Characterization of Rkr1, a nuclear RING-domain protein with functional connections to chromatin modification in *Saccharomyces cerevisiae*

by

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RNA Polymerase II (Pol II) transcription is a highly regulated process. Many factors associate with Pol II to ensure that transcription occurs as efficiently as possible. One of these factors is the Paf1 complex, which consists of the subunits Paf1, Ctr9, Rtf1, Cdc73, and Leo1. This complex has been shown to be important for the regulation of chromatin modifications that promote active transcription. Rkr1 was identified in a genetic screen to uncover factors that function in parallel with the Paf1 subunit Rtf1. My work has focused on characterizing a role for Rkr1 in transcription and chromatin function. I have shown that strains lacking *RKR1* have transcription-related phenotypes. Genetic analysis has shown that Rkr1 functions in parallel with Rtf1-dependent histone modifications, particularly histone H2B ubiquitylation and histone H3 lysine 4 methylation. Strains lacking *RKR1* have telomeric silencing defects, further connecting Rkr1 to chromatin function. Rkr1 is a nuclear protein that contains a RING domain at its extreme carboxy terminus. RING domain proteins often act as ubiquitin-protein ligases, which determine substrate specificity in the ubiquitylation pathway. Subsequent analyses have shown that Rkr1 does possess ubiquitin ligase activity *in vitro*, and mutational analysis shows that the RING domain of Rkr1 is required for *in vivo* activity. In an attempt to identify a functional process for Rkr1, a yeast two-hybrid screen was performed using an amino-terminal fragment of Rkr1 as bait. Twenty proteins were identified to interact with this region of Rkr1, many of which are functionally connected to transcription and chromatin. Microarray analysis shows that

Rkr1 is required for proper expression of a subset of genes in yeast. Taken together, my work has identified a new ubiquitylation pathway within the nucleus that acts to regulate transcription and chromatin function.

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PREFACE

First, I must thank Karen Arndt. Karen, you have been an amazing advisor, mentor and role model through the last 6 years. I want to thank you for taking me into your lab and teaching me how to be the best scientist I can be. Thank you for pushing me to speak at scientific meetings and for supporting me and my career choices. Rkr1 rocks!

I also need to thank my committee members, Jeff Brodsky, Jeffrey Lawrence, Valerie Oke, and John Woolford. Thank you for all of your insight and advice regarding my project. Thanks to Jeff Brodsky and his lab for allowing me to borrow equipment and reagents, and for helpful advice about the ubiquitin related experiments. Thanks to Jeffrey Lawrence for all of your advice throughout graduate school. You're musical talents are incredible, I will miss listening to you and Heather perform. Thanks to Valerie for being a great teacher; I learned a lot while we taught that first Genetics class. Thank you for providing many delicious baked goods throughout the years. Thanks to John for all of your help in troubleshooting the TAP purifications, and for your advice and support during my postdoc search.

I also need to thank many people who offered much appreciated advice, reagents and techniques that greatly advanced our progress with Rkr1. Thanks to Joe Martens for all of your advice at lab meetings, and especially for your help with the real time PCR. Thanks to Richard Gardner for many emails with support and advice regarding the ubiquitin ligase experiments and

helping us to identify substrates for Rkr1. Thanks to Greg Prelich for advice and for entertaining discussions at Yeast Meetings. Thanks to Fred Winston for sharing unpublished results, for supplying us with reagents and strains. Thank you for all of your support and encouragment with my postdoc search. I really appreciate all of your help!

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friendship and for positively representing the prokaryotic world even in the face of adversity. Thanks to Kathryn, Marcie and Alycia for all of the memories of the trips to Fort Lauderdale and Las Vegas—alligator farms, monkey squirrels, death-trap roller coaster rides, crazy circus shows and all. Thanks to all of you for your friendship through the years. I will miss you all so much, and I promise to visit as often as I can.

I also need to thank many other grad students in our department for helping me through the last 6 years. Thanks to all of the members of our class for your camaraderie and support, especially through our first year and through our comprehensive exams. I especially need to thank some of you for so much more. Thanks to Lisa Sproul, for being a great friend that I've known since the 6th grade—can you believe we've been in school this long? Sproulio, you never cease to amaze me with your poise through any situation, and your never-ending supply of enthusiasm about life and science. Here's to the "mountain girls"! I want to thank her fiancé, Jason Hoverman, for being a great friend as well. Thanks to Heather Hendrickson for your friendship, for sharing your musical talents, and especially for the discussions over the last few months in deciding what we want to do with our lives. Thanks to Laura Marinelli for your friendship and for always helping me to remember that "it's going to be ok". Go Penguins! Thanks to Steve Hancock for your friendship, your passion for ultimate frisbee, Pittsburgh sports and The Clarks. Even though I lost the bet, thanks for not being scared to wear a rockin' mullet to a Pirates' game! Thanks to Team Dalzell for throwing many memorable parties, including the infamous "JELLO" parties. Thanks to all of you for attending MANY Clarks' shows with me through the years. I will miss you all and I promise to keep in touch.

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that I would have missed many important deadlines without your help. Thanks to Natalie Marinzel for providing an endless supply of candy and for the entertaining discussions about Penn State. Thanks to Pat Dean for helping me with last minute or lost orders, and for all of your advice on life outside of the CLC complex.

I need to thank so many more people for making my time in graduate school so memorable. Thanks to Lori Bibb, Julia Van Kessel, Matt Bochman, and Justin Pruneski for your friendship and for so many memories, especially "fourties at four" and our New York City trip.

Most importantly, I want to thank my family for their constant support. I need to thank my parents, Clyde and Cynthia Braun. I also need to thank the rest of my family: my sister Staci and her husband Rich, my brother Joe, his wife Gretchen and their daughter Lyla, and my sister Stevie. Thank you for fostering my interest in science and for supporting and encouraging my decision to build a career in science. Thank you for asking questions about work and for distracting me when I needed it. Thank you to Mike Quigney for being the best friend I've ever had. Thank you for your support and advice about life and science. Thank you for challenging me to be a better person. I love you all!

1.0 INTRODUCTION

1.1 TRANSCRIPTION OCCURS IN THE CONTEXT OF NUCLEOSOMES

A regulated pattern of DNA/histone interactions (chromatin) condenses the DNA and helps to regulate the binding of non-histone proteins to the DNA. The access of these proteins, including DNA replication and repair factors, recombination and transcription factors, needs to be highly regulated to ensure the integrity of the genome. The state of the chromatin template can prove to be an obstacle to transcription, and many factors associate with RNA polymerase II and assist it in navigating through the nucleosomal template during transcription. This section will describe the nucleosome structure and post-translational modifications of histone proteins.

Table 1. RNA Polymerase II transcription and chromatin factors.

Factor	Characteristics
Asf1	Nucleosome assembly and disassembly factor; Overexpression causes derepression of silenced loci
Bre1	RING finger protein; recruits Rad6 to chromatin to catalyze the ubiquitylation of histone H2B at lysine 123; Ubiquitin protein ligase (E3)
Bur1/Bur2	Cyclin dependent kinase/cyclin pair; phosphorylates the CTD of RNA Pol II *in vitro*; Bur2 is required to recruit the Paf1 complex to chromatin
CAF1 complex (Cac1, Cac2 and Msi1)	Chromatin assembly complex; Incorporates newly synthesized histones into chromatin
Ccr-Not4 complex	Regulates transcription initiation, elongation and mRNA degradation; E3 activity in Not4 subunit
Chd1	Chromatin remodeling activity; Physically interacts with the Rtf1 subunit of the Paf1 complex; Physically associated with actively transcribed genes
COMPASS complex	Histone H3 K4 methyltransferase complex; Set1 is the catalytic subunit
CPF	Cleavage and polyadenylation factor; Involved in RNA Pol II transcription termination
CTD	C-terminal domain of the largest subunit of RNA Pol II; Subject to phosphorylation pattern changes during transcription
Ctk1	Phosphorylates the CTD of RNA Pol II at serine 2 at the 3' ends of genes
Dot1	Methyltransferase that targets histone H3 at lysine 79
DSIF complex	Mammalian transcription elongation complex that is homologous to yeast Spt4/Spt5 proteins
Esa1	Catalytic subunit of the NuA4 histone acetyltransferase complex; Acetylates several lysine residues of histone H4
FACT complex (Spt16, Pob3, Nhp6)	Transcription elongation complex; Important for maintaining proper chromatin structure during elongation
Fcp1	Phosphatase that targets the CTD of RNA Pol II

Gal4	Transcriptional activator of the *GAL* genes
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HHF1* and *HHF2	Genes that encode histone H4 proteins
HHT1* and *HHT2	Genes that encode histone H3 proteins
Hir proteins (Hir1, Hir2, Hir3, Hir4)	Family of proteins that are important for proper expression of the histone genes and proper incorporation of histones into chromatin
HTA1* and *HTA2	Genes that encode histone H2A proteins
HTB1* and *HTB2	Genes that encode histone H2B proteins
Htz1	Histone H2A variant in yeast; Homologous to mammalian variant H2A.Z
Kin28	Subunit of TFIIH; Phosphorylates the CTD of RNA Pol II at serine 5 during the transition from initiation to elongation
Lge1	RING domain protein; Associates with Bre1 to catalyze the ubiquitylation of histone H2B at lysine 123
Mediator complex	Transcriptional coactivator complex that interacts with both transcription activators and RNA Pol II to promote transcription
Nhp6	Subunit of FACT transcription elongation complex
NuA4	Histone acetyltransferase complex; Acetylates histones H2A and H4 at several lysine residues
Paf1 complex (Paf1, Ctr9, Rtf1, Cdc73, Leo1)	Transcription elongation complex; Subunits are required for post-translational histone modifications
Pob3	Subunit of FACT transcription elongation complex
P-TEFb	Mammalian CTD kinase; phosphorylates serine 2
Rad6	Ubiquitin-conjugating enzyme (E2); Ubiquitylates many proteins in yeast, including histone H2B at lysine 123
Rap1	DNA binding protein; Important for establishing telomeric silencing
Rpd3-Sin3	Histone deacetylase complex that regulates transcription and silencing; Recruited to actively transcribed genes by histone H3 lysine 36 methylation
RSC	Chromatin remodeling complex

SAGA	Transcription coactivator complex; Catalytic subunits Gcn5 and Ubp8 acetylate and deubiquitylate histones, respectively
Set1	Methyltransferase that targets histone H3 at lysine 4
Set2	Methyltransferase that targets histone H3 at lysine 36
Sir proteins (Sir1, Sir2, Sir3, Sir4)	Family of proteins that establish and maintain heterochromatin-like transcriptionally silenced regions of the yeast genome; Sir2 is a histone deacetylase
Spt10	Required for histone gene expression; Forms a heterodimer with Spt21; Contains a putative acetyltransferase domain
Spt21	Required for histone gene expression; Forms a heterodimer with Spt10
Spt16	Subunit of FACT; Required for proper chromatin structure during transcription elongation
Spt4/Spt5	Transcription elongation factors that are required for proper chromatin structure during elongation; Required for Paf1 complex association with actively transcribed genes
Spt6	Transcription elongation factor that is required for proper chromatin structure during elongation
Ssu72	CTD phosphatase in vitro; Targets serine 5 phosphorylation
SWI/SNF complex	ATP-dependent chromatin remodeling complex; Swi2/Snf2 is the catalytic ATPase subunit
SWR1	ATP-dependent chromatin remodeling complex; Exchanges Htz1 for H2A at euchromatic boundaries
TFIIA (2 subunits)	Transcription initiation factor; Coactivator
TFIIB (3 subunits)	Transcription initiation factor; Stabilizes TBP-TFIIA interaction
TFIID (TBP and TAFs; 15 subunits)	Transcription initiation factor; Binds to the TATA box within a promoter
TFIIE (2 subunits)	Transcription initiation factor; Stimulates TFIIH kinase activity
TFIIF (3 subunits)	Transcription initiation factor; Stimulates elongation

TFIIH (11 subunits)	Transcription initiation factor; Kin28 subunit phosphorylates the CTD of RNA Pol II
TFIIS	Transcription elongation factor; Stimulates RNA Pol II ribonuclease activity
Ume6	Transcriptional repressor; Recruits factors to create a chromatin state that is repressive to transcription

1.1.1 Nucleosome structure is dynamic and regulated at many levels

Amazingly, a eukaryotic cell facilitates the incorporation of two meters of DNA in a small nuclear space, without tangling or physically damaging the DNA. Cells condense DNA by wrapping it around histone proteins to form nucleosomes, which are further compacted in higher order structures to allow all of the DNA to fit into the nucleus in an ordered fashion. A nucleosome contains an octamer of histone proteins surrounded by 147 bp of DNA (WHITE *et al.* 2001). Most often, a histone octamer consists of two molecules each of histones H2A, H2B, H3 and H4 (WHITE *et al.* 2001). Histone H1 acts as a linker histone that helps to further condense chromatin by contacting DNA in between nucleosomes (WHITE *et al.* 2001). The histone octamer is thought to be formed in a stepwise fashion where a tetramer of H3/H4 molecules are incorporated, and then two separate H2A/H2B dimers are added to complete the nucleosome assembly process (reviewed in POLO and ALMOUZNI 2006).

The octamer is dynamic, and the histone proteins are incorporated into and dissociated from nucleosomes with the help of many factors, including chromatin remodeling factors (discussed in section 1.1.4) and histone chaperone proteins like Asf1 (TYLER *et al.* 1999), the histone regulatory (Hir) proteins (SHARP *et al.* 2001), and the heterotrimeric chromatin assembly factor-1 (CAF1) complex (SMITH and STILLMAN 1989). The histone chaperones are important for promoting proper nucleosomal interactions and preventing DNA and proteins from interacting with the histones prior to their incorporation into nucleosomes (reviewed in PARK and LUGER 2006). For example, Asf1 binds to H3/H4 dimers at the carboxy terminus of histone H3 to prevent histone H3 dimerization prior to nucleosome formation (POLO and ALMOUZNI 2006). These factors act to regulate histone deposition during DNA replication, but more recent data

suggest that they also act during DNA repair (EMILI *et al.* 2001) and transcription (JAMAI *et al.* 2007) to return the chromatin template to its original structure after these processes have been performed.

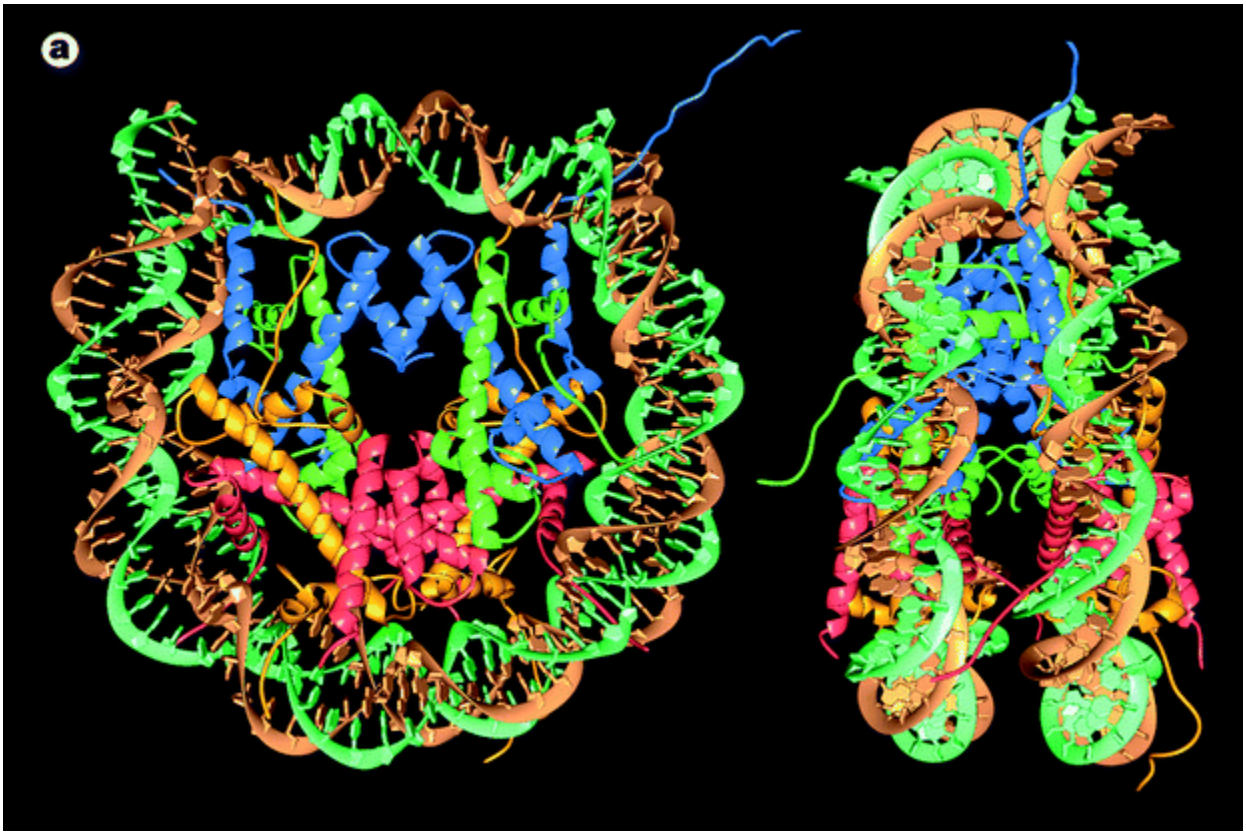
Expression of the histone genes in humans and yeast is regulated by similar mechanisms, although the proteins that regulate this process are not conserved. Human histones are encoded by many genes, including several variants of the four core histones that are dispersed throughout the genome (reviewed in GOVIN *et al.* 2005). Many of the histone variants are expressed in specific tissues, like H3t, which is thought to be expressed only in the testes (GOVIN *et al.* 2005). Yeast histones are encoded by two loci each, H2A (*HTA1* and *HTA2*), H2B (*HTB1* and *HTB2*), H3 (*HHT1* and *HHT2*) and H4 (*HHF1* and *HHF2*). These loci are found in divergently transcribed pairs, H2A and H2B are paired (*HTA1* and *HTB1*), as well as H3 and H4 (*HHT1* and *HHF1*). Histone gene expression peaks during S-phase. Spt10 and Spt21 are important for proper expression of a subset of the histone genes in yeast (DOLLARD *et al.* 1994; ERIKSSON *et al.* 2005; HESS *et al.* 2004; XU *et al.* 2005), and bind to specific DNA sequences within the promoters to promote transcription activation (ERIKSSON *et al.* 2005).

In all eukaryotes, the histone proteins fold in a distinct manner to create a globular central region that forms the core of the nucleosome, with the amino- and carboxy-terminal tails of the histones protruding from the core to surround the DNA (Figure 1). The core domain is structured into a three alpha helix domain called a "histone fold" (LUGER *et al.* 1997). The amino-terminal tails are unstructured and highly post-translationally modified, and more recently several modifications have been identified within the globular region of the histones (reviewed in SHILATIFARD 2006). Proteins involved in transcription, recombination, DNA repair and replication can gain access to the DNA via histone modification and chromatin remodeling

strategies. These processes will be described in more detail in the next few sections.

Figure 1. The structure of the nucleosome.

The left side of the figure illustratesthe nucleosomal structure. 146 basepairs of DNA is shown wrapped around an octamer of histones containing two molecules each of of histones H2A (yellow), H2B (red), H3 (blue) and H4 (green). The right side of the figure illustrates the nucleosomal structure that has been turned 90 degrees to the left. From Luger, K. *et al.* (1997) *Nature*. **389**:251-260.

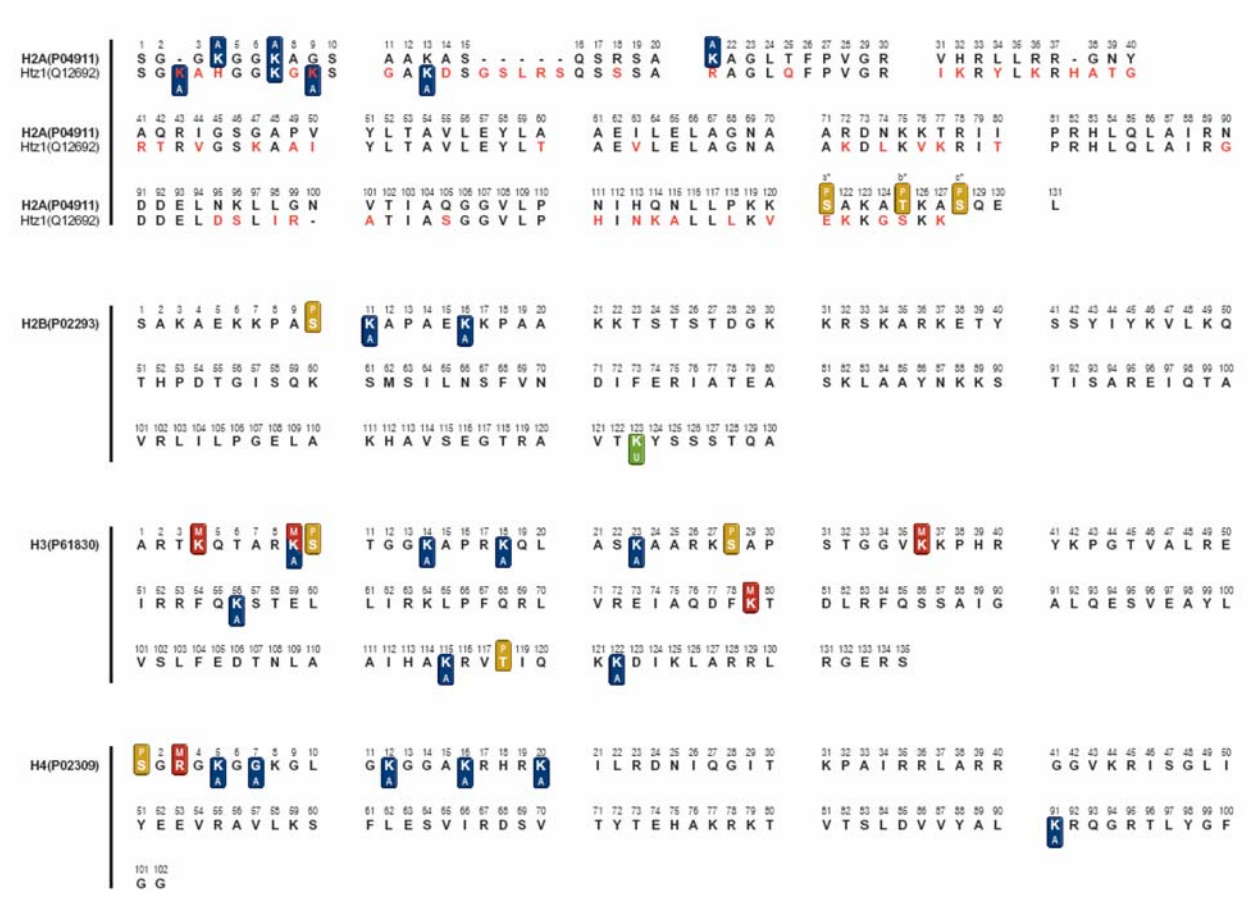

a

1.1.2 The histones are post-translationally modified

Each of the histones is subject to a variety of post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation (reviewed in PETERSON and LANIEL 2004) (Figure 2). All of these modifications are reversible, adding another level of regulation to chromatin function. Histone modifications can affect chromatin structure and function in a variety of ways (reviewed in LUGER 2006). Specifically, histone modifications can affect the recruitment of proteins that bind to specific histone modifications. Bromodomain containing proteins bind to acetylated histones (DHALLUIN *et al.* 1999; ZENG and ZHOU 2002). Analysis of eukaryotic proteins that contain chromodomains, WD40 domains, PhD domains and Tudor domains show that these proteins recognize specific methylated residues within the histones (HUYEN *et al.* 2004; KIM *et al.* 2006; LACHNER *et al.* 2001; WYSOCKA *et al.* 2005). Histone modifications can also alter histone-DNA interactions and histone-histone interactions to affect the localized chromatin structure (reviewed in LUGER 2006). The arrangements of particular sets of histone modifications is thought to form a readable "histone code", which acts to regulate chromatin structure at a given area in the genome (STRAHL and ALLIS 2000). In general, acetylation and methylation of histones is associated with active transcription, and hypo-methylated and hypo-acetylated histones are found in repressed and silenced genomic regions. However, histone methylation can have both positive and negative effects on transcription. In the next few sections, I will describe chromatin modifications that are associated with active transcription and how these modifications can be reversed.

Figure 2. The histones are post-translationally modified.

The primary amino acid sequences of the four core histones and Htz1 in *S. cerevisiae* are shown. The sites and types of post-translational modifications are shown. Blue marks represent sites that are acetylated, yellow marks represent phosphorylation sites, green marks represent ubiquitylation sites, and red marks represent methylation sites. From Abcam's website: (http://www.abcam.com/assets/pdf/chromatin/histone_modification_map_yeast.pdf).


H2A(P04911)
Htz1(Q12692)
H2B(P02293)
H3(P61830)
H4(P02309)

1.1.2.1 Histone acetylation

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate histone acetylation patterns. In general, HATs can catalyze acetylation of many lysine residues within the histones. Gcn5, the catalytic component of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex, is recruited to promoters by transcriptional activators (BHAUMIK *et al.* 2004; LEROY *et al.* 2006; UTLEY *et al.* 1998) and acetylates histones in this region to promote transcription (IMOBERDORF *et al.* 2006). In yeast, Gcn5 acetylates several lysines residues of different histones, including lysines 9, 14, 18, 23 and 27 of histone H3 and lysines 11 and 16 of histone H2B (reviewed in MILLAR and GRUNSTEIN 2006). Esa1 is the catalytic subunit of the NuA4 complex (ALLARD *et al.* 1999; SMITH *et al.* 1998). Esa1 acetylates histone H2A at lysine 7, and lysines 5, 8, 12, and 16 of histone H4, and lysine 14 of histone variant Htz1 (reviewed in MILLAR and GRUNSTEIN 2006). These modifications are also associated with actively transcribed genes (POKHOLOK *et al.* 2005). Rpd3 is one of the major HDACs in yeast, and acts to reverse the activities of Gcn5 and Esa1 (except histone H4 K16 acetylation) (reviewed in MILLAR and GRUNSTEIN 2006). Not surprisingly, Rpd3 activity is associated with transcriptional repression both in transcription initiation and transcription elongation. During elongation, Rpd3 (as part of the Rpd3S complex) is recruited to actively transcribing genes by histone H3 K36 tri-methylation, where it deacetylates histones to restore a repressed chromatin structure in the wake of RNA Pol II passage (CARROZZA *et al.* 2005; KEOGH *et al.* 2005).

1.1.2.2 Histone methylation

Lysine residues within the histones are also subject to methylation. Unlike HATs, histone methyltransferases (HMTs) in yeast only regulate the methylation of single residues

within the histones. Some methylation events cannot occur without prior addition of ubiquitin (a 76 amino acid moiety added as a post-translational modification) to histone H2B at lysine 123 (K123). Rad6, the ubiquitin-conjugating enzyme, is recruited to histone H2B at activated promoters by the ubiquitin-protein ligases Bre1 and Lge1 (HWANG *et al.* 2003; WOOD *et al.* 2003a). These proteins act together to catalyze the transfer of ubiquitin from Rad6 to histone H2B at lysine 123 (WOOD *et al.* 2003a). Histone H2B ubiquitylation at active genes is transient, and the ubiquitin protease Ubp8 (a component of SAGA) cleaves the ubiquitin moiety from K123 almost as quickly as the mark is added (DANIEL *et al.* 2004; HENRY *et al.* 2003). Ubiquitylation of histone H2B at lysine 123 is required for subsequent methylation of histone H3 at lysines 4 (K4) and 79 (K79) (SUN and ALLIS 2002). Although the functional purpose of this relationship is not well understood, the proteasome appears to be important for connecting these modifications. The proteasomal ATPases Rpt4 and Rpt6 are recruited to chromatin by histone H2B K123 ubiquitylation (EZHKOVA and TANSEY 2004). In the absence of Rpt4 and Rpt6, histone H3 methylation at K4 and K79 is lost, but histone H2B ubiquitylation remains intact (EZHKOVA and TANSEY 2004). Very recently, Tanny and colleagues showed that histone H2B ubiquitylation affects transcription and chromatin structure independently of its role in promoting histone H3 methylation in *S. pombe* (TANNY *et al.* 2007). H2B monoubiquitylation is required for proper transcription throughout the genome, and strains lacking H2B ubiquitylation have defects in cell growth and septation, while strains lacking histone H3 methylation did not exhibit these phenotypes (TANNY *et al.* 2007). This suggests that H2B ubiquitylation is not simply a mark that promotes H3 methylation, but instead is important for promoting transcription independent of its role in H3 methylation. Genome-wide studies have recently shown that histone H2B ubiquitylation is important for the proper expression of approximately 75 genes in

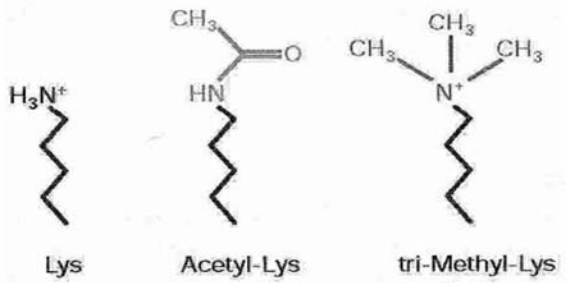
yeast (MUTIU *et al.* 2007). Many of these changes occur at genes within ten kilobases of a telomere and are thought to be the result of indirect effects on telomeric silencing (MUTIU *et al.* 2007). Interestingly, histone H2B ubiquitylation is important for the repression and activation of genes in yeast, and this modification has effects on the expression of a subset of genes independent of its role in promoting downstream methylation events on histone H3 (MUTIU *et al.* 2007).

Set1 (COMPASS subunit) and Dot1 catalyze the methylation of histone H3 at lysine 4 (K4) and 79 (K79), respectively (FENG *et al.* 2002; SANTOS-ROSA *et al.* 2002). Both of these modifications require the mono-ubiquitylation of histone H2B at K123 by Rad6, Bre1 and Lge1 (SUN and ALLIS 2002). Lysines can be mono-, di- or tri-methylated, with each of these modifications occurring in unique patterns within an actively transcribing gene (POKHOLOK *et al.* 2005) (Figure 3). Interestingly, K4 mono- and di-methylation are found throughout the genome (POKHOLOK *et al.* 2005). However, Set1 and K4 tri-methylation peaks at the 5' end of actively transcribed genes (BERNSTEIN *et al.* 2002; BRIGGS *et al.* 2001; KROGAN *et al.* 2003b; NG *et al.* 2003b; POKHOLOK *et al.* 2005; SANTOS-ROSA *et al.* 2002) (Figure 3). Dot1 and histone H3 K79 tri-methylation are also localized at actively transcribed genes (POKHOLOK *et al.* 2005). Recently, histone demethylases have been identified, consisting of a conserved family of proteins that contain JmjC domains. The yeast protein Jhd2 demethylates histone H3 that is tri-methylated at K4 (LIANG *et al.* 2007a; LIANG *et al.* 2007b). The demethylation process appears to be a slow one, as methylated histones have a half life of several hours, and are thought to provide a record of recent transcription (NG *et al.* 2003b).

Figure 3. Histone acetylation and methylation are most often marks of active transcription.

A) Covalent attachment of acetyl or methyl groups to lysine residues within histone proteins. From www.benbest.com/health/cancer.html. B) Lysines can be mono-, di-, or trimethylated. From Shilatifard A. (2006) *Annu. Rev. Biochem*. **75**:243-69. C) Chromatin immunoprecipitation experiments show that the pattern of chromatin modifications changes over an open reading frame. From Li, B. et al. (2007). *Cell*. **128**:707-719.

A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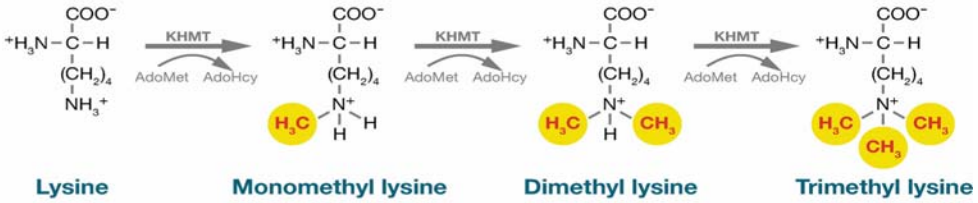
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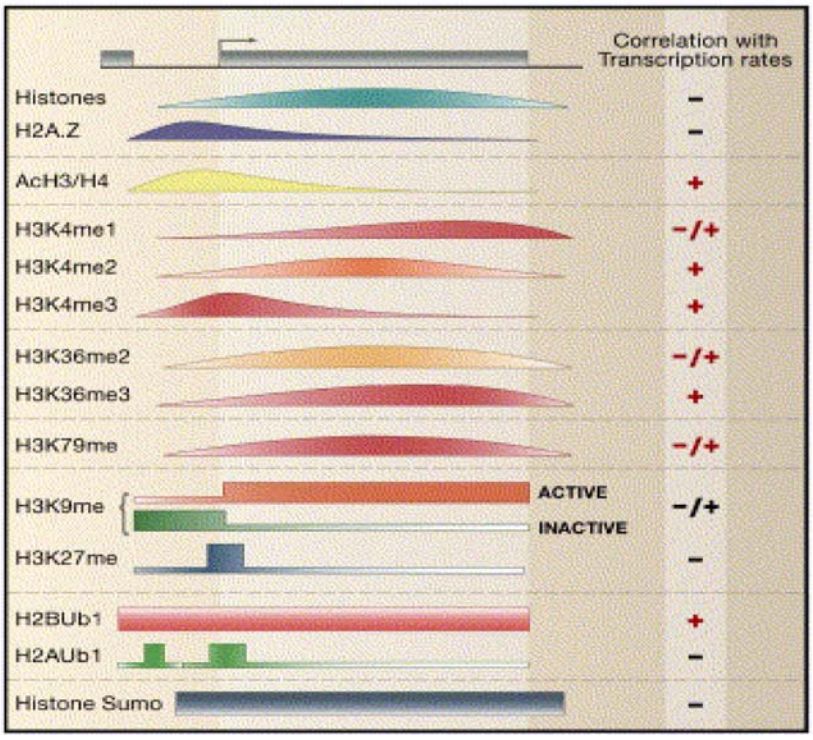
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H3K9me
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H2AUb1
Histone Sumo

Another HMT, Set2, is required for histone H3 lysine 36 (K36) methylation events (STRAHL *et al.* 2002). Similar to K4 and K79 methylation, histone H3 K36 mono- and di-methylation is found throughout the genome (POKHOLOK *et al.* 2005). K36 tri-methylation is associated with actively transcribed genes and is skewed towards the 3' end of genes (POKHOLOK *et al.* 2005; STRAHL *et al.* 2002). Very recently, evidence of K36 demethylating enzymes in yeast has been reported. Jhd1, a JmjC domain containing protein, has been shown to demethylate K36 mono- and di-methylation (TU *et al.* 2007). Rph1 (also a JmjC domain containing protein) demethylates K36 that is di- and tri-methylated (KLOSE *et al.* 2007).

1.1.3 Histone variants lead to changes in chromatin structure and function

In addition to post-translational modification of histones, variants of the core histones can be incorporated into nucleosomes to impact chromatin structure and function. Eukaryotic organisms encode non-allelic histone proteins that are similar in sequence to the core histones. Humans encode variants of histones H3 and H2A, including H3.3 and H2A.X (reviewed in LUGER 2003). Histone H3.3 is incorporated into nucleosomes in a replication-independent manner (AHMAD and HENIKOFF 2002). H2A.X is enriched in germline cells, and found in somatic cells of the thymus and spleen (MEISTRICH *et al.* 1985; NAGATA *et al.* 1991). H2A.X phosphorylation is associated with double-stranded break formation in mammalian cells (ROGAKOU *et al.* 1998). Interestingly, the yeast versions of the core histones H3 and H2A are more similar to the human histones H3.3 and H2A.X than the human core histone proteins (reviewed in LUGER 2003). This may be due to the fact that the majority of the yeast genome is transcriptionally active in rich medium, as opposed to the human genome which is highly repressed.

Eukaryotes also encode for a histone variant of H2A termed H2A.Z in mice and humans and Htz1 in yeast. Approximately 4% of all nucleosomes in mouse cells contain H2A.Z (WEST and BONNER 1980). Incorporation of this histone has been shown to alter the nucleosomal structure, and may have larger effects on intra- and inter-nucleosomal contacts (LUGER 2003). Specifically, nucleosomes containing H2A.Z have a more compact structure than nucleosomes containing H2A, however, these nucleosomes have decreased inter-nucleosomal interactions (LUGER 2003). Htz1 is exchanged with core H2A by the ATP-dependent activity of the SWR1 complex (MIZUGUCHI *et al.* 2004). Many studies in yeast have uncovered multiple functions for Htz1. Htz1 incorporation is enriched in euchromatin and has been shown to enhance the activity of boundary elements to prevent the spreading of silencing factors into euchromatic regions of the genome (MENEGHINI *et al.* 2003). Genome-wide experiments that combine chromatin immunoprecipitation and microarray techniques (ChIP-chip) have shown that two Htz1 containing nucleosomes are preferentially found flanking a nucleosome-free region within the promoters of transcriptionally silent genes (GUILLEMETTE *et al.* 2005; LI *et al.* 2005; RAISNER *et al.* 2005; ZHANG *et al.* 2005a). These promoters exhibit reduced levels of Htz1-containing nucleosomes upon transcription activation (GUILLEMETTE *et al.* 2005; LI *et al.* 2005; RAISNER *et al.* 2005; ZHANG *et al.* 2005a). Interestingly, Htz1 is acetylated at lysine 14 in the remaining nucleosomes within the promoters of active genes (MILLAR *et al.* 2006). The purpose of this acetylation event is not well understood, but may lead to removal of Htz1-containing nucleosomes, or act to promote transcription activation in another manner (BABIARZ *et al.* 2006; KEOGH *et al.* 2006; MILLAR *et al.* 2006).

1.1.4 Chromatin remodelers restructure and reposition nucleosomes

Many chromatin associated factors, including transcription, DNA replication and repair, and recombination factors require access to DNA sequences to accomplish their mission. Chromatin remodeling factors rearrange the nucleosome structure to allow or restrict access to the DNA. There are several classes of chromatin remodelers, and these complexes are recruited to chromatin through interactions with transcriptional activator proteins and specific post-translational modifications on the histones. Both chromatin remodeler and modifier complexes interact with RNA and DNA polymerases, defining chromatin structure as a critical component for proper modulation of many essential nuclear processes (reviewed in VAN VUGT *et al.* 2007).

Chromatin remodelers can restructure or reposition chromatin using ATPase subunits to insert histone variants into nucleosomes or to slide or eject nucleosomes (reviewed in VAN VUGT *et al.* 2007). The ATP-dependent chromatin remodeling complexes in *S. cerevisiae*, including SWI/SNF, RSC, ISW1, and INO80 complexes and Chd1, contain motifs that facilitate interactions with specific chromatin modifications. SWI/SNF and RSC complex subunits contain bromodomains, which bind to acetylated lysines (reviewed in HASSAN *et al.* 2002; WINSTON and ALLIS 1999). The ISW1 complex contains SANT and SLIDE domains that bind histone tails and linker DNA, respectively (reviewed in GRUNE *et al.* 2003). Chd1 contains a chromodomain, which recognizes methylated lysine residues (BANNISTER *et al.* 2001), and INO80 complex subunits contain DBINO domains that are predicted to interact with DNA (BAKSHI *et al.* 2004).

In yeast, DNA sequences position approximately 50% of the nucleosomes throughout the genome (SEGAL *et al.* 2006). Chromatin remodelers are used to reposition nucleosomes to allow or prevent access to the DNA (reviewed in VAN VUGT *et al.* 2007). Specifically, SWI/SNF

moves nucleosomes to allow the transcription machinery to access binding sites in the DNA (SCHNITZLER *et al.* 2001). Conversely, ISW1 spaces the nucleosomes relative to each other to promote a tightly packed chromatin structure that prevents non-histone protein-DNA interactions (BLANK and BECKER 1996; WHITEHOUSE and TSUKIYAMA 2006).

In the next two sections I will discuss two chromatin remodelers, the RSC complex and Chd1. These represent the only essential chromatin remodeling complex and the only single subunit chromatin remodeler, respectively, in yeast.

1.1.4.1 The RSC complex

SWI/SNF was the first ATP-dependent chromatin remodeling complex to be identified in yeast (PETERSON *et al.* 1994; PETERSON and HERSKOWITZ 1992). This 12 subunit complex is found at approximately 100-200 copies per cell, and is not essential for viability (CAIRNS *et al.* 1996; GHAEMMAGHAMI *et al.* 2003). Microarray analysis of strains grown in rich medium suggests that SWI/SNF is responsible for the proper expression of approximately 5% of the yeast genome (HOLSTEGE *et al.* 1998; SUDARSANAM *et al.* 2000).

The RSC (remodels the structure of chromatin) complex was identified based on sequence similarity to the SWI/SNF complex (CAIRNS *et al.* 1996). RSC is 10 times more abundant than SWI/SNF (CAIRNS *et al.* 1996), and contains 17 subunits, ten of which are essential for viability. Five subunits are paralogous with SWI/SNF subunits and three subunits are shared with SWI/SNF (CAIRNS *et al.* 1996; and reviewed in VAN VUGT *et al.* 2007). The catalytic subunit of RSC, Sth1, is similar in sequence to Snf2 of SWI/SNF, however Sth1 is essential (DU *et al.* 1998). *In vitro* experiments show that purified RSC complex exhibits DNA-dependent ATPase activity, as well as the ability to transfer histones onto a naked DNA template and remodel nucleosomes (KORNBERG and LORCH 1999; SAHA *et al.* 2002). Swi2 and Sth1 have

been shown to disrupt histone-DNA contacts within nucleosomal arrays, seen as altered sensitivity to DNase I (CAIRNS *et al.* 1996). Rsc1, Rsc2, Rsc4, and Sth1 contain bromodomains (Sth1 is the only of these proteins that contains a single bromodomain), and these proteins contain seven of the 15 bromodomains found in the yeast proteome (reviewed in VAN VUGT *et al.* 2007).

Two isoforms of RSC have been identified, one contains Rsc1 and the other contains Rsc2 (CAIRNS *et al.* 1999; NG *et al.* 2002b). Both Rsc1 and Rsc2 contain bromodomains, a bromo-associated homology domain and an AT hook domain (CAIRNS *et al.* 1999). A complete understanding of the functional significance of these two sub-complexes has yet to be elucidated. However, both *rsc1Δ* and *rsc2Δ* strains have reduced sporulation efficiency, but only *rsc1Δ* strains have defects in tetrad formation during sporulation (YUKAWA *et al.* 2002). Furthermore, overexpression of *RSC1* in a *rsc2Δ/rsc2Δ* mutant strain does not suppress the sporulation defect, and vice versa (BUNGARD *et al.* 2004; YUKAWA *et al.* 2002), further suggesting that these proteins differentially regulate the function of their respective RSC complexes.

Identification of human homologs of the SWI/SNF and RSC complexes has been complicated by the high levels of similarity shared by the subunits of these complexes (MUCHARDT and YANIV 2001). The mammalian homolog of Snf2 is Brg (MOHRMANN *et al.* 2004; WANG *et al.* 1996), and purification of Brg shows that it is found in two complexes, SWI/SNF-A (also known as BAF) and SWI/SNF-B (also known as PBAF) (MOHRMANN *et al.* 2004). PBAF contains Polybromo, a protein that contains six bromodomains and is similar in sequence with yeast Rsc1, Rsc2 and Rsc4 (MOHRMANN *et al.* 2004). Further work is needed to identify all components of the human RSC complex and characterize its functional role in chromatin structure.

1.1.4.2 Chd1

Chd1 (Chromodomain-ATPase/helicase-DNA binding protein 1) is a conserved ATP-dependent chromatin remodeling enzyme (FLAUS *et al.* 2006) that acts alone in *Drosophila* and yeast to remodel chromatin (LUSSER *et al.* 2005; TRAN *et al.* 2000). In *S. cerevisiae*, Chd1 is found at approximately 1600 proteins per cell (GHAEMMAGHAMI *et al.* 2003), and localizes to chromatin throughout the genome (TRAN *et al.* 2000). Chd1 is thought to interact with DNA in linker regions between nucleosomes (STOCKDALE *et al.* 2006) and reposition nucleosomes in an ATP dependent manner (TRAN *et al.* 2000). Strains lacking *CHD1* and genes encoding subunits of SWI/SNF are inviable (TRAN *et al.* 2000), suggesting that these two complexes functionally overlap *in vivo*. Like the SWI/SNF and RSC complexes, Chd1 can alter histone-DNA contacts, seen as altered DNase I sensitive sites (TRAN *et al.* 2000). However, the DNase I patterns of nucleosome arrays treated with SWI/SNF and Chd1 differ, suggesting that these enzymes function independently to create distinct changes in nucleosome position *in vivo* (TRAN *et al.* 2000).

Human and *S. cerevisiae* Chd1 contain two tandem chromodomains that have been shown to interact with Paf1 complex-dependent histone H3 K4 trimethylation through these domains (OKUDA *et al.* 2007; PRAY-GRANT *et al.* 2005; SIMS *et al.* 2005). However, the existence of this interaction in yeast is a topic of debate (PRAY-GRANT *et al.* 2005; SIMS *et al.* 2005). NMR structural studies and sequence alignments suggest that yeast Chd1 lacks several key residues found in the human Chd1 that are important for recognizing trimethylated lysine residues (OKUDA *et al.* 2007). Importantly, yeast Chd1 interacts with several transcription elongation factors, including the Paf1 complex, FACT and Spt4/5 (see section 1.2.4 for more details) (SIMIC *et al.* 2003), which may mediate its association with actively transcribed regions.

1.2 TRANSCRIPTION BY RNA POLYMERASE II IS A HIGHLY REGULATED PROCESS

1.2.1 RNA Pol II is a conserved, multi-subunit enzyme complex

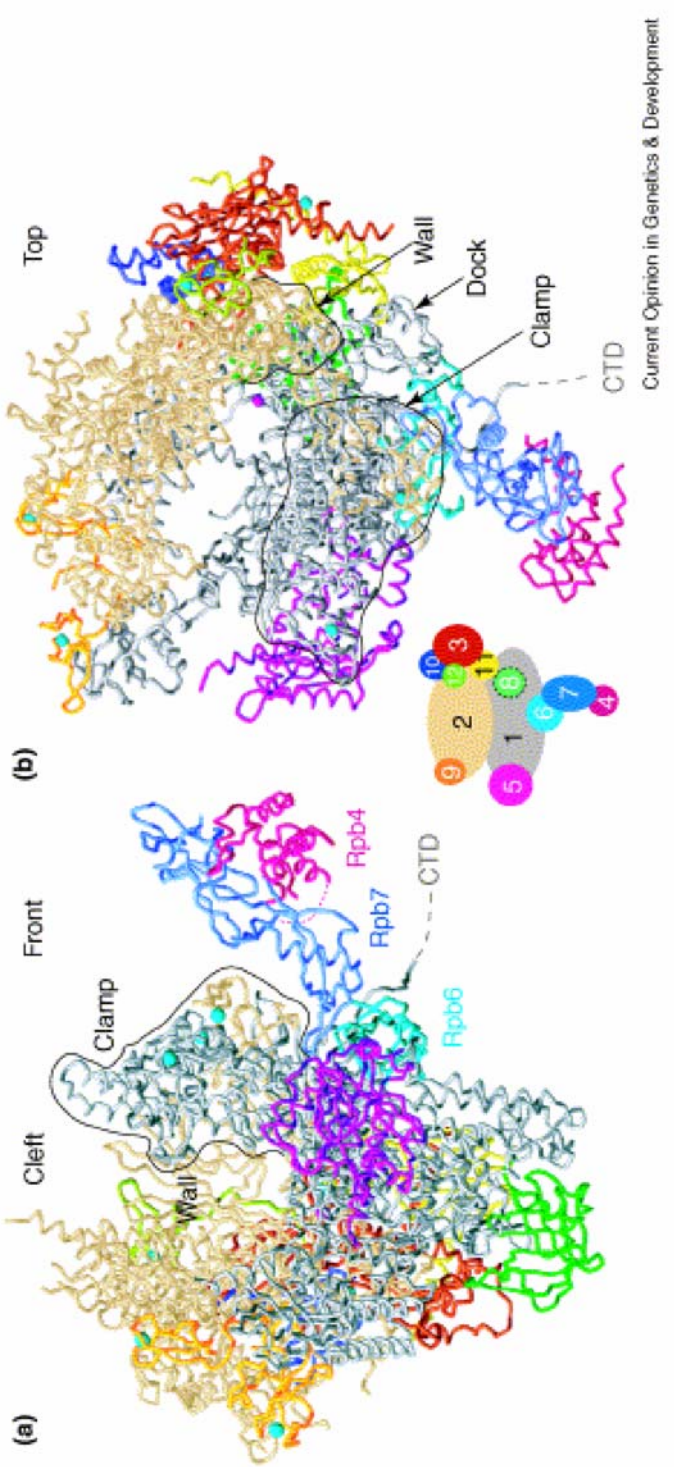
S. cerevisiae contains approximately 6000 protein coding genes (GOFFEAU *et al.* 1996), as well as genes that encode many untranslated RNAs. RNA polymerase II (Pol II) transcribes these protein coding genes (mRNAs), as well as small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). RNA Pol II in yeast is a twelve subunit complex (Rpb1-12) with a molecular mass of approximately 0.5 megadaltons (reviewed in CRAMER 2004). RNA Pol II subunits Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12 are shared among all three nuclear RNA polymerases (Pol I, II, and III) in eukaryotes (reviewed in MARTINEZ 2002). The subunits of RNA Pol II are highly conserved in eukaryotes, and studies of the RNA Pol II from *S. cerevisiae* have pioneered a structural understanding of the mechanism of transcription in eukaryotes (Figure 4). Rpb1 and Rpb2 are the largest subunits of Pol II, that together form the "clamp" and active site portions of the polymerase (CRAMER *et al.* 2000; CRAMER *et al.* 2001). The structure of RNA Pol II without Rpb4 and Rpb7 shows that the active site of the enzyme provides the foundation for a nine basepair DNA/RNA hybrid (GNATT *et al.* 2001). Rpb1 contains an unstructured C-terminal domain (CTD) that was not visible in the X-ray structures of RNA Pol II. I will discuss the CTD in more detail in section 1.2.2. Importantly, RNA Pol II has a multi-faceted surface that interacts with the DNA template and supplies a foundation for many interacting proteins that are important for regulating transcription (KETTENBERGER *et al.* 2004).

The subunits of eukaryotic RNA Pol II are somewhat conserved in archaea and prokaryotes (reviewed in GOEDE *et al.* 2006). However, archaeal RNA polymerase is more

similar to eukaryotic RNA Pol II than to the four subunit bacterial core RNA polymerase (GOEDE *et al.* 2006). Subcomplexes of the polymerases show similar interactions in archaeal and eukaryotic polymerases. Archaeal polymerase subunits E and F are homologous to eukaryotic Pol II subunits Rpb4 and Rpb7, which form a subcomplex that is not stably associated with the core polymerase (reviewed in GOEDE *et al.* 2006; TODONE *et al.* 2001). Interestingly, archaeal subunits E and F also form a complex, and archaeal F interacted with human Rpb7 to form a hybrid complex (WERNER *et al.* 2000). Archaeal D, N, L and P form a subcomplex as well, similar to RNA Pol II subcomplex made up of Rpb3, Rpb10, Rpb11, and Rpb12 (CRAMER *et al.* 2001; WERNER *et al.* 2000).

Figure 4. Structure of RNA Pol II.

RNA Polymerase II structure shown from the front (a) and top (b). Subunits are colored according to small scale in the middle of the figure. From Cramer, P. (2004) *Curr. Opin. Gen. Dev.* **14**:218-226.


(a)
Cleft
Wall
Clamp
Front
Rpb4
Rpb7
CTD
Rpb6
(b)
Top
Wall
Dock
Clamp
CTD
Current Opinion in Genetics & Development

Despite the structural complexity of RNA Pol II, this enzyme cannot function alone in the transcription process. RNA Pol II transcription has three main stages: 1) initiation, where the polymerase is recruited to an activated gene through physical interactions with many proteins, 2) elongation, where the nascent RNA chain grows as nucleotides are incorporated, and 3) termination, where the RNA is released, the polymerase dissociates from the DNA template, and the polymerase can be recycled at another promoter. Many factors, including promoter DNA sequences, activator and repressor proteins, the general transcription machinery, and association of accessory factors, are all important for regulating the individual steps of the transcription cycle. The next few sections will discuss what is currently known about how these factors coordinately regulate the transcription cycle.

1.2.2 The modification state of the CTD of RNA Pol II changes during the transcription cycle

Many proteins dynamically associate with RNA Pol II throughout the transcription cycle. It has become clear that many of these associations are regulated by the carboxy-terminal domain (CTD) of the largest subunit of RNA Pol II (Rpb1). The CTD is composed of a heptapeptide repeat (Tyr_1-Ser_2-Pro_3-Thr_4-Ser_5-Pro_6-Ser_7), and the number of repeats within the CTD increases with the complexity of the organism. The CTD of *S. cerevisiae* is made up of 26 or 27 repeats, *C. elegans* 34 repeats, *Drosophila* 42 repeats, and mice and humans 52 repeats (reviewed in HAMPSEY 1998). Only 8 repeats are required for viability in yeast (NONET *et al.* 1987; WEST and CORDEN 1995).

Many RNA processing factors bind to the CTD *in vitro*, and the CTD is required for efficient capping, splicing and polyadenylation *in vivo* (HIROSE and MANLEY 1998; HIROSE and

MANLEY 2000; MCCRACKEN *et al.* 1997; PROUDFOOT *et al.* 2002). These factors recognize specific phosphorylation patterns within the CTD (DAHMUS 1995; MANIATIS and REED 2002; PALANCADE and BENSAUDE 2003; reviewed in PROUDFOOT 2004; SIMS *et al.* 2004; ZORIO and BENTLEY 2004). RNA Pol II that contains a hypo-phosphorylated CTD is recruited to promoters through an interaction with the Mediator complex (described in more detail in section 1.2.3.3) (reviewed in BJORKLUND and GUSTAFSSON 2004; MALIK and ROEDER 2000; MYERS and KORNBERG 2000). The CTD is phosphorylated at position 5 in a TFIIH-dependent manner shortly after initiating transcription (KOMARNITSKY *et al.* 2000). As the polymerase moves along the open reading frame, serine 5 phosphorylation decreases and Ctk1-dependent serine 2 phosphorylation increases at the 3' end of the gene (KOMARNITSKY *et al.* 2000; MORRIS *et al.* 2005). These changes in the modification state of the polymerase are controlled by several kinases and phosphatases.

