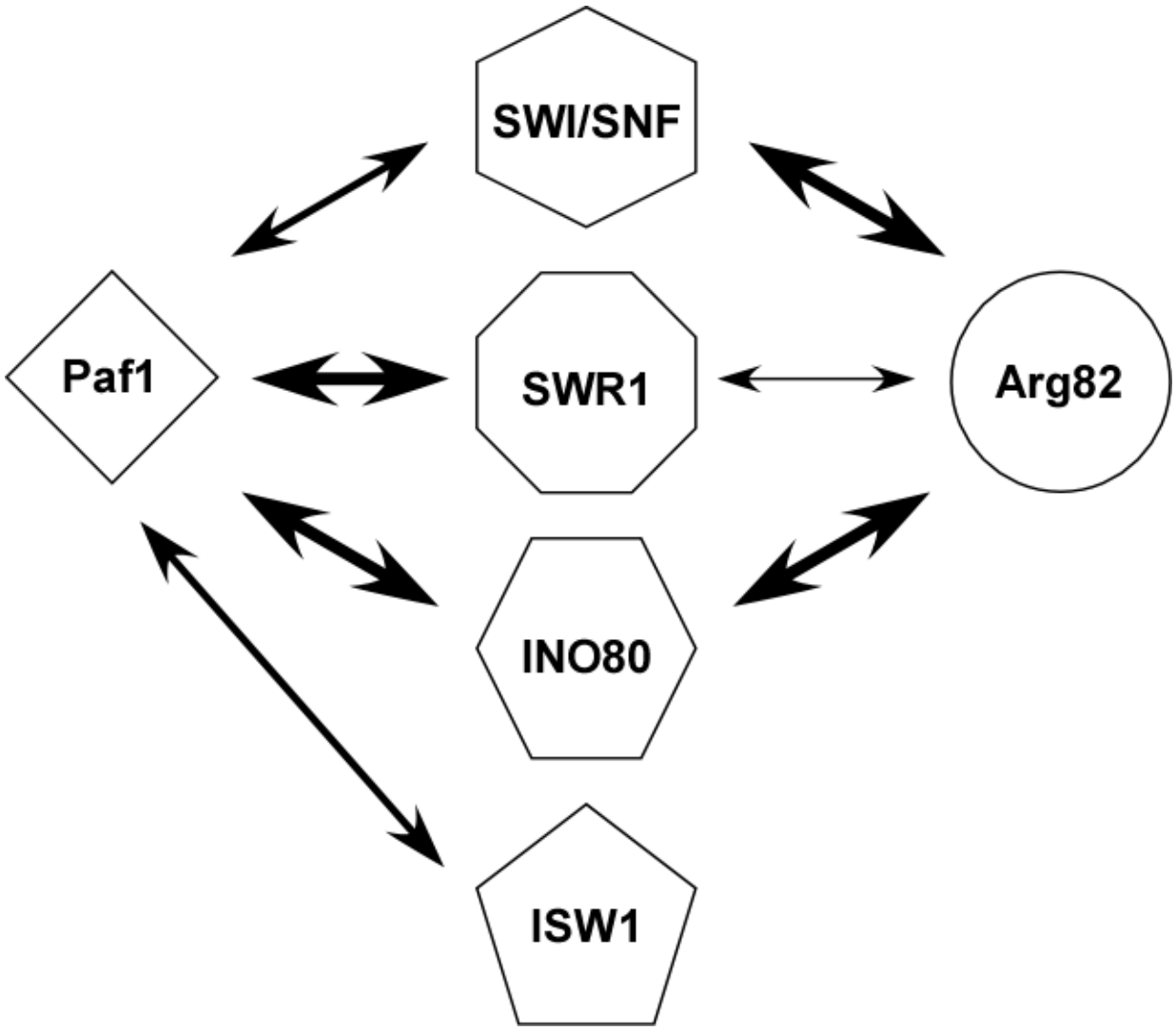

Figure 36. Genetic interactions between Paf1, Arg82, and chromatin remodeling complexes.

A schematic is shown representing genetic interactions that were observed between the indicated factors; the thickness of the line indicates the degree of interaction that was observed with the thickest lines signifying lethality; a *paf1* null allele showed a strong genetic interaction in a strain with an *ISW1* deletion, but no interaction was observed between *arg82Δ* and *isw1Δ.*

Additionally, individual disruption of the Paf1 complex, Arg82, or the INO80 and SWI/SNF chromatin remodeling complexes has a marked effect on at least two genes that are known to require proper chromatin remodeling for normal expression. This observation, in combination with my genetic data, may instead suggest that Arg82 and Paf1 both contribute to the normal function of the INO80, SWI/SNF, and SWR1 chromatin remodeling complexes. When both IP signaling and the Paf1 complex are concurrently disrupted, this may lead to widespread effects on chromatin remodeling that are too severe to support transcription at a level necessary to support cell viability. While the INO80, SWI/SNF, and SWR1 complexes are not essential when disrupted individually, deletion of the catalytic subunits of the INO80 or SWI/SNF complexes cause substantial growth defects. It is, therefore, likely that simultaneously inactivating or dampening the activity of all three of these chromatin remodeling complexes would have severe consequences for normal cell function.

5.0 DISCUSSION AND FUTURE DIRECTIONS

5.1 RTF1 IS COMPOSED OF FUNCTIONALLY DISTINCT REGIONS

A large number of accessory factors participate in mRNA synthesis by facilitating the recruitment and modification of Pol II, the cotranscriptional processing of the nascent transcript, the progression of Pol II along ORFs, and transcription termination. Accessory proteins that carry out distinct cotranscriptional events, such as transcript cleavage, can be recruited to discrete segments of a gene (BENTLEY 2005). In contrast, the Paf1 complex associates along the entirety of active ORFs (KIM *et al.* 2004a; MUELLER *et al.* 2004; SIMIC *et al.* 2003), suggesting that it functions throughout transcript elongation. My results indicate that the Paf1 complex component Rtf1 utilizes several independent mechanisms to orchestrate alterations to the chromatin template during gene expression. Using a collection of sequential *rtf1* deletion mutations, I demonstrated that discrete nonoverlapping segments of the protein are required for recruitment of Chd1 to active genes, histone modification, association with active ORFs, and physical interaction with the Paf1 complex; these regions of the protein have been given designations that reflect their functionalities: CID (Chd1-interaction domain), HMD (histone modification domain), OAR (ORF-association region), and PID (Paf1 complex interaction domain).

I observed that distinct transcriptional effects resulted from disruption of individual functions of Rtf1 (Figure 21). Combined with the observation that a complete deletion of *RTF1* causes more severe phenotypes than mutations that eliminate only one of its activities, these data suggest that the functions of Rtf1 are not entirely interdependent. Additionally, I observed that the most severe transcription-related phenotypes were caused by deletions in the HMD or OAR, which correspond to the most highly conserved regions of the protein; this observation suggests that promoting cotranscriptional histone modification is a critical means by which Rtf1 directs transcription.

Deletion of sequences required for either histone methylation or ORF association causes similar 6-AU sensitivity, but disruption of the association of Rtf1 with ORFs causes a more severe Spt$^-$ phenotype. This observation suggests that association of Rtf1 with active genes is required for a function in addition to H2B K123 ubiquitylation or H3 K4 and K79 methylation. Another indication that the 6-AU and Spt$^-$ phenotypes measure, at least partly, distinct functions of Rtf1 comes from the observation that Rtf1Δ5 causes a moderate Spt$^-$ phenotype but no sensitivity to 6-AU (Figure 14). Despite the Spt$^-$ phenotype resulting from deletion of this region, none of Rtf1's known functions are compromised in the absence of region 5; this may indicate that Rtf1 participates in an additional transcription-related function that has not been identified.

The OAR is the most highly conserved region of Rtf1; it contains 18 of the 26 residues that are invariant across *S. cerevisiae*, *S. pombe*, *H. sapiens*, and *C. elegans* (Figure 15). Recently, the structure of the corresponding region in human Rtf1 has been solved by NMR (DE JONG *et al.* 2008). Interestingly, the structure of a subdomain within the human OAR shows structural similarity to a number of nucleic acid binding proteins, including the PAZ domains of

Dicer and Argonaut (proteins involved in RNA interference), the Tudor domains of Sm and SMN proteins (proteins involved in assembly and function of the spliceosome), and bacterial transcription elongation factors including NusG. This observation led the authors to test whether Rtf1 possessed DNA binding activity; they found that the human Rtf1 OAR could bind single stranded DNA in vitro, but that it did not interact with double stranded DNA or RNA under similar conditions. The authors, therefore, proposed that the OAR may have a role in stabilizing the transcription "bubble" where the template and nontemplate strands of DNA have separated during active transcription.

Due to the conservation observed between the human and *S. cerevisiae* OAR domains, it will be beneficial to our understanding of Rtf1's function to determine whether the *S. cerevisiae* OAR also interacts with single stranded DNA. It is also possible that the OAR may be capable of interacting with double stranded DNA or RNA in the context of the full length protein, or in vivo. Rtf1 is known to physically interact with the Spt4/Spt5 transcription elongation complex, which mediates interaction of Rtf1 with Pol II. In light of my observation that the OAR is necessary for association of Rtf1 with active genes, it will be interesting to determine whether this region of the protein is also necessary for its physical interaction with the Spt4/Spt5 complex and/or Pol II and if these interactions are disturbed following treatment with DNase. Alternatively, the structure of the human Rtf1 OAR also resembled the structures of several proteins that contained Tudor domains, which have been proposed to possess histone methyl-lysine binding properties. No interaction was observed between the human OAR domain and un- or di-methylated H3 K4 (DE JONG *et al.* 2008), but it remains possible that the OAR of *S. cerevisiae* may bind mono- or tri- methylated H3 K4 or that it may interact with methylated H3 K36 or K79.

Previous analyses in the Arndt lab demonstrated that physical interaction with Chd1 is disrupted by deleting amino acids 3-30 of Rtf1 (region 1; CID) or by substituting phenylalanine for leucine at position 11 (Rtf1-1) (COSTA 2001; WARNER *et al.* 2007). I demonstrated that the *rtf1Δ1* mutation reduces association of Chd1 at the 5' ORFs of *PYK1* and *TEF2* (Figure 16), implying that the physical interaction between these two factors contributes to the normal recruitment of Chd1 to active genes. However, removing the CID from Rtf1 does not decrease Chd1 recruitment to the same extent as a complete *RTF1* deletion. This observation suggests that another function of Rtf1 may also contribute to Chd1 association with active genes. A likely candidate for this additional function is the role of Rtf1 in promoting histone modifications. *S. cerevisiae* Chd1 has been demonstrated to bind dimethylated H3 K4 through the second of the two chromodomains present near its N-terminus (PRAY-GRANT *et al.* 2005). Because H3 K4 Me^2 is an Rtf1-dependent histone modification that marks active ORFs, the HMD of Rtf1 may contribute to association of Chd1 with active genes by promoting the placement of this modification. However, conflicting reports exist regarding the validity of the physical interaction between *S. cerevisiae* Chd1 and H3 K4 Me^2. An additional study failed to detect interaction of the *S. cerevisiae* Chd1 chromodomains with H3 K4 Me^2 and demonstrated that *S. cerevisiae* lacks several key functional residues that mediate the known interaction between human CHD1 and an H3 K4 Me^2 peptide (OKUDA *et al.* 2007). Therefore, it may be useful to test the effect of all Rtf1 internal deletion mutants on the interaction of Chd1 with active genes.