1.2.2.1 CTD kinases

CTD phosphorylation occurs through the action of cyclin dependent kinases (CDKs) (reviewed in MEINHART *et al.* 2005). CDK7/cyclin H (Kin28 is the CDK in yeast) is a component of TFIIH that phosphorylates serine 5 of the CTD during transcription initiation (COIN and EGLY 1998; HENGARTNER *et al.* 1998; KOMARNITSKY *et al.* 2000). This activity is enhanced by the Mediator complex during initiation and promotes transcription elongation (GUIDI *et al.* 2004). CDK8/cyclin C (Srb10/11 in yeast) are components of a subset of Mediator complexes that phosphorylate serine 5 of the CTD (BORGGREFE *et al.* 2002; BOUBE *et al.* 2002; HENGARTNER *et al.* 1998; LIU *et al.* 2001) in order to repress transcription by preventing initiation complex formation (HENGARTNER *et al.* 1998).

CDK9/cyclin T are the core components of the positive transcription elongation factor-b

(P-TEFb) (PRICE 2000). P-TEFb was originally isolated though its ability to facilitate transcription of stalled elongating Pol II complexes in a CTD dependent manner (MARSHALL *et al.* 1996; MARSHALL and PRICE 1995). P-TEFb phosphorylates serine 2 of the CTD to promote transcription elongation (PRICE 2000; YAMAGUCHI *et al.* 2002; YAMAGUCHI *et al.* 1999). P-TEFb also phosphorylates human Spt5 to counteract the negative elongation function of this factor (WADA *et al.* 1998; YAMAGUCHI *et al.* 1998). Significantly, P-TEFb and Spt5 are required for Tat-mediated stimulation of HIV transcription *in vitro* (IVANOV *et al.* 2000; PRICE 2000; WADA *et al.* 1998; WU-BAER *et al.* 1998). The proposed homologs of P-TEFb in *S. cerevisiae* are Ctk1 and Bur1 (GUO and STILLER 2004; MURRAY *et al.* 2001; PRELICH 2002; PRELICH and WINSTON 1993). Keogh and colleagues propose that these proteins act separately to facilitate P-TEFb-like activity, where Ctk1 is the primary kinase for the CTD, and Bur1 may be the kinase for Spt5 (KEOGH *et al.* 2003). Interestingly, Ctk1 appears to affect transcription by regulating histone H3 K4 and K36 methylation (WOOD *et al.* 2007; XIAO *et al.* 2007). Specifically, strains lacking *CTK1* have increased levels of H3 K4 trimethylation in chromatin, while histone H2B ubiquitylation levels are unchanged (WOOD *et al.* 2007; XIAO *et al.* 2007). Ctk1 appears to regulate chromatin structure during transcription elongation by promoting histone H3 K36 methylation and preventing histone H3 K4 trimethylation (XIAO *et al.* 2007).

1.2.2.2 CTD phosphatases

There are two known CTD phosphatases, Fcp1 and Ssu72 (reviewed in MEINHART *et al.* 2005). Fcp1 is a conserved, essential phosphatase that targets the CTD of both free and actively transcribing Pol II (ARCHAMBAULT *et al.* 1997; ARCHAMBAULT *et al.* 1998; CHAMBERS and DAHMUS 1994; CHAMBERS and KANE 1996; CHAMBERS *et al.* 1995; KONG *et al.* 2005; LEHMAN and DAHMUS 2000). Fcp1 physically interacts with RNA Pol II subunits Rpb4/7 (CHAMBERS *et*

al. 1995; KAMENSKI *et al.* 2004; KIMURA *et al.* 2002), the phosphorylated CTD (YU *et al.* 2003), and TFIIF, which stimulates Fcp1 activity (ARCHAMBAULT *et al.* 1998; CHAMBERS *et al.* 1995; KAMADA *et al.* 2003; NGUYEN *et al.* 2003). Ssu72 is also a conserved and essential phosphatase that is believed to target the CTD of RNA Pol II *in vivo* (KRISHNAMURTHY *et al.* 2004). *In vitro* experiments show that Ssu72 can dephosphorylate recombinant CTD that is phosphorylated at serine 5, and serine 5 phosphorylation levels are increased in strains that are depleted of Ssu72 (KRISHNAMURTHY *et al.* 2004). Ssu72 activity is dependent on Pta1, and both proteins are subunits of the RNA cleavage and polyadenylation factor (CPF) (KRISHNAMURTHY *et al.* 2004; STEINMETZ and BROW 2003). Mammalian Ssu72 has been shown to bind the Pta1 homolog and Pol II (ST-PIERRE *et al.* 2005).

1.2.3 Transcription initiation

Transcription initiation requires that the RNA Pol II general transcription factors correctly recognize DNA elements within the promoter of a gene, and the assembly of these general transcription factors with RNA Pol II into a preinitiation complex. Initiation needs to be highly regulated to ensure that the cell reacts to its environment appropriately and in a timely fashion. This section will provide an overview of what regulates initiation, including promoter DNA sequences, activator and repressor proteins, and chromatin structure.

1.2.3.1 Promoter elements regulate transcription initiation

The promoter region of a gene contains core DNA sequences which are recognized by the RNA Pol II general transcription factors, as well as sequences that are recognized by gene specific activators and repressors (reviewed in SMALE and KADONAGA 2003). The core

promoter region consists of approximately 35 basepairs of DNA upstream and downstream of the transcription initiation site. This region contains binding sites for the general transcription factors (GTFs) and RNA Pol II. The TATA box, named for the conserved sequence of TATAAA that is found approximately 25-30 basepairs upstream of the transcription start site, is found at many metazoan gene promoters (reviewed in BREATHNACH and CHAMBON 1981). The TATA box is found approximately 40-120 basepairs from the transcription start site in 20% of promoters in *S. cerevisiae* (BASEHOAR *et al.* 2004; STRUHL 1989). The other 80% of the genome contains TATA-less promoters (BASEHOAR *et al.* 2004). The TATA-containing genes are most commonly regulated by stress and the expression of these genes is tightly controlled (BASEHOAR *et al.* 2004). The conserved initiator (Inr) element found in eukaryotes contains the DNA sequence in which transcription initiates (CORDEN *et al.* 1980; JAVAHERY *et al.* 1994; SMALE and BALTIMORE 1989; SMALE *et al.* 1990). This sequence consists of an adenosine at the transcription start site, a cytosine at the -1 position, and a few pyrimidines surrounding these nucleotides (reviewed in SMALE and KADONAGA 2003). This sequence is less conserved in yeast, but mutations around the transcription start site have been shown to result in the use of alternate transcription initiation sites (CHEN and STRUHL 1985; HAHN *et al.* 1985; MCNEIL and SMITH 1985; NAGAWA and FINK 1985). When the TATA box is located within 25-30 basepairs of the Inr element, these sequences synergistically activate transcription, but act independently if they are located more than 20 basepairs apart (O'SHEA-GREENFIELD and SMALE 1992). The TATA box and Inr coordinately direct the polarity of transcription (EMAMI *et al.* 1997; SMALE *et al.* 1990).

Another promoter element, the downstream promoter element (DPE), is important for TFIID binding at TATA-less promoters (reviewed in SMALE and KADONAGA 2003). This

element is conserved in metazoans, the core sequence is found downstream relative to the Inr element at basepairs +28-32 (KUTACH and KADONAGA 2000). The DPE and Inr work together to recruit TFIID to TATA-less promoters (KUTACH and KADONAGA 2000). One element found in promoters is not recognized by TFIID, but instead interacts with TFIIB (reviewed in SMALE and KADONAGA 2003). The TFIIB recognition element (BRE), is found immediately upstream of the TATA box in some promoters of both archaea and eukaryotes (LAGRANGE *et al.* 1998; QURESHI and JACKSON 1998). The consensus sequence G/C-G/C-G/A-C-G-C-C is found in human promoters (LAGRANGE *et al.* 1998). The carboxy-terminus of TFIIB interacts with this sequence, but yeast and plant homologs of TFIIB do not contain this region, suggesting the BRE element may not be important for initiation in these organisms (LAGRANGE *et al.* 1998; NIKOLOV *et al.* 1995; TSAI and SIGLER 2000). Various combinations of these promoter elements are found at promoters *in vivo* (reviewed in SMALE and KADONAGA 2003).

There are also DNA sequence elements that act at a distance from the site of transcription initiation (reviewed in SMALE and KADONAGA 2003). One class of these elements consists of the upstream activating and repressor sequences (UAS and URS, respectively). These sequences are bound by gene specific activator and repressor proteins that regulate recruitment of the general transcription machinery (described in section 1.2.3.2). Another DNA sequence that influences transcription initiation is known as an enhancer element. These sequences can be found kilobases upstream or downstream of the core promoter yet remain important for the regulation of transcription initiation. Interactions between factors bound at UAS/URS or enhancer sequences and the GTFs and Pol II act coordinately to control transcription initiation.

1.2.3.2 Transcriptional activators and repressors bind to DNA elements and recruit coactivators and corepressors

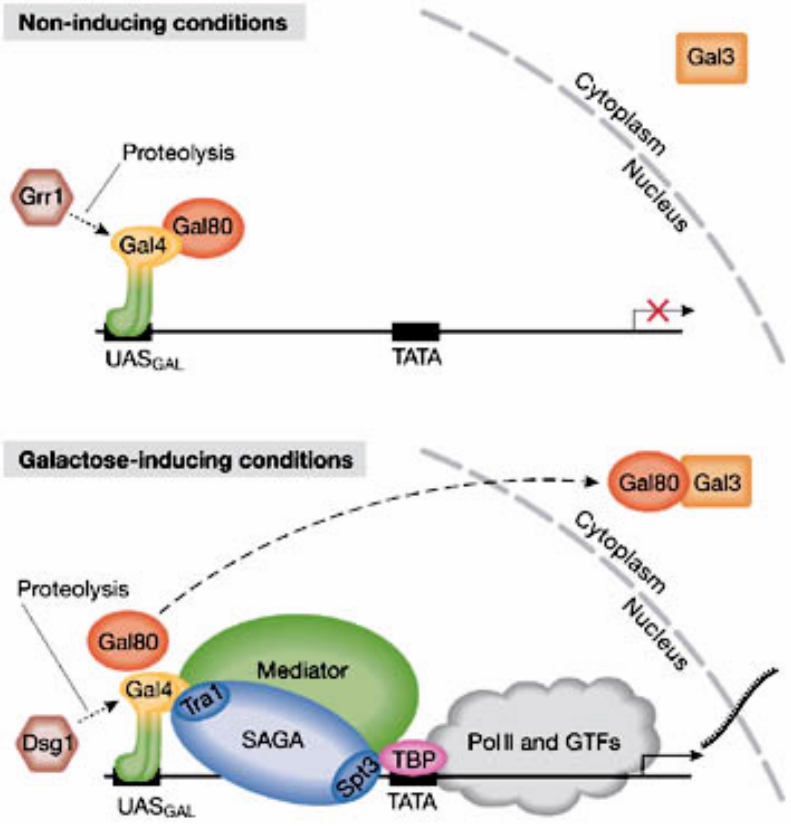
Transcriptional activators and repressors bind to upstream activating sequences (UAS) or upstream repressing sequences (URS), respectively, within the promoters of genes, often in response to environmental stimuli. Activator proteins recruit coactivator complexes (described in the section 1.2.3.3), chromatin remodeling complexes (discussed in section 1.1.4), and the general transcription machinery (discussed in section 1.2.3.4). Repressor proteins often recruit histone deacetylases and chromatin remodeling factors to create a chromatin structure that is repressive to transcription. There are many well characterized gene specific activators and repressors, which regulate a subset of genes in response to nutrient conditions or carbon sources. In this section, I will describe the modes of action for the model activator protein Gal4 and the model repressor protein Ume6.

Gal4 is an 881 amino acid protein that folds in such a way as to create two functional domains: a DNA-binding domain and an activation domain (reviewed in TRAVEN *et al.* 2006). Gal4 dimerizes, and together the DNA-binding domains recognize a 17 basepair sequence within a *GAL* promoter (MARMORSTEIN *et al.* 1992). The *GAL* genes encode for proteins that are required for galactose utilization, including membrane transporters and glycolytic enzymes. Therefore, these genes are repressed when yeast cells are grown in non-galactose conditions and activated when galactose is the primary carbon source. Under non-inducing conditions, Gal4 is bound to *GAL* promoters, but transcription activation is prevented by the binding of Gal80 to Gal4 (reviewed in TRAVEN *et al.* 2006). Gal80 prevents recruitment of TBP and TFIIB *in vitro* (WU *et al.* 1996), and SAGA recruitment *in vivo* (CARROZZA *et al.* 2002). Although the exact mechanism of disruption is unknown, under inducing conditions the cytoplasmic protein Gal3 is

recruited to the nucleus where Gal3 interacts with Gal80 and disrupts the Gal4-Gal80 complex (and reviewed in TRAVEN *et al.* 2006; ZENKE *et al.* 1996). Once Gal80 is removed, Gal4 can physically interact with TBP (MELCHER and JOHNSTON 1995; WU *et al.* 1996), TFIIB (WU *et al.* 1996), Mediator (ANSARI *et al.* 2002; JEONG *et al.* 2001; KOH *et al.* 1998), SAGA (BHAUMIK *et al.* 2004; BROWN *et al.* 2001), the chromatin remodeler SWI/SNF (YUDKOVSKY *et al.* 1999), and components of the proteasome (CHANG *et al.* 2001; GONZALEZ *et al.* 2002) (Figure 5). SAGA recruitment to the promoter is facilitated by a direct interaction with the SAGA subunit Tra1 with Gal4 bound to a promoter (BHAUMIK *et al.* 2004). Order of recruitment studies show that SAGA binds to promoters prior to the general transcription machinery, suggesting that a scaffold is created to facilitate transcription initiation (BHAUMIK and GREEN 2001; BRYANT and PTASHNE 2003). Mediator is also recruited to promoters by Gal4, but there is debate over whether or not Mediator recruitment depends on SAGA (BHAUMIK *et al.* 2004; BRYANT and PTASHNE 2003; KURAS *et al.* 2003; LARSCHAN and WINSTON 2005; LEMIEUX and GAUDREAU 2004). Recruitment of TBP and the general transcription machinery to *GAL* genes requires Gal4, SAGA and Mediator (BHAUMIK and GREEN 2001; BHAUMIK and GREEN 2002; DUDLEY *et al.* 1999; LARSCHAN and WINSTON 2001; LARSCHAN and WINSTON 2005). Interestingly, phosphorylation of Gal4 may regulate its interaction with Gal80, and ubiquitylation of Gal4 appears to be important for regulating transcription activation (reviewed in TRAVEN *et al.* 2006). The role of activator turnover in transcription activation will be discussed in section 14.4.1.

Figure 5. Gal4 recruits coactivators to activated promoters.

Mechanism of Gal4 activity in non-inducing and inducing conditions. Under non-inducing conditions, Gal80 interacts with Gal4 to prevent transcription coactivator recruitment by Gal4. Under inducing conditions (addition of galactose), Gal80 is released from Gal4 and Gal4 then recruits coactivators to promote transcription. See text for more information. From Traven, A. *et al.* (2006) *EMBO Reports*. **7**:496-499.


Non-inducing conditions
Gal3
Cytoplasm
Nucleus
Proteolysis
Grr1
Gal4
Gal80
UASGAL
TATA
Galactose-inducing conditions
Gal80
Gal3
Cytoplasm
Nucleus
Proteolysis
Gal80
Mediator
Gal4
Tra1
Dsg1
SAGA
Spt3
TBP
PolII and GTFs
UASGAL
TATA

Many transcriptional repressors control transcription by recruiting factors that condense the chromatin structure within bound promoters (reviewed in HAMPSEY 1998). Other repressors act to disrupt pre-initiation complex formation, but for simplicity I will only discuss the function of chromatin related repressors like Ume6. Ume6 binds to the *URS1* sequence within the promoters of meiosis specific genes during mitosis (reviewed in MITCHELL 1994). Ume6 recruits the histone deacetylase complex Sin3-Rpd3 (KADOSH and STRUHL 1997; KADOSH and STRUHL 1998a) which deacetylates two nucleosomes within the promoter (DECKERT and STRUHL 2001; KADOSH and STRUHL 1998b; RUNDLETT *et al.* 1998; SUKA *et al.* 2001). Ume6 also recruits the ISW2 chromatin remodeling complex which results in the formation of a nuclease-resistant chromatin structure within Ume6-bound promoters (GOLDMARK *et al.* 2000; KENT *et al.* 2001). Both Sin3-Rpd3 and ISW2 are required for Ume6-mediated repression, suggesting that the overall chromatin structure within the promoter controls transcription regulation (FAZZIO *et al.* 2001; GOLDMARK *et al.* 2000; KENT *et al.* 2001).

1.2.3.3 Transcription coactivators mediate interactions between activators and the general transcription factors

Coactivators are factors that mediate interactions between proteins bound at the UAS sequences upstream of a core promoter and the general transcription machinery. There are several well characterized coactivator complexes in yeast, including SAGA (Spt-Ada-Gcn5-acetyltransferase) Mediator, TFIIA, TBP-associated factors (TAFs), SWI/SNF, and ISW1 complexes. I will focus on the functions of SAGA and Mediator, and how each complex utilizes a unique mechanism to activate transcription.

SAGA contains 16-20 different proteins that form a complex with an approximate molecular mass of 1.8 megaDaltons (reviewed in TIMMERS and TORA 2005). There are three

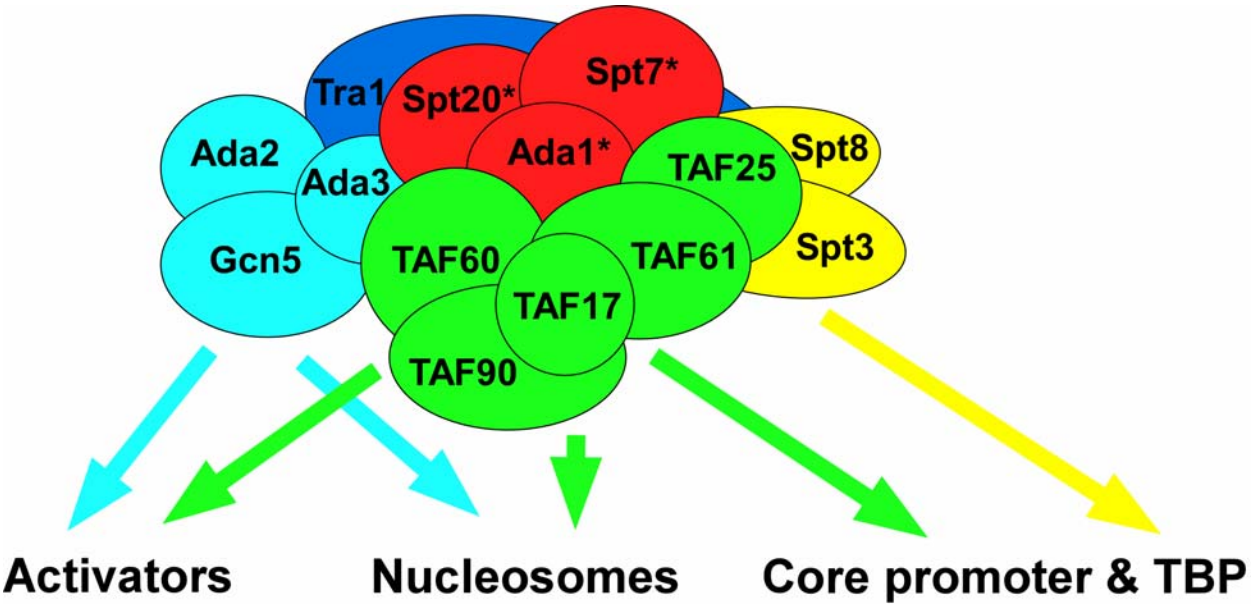
distinct subsets of proteins that make up the majority of the SAGA complex: 1) the Ada proteins (Ada1, Ada2, Ada3, Ada4 (Gcn5), and Ada5) which were identified in a genetic screen for proteins that interact with transcription activators Gcn4 and VP16 (BERGER *et al.* 1992; PINA *et al.* 1993), 2) the Spt proteins (Spt3, Spt7, Spt8, and Spt20) which were originally identified as suppressors of altered transcription initiation from Ty transposable elements within promoters (WINSTON *et al.* 1984), and 3) some of the TBP-associated factors (TAFs), (Taf5, Taf6, Taf9, Taf10, and Taf12), which are also subunits of TFIID (GRANT *et al.* 1998). SAGA also contains several other proteins, including the ataxia telangiectasia mutated (ATM)-related protein, Tra1 (GRANT *et al.* 1998; SALEH *et al.* 1998), the H2B-K123-ubiquitin protease Ubp8 (HENRY *et al.* 2003), Sgf11, which anchors Ubp8 to the SAGA complex (LEE *et al.* 2005b), Sgf29 and Sgf73 (unknown functions) (SANDERS *et al.* 2002; SHUKLA *et al.* 2006), and Sus1, a component of the mRNA export nuclear pore complex (RODRIGUEZ-NAVARRO *et al.* 2004). An alternative SAGA complex known as SLIK (SAGA-like) or SALSA contains Rtg2 and a truncated Spt7 protein but lacks Spt8 (PRAY-GRANT *et al.* 2002; STERNER *et al.* 2002; WU and WINSTON 2002).

SAGA acts as a coactivator by interacting with DNA-bound activators at promoters, modifying the chromatin structure, and recruiting the general transcription machinery (reviewed in MARTINEZ 2002) (Figure 6). Ada1, Spt7 and Spt20 are required for structural integrity of the complex (GRANT *et al.* 1998; STERNER *et al.* 1999; WU and WINSTON 2002) and Gcn5 and Ubp8 are the catalytic subunits, possessing histone acetyltransferase (HAT) activity (RUIZ-GARCIA *et al.* 1997) and ubiquitin protease activity (HENRY *et al.* 2003), respectively. Consistent with this, microarray experiments have shown that Gcn5 and Spt3 are required for the expression of 4% and 3% of yeast genes, but Spt20 is required for the expression of 10% of genes (LEE *et al.* 2000). While SAGA was initially identified as a histone acetyltransferase (GRANT *et al.* 1997),

which are characteristically associated with transcription activation, microarray analysis suggests that Gcn5 activity is important for the repression of a subset of yeast genes (HOLSTEGE *et al.* 1998).

Figure 6. The SAGA complex.

Ths SAGA complex contains several modules that interact with many other factors at the promoter of an activated gene. Arrows are color coordinated with SAGA subunits to represent interactions with factors at promoters. (*) represent subunits that are required for structural integrity of the complex. See text for additional information. Adapted from Martinez, E. (2002) *Plant Mol. Biol*. **50**:925-947.


Tra1
Spt20*
Spt7*
Ada2
Ada3
Ada1*
TAF25
Spt8
Gcn5
TAF60
TAF61
Spt3
TAF17
TAF90
Activators
Nucleosomes
Core promoter & TBP

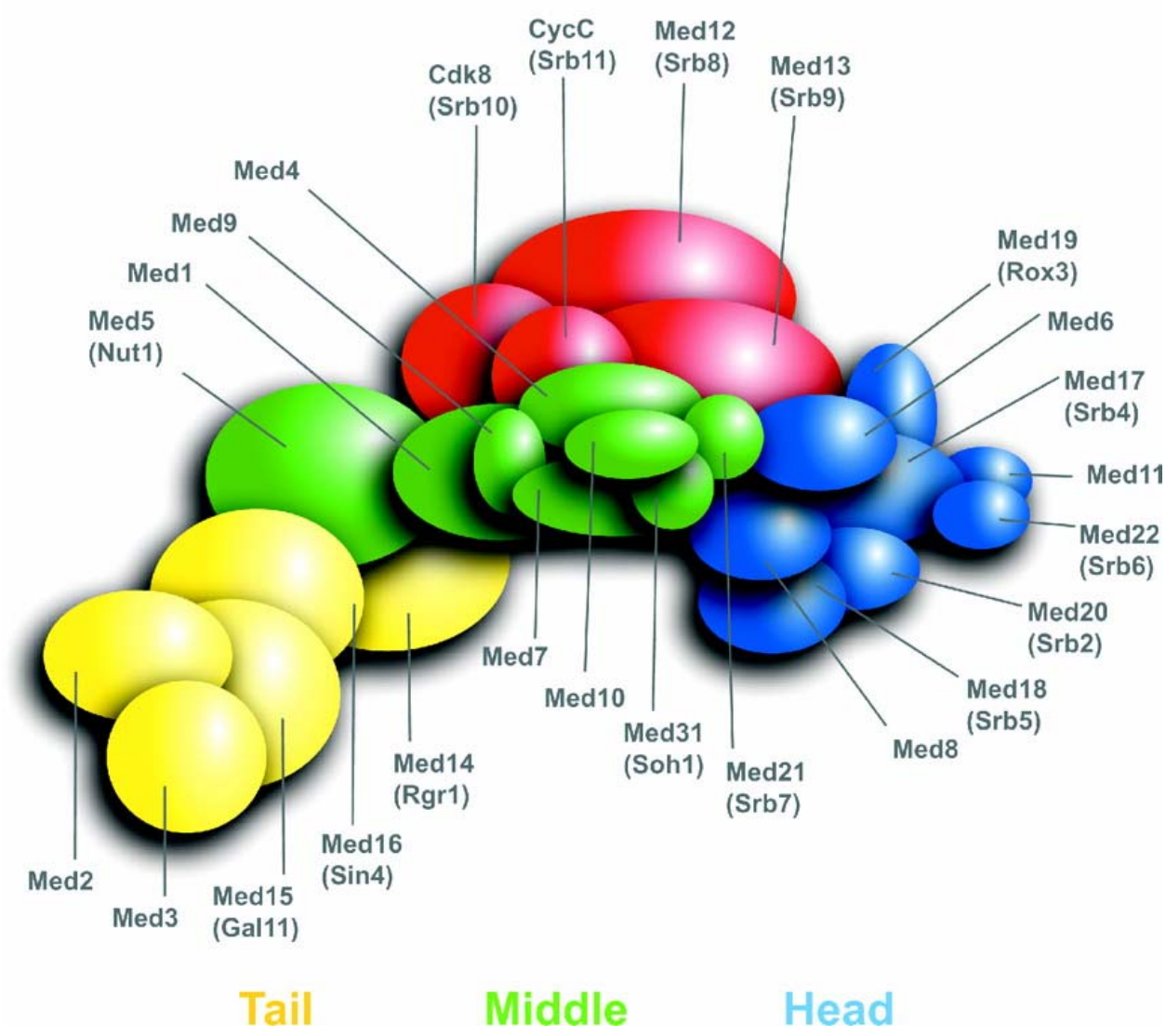
Mediator acts as a transcriptional coactivator by interacting with DNA-bound activators and recruiting RNA Pol II to activated genes (reviewed in KORNBERG 2005). Experiments for factors that stimulated *in vitro* RNA Pol II transcription demonstrated that Mediator contains approximately 20 proteins that fold together to form a complex with a total molecular mass of about 1 megaDalton (KIM *et al.* 1994) (Figure 7). Many of the subunits of Mediator were identified in genetic screens for factors involved in transcriptional regulation, including the Srb (suppressors of RNA polymerase B) proteins. Other components were identified by intensive biochemical purification and termed the Med (Mediator) proteins (MYERS *et al.* 1998). Mediator components are conserved throughout eukaryotes (BOUBE *et al.* 2002; BOURBON *et al.* 2004). Mediator is a modulated complex that consists of the "head", "middle", "tail", and Cdk8 (Srb8-11) subcomplexes (GUGLIELMI *et al.* 2004). This modular architecture is conserved from yeast to humans (GUGLIELMI *et al.* 2004). The head and middle modules contact RNA Pol II (DAVIS *et al.* 2002). The tail module physically contacts transcriptional activators (HAN *et al.* 1999; PARK *et al.* 2000). The Cdk8 subcomplex represses transcription by phosphorylating various gene-specific regulatory proteins (HIRST *et al.* 1999; NELSON *et al.* 2003) and the CTD of RNA Pol II (HOLSTEGE *et al.* 1998; LIAO *et al.* 1995).

Srb4 and Srb6 are "core" subunits of Mediator, and microarray analysis using a temperature-sensitive Srb4 mutant shows that over 5000 genes depend on Mediator for transcription (yeast contain approximately 6000 genes), and expression changes were similar to those seen in strains containing a temperature sensitive allele of *RPB1*, which encdes the largest subunit of RNA Pol II (HOLSTEGE *et al.* 1998; THOMPSON and YOUNG 1995). However, mutations in *SRB5* and *MED6* only affect expression of 16% and 10% of genes in yeast (HOLSTEGE *et al.* 1998). Consistent with a role for Mediator in regulating transcription initiation,

ChIP-chip analysis shows that Mediator is found at promoters and is associated throughout an actively transcribing gene (ZHU *et al.* 2006) as well as at the promoters of inactive genes (ANDRAU *et al.* 2006).

Figure 7. The Mediator complex.

The modular architecture of the Mediator complex. Head (blue), middle (green), tail (yellow), and Cdk8 (red) suncomplexes are shown. From Guglielmi *et al*. (2004) *NAR* **32**(18):5379-5391.


CycC
(Srb11)
Med12
(Srb8)
Med13
(Srb9)
Cdk8
(Srb10)
Med4
Med9
Med1
Med5
(Nut1)
Med19
(Rox3)
Med6
Med17
(Srb4)
Med11
Med22
(Srb6)
Med20
(Srb2)
Med18
(Srb5)
Med8
Med7
Med10
Med31
(Soh1)
Med21
(Srb7)
Med14
(Rgr1)
Med16
(Sin4)
Med2
Med3
Med15
(Gal11)
Tail
Middle
Head

1.2.3.4 The general transcription machinery coordinately regulates transcription initiation

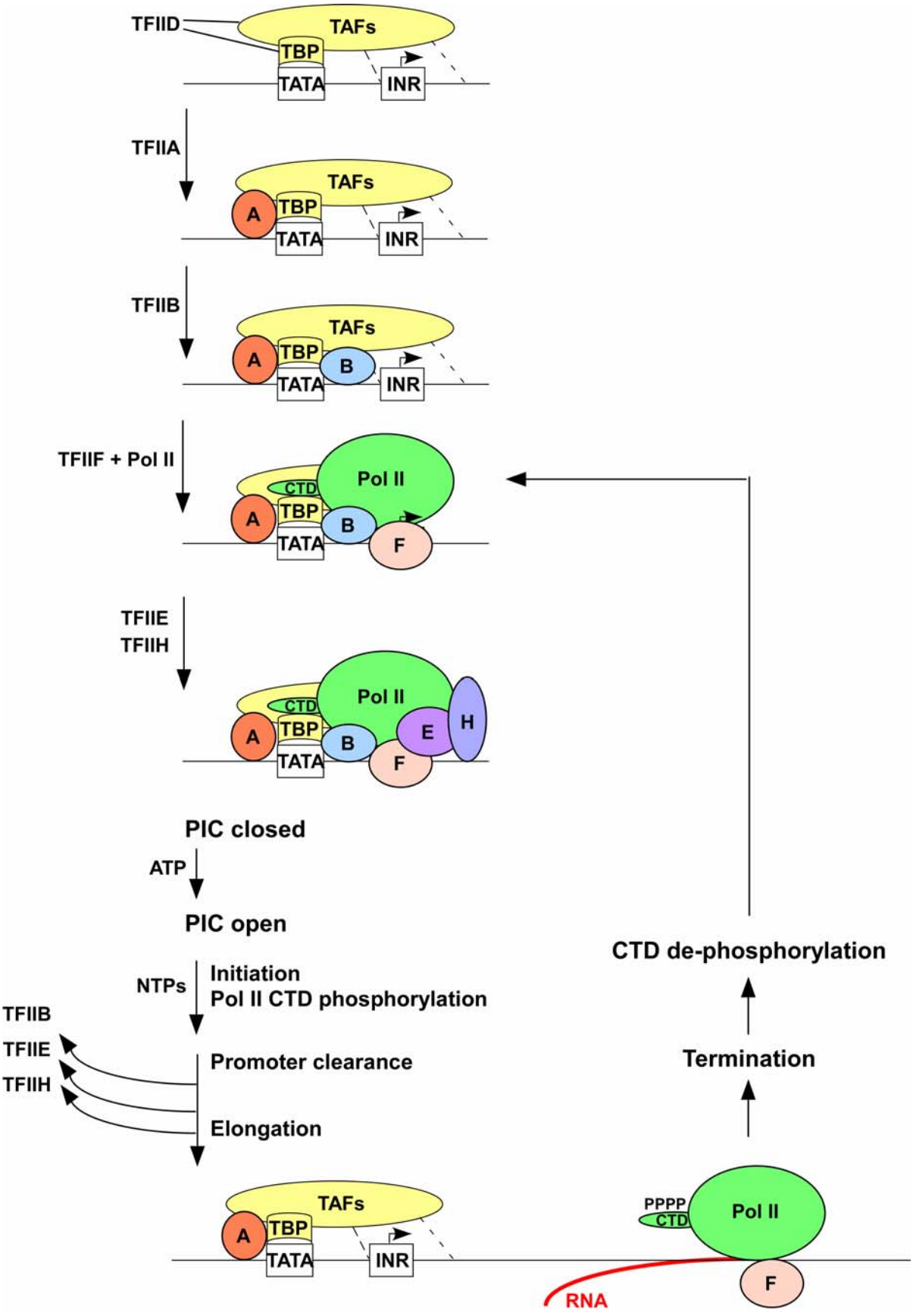
In vitro order of recruitment experiments have shown that members of the general transcription machinery can be recruited to an activated gene in a specific order (BURATOWSKI *et al.* 1989; FANG and BURTON 1996; HA *et al.* 1993) (Figure 8). The general transcription factors (GTFs) are the minimal components that are required for RNA Pol II transcription. The first step in preinitiation complex (PIC) formation involves the binding of the TATA-binding protein (TBP) to the TATA element within a promoter. Interestingly, TBP is also used to recruit RNA Pol I and Pol III to their respective promoters (KIM and ROEDER 1994). TBP associates with 14 other conserved proteins (TBP-associated factors, or TAFs) to compose TFIID (reviewed in BURLEY and ROEDER 1996; GREEN 2000). TBP binds the minor groove of the TATA box and induces an 80 degree bend in the DNA (LEE *et al.* 1991; WU *et al.* 2001b). The TAFs interact with the Inr and DPE elements around the TATA box (BURKE and KADONAGA 1997; and reviewed in BURLEY and ROEDER 1996; CHALKLEY and VERRIJZER 1999; OELGESCHLAGER *et al.* 1996; VERRIJZER and TJIAN 1996; WU *et al.* 2001a). TFIIA is recruited to TBP-DNA complexes (BISWAS *et al.* 2004). TFIIA was originally identified as a GTF (MATSUI *et al.* 1980; REINBERG and ROEDER 1987), but more recent evidence suggests that TFIIA is best described as a coactivator that directly contacts TBP (BISWAS *et al.* 2004). TFIIB is recruited to form a DNA-TBP-IIA-IIB ternary complex (BURATOWSKI *et al.* 1989), which then recruits TFIIF bound to RNA Pol II (FLORES *et al.* 1989). TFIIF is thought to interact with Pol II prior to PIC formation to prevent nonspecific binding of the polymerase to DNA and this interaction stabilizes the PIC once it is recruited to a promoter (reviewed in CONAWAY and CONAWAY 1993; GREENBLATT 1991). TFIIE enters the PIC after Pol II, but before TFIIH (BURATOWSKI *et al.* 1989; FLORES *et*

al. 1989). TFIIE physically interacts with unphosphorylated Pol II, TFIIF, and TFIIH (FLORES *et al.* 1989; MAXON and TJIAN 1994). TFIIH is the only GTF with known enzymatic activities, which include DNA-dependent ATPase (CONAWAY and CONAWAY 1989; ROY *et al.* 1994), ATP-dependent DNA helicase (SCHAEFFER *et al.* 1993; SERIZAWA *et al.* 1993), and CTD kinase activities (FEAVER *et al.* 1991; LU *et al.* 1992; SERIZAWA *et al.* 1992).

Once the PIC is assembled, RNA Pol II separates the strands of the DNA duplex, and begins transcription (reviewed in MARTINEZ 2002). Promoter clearance occurs after TFIIH-dependent CTD phosphorylation, at which point TFIIB, IIE and IIH contacts are disrupted, and the polymerase enters the open reading frame. TFIID and TFIIA remain bound to the promoter, and TFIIF remains bound to the polymerase. The polymerase then enters the elongation stage of transcription, which will be described in the next section. A study in yeast showed that Mediator components, TFIIA, IID, IIE and IIH are stabilized at the promoter through interactions with activators, suggesting that only TFIIB, IIF and a dephosphorylated Pol II are required for subsequent rounds of transcription at an activated promoter (YUDKOVSKY *et al.* 2000).

Figure 8. Recruitment of the general transcription machinery is highly ordered.

In vitro experiments defined the order of recruitment if the general transcription machinery. See text for additional information. Adapted from Martinez, E. (2002) *Plant Mol. Biol*. **50**:925-947.


TFIID
TAFs
TBP
TATA
INR
TFIIA
A
TFIIB
B
TFIIF + Pol II
Pol II
CTD
F
TFIIE
TFIIH
E
H
PIC closed
ATP
PIC open
NTPs
Initiation
Pol II CTD phosphorylation
Promoter clearance
Elongation
CTD de-phosphorylation
Termination
PPPP
RNA

1.2.4 Transcription elongation is facilitated by many proteins that associate with Pol II

Transcription elongation occurs as the polymerase incorporates nucleotides into a growing nascent RNA chain. RNA Pol II transcription of protein coding genes is coupled to mRNA processing, including capping, splicing, and polyadenylation of the transcript. Therefore, there is a dynamic association of many factors with the polymerase during the transcription cycle; different factors are required at the beginning of transcription as compared to the termination of transcription.

Both eukaryotic and bacterial transcription complexes can form stalled elongation complexes *in vitro* (LANDICK 1997; MOTE and REINES 1998; NUDLER *et al.* 1994), suggesting that there are factors that assist the polymerase in overcoming obstacles to transcription elongation *in vivo*. Many "elongation factors" have been characterized in eukaryotes and archaea. Interestingly, increasing information suggests that many eukaryotic elongation factors function to regulate chromatin modifications associated with active transcription (Figure 9). In the next few sections, I will describe the best characterized elongation factors in yeast and relevant disease connections in humans.

1.2.4.1 The Paf1 complex

The Paf1 complex is a nuclear complex, approximately 670 kiloDaltons in size, and minimally composed of 5 subunits, Paf1, Ctr9, Rtf1, Cdc73, and Leo1 (KROGAN *et al.* 2002; MUELLER and JAEHNING 2002; SQUAZZO *et al.* 2002). The members of this complex are conserved from *S. cerevisiae* to humans (ROZENBLATT-ROSEN *et al.* 2005; YART *et al.* 2005). Paf1 and Cdc73 were originally connected to transcription by purification of RNA Pol II transcription complexes that lacked the Mediator complex (SHI *et al.* 1997). The results of this

purification were confirmed by coimmunoprecipitation analysis that showed that the Paf1 complex members physically interact with RNA Pol II associated factors, including Spt4-5, yeast FACT and Chd1 (KROGAN *et al.* 2002; SIMIC *et al.* 2003; SQUAZZO *et al.* 2002). Genetic analysis also suggested that these proteins all worked in parallel to affect transcription (KROGAN *et al.* 2002; MUELLER and JAEHNING 2002; SIMIC *et al.* 2003; SQUAZZO *et al.* 2002). Quantitative Western blots show that the members of the Paf1 complex are found in equal amounts with RNA Pol II subunits, which led to the suggestion that there is one Paf1 complex associated with each transcribing Pol II complex (MUELLER *et al.* 2004). Subsequent analysis showed that the Paf1 complex is associated with all actively transcribed genes tested (MUELLER *et al.* 2004; SHELDON *et al.* 2005; SIMIC *et al.* 2003). Loss of individual Paf1 complex members leads to the altered expression of a subset of genes in yeast (PORTER *et al.* 2005; SHELDON 2005). Whereas the subunits of the Paf1 complex are found together on actively transcribed genes, the complex members do not act coordinately to affect transcription in the same manner, and data that I will discuss in the next few paragraphs show that the subunits have unique roles in promoting transcription.

Paf1 and Ctr9 are considered the integral subunits of the complex; loss of these factors leads to more severe phenotypes as compared to the loss of other subunits. Strains lacking *PAF1* or *CTR9* grow poorly, even on rich medium (BETZ *et al.* 2002). Strains lacking *RTF1*, *CDC73* or *LEO1* exhibit growth defects on medium containing 6-azauracil (6AU), although these strains grow slightly better on this medium compared to *paf1Δ* or *ctr9Δ* strains (BETZ *et al.* 2002; SQUAZZO *et al.* 2002). Strains that grow poorly on 6AU medium often have defects in transcription elongation because 6AU reduces nucleotide levels *in vivo*, increasing the need for transcription elongation factors that assist stalled elongation complexes (EXINGER and LACROUTE

1992). Strains lacking *PAF1*, *CTR9*, and to a lesser extent *RTF1*, have growth defects on medium lacking inositol (BETZ *et al.* 2002). The Ino$^-$ phenotype is associated with general transcription defects (HAMPSEY 1997). Strains lacking all Paf1 complex members exhibit an Spt$^-$ phenotype, which connects these factors to the regulation of transcription initiation (SQUAZZO *et al.* 2002). This phenotype is observed in mutant strains that restore transcription initiation to the proper TATA box in promoters that contain a competing TATA box within a Ty transposable element (WINSTON *et al.* 1984). Genetic interactions are seen in strains lacking members of the Paf1 complex and other transcription elongation factors, including Spt4, Spt5, and Spt16 (SQUAZZO *et al.* 2002).

Rtf1 was originally identified as a suppressor of a mutation in TBP that had altered DNA binding specificity (STOLINSKI *et al.* 1997). The graduate work of Patrick Costa (of the Arndt lab) showed that a *rtf1Δ* mutation was synthetically lethal with mutations in *POB3*, *FCP1*, and *CTK1*, which encode for a member of the FACT transcription elongation complex, an RNA Pol II CTD phosphatase, and an RNA Pol II CTD kinase, respectively (COSTA and ARNDT 2000). These results suggested that Rtf1 was important for regulating the transition from transcription initiation to elongation. The graduate work of Kathryn Sheldon of the Arndt lab showed that overexpression of *NAB3*, an essential factor involved in 3'-end formation of non-polyadenylated RNA Pol II transcripts, suppresses a conditional phenotype of *rtf1* mutant strains (SHELDON *et al.* 2005). Subsequent experiments showed that loss of Paf1 complex members results in transcription readthrough at the 3'-end of non-polyadenylated RNA Pol II transcripts, and *paf1Δ* strains have reduced amounts of Nab3 at actively transcribed genes (SHELDON *et al.* 2005). Work from the Jaehning lab showed that Paf1 is also important for 3'-end formation of RNA Pol II poly-adenylated transcripts (PENHEITER *et al.* 2005).

Interestingly, the Paf1 complex seems to ensure proper transcription by regulating the recruitment and activity of several histone modifying enzymes whose activity is associated with active transcription (Figure 9). Rtf1, Paf1 and Ctr9 are required for the recruitment and activity of Rad6 and Bre1, the ubiquitin-conjugating enzyme and ubiquitin-protein ligase that mono-ubiquitylate histone H2B at lysine 123 (K123) (NG *et al.* 2003b; WOOD *et al.* 2003b; XIAO *et al.* 2005). Because this ubiquitylation event is required for subsequent methylation of histone H3 at lysines 4 and 79, Paf1 complex members are also required for these methylation events (WOOD *et al.* 2003b). However, Paf1 and Ctr9 strains have decreased Rtf1 protein levels (MUELLER *et al.* 2004; SQUAZZO *et al.* 2002), which suggests that Rtf1 may be the primary factor that is responsible for the recruitment of the H2B ubiquitylation machinery.

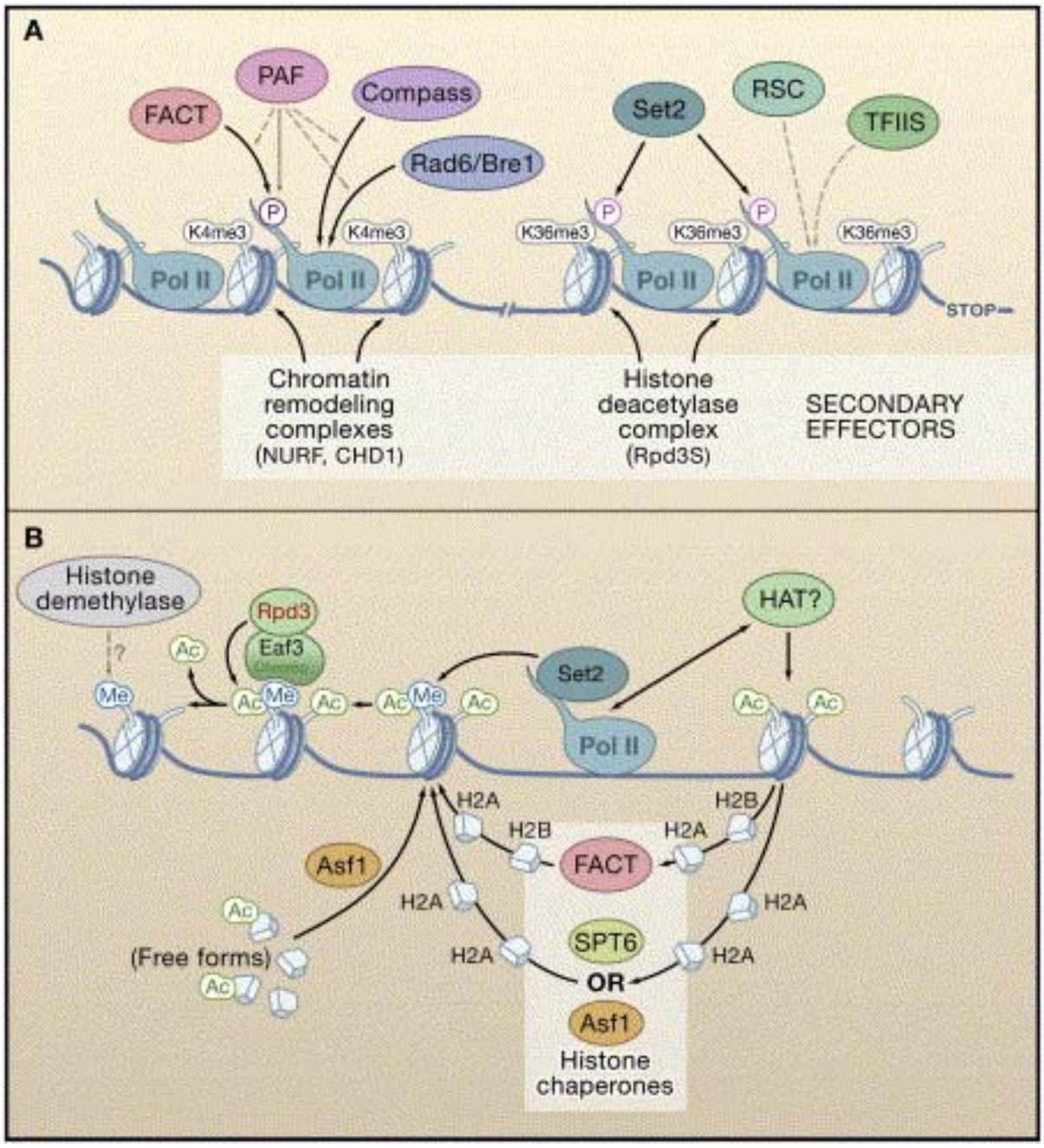
Paf1 and Ctr9 are also required for tri-methylation of histone H3 at lysine 36 (Y. Chu, R. Simic, M. Warner, K. Arndt and G. Prelich, submitted for publication). Other members of the Paf1 complex have no effect on K36 methylation. Specifically, Paf1 and Ctr9 are important for the recruitment of Set2, the enzyme that methylates histone H3 at K36. This result is interesting because mono-, di-, and tri-methylation of K36 are all performed by Set2, but loss of Paf1 or Ctr9 only affects the tri-methylation event at actively transcribing genes. Further analysis will be needed to fully understand this interesting result.

The Paf1 complex is conserved in eukaryotes. Cdc73 is 32% identical in sequence with the human tumor suppressor protein, parafibromin (ROZENBLATT-ROSEN *et al.* 2005). Mutations in this protein are associated with hyperparathyroidism-jaw tumor syndrome (CARPTEN *et al.* 2002; SHATTUCK *et al.* 2003). Immunoprecipitations show that parafibromin associates with the human homologs of Paf1, Ctr9 and Leo1, suggesting it is a member of the human Paf1 complex (ROZENBLATT-ROSEN *et al.* 2005; YART *et al.* 2005). In contrast to the Paf1 complex in yeast,

the human homolog of Rtf1 did not interact with parafibromin, suggesting that human Rtf1 may regulate transcription in a Paf1-independent manner in humans. However, similar to yeast, the human Paf1 complex physically associates with Rbbp5 and Ash2L, components of the human histone H3 K4 methyltransferase complex (ROZENBLATT-ROSEN *et al.* 2005). These results suggest that Rtf1 function may not be conserved through eukaryotes, or that human Rtf1 does not need to stably interact with the Paf1 complex to affect transcription.

Figure 9. Many transcription elongation factors regulate chromatin structure.

A) The state of the chromatin template during transcription elongation is regulated by factors that associate with RNA Pol II. The Paf1 complex associates with RNA Pol II that is phosphorylated at serine 5. This complex regulates the association of Rad6/Bre1, COMPASS, and FACT with chromatin, leading to an increase in H2B ubiquitylation and histone H3 K4 methylation at the 5'-end of ORFs. Set2 interacts with RNA Pol II that is phosphorylated within the CTD at serine 2. This leads to histone H3 K36 methylation at the 3'-ends of ORFs. Chromatin remodeling (eg. Chd1) and chromatin modifying (eg. Rpd3S) complexes are recruited to actively transcribed ORFs by the post-translational histone modifications mentioned above. B) Nucleosomal stability is highly regulated during transcription. Once the transcription complex has moved into the body of a gene where activator-dependent HATs no longer operate, other HATs are recruited to facilitate transcription through a chromatin template. The passage of the polymerase causes histone displacement. These histones are redeposited onto the DNA behind Pol II by histone chaperones and elongation factors, including FACT and Spt6. Alternatively, "new" histones in the nucleus are also available for nucleosome deposition. These newly deposited histones are hyperacetylated and are immediately methylated at histone H3 K36 by Set2. This modification recruits the Rpd3S deacetylase complex. Rpd3S HDAC activity results in the stabilization of the nucleosome. Methylation of H3K36 is eventually eliminated by a histone demethylases when the gene turns off. From Li, B. *et al.* (2007) *Cell*. **128**:707-719.


A
PAF
FACT
Compass
Rad6/Bre1
Set2
RSC
TFIIS
P
K4me3
K4me3
K36me3
K36me3
K36me3
Pol II
Pol II
Pol II
Pol II
STOP
Chromatin remodeling complexes (NURF, CHD1)
Histone deacetylase complex (Rpd3S)
SECONDARY EFFECTORS
B
Histone demethylase
?
Rpd3
Eaf3
Ac
Me
Set2
HAT?
Pol II
H2A
H2B
FACT
Asf1
(Free forms)
SPT6
OR
Asf1
Histone chaperones

1.2.4.2 Spt4/5, Spt6, and yeast FACT modulate chromatin structure during transcription

The *SPT* (suppressors of Ty) genes were identified in genetic screens to identify factors that suppressed initiation from an alternate TATA element within the promoters of yeast genes (WINSTON *et al.* 1984). Mutations in the *SPT* genes group into two classes; 1) those that cause phenotypes similar to mutations in *SPT15* (encodes TBP), and 2) those that cause phenotypes similar to histone gene mutations (the histone genes were identified in this screen as well). Several of the genes in the second class of factors are important for regulating chromatin modifications and structure during transcription, including Spt4, Spt5, Spt6 and Spt16.

Spt6 is a conserved elongation factor that is important for maintaining chromatin structure. Yeast Spt6 has been shown to physically interact with histones, and can assemble nucleosomes on plasmid DNA *in vitro* (BORTVIN and WINSTON 1996). Micrococcal nuclease assays show that *spt6* strains have genome-wide changes in chromatin structure (BORTVIN and WINSTON 1996). Strains expressing temperature sensitive alleles of *SPT6* have increased levels of transcription from cryptic TATA elements that are found within open reading frames, suggesting that Spt6 is important for reassembling nucleosomes that are displaced during transcription (KAPLAN *et al.* 2003) (Figure 9). Spt6 has been shown to physically interact with RNA Pol II, Spt5, and a novel protein Iws1 (interacts with Spt6) (KROGAN *et al.* 2002). Chromatin immunoprecipitation experiments have shown that *Drosophila* Spt6 colocalizes at genes with RNA Pol II upon induction of transcription (ANDRULIS *et al.* 2000; KAPLAN *et al.* 2000).

Spt4 and Spt5 form a tightly associated complex in yeast (KROGAN *et al.* 2002; SWANSON and WINSTON 1992) that is functionally connected to the regulation of transcription elongation (HARTZOG *et al.* 1998). Spt5 has also been shown to associate with RNA Pol II, transcription

initiation factors, RNA processing factors, and several transcription elongation factors, including Spt4, Pob3, TFIIS, and Spt6 (KROGAN *et al.* 2002; LINDSTROM *et al.* 2003). Spt4 physically interacts with RNA Pol II and Spt5 (KROGAN *et al.* 2002). Interestingly, Spt4 and Spt5 copurify with RNA Pol I and are important for proper expression of rRNA (SCHNEIDER *et al.* 2006a). Importantly, Spt4 (and possibly Spt5) is important for Paf1 complex recruitment to RNA Pol II after promoter clearance (QIU *et al.* 2006). Spt4 and Spt5 are conserved proteins, and the human homologs of these factors comprise the DSIF complex (WADA *et al.* 1998). This complex was identified as an inhibitor of transcription of elongation in cells exposed to the nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (WADA *et al.* 1998). *In vitro* experiments suggest that both the human (DSIF) and yeast (Spt4/5) proteins act to positively and negatively regulate transcription elongation (HARTZOG *et al.* 1998; WADA *et al.* 1998). Consistent with a positive role in transcription elongation, yeast and *Drosophila* Spt4 and Spt5 are localized at actively transcribed genes (ANDRULIS *et al.* 2000; KAPLAN *et al.* 2000; POKHOLOK *et al.* 2002). Genetic interactions with CTD truncations and CTD kinases and phosphatases suggest that Spt4 and Spt5 regulate elongation events that are controlled by physical interactions with the CTD of RNA Pol II (LINDSTROM and HARTZOG 2001; MURRAY *et al.* 2001).