Although I observed no transcription-related defects when the CID was deleted in an otherwise wild-type strain, the Arndt lab has previously shown that *rtf1-1* or deletion of *RTF1* or *CHD1* suppresses the Spt^- phenotype of the *spt15-122* mutation (STOLINSKI *et al.* 1997). I report here that this effect is also caused by *rtf1Δ1*. The similar effects of mutations in *RTF1* and

CHD1 in an *spt15-122* strain suggest that these factors elicit similar effects on chromatin structure. The interaction between Rtf1 and Chd1 may, therefore, be important for proper chromatin function particularly when the cell is sensitized to small changes in nucleosome positioning or stability, such as in a TBP mutant strain.

Additionally, I observed that the carboxy-terminus of Rtf1, defined by region 13 (PID), is necessary and sufficient for the interaction between Rtf1 and other Paf1 complex subunits. It is surprising that Rtf1Δ13 has apparently wild-type stability, because previous studies revealed a ten- to twenty- fold reduction in Rtf1 levels in crude extracts from *paf1Δ* and *ctr9Δ* strains (MUELLER *et al.* 2004; PORTER *et al.* 2005). My results indicate that a stable physical interaction with the Paf1 complex is not required for Rtf1 stability or that residues within Rtf1 that confer instability are deleted from the Rtf1Δ13 mutant protein. Consistent with a prior study, I demonstrated that interaction with Rtf1 mediates recruitment of Paf1 and Ctr9 to active ORFs (MUELLER *et al.* 2004).

Remarkably, disruption of the physical interaction between Rtf1 and the Paf1 complex causes only a mild Spt$^-$ phenotype and the known biochemical functions of Rtf1 remain largely intact. This observation suggests that Rtf1 retains function, independent of stable association with the Paf1 complex. In agreement with this idea, Rtf1 does not copurify with the human Paf1 complex (ROZENBLATT-ROSEN *et al.* 2005; YART *et al.* 2005; ZHU *et al.* 2005) and the carboxy-terminus is poorly conserved (Figure 15). Human Rtf1 may only interact with other Paf1 complex subunits while associated with chromatin or the human Paf1 complex may execute some functions independently of ORF association, as previously suggested from studies in yeast (MUELLER *et al.* 2004). Alternatively, Cdc73, which also promotes association of Paf1 and Ctr9 with active genes in yeast (MUELLER *et al.* 2004), may be the sole Paf1 complex subunit required

for this function in humans. The observation that only mild transcription-related phenotypes result when the CID or PID is removed from Rtf1 may also indicate that Rtf1 performs an additional unidentified role in the cell that is not transcription-related; the interaction between Rtf1 and Chd1 and/or other subunits of the Paf1 complex may ultimately be found to be important in this unidentified process.

The goal of analyzing the Rtf1 internal deletion mutants was to gain insight into the mechanism by which this protein functions. Through these analyses, I have demonstrated that the known roles of Rtf1 are mediated by distinct functional regions. At least 3 of these functional segments, the CID, OAR, and the PID, appear to be necessary to mediate protein-protein (or potentially protein-DNA) interactions. The Paf1 complex is believed to function as a platform for the recruitment of accessory factors during transcription elongation, and my data provide additional support for this idea in regard to the Rtf1 subunit of this complex. The exchange of interacting factors, allosteric changes in Rtf1's structure, or posttranslational modifications may control the association of Rtf1 with its binding partners. Alternatively, a catalytic role for Rtf1 itself cannot be completely ruled out. Structural analyses on full-length Rtf1 may reveal similarities to known enzymatic domains.

5.2 THE HISTONE MODIFICATION DOMAIN OF RTF1 IS NECESSARY AND SUFFICIENT TO PROMOTE RTF1-DEPENDENT HISTONE MODIFICATIONS

My analyses of the Rtf1 internal deletion series also revealed that a 90 amino acid segment spanning residues 63 through 152 (HMD) was necessary to promote Rtf1-dependent monoubiquitylation of histone H2B at K123 and methylation of histone H3 at K4 and K79. The

dependence of both H2B K123 Ub and its downstream histone methylation marks on a relatively small region of Rtf1 implied that the primary role of Rtf1 in histone modification may be to promote H2B K123 Ub. However, I created substitution mutations in the Rtf1 HMD and observed unique effects on Rtf1-dependent histone modifications; *rtf1 108-110A* and *rtf1 F80V F123S* eliminated all Rtf1-dependent histone modifications tested (H2B K123 Ub, di- and trimethylation of H3 K4 and dimethylation of H3 K79), while *rtf1 102-104A* affected only H3 K4 Me^3. These results suggested that Rtf1 may promote distinct Rtf1-dependent histone modifications by separate mechanisms.

However, it remains possible that the primary role of the HMD is to specifically promote H2B K123 Ub. In contrast to eliminating H2B K123 Ub, the *rtf1 102-104A* mutation may instead alter the genome-wide pattern of this modification. The progression of H3 K4 Me^2 to H3 K4 Me^3 may be particularly sensitive to proper patterns of H2B K123 Ub, and this effect may account for the specific loss of this modification in an *rtf1 102-104A* strain. ChIP analyses to explore the distribution of H2B K123 Ub within active genes will be useful in exploring this possibility. It is also possible that Rtf1 controls the deubiquitylation of H2B K123 Ub; deubiquitylation of H2B K123 has been demonstrated to be necessary for H3 K4 Me (WYCE *et al.* 2004). Examining association of the H2B deubiquitylating enzymes Ubp8 and Ubp10 with active genes in strains expressing Rtf1 HMD substitution mutations may reveal a role for Rtf1 in the recruitment of these enzymes.

Alternatively, factors have previously been identified that are necessary for the progression of H3 K4 Me^2 to H3 K4 Me^3; these factors include Not4, a subunit of the Ccr4-Not mRNA production and processing complex, and the Cps35, Cps40 and Cps60 subunits of the COMPASS complex (which contains the Set1 H3 K4 methyltransferase) (LEE *et al.* 2007;

MULDER *et al.* 2007; SCHNEIDER *et al.* 2005). Determining whether *rtf1 102-104A* impairs the recruitment or activity of these factors may provide insight into the specific effect of this mutation on H3 K4 Me^3. It has been reported previously that deletion of *RTF1* causes a more severe effect on H3 K4 Me^1 than is observed in the absence of Rad6 (the ubiquitin conjugating enzyme necessary for H2B K123 Ub) (DEHE *et al.* 2005). This observation supports the idea that Rtf1 may have effects on the activity of the Set1 methyltransferase that are independent of its role in H2B K123 Ub.

The effectiveness of transcriptional silencing at the telomeres is tightly linked to global patterns of histone modifications. Deletion of *RTF1* is known to disrupt this process (KROGAN *et al.* 2003; NG *et al.* 2003b) and I have demonstrated that partial deletion of the HMD or substitution mutations in this region that affect Rtf1-dependent histone modifications also compromise telomeric silencing. I also observed loss of telomeric silencing when regions of the Rtf1 OAR were deleted, even though these mutations led to only a minor decrease in H3 K4 Me^3 while other Rtf1-dependent H3 methylation marks remained at wild type levels. While these results may indicate that telomeric silencing is dramatically affected by slight perturbations in global histone modification patterns, it is more likely a reflection of the sensitivity of the *TEL-VR::URA3* reporter. To more accurately measure transcriptional activity at the telomeres, Northern blots could be performed to assess levels of *URA3* expression from the telomeric silencing reporter in strains harboring Rtf1 HMD mutations.

Additionally, I tested the effects of Rtf1 HMD mutations on three other transcription- or chromatin-related phenotypes: sensitivity to the base analog 6-AU, the Spt^- phenotype, and internal initiation from the *FLO8* locus. All HMD mutations tested (*rtf1Δ3*, *rtf1Δ4*, *rtf1 102-104A*, *rtf1 108-110A*, and *rtf1 F80V F123S*) caused similar sensitivity to 6-AU, indicating that

these mutations lead to a defect in transcription elongation. Partial deletions of the HMD (*rtf1Δ3* and *rtf1Δ4*) resulted in a very weak Spt$^-$ phenotype, but no effect on this phenotype is observed in strains expressing Rtf1 HMD substititution mutations. Conversely, the Rtf1 HMD substitution mutations allowed internal initiation from a cryptic promoter in the *FLO8* locus at a level equivalent to that observed for a complete deletion of *RTF1*, while partial deletions of the HMD led to a weaker effect on this phenotype.

The *FLO8* internal initiation phenotype and the Spt$^-$ phenotype have both been proposed to indicate that chromatin structure has been disrupted, allowing access to aberrant transcriptional start sites (KAPLAN *et al.* 2003). The *FLO8* internal promoter lies within the ORF, where Rtf1-dependent histone modifications are known to localize. Conversely, the Spt$^-$ phenotype typically results from utilizing alternate transcriptional start sites within the promoter, which is devoid of most Rtf1-dependent histone modifications. Mutations in the Rtf1 HMD allow internal initiation from *FLO8*, but cause a very mild or no Spt$^-$ phenotype; these observations may suggest that the HMD contributes mainly to the function of chromatin within active ORFs.

Another interesting observation made from these phenotypic studies is that partial deletions and substitution mutations in the HMD are not always functionally equivalent. I also observed that several HMD substitution mutations led to a decrease in the amount of total H3 that could be extracted from these strains; this effect is not caused by partial deletions of the HMD. These observations may indicate that Rtf1 substitution mutations are gain of function mutations that alter histone-DNA contacts. Consistent with the idea that the Paf1 complex may affect nucleosome stability on active genes, absence of Paf1 complex subunits results in an increase in levels of histone H3 present in the 5' ORFs of several genes (DEHE *et al.* 2005;

MARTON and DESIDERIO 2008). This increase in total histone H3 levels correlates with a decrease in levels of Pol II associated with the *GAL1* locus following induction and leads to decreased *GAL1* mRNA levels at 90 minutes post-induction (MARTON and DESIDERIO 2008; XIAO *et al.* 2005). ChIP analyses to determine if *rtf1 108-110A* and *rtf1 F80V F123S*, which both decreased the amount of total H3 present in whole cell extracts prepared by glass bead lysis, increase the level of total H3 present in the *GAL1* ORF upon gene induction will be useful in determining if Rtf1's role in histone modification contributes to nucleosome dynamics during transcription activation. It will also be interesting to determine if the Rtf1 CID contributes to histone eviction during the activation of gene transcription.

Similar studies to explore the effects of Rtf1 substitution mutations on H2A and H2B levels present in active ORFs following gene induction should also be performed. H2A/H2B dimers are more readily evicted from nucleosomes during transcription and may, therefore, be more strongly affected than histone H3 in the proposed ChIP analyses. Interestingly, the yFACT histone chaperone complex that contributes to H2A/H2B dynamics during transcription physically interacts with the Paf1 complex (SQUAZZO *et al.* 2002). It will be interesting to determine if yFACT requires the Rtf1 HMD for recruitment to recently induced genes or whether the HMD and yFACT physically interact.

I demonstrated that expressing the Rtf1 HMD as a fusion to the Gal4 DNA binding domain (HMD-GBD) in an *rtf1Δ* strain could restore global levels of H3 K79 Me^2 to those present in a wild type strain; expression of this construct also restored a modest level of global H3 K4 Me^3 in an *rtf1Δ* background and induced a 3-fold increase in this modification at the *GAL7* UAS under repressing conditions. These observations demonstrated that promoting Rtf1-dependent histone methylation is an intrinsic property of the HMD. Future experiments to

explore whether the HMD is also sufficient to promote H2B K123 Ub will further clarify the function of this region of Rtf1.

The ultimate goal of this experiment was to determine if the HMD could induce histone modifications in the absence of the transcription machinery. Although the *GAL7* locus should be silent under the conditions used in these experiments, it is possible that a low level of transcription is occurring under these conditions. Integration of Gal4 binding sites into an intergenic region and the inactive *PRM1* gene is currently underway. ChIP analysis to determine if Rtf1-dependent histone modifications are induced at these regions by expression of HMD-GBD will provide more conclusive data.

I also observed that expression of the HMD-GBD induced H3 K4 Me^3 and H3 K79 Me^2 in the 5' end of the *PYK1* ORF, a locus at which Gal4 binding sites have not been detected. This raises the possibility that Rtf1 may not need to be directly targeted to DNA to promote histone modification. Rtf1 may perhaps possess the ability to modify histones that are not incorporated into DNA.

To investigate this possibility, several experimental adjustments and additional lines of experimentation must be pursued. The HMD-GBD constructs used in these analyses were overexpressed from 2μ vectors. Because the HMD is fused to a DNA binding domain, overexpression of this fusion protein may allow nonspecific interaction with DNA on a genome-wide basis. This may lead to a diffuse, transient association of the HMD-GBD fusion throughout the genome at levels below the limits of detection of the ChIP assay. To address this issue, ChIP assays to determine if Rtf1-dependent histone modifications can be restored in an *rtf1Δ* background should be repeated utilizing constructs that express the HMD-GBD fusions from low copy vectors or inducible promoters to achieve lower expression levels of the fusion protein.

Additionally, the HMD alone can be expressed to determine if the absence of a DNA-binding domain affects the ability of this domain to induce Rtf1-dependent histone modifications. Preliminary NMR structural analyses indicate that the HMD is largely structured (collaboration between the laboratories of Dr. Andy VanDemark and Dr. Angela Gronenborn; personal communication); therefore, the HMD alone should be stably expressed in vivo. In the event that expression of the HMD alone cannot be achieved, it can be fused to a protein tag that does not possess DNA-binding activity, such as GFP. If Rtf1-dependent histone modifications are still induced in strains expressing HMD alone or HMD-GFP from low copy vectors, this would provide more convincing evidence that Rtf1 does not need to be associated with DNA to promote histone modification.

An alternative possibility is that the HMD itself possesses DNA binding activity; this activity may no longer be regulated when the HMD is overexpressed and is not present in the context of the full-length protein. This possibility could be tested by subjecting the HMD to the DNA-binding analyses that were performed to demonstrate that the human Rtf1 OAR possessed single stranded DNA binding activity (DE JONG *et al.* 2008). If DNA-binding activity is identified for the HMD, this may indicate that Rtf1 participates in histone modification by directly contacting DNA; this interaction may increase the accessibility of histone tails to histone modifying enzymes.

Although the HMD may interact with DNA, the possibility that it mediates protein-protein interactions should also be explored. Rtf1 is known to be necessary for the normal recruitment of the Set1-containing COMPASS complex and Rad6 to active genes (KROGAN *et al.* 2003; NG *et al.* 2003c; XIAO *et al.* 2005). It is also possible that the HMD physically interacts with the histones, histone deubiquitylating enzymes, or histone demethylases. Directed studies

to determine whether the HMD interacts directly with these factors, or with the yFACT histone chaperone complex, should provide useful information. The HMD may also interact with an intermediate factor that leads to the recruitment of these histone modifying enzymes. Several unbiased protein purification studies are currently underway to determine if HMD-interacting factors can be identified. The possibility that the HMD functions enzymatically also cannot be conclusively ruled out. Completion of the HMD structural analyses may reveal similarity to a known enzymatic fold, or other recognizable protein domain, and should significantly aid the investigation into understanding the mechanism by which Rtf1 directs histone modification at active genes.

5.3 GENETIC INTERACTIONS IMPLY CONNECTIONS BETWEEN THE INOSITOL POLYPHOSPHATE SIGNALING PATHWAY, TRANCRIPTION ELONGATION, AND CHROMATIN REMODELING

A screen for factors that become essential in the absence of Rtf1 uncovered mutations in several proteins that function during transcription elongation (COSTA and ARNDT 2000); this screen provided the first link between Rtf1 and transcription elongation. Interestingly, the same screen also identified mutations in Plc1 and Arg82, the first two enzymes in the inositol polyphosphate signaling pathway. The genetic interaction between mutations in these enzymes and Rtf1 suggested that IP signaling may also be connected to transcription elongation. To further investigate this phenomenon, I performed a series of genetic crosses to assess whether the genetic interaction between mutations in *ARG82* and *RTF1* was specific. I found that deletion of *ARG82* resulted in synthetic lethality or strong growth defects when combined with individual

deletions of four of the five members of the Paf1 complex; synthetic lethality resulted from combining a deletion of *ARG82* with a deletion of *PAF1* or *CTR9*. I also observed genetic interactions between deletion of *ARG82* and mutations in the Spt4/Spt5 complex. Together, these results further implied a connection between IP signaling and transcription elongation.

It is known that the Spt4/Spt5 complex is required for recruitment of the Paf1 complex to active genes (QIU *et al.* 2006), which may indicate that the link between IP signaling and transcription elongation may be mediated specifically through the Paf1 complex. It may be useful to extend this genetic analysis to determine if deletion of *ARG82* causes growth defects when combined with mutations in other known elongation factors, such as *CTK1*, *FCP1*, and the subunits of the yFACT complex, that have not been shown to affect the recruitment of the Paf1 complex to active genes. Additionally, the results of crosses between deletion of *ARG82* and mutations in the Bur1/Bur2 transcription elongation complex, which is known to affect association of the Paf1 complex with active ORFs, will provide further information concerning the connections between IP signaling and transcription elongation.

Arg82 is known to have at least three nuclear functions; it participates in the control of arginine-responsive genes as a subunit of the ArgR/Mcm1 transcriptional regulatory complex (BECHET *et al.* 1970), affects the activity and recruitment of several ATP-dependent chromatin remodeling complexes (SHEN *et al.* 2003; STEGER *et al.* 2003), and is necessary for the export of mRNA from the nucleus (YORK *et al.* 1999). My analyses demonstrated that the role of Arg82 as a transcriptional regulator or its function in mRNA export are likely not responsible for the synthetic lethality observed when *ARG82* and *PAF1* are simultaneously deleted. Arg82's role as a transcriptional regulator is independent of its kinase function (DUBOIS *et al.* 2000). To more conclusively demonstrate that the kinase function of Arg82 is specifically required in the absence

of *PAF1*, the effect of combining a deletion of *PAF1* with a mutation in the Arg82 kinase domain should be evaluated.

Additionally, it will be interesting to determine which function of the Paf1 complex is necessary in the absence of Arg82. This could be assessed by combining a deletion of *ARG82*, or the Arg82 kinase-dead mutant, with the Rtf1 internal deletion mutants. The results of this analysis would determine which of Rtf1's functions is necessary for normal cell growth when *ARG82* is disrupted. Paf1 and Ctr9 are known to have roles in trimethylation of histone H3 K36 and phosphorylation of elongating Pol II on Ser2 of CTD repeats (CHU *et al.* 2007; MUELLER *et al.* 2004). Genetic analyses to determine if the loss of Set2 (the H3 K36 methyltransferase) or Ctk1 (the CTD Ser2 kinase) cause growth defects in the absence of functional Arg82 kinase activity will also be informative.

The IP signaling pathway was linked to the activity of several chromatin remodeling complexes, including the INO80 and SWI/SNF complexes (SHEN *et al.* 2003; STEGER *et al.* 2003). I, therefore, expanded my genetic analyses to determine whether mutations in Arg82 or the Paf1 complex genetically interacted with disruptions of nonessential chromatin remodeling complexes. Strong synthetic genetic interactions are observed between deletion of *PAF1* or *ARG82* and disruption of the INO80, SWI/SNF, or SWR1 chromatin remodeling complexes. Disruption of these factors, with the exception of the SWR1 complex, was also found to impact expression of *SPL2* and *VTC3*. Interestingly, the IP signaling pathway, the Paf1 complex, and the SWI/SNF complex have all been implicated in the cellular stress response (FLICK and THORNER 1993; PORTER *et al.* 2002; SHIVASWAMY and IYER 2008). The connection between these 3 pathways may indicate that the cellular stress response is tightly controlled during all stages of transcription.

Several mechanisms could account for the synthetic lethality observed between factors involved in IP signaling, transcription elongation, and chromatin remodeling. All three processes may function in parallel for normal cell growth; this scenario is unlikely due to the pairwise synthetic lethality observed between disruptions in any two of these processes. Alternatively, the Paf1 complex and IP signaling may both contribute to the normal function of chromatin remodeling enzymes; when both transcription elongation and IP signaling are disrupted, chromatin remodeling is ineffective and results in cell inviability. A third possibility is that the IP signaling pathway affects chromatin remodeling and these two processes work in parallel to the effect of the Paf1 complex on transcription elongation; simultaneous disruption of transcription elongation and chromatin remodeling could result in severe effects on gene expression, which would have a dramatic effect on cell growth.

ChIP analyses will be useful in further investigating the connections between IP signaling, chromatin remodeling, and transcription elongation. It will be interesting to determine whether chromatin remodeling complexes are recruited to the ORFs of active genes; this may indicate that chromatin remodeling complexes directly affect transcription elongation. Candidate genes for these analyses will include *VTC3* and *SPL2*; expression of these genes was affected by deletion of *ARG82, PAF1,* or components of the INO80 and SWI/SNF chromatin remodeling complex. Defects in expression of *INO1* or recruitment of the INO80 and SWI/SNF to the promoters of several phosphate-responsive genes were identified in *arg82Δ* strains (STEGER *et al.* 2003); therefore, these genes would also represent ideal candidates for the proposed ChIP analyses. Additionally, it may be interesting to analyze the association of chromatin remodeling complexes with the bodies of heat-shock responsive genes. BRG1, the ATPase component of the human SWI/SNF complex, has been demonstrated to associate with both the promoter and

ORF of the *hsp70* gene following activation (COREY *et al.* 2003). If the INO80 and/or SWI/SNF complexes are found to associate with the bodies of genes in these analyses, the dependence of this association on the Paf1 complex should then be assessed; the results of these experiments should indicate whether the Paf1 complex has a direct effect on chromatin remodeling during transcription elongation.