Spt16 physically interacts with Pob3 and Nhp6 to form the yeast homolog of the human FACT (facilitates chromatin transcription) complex (ORPHANIDES *et al.* 1998). The human FACT complex contains Spt16 and SSRP1, which is similar in sequence to yeast proteins Pob3 and Nhp6 (BREWSTER *et al.* 2001). Spt16 physically interacts with RNA Pol II and transcription elongation factors, including the Paf1 complex and Spt4/5 (KROGAN *et al.* 2002). Chromatin immunoprecipitation experiments in yeast (MASON and STRUHL 2003) and *Arabidopsis*

(DUROUX *et al.* 2004) and immunofluorescence experiments in *Drosophila* (SAUNDERS *et al.* 2003) have shown that FACT is associated with actively transcribed genes. Interestingly, human FACT physically associates with the chromatin remodeling enzyme Chd1 (KELLEY *et al.* 1999) bound to histone H3 that is tri-methylated at K4 (reviewed in REINBERG and SIMS 2006), and knockdown of Chd1 protein levels in *Drosophila* reduces FACT recruitment to active genes (ADELMAN *et al.* 2006). FACT facilitates the removal of an H2A/H2B dimer during transcription (BELOTSERKOVSKAYA *et al.* 2003) (Figure 9), and strains containing mutations in FACT subunits exhibit aberrant initiation from cryptic TATA elements (KAPLAN *et al.* 2003), suggesting that FACT is important for maintaining proper chromatin structure during transcription (reviewed in REINBERG and SIMS 2006)). Interestingly, yeast FACT interacts with DNA polymerase α (SCHLESINGER and FORMOSA 2000; WITTMEYER and FORMOSA 1997; WITTMEYER *et al.* 1999), suggesting that FACT may modulate chromatin structure during many nuclear events, including transcription and DNA replication.

1.2.4.3 TFIIS

TFIIS, encoded by the gene *DST1* (also known as *PPR2*), is a 35 kiloDalton protein that stimulates transcription elongation *in vitro* and *in vivo* (KULISH and STRUHL 2001; SEKIMIZU *et al.* 1976). TFIIS is conserved throughout eukaryotes, and physically interacts with RNA Pol II (AGARWAL *et al.* 1991; HIRASHIMA *et al.* 1988; RAPPAPORT *et al.* 1988; REINBERG and ROEDER 1987; SHIMOARAISO *et al.* 1997). TFIIS is also homologous to GreA and GreB in bacteria (BORUKHOV *et al.* 1993). TFIIS and its bacterial homologs facilitate movement of the polymerase through an arrest site by stimulating the intrinsic RNA cleavage activity of RNA Pol II (KETTENBERGER *et al.* 2003; RUDD *et al.* 1994; WEILBAECHER *et al.* 2003). After cleavage, the transcript remains associated with RNA Pol II, and is realigned within the active site, where

the polymerase attempts elongation again (GU *et al.* 1993; KIREEVA *et al.* 2000; KOMISSAROVA and KASHLEV 1997). Strong synthetic phenotypes are observed in strains containing mutations in *DST1* and mutations in other elongation factors, including *CTK1* (JONA *et al.* 2001), *RTF1* (COSTA and ARNDT 2000), *SPT4*, *SPT5*, *SPT6* (HARTZOG *et al.* 1998), and *SPT16* (ORPHANIDES *et al.* 1999), suggesting that all of these factors functionally overlap to promote transcription elongation.

1.2.5 Transcription termination and 3'-end formation of the mRNA

Transcription termination occurs when the RNA polymerase releases the nascent RNA and dissociates from the DNA template (reviewed in ROSONINA *et al.* 2006). This process allows the polymerase to be recycled at another promoter, and prevents aberrant transcription of downstream genes. DNA or RNA elements at specific positions downstream of eukaryotic genes are recognized by eukaryotic RNA Pol I and Pol III and prokaryotic RNA polymerases and act to directly or indirectly disrupt the polymerase-DNA-RNA complex to terminate transcription (reviewed in HENKIN 2000; PAULE and WHITE 2000). Eukaryotic RNA Pol II transcription termination occurs stochastically and appears to be independent of *cis*-acting sequences (reviewed in ROSONINA *et al.* 2006). A full understanding of RNA Pol II termination is lacking, but it is known that termination is functionally connected to the 3' end formation of the nascent transcript. Transcription of the poly(A) sequence prompts the endonucleolytic cleavage of the transcript, which is released to be polyadenylated and translated, and the polymerase continues transcribing downstream of the poly(A) site, creating another uncapped transcript (reviewed in COLGAN and MANLEY 1997; ZHAO *et al.* 1999). This new transcript is targeted for degradation by Rat1/Xrn2 exonuclease, which promotes transcription termination (KIM *et al.* 2004b; WEST *et*

al. 2004). Rosonina and colleagues propose that after the poly(A) site the polymerase pauses, possibly due to conformational changes in accessory factors, which results in the recruitment of unknown termination factors that disrupt the polymerase-DNA-RNA interaction, leading to transcription termination (ROSONINA *et al.* 2006).

1.3 TRANSCRIPTIONAL SILENCING MECHANISMS IN YEAST

1.3.1 What is silencing?

Silenced chromatin has been characterized as a repressive chromatin structure that is established and maintained through physical interactions between non-histone proteins and histone proteins (reviewed in VAN LEEUWEN and GOTTSCHLING 2002). The chromatin structure within silenced regions in yeast is similar to that found in heterochromatin in humans and *Drosophila* (reviewed in RUSCHE *et al.* 2003). Specifically, the silenced regions in yeast are highly condensed throughout the cell cycle, associated with the nuclear periphery, and these regions have very little to no gene expression (VAN LEEUWEN and GOTTSCHLING 2002). Therefore, silencing mechanisms in yeast have been used as a model to understand heterochromatin function in higher eukaryotes. Although silencing is characterized by the loss of gene expression, factors involved in other processes related to chromatin, including DNA repair and replication are also excluded from silenced regions (GOTTSCHLING 1992; LOO and RINE 1994; SINGH and KLAR 1992). The amino-terminal tails of histones H3 and H4 within nucleosomes at silenced regions are hypo-methylated and hypo-acetylated (BRAUNSTEIN *et al.* 1993; SUKA *et al.* 2001). As one might expect, silenced chromatin contains highly ordered nucleosomes that are arranged in a

regularly spaced manner (RAVINDRA *et al.* 1999; WEISS and SIMPSON 1998).

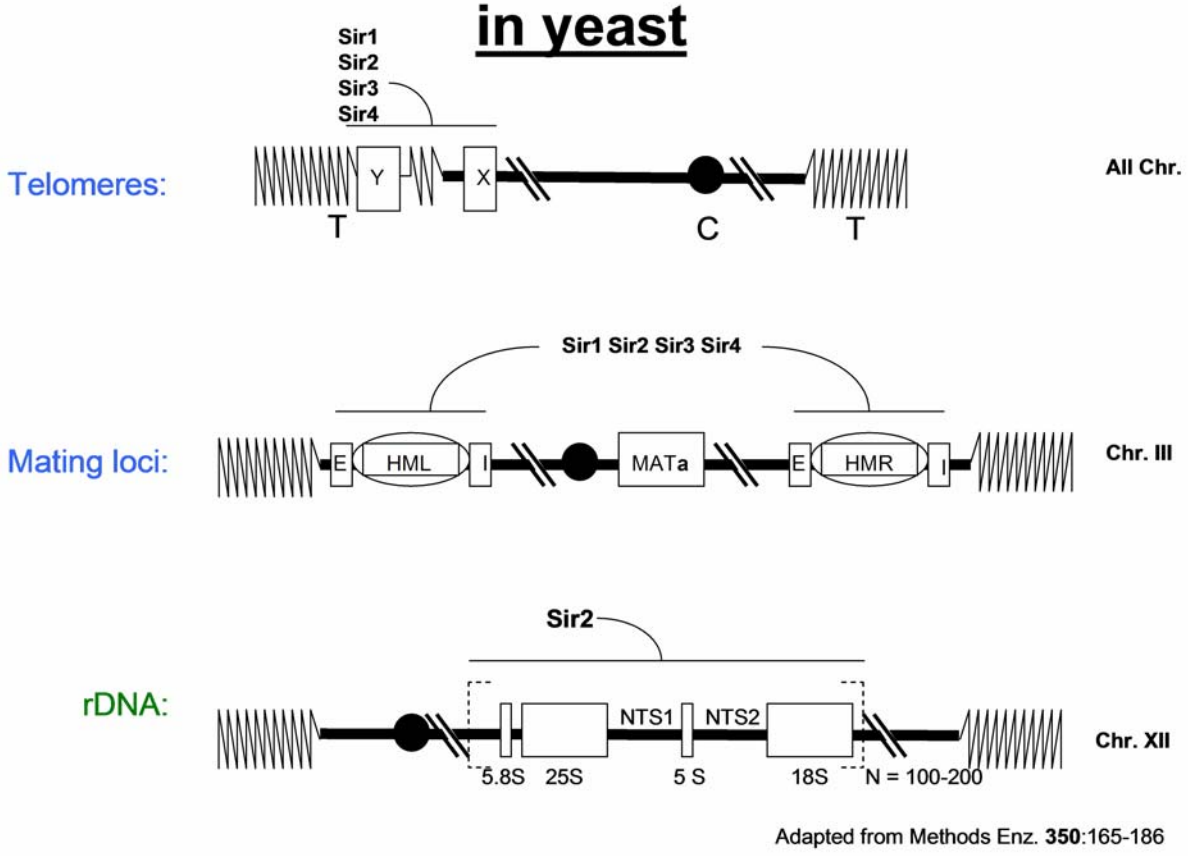
1.3.2 The Sir proteins are required to establish and maintain silencing

There are three regions of the yeast genome that utilize silencing mechanisms, and while the exact mechanism of silencing differs in all three regions, the proteins that establish and maintain silencing are common (Figure 10). The silent information regulator (Sir) proteins, Sir1-4, are required for silencing, although not all Sir proteins act at all three silenced regions (to be discussed further in subsequent sections). Sequence specific DNA binding proteins bind to silencer sequences at each silenced region and act to recruit the Sir proteins to a "starting" point for silencing. Sir1 is not required for silencing, but helps to assemble the other Sir proteins at silenced regions (PILLUS and RINE 1989; RUSCHE *et al.* 2002; ZHANG *et al.* 2002). Sir2 is an NAD^+-dependent histone deacetylase (IMAI *et al.* 2000) and is recruited to all of the silenced regions through physical interactions with sequence specific DNA-binding proteins (reviewed in RUSCHE *et al.* 2003). Sir3 and Sir4 require physical interactions with each other and hypo-acetylated histones to maintain silencing at telomeres and the silent mating loci (HECHT *et al.* 1995). Silencing is thought to spread from the silencing origin using a mechanism in which Sir2 deacetylates histone tails, creating a binding substrate for Sir3 and Sir4. As Sir2 continues to deacetylate histone tails in sequential nucleosomes as it moves away from the silencing origin, and Sir3 and Sir4 spread by binding these tails (reviewed in RUSCHE *et al.* 2003). Sir2 and Sir3 are found in limited concentrations *in vivo*, and silencing is affected by subtle changes in the protein levels of either factor. For example, overexpression of Sir3 enhances telomeric silencing (RENAULD *et al.* 1993). However, deletion of Sir4 decreases telomeric silencing (COCKELL *et al.* 1995), because Sir4 is required for Sir3 binding to this region.

Figure 10. Three regions of the genome are subjected to silencing.

Telomeres, mating loci and rDNA are subjected to silencing mechanisms in yeast. See text for additional details. Adapted from Van Leeuwen and Gottschling (2002) *Methods Enz.* **350**:165-186.

Three regions of the genome are silenced in yeast



Adapted from Methods Enz. **350**:165-186

1.3.3 The histone modification state in silenced regions differs from the modification state in actively transcribed regions

Post-translational modifications of histones play an important role in gene expression as well as transcriptional silencing. As described in section 1.1.2, histone acetylation and Rtf1-dependent histone modifications, specifically histone H3 K4 and K79 trimethylation, are found in euchromatic regions of the genome (POKHOLOK *et al.* 2005). Conversely, chromatin in silenced regions is hypo-acetylated and hypo-methylated. Interestingly, histone H3 lysine 9 (K9) methylation is characteristic of transcriptionally silenced regions in higher eukaryotes, but this modification is not found in *S. cerevisiae*. However, silencing defects appear in strains lacking certain factors required for global histone modifications, specifically Rad6 (SUN and ALLIS 2002), Set1 (BRYK *et al.* 2002; LAIBLE *et al.* 1997; NISLOW *et al.* 1997), Dot1 (NG *et al.* 2002a; SINGER *et al.* 1998; VAN LEEUWEN *et al.* 2002), or members of the Paf1 complex required for the activity of these enzymes (KROGAN *et al.* 2003a; NG *et al.* 2003a; WOOD *et al.* 2003b). Chromatin immunoprecipitation experiments in these strains show that the Sir proteins are diluted away from the normally silenced regions, due to the fact that the entire genome is hypo-methylated in these strains (NG *et al.* 2003a).

1.3.4 Three regions of the *S. cerevisiae* genome are subjected to silencing

In *S. cerevisiae*, telomeres, the silent mating loci, and rDNA repeats are subjected to silencing mechanisms. Each region uses distinct mechanisms to establish and maintain silencing, and I will describe these mechanisms in the next few sections.

1.3.4.1 Telomeric silencing

Telomeres are a specialized structure found on the ends of linear chromosomes. The purpose of this specialized structure is to maintain genomic integrity by protecting the ends of the chromosome from degradation or improper DNA fusions. Yeast telomeres are maintained in a mechanism that is similar, but not identical, to those used in higher eukaryotes. Unlike the rest of the genome, the DNA at the ends of the chromosome is not found in nucleosomes. The DNA sequence at the ends of the chromosome is made up of 350 +/- 75 basepairs of repetitive sequence (5'-TG_{1-3}-3') (WRIGHT *et al.* 1992). These repeats are bound by non-histone proteins, including Rap1 (CONRAD *et al.* 1990; GILSON *et al.* 1993; WRIGHT and ZAKIAN 1995), Cdc13 (BOURNS *et al.* 1998; TSUKAMOTO *et al.* 2001) and Hdf1 and Hdf2 (yeast Ku complex) (GRAVEL *et al.* 1998; MARTIN *et al.* 1999), resulting in the end of the chromosome folding back on itself, thereby protecting the end from exonucleases and illegitimate DNA fusions (DE BRUIN *et al.* 2000; STRAHL-BOLSINGER *et al.* 1997). This complex is structurally different from those found in higher eukaryotes because it does not include a DNA-strand invasion mechanism (DE BRUIN *et al.* 2000).

S. cerevisiae contain other repetitive DNA sequences that are found adjacent to the telomeric repeats described above, known as Y' and X (reviewed in THAM and ZAKIAN 2002). The DNA sequences adjacent to these telomeric repeats are found in nucleosomes (WRIGHT *et al.* 1992). Genes located in this region are subject to reversible transcriptional repression that is position and chromatin dependent, a consequence known as telomere position effect (TPE) (GOTTSCHLING *et al.* 1990). Transcriptional silencing in this region depends on many proteins, including those mentioned above and the four Sir proteins (Sir1-4) (HECHT *et al.* 1996; STRAHL-BOLSINGER *et al.* 1997) (Figure 10). Rap1 and the Ku complex bind to the telomeric repeats

(TG_{1-3}) and are thought to recruit Sir4 (HOPPE *et al.* 2002; LUO *et al.* 2002; MARTIN *et al.* 1999). Telomeric silencing is thought to spread as Sir2 deacetylates histone H3 and H4 tails, which provides high affinity binding sites for Sir3 and Sir4 (reviewed in RUSCHE *et al.* 2003; THAM and ZAKIAN 2002). Repetition of this deacetylation/tail-binding cycle results in a highly compact chromatin structure that excludes many DNA binding proteins, including endonucleases and transcriptional activators and repressors.

While the actual mechanism of silencing differs between budding yeast, fission yeast and humans, there are some generalizations that can be made about the silencing processes among these organisms. The mechanism of silencing is poorly characterized in humans (BAUR *et al.* 2001; KOERING *et al.* 2002). The telomeric DNA in all organisms is TG-rich and is bound by sequence-specific DNA binding proteins (reviewed in PERROD and GASSER 2003). Heterochromatin formation in higher eukaryotes is associated with histone H3 K9 methylation by Su(var)3-9 (MIAO and NATARAJAN 2005; SCHOTTA *et al.* 2002; STEWART *et al.* 2005). Reminiscent of *S. cerevisiae* Sir complex function, hypoacetylated histones with H3 K9 methylation are bound by HP1 in heterochromatic regions, which maintains the silenced state (JACOBS *et al.* 2001; SCHOTTA *et al.* 2002). Fission yeast contain histone H3 K9 methylation, an HP1 homolog known as Swi6, and use a telomeric silencing mechanism similar to humans (NAKAYAMA *et al.* 2001).

Telomeric silencing is more easily perturbed than mating loci silencing due to the fact that the *HM* genes have weak promoters that are more easily silenced (reviewed in VAN LEEUWEN and GOTTSCHLING 2002). Overexpression and loss of function mutations in many genes have been shown to disrupt telomeric silencing. Not surprisingly, strains that contain mutations that disrupt the cellular levels of the Sir proteins (APARICIO *et al.* 1991) or contain

mutations in the region of Rap1 that is important for interacting with Sir4 (KYRION *et al.* 1993; MORETTI and SHORE 2001) lose telomeric silencing. Overexpression or deletion of Dot1, the histone H3 K79 methyltranferase, or Set1, the histone H3 K4 methyltransferase, results in the loss of telomeric silencing (SINGER *et al.* 1998). These methylation events depend on the Paf1 complex member Rtf1, and not-suprisingly, strains lacking *RTF1* also lose telomeric silencing (NG *et al.* 2003a). These effects have been attributed to the redistribution of the Sir proteins from the telomeres to the rest of the genome, which is hypomethylated in the absence of these factors, much like the telomeric regions in wild type strains (NG *et al.* 2003a).

1.3.4.2 Silencing of the mating type cassettes

Haploid *S. cerevisiae* strains are either **a** or α mating types. Yeast secrete phereomones that correspond to their mating type in order to mate with cells of the opposite mating type. The mating type of a given strain is determined by the *MAT* locus, which can be either *MAT***a** or *MATα*. The *MAT* loci encode master transcriptional regulators which control the expression of a large number of genes. Yeast contain unexpressed versions of the **a** or α information at the silenced *HMR* and *HML* loci. Some yeast strains can switch mating types using a mechanism similar to gene conversion to enchange the gene found at the *MAT* locus with the gene found at either of the *HM* loci (reviewed in RUSCHE *et al.* 2003). This process utilizes the HO endonuclease. However, most laboratory yeast strains do not encode an active HO enzyme but the strains still maintain the silenced mating loci.

The *cis*-acting DNA sequences called the *E* and *I* silencers flank the *HML* and *HMR* loci, and are required for establishing and maintaining silencing in these regions (BRAND *et al.* 1985; FELDMAN *et al.* 1984; MAHONEY and BROACH 1989). Origin recognition complex (ORC) subunits, Abf1 and Rap1 bind to silencer sequences and recruit the Sir proteins to establish

silencing at these loci (BRAND *et al.* 1985; BUCHMAN *et al.* 1988; MAHONEY and BROACH 1989; MAHONEY *et al.* 1991; SHORE and NASMYTH 1987; SHORE *et al.* 1987) (Figure 10). All four Sir proteins (Sir1-4) are required for silencing at the *HML* and *HMR* loci (HABER and GEORGE 1979; HOPPER and HALL 1975; IVY *et al.* 1985; IVY *et al.* 1986; KLAR *et al.* 1979; RINE *et al.* 1979) and are recruited to these regions through physical interactions with the DNA binding proteins (RUSCHE *et al.* 2002; TRIOLO and STERNGLANZ 1996). Strains that lose silencing in this region express both mating pheromones and the haploid strain acts like a diploid (reviewed in LAURENSON and RINE 1992). Specifically, these cells do not mate with cells of the opposite mating type and do not respond to the mating pheromones.

Loss of function mutations have been shown to disrupt silencing of the mating loci. As stated above, the Sir proteins are required for establishing and maintaining silencing at the mating loci (HABER and GEORGE 1979; HOPPER and HALL 1975; IVY *et al.* 1985; IVY *et al.* 1986; KLAR *et al.* 1979; RINE *et al.* 1979). Mutations within *MCM10*, which encodes a DNA binding protein that is important for the initiation of DNA replication, have been shown to disrupt silencing of the mating loci (DOUGLAS *et al.* 2005). This is thought to be the result of the loss of a physical interaction between Sir2, Sir3 and Mcm10, which may be important for recruiting the Sir proteins to silenced region (DOUGLAS *et al.* 2005). Strains that contain mutations in the genes encoding Sas2, the catalytic subunit of the SAS histone acetyltransferase complex, or Asf1, a histone chaperone protein, exhibit defects in silencing of the mating loci (OSADA *et al.* 2005). While a mechanistic understanding of this observation is lacking, this suggests that SAS activity and Asf1 function are required for *HM* silencing (OSADA *et al.* 2005).

1.3.4.3 rDNA silencing

The ribosomal DNA (rDNA), found on chromosome XII in yeast, consists of 100-200 tandem

copies of 9.1 kb units that encode for ribosomal RNAs (reviewed in SMITH and BOEKE 1997). Each repeat encodes a copy of the 5S rRNA gene that is transcribed by RNA Pol III, and a copy of a 35S pre-rRNA gene that is transcribed by RNA Pol I (reviewed in SMITH and BOEKE 1997). The 35S pre-rRNA is ultimately processed into the 5.8S, 25S, and 18S rRNAs (reviewed in ZANCHIN and GOLDFARB 1999). These two genes are separated by non-transcribed spacer (NTS) regions (Figure 10). Because of the repetitive nature of this large genomic region, silencing mechanisms exist to prevent aberrant recombination of these essential genes. Interestingly, RNA Pol II transcribed genes are expressed when relocated to the rDNA repeats, suggesting that the rDNA is not completely repressed (KEIL and MCWILLIAMS 1993; SZOSTAK and WU 1980).

There appear to be inactive regions within the rDNA repeats that are associated with nucleosomes, and the actively transcribed repeats remain nucleosome-free (DAMMANN *et al.* 1993). Sir2 is the only Sir protein required for rDNA silencing (GOTTLIEB and ESPOSITO 1989), and strains lacking *SIR2* exhibit increased expression of rDNA repeats (SMITH and BOEKE 1997). Interestingly, while Sir3 and Sir4 are not involved in the spreading of silencing at rDNA, RNA Pol I has been shown to propagate Sir2 association within the rDNA in the direction of transcription (BUCK *et al.* 2002).

Several loss of function mutations have been shown to disrupt rDNA silencing. As stated above, strains lacking *SIR2* have rDNA silencing defects (GOTTLIEB and ESPOSITO 1989; SMITH and BOEKE 1997). Strains containing mutations in COMPASS subunits (histone H3 K4 methyltransferase complex) have been shown to disrupt rDNA silencing (MUELLER *et al.* 2006). Also, because the Paf1 complex is required for histone H3 K4 methylation by the COMPASS complex, strains that lack members of the Paf1 complex also have rDNA silencing defects (MUELLER *et al.* 2006). Mutations in the gene that encodes Ubp10, a histone H2B

deubiquitylating enzyme, also disrupt rDNA silencing (CALZARI *et al.* 2006). This silencing defect is the result of the loss of Sir2 association with the rDNA repeats (CALZARI *et al.* 2006).

1.4 THE UBIQUITIN-PROTEASOME PATHWAY IS CONNECTED TO RNA POL II TRANSCRIPTION

Surprisingly, components of the cellular machinery that are important for tagging and degrading aberrant proteins are functionally connected to the regulation of transcription. This section will discuss the components of the degradation machinery and how these factors are known to impact transcription at many levels.

Table 2. Ubiquitin related factors.

Factor	Characteristic
19S proteasome	"Cap" subcomplex of the 26S proteasome; Acts to specify which proteins are degraded; Unfolds proteins prior to entry into the 20S core
20S proteasome	Catalytic "core" subcomplex of the 26S proteasome; Three proteases degrade proteins within the central cavity of this complex
26S proteasome	Protein complex that selectively degrades misfolded or unwanted proteins within the cell; Utilizes several proteases to perform its function; Composed of the 19S and 20S subcomplexes
Bre1	RING finger protein; Recruits Rad6 to chromatin to catalyze the ubiquitylation of histone H2B at lysine 123; Ubiquitin protein ligase (E3)
Dsk2	Selectively targets poly-ubiquitylated proteins to the proteasome for destruction; Functionally overlaps with Rad23
DUB	De-ubiquitylating enzyme; removes ubiquitin groups that have been covalently attached to substrate proteins
E1 (Uba1)	Ubiquitin-activating enzyme; Activates ubiquitin through an ATP dependent process; Passes ubiquitin to E2s; Yeast contain 1 E1 enzyme
E2 (Ubc proteins)	Ubiquitin-conjugating enzyme; Receives ubiquitin from E1; Conjugates ubiquitin to substrate proteins with the help of E3s; Yeast contain 11 ubiquitin E2s
E3	Ubiquitin-protein ligase; Catalyzes the transfer of ubiquitin from the E2 to the substrate protein; Important for defining substrate specificity to E2s; Often contain a RING or HECT domain that interacts with the E2

HECT domain	Secondary structure formed by the chelation of 2 zinc ions with 8 conserved cysteine and histidine residues within a protein; Proteins that contain this domain often act as E3s to define substrate specificity to the ubiquitylation pathway; HECTs act as an intermediate in the ubiquitylation pathway
Not4	Ubiquitin protein ligase that targets EGD complex (transcription and ribosome related functions); Required for histone H3 K4 methylation, but not histone H2B ubiquitylation
Rad23	Selectively targets poly-ubiquitylated proteins to the proteasome for destruction; Functionally overlaps with Dsk2
Rad6	Ubiquitin-conjugating enzyme (E2); Ubiquitylates many proteins in yeast, including histone H2B at lysine 123
RING domain	Secondary structure formed by the chelation of 2 zinc ions with 8 conserved cysteine and histidine residues within a protein; Proteins that contain this domain often act as E3s to define substrate specificity to the ubiquitylation pathway
Rpn/Rpt proteins	19S proteasome proteins; Rpt proteins are AAA+ ATPases
San1	RING finger protein; Recruits Cdc34 to ubiquitylate misfolded or mutant transcription factors within the nucleus; Ubiquitin protein ligase (E3)
SUMO	Ubiquitin-like post-translational protein modification; This modification is often associated with transcriptional repression
Ubiquitin	76 amino acid post-translational modification; Mono-ubiquitylation changes the function of protein; Poly-ubiquitylation targets a protein for destruction

1.4.1 What is ubiquitylation?

Protein ubiquitylation is a highly regulated process that is often the first step in targeting a protein for degradation. Ubiquitin is a 76 amino acid peptide that is post-translationally added to a substrate protein at lysine residues. Ubiquitylation regulates many cellular processes including, but not limited to, cell cycle progression (KOEPP *et al.* 1999), transcription (BOEGER *et al.* 2005), and DNA repair (HOFMANN and PICKART 1999; SPENCE *et al.* 1995). Up to 20% of yeast proteins are targeted for ubiquitylation when the cells are grown in rich medium (PENG *et al.* 2003; WELCHMAN *et al.* 2005).

Proteins can be monoubiquitylated or polyubiquitylated. Monoubiquitylation can change the affinity of a given protein for another protein or change the enzymatic activity of the ubiquitylated protein (reviewed in FERDOUS *et al.* 2007; SOMESH *et al.* 2007). Polyubiquitylation occurs when ubiquitin chains are formed on a substrate protein. Ubiquitin contains seven conserved lysines itself (K6, 11, 27, 29, 33, 48, and 63), all of which are potential branching sites for polyubiquitylation chains. However, in yeast, only K27- and K48-linked ubiquitin chains are utilized in targeting proteins for degradation (GLICKMAN and CIECHANOVER 2002). Polyubiquitylation often targets a protein for degradation by the proteasome (PICKART 2001; VARSHAVSKY 1997) (see section 1.4.3 for more information).

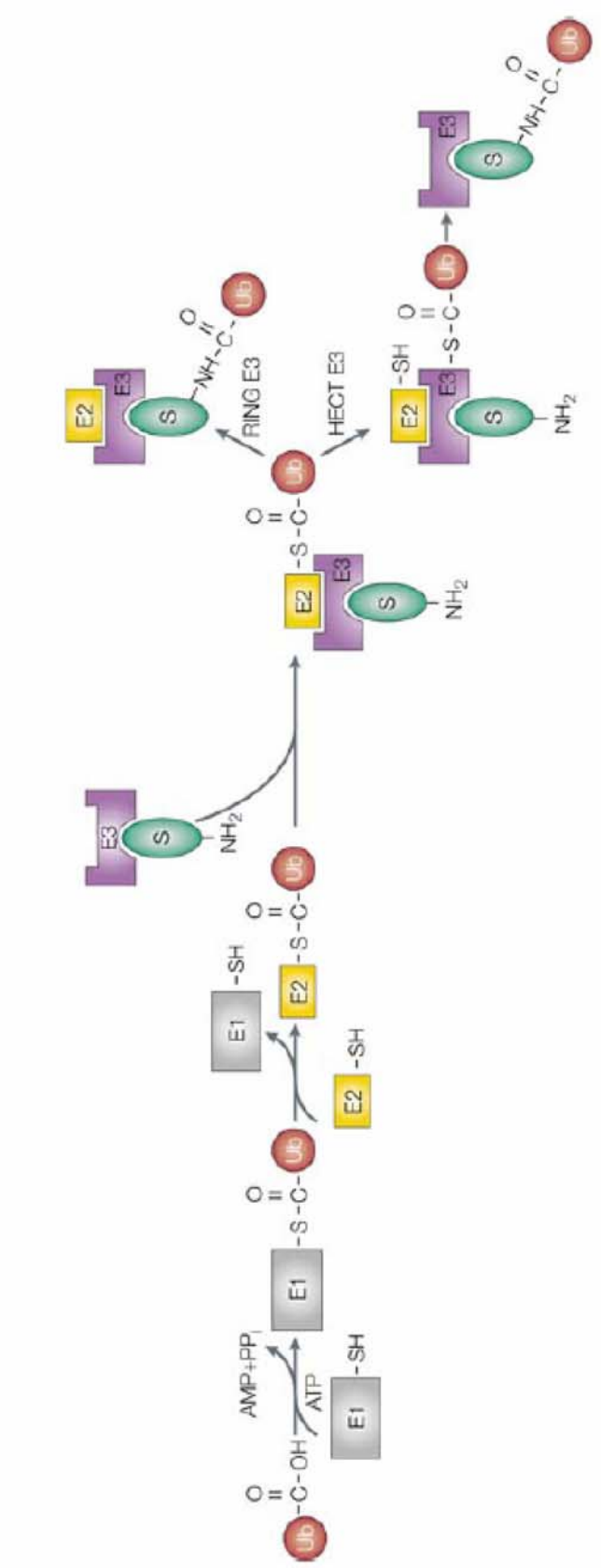
1.4.2 Components of the ubiquitylation pathway

Covalent attachment of ubiquitin to a substrate protein requires the coordinated functions of a ubiquitin-activating enzyme, a ubiquitin-conjugating enzyme, and a ubiquitin-protein ligase

(reviewed in FANG and WEISSMAN 2004; PICKART 2001) (Figure 11). While yeast contain only one ubiquitin-activating enzyme (Uba1) and eleven ubiquitin-conjugating enzymes (Ubc1-8, Ubc10, Ubc11 and Ubc13), there are many predicted ubiquitin-protein ligases, consistent with their proposed roles in determining substrate specificity (reviewed in PICKART 2001). Ubiquitylation is also reversible through the action of deubiquitylation enzymes. The next several paragraphs will describe the roles of each of these factors in the ubiquitylation pathway.

Figure 11. The ubiquitylation pathway.

Ubiquitylation of a substrate protein occurs through multiple steps. E1 (gray), E2 (purple), E3 (yellow), ubiquitin (red) and substrate (teal) proteins are shown. See text for additional details. From Fang, S. and A. Weissman. (2004) *Cell Mol Life Sci*. **61**:1546-61.


Ub
ATP
AMP+PP
E1
-SH
E2
E3
S
RING E3
HECT E3
NH2

1.4.2.1 Ubiquitin-activating enzymes

The ubiquitin activating enzyme (also known as E1) forms a thiol ester bond between the active site cysteine within the E1 and the carboxy-terminal glycine of ubiquitin (HAAS and ROSE 1982; HAAS *et al.* 1982). To accomplish this reaction, the E1 binds to Mg-ATP and then ubiquitin to form a ubiquitin adenylate intermediate that serves as the donor for the active site cysteine (HAAS and ROSE 1982; HERSHKO *et al.* 1983). Each E1 molecule carries two ubiquitin groups, one as an adenylate intermediate and the other at the active site through a thiol ester bond (reviewed in PICKART 2001). The active site ubiquitin is transferred to the ubiquitin-conjugating enzyme (Figure 11).

1.4.2.2 Ubiquitin-conjugating enzymes

Ubiquitin-conjugating enzymes (also known as E2s) contain a conserved 150 amino acid UBC domain that contains an active site cysteine that accepts the ubiquitin group from the E1 (PICKART 2001). Some E2s also contain amino-or carboxy-terminal extensions around the UBC domain that are required for E3 and substrate interactions (PICKART 2001). While there is a single E1 that is found throughout the cell, E2s confine some selection on substrate specificity through interactions with a subset of E3s and through localization within certain cellular compartments (HOCHSTRASSER 1996; PICKART 2001) (Figure 11). Transfer of ubiquitin to the E2 is rapid, and the downstream transfer to substrates is the rate limiting step in the ubiquitylation pathway (HAAS and BRIGHT 1988). The low number of E2s relative to E3s suggest that each E2 works with multiple E3s, however, *in vitro* experiments show that many E3s can catalyze the transfer of ubiquitin to substrates in combination with several E2 enzymes (FANG and WEISSMAN 2004).

1.4.2.3 Ubiquitin-protein ligases

Ubiquitin-protein ligases (also known as E3s) contain functional domains known as RING or HECT domains that interact with ubiquitin-conjugating enzymes (HUIBREGTSE *et al.* 1995; LORICK *et al.* 1999) (Figure 11). RING and HECT domains differ in both sequence and structure (BORDEN 2000; HUANG *et al.* 1999; ZHENG *et al.* 2000), but both domains interact with E2s to facilitate substrate ubiquitylation. For example, the mammalian E2 UbcH7 interacts with the HECT domain E3, E6-AP (HUANG *et al.* 1999; KUMAR *et al.* 1997; NUBER *et al.* 1996), and several RING containing E3s, including c-Cbl, HHARI, and H7-AP1 (MOYNIHAN *et al.* 1999; YOKOUCHI *et al.* 1999; ZHENG *et al.* 2000). RING domains and HECT domains facilitate ubiquitylation through distinct mechanisms that I will describe in the next few paragraphs.

The RING domain was first identified in a protein encoded by *Really Interesting New Gene 1* (FREEMONT *et al.* 1991). Unlike tandem zinc finger motifs, RING domains consist of eight critical amino acids, often 7 cysteine residues and one histidine residue, which bind two zinc ions to form a cross-brace structure (reviewed in JACKSON *et al.* 2000) (Figure 12). The consensus RING sequence is C-x_2-C-x_{9-39}-C-x_{1-3}-C/H-x_{2-3}-C/H-x_2-C-x_{4-48}-C-x_2-C, where C represents a cysteine residue, H represents a histidine residue, and x represents any amino acid (FANG and WEISSMAN 2004). However, some RING domains contain eight cysteines or amino acids other than cysteine and histidine in the zinc-binding positions (YANG *et al.* 2005).

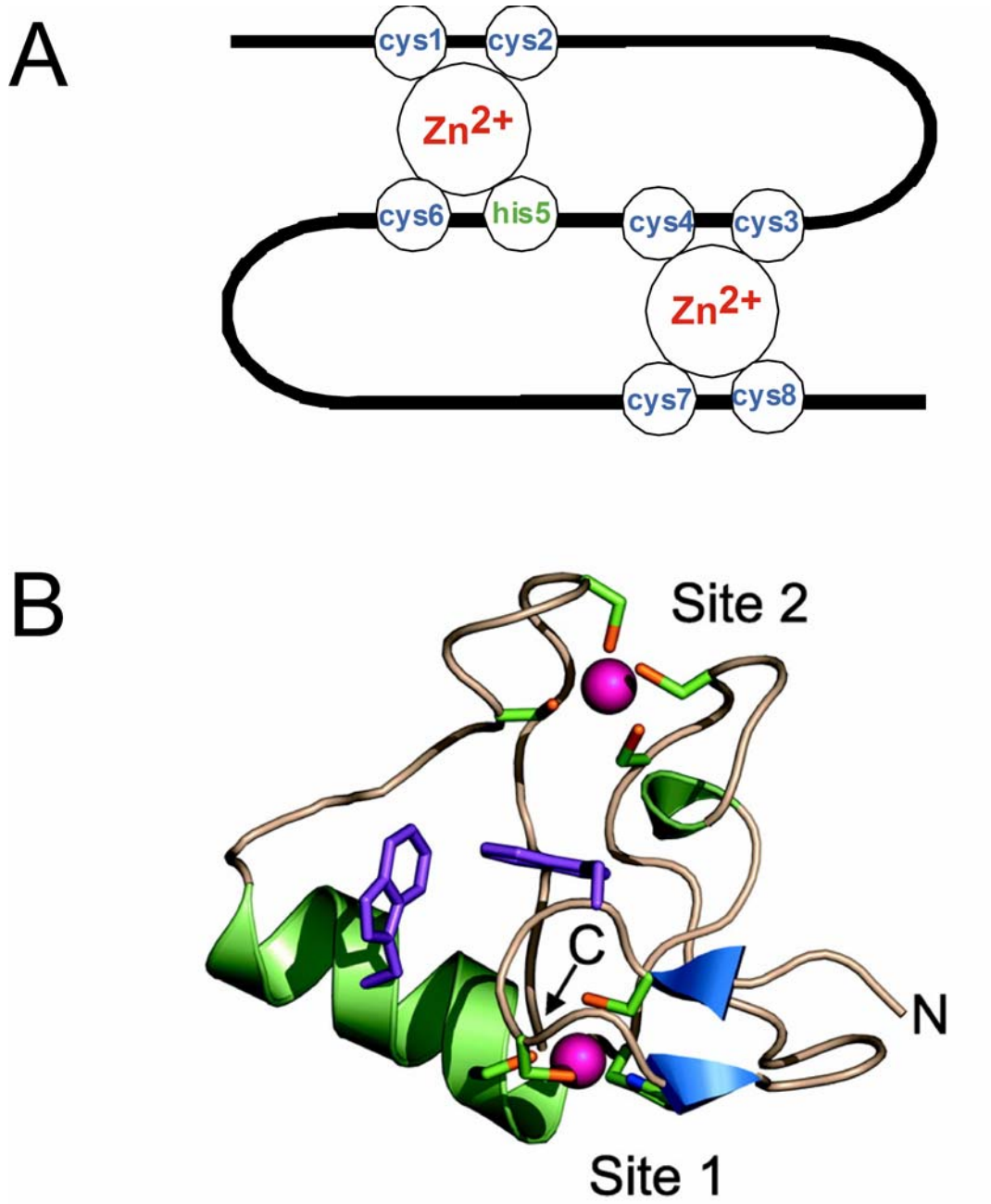
RING domain-containing E3s catalyze the transfer of ubiquitin from the E2 to the substrate by interacting with the E2 through the RING domain and interacting with the substrate in a region outside of the RING domain (FANG and WEISSMAN 2004). A general mechinsm for how RING E3s recognize their substrates is not understood (FANG and WEISSMAN 2004), although some E3s recognize specific sequences within their targets. Specifically, the anaphase

promoting complex (APC) recognizes its targets through motifs called "KEN boxes" (CHEN *et al.* 2002; DESHAIES 1999; HENDRICKSON *et al.* 2001) and "A boxes" (HARPER *et al.* 2002; LITTLEPAGE and RUDERMAN 2002).

HECT domain containing proteins were first identified as having homology with the carboxy terminus of E6-AP, which mediates the human papillomavirus encoded E6-dependent ubiquitylation of p53 in mammalian cells (SCHEFFNER *et al.* 1994). HECT proteins contain a conserved 350 amino acid domain at their carboxy termini (FANG and WEISSMAN 2004) while the amino termini interact with substrates and determine the cellular localization of the protein (WEISSMAN 2001). Unlike RING domain-containing E3s, HECT domain proteins are intermediates in the ubiquitylation pathway (reviewed in FANG and WEISSMAN 2004)). The amino terminus of the HECT protein interacts with the substrate while a conserved cysteine residue within the HECT domain accepts the ubiquitin group from the E2. The HECT protein then transfers ubiquitin to the substrate. A subset of HECT domain proteins contain WW domains that recognize PPXY or PPLP sequences within their substrates (MACIAS *et al.* 2002; SUDOL *et al.* 2001). WW domains consist of tryptophan-rich sequences that are approximately 35 amino acids in length (FANG and WEISSMAN 2004). Little is known about the process by which HECT domain proteins that do not contain WW domains interact with their substrates.

Figure 12. RING domain architecture.

A) Schematic representing RING domain architecture. Seven cysteines and one histidine residue chelate two zinc ions to form a cross-brace structure as shown. B) Solution structure of the RING domain from the Kaposi's sarcoma-associated herpesvirus protein K3. This RING domain consists of the C4HC3 pattern of conserved zinc chelating residues. Zinc ions are represented by magenta spheres, side chains of chelating amino acids are shown, and the conserved tryptophan residues found within RING domains are highlighted in purple. Alpha helices are green and beta sheets are blue. From Dodd, R. B. *et al*. (2004). *J. Biol. Chem*. **279**:53840-53847.


A
cys1
cys2
Zn2+
cys6
his5
cys4
cys3
Zn2+
cys7
cys8
B
Site 2
C
N
Site 1

1.4.2.4 Deubiquitylating enzymes

Deubiquitylating enzymes (DUBs) reverse the activities of E2s and E3s. Most DUBs are cysteine proteases, which means that the active site cysteine performs a nucleophilic attack on the bond that attaches ubiquitin to the substrate, forming an intermediate in which ubiquitin is covalently bound to the protease (reviewed in NIJMAN *et al.* 2005). These DUBs fall into one of two classes, 1) ubiquitin carboxy-terminal hydrolases (UCHs), 2) ubiquitin-specific processing proteases (UBPs), (reviewed in NIJMAN *et al.* 2005). The structures of these DUBs are distinct, despite the fact that both classes cleave ubiquitin (NIJMAN *et al.* 2005). Each of these classes of enzymes target specific types of ubiquitylated targets. For example, UBPs are important for cleaving and disassembling polyubiquitin chains (FANG and WEISSMAN 2004; NIJMAN *et al.* 2005).

The *S. cerevisiae* genome encodes for 17 putative DUBs, 16 of which fall into the UBP class (HOCHSTRASSER 1996). A significant amount of work has shown that Doa4 is a DUB in yeast with a wide range of functions. Doa4 works late in the ubiquitin-proteasome pathway by recycling ubiquitin from proteins targeted to the proteasome (PAPA and HOCHSTRASSER 1993). In fact, most of the Doa4 protein in the cell is physically associated with the proteasome (PAPA *et al.* 1999). Doa4 activity has been shown to be important for the degradation of many proteins in yeast, including the α2 repressor (PAPA and HOCHSTRASSER 1993). Doa4 overexpression leads to accelerated α2 repressor degradation, suggesting that Doa4 levels are limiting *in vivo* (PAPA and HOCHSTRASSER 1993).

Interestingly, mutations of specific DUBs in metazoans are correlated with disease, including embryonic development defects and tumor suppression and growth. This suggests that

DUBs do not act stochastically in the cell and at least some DUBs may target specific substrates (FANG and WEISSMAN 2004). Specifically, HAUSP (herpes virus-associated ubiquitin-specific protease) deubiquitylates p53 *in vitro* and *in vivo* and stabilizes p53 protein levels (LI *et al.* 2002).

1.4.3 The proteasome degrades ubiquitylated proteins

Once a protein has been polyubiquitylated, it is targeted for destruction by the 26S proteasome. Several factors in the cell ensure that this process is efficient and thorough. In this section, I will describe what happens to a protein after it is polyubiquitylated.

1.4.3.1 How do ubiquitylated proteins get to the proteasome?

Substrate proteins that have had a chain of at least four ubiquitin groups attached to them are targeted for destruction by the 26S proteasome (PICKART 2001; VARSHAVSKY 1997). The proteasome subunit Rpn10 (S5a in humans) binds to chains of ubiquitin at least 4 groups long through its ubiquitin-interacting motif (UIM) (DEVERAUX *et al.* 1994). Rpn10 functionally overlaps with related factors Dsk2 (Dph1 in *S. pombe*) and Rad23 (Rph23 in *S. pombe*) (CHEN and MADURA 2002; FUNAKOSHI *et al.* 2002; LAMBERTSON *et al.* 1999; RAO and SASTRY 2002; SCHAUBER *et al.* 1998; WILKINSON *et al.* 2001). These proteins are conserved in eukaryotes from yeast to humans (HARTMANN-PETERSEN *et al.* 2003). Dsk2 and Rad23 interact with polyubiquitylated proteins using ubiquitin-associated (UBA) domains (HOFMANN and BUCHER 1996) and transiently interact with the 19S proteasome subunit Rpn1 via ubiquitin-like (UBL) domains (ELSASSER *et al.* 2002). Thus, these proteins act as "destruction chaperones" by targeting polyubiquitylated proteins to the proteasome for degradation (HARTMANN-PETERSEN *et*

al. 2003).

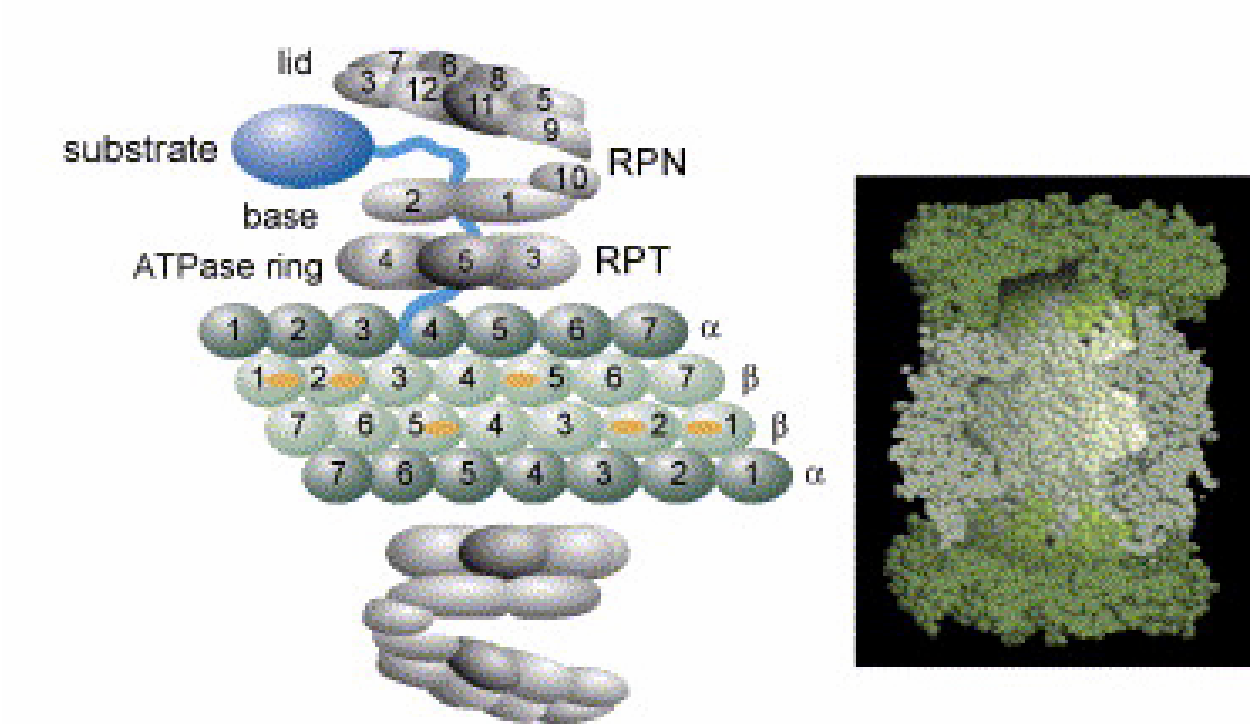
This is not the only mechanism that cells use to target ubiquitylated proteins for destruction. Several components of the ubiquitylation machinery in *S. cerevisiae* and humans have also been shown to physically interact with the proteasome. Specifically, the yeast E3, Ubr1, binds to the proteasome subunit Rpn2 (XIE and VARSHAVSKY 2000), and in humans, the E3 KIAA10 interacts with the proteasome subunit S2 (YOU and PICKART 2001). These results suggest that the ubiquitylation machinery may tag proteins for destruction at the same time that they take them to the proteasome.

1.4.3.2 The 26S proteasome selectively degrades ubiquitylated proteins

Studies using the model organism *Saccharomyces cerevisiae* led the way in determining that the proteasome degraded ubiquitylated proteins in eukaryotes. Yeast that contained a mutation that disrupted the chymotrypsin-like activity of cells led to an accumulation of ubiquitylated proteins (HEINEMEYER *et al.* 1991). The proteasome is made up of two sub-complexes, the 19S and 20S proteasomes (reviewed in PETERS 1994) (Figure 13). These complexes utilize distinct functions to tightly regulate protein degradation, which I will describe in the next few paragraphs.

Figure 13. The proteasome structure.

The proteasome is composed of 19S and 20S subunits. The 19S subunit consists of Rpt and Rpn proteins, and the 20S subunit consists of α and β proteins. The 20S subunits with yellow dots possess proteolytic activity. Space filling model represents the three-dimensional structure of the proteasome. From Wolf and Hilt (2004) *Biochim Biophys Acta*. **1695**:19-31.


lid
3
7
12
6
11
8
5
9
substrate
RPN
10
2
1
base
ATPase ring
4
5
3
RPT
1 2 3 4 5 6 7 α
1 2 3 4 5 6 7 β
7 6 5 4 3 2 1 β
7 6 5 4 3 2 1 α

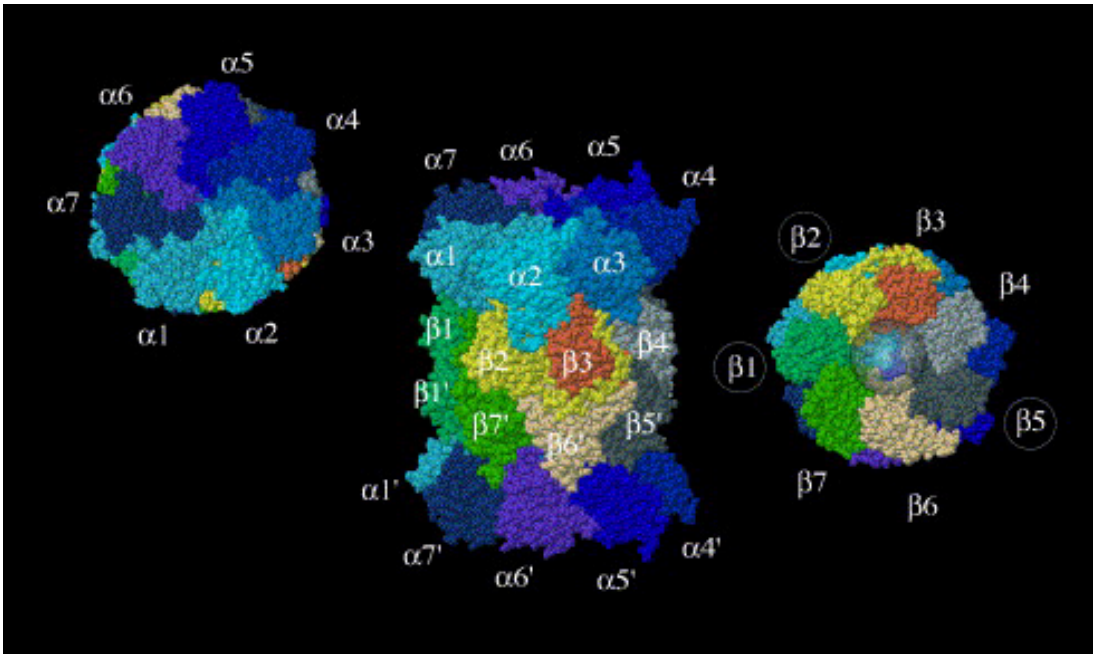
The 19S proteasome in yeast consists of more than 19 subunits (reviewed in WOLF and HILT 2004). The Rpn (regulatory particle non-ATPase) and Rpt (regulatory particle triple A protein) proteins of the 19S subunit form the lid and base subcomplexes, respectively (reviewed in WOLF and HILT 2004) (Figure 13). Rpn10 physically connects these two subcomplexes (reviewed in WOLF and HILT 2004). The 19S proteasome performs several functions to control protein degradation. It: 1) recognizes proteins that are tagged for degradation, 2) unfolds these proteins, 3) cleaves ubiquitin tags from proteins, 4) allows access to the 20S core, and 5) unfolds the protein prior to feeding it into the 20S core (reviewed in WOLF and HILT 2004). Rpn10 and Rpt5 have been shown to recognize polyubiquitylated proteins (DEVERAUX *et al.* 1994; ELSASSER *et al.* 2002; LAM *et al.* 2002). Alternatively, Dsk2 or Rad23 can chaperone ubiquitylated proteins to the proteasome via interactions with Rpn1 (ELSASSER *et al.* 2002; KIM *et al.* 2004a; SAEKI *et al.* 2002). Rpn11 is a DUB that is a component of the 19S proteasome (VERMA *et al.* 2002; YAO and COHEN 2002), but other DUBs, including Doa4 and Ubp6, have been shown to interact with the proteasome and remove ubiquitin from proteins that are to be degraded (LEGGETT *et al.* 2002; PAPA *et al.* 1999). The 19S subunit Rpt2 is thought to be the gatekeeper of the 20S core, since it is required for displacement of the α subunits from the 20S core (KOHLER *et al.* 2001). Little is known about how proteins are denatured prior to entering the 20S proteasome, but the ATPases Rpt1-6 are thought to provide this function to the 19S proteasome (RUBIN *et al.* 1998).

The 20S subunit of the proteasome structure is conserved in eukaryotes and prokaryotes, and consists of a barrel-shaped complex (HEGERL *et al.* 1991) formed by four stacks of α and β subunits with the α subunits on the outside layers and the β subunits within the inner layers (GROLL *et al.* 1997; GRZIWA *et al.* 1991; LOWE *et al.* 1995; PUHLER *et al.* 1992) (Figure 14).

There are seven different α and β subunits within each layer of the eukaryotic 20S proteasome (LOWE *et al.* 1995), which are encoded by 14 genes in yeast (HEINEMEYER *et al.* 1994). The 20S proteasome degrades proteins into small peptides and single amino acids that can be recycled into new proteins. Three different β subunits of the eukaryotic proteasome confer trypsin-like, chymotrypsin-like and peptidyl-glutamyl-peptide cleaving activities to degrade proteins (CHEN and HOCHSTRASSER 1996; GROLL *et al.* 1997; HEINEMEYER *et al.* 1997). The 20S proteasome can only degrade denatured proteins, limiting the destructive activity of this complex (reviewed in WOLF and HILT 2004).

Figure 14. The 20S proteasome structure.

The 20S proteasome is a barrel shaped structure consisting of layers of α and β subunits. Seven different α subunits and seven different β subunits comprise each layer. See text for additional details. From Wolf and Hilt. (2004) *Biochim Biophys Acta*. **1695**:19-31.