The Paf1 complex has been demonstrated to be necessary for the eviction of histones from the *GAL1* locus upon gene activation (MARTON and DESIDERIO 2008). It would be interesting to determine whether mutation of Arg82 or the INO80 and SWI/SNF complexes result in a similar defect. The Paf1 complex has several known connections to histone modification. A combined effect on histone modification and chromatin remodeling could result in the inability to sufficiently modify chromatin structure to allow gene expression to take place at levels necessary to support cell viability. In agreement with this idea, synthetic lethal interactions have been reported between mutations in the SAGA histone acetyltransferase complex and the SWI/SNF chromatin remodeling complex (ROBERTS and WINSTON 1997).

BIBLIOGRAPHY

AGGER, K., J. CHRISTENSEN, P. A. CLOOS and K. HELIN, 2008 The emerging functions of histone demethylases. Curr Opin Genet Dev **18:** 159-168.

AHN, S. H., M. KIM and S. BURATOWSKI, 2004 Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. Mol Cell **13:** 67-76.

AKANUMA, T., S. KOSHIDA, A. KAWAMURA, Y. KISHIMOTO and S. TAKADA, 2007 Paf1 complex homologues are required for Notch-regulated transcription during somite segmentation. EMBO Rep **8:** 858-863.

ALCAZAR-ROMAN, A. R., E. J. TRAN, S. GUO and S. R. WENTE, 2006 Inositol hexakisphosphate and Gle1 activate the DEAD-box protein Dbp5 for nuclear mRNA export. Nat Cell Biol **8:** 711-716.

ALCAZAR-ROMAN, A. R., and S. R. WENTE, 2008 Inositol polyphosphates: a new frontier for regulating gene expression. Chromosoma **117:** 1-13.

ALLARD, S., R. T. UTLEY, J. SAVARD, A. CLARKE, P. GRANT *et al.*, 1999 NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. Embo J **18:** 5108-5119.

ALLISON, L. A., M. MOYLE, M. SHALES and C. J. INGLES, 1985 Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell **42:** 599-610.

ARMSTRONG, J. A., 2007 Negotiating the nucleosome: factors that allow RNA polymerase II to elongate through chromatin. Biochem Cell Biol **85:** 426-434.

ARNDT, K. M., S. L. RICUPERO, D. M. EISENMANN and F. WINSTON, 1992 Biochemical and genetic characterization of a yeast TFIID mutant that alters transcription *in vivo* and DNA binding *in vitro*. Mol Cell Biol **12:** 2372-2382.

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN *et al.*, 1988 *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York.

BASEHOAR, A. D., S. J. ZANTON and B. F. PUGH, 2004 Identification and distinct regulation of yeast TATA box-containing genes. Cell **116:** 699-709.

BECHET, J., M. GREENSON and J. M. WIAME, 1970 Mutations affecting the repressibility of arginine biosynthetic enzymes in *Saccharomyces cerevisiae*. Eur J Biochem **12:** 31-39.

BELOTSERKOVSKAYA, R., S. OH, V. A. BONDARENKO, G. ORPHANIDES, V. M. STUDITSKY *et al.*, 2003 FACT facilitates transcription-dependent nucleosome alteration. Science **301:** 1090-1093.

BENTLEY, D. L., 2005 Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr Opin Cell Biol **17:** 251-256.

BERNSTEIN, B. E., E. L. HUMPHREY, R. L. ERLICH, R. SCHNEIDER, P. BOUMAN *et al.*, 2002 Methylation of histone H3 Lys 4 in coding regions of active genes. Proc Natl Acad Sci U S A **99:** 8695-8700.

BETZ, J. L., M. CHANG, T. M. WASHBURN, S. E. PORTER, C. L. MUELLER *et al.*, 2002 Phenotypic analysis of Paf1/RNA polymerase II complex mutations reveals connections to cell cycle regulation, protein synthesis, and lipid and nucleic acid metabolism. Mol Genet Genomics **268:** 272-285.

BLOCH, J. C., F. PERRIN and F. LACROUTE, 1978 Yeast temperature-sensitive mutants specifically impaired in processing of poly(A)-containing RNAs. Mol Gen Genet **165:** 123-127.

BRAUN, M. A., P. J. COSTA, E. M. CRISUCCI and K. M. ARNDT, 2007 Identification of Rkr1, a nuclear RING domain protein with functional connections to chromatin modification in *Saccharomyces cerevisiae*. Mol Cell Biol **27:** 2800-2811.

BRIGGS, S. D., M. BRYK, B. D. STRAHL, W. L. CHEUNG, J. K. DAVIE *et al.*, 2001 Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. Genes Dev **15:** 3286-3295.

BRIGGS, S. D., T. XIAO, Z. W. SUN, J. A. CALDWELL, J. SHABANOWITZ *et al.*, 2002 Gene silencing: trans-histone regulatory pathway in chromatin. Nature **418:** 498.

BUCHER, P., 1990 Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J Mol Biol **212:** 563-578.

BURATOWSKI, S., 2000 Snapshots of RNA polymerase II transcription initiation. Curr Opin Cell Biol **12:** 320-325.

BURATOWSKI, S., 2005 Connections between mRNA 3' end processing and transcription termination. Curr Opin Cell Biol **17:** 257-261.

BURATOWSKI, S., S. HAHN, L. GUARENTE and P. A. SHARP, 1989 Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56:** 549-561.

CAIRNS, B. R., Y. J. KIM, M. H. SAYRE, B. C. LAURENT and R. D. KORNBERG, 1994 A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. Proc Natl Acad Sci U S A **91:** 1950-1954.

CAIRNS, B. R., A. SCHLICHTER, H. ERDJUMENT-BROMAGE, P. TEMPST, R. D. KORNBERG *et al.*, 1999 Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. Mol Cell **4:** 715-723.

CAREY, M., B. LI and J. L. WORKMAN, 2006 RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. Mol Cell **24:** 481-487.

CARROZZA, M. J., B. LI, L. FLORENS, T. SUGANUMA, S. K. SWANSON *et al.*, 2005 Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell **123:** 581-592.

CHANDY, M., J. L. GUTIERREZ, P. PROCHASSON and J. L. WORKMAN, 2006 SWI/SNF displaces SAGA-acetylated nucleosomes. Eukaryot Cell **5:** 1738-1747.

CHANG, M., D. FRENCH-CORNAY, H. Y. FAN, H. KLEIN, C. L. DENIS *et al.*, 1999 A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling. Mol Cell Biol **19:** 1056-1067.

CHAUDHARY, K., S. DEB, N. MONIAUX, M. P. PONNUSAMY and S. K. BATRA, 2007 Human RNA polymerase II-associated factor complex: dysregulation in cancer. Oncogene **26:** 7499-7507.

CHU, Y., R. SIMIC, M. H. WARNER, K. M. ARNDT and G. PRELICH, 2007 *Regulation of histone modification and cryptic transcription by the Bur1 and Paf1 complexes*. EMBO J **26:** 4646-4656.

CLAPIER, C. R., K. P. NIGHTINGALE and P. B. BECKER, 2002 A critical epitope for substrate recognition by the nucleosome remodeling ATPase ISWI. Nucleic Acids Res **30:** 649-655.

CONRAD, M. N., J. H. WRIGHT, A. J. WOLF and V. A. ZAKIAN, 1990 RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell **63:** 739-750.

COREY, L. L., C. S. WEIRICH, I. J. BENJAMIN and R. E. KINGSTON, 2003 Localized recruitment of a chromatin-remodeling activity by an activator in vivo drives transcriptional elongation. Genes Dev **17:** 1392-1401.

COSTA, P. J., 2001 Characterization of the *Saccharomyces cerevisiae* Transcription Factor Rtf1. University of Pittsburgh Biological Sciences Ph.D. Thesis. **University of Pittsburgh; Pittsburgh, PA**.

COSTA, P. J., and K. M. ARNDT, 2000 Synthetic lethal interactions suggest a role for the *Saccharomyces cerevisiae* Rtf1 protein in transcription elongation. Genetics **156:** 535-547.

COTE, J., J. QUINN, J. L. WORKMAN and C. L. PETERSON, 1994 Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science **265:** 53-60.

COUTURE, J. F., and R. C. TRIEVEL, 2006 Histone-modifying enzymes: encrypting an enigmatic epigenetic code. Curr Opin Struct Biol **16:** 753-760.

CRAMER, P., 2004 RNA polymerase II structure: from core to functional complexes. Curr Opin Genet Dev **14:** 218-226.

CRAMER, P., D. A. BUSHNELL, J. FU, A. L. GNATT, B. MAIER-DAVIS *et al.*, 2000 Architecture of RNA polymerase II and implications for the transcription mechanism. Science **288:** 640-649.

CUI, Y., and C. L. DENIS, 2003 In vivo evidence that defects in the transcriptional elongation factors RPB2, TFIIS, and SPT5 enhance upstream poly(A) site utilization. Mol Cell Biol **23:** 7887-7901.

DANIEL, J. A., M. S. TOROK, Z. W. SUN, D. SCHIELTZ, C. D. ALLIS *et al.*, 2004 Deubiquitination of histone H2B by a yeast acetyltransferase complex regulates transcription. J Biol Chem **279:** 1867-1871.

DE JONG, R. N., V. TRUFFAULT, T. DIERCKS, E. AB, M. A. DANIELS *et al.*, 2008 Structure and DNA binding of the human Rtf1 Plus3 domain. Structure **16:** 149-159.

DEHE, P. M., and V. GELI, 2006 The multiple faces of Set1. Biochem Cell Biol **84:** 536-548.

DEHE, P. M., M. PAMBLANCO, P. LUCIANO, R. LEBRUN, D. MOINIER *et al.*, 2005 Histone H3 lysine 4 mono-methylation does not require ubiquitination of histone H2B. J Mol Biol **353:** 477-484.

DOWNS, J. A., S. ALLARD, O. JOBIN-ROBITAILLE, A. JAVAHERI, A. AUGER *et al.*, 2004 Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites. Mol Cell **16:** 979-990.

DUBOIS, E., V. DEWASTE, C. ERNEUX and F. MESSENGUY, 2000 Inositol polyphosphate kinase activity of Arg82/ArgRIII is not required for the regulation of the arginine metabolism in yeast. FEBS Lett **486:** 300-304.

DVIR, A., 2002 Promoter escape by RNA polymerase II. Biochim Biophys Acta **1577:** 208-223.

EISEN, J. A., K. S. SWEDER and P. C. HANAWALT, 1995 Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Res **23:** 2715-2723.

EL ALAMI, M., F. MESSENGUY, B. SCHERENS and E. DUBOIS, 2003 Arg82p is a bifunctional protein whose inositol polyphosphate kinase activity is essential for nitrogen and PHO gene expression but not for Mcm1p chaperoning in yeast. Mol Microbiol **49:** 457-468.

EL BAKKOURY, M., E. DUBOIS and F. MESSENGUY, 2000 Recruitment of the yeast MADS-box proteins, ArgRI and Mcm1 by the pleiotropic factor ArgRIII is required for their stability. Mol Microbiol **35:** 15-31.

EMRE, N. C., K. INGVARSDOTTIR, A. WYCE, A. WOOD, N. J. KROGAN *et al.*, 2005 Maintenance of low histone ubiquitylation by Ubp10 correlates with telomere-proximal Sir2 association and gene silencing. Mol Cell **17:** 585-594.

ERKINA, T. Y., and A. M. ERKINE, 2006 Displacement of histones at promoters of *Saccharomyces cerevisiae* heat shock genes is differentially associated with histone H3 acetylation. Mol Cell Biol **26:** 7587-7600.

EXINGER, F., and F. LACROUTE, 1992 6-Azauracil inhibition of GTP biosynthesis in *Saccharomyces cerevisiae*. Curr Genet **22:** 9-11.

EZHKOVA, E., and W. P. TANSEY, 2004 Proteasomal ATPases link ubiquitylation of histone H2B to methylation of histone H3. Mol Cell **13:** 435-442.

FANG, S. M., and Z. F. BURTON, 1996 RNA polymerase II-associated protein (RAP) 74 binds transcription factor (TF) IIB and blocks TFIIB-RAP30 binding. J Biol Chem **271:** 11703-11709.

FEATHERSTONE, M., 2002 Coactivators in transcription initiation: here are your orders. Curr Opin Genet Dev **12:** 149-155.

FENG, Q., H. WANG, H. H. NG, H. ERDJUMENT-BROMAGE, P. TEMPST *et al.*, 2002 Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr Biol **12:** 1052-1058.

FINGERMAN, I. M., H. C. LI and S. D. BRIGGS, 2007 A charge-based interaction between histone H4 and Dot1 is required for H3K79 methylation and telomere silencing: identification of a new trans-histone pathway. Genes Dev **21:** 2018-2029.

FLICK, J. S., and J. THORNER, 1993 Genetic and biochemical characterization of a phosphatidylinositol-specific phospholipase C in *Saccharomyces cerevisiae*. Mol Cell Biol **13:** 5861-5876.

FLORES, O., E. MALDONADO and D. REINBERG, 1989 Factors involved in specific transcription by mammalian RNA polymerase II. Factors IIE and IIF independently interact with RNA polymerase II. J Biol Chem **264:** 8913-8921.

GANGARAJU, V. K., and B. BARTHOLOMEW, 2007 Mechanisms of ATP dependent chromatin remodeling. Mutat Res **618:** 3-17.

GARDNER, R. G., Z. W. NELSON and D. E. GOTTSCHLING, 2005 Ubp10/Dot4p regulates the persistence of ubiquitinated histone H2B: distinct roles in telomeric silencing and general chromatin. Mol Cell Biol **25:** 6123-6139.

GENG, F., and W. P. TANSEY, 2008 Polyubiquitylation of histone H2B. Mol Biol Cell **19:** 3616-3624.

GHAEMMAGHAMI, S., W. K. HUH, K. BOWER, R. W. HOWSON, A. BELLE *et al.*, 2003 Global analysis of protein expression in yeast. Nature **425:** 737-741.

GNATT, A., 2002 Elongation by RNA polymerase II: structure-function relationship. Biochim Biophys Acta **1577:** 175-190.

GOFFEAU, A., B. G. BARRELL, H. BUSSEY, R. W. DAVIS, B. DUJON *et al.*, 1996 Life with 6000 genes. Science **274:** 546, 563-547.

GOLDMARK, J. P., T. G. FAZZIO, P. W. ESTEP, G. M. CHURCH and T. TSUKIYAMA, 2000 The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. Cell **103:** 423-433.

GOTTSCHLING, D. E., O. M. APARICIO, B. L. BILLINGTON and V. A. ZAKIAN, 1990 Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. Cell **63:** 751-762.

GOVIND, C. K., F. ZHANG, H. QIU, K. HOFMEYER and A. G. HINNEBUSCH, 2007 Gcn5 promotes acetylation, eviction, and methylation of nucleosomes in transcribed coding regions. Mol Cell **25:** 31-42.

GRANT, P. A., L. DUGGAN, J. COTE, S. M. ROBERTS, J. E. BROWNELL *et al.*, 1997 Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev **11:** 1640-1650.

GUZMAN, E., and J. T. LIS, 1999 Transcription factor TFIIH is required for promoter melting in vivo. Mol Cell Biol **19:** 5652-5658.

HA, I., S. ROBERTS, E. MALDONADO, X. SUN, L. U. KIM *et al.*, 1993 Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II. Genes Dev **7:** 1021-1032.

HANN, B. C., and P. WALTER, 1991 The signal recognition particle in *S. cerevisiae*. Cell **67:** 131-144.

HARTZOG, G. A., T. WADA, H. HANDA and F. WINSTON, 1998 Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in *Saccharomyces cerevisiae*. Genes Dev **12:** 357-369.

HECHT, A., T. LAROCHE, S. STRAHL-BOLSINGER, S. M. GASSER and M. GRUNSTEIN, 1995 Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell **80:** 583-592.

HENRY, K. W., A. WYCE, W. S. LO, L. J. DUGGAN, N. C. EMRE *et al.*, 2003 Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev **17:** 2648-2663.

HONG, L., G. P. SCHROTH, H. R. MATTHEWS, P. YAU and E. M. BRADBURY, 1993 Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 "tail" to DNA. J Biol Chem **268:** 305-314.

HUANG, H., A. KAHANA, D. E. GOTTSCHLING, L. PRAKASH and S. W. LIEBMAN, 1997 The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in *Saccharomyces cerevisiae*. Mol Cell Biol **17:** 6693-6699.

HWANG, W. W., S. VENKATASUBRAHMANYAM, A. G. IANCULESCU, A. TONG, C. BOONE *et al.*, 2003 A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. Mol Cell **11:** 261-266.

IIDA, T., and H. ARAKI, 2004 Noncompetitive counteractions of DNA polymerase epsilon and ISW2/yCHRAC for epigenetic inheritance of telomere position effect in *Saccharomyces cerevisiae*. Mol Cell Biol **24:** 217-227.

ISHIMI, Y., Y. OHBA, H. YASUDA and M. YAMADA, 1981 The interaction of H1 histone with nucleosome core. J Biochem **89:** 1881-1888.

JEANMOUGIN, F., J. D. THOMPSON, M. GOUY, D. G. HIGGINS and T. J. GIBSON, 1998 Multiple sequence alignment with Clustal X. Trends Biochem Sci **23:** 403-405.

JOHN, S., L. HOWE, S. T. TAFROV, P. A. GRANT, R. STERNGLANZ *et al.*, 2000 The something about silencing protein, Sas3, is the catalytic subunit of NuA3, a yTAF(II)30-containing HAT complex that interacts with the Spt16 subunit of the yeast CP (Cdc68/Pob3)-FACT complex. Genes Dev **14:** 1196-1208.

JONES, J. C., H. P. PHATNANI, T. A. HAYSTEAD, J. A. MACDONALD, S. M. ALAM *et al.*, 2004 C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats. J Biol Chem **279:** 24957-24964.

JONES, R. S., and W. M. GELBART, 1993 The Drosophila Polycomb-group gene Enhancer of zeste contains a region with sequence similarity to trithorax. Mol Cell Biol **13:** 6357-6366.

JONSSON, Z. O., S. K. DHAR, G. J. NARLIKAR, R. AUTY, N. WAGLE *et al.*, 2001 Rvb1p and Rvb2p are essential components of a chromatin remodeling complex that regulates transcription of over 5% of yeast genes. J Biol Chem **276:** 16279-16288.

JORGENSEN, P., N. P. EDGINGTON, B. L. SCHNEIDER, I. RUPES, M. TYERS *et al.*, 2007 The size of the nucleus increases as yeast cells grow. Mol Biol Cell **18:** 3523-3532.

JOSHI, A. A., and K. STRUHL, 2005 Eaf3 Chromodomain Interaction with Methylated H3-K36 Links Histone Deacetylation to Pol II Elongation. Mol Cell **20:** 971-978.

KAHANA, A., and D. E. GOTTSCHLING, 1999 DOT4 links silencing and cell growth in *Saccharomyces cerevisiae*. Mol Cell Biol **19:** 6608-6620.

KAPLAN, C. D., M. J. HOLLAND and F. WINSTON, 2005 Interaction between transcription elongation factors and mRNA 3'-end formation at the *Saccharomyces cerevisiae GAL10-GAL7* locus. J Biol Chem **280:** 913-922.

KAPLAN, C. D., L. LAPRADE and F. WINSTON, 2003 Transcription elongation factors repress transcription initiation from cryptic sites. Science **301:** 1096-1099.

KETTENBERGER, H., K. J. ARMACHE and P. CRAMER, 2003 Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage. Cell **114:** 347-357.

KETTENBERGER, H., K. J. ARMACHE and P. CRAMER, 2004 Complete RNA polymerase II elongation complex structure and its interactions with NTP and TFIIS. Mol Cell **16:** 955-965.

KIM, M., S. H. AHN, N. J. KROGAN, J. F. GREENBLATT and S. BURATOWSKI, 2004a Transitions in RNA polymerase II elongation complexes at the 3' ends of genes. Embo J **23:** 354-364.

KIM, M., N. J. KROGAN, L. VASILJEVA, O. J. RANDO, E. NEDEA *et al.*, 2004b The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. Nature **432:** 517-522.

KIM, T., and S. BURATOWSKI, 2007 Two *Saccharomyces cerevisiae* JmjC domain proteins demethylate histone H3 Lys36 in transcribed regions to promote elongation. J Biol Chem **282:** 20827-20835.

KIREEVA, M. L., B. HANCOCK, G. H. CREMONA, W. WALTER, V. M. STUDITSKY *et al.*, 2005 Nature of the nucleosomal barrier to RNA polymerase II. Mol Cell **18:** 97-108.

KIREEVA, M. L., W. WALTER, V. TCHERNAJENKO, V. BONDARENKO, M. KASHLEV *et al.*, 2002 Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. Mol Cell **9:** 541-552.

KIRMIZIS, A., H. SANTOS-ROSA, C. J. PENKETT, M. A. SINGER, M. VERMEULEN *et al.*, 2007 Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. Nature **449:** 928-932.

KIZER, K. O., H. P. PHATNANI, Y. SHIBATA, H. HALL, A. L. GREENLEAF *et al.*, 2005 A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol Cell Biol **25:** 3305-3316.

KLEFF, S., E. D. ANDRULIS, C. W. ANDERSON and R. STERNGLANZ, 1995 Identification of a gene encoding a yeast histone H4 acetyltransferase. J Biol Chem **270:** 24674-24677.

KOBOR, M. S., S. VENKATASUBRAHMANYAM, M. D. MENEGHINI, J. W. GIN, J. L. JENNINGS *et al.*, 2004 A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol **2:** E131.

KOCH, C., P. WOLLMANN, M. DAHL and F. LOTTSPEICH, 1999 A role for Ctr9p and Paf1p in the regulation G1 cyclin expression in yeast. Nucleic Acids Res **27:** 2126-2134.