α5
α6
α4
α7
α3
α1
α2
α7
α6
α5
α4
α1
α2
α3
β1
β2
β3
β4
β1'
β7'
β6'
β5'
α1'
α7'
α6'
α5'
α4'
β2
β3
β4
β1
β5
β7
β6

1.4.4 The ubiquitin-proteasome machinery functions within the nucleus

The ubiquitin-proteasome machinery localizes to the nucleus and affects many processes, including chromatin structure, DNA replication and repair, and transcription. In fact, some components of the ubiquitylation machinery are specific to the nucleus, like the E2 Cdc34 and several E3s, including San1 and Bre1 (described in more detail in section 1.4.4.2). In the next few sections I will focus on the functional connections between the ubiquitin-proteasome machinery and transcription. Specifically, I will spotlight several characterized nuclear, RING domain-containing proteins and their targets.

1.4.4.1 Proteolytic and nonproteolytic activities of the proteasome regulate many nuclear events

The proteasome itself has been shown to reside within the nucleus and regulate many nuclear processes, including DNA repair and transcription. Specifically, the proteasome has been shown to regulate gene expression through both proteolytic and nonproteolytic mechanisms (reviewed in COLLINS and TANSEY 2006). The 19S and 20S proteasome subunits localize to most genes in yeast in a transcription dependent manner, however not all genes were bound by both subunits, suggesting that each subunit affects transcription through a unique mechanism (SIKDER *et al.* 2006).

When arrested in transcription elongation by DNA damage, Pol II is polyubiquitylated and degraded as part of the repair process (BEAUDENON *et al.* 1999; BREGMAN *et al.* 1996; LUO *et al.* 2001; RATNER *et al.* 1998). *In vitro* experiments with highly purified components show that stalled elongation complexes are targeted for ubiquitylation by the ubiquitin-protein ligase

Def1 (SOMESH *et al.* 2005; WOUDSTRA *et al.* 2002). Serine 5 phosphorylation on the CTD of RNA Pol II (discussed in section 1.2.2) inhibits ubiquitylation, suggesting that the ubiquitylation machinery uses the modification state of the CTD as a signal to specifically target elongating RNA Pol II and not free or initiating Pol II during transcription-coupled DNA repair (SOMESH *et al.* 2005). Very recently, the HECT domain E3 Rsp5 was shown to bind to the CTD of RNA Pol II and direct ubiquitylation of the largest subunit of RNA Pol II (Rpb1) (SOMESH *et al.* 2007). Interestingly, this study shows that the E2, Ubc5, recognizes two different lysine residues within the body of Rpb1 (K330 and K695) and recruits Rsp5 to catalyze the codependent ubiquitylation of these sites (SOMESH *et al.* 2007). Strains containing mutations of these lysine residues are sensitive to 6-azauracil and UV irradiation, suggesting that these ubiquitylation modifications are important for regulating transcription elongation and DNA damage repair (SOMESH *et al.* 2007).

Several proteins important for Pol II transcription are modified by the protein ubiquitylation machinery within the nucleus (reviewed in LIPFORD and DESHAIES 2003; MURATANI and TANSEY 2003). Ubiquitylation of numerous transcriptional activators has been observed and for several of these, including the well-studied yeast activators Gcn4 and Gal4, ubiquitylation and proteasome-dependent proteolysis correlate with gene activation (LIPFORD *et al.* 2005; MURATANI and TANSEY 2003). However, whether proteolytic turnover of activator proteins is obligatory for activation remains a topic of debate (NALLEY *et al.* 2006). Very recently, Ferdous and colleagues showed that the proteasomal ATPases are recruited to promoters to encourage promoter escape and transcription elongation by disrupting activator-DNA interactions (FERDOUS *et al.* 2007). However, monoubiquitylation of the activator proteins prevents disruption of the activator-DNA contacts by the 19S proteasome, suggesting that monoubiquitylation increases the performance of the activator (FERDOUS *et al.* 2007).

Components of the proteasome 19S regulatory complex facilitate interactions between the SAGA histone acetyltransferase complex and transcriptional activators (LEE *et al.* 2005a). Specifically, direct interactions between the 19S proteasome and transcription activators can be detected at low levels (LEE *et al.* 2005a). SAGA is recruited to promoters by activators like Gal4 (see section 1.2.3.2), but this recruitment is increased in a dose-dependent manner by the 19S proteasome (LEE *et al.* 2005a). Direct interactions between the 19S proteasome and Gal4 are not detected *in vivo*, but the 19S proteasome physically recruits SAGA to promoters, where the 19S proteasome promotes physical interactions between Gal4 and SAGA and stimulates SAGA's histone acetyltransferase activity (LEE *et al.* 2005a).

Interestingly, the 19S proteasome is also important for several chromatin modifications. As described in section 1.1.2.2, histone H2B ubiquitylation is required for subsequent methylation of histone H3 at lysines 4 (K4) and 79 (K79). Surprisingly, the proteasomal ATPases Rpt4 and Rpt6 are required to couple these modifications (EZHKOVA and TANSEY 2004). In strains containing mutations that disrupt Rpt4 or Rpt6 function, histone H2B ubiquitylation occurs at similar levels to wildtype strains, but histone H3 K4 and K79 methylation is lost (EZHKOVA and TANSEY 2004). Histone H2B ubiquitylation is required to recruit the 19S proteasome to an activated gene (EZHKOVA and TANSEY 2004). Consistent with a role for these modifications in maintaining telomeric and mating loci silencing (discussed in section 1.3.4.1), strains expressing mutant Rpt4 or Rpt6 have defects in silencing both of these regions (EZHKOVA and TANSEY 2004). However, strains containing mutations in the catalytic subunits of the proteasome, including Rpn4, Rpn10 or Pre1, did not disrupt silencing, suggesting that Rpt4 and Rpt6 affect chromatin modification in a proteolysis-independent manner (EZHKOVA and TANSEY 2004). Ezhkova and colleagues propose that H2B ubiquitylation recruits

the 19S proteasome to promoters, and once transcription begins, the 19S proteasome traverses an open reading frame with RNA Pol II to facilitate Set1 and Dot1 (the K4 and K79 methyltransferases, respectively) activity (EZHKOVA and TANSEY 2004).

1.4.4.2 Nuclear ubiquitin-protein ligases

Several nuclear RING domain-containing ubiquitin protein ligases have been characterized in yeast. These include Rad5 and Rad18, which direct the ubiquitylation of PCNA during DNA damage repair (HOEGE *et al.* 2002). The E3s Rad5 and Rad18 recruit the E2s Ubc13-Mms2 and Rad6, respectively, to chromatin (BAILLY *et al.* 1994; ULRICH and JENTSCH 2000). Rad6 and Rad18 facilitate monoubiquitylation of proliferating cell nuclear antigen (PCNA), and Ubc13-Mms2 and Rad5 facilitate lysine 63-linked polyubiquitylation of PCNA at the same lysine residue (HOEGE *et al.* 2002). PCNA is a DNA polymerase sliding clamp that participates in DNA replication and repair (FUKUDA *et al.* 1995). These modifications are conserved in yeast and humans, and are required for DNA repair (HOEGE *et al.* 2002). While the exact mechanism is unclear, the different modification states are proposed to affect the function of PCNA (HOEGE *et al.* 2002).

San1 is an E3 that is required for the degradation of certain misfolded nuclear proteins (DASGUPTA *et al.* 2004; GARDNER *et al.* 2005). San1 (Sir antagonist 1) was originally identified as a suppressor of a mutation in *SIR4*, a protein required for silencing at telomeres and mating loci (RINE and HERSKOWITZ 1987; SCHNELL *et al.* 1989). San1 contains a noncanonical C3HGC3 RING domain, which possesses ubiquitin-protein ligase activity *in vitro* (DASGUPTA *et al.* 2004; GARDNER *et al.* 2005). A functional understanding of the suppression phenotype came when it was shown that mutations in *SAN1* stabilize Sir4 protein levels, and that San1, along with the E2 Cdc34, is important for targeting mutant Sir4 proteins for destruction by the proteasome

(DASGUPTA *et al.* 2004; GARDNER *et al.* 2005). Microarray experiments were performed on strains lacking *SAN1*. The results show that San1 is not important for the expression of genes when the cells are grown in rich medium (DASGUPTA *et al.* 2004; GARDNER *et al.* 2005). However, when *san1Δ* strains were grown in minimal medium, the expression of 30 genes is affected, with most of these genes encoding proteins involved in cellular stress response (GARDNER *et al.* 2005). These results suggest that San1 is required to degrade mutant proteins *in vivo*, preventing cellular stress by reducing the buildup of aberrant proteins in the nucleus (GARDNER *et al.* 2005).

BRE1 encodes an E3 that, in cooperation with the RING domain protein Lge1, ubiquitylates histone H2B at lysine 123 (K123) (HWANG *et al.* 2003; WOOD *et al.* 2003a). The significance of this modification is described in more detail in section 1.1.2.2. In brief, this modification is associated with active transcription, and is required for subsequent di- and trimethylation of histone H3 at lysines 4 and 79 (WOOD *et al.* 2003b).

The conserved Ccr-Not complex contains nine subunits that regulate transcription at many levels. The Not4 subunit of this complex contains a noncanonical C4C4 RING domain (HANZAWA *et al.* 2001). The E2s Ubc4 and Ubc5 interact with Not4 in a yeast two-hybrid screen (ALBERT *et al.* 2002; WINKLER *et al.* 2004). In yeast, Not4 ubiquitylates the EGD (enhancer of Gal4 DNA binding) complex, which is composed of the subunits Egd1 and Egd2 (PANASENKO *et al.* 2006). The EGD complex is involved in ribosomal and transcription related functions in yeast (MONCOLLIN *et al.* 1986; PARTHUN *et al.* 1992; QUELO *et al.* 2004; ZHENG *et al.* 1990; ZHENG *et al.* 1987). Not4 ubiquitylates both subunits, and proper cellular localization of Egd2 depends on this modification (PANASENKO *et al.* 2006). Interestingly, the Ccr-Not complex was recently connected to Paf1 complex-dependent chromatin modification. The Not

proteins were shown to be required for histone H3 lysine 4 trimethylation, but not histone H3 lysine 79 methylation (MULDER *et al.* 2007). Specifically, Not4 is required for histone H2B ubiquitylation via recruitment of the Paf1 complex (MULDER *et al.* 2007), which is required for recruitment and activity of Rad6 and Bre1, the proteins that are responsible for ubiquitylating H2B at lysine 123 (described in more detail in section 1.2.4.1 and section 1.1.2.2).

1.5 THESIS AIMS

A former graduate student in the Arndt lab, Patrick Costa, performed a synthetic lethal screen to identify a cellular function for Rtf1. This screen identified nine complementation groups, eight of which are connected to transcription. These results provided a great deal of support for a role for Rtf1 in the regulation of transcription elongation. This screen also identified a mutation in an uncharacterized open reading frame, *YMR247c*, as synthetically lethal with *rtf1Δ*. The goal of my thesis work is to characterize this novel gene and to gain an understanding of the function of the encoded protein. Through information obtained in my work, I was able to rename *YMR247c* as *RKR1*, or RING domain mutant killed by *rtf1Δ*. I will refer to this gene as *RKR1* throughout this document.

When I began my thesis work, very little information was available regarding *RKR1*. I performed several experiments that addressed basic questions regarding Rkr1, including determining the cellular localization of this protein and extending the genetic analysis to connect *RKR1* to transcription and Rtf1 function. I used multiple approaches to gain a functional understanding of the role of Rkr1 *in vivo*, including database analyses, genetic and biochemical approaches, as well as microarray analysis to determine if Rkr1 affected global transcription. All

of these approaches characterized a role for Rkr1 as a nuclear ubiquitin-protein ligase that works in parallel with Rtf1-dependent histone modifications to affect the expression of a subset of genes in yeast.

2.0 GENETIC ANALYSIS TO INVESTIGATE A FUNCTION FOR RKR1

2.1 INTRODUCTION

Functional analysis of the Paf1 transcription elongation complex has suggested that the Rtf1 subunit is important for regulating events during transcription. Much of these data came from the dissertation research of Patrick Costa, a former member of the Arndt lab. This work showed that Rtf1 was part of the Paf1 complex and was functionally important for the regulation of transcription elongation (COSTA and ARNDT 2000). Subsequent analyses from other labs demonstrated that Rtf1 primarily affects transcription by regulating the post-translational modification of histones (NG *et al.* 2003a; WOOD *et al.* 2003b; XIAO *et al.* 2005). Rtf1 is required for histone H2B ubiquitylation at lysine 123 and subsequent methylation of histone H3 at lysines 4 and 79 (NG *et al.* 2003a; WOOD *et al.* 2003b). These modifications, like Rtf1, are associated with actively transcribing genes (POKHOLOK *et al.* 2005; XIAO *et al.* 2005). Rtf1 is important for the recruitment and activity of Rad6 and Bre1, which, in association with Lge1, are the ubiquitin conjugating enzyme and ubiquitin-protein ligase that are responsible for H2B ubiquitylation at lysine 123 (NG *et al.* 2003a; WOOD *et al.* 2003b). Set1 and Dot1 methylate histone H3 at lysines 4 and 79 (FENG *et al.* 2002; SANTOS-ROSA *et al.* 2002), respectively, only after histone H2B is ubiquitylated at lysine 123 (WOOD *et al.* 2003a). Therefore, these methylation events are also Rtf1-dependent.

One method used to gain a better understanding of Rtf1's function in the cell was a synthetic lethal screen. This screen identified a nonsense mutation in *YMR247c* as synthetically lethal with the loss of *RTF1*. Since synthetic lethality often arises from the loss of factors that function in parallel pathways (OOI *et al.* 2006), we predict that Ymr247c functions in parallel with Rtf1 to regulate a common, essential process. The *ymr247c* mutation that was identified in the *rtf1Δ* synthetic lethal screen is predicted to encode a protein that is truncated at amino acid 1133 and lacks a conserved RING domain at the extreme carboxy-terminus (see Chapter 3 for details). Because this RING domain is the only identifiable functional domain or motif in the protein, we renamed *YMR247c* as *RKR1*, or RING domain mutant killed by *rtf1Δ*.

Genetic interactions can often provide insight into the function of a gene product in the cell. To gain further insight into which function of Rtf1 is required when Rkr1 is absent, I performed extensive genetic analysis through directed crosses. The results of these analyses suggest that Rkr1 functions in parallel with Rtf1-dependent chromatin modifications. My studies do not indicate that Rkr1 directly affects the post-translational modification of histones, but loss of Rkr1 disrupts telomeric silencing, a process that depends heavily on the proper arrangement of histone modifications in euchromatin and heterochromatin.

2.2 METHODS

2.2.1 Genetic methods

The strains used in this chapter are listed in Table 3. With the exception of O660 and KA102-106, OKA91 and OKA92, all strains are isogenic to FY2, a $GAL2^{+}$ derivative of S288C

(WINSTON *et al.* 1995). *RKR1*, *RTF1*, *PAF1*, *BRE1*, *LGE1*, *DOT1*, *SET1* and *SET2* disruptions were created by a PCR-based method using the *HIS3*-marked plasmid pRS303 and the *kanMX4*-marked plasmid pRS400 (AUSUBEL 1988). The primers used to amplify these deletion cassettes are listed in Table 4. In each case, disruptions were made in diploid strains and confirmed by PCR or Southern analysis. Haploid mutant progeny were obtained by tetrad dissection. All disruptions remove the entire open reading frame and replace it with the indicated selectable marker. Genetic crosses, tetrad analyses, and yeast transformations were performed using standard methods (GIETZ and WOODS 2002; ROSE 1990).

Table 3. Strains used in Chapter 2.

Strain	Genotype
FY245	*MAT***a** *spt4Δ::URA3 ura3-52 trp1Δ63*
FY406	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6 = *URA3 HTA1-HTB1 CEN/ARS*]
FY623	*MATα rad6Δ::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52 suc2ΔUAS (-1900/-390)*
FY632	*MAT***a**/α *his4-917δ/ his4-917δ lys2-173R2/ lys2-173R2 leu2Δ1/ leu2Δ1 ura3-52/ura3-52 trp1Δ63/ trp1Δ63*
FY896	*MAT***a** *spt10Δ::TRP1 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 suc2ΔUAS (-1900/-390)*
FY2199	*MAT***a** *spt21Δ::HIS3 his3Δ200 lys2-128δ leu2Δ0 ura3Δ0*
GHY1094	*MATα ctr9Δ::kanMX4 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52*
KA104	*MATα TELVR::URA3 lys2-128δ ura3-52*
KA105	*MATα spt10Δ201::HIS3 TELVR::URA3 his3Δ200 lys2-128δ ura3(-52 or Δ0) trp(1Δ63 or 1*$^{-}$*)* [pMB27 = *SPT10 TRP1 CEN/ARS*]
KA106	*MATα spt10Δ201::HIS3 TELVR::URA3 his3Δ200 lys2-128δ ura3(-52 or Δ0) trp(1Δ63 or 1*$^{-}$*)* [pMB27 = *SPT10 TRP1 CEN/ARS*]
KA21	*MATα srb5Δ::HisG-URA3 his3Δ200 leu2 ura3-52 trp1Δ63 arg4-12*
KY303	*MATα his4-912δ lys2-128*δ *leu2Δ1 ura3-52*
KY423	*MAT***a** *his3Δ200 ura3-52 trp1Δ63*
KY425	*MAT***a** *rtf1Δ100::URA3 his4-912δ lys2-128*δ *ura3-52*
KY581	*MATα ctk1Δ::HIS3 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52* [pRS316-*CTK1*]
KY595	*MAT***a** *ura3-52*
KY661	*MAT***a** *his3Δ200 his4-912δ lys2-128*δ *leu2Δ1 ura3-52*
KY685	*MATα paf1Δ::URA3 his4-912δ lys2-128*δ *leu2Δ(0 or 1) ura3(Δ0 or -52)*
KY714	*MAT***a** *ppr2Δ::HISG-URA3 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52*
KY715	*MAT***a** *spt5-194 his3Δ200 leu2Δ1 ura3-52*
KY766	*MATα his3Δ200 lys2-128*δ *leu2Δ1 ura3-52*
KY802	*MAT***a** *paf1Δ::URA3 his3Δ200 lys2-173R2 ura3(-52 or Δ0)*
KY806	*MAT***a** *leo1Δ::URA3 his3Δ200 lys2-173R2 ura3-52*
KY811	*MATα (hht1-hhf1)Δ::LEU2 (hht2-hhf2)Δ::kanMX4 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52 trp1Δ63* [pDM1 = *HHT2-HHF2 URA3, CEN/ARS*]
KY858	*MAT***a** *spt10Δ201::HIS3 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52* [pFW217 = *URA3, SPT10, CEN/ARS*]
KY903	*MAT***a** *dot1Δ::HIS3 his3Δ200 his4-912δ lys2-128δ leu2Δ1 ura3-52*
KY907	*MAT***a** *set1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY912	*MAT***a** *set2Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52*
KY957	*MAT***a** *rtf1Δ101::LEU2 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY960	*MAT***a** *rtf1 Δ::LEU2 rkr1Δ::kanMX4 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pKA69 = *RTF1 URA3 CEN/ARS*]
KY968	*MAT***a** *bre1Δ::kanMX4 his3Δ200 ura3-52*
KY981	*MAT***a** *rkr1Δ::kanMX4 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6 = *HTA1-HTB1 URA3*

	CEN/ARS]
KY982	*MAT***a** *rtf1Δ::kanMX4 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63* [pSAB6 = *HTA1-HTB1 URA3 CEN/ARS*]
KY1023	*MAT***a** *his3Δ200 trp1Δ63*
KY1064	*MATα rkr1Δ::HIS3 (hht1-hhf1)Δ::LEU2 (hht2-hhf2)Δ::kanMX4 his3Δ200 lys2-128*δ, *leu2Δ1 ura3-52 trp1Δ63* [pDM1 = *HHT2-HHF2 URA3 CEN/ARS*]
KY1072	*MATα siz2Δ::kanMX4 his4-912δ lys2-128*δ *ura3-52 trp1Δ63 suc2ΔUAS (-1900/-390)*
KY1123	*MAT***a** *lge1Δ::kanMX4 rkr1Δ::HIS3 his3Δ200 leu2Δ1 trp1Δ63*
KY1124	*MATα lge1Δ::kanMX4 his3Δ200 leu2Δ1 trp1Δ63*
KY1131	*MATα bre1Δ::kanMX4 his3Δ200 ura3-52 trp1Δ63*
KY1141	*MAT***a** *bre1Δ::kanMX4 rkr1Δ::HIS3 his3Δ200 leu2Δ1*
KY1143	*MATα rkr1Δ::kanMX4 rtf1-108-110A leu2Δ1 ura3-52 trp1Δ63*
KY1162	*MATα cdc73Δ::kanMX4 his3Δ200 leu2Δ0 ura3-52*
KY1163	*MATα spt5-242 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52*
KY1164	*MATα spt6-14 his3Δ200 lys2-128*δ *leu2Δ1*
KY1165	*MAT***a** *spt16-197 his3Δ200 his4-912δ leu2Δ1 ura3-52*
KY1166	*MAT***a** *rkr1Δ::kanMX4 his3Δ200 leu2Δ1 trp1Δ63*
KY1168	*MATα rkr1Δ::kanMX4 his3Δ200 trp1Δ63*
KY1171	*MATα rkr1Δ::HIS3 his3Δ200 his4-912δ leu2Δ1*
KY1172	*MATα rkr1Δ::kanMX4 leu2Δ1 ura3-52 trp1Δ63*
KY1173	*MAT***a***/α RKR1/rkr1Δ::HIS3 his3Δ200/ his3Δ200 LEU2/ leu2Δ1 URA3/ ura3-52 TRP1/trp1Δ63*
KY1174	*MATα rtf1Δ3 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 arg4-12*
KY1175	*MATα rtf1Δ4 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 arg4-12*
KY1176	*MATα rkr1Δ::HIS3 his3Δ200 his4-912*δ *lys2-128*δ *leu2Δ1 ura3-52 trp1Δ63*
KY1177	*MATα rkr1Δ::HIS3 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52*
KY1178	*MAT***a** *rkr1Δ::HIS3 his3Δ200 lys2-128*δ *leu2Δ1 ura3-52*
KY1179	*MATα rkr1Δ::HIS3 his3Δ200 his4-912*δ *lys2-128*δ *leu2Δ1 trp1Δ63*
KY1180	*MAT***a** *rkr1Δ::HIS3 his3Δ200 lys2-128*δ *leu2Δ1 trp1Δ63*
KY1222	*MATα rkr1Δ::kanMX4 set1Δ::HIS3 his3Δ200 leu2Δ1 ura3-52* [pMB77 = *rkr1-C1508A LEU2 CEN/ARS*]
MBY21	*MAT***a** *rkr1Δ::kanMX, his3Δ200 leu2Δ1*
MBY251	*MAT***a** *rkr1Δ::kanMX4, rtf1Δ3, his4-912*δ *lys2-128*δ *leu2Δ1, ura3-52 trp1Δ63*
MBY260	*MATα rkr1Δ::kanMX4 rtf1-102-104A his4-912*δ *lys2-128*δ *leu2Δ1 ura3-52 trp1Δ63*
MBY264	*MAT***a** *rkr1Δ::kanMX4 rtf1-F123S his4-912*δ *lys2-128*δ *leu2Δ1*
MBY30	*MAT***a** *rkr1Δ::kanMX4 ura3-52*
MBY31	*MATα rkr1Δ::kanMX4 his3Δ200, leu2Δ1 trp1Δ63*
MBY36	*MATα rkr1Δ::kanMX4 his3Δ200 trp1Δ63*
MBY40	*MATα rkr1Δ::kanMX4 his3Δ200 lys2-128*δ *ura3-52*
MHY178	*MAT***a** *rtf1Δ3 his4-912*δ *lys2-128*δ *leu2Δ1 ura3-52 trp1Δ63*

MHY182	*MAT***a** *rtf1-102-104A his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
MHY186	*MAT***a** *rtf1-F123S his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
MHY204	*MATα rtf1-108-110A his4-912δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*
O660	*MATα TELVR::URA3 his3^{-} ura3-52 trp1^{-}*
OKA91	*MATα hmr***a***::URA3 his3Δ200, lys2-801, leu2Δ1, ura3-52, trp1Δ63, ade2-101*
OKA92	*MATα hmlα::URA3 his3Δ200, lys2-801, leu2Δ1, ura3-52, trp1Δ63, ade2-101*
SHY15	*MAT***a** *rkr1Δ::HIS3 his3Δ200 his4-912δ lys2-128δ leu2Δ1 ura3-52*
SHY16	*MATα rkr1Δ::HIS3 his3Δ200 his4-912δ lys2-128δ leu2Δ1*

FY, GHY, KY, MBY, MHY and SHY strains were generated in the labs of Fred Winston, Grant Hartzog, or Karen Arndt, or made by Maggie Braun, Marcie Warner or Steve Hancock respectively. OKA strains have been obtained from other labs.

2.2.2 Plasmids

pPC65, a pRS314 (SIKORSKI and HIETER 1989) derivative of a library plasmid (pPC62) that was obtained through complementation of the *SL505* mutation, contains a 7447 bp insert which includes *tA(AGC)M2* (alanine tRNA), *RKR1*, *SNR86,* and part of *FAA4*. pMB11 is identical to pPC65, except for the addition of the triple HA epitope sequence (3xHA) at the amino terminus of Rkr1. To introduce the epitope tag, Stephen Hancock created pSH3 by subcloning the XhoI/AatII fragment of pPC65 (containing the promoter/5' end of *RKR1*) into pRS406 (SIKORSKI and HIETER 1989). Stephen Hancock used site-directed mutagenesis (Stratagene Quikchange) to introduce an NdeI site at the ATG of *RKR1* in pSH3, and I inserted a PCR fragment encoding the 3xHA tag flanked by NdeI sites at this site. I subcloned the XhoI/AatII fragment back into pPC65 to create pMB11. pMB26, which contains *HTA1*, *FLAG-htb1-K123R*, and *HIS3*, was created by replacing a 737 bp XhoI/SphI fragment from a plasmid (NG *et al.* 2002c) containing *FLAG-HTB1* with a 737 bp fragment containing *htb1-K123R* (NG *et al.* 2002c). The presence of *FLAG-htb1-K123R* in pMB26 was confirmed by DNA sequencing. Derivatives of pRS314 that contain *HHT2* and *myc-HHF2* (pNOY436) (KEENER *et al.* 1997), wild-type *HHT2* and *HHF2* (pJH18) (HSU *et al.* 2000) or *hht2-K4R* and *HHF2* (BRIGGS *et al.* 2001) were described previously. pMB27, which contains *SPT10* and *TRP1*, was created by ligating a 2503 bp SpeI/XhoI fragment from pGN1101 (NATSOULIS *et al.* 1994) to SpeI/XhoI digested pRS314 (SIKORSKI and HIETER 1989).

2.2.3 Media

Rich (YPD), YPGlycerol (YPG), synthetic complete (SC), synthetic dextrose (SD), 5-fluoro-orotic acid (5-FOA) and sporulation media were prepared as described (ROSE 1990). Media lacking (-Ino) or containing 200 μM inositol (+Ino) were prepared with yeast nitrogen base that contained ammonium sulfate but lacked inositol (Q-Bio Systems). NaCl, LiCl, caffeine, and hydroxyurea were added to YPD medium to final concentrations of 1.4 M, 0.3 M, 15 mM, or 100mM, respectively. 6-azauracil (6AU) was added to SC-Ura medium to a final concentration of 50 μg/ml. G418 medium for selection of yeast strains expressing the *kanMX4* gene was prepared as described previously (JAUERT *et al.* 2005).

2.2.4 Growth assays

Saturated cultures of each strain were grown in appropriate media. Cells were collected by centrifugation, and cell pellets were washed two times with sterile water. Ten-fold serial dilutions (1 x 10^8 cells/ml to 1 x 10^4 cells/ml) were made in sterile water and two or three microliters of each dilution were spotted onto appropriate media. Spots were allowed to dry and plates were incubated at 30^{o}C for 3-5 days. YPD and YPG plates were used in every assay to ensure even spotting and to assay for petite cells, respectively. Specific strains and plasmids used in individual experiments are described in the figure legends.

2.2.5 Sporulation efficiency assay

Strains KY1177 (*rkr1Δ*), KY1180 (*rkr1Δ*), KY766 (WT), and KY423 (WT) were used in these

assays. Liquid sporulation medium (2.45 g potassium acetate in 250 ml ddH_2O) was used to determine sporulation efficiency. Amino acids were added to cultures to supplement auxotrophies. Cultures were inoculated and incubated at room temperature overnight prior to incubation at 30°C for 2 additional days. Undiluted, unsonicated cultures were counted with the hemacytometer. Sporulation efficiency was calculated by dividing the number of triads and tetrads in 20 squares by the total number of cells in 20 squares. Cultures were counted 3, 5, and 8 days post-inoculation.

2.2.6 Indirect immunofluorescence

Yeast strain FY632 was transformed with pPC65 (untagged *RKR1*) or pMB11 (*3xHA-RKR1*) and transformants were grown in SC medium lacking tryptophan to a density of approximately 1 x 10^7 cells/ml. A total of 1.4 x 10^8 cells were processed for indirect immunofluorescence as described previously (SIKORSKI and HIETER 1989). The primary antibody, anti-HA (Roche), was added at a 1:3000 dilution, and the secondary antibody, Cy3-conjugated goat anti-mouse IgG (Molecular probes Alexa Fluor 488), was added at a 1:250 dilution. DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) was used to stain the DNA, as described previously (PRINGLE *et al.* 1989). Cells were visualized with an Olympus BX60 epifluorescence microscope and photographed with QED *in vivo* software.

2.2.7 Northern analysis

MBY30 and KY595 were grown to approximately 1 x 10^7 cells/ml in SD medium supplemented with 200 μM inositol. Cells were washed into inositol starvation medium and aliquots were

taken from the culture for analysis at 2, 4 and 6 hours after induction. RNA isolation and Northern analysis were performed as described previously (SHIRRA and ARNDT 1999). The *INO1* probe was synthesized by random-prime labeling of a PCR fragment containing *INO1*. The *SCR1* probe (loading control) was synthesized by random-prime labeling of a PCR fragment containing *SCR1*. Phosphorimaging analysis was performed with Image Gauge software.

2.2.8 Chromatin immunoprecipitation (ChIP) analysis

KY811 and KY1064 were transformed with *TRP1*-marked *CEN/ARS* plasmids expressing either myc-tagged histone H4 (pNOY436) (KEENER *et al.* 1997) or untagged H4 (BRIGGS *et al.* 2001). Transformants were passaged on medium containing 5FOA to select against the *URA3*-marked plasmid that expressed wildtype histone H4 in these strains. $5FOA^R$ colonies were grown to 0.9–2.0 x 10^7 cells/ml (early log-phase) in 250 ml SD minimal medium supplemented with lysine and uracil (and histidine for wildtype cultures). Cells were treated with formaldehyde and collected as described previously (SIMIC *et al.* 2003). Cells were lysed and the chromatin fraction was sonicated to obtain 200-500 basepair chromatin fragments. Histones were immunoprecipitated from 700 µl of chromatin with 2 µl anti-histone H2B (Upstate), 3 µl anti-histone H3 (Abcam), 2 µl anti-tetra acetylated histone H4 (Upstate), or preconjugated myc-agarose beads (to immunoprecipitate histone H4) (Santa Cruz). Protein-A secondary beads (Amersham Biosciences) were used to immunoprecipitate H2B, H3, and tetra-acetylated H4 immune complexes. Immunoprecipitates were washed and DNA was purified from chromatin after the crosslinks were reversed with heat. Quantitative PCR was performed using primers that hybridize at chromosomal regions 0.2, 3.5, and 20 kb from the telomere on the right arm of chromosome VI, as well as primers that hybridize at the 3' end of the *GAL1* open reading frame

(primer sequences can be found in Table 4). Phosphorimaging analysis was performed with Image Gauge software. Immunoprecipitated (IP) samples were quantitiated relative to input signal for each culture.

Table 4. Primers used in Chapter 2 studies.

Oligo name	Used for:	Sequence (5' to 3')
SHO1	*rkr1Δ*	GGCAAGGTATAGGGCTGGATTGTATAATTTGTAGAGAGCACAGATTG TACTGAGAGTGCAC
SHO2	*rkr1Δ*	GTTCAAGCAATAGTTGGTTCTTAATGTCGTTTGGTGGAATCCTGTGCG GTATTTCACACCG
MBO34	*bre1Δ*	GTTGGATGGATGTTGTTTGAACGGTAAACTAACAGAGAGCCTGTGCG GTATTTCACACCG
MBO35	*bre1Δ*	CTCACCCGGCCGCCCAAAGTATTATATGAATCTTTAGGGCAGATTGTA CTGAGAGTGCAC
MBO41	*rtf1Δ*	GATGCCATTGCTGACTTGAA
MBO42	*rtf1Δ*	CTGAATCTGGCAAAGCCTTC
MBO97	*lge1Δ*	GTTTTAAGGGGCGGGGCAAGAGTGGCGGGGAATTCCGCCGCTGTGCG GTATTTCACACCG
MBO98	*lge1Δ*	CTTGCGTTTACGTAGTTTATCTATTTATAGGTACGGTATACAGATTGT ACTGAGAGTGCAC
MBO139	ChIP 0.2 kb	CAAGCGGCTGGACTACTTTCTGG
MBO140	ChIP 0.2 kb	CAATTTTATGTAGATATCCACCAC
MBO141	ChIP 20 kb	GGCTGCTTCTATCACTACGCGTC
MBO142	ChIP 20 kb	CGTCTGTGAAAACAGATTTAATG
MBO151	ChIP 3.5 kb	CAACCATCTAGTAGCCAATGTTTGC
MBO152	ChIP 3.5 kb	GATAAAGCAGAATCATTTCCGCTG

ChIP primer sequence distances are relative to the telomere on the right arm of chromosome VI.

2.3 RESULTS

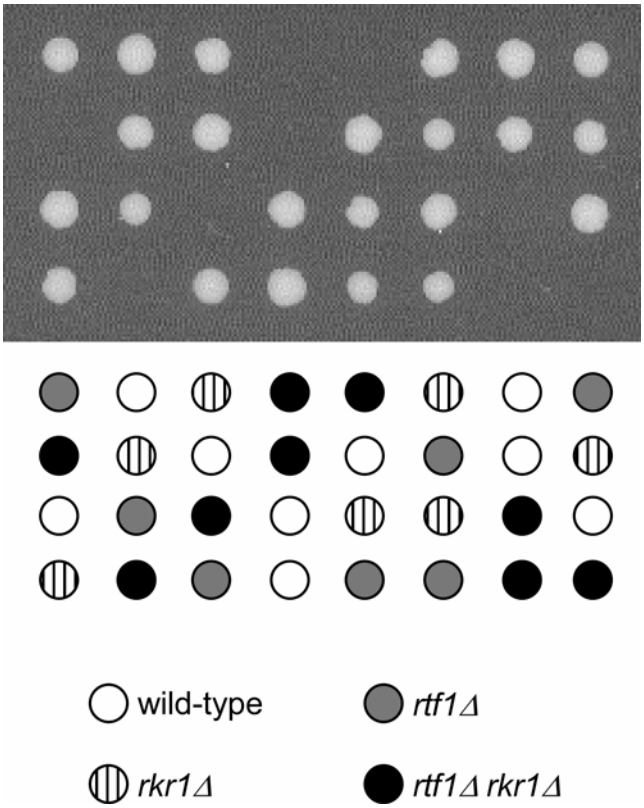
2.3.1 *rtf1Δ* is synthetically lethal with *rkr1Δ*

The Paf1 complex functions in transcription elongation, RNA 3' end formation, and histone modification. Original support for a role of the Paf1 complex in transcription elongation came from a genetic screen for mutations that cause lethality in combination with an *rtf1Δ* mutation (COSTA and ARNDT 2000). This screen identified synthetic lethal mutations in *CTK1*, *FCP1*, and *POB3*, which encode an RNA Pol II CTD kinase, an RNA Pol II CTD phosphatase, and a component of the FACT transcription elongation complex, respectively. In addition to these well-characterized RNA Pol II transcription factors, this screen also identified a synthetic lethal mutation, the *SL505* mutation, in a previously uncharacterized gene, *RKR1*. *RKR1* is a 4686 bp open reading frame that is predicted to encode a protein of 1562 amino acids. Patrick Costa used linkage analysis to confirm that *RKR1* contained the *SL505* mutation, and Steve Hancock performed DNA sequence analysis of a gap-repaired plasmid containing the *RKR1-SL505* mutation and showed that it led to a premature stop codon at amino acid 1133 (BRAUN *et al.* 2007).

I confirmed the results of the plasmid-based synthetic lethal screen using tetrad analysis of spores derived from diploid strains that were doubly heterozygous for complete deletions of *RTF1* and *RKR1*. No viable haploid *rtf1Δ rkr1Δ* strains were obtained after tetrad dissection (Figure 15). This synthetic lethal interaction indicates that Rkr1 and Rtf1 regulate similar processes in yeast.

Figure 15. Strains lacking *RTF1* and *RKR1* are inviable.

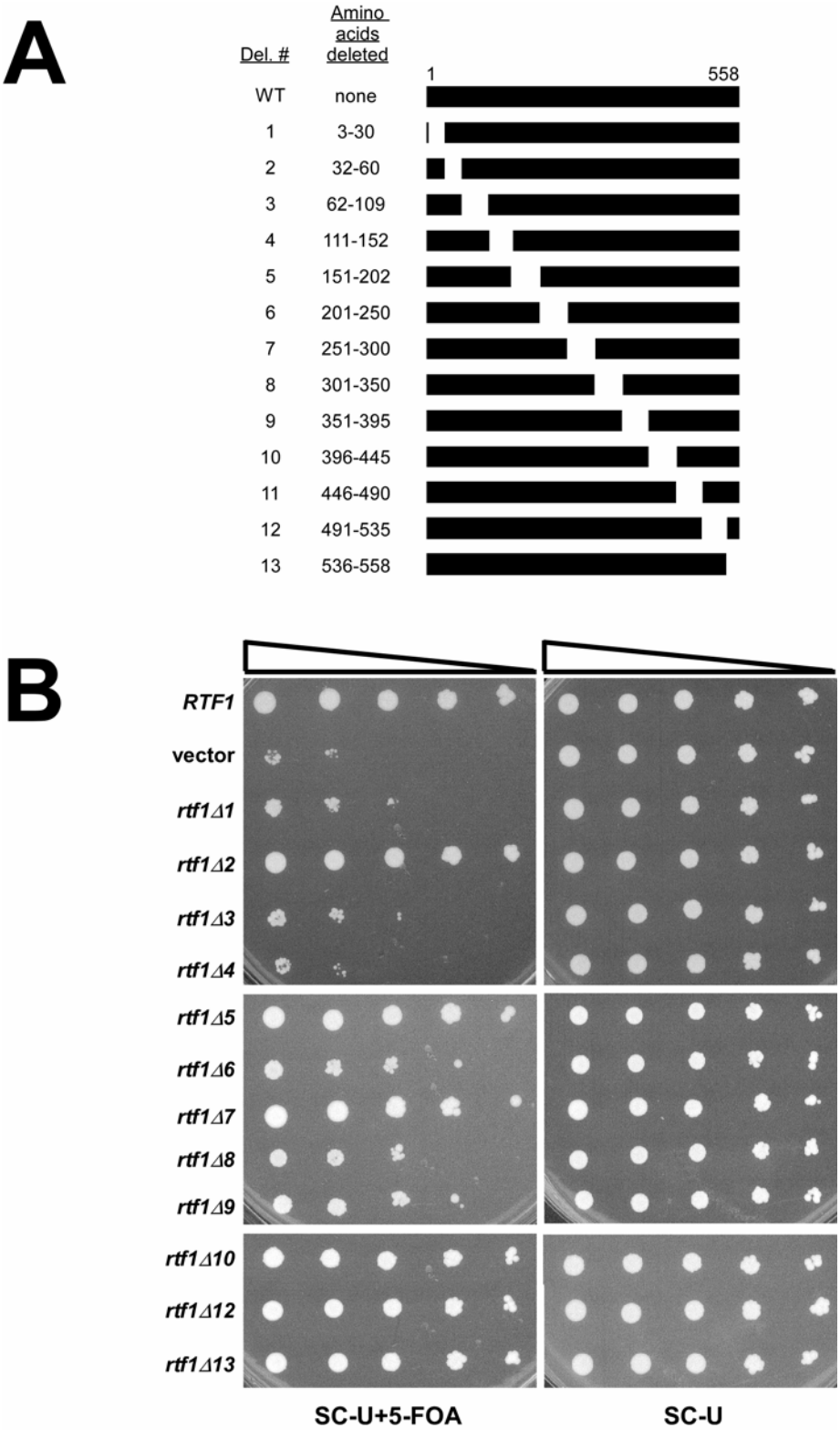
Yeast strains of opposite mating types containing deletions of *RKR1* (KY1172) or *RTF1* (KY957) were mated and sporulated. Tetrads were dissected and incubated for 3 days at 30° C.


wild-type
rtf1Δ
rkr1Δ
rtf1Δ rkr1Δ

To better define why Rtf1 is required for viability when Rkr1 is absent, I took advantage of a set of *rtf1* internal deletion mutations that disrupt specific functions of the protein (M. H. Warner, K. L. Roinick, and K. M. Arndt, submitted for publication). Discrete regions of Rtf1 are important for its association with actively transcribed genes, interactions with other members of the Paf1 complex, and post-translational histone modifications. Strains lacking *RKR1* and *RTF1*, which were kept alive with a *URA3*-marked plasmid containing *RTF1*, were transformed with *TRP1*-marked plasmids containing thirteen individual deletions of *RTF1* (#1-13) (Figure 16). I counter-selected the *URA3*-marked plasmid by growing these transformants on medium containing 5-FOA. Growth defects were seen in *rkr1Δ rtf1Δ* strains that expressed *rtf1Δ1, Δ3, Δ4, Δ6, Δ8 or Δ9* (Figure 16). Strains expressing *rtf1Δ1*, *Δ3* or *Δ4* have the most severe growth defects, while strains expressing *rtf1Δ6, Δ8* or *Δ9* grow less well. This suggests that the functions of Rtf1 that are associated with regions 1, 3 and 4 are more important for growth in strains lacking *RKR1*. The significance of these growth defects will be discussed separately in the next few paragraphs. Interestingly, regions 6-9 are important for the association of the Paf1 complex with RNA Pol II during transcription (M. H. Warner, K. L. Roinick, and K. M. Arndt, submitted for publication), suggesting that low levels of Rtf1 chromatin association provide enough function to sustain viability to *rkr1Δrtf1Δ* strains.

Figure 16. Specific regions of Rtf1 are required when Rkr1 is absent.

A) Thirteen deletion mutations were generated throughout the *RTF1* coding sequence (Rtf1Δ1-13). Numbers on the left describe the amino acids that are lost with the deletion mutation. B) An *rtf1Δ rkr1Δ* strain (KY960) was transformed with *TRP1*-marked plasmids expressing wild type *RTF1*, each of the *rtf1Δ*s, or vector alone (Rtf1Δ11 was omitted for technical reasons). Transformants were serially diluted and spotted onto medium containing or lacking 5FOA. Plates were incubated at 30° C for 3 days.


A
Del. #
Amino acids deleted
1
558
WT
none
1
3-30
2
32-60
3
62-109
4
111-152
5
151-202
6
201-250
7
251-300
8
301-350
9
351-395
10
396-445
11
446-490
12
491-535
13
536-558
B
RTF1
vector
rtf1Δ1
rtf1Δ2
rtf1Δ3
rtf1Δ4
rtf1Δ5
rtf1Δ6
rtf1Δ7
rtf1Δ8
rtf1Δ9
rtf1Δ10
rtf1Δ12
rtf1Δ13
SC-U+5-FOA
SC-U

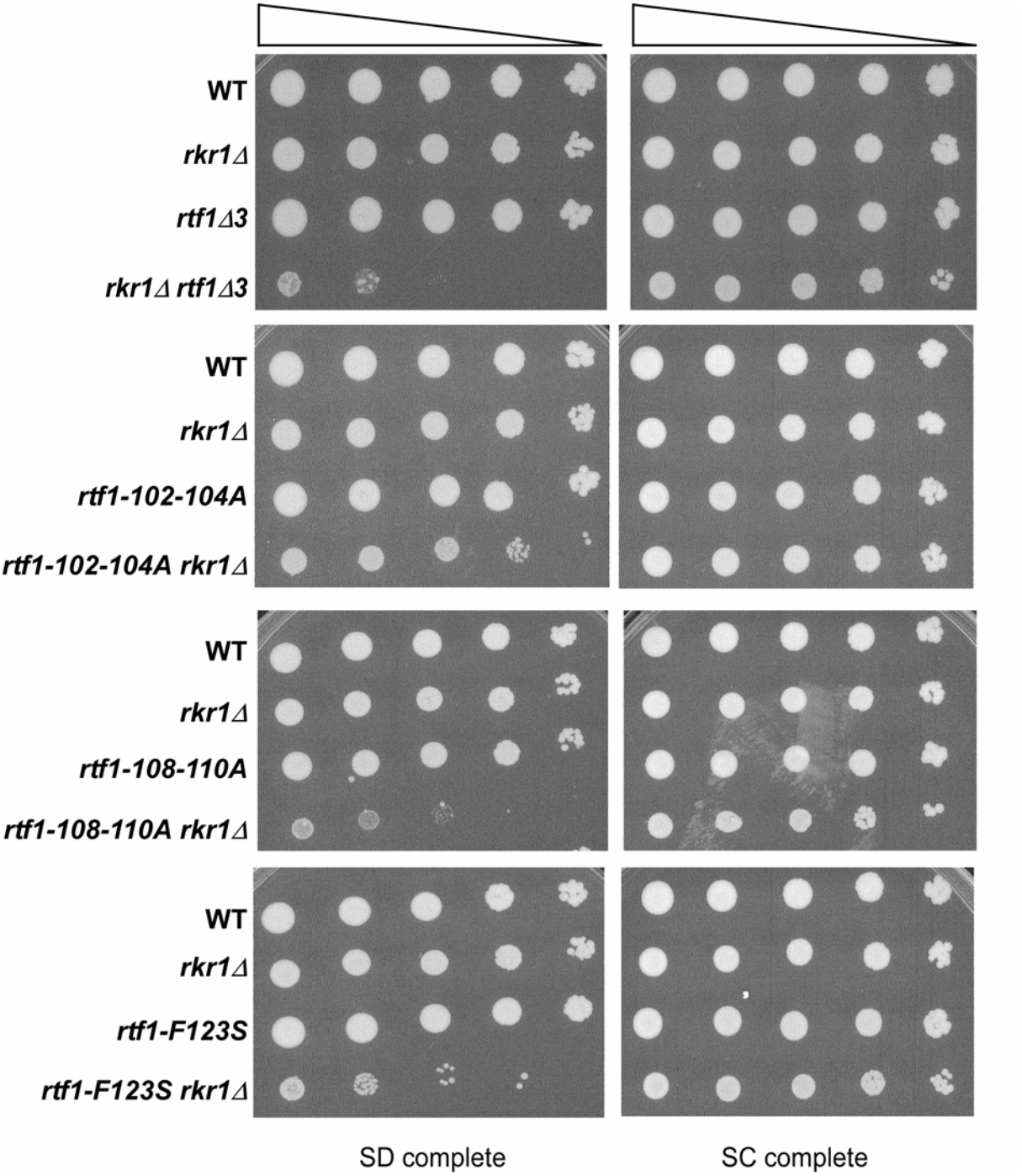
To confirm the growth defects seen in these transformants, I crossed strains lacking *RKR1* to strains containing integrated forms of the different *rtf1* deletion mutations and found that the *rtf1Δ3* and *rtf1Δ4* mutations, which together eliminate amino acids 62-152 from the Rtf1 protein, show a strong genetic interaction with *rkr1Δ*. Specifically, the *rkr1Δ rtf1Δ3* and *rkr1Δ rtf1Δ4* double mutants grow very poorly on minimal synthetic dextrose medium (Figure 17). Notably, deletion of amino acids 62-152 in Rtf1 eliminates histone H2B ubiquitylation and histone H3 K4 and K79 methylation (M. H. Warner, K. L. Roinick, and K. M. Arndt, submitted for publication). The results of these analyses suggest that Rkr1 functions in parallel with Rtf1-dependent chromatin modifications.

Marcie Warner generated several site-directed alanine scanning mutations within the region covered by deletions 3 and 4 of Rtf1. Groups of 3 charged amino acids were mutated to alanines with the goal of disrupting protein-protein interaction sites, important for histone modification. In a separate study, Jill Dembowski generated mutations in *RTF1* with PCR-based mutagenesis, and characterized phenylalanine at position 123 (F123) as being required for histone H3 K4 methylation. To determine whether these residues within Rtf1 are required in the absence of Rkr1, I crossed *rkr1Δ* strains by strains that contained integrated versions of *rtf1-102-104A*, *rtf1-108-110A*, and *rtf1-F123S*. Interestingly, *rkr1Δ rtf1-108-110A and rkr1Δ rtf1-F123S* double mutant strains grow poorly on minimal medium, while *rkr1Δ rtf1-102-104A* double mutant strains grow as well as either parent strain on minimal medium (Figure 17). Marcie Warner has shown that strains containing the *rtf1-102-104A* mutation contain reduced levels of H3 K4 methylation, while *rtf1-108-110A* and *rtf1-F123S* mutant strains lose this histone modification. Interestingly, the *rtf1-102-104A* and *rtf1-108-110A* mutations result in approximately a two-fold reduction in histone H3 K79 methylation, and the *rtf1-F123S* mutation

results in approximately a ten-fold reduction in K79 methylation, while strains containing *rtf1Δ4* lose this modification entirely (M. H. Warner and K. M. Arndt, unpublished observations). These results further suggest that Rkr1 functions in parallel with Rtf1-dependent histone modifications.

Figure 17. Genetic interactions suggest that Rkr1 functions in parallel with Rtf1 dependent histone modifications.

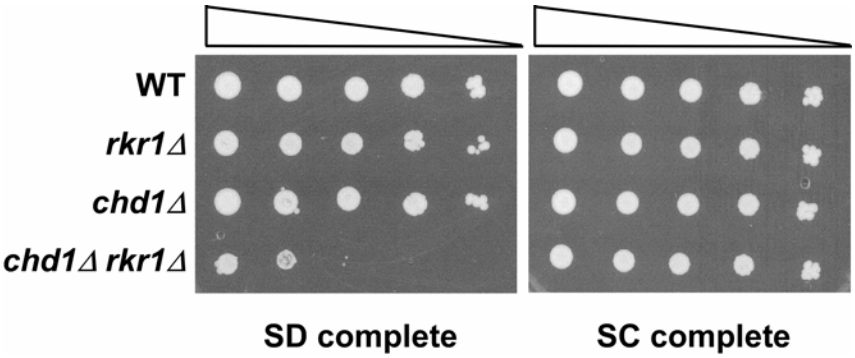
Serial dilutions of strains with the genotypes listed on the left of the figure were serially diluted and spotted onto SD or SC complete medium. Plates were incubated at 30° C for 3 days. Wild type and *rkr1Δ* strains for all panels are a wild type strain derived from crossing KY1023 and KY1072, and MBY36, respectively. Other strains used (top to bottom): MHY178, MBY251, MHY182, MBY260, MHY204, KY1143, MHY186, and MBY264, respectively.


WT
rkr1Δ
rtf1Δ3
rkr1Δ rtf1Δ3
WT
rkr1Δ
rtf1-102-104A
rtf1-102-104A rkr1Δ
WT
rkr1Δ
rtf1-108-110A
rtf1-108-110A rkr1Δ
WT
rkr1Δ
rtf1-F123S
rtf1-F123S rkr1Δ
SD complete
SC complete

Synthetic growth defects were seen in *rkr1Δ rtf1Δ* transformants expressing *rtf1Δ1*. The *rtf1Δ1* mutation results in an Rtf1 protein that lacks amino acids 3-30 (Figure 16). This region of Rtf1 is required for a physical interaction with the chromatin remodeler Chd1 (M. H. Warner, K. L. Roinick, and K. M. Arndt, submitted for publication). To confirm the results of the plasmid-based assay, I also crossed *rkr1Δ* strains by strains lacking *CHD1*. Double mutant strains grow very poorly on minimal medium (Figure 18). This suggests that Rkr1 is important for growth on minimal medium in the absence of Chd1 chromatin remodeling activity. The genetic interaction between *RKR1* and *CHD1* further connects Rkr1 function to chromatin function.

Figure 18. Genetic interactions suggest that Rkr1 functions in parallel with Chd1.

Strains with the genotypes listed on the left were serially diluted and spotted onto SD or SC complete medium. Plates were incubated at 30° C for 3 days.


WT
rkr1Δ
chd1Δ
chd1Δ rkr1Δ
SD complete
SC complete

2.3.2 *RKR1* is not synthetically lethal with other members of the Paf1 complex or other transcription elongation factors

To determine if the genetic interaction between *RKR1* and *RTF1* extended to the remaining members of the Paf1 complex, strains lacking *RKR1* were crossed by strains containing mutations in the genes that encode for the individual members of the Paf1 complex. Interestingly, although *rkr1Δ paf1Δ* and *rkr1Δ ctr9Δ* double mutants grow very slowly, only *rkr1Δ rtf1Δ* double mutants are inviable (Table 5). This result is particularly striking because deletion of *PAF1* or *CTR9* generally causes stronger mutant phenotypes than deletion of any other member of the Paf1 complex, including *RTF1* (BETZ *et al.* 2002; SQUAZZO *et al.* 2002). Therefore, the synthetic lethality between *RKR1* and *RTF1* most likely relates to a function of the Paf1 complex that is primarily carried out by Rtf1, potentially histone H2B ubiquitylation or histone H3 K4 or K79 methylation.

The Paf1 complex physically and genetically interacts with several transcription elongation factors, including Spt4-Spt5, and Spt16-Pob3 (yFACT) (SQUAZZO *et al.* 2002). To determine if Rkr1 is involved in transcription elongation, I performed genetic crosses with *rkr1Δ* strains and strains containing mutations in genes that encode transcription elongation factors, including *SPT4*, *SPT5*, *SPT6*, *SPT16* and *PPR2*, which encodes TFIIS. While I observed several enhanced mutant phenotypes in the double mutant strains, I found no severe genetic interactions to indicate that Rkr1 is solely or primarily involved in transcription elongation (Table 5).

Table 5. Genetic interactions suggest that Rkr1 does not primarily function to promote elongation.

Mutation[a]	Phenotypes[b]
rtf1Δ rkr1Δ	Dead
paf1Δ rkr1Δ	Slow growth
ctr9Δ rkr1Δ	Slow growth
cdc73Δ rkr1Δ	$NaCl^-$, $Caff^-$
leo1Δ rkr1Δ	None
spt4Δ rkr1Δ	$LiCl^-$
spt5-194 rkr1Δ	$LiCl^-$, $NaCl^-$
spt5-242 rkr1Δ	$LiCl^-$, $NaCl^-$
spt6-14 rkr1Δ	Increased $6AU^S$, $NaCl^-$, $LiCl^-$
spt16-197 rkr1Δ	$LiCl^-$, $NaCl^-$, enhanced Ino^-
ppr2Δ rkr1Δ	$Caff^-$

[a] The parents for the crosses in the order listed are: KY1172 and KY957, KY1177 and KY802, KY1180 and GHY1094, KY1180 and KY1162, KY1177 and KY806, KY1178 and a strain derived from FY245, KY1179 and KY715, KY1180 and KY1163, KY1180 and KY1164, KY1179 and KY1165, KY1176 and KY714.