KOHLER, A., and E. HURT, 2007 Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cell Biol **8:** 761-773.

KOMARNITSKY, P., E. J. CHO and S. BURATOWSKI, 2000 Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev **14:** 2452-2460.

KORNBERG, R. D., 2005 Mediator and the mechanism of transcriptional activation. Trends Biochem Sci **30:** 235-239.

KORNBERG, R. D., and J. O. THOMAS, 1974 Chromatin structure; oligomers of the histones. Science **184:** 865-868.

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

KREBS, J. E., 2007 Moving marks: dynamic histone modifications in yeast. Mol Biosyst **3:** 590-597.

KROGAN, N. J., J. DOVER, S. KHORRAMI, J. F. GREENBLATT, J. SCHNEIDER *et al.*, 2002a COMPASS, a histone H3 (Lysine 4) methyltransferase required for telomeric silencing of gene expression. J Biol Chem **277:** 10753-10755.

KROGAN, N. J., J. DOVER, A. WOOD, J. SCHNEIDER, J. HEIDT *et al.*, 2003 The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol Cell **11:** 721-729.

KROGAN, N. J., M. KIM, S. H. AHN, G. ZHONG, M. S. KOBOR *et al.*, 2002b RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. Mol. Cell. Biol. **22:** 6979-6992.

KUNKEL, T. A., J. D. ROBERTS and R. A. ZAKOUR, 1987 Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol **154:** 367-382.

KUTACH, A. K., and J. T. KADONAGA, 2000 The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters. Mol Cell Biol **20:** 4754-4764.

LANDRY, J., A. SUTTON, S. T. TAFROV, R. C. HELLER, J. STEBBINS *et al.*, 2000 The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A **97:** 5807-5811.

LARIBEE, R. N., N. J. KROGAN, T. XIAO, Y. SHIBATA, T. R. HUGHES *et al.*, 2005 BUR kinase selectively regulates H3 K4 trimethylation and H2B ubiquitylation through recruitment of the PAF elongation complex. Curr Biol **15:** 1487-1493.

LARIBEE, R. N., Y. SHIBATA, D. P. MERSMAN, S. R. COLLINS, P. KEMMEREN *et al.*, 2007 CCR4/NOT complex associates with the proteasome and regulates histone methylation. Proc Natl Acad Sci U S A **104:** 5836-5841.

LAURENT, B. C., M. A. TREITEL and M. CARLSON, 1991 Functional interdependence of the yeast SNF2, SNF5, and SNF6 proteins in transcriptional activation. Proc Natl Acad Sci U S A **88:** 2687-2691.

LAURENT, B. C., X. YANG and M. CARLSON, 1992 An essential *Saccharomyces cerevisiae* gene homologous to SNF2 encodes a helicase-related protein in a new family. Mol Cell Biol **12:** 1893-1902.

LEE, J. S., A. SHUKLA, J. SCHNEIDER, S. K. SWANSON, M. P. WASHBURN *et al.*, 2007 Histone crosstalk between H2B monoubiquitination and H3 methylation mediated by COMPASS. Cell **131:** 1084-1096.

LEVCHENKO, V., B. JACKSON and V. JACKSON, 2005 Histone release during transcription: displacement of the two H2A-H2B dimers in the nucleosome is dependent on different levels of transcription-induced positive stress. Biochemistry **44:** 5357-5372.

LI, B., M. CAREY and J. L. WORKMAN, 2007a The role of chromatin during transcription. Cell **128:** 707-719.

LI, B., M. GOGOL, M. CAREY, D. LEE, C. SEIDEL *et al.*, 2007b Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. Science **316:** 1050-1054.

LI, B., L. HOWE, S. ANDERSON, J. R. YATES, 3RD and J. L. WORKMAN, 2003 The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. J Biol Chem **278:** 8897-8903.

LICATALOSI, D. D., G. GEIGER, M. MINET, S. SCHROEDER, K. CILLI *et al.*, 2002 Functional interaction of yeast pre-mRNA 3' end processing factors with RNA polymerase II. Mol Cell **9:** 1101-1111.

LINDSTROM, D. L., and G. A. HARTZOG, 2001 Genetic interactions of Spt4-Spt5 and TFIIS with the RNA polymerase II CTD and CTD modifying enzymes in *Saccharomyces cerevisiae*. Genetics **159:** 487-497.

LINDSTROM, D. L., S. L. SQUAZZO, N. MUSTER, T. A. BURCKIN, K. C. WACHTER *et al.*, 2003 Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol **23:** 1368-1378.

LOGAN, J., E. FALCK-PEDERSEN, J. E. DARNELL, JR. and T. SHENK, 1987 A poly(A) addition site and a downstream termination region are required for efficient cessation of transcription by RNA polymerase II in the mouse beta maj-globin gene. Proc Natl Acad Sci U S A **84:** 8306-8310.

LORCH, Y., and R. D. KORNBERG, 1985 A region flanking the GAL7 gene and a binding site for GAL4 protein as upstream activating sequences in yeast. J Mol Biol **186:** 821-824.

LOWELL, J. E., D. Z. RUDNER and A. B. SACHS, 1992 3'-UTR-dependent deadenylation by the yeast poly(A) nuclease. Genes Dev **6:** 2088-2099.

LUGER, K., A. W. MADER, R. K. RICHMOND, D. F. SARGENT and T. J. RICHMOND, 1997 Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389:** 251-260.

LUND, M. K., and C. GUTHRIE, 2005 The DEAD-box protein Dbp5p is required to dissociate Mex67p from exported mRNPs at the nuclear rim. Mol Cell **20:** 645-651.

MARTINEZ, E., 2002 Multi-protein complexes in eukaryotic gene transcription. Plant Mol Biol **50:** 925-947.

MARTON, H. A., and S. DESIDERIO, 2008 *The Paf1 complex promotes displacement of histones upon rapid induction of transcription by RNA polymerase II.* BMC Mol Biol. **9**.

MASON, P. B., and K. STRUHL, 2003 The FACT complex travels with elongating RNA polymerase II and is important for the fidelity of transcriptional initiation in vivo. Mol Cell Biol **23:** 8323-8333.

MAVRICH, T. N., I. P. IOSHIKHES, B. J. VENTERS, C. JIANG, L. P. TOMSHO *et al.*, 2008 A barrier nucleosome model for statistical positioning of nucleosomes throughout the yeast genome. Genome Res **18:** 1073-1083.

MCGINTY, R. K., J. KIM, C. CHATTERJEE, R. G. ROEDER and T. W. MUIR, 2008 Chemically ubiquitylated histone H2B stimulates hDot1L-mediated intranucleosomal methylation. Nature **453:** 812-816.

MESSENGUY, F., E. DUBOIS and C. BOONCHIRD, 1991 Determination of the DNA-binding sequences of ARGR proteins to arginine anabolic and catabolic promoters. Mol Cell Biol **11:** 2852-2863.

MICHELL, R. H., N. M. PERERA and S. K. DOVE, 2003 Morton Medal Lecture. New insights into the roles of phosphoinositides and inositol polyphosphates in yeast. Biochem Soc Trans **31:** 11-15.

MILLAR, C. B., and M. GRUNSTEIN, 2006 Genome-wide patterns of histone modifications in yeast. Nat Rev Mol Cell Biol **7:** 657-666.

MILLER, T., N. J. KROGAN, J. DOVER, H. ERDJUMENT-BROMAGE, P. TEMPST *et al.*, 2001 COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc Natl Acad Sci U S A **98:** 12902-12907.

MINVIELLE-SEBASTIA, L., K. BEYER, A. M. KRECIC, R. E. HECTOR, M. S. SWANSON *et al.*, 1998 Control of cleavage site selection during mRNA 3' end formation by a yeast hnRNP. Embo J **17:** 7454-7468.

MIZUGUCHI, G., X. SHEN, J. LANDRY, W. H. WU, S. SEN *et al.*, 2004 ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science **303:** 343-348.

MORETTI, P., K. FREEMAN, L. COODLY and D. SHORE, 1994 Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev **8:** 2257-2269.

MORILLON, A., N. KARABETSOU, J. O'SULLIVAN, N. KENT, N. PROUDFOOT *et al.*, 2003 Isw1 chromatin remodeling ATPase coordinates transcription elongation and termination by RNA polymerase II. Cell **115:** 425-435.

MORRISON, A. J., J. HIGHLAND, N. J. KROGAN, A. ARBEL-EDEN, J. F. GREENBLATT *et al.*, 2004 INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. Cell **119:** 767-775.

MUELLER, C. L., and J. A. JAEHNING, 2002 Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex. Mol Cell Biol **22:** 1971-1980.

MUELLER, C. L., S. E. PORTER, M. G. HOFFMAN and J. A. JAEHNING, 2004 The Paf1 complex has functions independent of actively transcribing RNA polymerase II. Mol Cell **14:** 447-456.

MUELLER, J. E., M. CANZE and M. BRYK, 2006 The requirements for COMPASS and Paf1 in transcriptional silencing and methylation of histone H3 in *Saccharomyces cerevisiae*. Genetics **173:** 557-567.

MUHLRAD, D., R. HUNTER and R. PARKER, 1992 A rapid method for localized mutagenesis of yeast genes. Yeast **8:** 79-82.

MULDER, K. W., A. B. BRENKMAN, A. INAGAKI, N. J. VAN DEN BROEK and H. T. TIMMERS, 2007 Regulation of histone H3K4 tri-methylation and PAF complex recruitment by the Ccr4-Not complex. Nucleic Acids Res **35:** 2428-2439.

MURATANI, M., and W. P. TANSEY, 2003 How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol **4:** 192-201.

MURPHY, R., J. L. WATKINS and S. R. WENTE, 1996 GLE2, a *Saccharomyces cerevisiae* homologue of the Schizosaccharomyces pombe export factor RAE1, is required for nuclear pore complex structure and function. Mol Biol Cell **7:** 1921-1937.

NAGY, P. L., J. GRIESENBECK, R. D. KORNBERG and M. L. CLEARY, 2002 A trithorax-group complex purified from *Saccharomyces cerevisiae* is required for methylation of histone H3. Proc Natl Acad Sci U S A **99:** 90-94.

NG, H. H., D. N. CICCONE, K. B. MORSHEAD, M. A. OETTINGER and K. STRUHL, 2003a Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. Proc Natl Acad Sci U S A **100:** 1820-1825.

NG, H. H., S. DOLE and K. STRUHL, 2003b The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J Biol Chem **278:** 33625-33628.

NG, H. H., F. ROBERT, R. A. YOUNG and K. STRUHL, 2003c Targeted recruitment of Set1 histone methyltransferase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell **11:** 709-719.

NG, H. H., R. M. XU, Y. ZHANG and K. STRUHL, 2002 Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. J Biol Chem **277:** 34655-34657.

NICKEL, B. E., and J. R. DAVIE, 1989 Structure of polyubiquitinated histone H2A. Biochemistry **28:** 964-968.

NOGALES, E., 2000 Recent structural insights into transcription preinitiation complexes. J Cell Sci **113 Pt 24:** 4391-4397.