[b] All phenotypes listed correspond to the synthetic phenotypes observed for the double mutant strains. The $6AU^S$ phenotype caused by *spt6-14* and the Ino^- phenotype caused by *rkr1Δ* are enhanced in the double mutants as indicated. Slow growth, small colonies after 3-5 days of growth at 30°C; $NaCl^-$, sensitive to 1.4 M NaCl; $LiCl^-$, sensitive to 0.3 M LiCl; $Caff^-$, sensitive to 15 mM caffeine.

2.3.3 Strains lacking *RKR1* have phenotypes that suggest a role for Rkr1 in transcription and sporulation

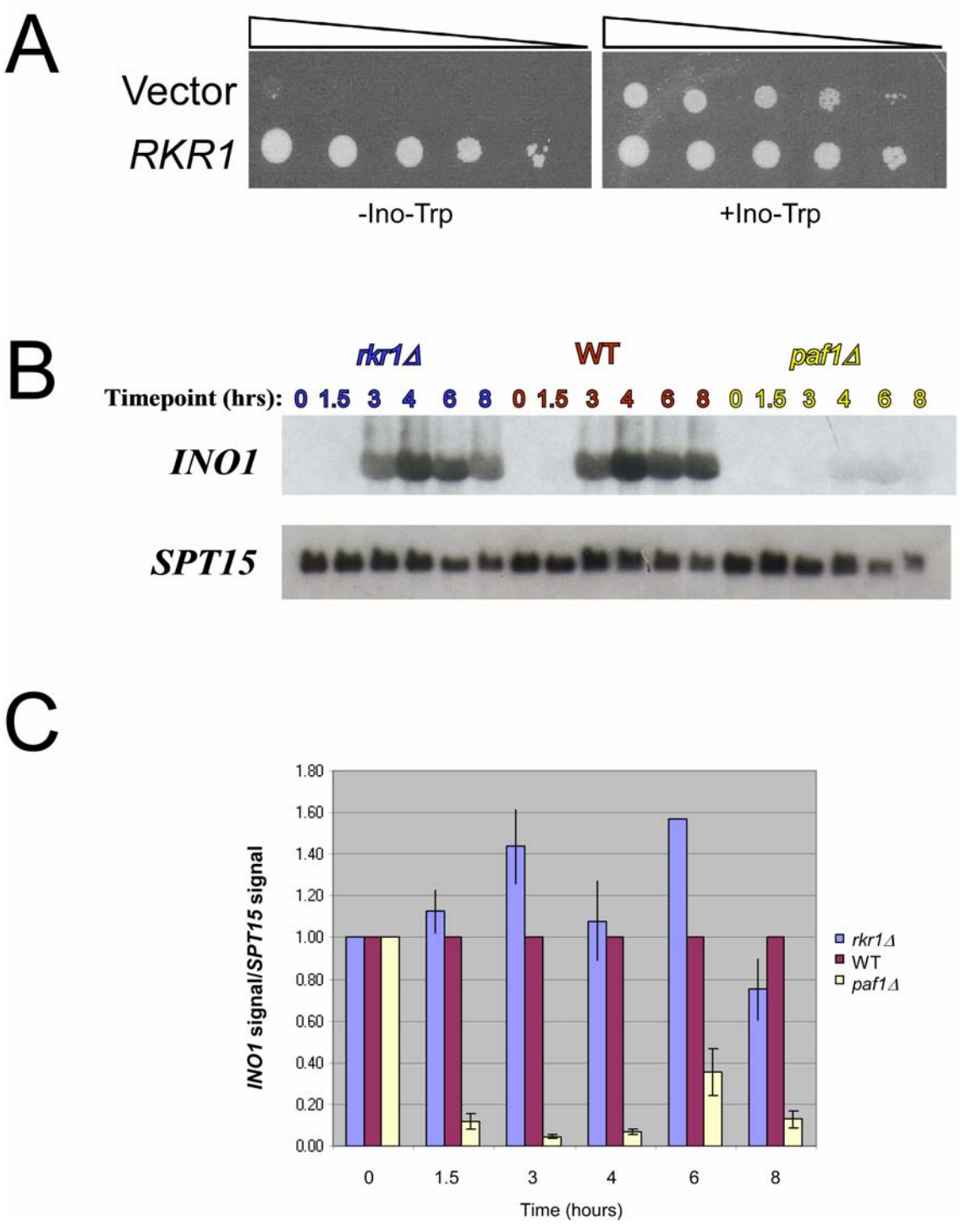
In an attempt to identify a cellular process that requires Rkr1, *rkr1Δ* strains were exposed to a wide range of phenotypic tests. *rkr1Δ* mutants were tested for their ability to grow on media lacking inositol or on media containing 6-azauracil, caffeine, formamide, hydroxyurea, benomyl, sucrose, raffinose, glycerol, or high concentrations of salt (sodium chloride or lithium chloride). These strains were also assayed for growth defects at 37° and 15°C on YPD. For most of these phenotypes, the *rkr1Δ* strains appeared similar to wild type. However, I observed that *rkr1Δ* strains grow poorly on media lacking inositol, a phenotype associated with general defects in transcription (Figure 19) (HAMPSEY 1997). This phenotype correlates well with the genetic interaction with *RTF1*, as defects in the Paf1 complex also cause inositol auxotrophy (BETZ *et al.* 2002).

Strains that are Ino^- often have defects in transcribing *INO1*, which encodes a protein that is important for the production of inositol phosphates and inositol containing phospholipids (reviewed in SHIRRA *et al.* 2005) . This gene is induced in low inositol conditions, and previous studies have shown that loss of other transcription factors, including members of the Paf1 complex, affect *INO1* expression (ARNDT *et al.* 1995; BETZ *et al.* 2002; GANSHEROFF *et al.* 1995; ROBERTS and WINSTON 1996; SCAFE *et al.* 1990). To better characterize the cause of the poor growth on media lacking inositol, I assayed the transcription of *INO1* in *rkr1Δ* strains. Northern analysis shows that, upon induction, *INO1* is expressed at similar levels in wild type and *rkr1Δ* strains (Figure 19). While the cause of the inositol auxotrophy in *rkr1Δ* strains remains unknown, this phenotype appears to be unrelated to defects in *INO1* expression. Further analysis

to investigate genome-wide transcription defects in *rkr1Δ* strains is described in Chapter 5.

Figure 19. Strains lacking *RKR1* grow poorly on medium lacking inositol.

A) A *rkr1Δ* strain (KY1168) was transformed with *TRP1*-marked plasmids expressing *RKR1* or empty vector. Transformants were serially diluted and spotted onto medium that lacked tryptophan and either lacked or contained inositol. Plates were incubated at 30° C for three days. B) Wild type (KY661), *rkr1Δ* (SHY16), and *paf1Δ* (KY685) strains were grown to early log phase in medium containing inositol, and then shifted into medium lacking inositol. Cells were collected at the indicated times and RNA was prepared. Northern blots were performed with probes to *INO1* and *SPT15* (loading control). A representative Northern blot is shown. C) Quantitation of *INO1* Northerns. The results of two independent experiments are shown. Error bars represent the maximum and minimum levels of expression detected in the two experiments.


A
Vector
RKR1
-Ino-Trp
+Ino-Trp
B
rkr1Δ
WT
paf1Δ
Timepoint (hrs): 0 1.5 3 4 6 8 0 1.5 3 4 6 8 0 1.5 3 4 6 8
INO1
SPT15
C
INO1 signal/SPT15 signal
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
1.5
3
4
6
8
Time (hours)
rkr1Δ
WT
paf1Δ

In the process of creating double mutant strains for genetic analysis, *rkr1Δ* homozygous diploids were created. It was observed that these diploids failed to sporulate. This finding was confirmed by quantitating the sporulation frequency of wild type (*RKR1*$^{+}$/*RKR1*$^{+}$), heterozygous (*RKR1*$^{+}$/*rkr1Δ*) and homozygous (*rkr1Δ*/*rkr1Δ*) diploid strains. The data show that homozygous *rkr1Δ* strains do not sporulate, and heterozygous *rkr1Δ* strains sporulate at a lower frequency compared to wild type strains (Table 6). Interestingly, diploid strains that contain homozygous mutations in genes for the members of the Paf1 complex members also fail to sporulate (ENYENIHI and SAUNDERS 2003).

Table 6. Strains lacking *RKR1* do not sporulate.

Cross	Total # cells	# Triads/tetrads	% Sporulation
*MAT***a** *rkr1Δ* x *MATα rkr1Δ*	849	0	0
*MAT***a** *rkr1Δ* x *MATα* WT	613	27	4.5 +/- 1.7
*MAT***a** WT x *MATα rkr1Δ*	449	26	6.7 +/- 3.3
*MAT***a** WT x *MATα* WT	936	119	11.45 +/- 3.2

Strains used for these crosses are as follows (top to bottom): KY1180 and KY1177, KY1180 and KY766, KY423 and KY1177, KY423 and KY766, respectively. Error values represent the difference in percent sporulation in two independently grown cultures of each diploid.

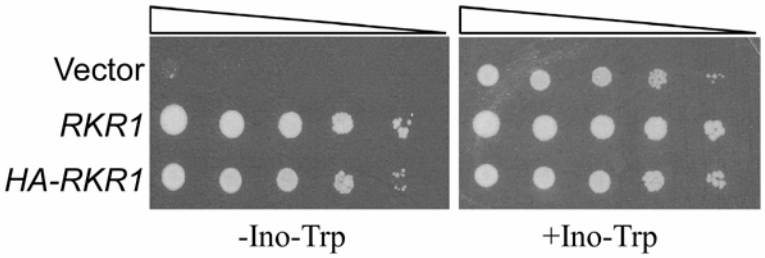
2.3.4 Rkr1 is a nuclear protein

A large-scale study has been performed to determine the subcellular localization of all yeast proteins (HUH *et al.* 2003). This study involved the construction of carboxy-terminal GFP fusions for the majority of open reading frames in yeast and microscopy to detect localization of the GFP signal. Rkr1 was not localized to any cellular compartment in this study, possibly because incorporation of the GFP tag at the carboxy terminus disrupted the structure and/or function of the RING domain. Database analyses did not reveal any cellular localization signals within the primary amino acid sequence of Rkr1. Therefore, I used indirect immunofluorescence to determine the cellular localization of Rkr1. To detect Rkr1, I constructed an amino-terminal HA-epitope tagged version of the protein. This tag does not appear to disrupt activity as determined by complementation of the Ino^- phenotype of a *rkr1Δ* strain (Figure 20), and immunoblotting analysis showed that HA-Rkr1 migrates in denaturing gels at its predicted molecular mass of approximately 180 kilodaltons (Figure 20). Indirect immunofluorescence experiments showed that HA-Rkr1 is localized to the nucleus (Figure 20).

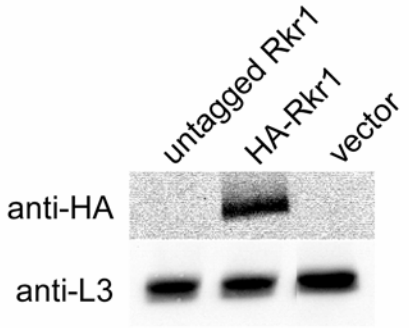
Figure 20. Rkr1 is a nuclear protein.

A) *HA-RKR1* complements a *rkr1Δ* mutation. A *rkr1Δ* strain (KY1168) was transformed with *TRP1*-marked plasmids expressing untagged or HA-tagged *RKR1*, or empty vector. Transformants were serially diluted and spotted onto medium containing tryptophan and either lacking or containing inositol. Plates were incubated at 30° C for 3 days. B) Immunoblot analysis shows that HA-Rkr1 is expressed. C) Indirect immunofluorescence analysis shows that Rkr1 is a nuclear protein. See methods section for more information.

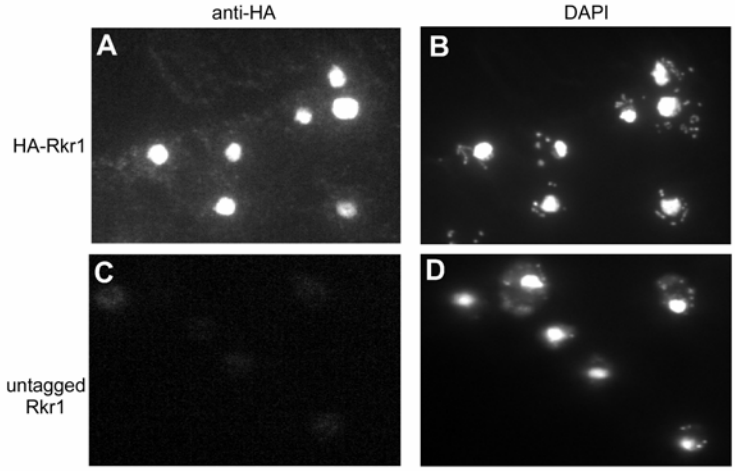
A


Vector
RKR1
HA-RKR1
-Ino-Trp
+Ino-Trp

B


untagged Rkr1
HA-Rkr1
vector
anti-HA
anti-L3

C


anti-HA
DAPI
A
B
C
D
HA-Rkr1
untagged Rkr1

2.3.5 *RKR1* genetically interacts with factors involved in chromatin modification

To further investigate a potential connection between *RKR1* and post-translational histone modifications, *rkr1Δ* strains were crossed with strains lacking specific histone modifying enzymes. Rad6 is the ubiquitin-conjugating enzyme and Bre1, in association with Lge1, is the ubiquitin-protein ligase required for histone H2B K123 ubiquitylation (HWANG *et al.* 2003; ROBZYK *et al.* 2000; WOOD *et al.* 2003a). Set1, Set2, and Dot1 are the methyltransferases responsible for methylating K4, K36, and K79 of histone H3, respectively (FENG *et al.* 2002; SANTOS-ROSA *et al.* 2002; STRAHL *et al.* 2002). The Paf1 complex is required for each of these modifications (KROGAN *et al.* 2003a; KROGAN *et al.* 2003b; NG *et al.* 2003a; NG *et al.* 2003b; WOOD *et al.* 2003b). Interestingly, *rkr1Δ rad6Δ* double mutants exhibit a strong synthetic growth defect (Table 7). Further genetic analysis using strains lacking both *RKR1* and the H2B ubiquitylation site (*htb1-K123R*) indicates that Rkr1 is important for cell growth in the absence of H2B ubiquitylation (Figure 21). Moreover, *rkr1Δ* strains lacking *BRE1*, *LGE1,* or *SET1* grow very poorly on SD medium, similar to the *rtf1Δ3* or *Δ4* genetic interactions (Figure 21 and data not shown). Defective growth on SD medium was also observed for a *rkr1-C1508A set1Δ* double mutant strain, indicating a requirement for the Rkr1 RING domain when Set1 is absent (Table 7). Because Set1 has been shown to methylate substrates other than histone H3 K4 (ZHANG *et al.* 2005b), I examined the phenotype of a *rkr1Δ* strain in which histone H3 K4 could not be methylated (*hht2-K4R*). Similar to a *rkr1Δ set1Δ* strain, the *rkr1Δ hht2-K4R* strain grows poorly on SD medium (Figure 21), suggesting that the synthetic phenotype between *rkr1Δ* and *set1Δ* is due to a lack of histone H3 K4 methylation.

Table 7. Genetic interactions between *RKR1* and genes encoding proteins involved in chromatin modification.

Genotypes[a]	Phenotypes[b]
rtf1Δ3 rkr1Δ	SD^-
rtf1Δ4 rkr1Δ	SD^-
rad6Δ rkr1Δ	Slow growth, SD^-, Gly^-
bre1Δ rkr1Δ	SD^-, Gly^-
lge1Δ rkr1Δ	SD^-
set1Δ rkr1Δ	SD^-, HU^S, $NaCl^-$
set1Δ rkr1-C1508A	SD^{-c}
dot1Δ rkr1Δ	None
set2Δ rkr1Δ	None
gcn5Δ rkr1Δ	$NaCl^-$. Enhanced Ino^-

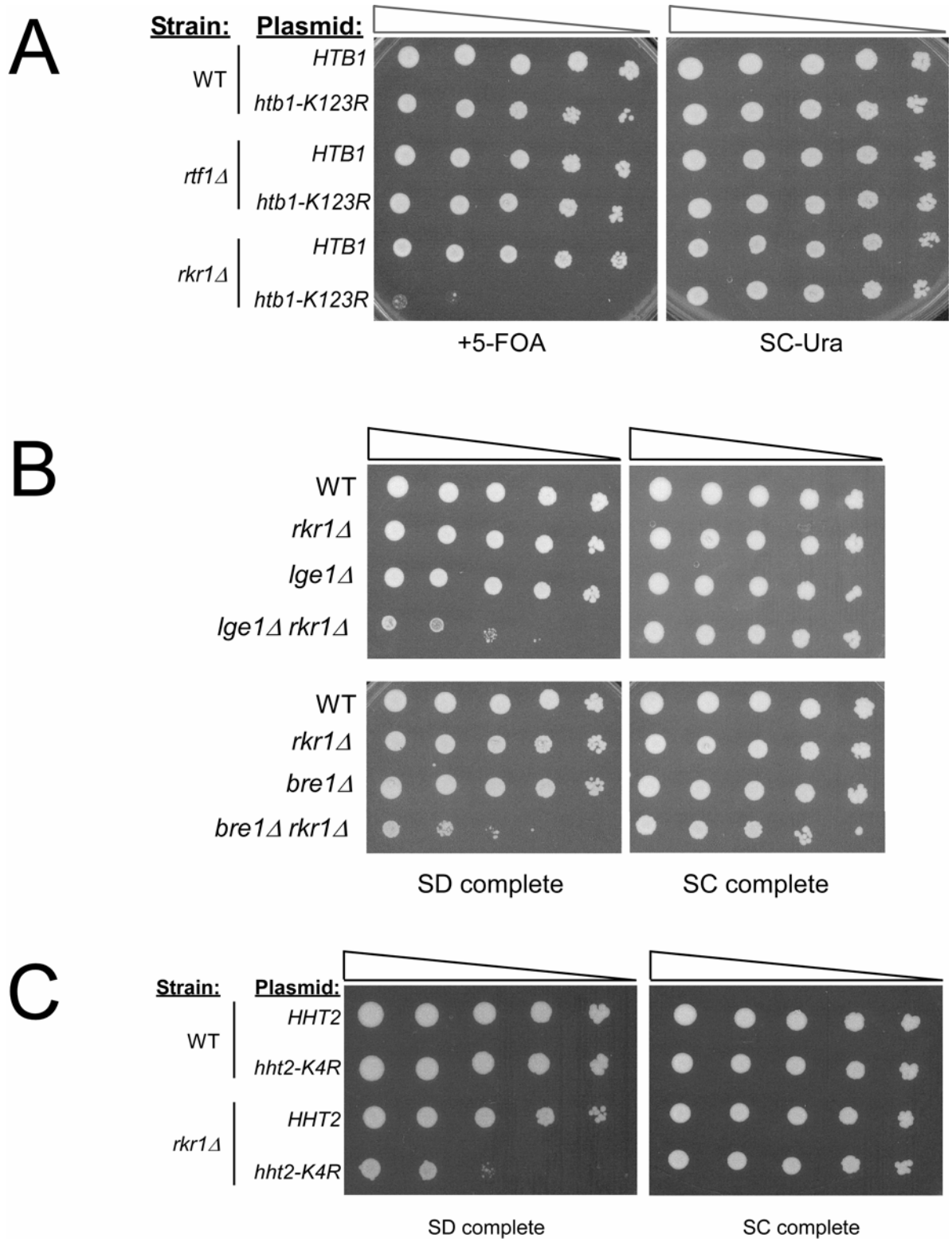
[a] Double mutants were generated from the following genetic crosses unless stated otherwise: KY1166 and KY1174, KY1166 and KY1175, MBY30 and FY623, KY1171 and KY968, the *lge1Δ* was created in a *RKR1/rkr1Δ* diploid (KY1173) prior to sporulation and tetrad dissection, KY1168 and KY907, the *set1Δ rkr1-C1508A* double mutant (KY1222) was generated by transformation, KY1168 and KY903, KY1168 and KY912, FY896 and KY1168, and FY2199 and KY1168.

[b] All phenotypes listed correspond to the synthetic phenotypes observed for the double mutant strains. SD^-, poor growth on SD media; Slow growth, small colonies after 7 days of growth at 30^o C on YPD; Gly^-, inviable on YP glycerol media; HU^S, sensitive to 100 mM hydroxyurea; $NaCl^-$, sensitive to 1.4 M NaCl.

[c] Growth on SD medium was the only phenotype tested for this double mutant.

Figure 21. Genetic interactions between *RKR1* and genes that encode proteins that affect histone H2B ubiquitylation and histone H3 K4 methylation.

A) Wild type (KY406), *rtf1Δ* (KY982), and *rkr1Δ* (KY981) strains that lacked the genomic copies of histone H2B genes were transformed with *URA3*-marked plasmids expressing either wildtype or K123R derivatives of a gene encoding histone H2B (*HTB1*). Transformants were serially diluted and spotted onto medium either containing or lacking 5FOA. Plates were incubated at 30° C for 3 days. B) Strains with the genotypes listed on the left were serially diluted and spotted onto SD or SC complete medium. Plates were incubated at 30° C for 3 days. Strains used (from top to bottom): KY595, MBY31, KY1124, KY1123, a wild type strain derived from crossing KY1023 and KY1072, MBY36, KY1131 and KY1141, respectively. C) Wild type (KY811) and *rkr1Δ* (KY1064) strains that lacked the genomic copies of histone H3 genes were transformed with *TRP1*-marked plasmids expressing either wild type or K4R derivatives of a gene encoding histone H3 (*HHT2*). Transformants were serially diluted and spotted onto SD or SC complete medium. Plates were incubated at 30° C for 3 days.

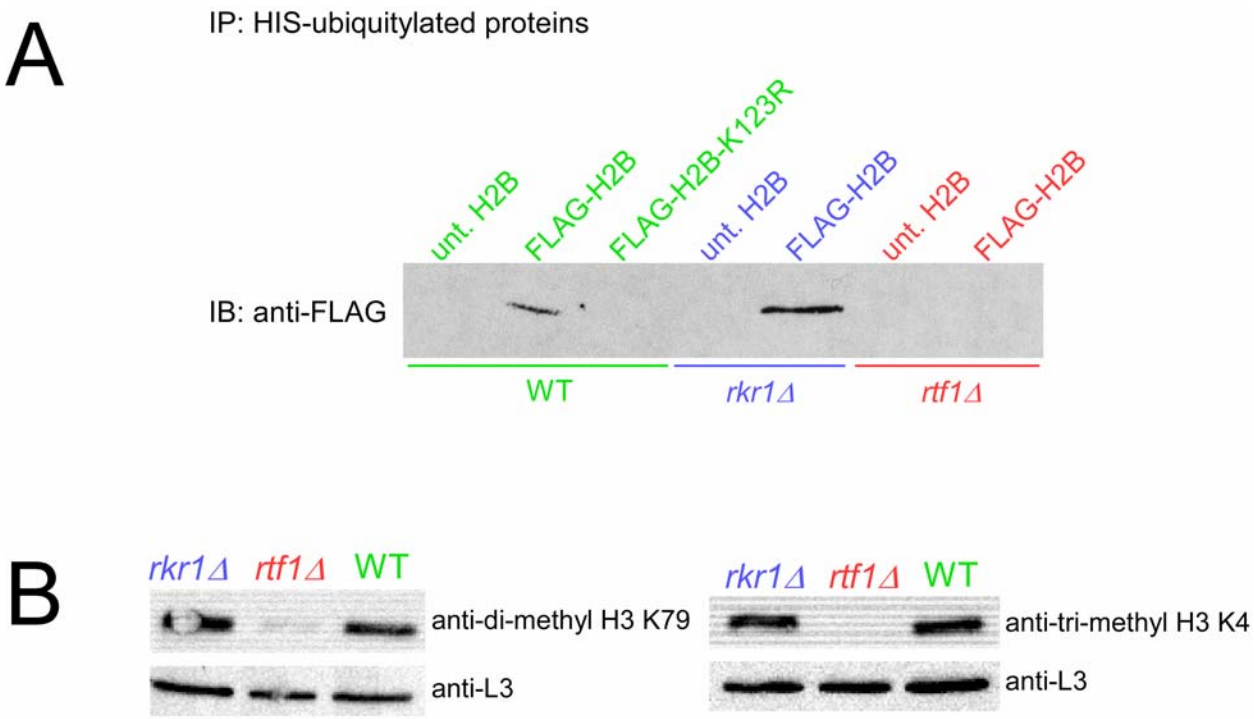

A
Strain:
Plasmid:
WT
HTB1
htb1-K123R
rtf1Δ
HTB1
htb1-K123R
rkr1Δ
HTB1
htb1-K123R
+5-FOA
SC-Ura
B
WT
rkr1Δ
lge1Δ
lge1Δ rkr1Δ
WT
rkr1Δ
bre1Δ
bre1Δ rkr1Δ
SD complete
SC complete
C
Strain:
Plasmid:
WT
HHT2
hht2-K4R
rkr1Δ
HHT2
hht2-K4R
SD complete
SC complete

While deletion of *RKR1* causes significant growth defects in strains defective for histone H2B K123 ubiquitylation or H3 K4 methylation, Rkr1 itself does not appear to affect these modifications. Histone H3 K4 trimethylation and K79 dimethylation as well as histone H2B K123 ubiquitylation occur at wild-type levels in strains lacking *RKR1* (Figure 22). Taken together, my findings suggest that Rkr1 acts in parallel with Rtf1-dependent histone modifications.

Although many strong genetic interactions between Rkr1 and factors that are important for some Rtf1-dependent histone modifications are observed, *RKR1* does not genetically interact with all factors involved in histone modification. Interestingly, while histone H3 K4 and K79 methylation both require histone H2B K123 ubiquitylation, no strong genetic interactions were observed in *rkr1Δ dot1Δ* double mutant strains. Paf1 and Ctr9 are required for histone H3 K36 trimethylation (KROGAN *et al.* 2003b), which is found on histones in active genes (POKHOLOK *et al.* 2005); however, no strong genetic interactions were observed in *rkr1Δ set2Δ* double mutant strains. Furthermore, *rkr1Δ gcn5Δ* double mutant strains show only mild phenotypes (Table 7). Gcn5 is the catalytic subunit of the SAGA histone acetyltransferase complex (RUIZ-GARCIA *et al.* 1997), and its modifications are associated with transcriptional activity (POKHOLOK *et al.* 2005). These data further support the idea that Rkr1 functions in parallel with specific Rtf1-dependent histone modifications and not all histone modifications that are associated with active transcription.

Figure 22. Rtf1-dependent histone modifications are not dirupted in *rkr1Δ* strains.

A) Wild type (KY943), *rkr1Δ* (KY981), and *rtf1Δ* (KY982) strains that lacked the genomic copies of histone H2B were transformed with plasmids that express either untagged wild type H2B, FLAG-tagged wild type H2B, or FLAG-tagged H2B–K123R, as well as plasmids that express HIS-tagged ubiquitin. Ubiquitylated proteins were isolated using a nickel resin and enriched proteins were separated on a 7-20% gradient SDS-PAGE gel. Immunoblot analysis was performed using antibodies to FLAG-H2B. Experiments using untagged ubiquitin were performed in parallel and no FLAG signal was detected using immunoblot analysis (data not shown). B) Wild type (KY303), *rtf1Δ* (KY425) and *rkr1Δ* (SHY15) strains were grown in rich medium and cell lysates were collected. Immunoblot analysis was performed with antibodies specific to histone H3 K4 trimethylation, anti-histone H3 K79 dimethylation, and ribosomal protein L3 (loading control).

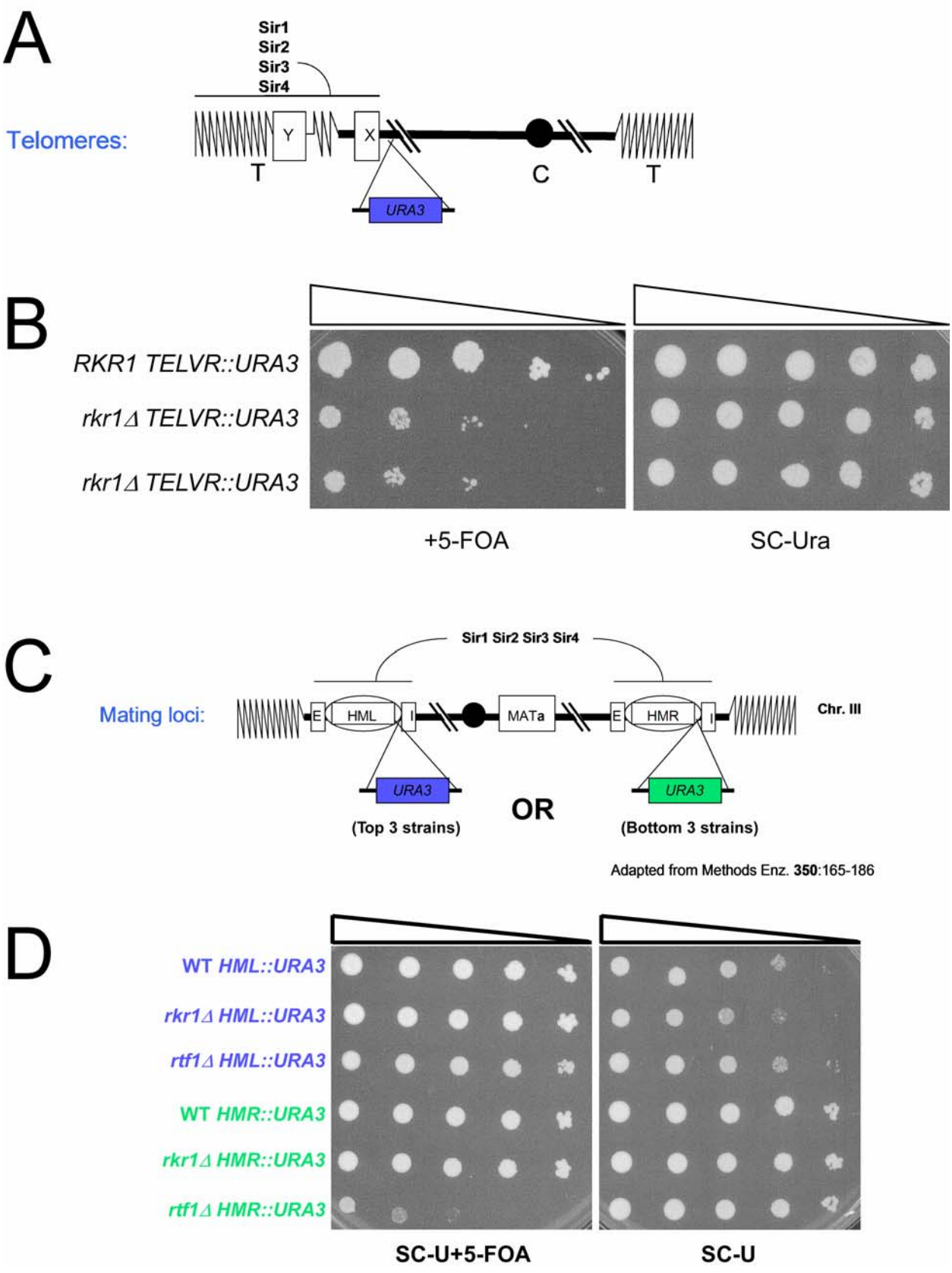

A
IP: HIS-ubiquitylated proteins
unt. H2B
FLAG-H2B
FLAG-H2B-K123R
unt. H2B
FLAG-H2B
unt. H2B
FLAG-H2B
IB: anti-FLAG
WT
rkr1Δ
rtf1Δ
B
rkr1Δ rtf1Δ WT
anti-di-methyl H3 K79
anti-L3
rkr1Δ rtf1Δ WT
anti-tri-methyl H3 K4
anti-L3

2.3.6 Strains lacking *RKR1* have defects in telomeric silencing

Transcriptional silencing of genes that are positioned near telomeres requires a particular histone modification profile. Telomeric chromatin is enriched in hypo-acetylated and hypo-methylated histones, which provide interaction sites for the Sir proteins (reviewed in RUSCHE *et al.* 2003)). Strains lacking Paf1 complex members exhibit defects in telomeric silencing (KRISHNAMURTHY *et al.* 2004; NG *et al.* 2003a), most likely because the genome-wide loss of histone H3 methylation leads to the redistribution of Sir proteins away from their normal sites of action (SANTOS-ROSA *et al.* 2004). To determine if Rkr1 is important for telomeric silencing, wild-type and *rkr1Δ* strains that contain a telomeric *URA3* reporter gene were constructed. These strains were serially diluted and plated to medium containing 5-FOA. Wild-type strains grow robustly on this medium, indicating silencing of the telomeric *URA3* reporter (Figure 23). However, strains lacking *RKR1* grow poorly on the 5-FOA medium, suggesting that telomeric silencing is disrupted in the *rkr1Δ* strains (Figure 23). In contrast, transcriptional silencing at the rDNA and silent mating type loci occur normally in *rkr1Δ* strains (Figure 23 and data not shown). These regions utilize different mechanisms to facilitate silencing (described in the section 1.3.4), which may explain why Rkr1 only affects one type of silencing. A role in telomeric silencing is consistent with the idea that Rkr1 modulates chromatin structure or function.

Figure 23. Strains lacking *RKR1* have telomeric silencing defects.

A) The telomeres of all chromosomes are silenced by the Sir family of proteins. See section 1.3.4.1 of the Introduction for additional information. B) Wild type and *rkr1Δ* strains that contained the *URA3* gene positioned near the right arm of chromosome VI (O660, KA105 and KA106, respectively) were serially diluted and spotted onto medium that either contained or lacked 5FOA. Strains were incubated at 30° C for 3 days. C) The mating loci in yeast are subject to silencing by the Sir proteins. See Introduction for additional information. D) Wild type (OKA91 and OKA92), *rtf1Δ* and *rkr1Δ* strains (derived from OKA91 and OKA92) that contained the *URA3* gene positioned near the *HML* or *HMR* locus were serially diluted and spotted onto medium that either contained or lacked 5FOA. Strains were incubated at 30° C for 3 days. *rtf1Δ* strains serve as positive controls for *HM* silencing assay. *HMR* silencing is more easily perturbed due to differences in the way the *URA3* gene was integrated at the *HMR* and *HML* loci (SINGER *et al.* 1998).


A
Sir1
Sir2
Sir3
Sir4
Telomeres:
Y
X
T
C
T
URA3
B
RKR1 TELVR::URA3
rkr1Δ TELVR::URA3
rkr1Δ TELVR::URA3
+5-FOA
SC-Ura
C
Sir1 Sir2 Sir3 Sir4
Mating loci:
E
HML
I
MATa
E
HMR
I
Chr. III
URA3
URA3
OR
(Top 3 strains)
(Bottom 3 strains)
Adapted from Methods Enz. 350:165-186
D
WT HML::URA3
rkr1Δ HML::URA3
rtf1Δ HML::URA3
WT HMR::URA3
rkr1Δ HMR::URA3
rtf1Δ HMR::URA3
SC-U+5-FOA
SC-U

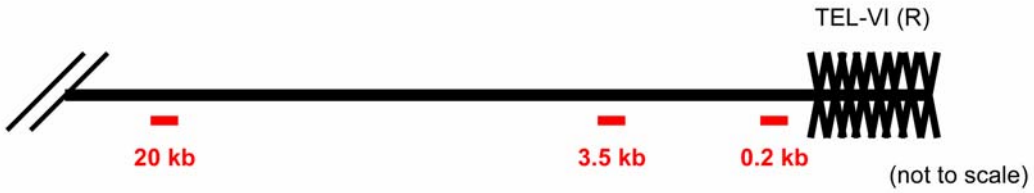
2.3.6.1 Histone deposition at telomeres is not affected in *rkr1Δ* strains

In an attempt to determine the cause of the telomeric silencing defects in *rkr1Δ* strains, histone deposition at telomeres was quiantified in wild type and *rkr1Δ* strains. Using chromatin immunoprecipitation (ChIP) analysis, the levels of histones H2B, H3 and myc-tagged H4 were determined at three different distances (0.2 kb, 3.5 kb, and 20 kb) from the telomere on the right arm of chromosome VI (Figure 24). As a control, the levels of histones at the *GAL1* gene were quantified. This gene is strongly repressed in the glucose conditions used for this experiment and therefore should be associated with high levels of histones. The results show that histone deposition at telomeres is not reduced in *rkr1Δ* strains (Figure 24). The levels of histones at the telomeres are similar to the levels found at the repressed *GAL1* gene (data not shown). While the cause of the telomeric silencing defect is currently unknown, it appears to not be due to loss of the core histones at telomeric regions.

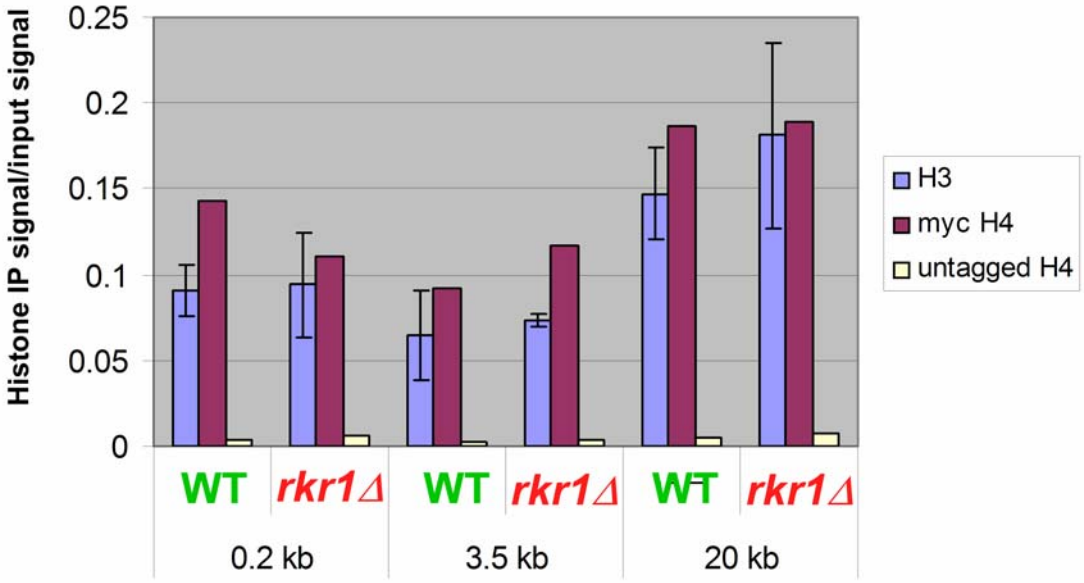
Figure 24. Histone deposition at telomere VI is not affected by the loss of Rkr1.

Wild type (KY811) and *rkr1Δ* (KY1064) strains were transformed with plasmids expressing either myc-tagged histone H4 or untagged histone H4. ChIP experiments were performed using antibodies directed towards myc-tagged histone H4 and total H3. Radioactive PCR was performed using primers that amplify sequences at 0.2, 3.5 and 20 kb from the telomere. Signals for myc-H4 and total H3 were quantitated relative to input. H3 data represent results from 3 independent experiments, and myc-H4 data represent the results from 2 independent experiments. Error bars represent the standard error in H3 ChIP experiments.

Positions of primer pairs along chromosome VI



Histone deposition at telomere VI (R) in minimal medium



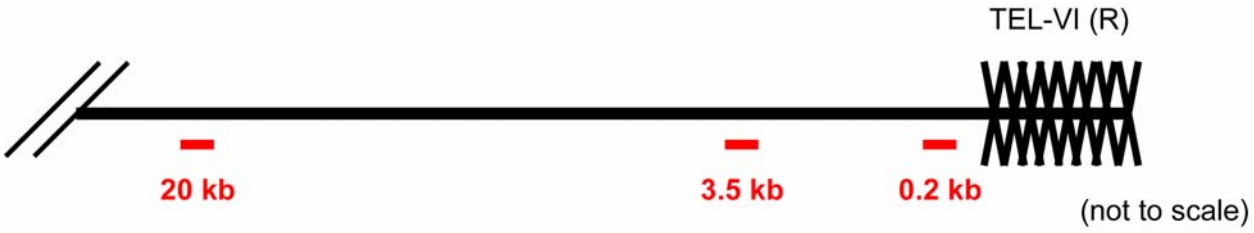
2.3.6.2 Telomeric histone H4 acetylation is not affected by the loss of *RKR1*

Telomeric silencing is a process that depends heavily on the proper modification of histones (reviewed in RUSCHE *et al.* 2003)). Silenced regions are hypomethylated and hypoacetylated, and this hypo-modified state is maintained by the Sir proteins (reviewed in section 1.3). Specifically, Sir2 is a histone deacetylase (HDAC) that reduces histone H4 acetylation in silenced regions (IMAI *et al.* 2000). To determine if Sir2 function was disrupted in *rkr1Δ* strains, ChIP analysis was performed with anti-tetra-acetylated histone H4 antibody. The same primer sets described in the previous section (0.2, 3.5 and 20 kb from the telomere) were used to amplify DNA that was immunoprecipitated as part of acetylated nucleosomes. As expected, histone acetylation increases in correlation with increasing distance from the telomere (Figure 25). However, histone H4 acetylation at the telomeres is not disrupted in *rkr1Δ* strains (Figure 25). Therefore, the mechanistic basis for the telomere silencing defect of *rkr1Δ* strains remains unknown and will require further investigation.

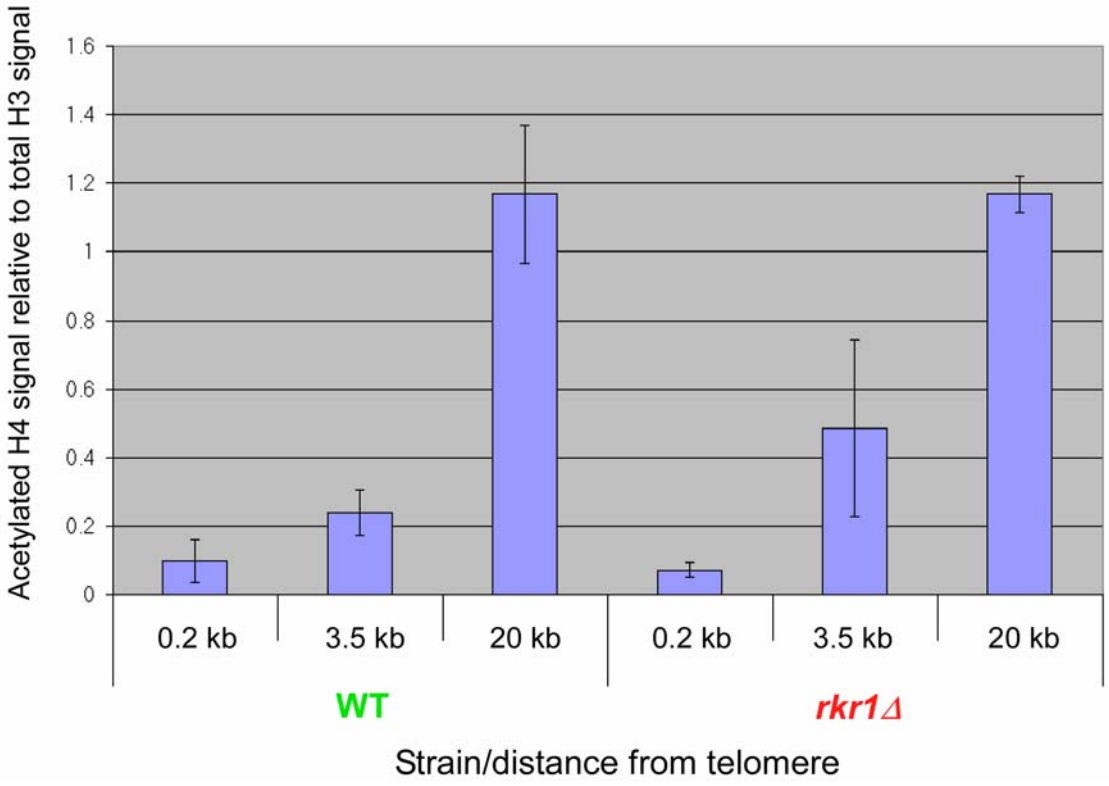
Figure 25. Histone H4 acetylation at telomere VI is not affected by the loss of Rkr1.

Chromatin immunoprecipitation experiments were performed in wild type (KY811) and *rkr1Δ* (KY1064) strains using antibodies directed towards tetra-acetylated histone H4 (K5, K8, K12 and K16 acetylated) and total H3. Radioactive PCR was performed using primers that amplify sequences at 0.2, 3.5 and 20 kb from the telomere. Signals for acetylated H4 and total H3 were quantitated relative to input, and Ac H4 signal was calculated relative to total H3.

Positions of primer pairs along chromosome VI



Tetra-acetylated H4 at Telomere VI (R) in minimal medium



2.3.7 *RKR1* genetically interacts with other factors involved in transcription

While much of the information that I have discussed so far suggests that Rkr1 is functionally connected to specific Rtf1-dependent chromatin modifications, I have also uncovered other genetic interactions that support a role for Rkr1 in chromatin function and transcription. While providing more insight into the role of Rkr1 *in vivo*, these studies are still at their beginning stages, and will require more work to fully understand their meaning.

2.3.7.1 Deletion of *RKR1* is synthetically lethal with a deletion of *SPT10*, a gene encoding a potential histone acetyltransferase

SPT10 is one of many *SPT* genes that were originally identified in a screen for suppressors of transposable element insertion mutations in yeast (FASSLER and WINSTON 1988; NATSOULIS *et al.* 1994). Strains lacking Spt10 exhibit global changes in chromatin structure and gene expression (ERIKSSON *et al.* 2005; XU *et al.* 2005). Spt10 and its interacting partner Spt21 bind to the promoters of histone genes and activate their transcription, providing a potential explanation for the broad transcriptional effects of *spt10* mutations (DOLLARD *et al.* 1994; ERIKSSON *et al.* 2005; HESS *et al.* 2004; XU *et al.* 2005). Interestingly, Spt10 contains a predicted acetyltransferase domain (NEUWALD and LANDSMAN 1997) that is required for its transcription activation activity (HESS *et al.* 2004), and *spt10* mutants have reduced histone H3 K56 acetylation at histone gene promoters (XU *et al.* 2005). However, direct acetylation of histones by Spt10 has yet to be demonstrated (HESS *et al.* 2004; XU *et al.* 2005), and recent studies indicate that global levels of histone H3 K56 acetylation are greatly reduced in strains lacking Rtt109 or Asf1 (DRISCOLL *et al.* 2007; HAN *et al.* 2007a; HAN *et al.* 2007b; TSUBOTA *et al.* 2007), but not Spt10 (SCHNEIDER *et al.* 2006b). A synthetic lethal screen involving *spt10Δ*

identified mutations in *RKR1* (D. Hess and F. Winston, personal communication). I confirmed this result by tetrad analysis using complete deletions of *RKR1* and *SPT10* (Figure 26).

Because Spt10 has a well-studied role in histone gene transcription, Elia Crisucci measured histone mRNA levels in a *rkr1Δ* strain using RT-PCR analysis and oligonucleotide primers that distinguish among the highly related histone genes (HESS *et al.* 2004). Elia found that all of the histone genes are expressed at nearly wild-type levels in the *rkr1Δ* strain (BRAUN *et al.* 2007), while *HTA2*, *HTB2*, and *HHF2* mRNA levels are significantly decreased in the *spt10Δ* strain, as expected from earlier studies (ERIKSSON *et al.* 2005; HESS *et al.* 2004). I confirmed by immunoblotting analysis that histone H2B, H3, and H4 levels are unaffected by the *rkr1Δ* mutation (Figure 26; H2A levels were not tested). Therefore, the inviability of *rkr1Δ spt10Δ* double mutant strains is most likely not due to insufficient histone gene expression. Consistent with this idea, *rkr1Δ spt21Δ* double mutants are viable (Table 7), even though *spt21Δ* and *spt10Δ* mutations both reduce histone mRNA levels (DOLLARD *et al.* 1994; HESS *et al.* 2004).

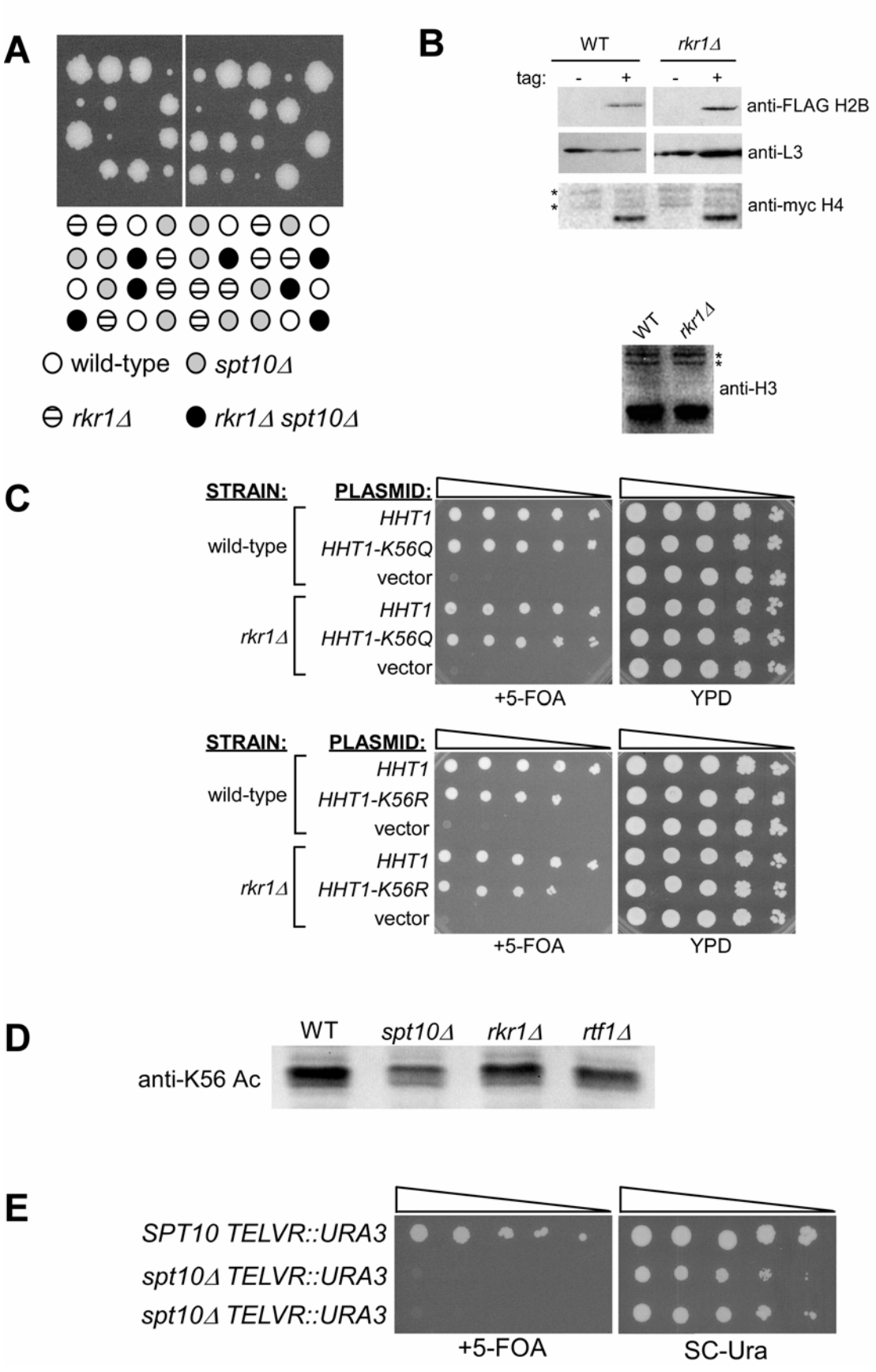
To determine if Rkr1 is essential in the absence of Spt10 because histone H3 K56 acetylation is defective, I performed a plasmid shuffle experiment with $RKR1^{+}$ and *rkr1Δ* strains that lacked both chromosomal copies of the genes for histones H3 and H4 and carried a *URA3*-marked *HHT2-HHF2 CEN/ARS* plasmid. Cells were transformed with a *TRP1*-marked plasmid that expressed a histone H3 derivative in which K56 was replaced with arginine or glutamine (MASUMOTO *et al.* 2005). Following growth on synthetic medium containing 5-FOA, the histone H3 K56R or K56Q derivative was the only version of histone H3 available in the cell. The results of the plasmid shuffle revealed that *rkr1Δ* strains grow as well as $RKR1^{+}$ strains in the presence of the histone H3 K56R and K56Q derivatives (Figure 26). In addition, histone H3 K56

acetylation occurs at wild-type levels in *rkr1Δ* cells (Figure 26). Therefore, the basis for the synthetic lethality between *rkr1Δ* and *spt10Δ* remains unclear but it appears not to be related to Spt10's proposed role in histone H3 K56 acetylation (XU *et al.* 2005).

Since loss of Rkr1 or Rtf1 alleviates telomeric silencing, I wanted to determine if loss of *SPT10* also causes this phenotype. Strains lacking *SPT10* and containing the telomeric *URA3* reporter gene were constructed and plated on 5-FOA medium to monitor *URA3* expression. Consistent with a role for Spt10 in chromatin structure or function, the *spt10Δ* strains grow extremely poorly in the presence of 5-FOA, indicating a strong defect in telomeric silencing (Figure 26).

Figure 26. *rkr1Δ* is synthetically lethal with *spt10Δ*.

A) *rkr1Δ* (MBY40) and *spt10Δ* (FY896) strains of opposite mating types were mated and sporulated. Tetrads were dissected and incubated at 30° C for 5 days. B) Immunoblot analysis of histone levels show that the core histones are expressed at wild type levels in a *rkr1Δ* strain. Immunoblot analysis using antibodies toward the ribosomal protein L3 serve as a loading control for the FLAG-H2B immunoblot. Asterisks represent cross-reacting bands that serve as loading controls for the H3 and myc-H4 immunoblots. C) Plasmid shuffle experiment shows that *rkr1Δ* is not essential in the absence of histone H3 K56 acetylation. D) Immunoblot analysis shows that histone H3 K56 acetylation is not defective in *rkr1Δ* strains. The anti-K56 Ac antibody was the kind gift of Dr. Alain Verrault (University of Montreal). E) Strains lacking *SPT10* have telomeric silencing defects. Wild type (KA104) and *spt10Δ* (KA106 and KA105) strains that contain the telomeric *URA3* gene were serially diluted and spotted onto medium containing 5FOA or control medium. Plates were incubated at 30° C for 5 days.