NORDICK, K., M. G. HOFFMAN, J. L. BETZ and J. A. JAEHNING, 2008 Direct interactions between the Paf1 complex and a cleavage and polyadenylation factor are revealed by dissociation of Paf1 from RNA polymerase II. Eukaryot Cell **7:** 1158-1167.

OHDATE, H., C. R. LIM, T. KOKUBO, K. MATSUBARA, Y. KIMATA *et al.*, 2003 Impairment of the DNA binding activity of the TATA-binding protein renders the transcriptional function of Rvb2p/Tih2p, the yeast RuvB-like protein, essential for cell growth. J Biol Chem **278:** 14647-14656.

OKUDA, M., M. HORIKOSHI and Y. NISHIMURA, 2007 Structural polymorphism of chromodomains in Chd1. J Mol Biol **365:** 1047-1062.

ORPHANIDES, G., T. LAGRANGE and D. REINBERG, 1996 The general transcription factors of RNA polymerase II. Genes Dev **10:** 2657-2683.

ORPHANIDES, G., G. LEROY, C. H. CHANG, D. S. LUSE and D. REINBERG, 1998 FACT, a factor that facilitates transcript elongation through nucleosomes. Cell **92:** 105-116.

ORPHANIDES, G., W. H. WU, W. S. LANE, M. HAMPSEY and D. REINBERG, 1999 The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. Nature **400:** 284-288.

OSADA, S., A. SUTTON, N. MUSTER, C. E. BROWN, J. R. YATES, 3RD *et al.*, 2001 The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASF1. Genes Dev **15:** 3155-3168.

OSLEY, M. A., 2006 Regulation of histone H2A and H2B ubiquitylation. Brief Funct Genomic Proteomic **5:** 179-189.

PALANCADE, B., and O. BENSAUDE, 2003 Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. Eur J Biochem **270:** 3859-3870.

PAVRI, R., B. ZHU, G. LI, P. TROJER, S. MANDAL *et al.*, 2006 Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. Cell **125:** 703-717.

PENHEITER, K. L., T. M. WASHBURN, S. E. PORTER, M. G. HOFFMAN and J. A. JAEHNING, 2005 A posttranscriptional role for the yeast Paf1-RNA polymerase II complex is revealed by identification of primary targets. Mol Cell **20:** 213-223.

PETERSON, C. L., A. DINGWALL and M. P. SCOTT, 1994 Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement. Proc Natl Acad Sci U S A **91:** 2905-2908.

PETERSON, C. L., and I. HERSKOWITZ, 1992 Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription. Cell **68:** 573-583.

PETERSON, C. L., W. KRUGER and I. HERSKOWITZ, 1991 A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. Cell **64:** 1135-1143.

PHATNANI, H. P., J. C. JONES and A. L. GREENLEAF, 2004 Expanding the functional repertoire of CTD kinase I and RNA polymerase II: novel phosphoCTD-associating proteins in the yeast proteome. Biochemistry **43:** 15702-15719.

POKHOLOK, D. K., N. M. HANNETT and R. A. YOUNG, 2002 Exchange of RNA polymerase II initiation and elongation factors during gene expression *in vivo*. Mol Cell **9:** 799-809.

POKHOLOK, D. K., C. T. HARBISON, S. LEVINE, M. COLE, N. M. HANNETT *et al.*, 2005 Genome-wide map of nucleosome acetylation and methylation in yeast. Cell **122:** 517-527.

PORTER, S. E., K. L. PENHEITER and J. A. JAEHNING, 2005 Separation of the *Saccharomyces cerevisiae* Paf1 complex from RNA polymerase II results in changes in its subnuclear localization. Eukaryot Cell **4:** 209-220.

PORTER, S. E., T. M. WASHBURN, M. CHANG and J. A. JAEHNING, 2002 The yeast Paf1-RNA polymerase II complex is required for full expression of a subset of cell cycle-regulated genes. Eukaryot Cell **1:** 830-842.

PRATHER, D., KROGAN, N.J., EMILI, A., GREENBLATT, J.F., WINSTON, F., 2005 *Identification and characterization of Elf1, a conserved transcription elongation factor in Saccharomyces cerevisiae.* Mol Cell Biol **25:** 10122-10135.

PRAY-GRANT, M. G., J. A. DANIEL, D. SCHIELTZ, J. R. YATES, 3RD and P. A. GRANT, 2005 Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. Nature **433:** 434-438.

PREKER, P. J., M. OHNACKER, L. MINVIELLE-SEBASTIA and W. KELLER, 1997 A multisubunit 3' end processing factor from yeast containing poly(A) polymerase and homologues of the subunits of mammalian cleavage and polyadenylation specificity factor. Embo J **16:** 4727-4737.

PRELICH, G., 2002 RNA polymerase II carboxy-terminal domain kinases: emerging clues to their function. Eukaryot Cell **1:** 153-162.

QIU, H., C. HU, C. M. WONG and A. G. HINNEBUSCH, 2006 The Spt4p subunit of yeast DSIF stimulates association of the Paf1 complex with elongating RNA polymerase II. Mol Cell Biol **26:** 3135-3148.

RANDO, O. J., T. H. CHI and G. R. CRABTREE, 2003 Second messenger control of chromatin remodeling. Nat Struct Biol **10:** 81-83.

ROBERTS, S. M., and F. WINSTON, 1997 Essential functional interactions of SAGA, a *Saccharomyces cerevisiae* complex of Spt, Ada, and Gcn5 proteins, with the Snf/Swi and Srb/mediator complexes. Genetics **147:** 451-465.

ROBZYK, K., J. RECHT and M. A. OSLEY, 2000 Rad6-dependent ubiquitination of histone H2B in yeast. Science **287:** 501-504.

ROEDER, G. S., and G. R. FINK, 1982 Movement of yeast transposable elements by gene conversion. Proc Natl Acad Sci U S A **79:** 5621-5625.

ROGUEV, A., D. SCHAFT, A. SHEVCHENKO, W. W. PIJNAPPEL, M. WILM *et al.*, 2001 The *Saccharomyces cerevisiae* Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. Embo J **20:** 7137-7148.

ROSE, M. D., F. WINSTON and P. HIETER, 1990 *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

ROTHSTEIN, R., 1991 Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol **194:** 281-301.

ROUGEMAILLE, M., T. VILLA, R. K. GUDIPATI and D. LIBRI, 2008 mRNA journey to the cytoplasm: attire required. Biol Cell **100:** 327-342.

ROZENBLATT-ROSEN, O., C. M. HUGHES, S. J. NANNEPAGA, K. S. SHANMUGAM, T. D. COPELAND *et al.*, 2005 The parafibromin tumor suppressor protein is part of a human Paf1 complex. Mol Cell Biol **25:** 612-620.

RUDD, M. D., M. G. IZBAN and D. S. LUSE, 1994 The active site of RNA polymerase II participates in transcript cleavage within arrested ternary complexes. Proc Natl Acad Sci U S A **91:** 8057-8061.

RUSCHE, L. N., A. L. KIRCHMAIER and J. RINE, 2003 The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. Annu Rev Biochem **72:** 481-516.

RYAN, K., O. CALVO and J. L. MANLEY, 2004 Evidence that polyadenylation factor CPSF-73 is the mRNA 3' processing endonuclease. Rna **10:** 565-573.

SAIARDI, A., H. ERDJUMENT-BROMAGE, A. M. SNOWMAN, P. TEMPST and S. H. SNYDER, 1999 Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr Biol **9:** 1323-1326.

SANTOS-ROSA, H., A. J. BANNISTER, P. M. DEHE, V. GELI and T. KOUZARIDES, 2004 Methylation of H3 lysine 4 at euchromatin promotes Sir3p association with heterochromatin. J Biol Chem **279:** 47506-47512.

SANTOS-ROSA, H., H. MORENO, G. SIMOS, A. SEGREF, B. FAHRENKROG *et al.*, 1998 Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores. Mol Cell Biol **18:** 6826-6838.

SANTOS-ROSA, H., R. SCHNEIDER, A. J. BANNISTER, J. SHERRIFF, B. E. BERNSTEIN *et al.*, 2002 Active genes are tri-methylated at K4 of histone H3. Nature **419:** 407-411.

SCHNEIDER, D. A., S. L. FRENCH, Y. N. OSHEIM, A. O. BAILEY, L. VU *et al.*, 2006 RNA polymerase II elongation factors Spt4p and Spt5p play roles in transcription elongation by RNA polymerase I and rRNA processing. Proc Natl Acad Sci U S A **103:** 12707-12712.

SCHNEIDER, J., A. WOOD, J. S. LEE, R. SCHUSTER, J. DUEKER *et al.*, 2005 Molecular regulation of histone H3 trimethylation by COMPASS and the regulation of gene expression. Mol Cell **19:** 849-856.

SCHULTZ, M. C., D. J. HOCKMAN, T. A. HARKNESS, W. I. GARINTHER and B. A. ALTHEIM, 1997 Chromatin assembly in a yeast whole-cell extract. Proc Natl Acad Sci U S A **94:** 9034-9039.

SEGREF, A., K. SHARMA, V. DOYE, A. HELLWIG, J. HUBER *et al.*, 1997 Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. Embo J **16:** 3256-3271.

SHAHBAZIAN, M. D., K. ZHANG and M. GRUNSTEIN, 2005 Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1. Mol Cell **19:** 271-277.

SHEARS, S. B., 2000 Transcriptional regulation: a new dominion for inositol phosphate signaling? Bioessays **22:** 786-789.

SHELDON, K. E., D. M. MAUGER and K. M. ARNDT, 2005 A Requirement for the *Saccharomyces cerevisiae* Paf1 complex in snoRNA 3' end formation. Mol Cell **20:** 225-236.

SHEN, X., G. MIZUGUCHI, A. HAMICHE and C. WU, 2000 A chromatin remodelling complex involved in transcription and DNA processing. Nature **406:** 541-544.

SHEN, X., H. XIAO, R. RANALLO, W. H. WU and C. WU, 2003 Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. Science **299:** 112-114.

SHI, X., M. CHANG, A. J. WOLF, C. H. CHANG, A. A. FRAZER-ABEL *et al.*, 1997 Cdc73p and Paf1p are found in a novel RNA polymerase II-containing complex distinct from the Srbp-containing holoenzyme. Mol Cell Biol **17:** 1160-1169.

SHI, Y., F. LAN, C. MATSON, P. MULLIGAN, J. R. WHETSTINE *et al.*, 2004 Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell **119:** 941-953.

SHILATIFARD, A., 2006 Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu Rev Biochem **75:** 243-269.

SHIRRA, M. K., S. E. ROGERS, D. E. ALEXANDER and K. M. ARNDT, 2005 The Snf1 protein kinase and Sit4 protein phosphatase have opposing functions in regulating TATA-binding protein association with the *Saccharomyces cerevisiae INO1* promoter. Genetics **169:** 1957-1972.

SHIVASWAMY, S., and V. R. IYER, 2008 Stress-dependent dynamics of global chromatin remodeling in yeast: dual role for SWI/SNF in the heat shock stress response. Mol Cell Biol **28:** 2221-2234.