A
wild-type
spt10Δ
rkr1Δ
rkr1Δ spt10Δ
B
WT
rkr1Δ
tag:
- + - +
anti-FLAG H2B
anti-L3
anti-myc H4
WT
rkr1Δ
anti-H3
C
STRAIN:
PLASMID:
wild-type
rkr1Δ
HHT1
HHT1-K56Q
vector
+5-FOA
YPD
HHT1-K56R
D
WT
spt10Δ
rkr1Δ
rtf1Δ
anti-K56 Ac
E
SPT10 TELVR::URA3
spt10Δ TELVR::URA3
+5-FOA
SC-Ura

2.3.7.2 *RKR1* shows genetic interactions with other transcription factors that were identified in the synthetic lethal screen with *rtf1Δ*

Patrick Costa identified nine complementation groups in his synthetic lethal screen with *rtf1Δ* (Table 8). These groups, except for the group containing *RKR1*, contained genes that all suggested Rtf1 was important for the regulation of transcription. Genetic analysis was used to determine if Rkr1 was functionally connected to any of the other genes that were identified in the *rtf1Δ* synthetic lethal screen. Interestingly, *rkr1Δ* is synthetically lethal with *srb5Δ* (Figure 27). Srb5 is a component of the Mediator complex, which is important for establishing connections between transcriptional activators and the general transcription machinery (reviewed in section 1.2.3.3). This genetic interaction suggests that Rkr1 may play a role in the regulation of transcription initiation.

In the course of these genetic studies, I observed that *rkr1Δ ctk1Δ* strains are very slow growing (Figure 27). Ctk1 is a kinase that is primarily responsible for phosphorylating the carboxy-terminal domain (CTD) of the largest subunit of RNA Pol II (Rpb1) (PATTURAJAN *et al.* 1999). The CTD in yeast is composed of 26 tandem copies of a heptapeptide repeat (YSPTSPS). Ctk1 phosphorylates the serine at position two of the repeat. This modification occurs while the polymerase is at the 3' end of transcribing genes and is important for the proper recruitment of many factors that are involved in late stages of mRNA processing, including the poly-adenylation factors (AHN *et al.* 2004). The cause of this interesting genetic interaction remains unknown, but supports a role for Rkr1 in RNA Pol II transcription elongation.

Double mutant strains were constructed that contain *rkr1Δ* and *arg82Δ*, *plc1Δ*, *swi4Δ*, *swi6Δ*, or *fcp1-110* mutations (data not shown). No detectable phenotypes were observed in the double mutants. Due to the genetic instability of *pob3-272* strains, genetic interactions were not

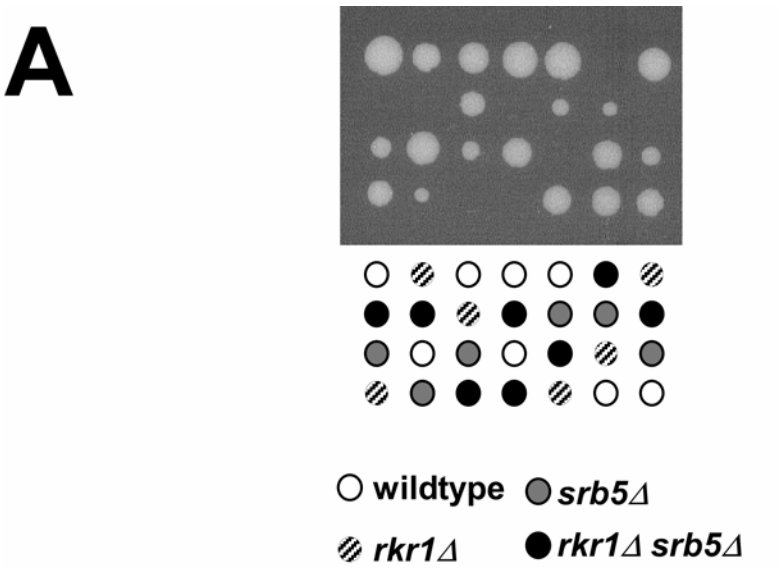
tested between *RKR1* and *POB3*.

Table 8. List of genes that are synthetically lethal with *rtf1Δ*.

Complementation group (# isolates)	Gene	Function
A (4)	*SWI6*	Transcriptional activator
B (2)	*SWI4*	Transcriptional activator
C (2)	*PLC1*	Signal transduction
D (1)	*SRB5*	Mediator component
E (1)	*CTK1*	Pol II CTD kinase
F (1)	*FCP1*	Pol II CTD phosphatase
G (1)	*POB3*	FACT, elongation factor
H (1)	*ARG82*	Signal transduction
I (1)	*RKR1*	Ubiquitin-protein ligase, Functionally connected to chromatin function

Figure 27. *rkr1Δ* is synthetically lethal with *srb5Δ* and *rkr1Δ ctk1Δ* strains grow very poorly

A) *rkr1Δ* (KY1178) and *srb5Δ* (KA21) strains of opposite mating types were mated and sporulated. Tetrads were dissected and incubated at 30° C for 4 days. B) A *ctk1Δ* strain that was transformed with a *URA3*-marked plasmid expressing *CTK1* (KY581) was crossed to a *rkr1Δ* (MBY21) strain of opposite mating type. The resulting diploid was plated to medium containing 5-FOA to counter-select for the *URA3*-marked *CTK1* plasmid. The resulting *ura3*$^{-}$ strain was sporulated. Tetrads were dissected and incubated at 30° C for 7 days.


A
wildtype
srb5Δ
rkr1Δ
rkr1Δ srb5Δ
B
Plate #1
Plate #2
wildtype
ctk1Δ
rkr1Δ
rkr1Δ ctk1Δ

2.4 CONCLUSIONS

To try to determine a function for Rkr1 *in vivo*, we turned to the "awesome power of yeast genetics". Strains lacking *RKR1* have phenotypes that indicate that Rkr1 globally affects transcription and sporulation (inositol auxotrophy and Spo$^-$, respectively). Indirect immunofluorescence experiments show that Rkr1 is a nuclear protein. The results of Patrick Costa's synthetic lethal screen were confirmed to show that *rkr1Δ* is synthetically lethal with *rtf1Δ*. Further analysis suggests that Rkr1 functions in parallel with Rtf1-dependent histone modifications. Strains lacking *RKR1* in combination with *RAD6*, *BRE1*, or *LGE1* are sick and grow poorly on minimal medium. Strains lacking *RKR1* where histone H2B ubiquitylation at K123 is disrupted exhibit a strong growth defect, suggesting that Rkr1 functions in parallel with histone H2B K123 ubiquitylation. Also, *rkr1Δ set1Δ* strains grow poorly on minimal medium, but *rkr1Δ dot1Δ* strains show no detectable phenotypes. These observations suggest that Rkr1 functions in parallel with histone H3 K4 methylation but not histone H3 K79 methylation, yet both of these modifications depend on histone H2B K123 ubiquitylation (Figure 28).

Strains lacking Paf1 complex members have defects in telomeric silencing, due to loss of global histone H3 methylation marks (SANTOS-ROSA *et al.* 2004). My results indicating that strains lacking *RKR1* also have this phenotype support the idea that Rkr1 affects chromatin modifications and/or function. Histone H4 acetylation at the telomere is found at similar levels in wildtype and *rkr1Δ* strains; and histone deposition at the telomeres is not affected in *rkr1Δ* strains. The cause of the telomeric silencing defect in a *rkr1Δ* strain is therefore unknown.

No genetic interactions were found to suggest that Rkr1 functions primarily in

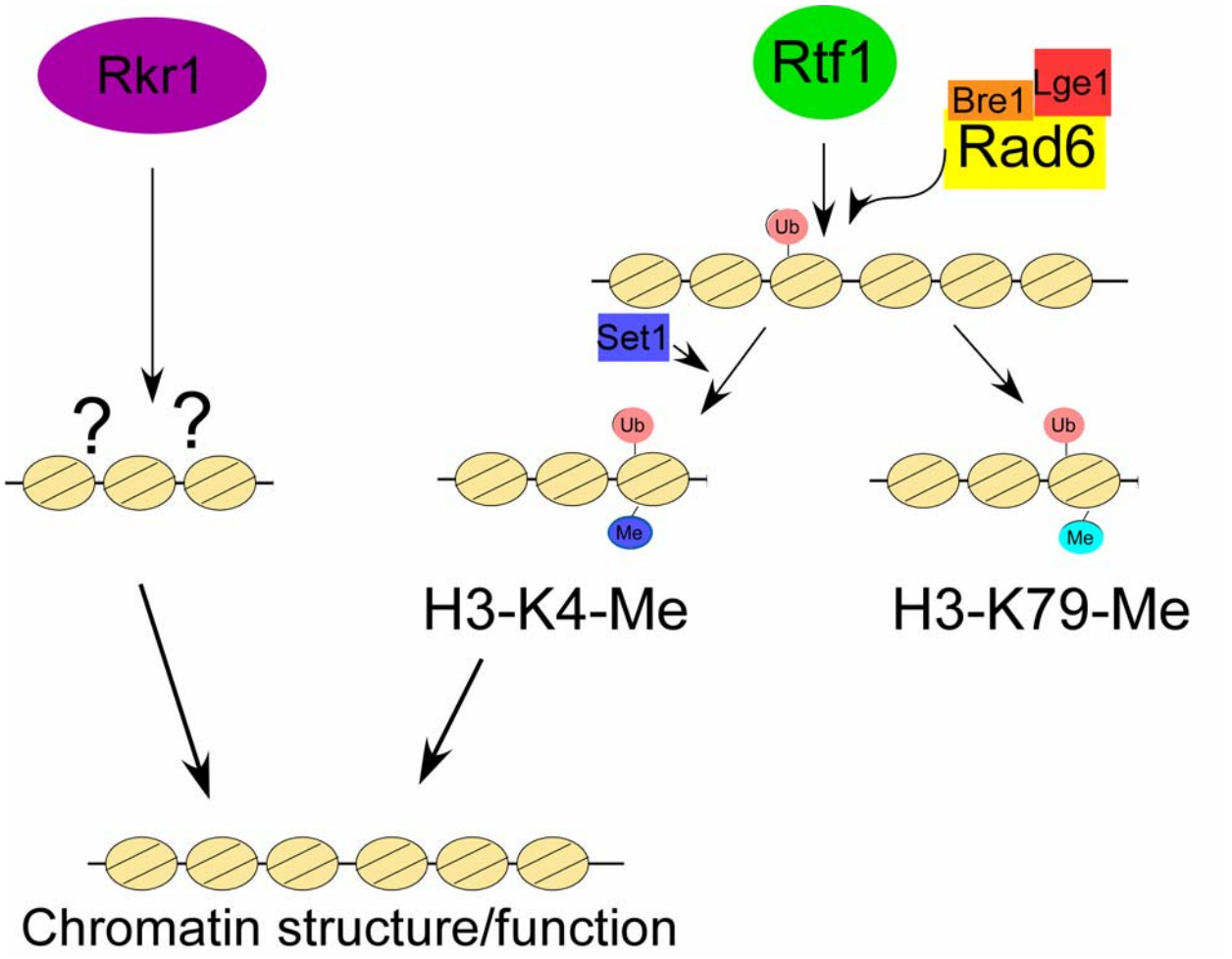
transcription elongation. While *rkr1Δ paf1Δ* and *rkr1Δ ctr9Δ* strains are sick, strains lacking *RKR1* and other elongation factors like *CDC73*, *LEO1*, *SPT4*, *SPT5*, *SPT6*, *SPT16* or TFIIS do not show strong synthetic phenotypes. The synthetic lethality between *RKR1* and *RTF1* suggests that Rkr1 functions in parallel with a role of the Paf1 complex that is primarily carried out by Rtf1, most likely histone H2B ubiquitylation and histone H3 K4 methylation.

Preliminary results from the Winston lab were confirmed to show that strains lacking *RKR1* and *SPT10* are inviable. This result suggests that this strong genetic interaction is probably not due to the known roles for Spt10 (histone gene expression and K56 acetylation), and may instead be due to an as-yet-undiscovered role of Spt10. While I have shown that strains lacking *RKR1* have wild type histone mRNA and histone protein levels, histone deposition in the genome or subsequent modification may be disrupted in these strains. My analysis shows that histone H3 K56 acetylation occurs at wild type levels in *rkr1Δ* strains. Strains lacking *SPT10* or *RKR1* have defects in telomeric silencing, a process that depends heavily on proper chromatin structure. These data further support a role for Rkr1 in chromatin structure and/or function.

I also observe genetic interactions between *rkr1Δ* and *srb5Δ* and *ctk1Δ*. Strains lacking *RKR1* and *SRB5* are inviable, while strains lacking *RKR1* and *CTK1* are sick. Srb5 is a subunit of the Mediator coactivator complex and Ctk1 is a kinase that targets the CTD of RNA Pol II. While further characterization is needed to fully understand the meaning of these interactions, they functionally connect Rkr1 to transcription.

Figure 28. Model for Rkr1 function based on genetic interactions.

Based on the genetic interactions described in Chapter 2, I believe that Rkr1 functions in parallel with Rtf1-dependent histone modifications to affect chromatin structure and/or function. Specifically, Rkr1 appears to function in parallel with histone H2B ubiquitylation and histone H3 K4 methylation, but not histone H3 K79 methylation. Rad6/Bre1/Lge1 and Set1 are the H2B ubiquitylation machinery and the H3 K4 methyltransferase, respectively.


Rkr1
Rtf1
Bre1
Lge1
Rad6
Ub
Set1
?
?
Ub
Me
Ub
Me
H3-K4-Me
H3-K79-Me
Chromatin structure/function

3.0 FUNCTIONAL ANALYSIS OF RKR1 USING DATABASE ANALYSIS AND *IN VITRO* ASSAYS

3.1 INTRODUCTION

Covalent attachment of ubiquitin to a substrate protein requires the coordinated functions of a ubiquitin-activating enzyme, a ubiquitin-conjugating enzyme, and a ubiquitin-protein ligase (reviewed in FANG and WEISSMAN 2004). While yeast contain only one ubiquitin-activating enzyme and eleven ubiquitin-conjugating enzymes, there are many predicted ubiquitin-protein ligases, consistent with their proposed roles in determining substrate specificity (reviewed in PICKART 2001). Ubiquitin-protein ligases contain functional domains known as RING or HECT domains that interact with ubiquitin-conjugating enzymes (HUIBREGTSE *et al.* 1995; LORICK *et al.* 1999). Several nuclear RING domain-containing ubiquitin-protein ligases have been characterized in yeast. These include Rad5 and Rad18 which direct the ubiquitylation of PCNA during DNA damage repair (HOEGE *et al.* 2002), San1 which is required for the degradation of certain misfolded nuclear proteins (DASGUPTA *et al.* 2004; GARDNER *et al.* 2005), Bre1 which ubiquitylates histone H2B at K123 (HWANG *et al.* 2003; WOOD *et al.* 2003a), and Not4 which is a component of the multi-functional Ccr4-Not complex (PANASENKO *et al.* 2006).

In parallel with the studies described in Chapter 2, I performed several database analyses to identify functional domains or motifs within the primary amino acid sequence of Rkr1. These

analyses show that Rkr1 contains a conserved RING domain at its extreme carboxy terminus. I show that this RING domain is required for Rkr1's *in vivo* function, and that this domain has ubiquitin-protein ligase activity *in vitro*. This information, combined with results that I described in Chapter 2, suggests that Rkr1 may use its ubiquitin-protein ligase activity to ubiquitylate nuclear proteins, which directly or indirectly affect chromatin modifications or function.

3.2 METHODS

3.2.1 Plasmids

Standard cloning techniques were used to construct all plasmids (AUSUBEL 1988). pMB66 was created by site-directed mutagenesis using pMB11 (described in section 2.2.2) as a template to replace cysteine 1508 of Rkr1 with alanine. pMB30 was created by ligating a 1137 bp blunt-ended BanI/StuI fragment from pPC65 to SmaI digested pGEX-3X (SMITH and JOHNSON 1988) to generate a GST fusion protein containing amino acids 1251-1562 of Rkr1. pMB81 was created by site-directed mutagenesis of pMB30, replacing cysteine 1508 of Rkr1 with an alanine. pMB81 was sequenced throughout the *RKR1* ORF to ensure that no secondary mutations were created.

3.2.2 Media

Media lacking (-Ino) or containing 200 μM inositol (+Ino) were prepared with yeast nitrogen

base that contained ammonium sulfate but lacked inositol (Q-Bio Systems). Ampicillin was added to Luria Broth (LB) medium as necessary to a final concentration of 50 µg/ml.

3.2.3 Sequence analysis.

PROSITE (http://ca.expasy.org/prosite) sequence analysis tools were used to predict the presence of any functional motifs or domains within the primary amino acid sequence of Rkr1. A RING domain is predicted at the carboxy terminus of Rkr1, consisting of amino acids 1508-1554. The sequence alignment in Figures 29 and 30 were obtained from BLAST (http://www.ncbi.nlm.nih.gov/BLAST) searches, using *S. cerevisiae* Rkr1 protein sequence in its entirety as the query. Sequences of the most similar proteins from *H. sapiens* (accession # NP_056380.1; E value = 4 e-41), *M. musculus* (accession # XP_982690.1; E value = 7 e-42), *D. melanogaster* (accession # NP_730427; E value = 1 e-21), *A. thaliana* (accession # NP_200649.1; E value = 2 e-36), and *S. pombe* (accession # CAA20765.1; E value = 3 e-51) were aligned with *S. cerevisiae* Rkr1 sequence using the Clustal W program (http://www.ebi.ac.uk/clustalw). The alignments were copied into Jalview (http://www.jalview.org) and purple shading was added using a threshold of 50% sequence identity.

3.2.4 Growth assays

Growth assays were performed as described in the methods section of Chapter 2 (section 2.2).

3.2.5 Immunoblotting analysis.

Transformed cells were grown under selective conditions to a density of approximately 4 x 10^7 cells/ml. Whole cell extracts were prepared by glass bead lysis in lysis buffer (100 mM sodium acetate, 20 mM HEPES, pH 7.4, 2 mM magnesium acetate, 10 mM EDTA, 10% glycerol, 1 mM DTT, plus protease inhibitors), as previously described (SHIRRA *et al.* 2005). Proteins (25 μg extract per lane) were separated on 7.5% SDS polyacrylamide gels and transferred to nitrocellulose membranes. The following antibodies were used: anti-HA (1:3000 dilution; Roche), anti-L3 (1:5000 dilution) (VILARDELL and WARNER 1997), anti-FLAG M2 (1:1000; Sigma), anti-ubiquitin (1:50) (KAHANA and GOTTSCHLING 1999), anti-GST (1:500; Invitrogen). HRP-conjugated secondary antibodies (Amersham Biosciences) were used at a 1:5000 dilution. Immunoreactive proteins were detected by chemiluminescence (Perkin Elmer) and visualized with a Kodak 440CF digital imaging station.

3.2.6 Purification of recombinant proteins.

BL21 (DE3) cells were transformed separately with plasmids expressing wild type (pMB30) or mutant (pMB81) GST-Rkr1 fusion proteins, or GST alone (pGEX-3X). Cells were grown at 37°C and induced for 1.5 hours at OD_{600} of 0.6-0.7 with 0.3 mM IPTG. Cell lysates were made by sonication in a buffer containing 50 mM Tris-Cl, pH 8.0 and 1 mM EDTA as described previously (DASGUPTA *et al.* 2004). Lysate (10 ml) was incubated for 1 hr at 4^o C with 1 ml prewashed GST Sepharose beads in the presence of 1% Triton X-100 and protease inhibitors. Sepharose beads were pelleted and washed 3 times with phosphate-buffered saline (PBS) pH 7.3 (AUSUBEL 1988) plus 10% glycerol, 1% Triton X-100 and protease inhibitors. Fusion proteins

were eluted in PBS plus 1% Triton X-100 and 50 mM glutathione for 10 minutes at room temperature. Eluate protein amounts were estimated by comparing Coomassie staining intensities of eluted proteins to those of known amounts of BSA.

3.2.7 *In vitro* ubiquitylation assays

Ubiquitylation assays were performed as previously described (GARDNER *et al.* 2005), with some modifications. Ubiquitin-activating enzyme (yeast recombinant Uba1), ubiquitin-conjugating enzyme (human recombinant UbcH5a) and ubiquitin (human recombinant) were purchased from Boston Biochem. 100 ng Uba1, 200 ng UbcH5a, and ~500 ng GST fusion proteins were combined in reaction buffer (50 mM Tris-Cl, pH 7.5, 2 mM ATP, 2.5 mM $MgCl_2$, 0.5 mM DTT) with 2.5 μg ubiquitin in 30 μl reactions. Reactions were incubated for 1.5 hrs at 30^o C. Proteins were separated on a 7-20% gradient SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblotting analysis was performed with anti-ubiquitin antibody (KAHANA and GOTTSCHLING 1999) at a 1:50 dilution to detect ubiquitin-conjugated substrates and anti-GST antibody (Invitrogen) at a 1:500 dilution to detect and normalize levels of GST fusion proteins. HRP-conjugated secondary antibodies (Amersham Biosciences) were used at 1:5000 dilutions. Chemiluminescent signals (Perkin Elmer) were detected with a Kodak 440CF digital imaging station.

3.3 RESULTS

3.3.1 Database analyses reveal that Rkr1 contains a RING domain at its extreme carboxy terminus

In parallel with the genetic analyses, I performed several database searches in an effort to identify a cellular role for Rkr1. BLAST analysis showed that the protein is conserved with other uncharacterized proteins of similar size in many eukaryotes, including humans (Figure 29). There is significant homology throughout the protein, but a region at the carboxy terminus is most highly conserved (Figure 30). Sequence analysis indicated that a RING domain exists in this region, between amino acids 1508 and 1554 (Figure 30). Importantly, the same proteins are identified at a significant level if BLAST analysis is performed with the *S. cerevisiae* Rkr1 sequence without the RING domain (data not shown). This suggests that the proteins from other eukaryotes are mostl likely true Rkr1 homologs.

RING domains consist of eight critical amino acids, often 7 cysteine residues and one histidine residue, that bind two zinc ions to form a cross-brace structure (reviewed in JACKSON *et al.* 2000) (see Figure 12 in the Introduction). The RING domain of Rkr1 is of the C4HC3 type and is the only recognizable domain or motif within the protein. RING domains bind to ubiquitin-conjugating enzymes (E2s) to catalyze the transfer of ubiquitin from the E2 to the substrate. In most RING domains, the cysteine and histidine residues are arranged in either a C3HC4 or C3H2C3 sequence (reviewed in PICKART 2001). The C4HC3 pattern of the Rkr1 RING domain appears to be less common (DODD *et al.* 2004; HASSINK *et al.* 2005). The solution structure of the C4HC3 RING domain of the Kaposi's sarcoma-associated herpesvirus K3 protein has been solved (DODD *et al.* 2004) (Figure 12 in the Introduction). Chemical mutagenesis and

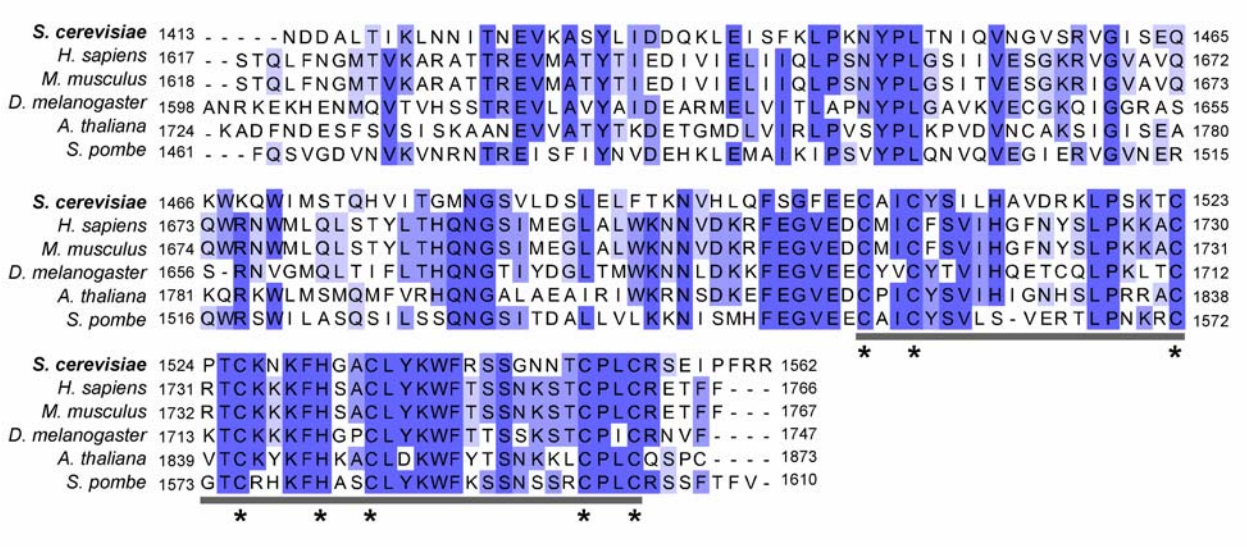
two-hybrid analysis showed that this non-canonical RING domain interacts with ubiquitin-conjugating enzymes on the same face of the RING domain as classical RING domains (DODD *et al.* 2004).

Figure 29. Rkr1 protein sequence is conserved among eukaryotes.

Alignment of primary amino acid sequence for *S. cerevisiae* Rkr1 and homologs identified in *H. sapiens*, *M. musculus*, *D. melanogaster*, *A. thaliana*, and *S. pombe* (top to bottom, respectively). The individual residues are not meant to be read. Instead, this figure is shaded to 50% sequence identity, with darker shades of blue representing higher degrees of conservation. Note the conservation at the amino terminus as well as the higher level of conservation at the carboxy terminus.

Figure 30. The RING domain of Rkr1 is highly conserved.

A) Rkr1 contains a conserved RING domain at its extreme carboxy terminus. Enlargement of the alignment shown in Figure 29. The last 150 amino acids from each of the organisms listed is shaded at 50% sequence identity, with darker shading representing higher conservation. The residues within the RING domain are underlined, and those residues that are critical for RING domain formation are marked with asterisks.

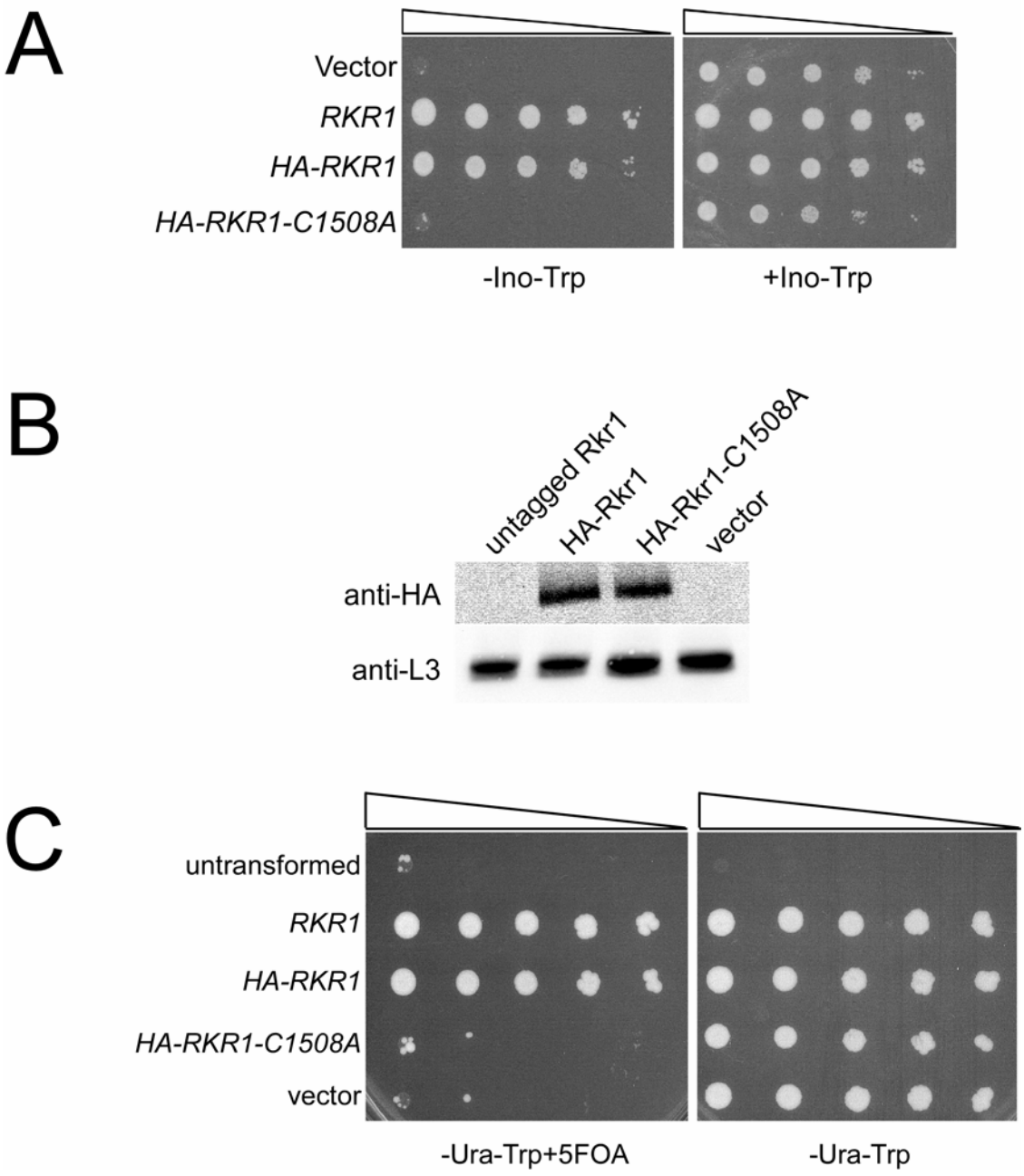

S. cerevisiae 1413 -----NDDALTIKLNNITNEVKASYLIDDQKLEISFKLPKNYPLTNIQVNGVSRVGISEQ 1465
H. sapiens 1617 --STQLFNGMTVKARATTREVMATYTIEDIVIELIIQLPSNYPLGSIIVESGKRVGVAVQ 1672
M. musculus 1618 --STQLFNGMTVKARATTREVMATYTIEDIVIELIIQLPSNYPLGSITVESGKRIGVAVQ 1673
D. melanogaster 1598 ANRKEKHENMQVTVHSSTREVLAVYAIDEARMELVITLAPNYPLGAVKVECGKQIGGRAS 1655
A. thaliana 1724 -KADFNDESFSVSISKAANEVVATYTKDETGMDLVIRLPVSYPLKPVDVNCAKSIGISEA 1780
S. pombe 1461 ---FQSVGDVNVKVNRNTREISFIYNVDEHKLEMAIKIPSVYPLQNVQVEGIERVGVNER 1515
S. cerevisiae 1466 KWKQWIMSTQHVITGMNGSVLDSLELFTKNVHLQFSGFEECAICYSILHAVDRKLPSKTC 1523
H. sapiens 1673 QWRNWMLQLSTYLTHQNGSIMEGLALWKNNVDKRFEGVEDCMICFSVIHGFNYSLPKKAC 1730
M. musculus 1674 QWRNWMLQLSTYLTHQNGSIMEGLALWKNNVDKRFEGVEDCMICFSVIHGFNYSLPKKAC 1731
D. melanogaster 1656 S-RNVGMQLTIFLTHQNGTIYDGLTMWKNNLDKKFEGVEECYVCYTVIHQETCQLPKLTC 1712
A. thaliana 1781 KQRKWLMSMQMFVRHQNGALAEAIRIWKRNSDKEFEGVEDCPICYSVIHIGNHSLPRRAC 1838
S. pombe 1516 QWRSWILASQSILSSQNGSITDALLVLKKNISMHFEGVEECAICYSVLS-VERTLPNKRC 1572
S. cerevisiae 1524 PTCKNKFHGACLYKWFRSSGNNTCPLCRSEIPFRR 1562
H. sapiens 1731 RTCKKKFHSACLYKWFTSSNKSTCPLCRETFF--- 1766
M. musculus 1732 RTCKKKFHSACLYKWFTSSNKSTCPLCRETFF--- 1767
D. melanogaster 1713 KTCKKKFHGPCLYKWFTTSSKSTCPICRNVF---- 1747
A. thaliana 1839 VTCKYKFHKACLDKWFYTSNKKLCPLCQSPC---- 1873
S. pombe 1573 GTCRHKFHASCLYKWFKSSNSSRCPLCRSSFTFV- 1610

3.3.2 Mutational analysis shows that the RING domain of Rkr1 is required for *in vivo* function

To determine if the RING domain is important for the *in vivo* function of Rkr1, I mutated the first cysteine of the RING domain, changing it to an alanine (Rkr1-C1508A). Similar substitutions have been shown to disrupt the functions of other RING domain-containing proteins (DASGUPTA *et al.* 2004; TAKAGI *et al.* 2005). Strains lacking the genomic copy of *RKR1* were transformed with plasmids expressing wild-type and C1508A forms of Rkr1. Growth assays showed that strains expressing wildtype Rkr1 grow on media lacking inositol, while strains expressing Rkr1-C1508A grow as poorly as *rkr1Δ* strains (Figure 31). Furthermore, using a plasmid shuffle experiment, I showed that the Rkr1-C1508A derivative fails to complement the synthetic lethality between *rtf1Δ* and *rkr1Δ* (Figure 31). Immunoblotting analysis demonstrated that the inability of Rkr1- C1508A to complement the *rkr1Δ* allele is not due to instability of the Rkr1 mutant protein (Figure 31). Together, these data demonstrate that the conserved RING domain of Rkr1 is required for the function of the protein *in vivo*.

Figure 31. The RING domain of Rkr1 is required for *in vivo* function.

A) *HA-RKR1-C1508A* does not complement a *rkr1Δ* mutation. A *rkr1Δ* strain (KY1168) was transformed with *TRP1*-marked plasmids expressing untagged *RKR1*, HA-tagged *RKR1*, HA-tagged *RKR1-C1508A,* or empty vector. Transformants were serially diluted and spotted onto medium lacking tryptophan and either lacking or containing inositol. Plates were incubated at 30° C for 3 days. B) Immunoblot analysis shows that HA-Rkr1-C1508A is expressed at levels equivalent to HA-Rkr1. L3 levels serve as loading controls. C) A *rkr1Δ rtf1Δ* (KY960) strain was transformed with *TRP1*-marked plasmids expressing either untagged, HA-tagged wildtype, or HA-tagged RING mutant derivatives of Rkr1. An untransformed strain (KY960) was also spotted to show the requirement for the *RTF1* plasmid for survival. Transformants were serially diluted and spotted onto medium either containing or lacking 5FOA. Plates were incubated at 30° C for 3 days.

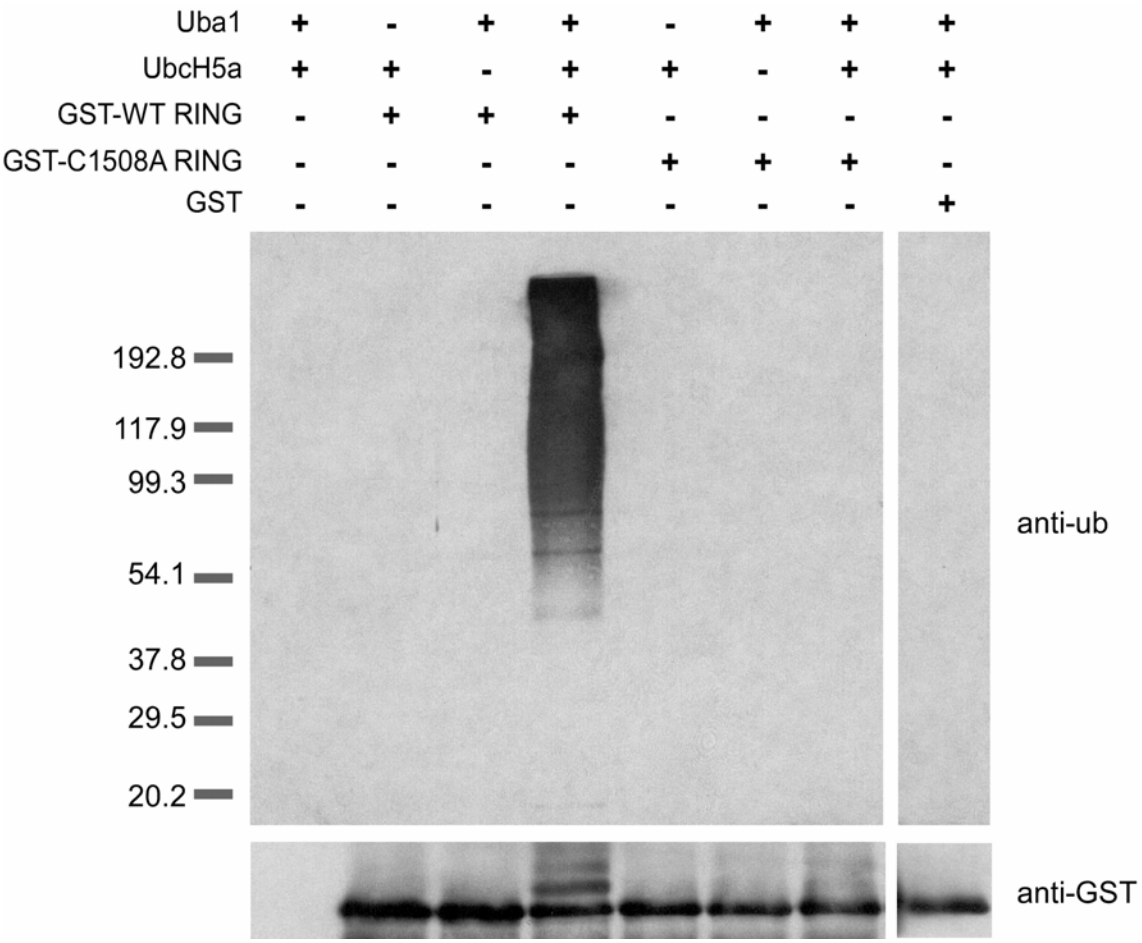

A
Vector
RKR1
HA-RKR1
HA-RKR1-C1508A
-Ino-Trp
+Ino-Trp
B
untagged Rkr1
HA-Rkr1
HA-Rkr1-C1508A
vector
anti-HA
anti-L3
C
untransformed
RKR1
HA-RKR1
HA-RKR1-C1508A
vector
-Ura-Trp+5FOA
-Ura-Trp

3.3.3 The RING domain of Rkr1 possesses ubiquitin-protein ligase activity *in vitro*

Proteins that contain a RING domain often possess ubiquitin-protein ligase activity (FANG and WEISSMAN 2004). Within the ubiquitylation pathway, RING-domain ubiquitin-protein ligases are thought to bring the ubiquitin-conjugating enzyme and substrate together to facilitate the transfer of ubiquitin to the substrate (FANG and WEISSMAN 2004). Since the only identified domain or motif in Rkr1 is the carboxy-terminal RING domain, I tested Rkr1 for this activity. Because attempts to express full-length, recombinant Rkr1 were unsuccessful, GST fusions to the carboxy terminus of Rkr1 (amino acids 1251-1562) were constructed. Wild-type Rkr1 and Rkr1-C1508A GST fusions, as well as GST alone, were purified from bacteria. *In vitro* ubiquitin-protein ligase assays were performed using recombinant ubiquitin, ubiquitin-activating enzyme (yeast Uba1), ubiquitin conjugating enzyme (human UbcH5a), and GST-RING proteins. These conditions have been used previously to show that other RING domain-containing proteins can facilitate polyubiquitylation (GARDNER *et al.* 2005). Reactions lacking individual components of the ubiquitylation pathway were performed to show that all components of the pathway are required for efficient ubiquitylation. The results of my experiments show that the RING domain of Rkr1 has ubiquitin-protein ligase activity that is dependent on the presence of the first cysteine residue in the RING motif (Figure 32). Neither the Rkr1-C1508A fusion protein nor GST yielded detectable levels of protein ubiquitylation.

Figure 32. The RING domain of Rkr1 possesses ubiquitin protein ligase activity in vitro.

Purified, recombinant proteins were added in various combinations to reactions containing ATP and ubiquitin. Reactions were separated on a 7-20% gradient SDS polyacrylamide gel and immunoblot analysis was performed using anti-ubiquitin and anti-GST antibodies. See methods and text for additional information.


Uba1 + - + + - + + +
UbcH5a + + - + + - + +
GST-WT RING - + + + - - - -
GST-C1508A RING - - - - + + + -
GST - - - - - - - +
192.8
117.9
99.3
54.1
37.8
29.5
20.2
anti-ub
anti-GST

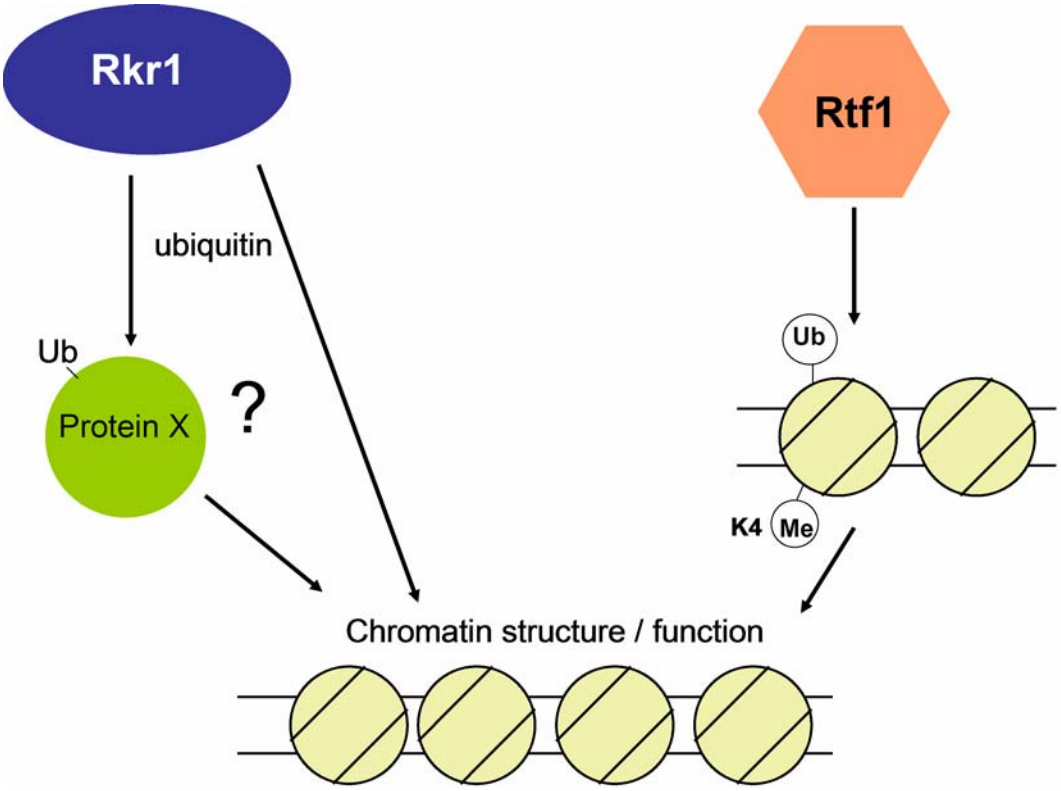
3.4 CONCLUSIONS

In my attempts to determine an *in vivo* function for Rkr1, I performed several database analyses. My analysis shows that Rkr1 is conserved in eukaryotes, including humans. The RING domain at the extreme carboxy terminus is the most highly conserved region of the protein, but there is also significant homology at the amino terminus of Rkr1. The RING domain is the only recognizable domain in Rkr1. To determine if the RING domain is required for Rkr1's *in vivo* function, I used site-directed mutagenesis to destroy this structural domain. Rkr1-C1508A fails to complement the Ino^- phenotype of a *rkr1Δ* strain. In Chapter 2 I showed that this RING domain mutation fails to complement a *rkr1Δ* for synthetic lethality with *rtf1Δ* and fails to complement the SD^- phenotype of a *set1Δ rkr1Δ* strain. These results suggest that the RING domain is required for the *in vivo* function of Rkr1.

Biochemically, proteins that contain RING domains often act as ubiquitin-protein ligases (JACKSON *et al.* 2000). I used an *in vitro* ubiquitylation assay to show that the RING domain of Rkr1 can catalyze efficient poly-ubiquitylation. This activity is dependent on a functional RING domain, as Rkr1-C1508A RING domain does not catalyze detectable levels of poly-ubiquitylation *in vitro*. These data suggest that Rkr1 may function as a ubiquitin-protein ligase *in vivo*, possibly targeting factors that affect chromatin structure or function (Figure 33). In Chapter 4, I will describe several nonbiased approaches that we took to uncover targets of this ubiquitin ligase activity.

Figure 33. Model for Rkr1 activity *in vivo*.

Based on the data that were presented in Chapters 2 and 3, I believe that Rkr1 uses its ubiquitin ligase activity to either directly or indirectly affect chromatin structure and/or function in a manner that parallels the function of Rtf1-dependent histone H2B ubiquitylation and histone H3 K4 methylation.


Rkr1
Rtf1
ubiquitin
Ub
Protein X
?
Ub
K4
Me
Chromatin structure / function

4.0 IDENTIFICATION OF PROTEINS THAT PHYSICALLY INTERACT WITH RKR1

4.1 INTRODUCTION

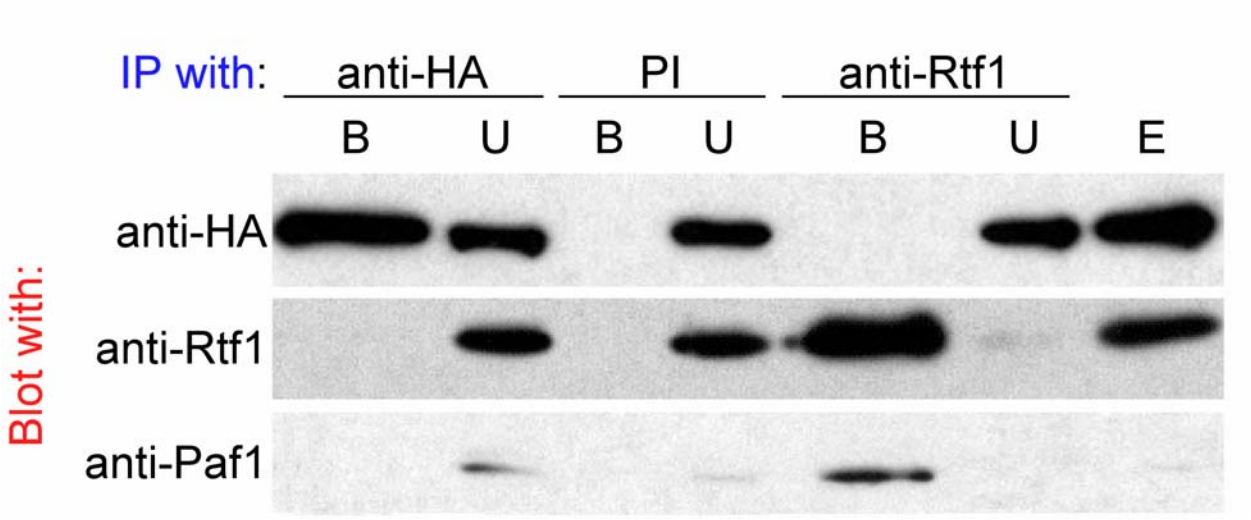
Rkr1 has been shown to physically interact with two protein complexes in yeast. Work from the laboratory of Raymond Deshaies has shown that Rkr1 and the Paf1 complex copurify with the 19S cap of the proteasome, but only in the absence of ATP (VERMA *et al.* 2000). This study purified FLAG-tagged Rpt1 (a subunit of the 19S proteasome) and performed mass spectroscopy to determine the identity of copurifying proteins. Interestingly, Rtf1 contains a non-canonical FLAG sequence, which may explain why the Paf1 complex was purified with a FLAG-purified protein. I performed coimmunoprecipitation experiments and detected no physical interaction between Rkr1 and Rtf1 or Paf1 (Figure 34). This suggests that Rkr1 may physically interact with the 19S proteasome in a Paf1 complex-independent manner. A second study, performed in the laboratory of Andrew Link, purified ribosomes using three different approaches with the hope of identifying previously uncharacterized translation-machinery-associated factors (FLEISCHER *et al.* 2006). They 1) fractioned 40S, 60S and 80S ribosomes using sucrose gradients, 2) purified ribosomes with increasing salt concentrations using discontinuous sucrose gradients, and 3) purified ribosomes and applied washes containing increasing salt concentrations to dissociate regulatory factors from core ribosomes. These studies were all

followed by mass spectroscopic analysis to identify any proteins that physically interact with the ribosome. Rkr1 copurified with 60S ribosomes in the sucrose gradients and purified with ribosomes washed with 1M ammonium chloride. These data suggest that Rkr1 may associate with ribosomes, although we have yet to obtain a functional understanding of this interaction.

To complement the genetic analysis described in Chapter 2, I investigated Rkr1's physical interactions *in vivo*. The results of these studies could confirm previously identified interactions, lead us to a new functional complex, or identify possible substrates for Rkr1's ubiquitin ligase activity. However, identification of ubiquitin ligase substrates presents a difficult problem, since these proteins are most often destroyed as a result of the interaction. In an attempt to circumvent the degradation of proteins that interact with wild type Rkr1, two different regions of Rkr1 were used as bait in separate yeast two-hybrid screens. We used a conserved amino-terminal fragment as well as a carboxy-terminal fragment that contains the RING domain as baits. We identified 20 proteins that interact with the amino terminus of Rkr1. I performed preliminary experiments to determine if these proteins are substrates of Rkr1's ubiquitin ligase activity. No interactions with the carboxy-terminal fusion were identified, most likely due to the ubiquitylation and degradation of any interacting proteins.

Figure 34. Coimmunoprecipitation experiments do not detect a physical interaction between Rkr1 and Rtf1.

Coimmunoprecipitation experiments were performed to detect physical interactions between HA-Rkr1 and Rtf1 or Paf1. Immunoprecipitations were performed using either anti-HA or anti-Rtf1 antibodies, or preimmune serum as a control for the Rtf1 antibody. Immunoblot analysis was performed using antibodies anti-HA, anti-Rtf1 and anti-Paf1 antibodies. Immunoblot analysis shows that no physical interactions were detected. However, a physical interaction was detected between Rtf1 and Paf1 as expected. B= bound fraction, U=unbound fraction, E=cell extract, PI=pre-immune serum.


IP with:
anti-HA
PI
anti-Rtf1
B
U
B
U
B
U
E
Blot with:
anti-HA
anti-Rtf1
anti-Paf1

Ubiquitin-protein ligases have been shown to work both in the context of a complex or by themselves. Ligases that act alone presumably interact only briefly with their partner ubiquitin conjugating enzyme and the substrate(s). There are examples of both types of ligases that act within the context of the nucleus. Bre1 and Lge1, both RING domain containing proteins, interact with each other and the ubiquitin-conjugating enzyme Rad6 to facilitate histone H2B ubiquitylation at lysine 123 (HWANG *et al.* 2003; WOOD *et al.* 2003a). The Skp1-Cullin-F-Box (SCF) complex incorporates many E3s to define substrates that are ubiquitylated throughout the cell cycle (SKOWYRA *et al.* 1997). Rad18 works with the E2 Ubc2 to target proteins involved in DNA repair for ubiquitylation (BAILLY *et al.* 1994). In parallel with two-hybrid analysis, I purified Rkr1 from yeast under native conditions to attempt to define a functional complex for Rkr1. The results of this analysis suggest that Rkr1 does not maintain any stable interactions *in vivo*, and support a model in which the proteins that interact in the two-hybrid screen are potential targets of Rkr1's ubiquitin ligase activity.

4.2 METHODS

4.2.1 Plasmids

pMB11, pMB66, and pPC65 were described in Chapter 2 methods section (section 2.2). pMB67 and pMB68 were created by PCR cloning. The protein-A-TEV portion of the modified TAP tag was amplified using PCR with primers that hybridized to the protein-A-TEV sequence in pBS1761 (PUIG *et al.* 2001). The 5' primer added an NdeI restriction sequence. The 3' primer added sequences that are identical to those found in the 5' FLAG sequence. In a separate

set of reactions, the 3xFLAG sequence was PCR amplified using a 5' primer that added sequences that are identical to those found at the 3' end of the protein-A-TEV PCR product. The 3' primer added an NdeI restriction site. These PCR products were gel purified and combined into a PCR reaction, where they would hybridize to each other and prime a longer PCR product that contained protein-A-TEV-3xFLAG, with NdeI restriction sites on the 5' and 3' ends of the product. This product was purified, digested with NdeI, and ligated into pSH3 (which contains the 5' end of *RKR1*) that had been linearized at the NdeI site at the ATG codon of *RKR1*. This plasmid was sequenced over the new tag to confirm that no mutations were introduced by PCR. An XhoI/AatII fragment from the resulting plasmid was subcloned into XhoI/AatII digested pMB11 and pMB66, to create plasmids containing modified TAP-tagged, full-length, wildtype *RKR1* (pMB67), and modified TAP-tagged, full-length *RKR1-C1508A* (pMB68), respectively.

4.2.2 Yeast Strains and Media

Yeast strains used in these studies are listed in Table 9. TAP-tagged strains that were used in the coimmunoprecipitations and cycloheximide-chase experiments are from the TAP-tag collection (GHAEMMAGHAMI *et al.* 2003), and are not isogenic with FY/KY strains. Yeast strains were generated as described in Chapter 2 methods section (section 2.2). *RSC8* was tagged by transforming a PCR fragment that contained 3xHA sequence and the *kanMX* selectable marker into MBY193. The following primers were used for amplifying the tag for Rsc8: MBO71 (5' tgcaaaattgcagccccaggtgtacaaaccgtggtcattgCGGATCCCCGGGTTAATTAA 3'), MBO72 (5'tattagaaatataaaatatgacaatagtacactttgtaggGAATTCGAGCTCGTTTAAAC 3'). YPD medium was made as described in the Methods section of Chapter 2 (section 2.2).

Table 9. Table of Strains for Chapter 4.

Strain	Genotype
KY1241	*MATα rkr1Δ::HIS3*, *RSC8-HA-kanMX4*, *his3Δ200*, *leu2Δ1*, *ura3-52*, *trp1Δ63*
KY1242	*MAT***a** *RSC8-HA-kanMX4*, *his3Δ200*, *leu2Δ1*, *ura3-52*, *trp1Δ63*
KY592	*MATα leu2Δ1*, *ura3-52*, *trp1Δ63*
MBY151	*MAT***a** *gal4Δ*, *gal80Δ*, *LYS2::GAL1-HIS3*, *GAL2-ADE2*, *met2::GAL7-LacZ*, *his3Δ200*, *leu2-3*, *112*, *ura3-52*, *trp1-901* [pGBT9 = *GAL4 DBD*, *TRP1*, 2μ]
MBY152	*MAT***a** *gal4Δ*, *gal80Δ*, *LYS2::GAL1-HIS3*, *GAL2-ADE2*, *met2::GAL7-LacZ*, *his3Δ200*, *leu2-3*, *112*, *ura3-52*, *trp1-901* [pGBT9+RKR1-N-term = *GAL4 DBD-RKR1 N-term*, *TRP1*, 2μ]
MBY168	*MAT***a** *rkr1Δ::kanMX4*, *his3Δ200*, *leu2Δ1*, *ura3-52*, *trp1Δ63*
MBY205	*MAT***a** *KGD2-TAP-HIS3MX6*, (*his3Δ200*?), *lys2-128δ*, *leu2Δ1*, *ura3*(*Δ0* or *-52*), *trp1Δ63*
MBY226	*MAT***a** *HPA3-TAP-HIS3MX6*, (*his3Δ200*?), *ura3*(*Δ0* or *-52*), *trp1Δ63*
MBY243	*MAT***a** *STE12-TAP-HIS3MX6*, *his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY244	*MAT***a** *MAC1-TAP-HIS3MX6*, *his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY245	*MAT***a** *GZF3-TAP-HIS3MX6*, *his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY246	*MAT***a** *DSK2-TAP-HIS3MX6*, *his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY247	*MAT***a** *STE12-TAP-HIS3MX6*, *rkr1Δ::kanMX4his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY248	*MAT***a** *MAC1-TAP-HIS3MX6*, *rkr1Δ::kanMX4his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY249	*MAT***a** *GZF3-TAP-HIS3MX6*, *rkr1Δ::kanMX4his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY250	*MAT***a** *DSK2-TAP-HIS3MX6*, *rkr1Δ::kanMX4his3Δ1*, *leu2Δ0*, *ura3Δ0*, *met15Δ0*
MBY36	*MATα rkr1Δ::kanMX4*, *his3Δ200*, *trp1Δ63*

4.2.3 Coimmunoprecipitation experiments

2 mg of total protein extract were raised to 750 µl volume with 1x lysis buffer (100 mM sodium acetate, 20 mM HEPES, pH 7.4, 2 mM magnesium acetate, 10 mM EDTA, 10% glycerol, 1 mM DTT, plus protease inhibitors) and Tween 20 was added to 0.05% final concentration. IgG-sepharose beads were prewashed with 1x lysis buffer and 25 µl were added per IP reaction. Reactions were mixed at 4^{o} C for 2 hours. Immune complexes were collected by centrifugation at 5000 rpm and washed 5 times with 1 ml lysis buffer plus 200 mM NH_4OAc plus 0.05% Tween 20. Immunoprecipitated proteins were eluted from beads in 1x sample buffer (80 mM Tris-Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 100 mM DTT, 10% glycerol, 2 mM PMSF) by boiling at 100^{o} C for 3 minutes. Samples were loaded onto SDS polyacrylamide gels prior to being transferred to nitrocellulose for immunoblot analysis.

4.2.4 Immunoblot analysis

Whole cell extracts were prepared as described in Chapter 2 methods. Proteins (25 µl extract/immunoprecipitate or the entire *in vitro* ubiquitylation reaction) were separated on 10% or 7-20% gradient SDS polyacrylamide gels and transferred to nitrocellulose membranes. The following antibodies were used: anti-TAP (peroxidase-anti-peroxidase) (1:3000 dilution; Sigma), anti-HA (1:3000 dilution; Roche), and anti-Sec61 (1:1000 dilution; a gift of the Brodsky lab). HRP-conjugated secondary antibodies (Amersham Biosciences) were used at a 1:5000 dilution. Immunoreactive proteins were detected by chemiluminescence (Perkin Elmer) and visualized with a Kodak 440CF digital imaging station.

4.2.5 Cycloheximide-chase experiments

Cycloheximide-chase experiments were performed as described previously (GARDNER *et al.* 1998). Briefly, 10 ml cultures of MBY245 (Gzf3-TAP, *RKR1*$^+$)**,** MBY249 (Gzf3-TAP, *rkr1Δ*), and KY592 (untagged Gzf3, *RKR1*$^+$) or KY1241 (Rsc8-HA, *rkr1Δ*), KY1242 (Rsc8-HA, *RKR1*$^+$) and another strain derived from the same cross (untagged Rsc8, *RKR1*$^+$) were grown to 1 x 10^7 cells/ml (early log-phase) in YPD, SC complete, or SD minimal medium. Cycloheximide was added to 50 µg/ml final concentration. Cultures were mixed and the zero timepoint sample (2 ml) was collected by centrifugation. The rest of the culture was incubated at 30^o C for 90 minutes, and 2 ml of each culture were collected at 30, 60 and 90 minutes. Samples from each timepoint were processed as follows. Cells were harvested in 2 ml round-bottomed screw-capped tubes. Cells were resuspended in 200 µl SUMEB buffer (1% SDS, 8M Urea, 10 mM MOPS, pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) plus protease inhibitors. 100 µl glass beads were added, and samples were shaken for 3 minutes on setting #7 in the multi-tube vortexer. Samples were incubated at 100^o C for 10 minutes prior to centrifugation at 13,000 rpm for 5 minutes. Supernatants were transferred to new Eppendorf tubes and stored at 4^o C until all samples were collected and processed. 25 µl of each sample was loaded onto 10% SDS polyacrylamide gels and transferred to nitrocellulose prior to immunoblot analysis.

4.2.6 Modified TAP purification

Strains lacking *RKR1* (MBY36) that were transformed with pPC65 (untagged Rkr1), pMB67 (protein A-TEV-3xFLAG-Rkr1), or pMB68 (protein A-TEV-3xFLAG-Rkr1-C1508A) were grown to 4-5 x 10^7 cells/ml (mid-log-phase) and cells were collected by centrifugation (4000

rpm, 4 minutes, 4° C) in a GS3 rotor. Cells were resuspended in 2 ml cold sterile water per gram weight of wet cells. Cells were transferred to a 50 ml Falcon tube, and washed 1 time with 1.3 volumes TAP-MB1 (50 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM β-mercaptoethanol) without protease inhibitors and washed 1 time with 1.3 volumes TAP-MB plus protease inhibitors. Cell pellets were frozen in liquid nitrogen. Cell lysates were made using a Krups coffee grinder with dry ice pellets. The cell pellets/dry ice were ground for 2.5 minutes. The powder was transferred to a sterile beaker and thawed at room temperature. Once thawed, 0.8 volumes of TAP-MB1 plus protease inhibitors were added to each sample. Samples were transferred to Oak Ridge tubes and centrifuged at 15,000 rpm for 20 minutes at 4° C (SA600 rotor). Supernatant was separated by ultracentrifugation at 33,500 rpm, for 1 hour at 4° C (Ti70i rotor). Soluble fractions of the extracts were mixed with pre-washed IgG-sepharose (Amersham Biosciences) in batch at 4° C for 1 hour. Immune complexes were collected by centrifugation and transferred to siliconized microfuge tubes. The complexes were washed 4 times with TAP-MB2 (50 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM β-mercaptoethanol, 0.05% NP-40) plus protease inhibitors and 5 times with TAP-MB2 without protease inhibitors. Bead-protein complexes were resuspended in 200 μl TAP-MB2 plus 10 units of AcTEV protease (Invitrogen), and incubated at 16° C for 2 hours. Cleaved protein complexes (supernatants) were collected after centrifugation (1 minute, 2000 rpm, 4° C). Supernatant was mixed with 100 μl pre-washed FLAG-M2 resin in siliconized microfuge tubes for 2 hours at 4° C. Resin was collected by centrifugation (2000 rpm, 1 minute, 4° C) and the resin was washed 5 times with 1 ml TAP-MB2 without protease inhibitors and 5 times with 1 ml TAP-MB3 (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, and 10 μM $ZnCl_2$). Samples were mixed for 4 minutes at 4° C each wash. Rkr1 protein complexes were eluted with

20 µl of 10 mg/ml FLAG peptide (University of Pittsburgh Peptide Synthesis Facility) plus 80 µl TAP-MB4 (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 10 µM $ZnCl_2$). Samples were rotated at room temperature for 30 minutes. Elution was repeated 1 time at 4° C. Elutions were combined and half of each sample was TCA precipitated prior to loading on 7-20% SDS polyacrylamide gels. Gels were silver stained as described in the next section (section 4.2.7).

4.2.7 Silver staining SDS-PAGE gels

SDS polyacrylamide gels were silver stained using a rapid, low background protocol obtained from Jeff Brodsky. Briefly, gels were fixed for at least 30 minutes in 50% ethanol, 12% acetic acid, and 0.5 ml/L formaldehyde. Gels were washed in 50% ethanol for 20-40 minutes and washed quickly with 0.01% sodium thiosulfate. Gels were stained with fresh 0.1% silver nitrate for 20 minutes and developed with 0.28 M sodium carbonate, 0.0002% sodium thiosulfate, and 240 µl/0.5 L formaldehyde. The development was stopped by soaking the gel in 10 mM EDTA.

4.3 RESULTS

4.3.1 A yeast two-hybrid screen shows that Rkr1 physically interacts with proteins involved in chromatin function, transcription, and ubiquitylation

Our laboratory performed two separate yeast two-hybrid screens using different fragments of Rkr1 fused to the Gal4 DNA binding domain as bait. The first screen, performed by Alexis Carulli, used a fusion that contained amino acids 1251-1562 of Rkr1 as bait. This region of Rkr1

contains the RING domain that is predicted to interact with ubiquitin-conjugating enzymes and ~275 amino acids amino-terminal to the RING domain. This screen detected no physical associations with Rkr1, presumably because proteins were degraded as a result of the interaction. Rebecca Gonda performed another two-hybrid screen using a fusion that contained amino acids 1-257 of Rkr1 as bait. This portion of Rkr1 is conserved in eukaryotes and may serve as a substrate binding region (Figure 29 in Chapter 3). This screen identified 20 proteins that interact with Rkr1 (Table 10).

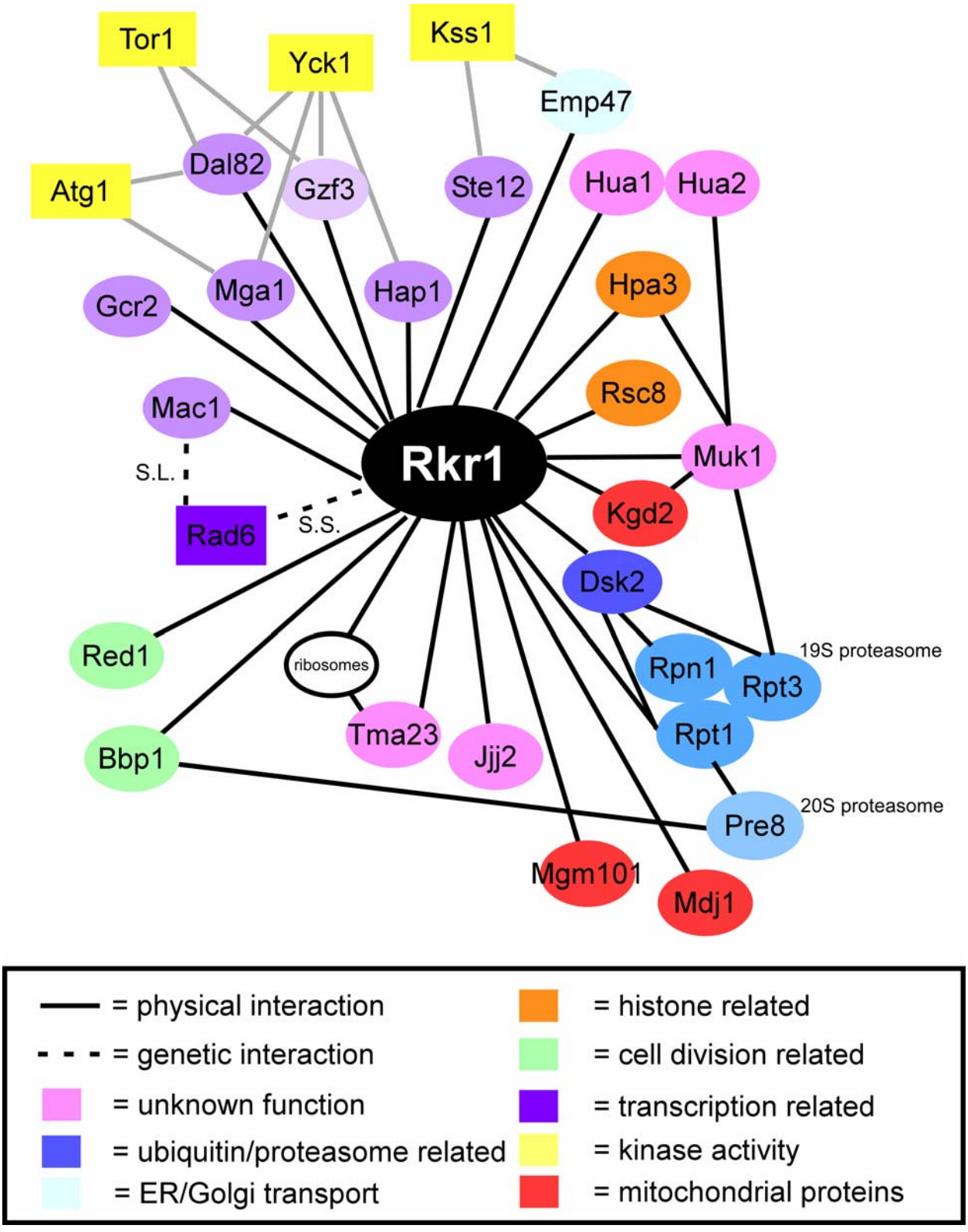
The proteins that interact with the amino terminus of Rkr1 fall into several interesting classes. There are proteins that modify and remodel chromatin (Hpa3 and Rsc8, respectively), and several transcription activators and a repressor (Gcr2, Dal82, Mac1, Mga1, Ste12, Hap1, and Gzf3, respectively). One interacting protein, Dsk2, is important for transporting polyubiquitylated proteins to the proteasome. The interacting proteins Bbp1 and Red1 are important for cell division. The amino terminus of Rkr1 also interacts with a few mitochondrial proteins (Mgm101, Kgd2, Mdj1), a protein involved in ER-Golgi transport (Emp47), and several uncharacterized proteins (Jjj2, Tma23, Hua1, and Muk1). Table 10 also contains brief descriptions of the known functions of each of these proteins. Figure 35 illustrates known physical and genetic interactions among these proteins. The next few sections describe experiments that I performed to follow up these results.

Table 10. Proteins that physically interact with the amino terminus of Rkr1, as determined by a two-hybrid screen.

Protein	# isolates	Group	Description
Hpa3	5	Histone	Histone and other protein acetyltransferase; acetylates histones weakly in vitro
Rsc8	1	Histone	Remodel the structure of chromatin 8; Subunit Rsc complex
Gcr2	2	Transcription regulator	Glycolysis regulatory protein 2; activator of glycolytic genes
Dal82	1	Transcription regulator	Degradation of Allentoin 82; positive and negative transcription regulator, activates allophanate- inducible genes, DNA binding protein
Gzf3	1	Transcription regulator	Gata Zinc Finger protein 3; Dal80 homolog; negative regulator of nitrogen response genes
Mac1	2	Transcription regulator	Metal-binding transcriptional activator 1; Copper-sensing factor involved in regulation of genes required for high affinity Cu transport
Mga1	1	Transcription regulator	
Ste12	1	Transcription regulator	Sterile 12; Transcription activator of mating and pseudohyphal/invasive growth genes
Hap1	1	Transcription regulator	Heme Activator Protein 1; Zn-finger transcription factor of Zn(2)-Cys(6) binuclear cluster
Dsk2	3	Ubiquitin	Nuclear enriched ubiquitin-like poly-ubiquitin binding protein; Interacts with the proteasome
Bbp1	1	Cell division	Bfr1 binding protein 1; essential; required for spindle pole body duplication
Red1	2	Cell division	Reductional division 1; involved in centrosome segregation in first meiotic division, component of axial elements in synaptonemal complex
Mgm101	2	Mitochondrial	Mitochodrial genome maintenance 101; binds to DNA, involved in DNA repair
Kgd2	3	Mitochondrial	Alpha-ketoglutarate dehydrogenase 2; catalyzes step in TCA cycle; DNA-binding activity; phosphotidyl-inositol binding; important in maintaining stability of mitochondrial DNA
Mdj1	2	Mitochondrial	Member of the DnaJ family of molecular chaperones 1; DnaJ homolog; involved in folding proteins in mitochondrial matrix; interacts w/ Rad6 (2-hyb)
Emp47	1	ER/Golgi	Glycolytic transport; Integral ER-derived COPII-coated residues; Ubiquitylated *in vivo*
Jjj2	1	Uncharacterized	Contains J-domain; homolog to DnaJ protein; Similar to Hlj1; null is Spo-
Tma23	1	Uncharacterized	Nucleolar, contains putative RNA binding domain; Nucleoloar protein that copurifies with ribosomes
Hua1	1	Uncharacterized	Zn-finger domain; Similar to type 1 J-proteins; Possible role in actin patch assembly
Muk1	1	Uncharacterized	Localized to cytoplasm; possible role in transcription regulation

Figure 35. Network of physical and genetic interactions among the proteins identified in the yeast two-hybrid screen with Rkr1.

Many of the proteins that physically interact with Rkr1 in a yeast two-hybrid screen actually physically and genetically interact with each other and other functional complexes, including the proteasome and ribosomes. Physical interactions are designated by a solid black line, genetic interactions are represented by dashed black lines, and enzyme-substrate interactions are depicted by solid gray lines. Synthetically lethal (SL) and synthetically sick (SS) interactions are shown. The proteins that physically interact with Rkr1 fall into several functional classes, which are designated with similarly colored circles (see legend within the figure).


Tor1
Yck1
Kss1
Emp47
Dal82
Atg1
Gzf3
Ste12
Hua1
Hua2
Mga1
Hap1
Hpa3
Gcr2
Rsc8
Mac1
S.L.
Rkr1
Muk1
Rad6
S.S.
Kgd2
Dsk2
Red1
ribosomes
19S proteasome
Rpn1
Rpt3
Tma23
Jjj2
Rpt1
Bbp1
20S proteasome
Pre8
Mgm101
Mdj1
= physical interaction
= genetic interaction
= unknown function
= ubiquitin/proteasome related
= ER/Golgi transport
= histone related
= cell division related
= transcription related
= kinase activity
= mitochondrial proteins

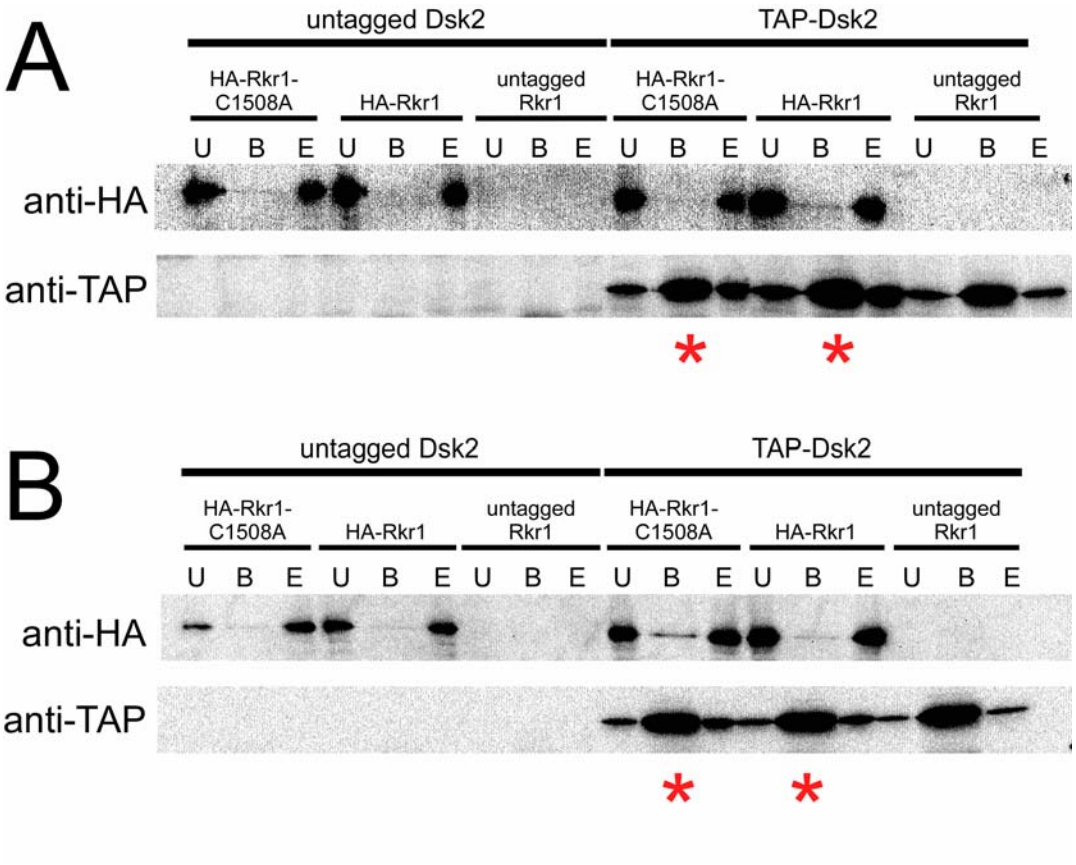
4.3.1.1 Dsk2 physically interacts with Rkr1

The amino terminus of Rkr1 interacts with 2 different Gal4-activation domain fusions to Dsk2. One fusion contains amino acids 83-373 and the other contains amino acids 85-373 (*DSK2* encodes a 373 amino acid protein). Dsk2 contains a ubiquitin-like (UBL) domain at its amino terminus and a ubiquitin-associated (UBA) domain at its carboxy terminus. The UBL domain interacts with the 19S proteasome subunit Rpn1, and the UBA domain associates with polyubiquitin chains on other proteins (FUNAKOSHI *et al.* 2002), and thus Dsk2 acts as a "destruction chaperone" by targeting polyubiquitylated proteins to the proteasome. Hartmann-Petersen *et al*. suggest that Dsk2 may "pick up" polyubiquitylated proteins just as they are tagged for destruction, and therefore may interact with the ubiquitylation machinery (HARTMANN-PETERSEN *et al.* 2003). The human homolog of Dsk2 has been shown to interact with a ubiquitin-protein ligase, E6AP (KLEIJNEN *et al.* 2000). The physical interaction between Rkr1 and Dsk2 suggests that Rkr1 may be important for the polyubiquitylation of proteins in yeast, and these substrates are targeted to the proteasome by Dsk2.

It is possible that this physical interaction should be somewhat stable and detectable in coimmunoprecipitation experiments if Dsk2 contacts Rkr1 and is not a target of its ubiquitin-protein ligase activity. I therefore performed coimmunoprecipitation experiments to detect a physical interaction between Rkr1 and Dsk2. The results of these experiments suggest that Rkr1 and Dsk2 do physically interact, although whether or not a functional RING domain is required for this interaction is not known (Figure 36). In the first of two experiments, a very weak interaction between Rkr1-C1508A and Dsk2 was detected, and the second experiment showed that Rkr1-C1508A interacted with Dsk2 more robustly (Figure 36).

Figure 36. Rkr1 physically interacts with Dsk2.

Coimmunoprecipitation experiments with TAP-Dsk2 and HA-Rkr1 were performed as described in the Methods section (Immunoprecipitate TAP-Dsk2 and immunoblot for HA-Rkr1). Immunoblot analysis was performed using anti-HA antibody to detect Rkr1 and anti-TAP antibody to detect Dsk2. (*) represents relevant coimmunoprecipitation lanes. A) Experiment #1. B) Experiment #2. B= bound fraction, U=unbound fraction, E=cell extract

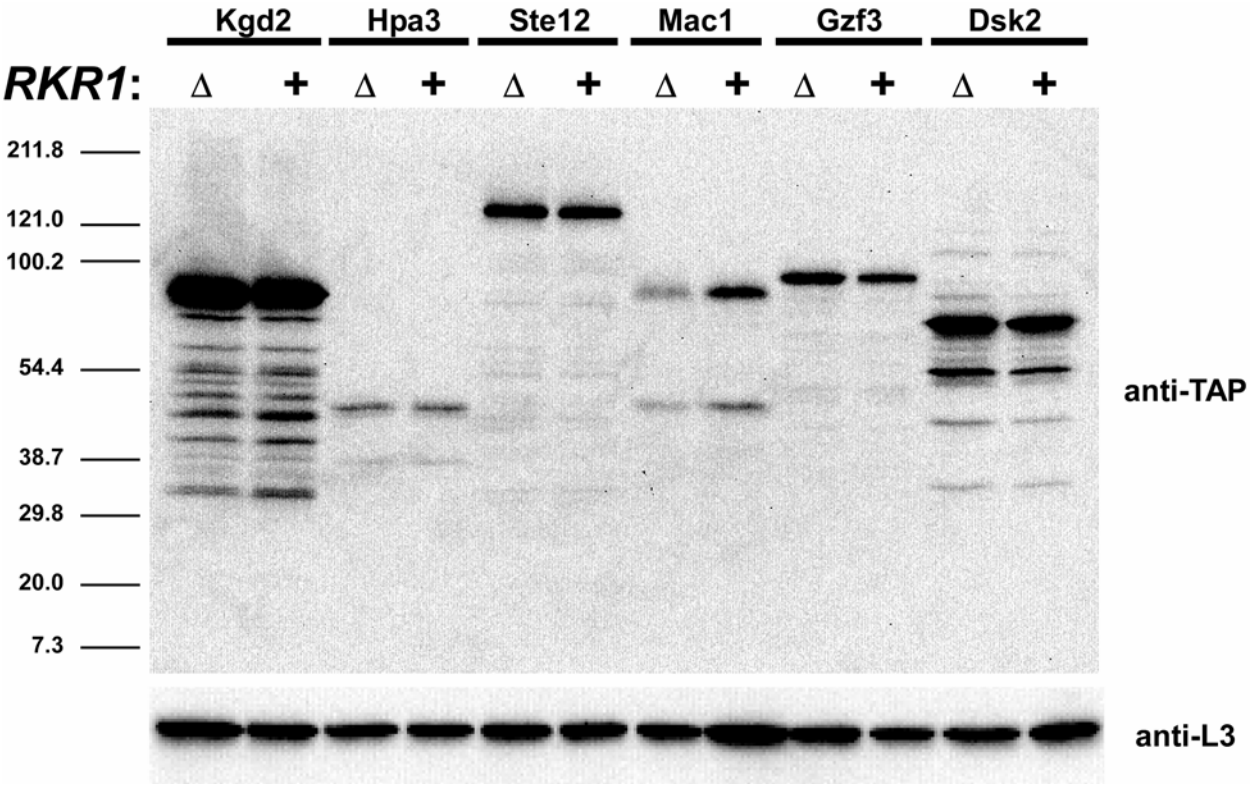

A
untagged Dsk2
TAP-Dsk2
HA-Rkr1-C1508A
HA-Rkr1
untagged Rkr1
HA-Rkr1-C1508A
HA-Rkr1
untagged Rkr1
U B E U B E U B E U B E U B E U B E U B E
anti-HA
anti-TAP
*
*
B
untagged Dsk2
TAP-Dsk2
HA-Rkr1-C1508A
HA-Rkr1
untagged Rkr1
HA-Rkr1-C1508A
HA-Rkr1
untagged Rkr1
U B E U B E U B E U B E U B E U B E U B E
anti-HA
anti-TAP
*
*

4.3.1.2 The global levels of proteins identified in the two-hybrid screen are not significantly affected by the loss of Rkr1 in rich medium

The results of the two-hybrid screen show that the amino terminus of Rkr1 interacts with 20 proteins. We hypothesized that these proteins may be ubiquitylated and subsequently degraded in a Rkr1-dependent manner. I used immunoblot analysis to determine if global levels of these proteins increased in the absence of Rkr1. These experiments were performed using carboxy-terminal TAP-tagged proteins that were obtained from the TAP-tagged collection (**ref***). Strains were grown in rich medium (YPD) to log-phase prior to lysis in sample buffer. Cell extracts were loaded onto SDS-PAGE gels and proteins were transferred to nitrocellulose for immunoblot analysis. The results of this analysis show that the levels of the proteins that were assayed in these experiments are not affected by the loss of Rkr1 (Figure 37). This suggests that Rkr1 may not be responsible for degrading these proteins in rich medium. It is possible that a redundant ubiquitin ligase may act to destroy these proteins, or Rkr1 may not be important for degrading these proteins at all.

Figure 37. The levels of proteins that physically interact with Rkr1 are not significantly affected by the loss of Rkr1.

rkr1Δ strains that express TAP-tagged versions of the proteins listed across the top of the figure were transformed with plasmids that either contained wild type *RKR1* or empty vector. Total cell lysates were separated on a 7-20% gradient SDS-PAGE gel. Immunoblot analysis of cell lysates from these strains was performed with antibodies to detect the TAP-tag fusion proteins and L3 (loading control).


Kgd2
Hpa3
Ste12
Mac1
Gzf3
Dsk2
RKR1: Δ + Δ + Δ + Δ + Δ + Δ +
211.8
121.0
100.2
54.4
38.7
29.8
20.0
7.3
anti-TAP
anti-L3

4.3.1.3 Rsc8 and Gzf3 are not degraded in a Rkr1-dependent manner in rich medium

The amino terminus of Rkr1 interacts with amino acids 341-557 of Rsc8 (*RSC8* encodes a 557 amino acid protein). Rsc8 is a component of the ATP-dependent RSC chromatin remodeling complex (CAIRNS *et al.* 1996). The RSC complex was identified by homology to the SWI/SNF chromatin remodeling complex, and biochemical purification of RSC shows that there are 17 subunits (CAIRNS *et al.* 1996). This complex interacts with acetylated nucleosomes (HASSAN *et al.* 2002; WINSTON and ALLIS 1999) to facilitate transcription (CAREY *et al.* 2006). Rsc8 is 30% identical and 52% similar to the Swi3 component of the SWI/SNF complex (CAIRNS *et al.* 1996). Rsc8 contains a SWIRM domain, which is important for maintaining associations with other RSC complex members and nucleosomes (DA *et al.* 2006), and a ZZ-type zinc finger, which may be important for protein-protein interactions (CAIRNS *et al.* 1996).

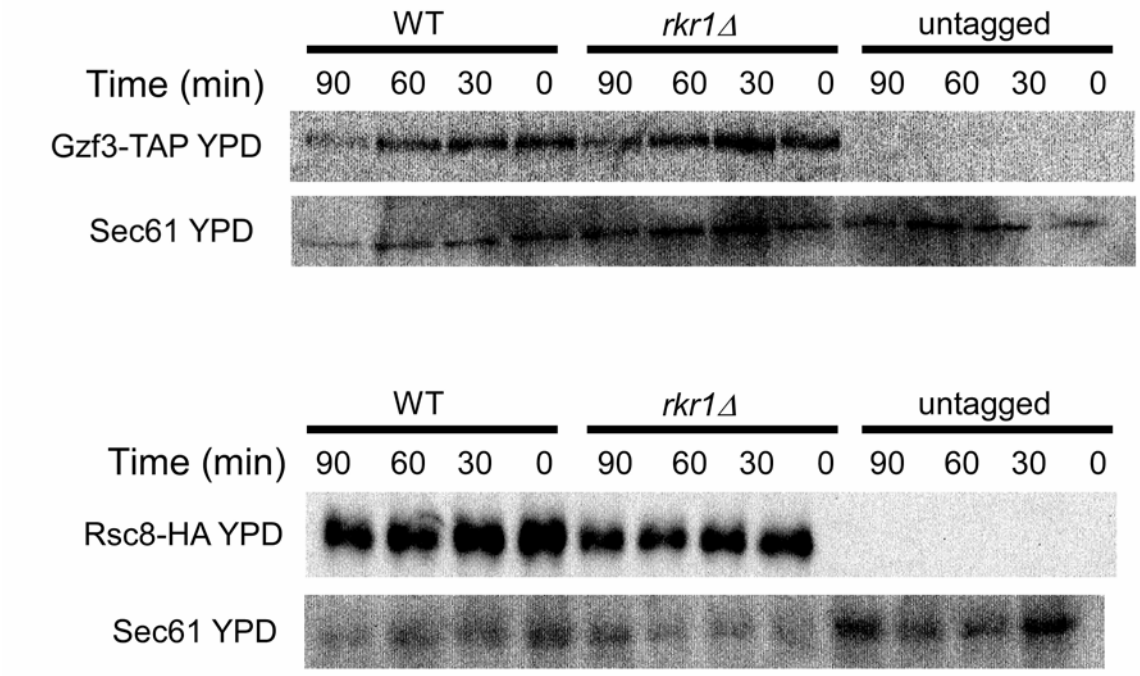
The amino terminus of Rkr1 interacts with the carboxy-terminal 132 amino acids of Gzf3 (amino acids 419-551). Gzf3 is a RNA Pol II transcription factor that represses nitrogen catabolite genes (SOUSSI-BOUDEKOU *et al.* 1997). Gzf3 contains a zinc finger which binds to GATA DNA sequences in promoter elements of nitrogen responsive genes (SOUSSI-BOUDEKOU *et al.* 1997). Gzf3 is similar in sequence and function to Dal80, which also represses nitrogen responsive genes (SOUSSI-BOUDEKOU *et al.* 1997).

To determine if Rkr1 was important for the degradation of Rsc8 and Gzf3, I performed cycloheximide-chase experiments using *rkr1Δ* strains that express carboxy-terminally HA-tagged Rsc8 and TAP-tagged Gzf3. Strains were grown to log-phase in rich medium and cycloheximide was added to stop protein synthesis. Samples were collected over a 90 minute period, and extracts were made from cells collected at each point. Immunoblot analysis of these strains shows that Rsc8 and Gzf3 protein levels are not stabilized by the loss of Rkr1 (Figure 38).

Because differences in gene expression were observed in *rkr1Δ* strains grown in SD minimal medium (see Chapter 5), but not rich medium, Rkr1 may only affect the stabilization of these factors under certain growth conditions. Future experiments are required to investigate this possibility.

Figure 38. Rsc8 and Gzf3 protein levels are not affected by the loss of Rkr1 in rich medium.

Cycloheximide chase experiments were performed as described in the methods section. Time (minutes) after cycloheximide addition is noted above each lane. Sec61 (endoplasmic reticulum membrane protein) immunoblots are used for loading controls (antibody is described in (STIRLING *et al.* 1992)).


WT
rkr1Δ
untagged
Time (min)
90 60 30 0 90 60 30 0 90 60 30 0
Gzf3-TAP YPD
Sec61 YPD
WT
rkr1Δ
untagged
Time (min)
90 60 30 0 90 60 30 0 90 60 30 0
Rsc8-HA YPD
Sec61 YPD

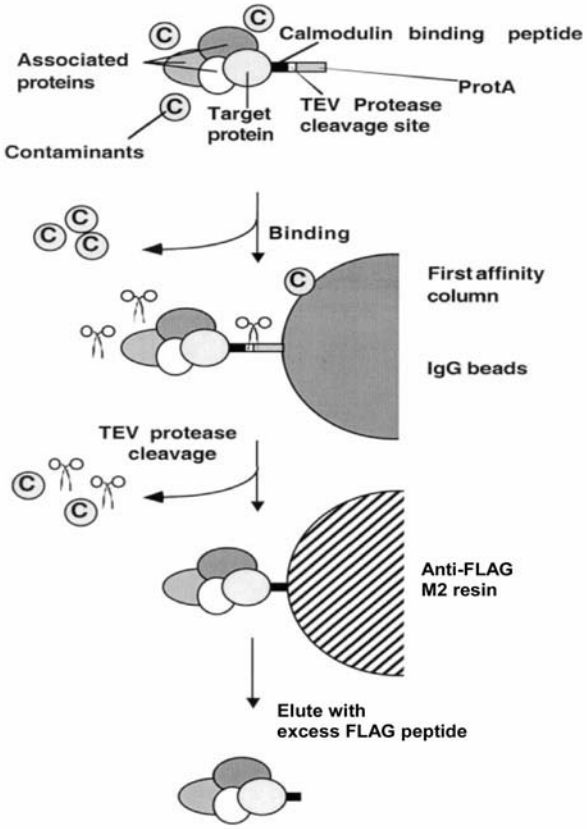
4.3.2 Modified TAP purification

In parallel with the yeast two-hybrid screen, I isolated Rkr1 using a technique that many other labs have used to purify proteins in native complexes from yeast (PUIG *et al.* 2001). Rkr1 was amino-terminally tagged with the traditional TAP tag, which contains a protein-A module and calmodulin binding peptide separated by a TEV cleavage site (PUIG *et al.* 2001). This tag was added to the amino-terminus of Rkr1 to avoid disrupting the RING domain at the carboxy terminus. Using this approach, no proteins copurified with Rkr1. I also had difficulty eluting Rkr1 from the calmodulin column. To overcome the elution difficulties, I created a new tag that I will refer to as the modified TAP tag. This tag contains the protein-A module and 3xFLAG peptide, separated by the TEV cleavage site. This method allows for elution of purified proteins using excess FLAG peptide, which should overcome the previous elution difficulties. To enhance for interacting proteins that may be destroyed by wildtype Rkr1, I also tagged Rkr1-C1508A for purification. Using this method I was able to purify both forms of Rkr1, however, I saw no co-purifying proteins. I tried many different buffer conditions (chloride and acetate buffers with high and low salt concentrations) and I tried purifying Rkr1 from up to 18 liters of cells to enhance for minute levels of interacting proteins. The results of the purification experiments showed that no proteins (detectable by silver stain) co-purify with either wildtype or mutant Rkr1 (Figure 39). These results suggest that Rkr1 may not stably interact with any proteins in yeast.

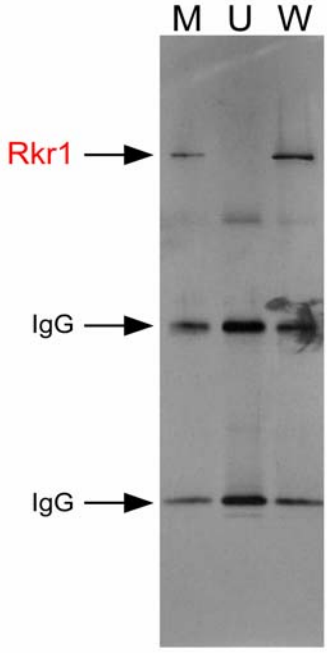
Figure 39. Purification of Rkr1.

A) Modified TAP purification strategy. See Methods section for more information. B) Wild type and C1508A derivatives of Rkr1 were purified using the modified TAP tag strategy. Half of the eluates (representing 1 liter of cells) were TCA precipitated and proteins were separated in a 7-20% gradient SDS-PAGE gel. The gel was silver stained to identify purified proteins.

A



B



M=Rkr1-C1508A
U=untagged Rkr1
W=Rkr1

4.4 CONCLUSIONS

This chapter describes our attempts to characterize Rkr1's physical interactions. A yeast two-hybrid screen identified 20 proteins that physically interact with the amino terminus of Rkr1. These proteins fall into several functional classes that are involved in chromatin function, transcription regulation, and ubiquitylation. These proteins may be targets of Rkr1's ubiquitin ligase activity, but my results show that steady-state levels of many of these proteins are not increased in *rkr1Δ* strains grown in rich medium. Further analysis is required to determine if Rkr1 ubiquitylates these proteins under specific conditions.

The amino terminus of Rkr1 interacted with Dsk2 in the two-hybrid screen. Dsk2 is important for binding poly-ubiquitylated proteins and transporting them to the proteasome for destruction (reviewed in HARTMANN-PETERSEN *et al.* 2003). Coimmunoprecipitation experiments show that Rkr1 and Dsk2 do physically interact. This connects Rkr1's function to the ubiquitin-proteasome pathway, and further supports a model in which Rkr1 acts as a ubiquitin ligase to target proteins within the nucleus for destruction.

The two-hybrid screen also showed that Rkr1 interacts with Rsc8 and Gzf3. Rsc8 is a subunit of an essential chromatin remodeling complex (CAIRNS *et al.* 1996), and Gzf3 is a transcriptional repressor that regulates nitrogen responsive genes (SOUSSI-BOUDEKOU *et al.* 1997). Interestingly, several Gzf3 regulated genes are affected by the loss of *RKR1* when the strains are grown in minimal medium (see Chapter 5). The results of my cycloheximide-chase experiments show that Rkr1 may not be important for degrading these proteins in rich medium.

The proteins identified in the yeast two-hybrid screen may be targets of Rkr1's ubiquitin ligase activity, but more analysis is required to fully understand these interactions.

I purified Rkr1 from yeast using a gentle method that allows for native purification of protein complexes (PUIG *et al.* 2001). Because I had difficulty eluting Rkr1 with traditional TAP tag purification, I created the modified TAP tag. Biochemical purification of wild type and mutant Rkr1 with this method failed to show any copurifying proteins. This suggests that Rkr1 does not stably associate with any proteins in yeast.

5.0 IDENTIFICATION OF YEAST GENES REGULATED BY RKR1 USING MICROARRAY ANALYSIS

5.1 INTRODUCTION

The goal of my research has been to investigate the synthetic lethal interaction caused by the combined loss of Rkr1, a previously uncharacterized protein, and Rtf1, a member of the Paf1 transcription elongation complex. Multiple parallel pathways often act coordinately to elicit proper transcription of downstream genes. Synthetic lethality is believed to result when parallel pathways that impinge on an essential process are disrupted simultaneously. A former graduate student in the Arndt lab, Kathryn Sheldon, demonstrated that members of the Paf1 complex are essential for normal transcription of a subset of genes in yeast. The synthetic lethality observed in *rkr1Δ rtf1Δ* strains suggests that Rkr1 may also be linked to gene expression. My previous observations that Rkr1 localizes to the nucleus and that strains lacking *RKR1* have phenotypes indicative of general transcription defects (see Chapter 2 for details) further support a role for Rkr1 in transcription.

To determine if Rkr1 affected the expression of any genes in yeast, I collaborated with Martin Schmidt's lab to perform microarray experiments. This analysis shows that Rkr1 affects the expression of a subset of genes in yeast. Further analysis with real-time PCR confirms the results of the microarrays. Interestingly, the microarray data indicate that some genes are

oppositely affected by the loss of *RKR1* and the loss of Paf1 complex members. Taken as a whole, Rkr1 appears to affect the expression of a subset of genes in yeast, and Rkr1 and the Paf1 complex oppositely regulate the expression of a fraction of these genes.

5.2 METHODS

5.2.1 Strains and media

Strains used in these studies are listed in Table 11. For all analyses, strains were grown to 1-2 x 10^7 cells/ml (early log-phase) in 10 ml rich medium or minimal medium that was supplemented with appropriate amino acids. Cells were collected by centrifugation, and total RNA was collected as described in the next section.

Table 11. Table of strains used in Chapter 5.

Strain	**Genotype**
KY592	*MATα leu2Δ1*, *ura3-52*, *trp1Δ63*
KY595	*MAT***a** *ura3-52*
KY694	*MATα ctr9Δ::kanMX4*, *leu2Δ1*, *ura3-52*, *trp1Δ63*
KY951	*MATα rtf1Δ::kanMX4*, *lys2-128δ*, *leu2Δ1*, *ura3-52*, *trp1Δ63*
MBY169	*MATα rkr1Δ::kanMX4*, *leu2Δ1*, *ura3-52*, *trp1Δ63*
MBY30	*MAT***a** *rkr1Δ::kanMX4*, *ura3-52*

5.2.2 RNA isolation, cDNA and cRNA synthesis for microarrays

All experiments were performed in triplicate. Total RNA was isolated from wild type (KY595) and *rkr1Δ* (MBY30) strains as previously described (ARNDT *et al.* 1995). RNeasy kits (Qiagen) were used to DNase treat the RNA prior to cDNA synthesis. cDNA synthesis (Invitrogen Superscript) was performed according to kit directions using 7 µg of RNA and oligo dT primers. cRNA was created using the Enzo kit with half (5 µl) of the cDNA reaction. cRNA was purified using RNeasy kits (Qiagen). Biotin labeling of cRNA and array hybridizations were performed at the University of Pittsburgh Medical School Core Microarray Facility. GeneChip Yeast Genome S98 arrays from Affymetrix were used for hybridizations. The hybridized array was stained and scanned. The amount of light emitted from each target spot at 570 nm is proportional to the amount of bound target at each location on the array.

5.2.3 Data analysis for microarrays

We were provided with a Microsoft Excel file that contained values representing the relative intensities for each of the array targets (representing all ORFs in yeast). Values representing spot intensities in three independent *rkr1Δ* strains were subtracted from respective wildtype strain values, providing three datasets. These values were averaged to provide a list of changes over the three experiments. These values (and their corresponding gene name) were sorted numerically from highest to lowest. These numerical differences provide a $\log_2$ based representation of the fold change in gene expression between the two datasets. Any values (positive or negative) that were larger than 0.8 (representing at least a 1.75 fold change in

expression) were considered significant and these genes were considered for further analysis and confirmed by real-time PCR (see below).

5.2.4 Significance analysis of microarray (SAM)

This Microsoft Excel based program was downloaded from http://www-stat.Stanford.EDU/~tibs/SAM. The data were entered using the "two-class paired" format, which compares the numbers for wild type and *rkr1Δ* strains separately for each experiment, instead of averaging all three datasets for wildtype and *rkr1Δ* strains together. The genes highlighted in Tables 13 and 14 were found to be significantly affected at a user-defined delta value of 0.276, with a false discovery rate of 15.2%, and the minimum fold change was set to 1.75. This means that the genes listed that have a q-value of 4.87 have a 4.87% chance of being a false positive gene.

5.2.5 Real-time PCR analysis

Strains were grown to 1 x 10^7 cells/ml (early log-phase) in 10 ml of SD minimal medium supplemented with required amino acids. RNA was extracted as described (ARNDT *et al.* 1995) and treated with DNase (Ambion). cDNA was synthesized (Invitrogen Superscript) using 1 µg of RNA and 250 ng random hexamers in 20 µl reactions. Primers for PCR were designed using the Primer Express Software version 3.0 (instructions available at http://docs.appliedbiosystems.com/pebiodocs/04362460.pdf) and their sequences are listed in Table 12. Primer efficiencies were calculated using methods described on the Applied Biosystems website (www.appliedbiosystems.com). Quantitative PCR analysis in real time was

performed with Applied Biosystems 7300 system to detect SYBR green fluorescence intensities. Ten-fold dilutions of cDNA, 0.5 μM primer, and SYBR green 2x PCR mix (Applied Biosystems) were used in 20 μl reactions. All reactions were performed in triplicate. PCR conditions were performed as follows: 1 cycle of 94^{o} C for 10 minutes, followed by 40 cycles of 94^{o} C for 15 seconds, 60^{o} C for 30 seconds, and 72^{o} C for 45 seconds. Data were collected during the 72^{o} C stage. Dissociation curves show that only one PCR product was created in each reaction. Relative Ct values for a given gene were calculated by dividing the change in expression at that given gene, like *ZRT1*, by the change in expression at a control gene, *SCR1*.

Table 12. Table of primers used for real time PCR.

Primer name	Gene	Sequence (5' to 3')
MBO178	*SCR1* (forward)	CGCACCGTGCCCTGTT
MBO179	*SCR1* (reverse)	AGCTCTGCCCAGGACAAATTT
MBO180	*ZRT1* (forward)	CAAAGTATTTTGGTTCCGGTGTT
MBO181	*ZRT1* (reverse)	GTACCACCAATCGCACCATAAG
MBO182	*DUR3* (forward)	AGCATGTCTTGCGGTGGAA
MBO183	*DUR3* (reverse)	TTTGCCTGGAACGAAGTAAGTG
MBO184	*BNA2* (forward)	GGTTTATGGCGCACGCTTAT
MBO185	*BNA2* (reverse)	CAACAGTGGCCTTGCAATACA
MBO186	*HXK1* (forward)	AAGGTTTGACAAAGAAGGGAGGTA
MBO187	*HXK1* (reverse)	CCCAAATCAATGGCCAAATAGT

5.3 RESULTS

5.3.1 Microarray analysis shows that Rkr1 affects the expression of a subset of genes in yeast

Many accessory transcription factors have been shown to affect only a subset of yeast genes, and not genome-wide transcription, presumably due to redundancy of many of these factors. Strains lacking *RKR1* are Ino$^-$, a phenotype indicative of general transcription defects (HAMPSEY 1997) (see Chapter 2). I also uncovered physical interactions between Rkr1 and several transcriptional regulators (see Chapter 4 for details). To investigate the global role of Rkr1 in transcription, I performed DNA microarray analysis using Affymetrix Microarrays in collaboration with Martin Schmidt's lab in the Department of Molecular Genetics and Biochemistry at the University of Pittsburgh Medical School. Total RNA isolated from wild type and *rkr1Δ* strains (grown in triplicate) was used to make cDNA. Rhonda McCartney, of the Schmidt lab, used *in vitro* transcription to generate cRNA, which was biotinylated and hybridized to Affymetrix arrays that contained oligonucleotides that represented every open reading frame (ORF) in yeast. The Microarray Core Facility generated target intensity profiles for both wild type and *rkr1Δ* strains.

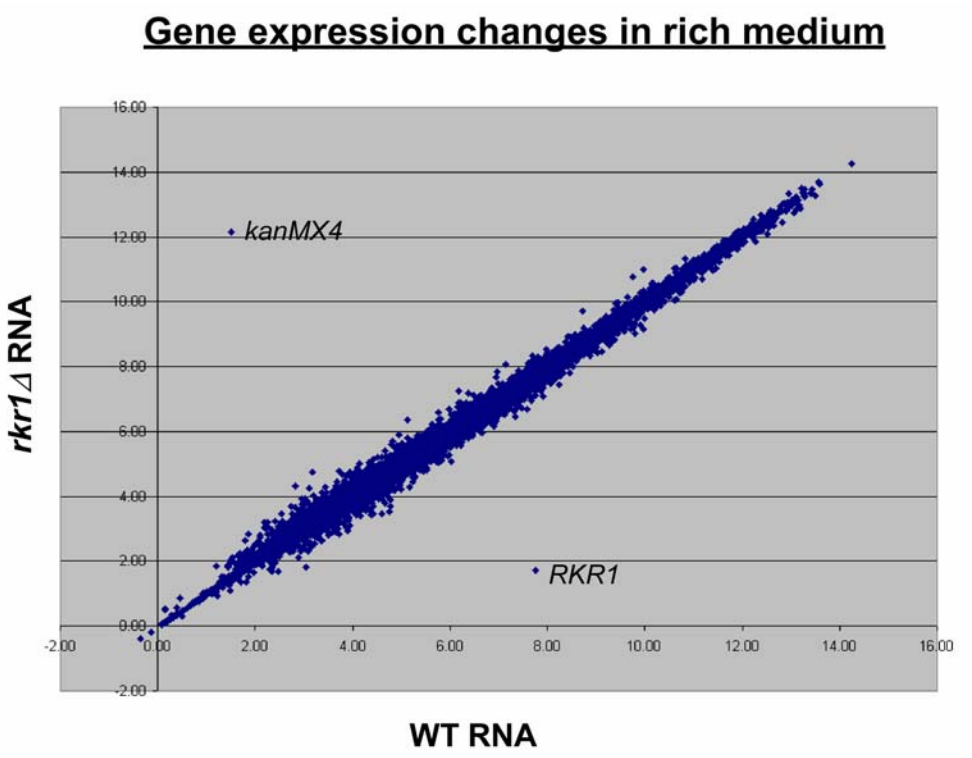
My initial attempts to investigate gene expression profiles in *rkr1Δ* strains were performed using RNA from strains grown in rich medium. However, I detected no changes in gene expression (greater then 1.2 fold) under these conditions (Figure 40). I attempted to determine if there were conditions under which I could see Rkr1-dependent gene expression changes. I recalled the observation that *rkr1Δ* strains that have defects in histone modifications

grew very poorly on minimal medium (see Chapter 2 for details). Also, Richard Gardner had performed microarray experiments to characterize San1, another nuclear, ubiquitin-protein ligase that is important for degradation of mutant transcription factors and mutant silencing factors (GARDNER *et al.* 2005). Dr. Gardner could not detect significant changes in gene expression when *san1Δ* strains were grown in rich medium, but determined that the expression of many stress response genes changed when *san1Δ* strains were grown in minimal medium (GARDNER *et al.* 2005). For these reasons, I decided to investigate gene expression profiles in *rkr1Δ* strains that were grown in minimal medium. I again collaborated with the Schmidt lab to perform these experiments, and we obtained target intensity profiles for strains grown in minimal medium from the Core Facility. I calculated the relative amount of gene expression in wild type and *rkr1Δ* strains and found a subset of affected genes (Figure 40). Genes that are affected greater than 1.75 fold (arbitrary value) are listed in Tables 13 and 14. At this cutoff, Rkr1 appears to be important for the expression of 34 genes and Rkr1 appears to be important for the repression of 76 genes in yeast (Tables 13 and 14). The positioning of these genes in yeast suggests that Rkr1 does not affect the expression of genes located at a particular genomic location, i.e. telomeric regions (data not shown).

Figure 40. Rkr1 affects the expression of a subset of yeast genes when strains are grown in minimal medium.

A) Scatterplot representing changes in gene expression between wild type and *rkr1Δ* strains grown in rich medium. Each dot represents a gene. If a given gene is expressed equally in wild type and *rkr1Δ* strains, it will fall along a diagonal line with a slope of 1.0. The majority of genes fall along this line when strains are grown in rich medium. *RKR1* and *kanMX4* (drug resistance gene that replaces *RKR1* in the null) are marked. B) Scatterplot representing changes in genes expression between wild type and *rkr1Δ* strains grown in minimal medium. Each dot represents a gene. If a given gene is expressed equally in wild type and *rkr1Δ* strains, it will fall along a diagonal line with a slope of 1.0. Several genes fall off of this line when strains are grown in minimal medium. Pink dots represent the genes whose expression changes greater than 1.75 fold. *RKR1* and *kanMX4* (drug resistance gene that replaces *RKR1* in the null) are marked.

A


Gene expression changes in rich medium
kanMX4
RKR1
rkr1Δ RNA
WT RNA
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-2.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00

B

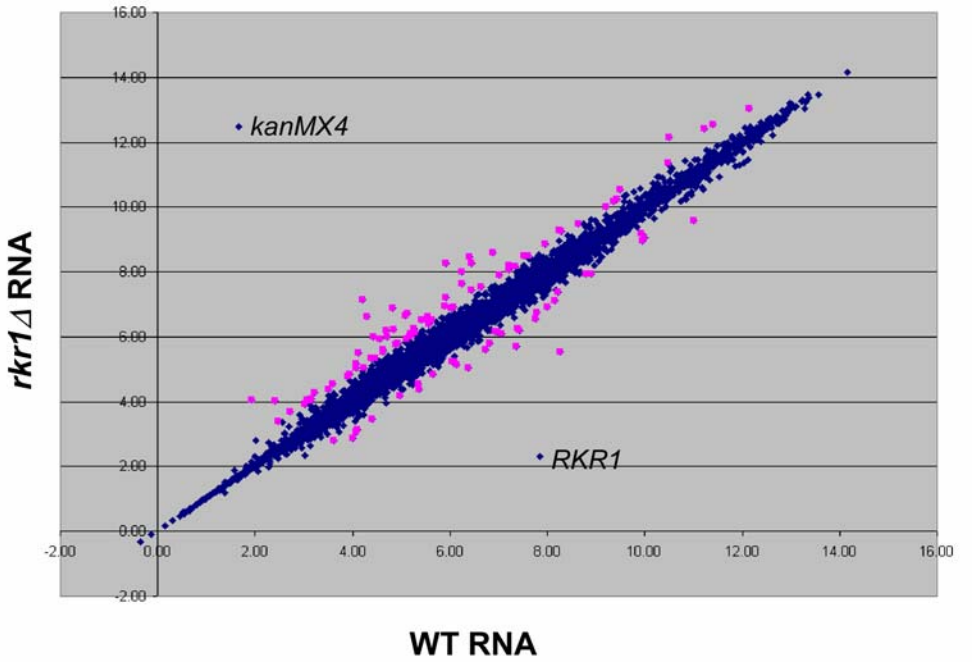

Gene expression changes in minimal medium
kanMX4
RKR1
rkr1Δ RNA
WT RNA
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-2.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00

Table 13. . List of genes that require Rkr1 for full expression.