SHOGREN-KNAAK, M., H. ISHII, J. M. SUN, M. J. PAZIN, J. R. DAVIE *et al.*, 2006 Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science **311:** 844-847.

SIF, S., 2004 ATP-dependent nucleosome remodeling complexes: enzymes tailored to deal with chromatin. J Cell Biochem **91:** 1087-1098.

SIKORSKI, R. S., and P. HIETER, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19-27.

SIMIC, R., D. L. LINDSTROM, H. G. TRAN, K. L. ROINICK, P. J. COSTA *et al.*, 2003 Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. Embo J **22:** 1846-1856.

SIMS, R. J., 3RD, S. MILLHOUSE, C. F. CHEN, B. A. LEWIS, H. ERDJUMENT-BROMAGE *et al.*, 2007 Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. Mol Cell **28:** 665-676.

SMALE, S. T., and J. T. KADONAGA, 2003 The RNA polymerase II core promoter. Annu Rev Biochem **72:** 449-479.

SMITH, D. B., and K. S. JOHNSON, 1988 Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:** 31-40.

SMITH, J. S., C. B. BRACHMANN, I. CELIC, M. A. KENNA, S. MUHAMMAD *et al.*, 2000 A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci U S A **97:** 6658-6663.

SQUAZZO, S. L., P. J. COSTA, D. L. LINDSTROM, K. E. KUMER, R. SIMIC *et al.*, 2002 The Paf1 complex physically and functionally associates with transcription elongation factors *in vivo*. Embo J **21:** 1764-1774.

STEGER, D. J., E. S. HASWELL, A. L. MILLER, S. R. WENTE and E. K. O'SHEA, 2003 Regulation of chromatin remodeling by inositol polyphosphates. Science **299:** 114-116.

STEINMETZ, E. J., N. K. CONRAD, D. A. BROW and J. L. CORDEN, 2001 RNA-binding protein Nrd1 directs poly(A)-independent 3'-end formation of RNA polymerase II transcripts. Nature **413:** 327-331.

STERNER, D. E., and S. L. BERGER, 2000 Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev **64:** 435-459.

STOLINSKI, L. A., D. M. EISENMANN and K. M. ARNDT, 1997 Identification of *RTF1*, a novel gene important for TATA site selection by TATA box-binding protein in *Saccharomyces cerevisiae*. Mol Cell Biol **17:** 4490-4500.

STRAHL, B. D., and C. D. ALLIS, 2000 The language of covalent histone modifications. Nature **403:** 41-45.

STRAHL, B. D., P. A. GRANT, S. D. BRIGGS, Z. W. SUN, J. R. BONE *et al.*, 2002 Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Mol Cell Biol **22:** 1298-1306.

STRUHL, K., 1989 Molecular mechanisms of transcriptional regulation in yeast. Annu Rev Biochem **58:** 1051-1077.

SUDARSANAM, P., V. R. IYER, P. O. BROWN and F. WINSTON, 2000 Whole-genome expression analysis of snf/swi mutants of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A **97:** 3364-3369.

SUN, Z. W., and C. D. ALLIS, 2002 Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature **418:** 104-108.

SWANSON, M. S., E. A. MALONE and F. WINSTON, 1991 *SPT5*, an essential gene important for normal transcription in *Saccharomyces cerevisiae*, encodes an acidic nuclear protein with a carboxy-terminal repeat. Mol Cell Biol **11:** 4286.

TABANCAY, A. P., JR., and S. L. FORSBURG, 2006 Eukaryotic DNA replication in a chromatin context. Curr Top Dev Biol **76:** 129-184.

TENNEY, K., M. GERBER, A. ILVARSONN, J. SCHNEIDER, M. GAUSE *et al.*, 2006 Drosophila Rtf1 functions in histone methylation, gene expression, and Notch signaling. Proc Natl Acad Sci U S A **103:** 11970-11974.

TRAN, H. G., D. J. STEGER, V. R. IYER and A. D. JOHNSON, 2000 The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. Embo J **19:** 2323-2331.

TSCHIERSCH, B., A. HOFMANN, V. KRAUSS, R. DORN, G. KORGE *et al.*, 1994 The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. Embo J **13:** 3822-3831.

TSUBOTA, T., C. E. BERNDSEN, J. A. ERKMANN, C. L. SMITH, L. YANG *et al.*, 2007 Histone H3-K56 acetylation is catalyzed by histone chaperone-dependent complexes. Mol Cell **25:** 703-712.

TSUKADA, Y., J. FANG, H. ERDJUMENT-BROMAGE, M. E. WARREN, C. H. BORCHERS *et al.*, 2006 Histone demethylation by a family of JmjC domain-containing proteins. Nature **439:** 811-816.

VAN ATTIKUM, H., O. FRITSCH, B. HOHN and S. M. GASSER, 2004 Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair. Cell **119:** 777-788.

VAN LEEUWEN, F., P. R. GAFKEN and D. E. GOTTSCHLING, 2002 Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell **109:** 745-756.

VAN VUGT, J. J., M. RANES, C. CAMPSTEIJN and C. LOGIE, 2007 The ins and outs of ATP-dependent chromatin remodeling in budding yeast: biophysical and proteomic perspectives. Biochim Biophys Acta **1769:** 153-171.

VILARDELL, J., and J. R. WARNER, 1997 Ribosomal protein L32 of *Saccharomyces cerevisiae* influences both the splicing of its own transcript and the processing of rRNA. Mol Cell Biol **17:** 1959-1965.

WADA, T., T. TAKAGI, Y. YAMAGUCHI, A. FERDOUS, T. IMAI *et al.*, 1998 DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. Genes Dev **12:** 343-356.

WARNER, M. H., K. L. ROINICK and K. M. ARNDT, 2007 Rtf1 is a multifunctional component of the Paf1 complex that regulates gene expression by directing cotranscriptional histone modification. Mol Cell Biol **27:** 6103-6115.

WEAKE, V. M., and J. L. WORKMAN, 2008 Histone ubiquitination: triggering gene activity. Mol Cell **29:** 653-663.

WEILBAECHER, R. G., D. E. AWREY, A. M. EDWARDS and C. M. KANE, 2003 Intrinsic transcript cleavage in yeast RNA polymerase II elongation complexes. J Biol Chem **278:** 24189-24199.

WEIRICH, C. S., J. P. ERZBERGER, J. S. FLICK, J. M. BERGER, J. THORNER *et al.*, 2006 Activation of the DExD/H-box protein Dbp5 by the nuclear-pore protein Gle1 and its coactivator InsP6 is required for mRNA export. Nat Cell Biol **8:** 668-676.

WEST, M. L., and J. L. CORDEN, 1995 Construction and analysis of yeast RNA polymerase II CTD deletion and substitution mutations. Genetics **140:** 1223-1233.

WHITEHOUSE, I., O. J. RANDO, J. DELROW and T. TSUKIYAMA, 2007 Chromatin remodelling at promoters suppresses antisense transcription. Nature **450:** 1031-1035.

WILLIAMS, S. K., D. TRUONG and J. K. TYLER, 2008 Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. Proc Natl Acad Sci U S A **105:** 9000-9005.

WINSTON, F., and M. CARLSON, 1992 Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. Trends Genet **8:** 387-391.

WINSTON, F., C. DOLLARD and S. L. RICUPERO-HOVASSE, 1995 Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast **11:** 53-55.

WITTSCHIEBEN, B. O., G. OTERO, T. DE BIZEMONT, J. FELLOWS, H. ERDJUMENT-BROMAGE *et al.*, 1999 A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol Cell **4:** 123-128.

WOOD, A., N. J. KROGAN, J. DOVER, J. SCHNEIDER, J. HEIDT *et al.*, 2003a Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. Mol Cell **11:** 267-274.

WOOD, A., J. SCHNEIDER, J. DOVER, M. JOHNSTON and A. SHILATIFARD, 2003b The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. J Biol Chem **278:** 34739-34742.

WOODAGE, T., M. A. BASRAI, A. D. BAXEVANIS, P. HIETER and F. S. COLLINS, 1997 Characterization of the CHD family of proteins. Proc Natl Acad Sci U S A **94:** 11472-11477.

WOYCHIK, N. A., and M. HAMPSEY, 2002 The RNA polymerase II machinery: structure illuminates function. Cell **108:** 453-463.

WYCE, A., K. W. HENRY and S. L. BERGER, 2004 H2B ubiquitylation and de-ubiquitylation in gene activation. Novartis Found Symp **259:** 63-73; discussion 73-67, 163-169.

WYCE, A., T. XIAO, K. A. WHELAN, C. KOSMAN, W. WALTER *et al.*, 2007 H2B ubiquitylation acts as a barrier to Ctk1 nucleosomal recruitment prior to removal by Ubp8 within a SAGA-related complex. Mol Cell **27:** 275-288.

XIAO, T., C. F. KAO, N. J. KROGAN, Z. W. SUN, J. F. GREENBLATT *et al.*, 2005 Histone H2B ubiquitylation is associated with elongating RNA polymerase II. Mol Cell Biol **25:** 637-651.

YART, A., M. GSTAIGER, C. WIRBELAUER, M. PECNIK, D. ANASTASIOU *et al.*, 2005 The HRPT2 tumor suppressor gene product parafibromin associates with human PAF1 and RNA polymerase II. Mol Cell Biol **25:** 5052-5060.

YORK, J. D., A. R. ODOM, R. MURPHY, E. B. IVES and S. R. WENTE, 1999 A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science **285:** 96-100.

YOW, G. Y., T. UO, T. YOSHIMURA and N. ESAKI, 2004 D-amino acid N-acetyltransferase of *Saccharomyces cerevisiae*: a close homologue of histone acetyltransferase Hpa2p acting exclusively on free D-amino acids. Arch Microbiol **182:** 396-403.

YUAN, G. C., Y. J. LIU, M. F. DION, M. D. SLACK, L. F. WU *et al.*, 2005 Genome-scale identification of nucleosome positions in *S. cerevisiae*. Science **309:** 626-630.

YUDKOVSKY, N., J. A. RANISH and S. HAHN, 2000 A transcription reinitiation intermediate that is stabilized by activator. Nature **408:** 225-229.

ZENG, L., and M. M. ZHOU, 2002 Bromodomain: an acetyl-lysine binding domain. FEBS Lett **513:** 124-128.

ZHANG, H., D. N. ROBERTS and B. R. CAIRNS, 2005 Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. Cell **123:** 219-231.

ZHAO, J., L. HYMAN and C. MOORE, 1999 Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. Microbiol Mol Biol Rev **63:** 405-445.

ZHU, B., S. S. MANDAL, A. D. PHAM, Y. ZHENG, H. ERDJUMENT-BROMAGE *et al.*, 2005 The human PAF complex coordinates transcription with events downstream of RNA synthesis. Genes Dev **19:** 1668-1673.

ZLATANOVA, J., and A. THAKAR, 2008 H2A.Z: view from the top. Structure **16:** 166-179.

ZOFALL, M., J. PERSINGER, S. R. KASSABOV and B. BARTHOLOMEW, 2006 Chromatin remodeling by ISW2 and SWI/SNF requires DNA translocation inside the nucleosome. Nat Struct Mol Biol **13:** 339-346.