Gene	Fold change	Standard deviation
ERG10	-1.68	1.16
PUT1	-1.73	1.09
RGS2	-1.74	1.29
PRM1	-1.74	1.21
YPR157W	-1.75	1.14
YRF1-5	-1.75	1.30
CAD1	-1.75	1.53
YLR126C	-1.75	1.07
YIL080W	-1.78	1.15
DAL7	-1.79	1.27
AGA2	-1.80	1.18
TPO4	-1.90	2.86
DAL5	-1.91	1.06
SUL1	-1.92	1.66
STP4	-1.94	1.33
INO1	-1.95	3.81
OPT1	-1.97	1.27
RMD6	-1.97	1.89
HSP30	-2.00	1.96
PRY1	-2.00	1.05
MUP1	-2.01	1.43
RPI1	-2.03	1.34
AGA1	-2.05	1.24
MDH2	-2.12	1.39
DAL80	-2.18	1.58
PHO84	-2.19	1.37
YJR088C	-2.20	1.14
DUR3	-2.33	1.19
WSC4	-2.35	1.02
DAL4	-2.52	1.31
ITR1	-2.65	1.94
YPL095C	-3.16	1.45
ZRT1	-6.51	2.23

Genes that are shaded gray were found to be significant by SAM analysis.

Table 14. List of genes that require Rkr1 for their repression.

Gene	Fold change	Standard deviation
YOL053C	7.69	1.64
YGP1	5.11	1.33
HXK1	4.97	1.33
SOL4	4.36	1.23
YCL042W	4.21	1.67
GLK1	4.17	1.37
PNC1	3.60	1.20
HSP82	3.38	1.41
COS8	3.27	1.67
HSP12	3.15	2.69
HXT5	3.05	1.44
MSC1	3.02	1.20
HSP104	2.96	1.30
RPP1B	2.93	1.24
YDR034W	2.87	1.43
AHA1	2.64	1.26
COS8	2.64	1.90
COS8	2.63	1.73
HSP78	2.61	1.46
HOR2	2.45	2.32
YLR327C	2.42	1.27
SSA1	2.28	1.36
SSA1	2.22	1.36
TFS1	2.18	1.24
PGM2	2.16	1.21
HSP26	2.16	1.48
INH1	2.13	1.24
GPH1	2.10	1.08
YBR085C	2.07	1.46
ALD6	2.06	1.46
PYC1	2.05	1.16
YMR196W	2.05	1.32
CAR2	2.01	1.67
OXA1	2.01	4.27
GNA1	2.00	3.43
ZTA1	1.98	1.27
CIT1	1.98	1.11
ALD4	1.97	1.12
YFR016C	1.97	3.33
YHR087W	1.96	1.16
TSA2	1.94	1.18
YNL274C	1.93	1.11
COS5	1.93	1.32
SSA4	1.91	1.35
NCA3	1.91	1.62
DCS2	1.90	1.38
AMS1	1.89	1.20
GSY2	1.88	1.29
COS7	1.87	1.38

Gene	Fold change	Standard deviation
GPG1	1.86	1.35
BNA6	1.86	3.22
YER163C	1.86	1.88
BNA2	1.86	1.38
SSA2	1.86	1.23
HXT2	1.85	1.49
GPD1	1.85	1.34
TVP15	1.85	1.27
GAD1	1.85	1.35
TPK1	1.84	1.49
TPS1	1.83	1.50
TSL1	1.83	1.26
YBR056W	1.83	1.57
UBP5	1.82	2.51
YBR230C	1.82	1.15
COS1	1.82	1.21
KAR2	1.82	1.06
LSP1	1.81	1.22
YMR31	1.80	2.35
COS5	1.79	1.22
YHR138C	1.78	1.38
EMI2	1.77	1.36
YFL044C	1.77	1.58
IDH2	1.77	1.51
RAD51	1.76	1.82
STI1	1.76	1.06
SEC53	1.76	2.17
ATG18	1.75	1.81

Genes that are shaded gray were found to be significant by SAM analysis.

5.3.2 SAM analysis suggests that Rkr1 significantly affects the expression of 73 genes in yeast

In parallel with the data analysis described above, I performed "significance analysis of microarrays" (SAM) analysis on the three datasets. This program uses statistical analysis to determine significant changes in gene expression in microarray data sets (TUSHER *et al.* 2001). SAM calculates a value for each gene that represents a correlation in gene expression in different conditions, in this case, gene expression in wild type versus *rkr1Δ* strains. The level of significance is user-defined by a delta value, which determines the false-positive rate. The user can also define the minimal fold-change, to ensure that all outputs are affected by a specified fold-change. I entered $\log_2$-based values that were collected from the University of Pittsburgh Microarray Core Facility. I chose to pair my experiments because they were performed on different days with different reagents, and while the changes in gene expression were the same (up- versus down-regulated), the actual values for the different experiments were different enough to cause a large standard deviation, which may affect the output of the program. I also tried many different delta values, and fold-change values. With a delta value of 0.28 and a fold-change value of 1.75, SAM analysis determined that there are 75 genes affected by the loss of Rkr1 (Tables 13 and 14), with a false-positive rate of 15.2%. More specifically, 49 genes are up-regulated and 24 genes are down-regulated in a *rkr1Δ* strain. Most of these genes are found in the list of genes that are affected greater than 1.75 fold using mathematical ranking of expression changes that I described in the last section (Tables 13 and 14). These results provide statistical significance for the observed Rkr1-dependent effects on the expression of a subset of genes in yeast.

5.3.3 Real-time PCR confirms that several genes are affected by the loss of *RKR1*

Real-time PCR was performed to confirm the changes in gene expression at four yeast genes that were detected in the microarray experiments. First, I chose to investigate expression changes at *ZRT1* and *HXK1*, which were the two genes most affected by the loss of *RKR1* (Table 15). In microarray experiments, *ZRT1* expression is 6.5 fold decreased, and *HXK1* expression is 5 fold increased in *rkr1Δ* strains compared to wild type strains. *ZRT1* encodes a membrane protein that acts as a high affinity zinc transporter in yeast and this gene is up-regulated in response to zinc limiting conditions in wild type strains (ZHAO and EIDE 1996). *HXK1* encodes a cytoplasmic hexokinase isoenzyme which acts in the glucose degradation pathway (KOPETZKI *et al.* 1985).

I also chose to follow up expression changes of *DUR3*. According to microarray experiments, *DUR3* expression is 2.3 fold decreased in *rkr1Δ* strains (Table 15). This gene encodes a membrane urea transporter and its expression is regulated by the nitrogen-catabolic pathway (ELBERRY *et al.* 1993). In wild type cells, *DUR3* expression is induced by allophanate, the last intermediate in the allantoin degradative pathway (ELBERRY *et al.* 1993). Interestingly, this gene is repressed by Gzf3 and activated by Dal82, two proteins that interact with the amino terminus of Rkr1 in a yeast-two hybrid screen (see Chapter 4 for details).

The fourth gene that I chose to follow up is *BNA2*. *BNA2* expression is increased 1.85 fold in *rkr1Δ* strains compared to wild type strains (Table 15). *BNA2* encodes an enzyme that acts in the nicotinamide-adenine-dinucleotide (NAD) biosynthesis pathway (FUKUZUMI *et al.* 2001). Interestingly, strains lacking the Paf1 complex member, Ctr9, have decreased expression of *BNA1* and *BNA6* and increased expression of *ZRT1* in rich medium (SHELDON 2005) (see the next section for further analysis). *BNA1* and *BNA6* encode enzymes that also act in the NAD biosynthesis pathway downstream of Bna2 (PANOZZO *et al.* 2002) (Figure 41).

I isolated RNA from wild type and *rkr1Δ* strains (grown in triplicate) in minimal medium and generated cDNA. I used the cDNA in real-time PCR reactions with primers specific to *ZRT1*, *HXK1*, *DUR3*, *BNA2*, and *SCR1* (internal control). The results of this analysis show that all genes tested are affected by the loss of Rkr1 (Table 15). While the absolute numbers are different, the trends are consistent between microarray and real-time PCR results, which suggests that Rkr1 does affect the expression of a subset of genes in yeast.

Figure 41. The NAD biosynthesis pathway in yeast.

Schematic view of the intermediates, enzymes and genes involved in NAD^+ biosynthesis via the kynurenine pathway or by direct incorporation of nicotinic acid. Note that Bna1, Bna2 and Bna6 all act in this pathway. From Panozzo, C. *et al*. (2002) *FEBS Letters*. 517:97-102.

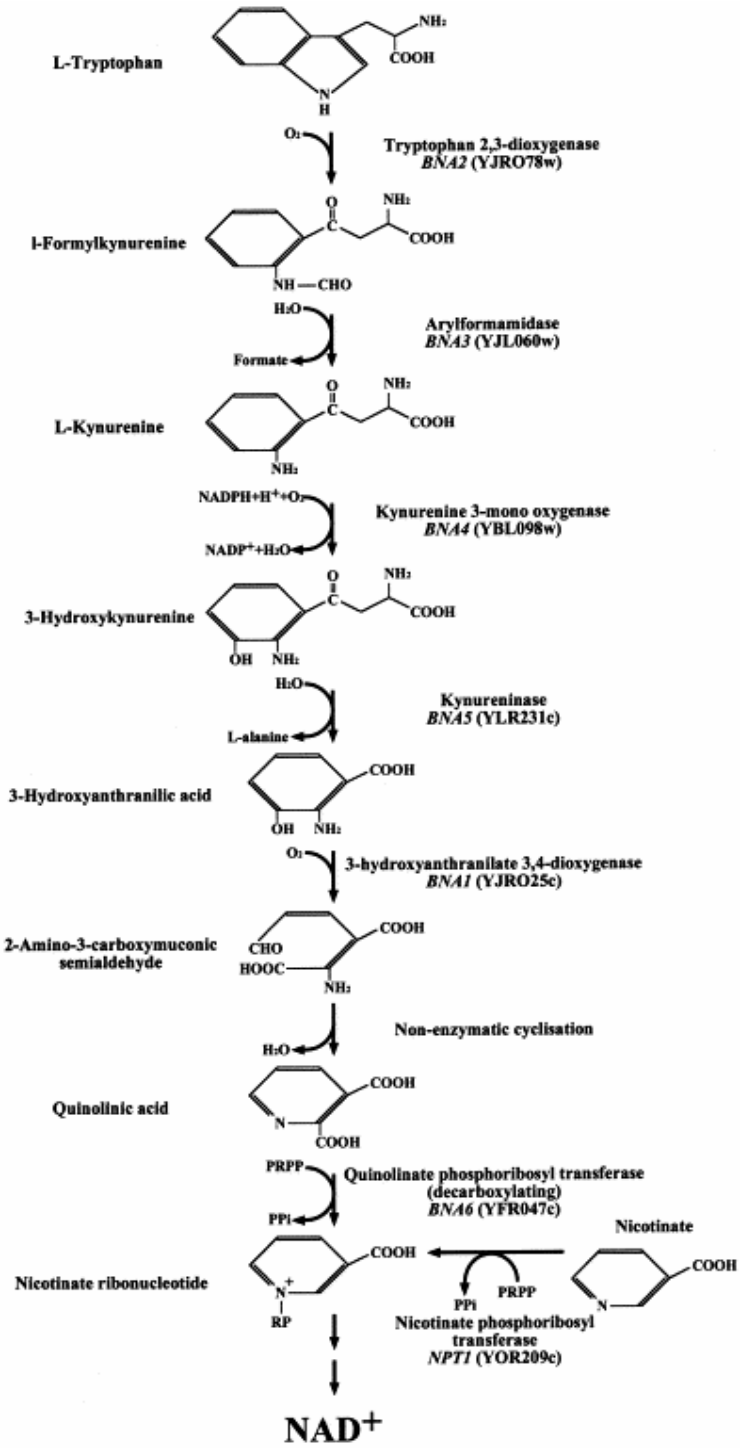

L-Tryptophan
Tryptophan 2,3-dioxygenase
BNA2 (YJRO78w)
l-Formylkynurenine
Arylformamidase
BNA3 (YJL060w)
Formate
L-Kynurenine
Kynurenine 3-mono oxygenase
BNA4 (YBL098w)
3-Hydroxykynurenine
Kynureninase
BNA5 (YLR231c)
L-alanine
3-Hydroxyanthranilic acid
3-hydroxyanthranilate 3,4-dioxygenase
BNA1 (YJRO25c)
2-Amino-3-carboxymuconic semialdehyde
Non-enzymatic cyclisation
Quinolinic acid
Quinolinate phosphoribosyl transferase (decarboxylating)
BNA6 (YFR047c)
Nicotinate
Nicotinate ribonucleotide
Nicotinate phosphoribosyl transferase
NPT1 (YOR209c)
NAD+

Table 15. Confirmation of microarray results with realtime PCR.

Gene	Array results	Real-time results
HXK1	**5x up**	**2.2x up**
ZRT1	**6.5x down**	**4.5x down**
DUR3	**2.3x down**	**3.5x down**
BNA2	**1.9x up**	**1.6x up**

Values shown represent the fold change of gene expression in *rkr1Δ* strains compared to wild type levels. Strains used are KY595 (wild type) and MBY30 (*rkr1Δ*).

5.3.4 Some yeast genes appear to be oppositely affected by the loss of Rkr1 and Rtf1

Surprisingly, Rkr1 affected the expression of genes that Kathryn Sheldon had shown to be affected by the loss of Ctr9 (SHELDON 2005). Specifically, these genes were oppositely affected by the loss of Rkr1 and Ctr9. For example, *ZRT1* expression is decreased in a *ctr9Δ* strain, but increased in a *rkr1Δ* strain (Table 16). Also, *BNA2* expression was increased in *rkr1Δ* strains, while *BNA1* and *BNA6* expression decreased in *ctr9Δ* strains (Table 16). Because Dr. Sheldon's data came from strains grown in rich medium and my data came from strains grown in minimal medium, I used real-time PCR to compare expression of *ZRT1*, *HXK1*, *DUR3*, and *BNA2* in wild type, *rtf1Δ*, *ctr9Δ* and *rkr1Δ* strains grown in minimal medium (Table 16). The results of this analysis show that *DUR3* appears to be oppositely regulated by Rkr1 and the Paf1 complex members Rtf1 and Ctr9 in minimal medium. *ZRT1* may be oppositely regulated as well, but the increased expression in *rtf1Δ* and *ctr9Δ* strains is minimal. *HXK1* and *BNA2* expression increased in all mutant strains tested compared to wild type, although *BNA2* levels were more affected by the loss of the Paf1 complex members than by the loss of Rkr1. Although the mechanism of this phenomenon is currently unknown, it appears as though Rkr1 and Paf1 complex oppositely regulate the expression of a subset of genes in yeast.

Table 16. Real-time PCR shows that Rkr1, Rtf1 and Ctr9 regulate an overlapping set of genes in minimal medium.

	ZRT1	***HXK1***	***DUR3***	***BNA2***
rkr1Δ	**4.4x down**	**2.2x up**	**3.5x down**	**1.6x up**
rtf1Δ	**0.99 (no change)**	**4.3x up**	**5.7x up**	**3.9x up**
ctr9Δ	**1.5x up**	**3.3x up**	**2.9x up**	**13.7x up**

*All values represent fold change in expression relative to wild type strains grown in minimal medium. Strains used are KY592 (wild type), KY951 (*rtf1Δ*), MBY169 (*rkr1Δ*), and KY694 (*ctr9Δ*).

5.3.5 Information obtained in the yeast two-hybrid screen overlaps with information obtained in microarray experiments

Interestingly, I observed an overlap in the yeast two-hybrid screen and microarray datasets. Gzf3 and Dal82 negatively and positively regulate many genes involved in nitrogen metabolism, respectively. The expression of many of the genes that are regulated by these factors was decreased in strains lacking *RKR1* (Table 17).

Table 17. An overlap in the microarray and yeast-two hybrid screen datasets.

Two-hybrid screen proteins

Protein	# hits	Description
Gzf3	1	Transcription repressor
Dal82	1	Transcription activator

Microarray genes

Gene	Fold decrease in *rkr1Δ*
DAL7	1.8
DAL5	1.9
DAL80	2.2
DUR3	2.3
DAL4	2.4

5.4 CONCLUSIONS

This chapter describes my attempts to identify a role for Rkr1 in transcription in yeast. I used Affymetrix microarray analysis to assess transcription levels of every gene in yeast in wild type and *rkr1Δ* strains. When strains were grown in rich medium, I saw no attenuation in the expression of any genes. However, when strains were grown in minimal medium, I detected that Rkr1 affects the expression of a subset of genes, specifically 34 genes are up-regulated greater than 1.75 fold, and 76 genes are down-regulated greater than 1.75 fold in strains lacking *RKR1*. The significance analysis of microarrays (SAM) program determined that many of these genes are significantly affected by the loss of Rkr1.

I used real-time PCR to confirm changes in gene expression in strains lacking *RKR1* compared to wild type strains. The results show that Rkr1 is important for wild type expression of all four genes tested with this method, *ZRT1*, *HXK1*, *DUR3*, and *BNA2*. Interestingly, I noticed that Rkr1 and the Paf1 complex oppositely affect the expression of a subset of genes in yeast. Specifically, *DUR3* expression increased in *rkr1Δ* strains, and decreased in *rtf1Δ* and *ctr9Δ* strains. While I have yet to uncover the mechanism that is responsible for this result, I can conclude that Rkr1 affects the transcription of a subset of genes and that the expression of some of these genes is oppositely regulated by the Paf1 complex in yeast.

6.0 DISCUSSION

6.1 SUMMARY OF RESULTS

In my work, I have characterized a previously unstudied nuclear RING domain protein, Rkr1, and provided evidence to suggest a role for this protein in transcription and chromatin function. Rkr1 was identified in a genetic screen for factors that become essential in the absence of the Paf1 complex component Rtf1, a protein required for several histone modifications that mark active genes. Several lines of evidence implicate Rkr1 in the regulation of chromatin structure or function (Table 18). In addition to Rtf1, Rkr1 exhibits genetic interactions with several other factors involved in chromatin modification. Specifically, Rkr1 exhibits strong genetic interactions with factors required for histone H2B ubiquitylation and histone H3 K4 methylation. Additional evidence linking *RKR1* to chromatin modification is its synthetic lethal relationship with *SPT10*, a gene that encodes a protein that is required for normal expression of the histones. Consistent with a role in chromatin structure and histone modification, I show that strains lacking either *RKR1* or *SPT10* have defects in telomeric silencing.

From data obtained in my *in vitro* experiments, I propose that the RING domain of Rkr1 is required for the ubiquitylation of one or more nuclear proteins. A yeast two-hybrid screen identified twenty proteins that physically interact with Rkr1, many of which are involved in transcription and chromatin function. Microarray experiments show that Rkr1 is required for the

proper expression of a subset of genes in yeast. In summary, my results indicate the existence of a new nuclear protein ubiquitylation pathway that is functionally connected to chromatin and transcription.

These results have established a foundation for many future experiments that will continue to elucidate Rkr1's role in transcription and chromatin function. In this section I will briefly discuss the results that have been described in the previous chapters and address questions that arise from the data that have been presented.

Table 18. Summary of evidence that suggests a role for Rkr1 in regulating chromatin function.

Telomeric silencing defect in *rkr1Δ* strain
Inositol auxotrophy of *rkr1Δ* strains
Sporulation defect in *rkr1Δ*/*rkr1Δ* diploids
Nuclear localization of Rkr1
Genetic interactions with histone modifiers *RAD6*/*BRE1* and *SET1*
Physical interactions with *RSC8* (chromatin remodeling complex)
Genetic interaction with *CHD1* (chromatin remodeler)
Genetic interaction with *SPT10* (activates histone gene expression)

6.2 GENETIC ANALYSIS SUGGESTS THAT RKR1 IS FUNCTIONALLY CONNECTED TO CHROMATIN MODIFICATIONS THAT MARK ACTIVE TRANSCRIPTION

6.2.1 How can we interpret the synthetic lethality observed in *rkr1Δ rtf1Δ* strains?

Strains lacking *RTF1*, but not other members of the Paf1 complex, are inviable when *RKR1* is deleted. This observation is noteworthy because mutant phenotypes and protein stabilities suggest that Paf1 and Ctr9 are integral members of the Paf1 complex, while Rtf1 appears to be a more peripheral member of the complex (BETZ *et al.* 2002; MUELLER *et al.* 2004; SQUAZZO *et al.* 2002). Interactions between *rtf1Δ* and *rkr1Δ* indicate that Rtf1 performs a function for which Paf1 and Ctr9 are not sufficient. Importantly, Rtf1 appears to be the only member of the complex that is essential for normal H3 K4 and K79 methylation (M.H. Warner and K.M. Arndt, unpublished observations). Although Paf1 has been shown to be important for these modifications (KROGAN *et al.* 2003a; NG *et al.* 2003a; NG *et al.* 2003b), *paf1Δ* strains have significantly reduced levels of Rtf1 protein (MUELLER *et al.* 2004; SQUAZZO *et al.* 2002), which may explain the decreased levels of H3 K4 and K79 methylation in the absence of Paf1. The synthetic lethal interaction between *RTF1* and *RKR1* is also specific to *RKR1*; deletion of *SAN1*, a gene encoding another nuclear RING protein, exhibits no synthetic interactions with *rtf1Δ* (data not shown).

I have also observed that removal of residues 62-152 of Rtf1 (*rtf1Δ3* and *Δ4*), which are responsible for histone H2B ubiquitylation and histone H3 K4 and K79 methylation, causes

severe growth defects on minimal medium when combined with the loss of *RKR1*. Genetic results from my work indicate that removing amino acids 62-152 of Rtf1 interferes with Rad6/Bre1 activity, as similar phenotypes are observed with *rad6Δ rkr1Δ* and *bre1Δ rkr1Δ* strains. These results suggest that the synthetic lethality of *rtf1Δ rkr1Δ* strains is largely due to the combined loss of the Rtf1-dependent chromatin modifications and yet unknown Rkr1-dependent chromatin function(s). However, it is not clear why *rtf1Δ3 rkr1Δ* and *rtf1Δ4 rkr1Δ* double mutants are viable, while *rkr1Δ* cells containing a complete deletion of *RTF1* are not. Presumably the synthetic lethality between *rtf1Δ* and *rkr1Δ* is due to the absence of more than one activity of Rtf1. Considering the genetic interaction between *RKR1* and *CHD1*, a chromatin remodeler that physically interacts with Rtf1 (SIMIC *et al.* 2003), the synthetic lethality between *rkr1Δ* and *rtf1Δ* may be due to the loss of a chromatin structure that is conducive to active transcription. The failure to transcribe essential genes effectively may result in lethality.

The poor growth phenotype that is observed on SD minimal medium (but not SC medium) could be due to a number of factors. SD medium consists of ammonium sulfate, yeast nitrogen base, and glucose, and is supplemented with the minimal amino acids that yeast require to survive. SC medium contains ammonium sulfate, yeast nitrogen base, glucose, and a mixture containing all amino acids along with other compounds, including para-aminobenzoic acid (PABA; component of B vitamin complexes in yeast), uracil, and inositol. It is possible that the SD^- strains that I have described could have defects in transcribing the genes that are required to synthesize the components that are found in SC but not SD medium. To determine what these compounds are, SC medium that lacks individual components (or groups of components) that are not found in SD medium could be created and we could investigate growth of *rkr1Δ rtf1Δ3* strains on these media. These strains should grow more poorly on the SC deficient medium than

wild type or single mutant strains. We could also investigate the transcription profiles of double mutant strains (eg. *rkr1Δ rtf1Δ3*) grown in minimal medium and compare gene expression to single mutant and wild type strains. This information could uncover a more global role for Rkr1 in gene expression and assist us in understanding Rkr1's function *in vivo*.

6.2.2 Why is telomeric silencing disrupted in *rkr1Δ* strains?

To gain a better understanding of Rkr1's role in chromatin function, I investigated Rkr1's role in telomeric silencing. This process heavily depends on the proper distribution of chromatin modifications, where the silenced chromatin contains hypomethylated and hypoacetylated histones (reviewed in RUSCHE *et al.* 2003). Consistent with a role for Rkr1 in chromatin structure and/or function, strains lacking Rkr1 exhibit telomeric silencing defects (Chapter 2). However, silencing is not disrupted at the mating loci or ribosomal DNA in *rkr1Δ* strains. Chromatin immunoprecipitations (ChIPs) show that incorporation of histones H3 and H4 and histone H4 acetylation are unaffected in *rkr1Δ* strains, which suggests that histone H3 and H4 deposition and acetylation do not play a role in the Rkr1-dependent telomeric silencing mechanism. Silencing at telomeres appears to be more easily perturbed than silencing at the other two silenced regions of the genome, suggesting that Rkr1 is not causing a severe disturbance in chromatin function *in vivo*. This idea is supported by the lack of strong transcription-related phenotypes in *rkr1Δ* strains.

There are a few possible explanations for why only telomeric silencing is disrupted in *rkr1Δ* strains. First, Rkr1 could be important for the deposition of other histones into chromatin, particularly at telomeric boundaries. Specifically, the H2A variant Htz1 is important for establishing a "boundary element" between euchromatin and the telomeres (MENEGHINI *et al.*

2003). Rkr1 may be important for proper positioning of this histone variant into chromatin. Genetic analysis of *rkr1Δ* and *rtf1Δ* strains that lack *HTZ1* or SWR1 complex members (required for Htz1 incorporation into chromatin) could determine if this hypothesis is viable. Alternatively, Rkr1 may be important for histone modification(s) throughout the genome, and the telomeric silencing defects may be due to loss of those marks. Support for this model is found in the genetic data that Rkr1 functions in parallel with transcriptionally active chromatin marks. Rkr1 may affect telomeric silencing through a mechanism that is similar to Rtf1, where the loss of global chromatin modifications dilutes the Sir proteins from the telomeres. However, I do not favor this idea because Sir2 dependent histone H4 acetylation patterns are not disrupted in *rkr1Δ* strains (Chapter 2), suggesting that Rkr1 does not significantly affect Sir protein association with the telomeres. A targeted approach using Western analysis or genetic analysis with strains containing mutations in known histone modifiers or mutations in the histone genes could be used to investigate Rkr1's role in transcriptionally active histone modifications. Additional insight into Rkr1's role in telomeric silencing could be gained from studies to determine if overexpression of Rkr1 could suppress telomeric silencing defects caused by the loss of other proteins involved in silencing, including Set1, Rtf1, and the Sir proteins. Suppression of a silencing defect could narrow Rkr1's function in telomeric silencing.

Another possible explanation for the telomeric silencing defect could be that telomere length is disrupted in *rkr1Δ* strains. A study of nonessential yeast genes that are required for proper telomere length identified many transcription factors, including components of Mediator, Rad6/Bre1 and members of the Paf1 complex (all but *CTR9*) (GATBONTON *et al.* 2006). This study made use of the "deletion collection" of yeast strains, which consists of approximately 4800 strains that each contain a separate deletion of a nonessential open reading frame. While

this study showed that loss of the Paf1 complex or loss of Rad6/Bre1 results in shortened telomeres, Rkr1 was not identified as a factor that is required for proper telomere length. However, our lab does not regularly use the deletion collection strains. Because of the functional connection between Rkr1 and the Paf1 complex and H2B ubiquitylation, I believe that it is possible that Rkr1 may also regulate telomere length. While I do not know how or why the Paf1 complex or Rad6/Bre1 affect telomere length, I could postulate that the loss of the Sir proteins from the telomeres results in an "unraveling" of the telomeric chromatin structure, which could affect the recruitment and/or activity of telomerase or DNA rearrangements involving the telomeres. There could also be an unknown Paf1-dependent mechanism that affects telomere length maintenance, and this process could involve Rkr1.

6.2.3 What do the genetic interactions between *RKR1* and factors involved in chromatin modification tell us?

Several lines of evidence implicate Rkr1 in the regulation of chromatin structure or function. Rkr1 exhibits strong genetic interactions with factors required for histone H2B ubiquitylation and histone H3 K4 methylation. Specifically, *rkr1Δ* strains containing an amino acid replacement for lysine 123 on histone H2B grow very poorly. In addition, *rkr1Δ set1Δ* and *rkr1Δ hht2-K4R* double mutants grow very poorly on minimal media. In contrast, no synthetic phenotypes were observed between *rkr1Δ* and *dot1Δ*, which methylates histone H3 at K79. This is surprising because both histone H3 K4 and K79 methylation depend on histone H2B K123 ubiquitylation. Therefore, my data suggest that these modifications have distinct functions *in vivo* and that Rkr1 is most strongly connected to histone H2B ubiquitylation.

Interestingly, strains that lack *RKR1* and contain point mutations within *RTF1* that disrupt

histone modifications exhibit genetic interactions. Specifically, *rkr1Δ* strains that contain the *rtf1-108-110A* and *rtf1-F123S* mutations, but not the *rtf1-102-104A* mutation, grow poorly on SD minimal medium. The *rtf1-108-110A* and *rtf1-F123S* mutations disrupt histone H3 K4 methylation but not K79 methylation, but the *rtf1-102-104A* mutation only moderately disrupts histone H3 K4 methylation levels (M. Warner, K. M. Arndt, unpublished observations). The status of histone H2B ubiquitylation levels has not been investigated in these strains, but the loss of K4 methylation suggests that H2B ubiquitylation will be disrupted. These observations further support a model in which Rkr1 functions in parallel with histone H2B ubiquitylation and histone H3 K4 methylation, but not histone H3 K79 methylation.

6.2.3.1 Does Rkr1 function to regulate histone modification(s)?

I observe strong genetic interactions between *RKR1* and genes encoding histone modification enzymes, specifically those enzymes that are required for histone H2B ubiquitylation and H3 K4 methylation. These histone modifications are Rtf1-dependent and are associated with actively transcribing genes (KROGAN *et al.* 2003a; KROGAN *et al.* 2003b; NG *et al.* 2003a; NG *et al.* 2003b; POKHOLOK *et al.* 2005; WOOD *et al.* 2003b). This suggests that Rkr1 functions in parallel with histone modifications that are associated with active transcription, possibly by mediating chromatin modifications that are associated with activate transcription, Specifically, histone acetylation is also associated with actively transcribing genes (POKHOLOK *et al.* 2005). It is possible that Rkr1 is required for histone acetylation at actively transcribed genes. However, I did not observe any genetic interactions between *RKR1* and *GCN5*, a histone acetyltransferase that functions at activated genes (IMOBERDORF *et al.* 2006; POKHOLOK *et al.* 2005). In addition, histone H4 acetylation levels are not altered in a *rkr1Δ* strains at the telomere on the right arm of chromosome VI (Chapter 2) This suggests that Rkr1 may not function in

histone H4 acetylation or Gcn5-dependent histone acetylation, but may affect transcription and chromatin function another way, perhaps by working with another histone acetyltransferase that is recruited to actively transcribing genes.

Further genetic analysis will be required to gain a better understanding of Rkr1's role in chromatin structure and function. Genetic analysis using strains that express histones that lack the amino-terminal tails could provide insight into which histone tails (and possibly which modifications) are required in the absence of *RKR1*. Subsequent analysis using strains that contain point mutations in the histone genes could identify the residues that function in parallel with Rkr1. Genetic analysis using *rkr1Δ* strains and strains that lack histone chaperones could uncover a role for Rkr1 in chromatin architecture and formation.

6.2.4 What do the phenotypes that are observed in *rkr1Δ* strains mean?

During the course of my work, I discovered that homozygous *rkr1Δ* diploid strains do not sporulate. This phenotype has been described for dipoid yeast strains that lack other factors that regulate transcription and chromatin function, including *BRE1*, *RAD6*, *CTK1*, *GCN5*, *LEO1*, *PAF1*, *RTF1*, *SPT4*, and *SPT10* (ENYENIHI and SAUNDERS 2003). A global study identified 334 genes that are required for sporulation, including those genes that are listed above (ENYENIHI and SAUNDERS 2003). However, *RKR1* was not shown to be required for sporulation in this study. The discrepancy in the data may be explained by genetic variation in the strain backgrounds, or, is more likely due to an error in the *rkr1Δ* in the commercially available deletion collection. Therefore, the failure to identify *RKR1* in global sporulation studies may not be surprising. Further characterization of Rkr1's function is needed to appreciate the mechanism by which Rkr1 regulates sporulation.

rkr1Δ strains grow poorly on medium lacking inositol. This phenotype is associated with general transcription defects (HAMPSEY 1997), and strains that exhibit this phenotype often have defects in expressing inositol biosynthesis (*INO*) genes. Microarray analysis identified *INO1* as a gene that has decreased expression in a *rkr1Δ* strain grown in minimal medium, suggesting that Rkr1 is required for *INO1* expression. However, directed analysis of *INO1* expression (Northern analysis) in *rkr1Δ* strains grown in minimal medium did not detect changes in *INO1* expression compared to wild type strains. I currently do not understand the observed difference between the phenotype and *INO1* expression studies. A possible explanation could be that this gene can be affected by cell density at the time of induction. Further understanding of Rkr1's role in chromatin function should help to elucidate an explanation for this phenotype.

6.2.5 How can we explain the genetic interactions between *RKR1* and transcription factors?

6.2.5.1 Why are *rkr1Δ spt10Δ* strains inviable?

Additional evidence linking *RKR1* to chromatin modification is its synthetic lethal relationship with *SPT10*. Spt10 regulates the transcription of many genes in yeast, particularly histone genes, and is important for histone H3 K56 acetylation at histone gene promoters *in vivo* (DOLLARD *et al.* 1994; HESS *et al.* 2004; XU *et al.* 2005). However, a recent report indicates that Spt10 is not required for global histone H3 K56 acetylation (SCHNEIDER *et al.* 2006b). Here, I show that Rkr1 does not affect histone gene expression nor does it regulate a process that overlaps with histone H3 K56 acetylation, supporting the idea that Spt10 has functions independent of histone H3 K56 acetylation.

Strains lacking *SPT10* are synthetically lethal with the loss of the histone chaperones

including *CAF1* and the *HIR* genes, suggesting that Spt10 functions in parallel with histone incorporation into chromatin (D. Hess and F. Winston, personal communication). The synthetic lethality between *RKR1* and *SPT10* suggests that Rkr1 could be important for histone deposition into chromatin. Strains lacking Spt10 have reduced histone levels (DOLLARD *et al.* 1994; ERIKSSON *et al.* 2005; HESS *et al.* 2004), but our work has shown that *rkr1Δ* strains express the histone genes at levels that are similar to those observed in wild type strains. If Rkr1 is important for incorporation of histones into chromatin, the inviability of a *rkr1Δ spt10Δ* strain could be explained by a synergistic effect on chromatin formation, where low levels of histone proteins (*spt10Δ*) are poorly incorporated into chromatin (*rkr1Δ*), resulting in disruption of chromatin structure that is too severe to sustain viability. However, chromatin immunoprecipitation analysis shows that *rkr1Δ* strains have wild type levels of histones H3 and H4 incorporated at telomere-proximal locations, suggesting that Rkr1 may not be required for histone incorporation in this region. Genetic analysis using strains lacking *RKR1* and strains containing mutations in histone chaperone genes could be used to determine if Rkr1 is connected to histone incorporation into chromatin.

6.2.5.2 Why are *rkr1Δ srb5Δ* strains inviable?

Strains lacking *RKR1* and *SRB5* are inviable (Chapter 2). Srb5 is a subunit of the Mediator coactivator complex. While I do not understand the basis for this genetic interaction, it implies that Rkr1 is important for proper transcription initiation. Strains lacking *RTF1* that contain a point mutation in *SRB5* (*srb5-77*) are also inviable, but *rtf1Δ srb5Δ* strains are not (COSTA 2001). Subsequent analysis showed that the *rtf1Δ srb5-77* genetic interaction was specific; *RTF1* did not genetically interact with other Mediator subunits. Strains that contain the *srb5-77* mutation are sensitive to 6 azauracil (COSTA 2001), suggesting that these strains have

transcription elongation defects. Genetic analysis using *rkr1Δ srb5-77* double mutant strains has yet to be performed. A genetic interaction with *srb5-77* would implicate Rkr1 in the regulation of transcription.

Mediator is composed of three modules, known as head, middle and tail domains (GUGLIELMI *et al.* 2004). The head and middle domains are important for physical interactions with RNA Pol II (DAVIS *et al.* 2002). The tail domain has been shown to physically interact with activators (HAN *et al.* 1999; PARK *et al.* 2000). Srb5 is part of the head domain (GUGLIELMI *et al.* 2004). Genetic analysis can be used to determine if other domains/activities of Mediator are required for viability in the absence of Rkr1.

6.2.5.3 Why do *rkr1Δ ctk1Δ* strains grow poorly?

Genetic analyses uncovered an interaction between *RKR1* and *CTK1*, which encodes a CTD kinase. Specifically *rkr1Δ ctk1Δ* strains grow poorly on rich medium. Currently a mechanism to explain this observation is unknown. Perhaps Rkr1 and Ctk1 are both required for optimal expression of a subset of essential genes in yeast. Interestingly, Ctk1 is required for Set2 recruitment to an actively transcribed gene (KROGAN *et al.* 2003b), suggesting that the sickness of *rkr1Δ ctk1Δ* strains may be due to combined effects of the loss of the unknown Rkr1-dependent chromatin function and decreased H3 K36 methylation levels. We have yet to determine if Rkr1 is important for H3 K36 tri-methylation. However, no genetic interactions are observed in *rkr1Δ set2Δ* strains (Chapter 2), suggesting that Rkr1 does not function in parallel with histone H3 K36 methylation. Further genetic analysis using strains that lack *RKR1* and other CTD kinases and the CTD phosphatases could help uncover the cause of the poor growth of *rkr1Δ ctk1Δ* strains.

6.3 RKR1 IS A NUCLEAR, RING DOMAIN CONTAINING PROTEIN

6.3.1 Rkr1 contains a functionally important RING domain

Database analyses show that the only identifiable domain or motif in Rkr1 is a RING domain at the extreme carboxy terminus of the protein (Chapter 3). Mutational analysis shows that the RING domain is required for Rkr1 function *in vivo*. Although the mechanism that links Rkr1 to chromatin is unknown, the presence of a RING domain suggests that Rkr1 may post-translationally modify transcription factors or chromatin components to alter their activity or stability.

In most RING domains, the cysteine and histidine residues are arranged in either a C3HC4 or C3H2C3 sequence (PICKART 2001). The C4HC3 pattern of the Rkr1 RING domain appears to be less common (DODD *et al.* 2004; HASSINK *et al.* 2005). However, the solution structure of the C4HC3 RING domain of the Kaposi's sarcoma-associated herpesvirus K3 protein has been solved (DODD *et al.* 2004). Chemical mutagenesis and two-hybrid analysis showed that this non-canonical RING domain interacts with ubiquitin conjugating enzymes on the same face of the RING domain as classical RING domains (DODD *et al.* 2004).

RING domains bear sequence and structural similarity to another protein interaction domain, the plant homeodomain (PHD) finger (BIENZ 2006). Interestingly, PHD fingers have recently been shown to interact with methylated lysine residues on histones (SIMS and REINBERG 2006). However, amino acids outside of the eight critical cysteine and histidine residues indicate that Rkr1 contains a RING domain and not a PHD finger. The RING domain of Rkr1 contains a tryptophan four residues after the cysteine in the sixth position of the Cys/His sequence, and this amino acid is highly conserved among RING domain proteins (DODD *et al.* 2004). In contrast,

PHD fingers contain an invariant tryptophan two positions amino-terminal to the seventh cysteine, a residue not found in Rkr1 (AASLAND *et al.* 1995).

6.3.2 The RING domain of Rkr1 possesses ubiquitin ligase activity

RING domain containing proteins often are ubiquitin-protein ligases that define substrate specificity within the ubiquitylation pathway. *In vitro* experiments using purified E1 and E2 components of the ubiquitylation machinery combined with purified GST fusions to the RING domain of Rkr1 show that GST-Rkr1 can promote polyubiquitylation of itself, indicating that Rkr1 is most likely a ubiquitin ligase *in vivo*.

I currently do not know the E2(s) with which Rkr1 functions *in vivo*, but several experiments could determine which ubiquitylation pathway utilizes Rkr1. Directed yeast two-hybrid experiments with Gal4-DNA binding domain fusions to Rkr1 and Gal4 activation domain fusions to the E2s should detect specific but transient interactions. Also, directed double mutant analysis using *rtf1Δ* strains that contain mutations in the genes that encode the 11 ubiquitin E2s in yeast should yield the E2 that functions with Rkr1. I would expect that an *rtf1Δ* strain that lacks the gene encoding an E2 would be inviable if a specific E2 functions with Rkr1. *In vitro* experiments using purified E2 from yeast could confirm the results of the genetic analysis.

6.3.2.1 What type(s) of ubiquitylation does Rkr1 catalyze?

Rkr1 may catalyze mono- or polyubiquitylation of substrates *in vivo*. The results of the *in vitro* assay suggest that the RING domain of Rkr1 can catalyze protein polyubiquitylation, however the amino-terminal portion may be important for regulating ligase function *in vivo*. If Rkr1 catalyzes polyubiquitylation, it would be interesting to determine which type(s) of ubiquitin

chains are formed. Ubiquitin can be conjugated to itself to form polyubiquitin chains linked at one of six lysine residues within the ubiquitin protein sequence. Linkages at lysines 48 and 63 appear to be the most common types found *in vivo*, both leading to proteasomal destruction of the conjugated substrates. Purified ubiquitin proteins that are mutated at the individual lysine residues could be used in *in vitro* ubiquitylation reactions. Loss of signal in reactions that contain ubiquitin with a particular lysine substitution would suggest that Rkr1 catalyzes that type of polyubiquitylation.

6.3.2.2 What are the targets of Rkr1's ubiquitin ligase activity?

Finding targets of this activity is a very difficult process because any protein that interacts with Rkr1 *in vivo* is probably polyubiquitylated and targeted for destruction by the proteasome. We have established a collaboration with Richard Gardner's lab at the University of Washington to use a biochemical approach to identify Rkr1 substrates. These experiments involve using mass spectroscopic analysis to identify increased protein levels in *rkr1Δ* strains compared to wild type strains. The proteins found at higher levels are potential substrates for the ubiquitin ligase.

I propose that the conserved amino-terminal region serves as a substrate recognition module within Rkr1. If this is true, then the proteins identified to interact in our yeast two-hybrid screen (Chapter 4) may be substrates of Rkr1's ubiquitin ligase activity. Further experiments (described in section 6.4.1) will be needed to determine is these proteins are indeed ubiquitylated in a Rkr1-dependent manner. This can be done by expressing untagged or HIS-tagged ubiquitin in strains expressing tagged version of a protein of interest. We can purify all ubiquitylated proteins with a nickel column, and then perform immunoblot analysis to detect our protein of interest in the ubiquitin-enriched proteins.

The amino terminus of Rkr1 is conserved in eukaryotes. Mutational analysis of this

region of the protein could provide insight into the function of this region *in vivo*. It would be interesting to determine if expression of an amino-terminally truncated Rkr1 in yeast could complement the lethality of a *rkr1Δ rtf1Δ* strain or lead to dominant negative effects in a wild type strain. Complementation would suggest that the amino terminus of Rkr1 provides little or no functional specificity to Rkr1 *in vivo*. Dominant negative effects would suggest that the amino terminus of Rkr1 is important for regulating substrate specificity.

6.3.2.3 Could Rkr1 act as a ligase for ubiquitin-like modifications?

From data obtained through genetic analysis and my *in vitro* experiments, I propose that the RING domain of Rkr1 is required for the ubiquitylation of one or more nuclear proteins. Protein sumoylation, a ubiquitin-like modification, is also facilitated by RING domain-containing proteins (HAY 2005). While it remains possible that Rkr1 could act as a SUMO-protein ligase *in vivo*, our lab has obtained genetic and biochemical data that do not support this idea. For example, we do not see any genetic interactions in strains containing mutations in *RTF1* and *UBC9*, the gene encoding the sole SUMO-conjugating enzyme in yeast. Rkr1 does not physically interact with SUMO (tested by directed yeast two-hybrid screen), arguing against a role for Rkr1 as a SUMO ligase. Moreover, Cheng *et al.* recently reported that SUMO-protein ligases, such as Siz1, Siz2 and Zip3, have a unique variant RING sequence, C3HCHC2 (CHENG *et al.* 2006).

6.4 RKR1 PHYSICALLY INTERACTS WITH PROTEINS INVOLVED IN UBIQUITYLATION, TRANSCRIPTION AND CHROMATIN FUNCTION

The amino terminus of Rkr1 physically interacts with 20 proteins in a yeast two-hybrid screen (Chapter 4). These proteins fall into several functional classes, including transcription and chromatin related factors, and the ubiquitin related factor, Dsk2. These physical interactions further support a model in which Rkr1 functions in parallel with Rtf1 using its ubiquitin ligase activity to affect chromatin function and transcription. Future experiments will be aimed at confirming these physical interactions with full-length Rkr1.

Biochemical purification of Rkr1 suggests that Rkr1 is not part of a stably associated complex *in vivo*. This observation supports a model in which the proteins that were shown to interact with Rkr1 in the yeast two-hybrid screen are most likely targets of its ubiquitin ligase activity.

6.4.1 Are any of the proteins that interact with Rkr1 targets of its ubiquitin ligase activity?

Many experiments can be performed to determine if the proteins that interact with the amino terminus of Rkr1 are ubiquitylation substrates. Cycloheximide chase experiments can be used to determine if these proteins are degraded at faster rates in wild type strains compared to *rkr1Δ* strains. Preliminary experiments using Gzf3 and Rsc8 suggest that Rkr1 is not important for the degradation of these proteins in rich medium. However, Rkr1 may be important for the degradation of these proteins only when cells are stressed. Alternatively, Rkr1 may be important for the degradation of only mutant versions of these proteins, which comprise a small fraction of

the total protein in the cell. Cycloheximide chase experiments using strains that are grown in minimal medium or strains that are expressing mutant versions of these proteins may clarify Rkr1's function *in vivo*.

6.4.2 Physical connections between Rkr1 and the proteasome support a role for Rkr1 in ubiquitylation *in vivo*.

Rkr1 physically interacts with Dsk2 (Chapter 4), a protein that contacts polyubiquitylated proteins and targets them to the proteasome for destruction (HARTMANN-PETERSEN and GORDON 2004). Further characterization is necessary to determine if a functional Rkr1 RING domain is required for this interaction. This interaction supports a functional role for Rkr1 in protein ubiquitylation *in vivo*.

Rkr1 has been shown to physically interact with the 19S proteasome in the absence of ATP (VERMA *et al.* 2000). This interaction also supports a role for Rkr1 in protein ubiquitylation *in vivo*. However, this interaction has not been confirmed in our lab. Coimmunoprecipitation experiments could confirm the stability of this interaction. The 19S proteasome has been shown to associate with actively transcribed genes, and to be required for connecting histone H2B ubiquitylation with histone H3 K4 and K79 methylation. Chromatin immunoprecipitation experiments could be used to investigate whether Rkr1 is required for the association with the 19S proteasome with chromatin. However, this model is improbable because histone H3 K4 and K79 methylation occur at wild type levels in *rkr1Δ* strains (Chapter 2), although Rkr1 could recruit the proteasome to chromatin by mediating the ubiquitylation of other chromatin-associated proteins.

6.5 RKR1 IS IMPORTANT FOR THE PROPER EXPRESSION OF A SUBSET OF GENES IN YEAST

Microarray analysis (see Chapter 5) shows that Rkr1 is required for the proper expression of a subset of genes in yeast. Specifically, 50 genes are up-regulated more than 1.75 fold and 25 genes are down-regulated more than 1.75 fold in a *rkr1Δ* strain. These genes fall into many functional classes, and are found throughout the genome, suggesting that Rkr1 does not have strong effects on transcription in vivo.

6.5.1 How does Rkr1 affect gene expression in yeast?

This study provides the basis for many future experiments that can clarify Rkr1's role in transcription. At this point, I can envision two models for why some genes are mis-regulated in *rkr1Δ* strains. The first model is that Rkr1 is required for proper chromatin architecture in yeast. Defects in gene expression may be detected if Rkr1 is important for establishing or maintaining proper chromatin structure within promoters. Defects in this process could lead to access or occlusion of transcription factor binding sites that are important for the proper expression of downstream genes. This model is supported by evidence that Rkr1 is important for telomeric silencing, a process that is influenced by genome wide defects in either chromatin modification or chromatin structure. However, preliminary MNase digestion experiments suggest that strains lacking *RKR1* do not have detectable changes in global chromatin structure compared to chromatin from a wild type strain (K. Klucevsek, M. Braun, and K. Arndt, unpublished obervations). This suggests that Rkr1 may not affect gene expression through global chromatin structure. However, Rkr1 physically interacts with Rsc8, a component of the RSC chromatin

remodeling complex, and genetically interacts with another chromatin remodeler, Chd1. These interactions suggest that Rkr1 may affect gene expression by influencing the activity of a chromatin remodeling complex. Support for this model also comes from the genetic interaction with *SPT10*. Strains lacking *SPT10* have reduced histone levels, and also have global changes in chromatin structure (DOLLARD *et al.* 1994; HESS *et al.* 2004; NATSOULIS *et al.* 1994). This genetic interaction may suggest that Rkr1 is important for regulating the insertion of histones into chromatin, which would have a downstream effect on gene expression. Investigating Rkr1's role in chromatin structure by repeating the global MNase assays could help us to understand if Rkr1 plays a role in chromatin structure. We could also direct these studies to look at chromatin architecture at specific genes by combining MNase digestions and Southern analysis.

The second model for Rkr1's role in gene expression proposes that Rkr1 is important for recruitment and/or activity of the transcription factors that regulate the expression of the genes that were identified by microarray analysis. This model is supported by overlap in the yeast two-hybrid and microarray datasets. Gzf3 and Dal82, which physically interact with Rkr1 in a yeast two-hybrid screen, regulate the expression of nitrogen responsive genes in yeast (ELBERRY *et al.* 1993; SOUSSI-BOUDEKOU *et al.* 1997). Most of the genes regulated by these proteins were downregulated in strains lacking *RKR1* (Chapter 5). ChIP experiments could be used to determine if these proteins are properly recruited to their target genes in strains lacking *RKR1*. At this point, I cannot favor either model, simply due to a lack of information. These models need not be mutually exclusive either. Rkr1 may directly affect the recruitment of transcription factors by influencing chromatin remodeling activities at gene promoters.

6.5.2 Is Rkr1 directly involved in regulating any of the genes identified by microarray?

I have tried to determine if Rkr1 localizes to activated genes, including *INO1* and *GAL1/10* by ChIP analysis. These genes can be induced by the removal of inositol from the medium or addition of galactose to the medium, respectively. I was unable to detect Rkr1 localization at these genes, either at the promoter or within the open reading frame (data not shown). These results suggest that Rkr1 may not be associated with all genes in yeast, or that Rkr1 may be present at very low levels on chromatin. Work from the laboratory of Dr. Michael Grunstein show that the association of the histone deacetylase Rpd3 with chromatin was only detected after crosslinking proteins to proteins with dimethyl adipimidate (DMA) and then crosslinking proteins to DNA with formaldehyde (KURDISTANI and GRUNSTEIN 2003). It is definitely possible for a protein to associate with chromatin and not be detected by conventional formaldehyde ChIP analysis. We could perform ChIP experiments using the highly sensitive method described above to determine if Rkr1 associated with chromatin at genes identified by microarray analysis, or use other methods that do not rely on assaying specific loci.

6.6 OVERALL MODEL FOR RKR1 ACTIVITY

In summary, my results indicate the existence of a new nuclear protein ubiquitylation pathway that is functionally connected to chromatin and transcription. Based on the findings described above, I hypothesize that the ubiquitin-protein ligase activity of Rkr1 operates in parallel with Rtf1 and several histone modifications to regulate a common, essential process. Rkr1's ubiquitylation activity may be important for establishing a chromatin structure that is needed for highly efficient transcription. Alternatively, Rkr1 may be important for the proper recruitment and/or activity of transcription activators or repressors. Although the best-characterized functions for Rtf1 relate to transcription, my data do not exclude the possibility that the synthetic lethal relationship between Rtf1 and Rkr1 is due to their involvement in other essential processes, which remain to be identified. Future experiments will aim to expose the *in vivo* target(s) of Rkr1's ubiquitin-protein ligase activity and explain how the target(s) relates to chromatin structure or function. Finally, because Rkr1 is a conserved protein, further studies in yeast should illuminate the role of homologous proteins in humans and other eukaryotes.

APPENDIX A

HIGH COPY SUPPRESSORS OF RKR1-RTF1 SYNTHETIC LETHALITY

Proteins expressed at elevated levels can suppress growth defects of null mutant strains if the over-expressed proteins can restore the function of the pathway controlled by the mutant protein. To obtain high-copy number suppressors of *rtf1Δ*/*rkr1Δ* synthetic lethality, MBY186 (*rkr1Δ::kanMX4, rtf1Δ::ARG4, leu2Δ1, ura3-52, trp1Δ63, arg4-12*, [pKA61 = *RTF1/URA3/CEN/ARS*]) was transformed with a high-copy number, *LEU2*-marked library that contained 9-12 kb inserts of genomic DNA fragments. Approximately 13,500 colonies were screened to ensure that ≥99% of the genome would be covered in the library. Transformants were selected on SC-Leu dropout medium. Transformants were replica printed to YPG, SC-Ura-Leu, SC-Ura-Leu+5FOA, and SC-Ura to determine which colonies could lose pKA61 and survive with the high-copy plasmid. One hundred and thirty one 5FOA resistant colonies were patched to SC-Leu and replica printed to confirm resistance. All colonies were 5FOA resistant upon re-printing. *LEU2*-marked plasmids were recovered from transformants using the Smash and Grab method (HOFFMAN and WINSTON 1987) on cultures grown in SC-Leu medium. Plasmids were re-transformed into MBY186 to confirm the suppression phenotype. Forty eight plasmids confirmed 5FOA resistance upon re-transformation. PCR confirmed that 12 of these inserts contained *RKR1* and 9 contained *RTF1*. DNA sequencing showed that there were 6 unique plasmids that conferred 5FOA resistance to MBY186 (Table 19). The number of times each insert was obtained is listed in Table 19.

Table 19. High-Copy-Number suppressors of *rtf1Δ/rkr1Δ* synthetic lethality.

Insert #	# Isolates	Gene name	Gene description
1	7	*LSM4*	mRNA processing factor; if nuclear, part of snRNP; if cytoplasmic, important for mRNA decay
2	3	*MRPL3*	Mitochondrial ribosomal protein (large subunit)
		CSI1	Subunit of Cop9 signalsome; required for deneddylation or removal of ubiquitin-like Rub1 from Cdc53 (cullin); involved in adapting to pheromone signaling
		PEX12	Contains a C3HC4 type RING domain; peroxisomal membrane protein
		YMR027w	Uncharacterized; Increased expression reduces Ty transposition
		TAP42	Tor signaling pathway component; associates physically with Sit4 and PP2A
		FAR8	Pheromone response
3	1	*YNL224c*	Uncharacterized; Physically interacts with Prp43 (RNA helicase in spliceosome)
		CNM67	Spindle pole body component
		YNL226w	Uncharacterized; Physically interacts with Nip7 (nucleolar protein)
		JJJ1	J-domain containing protein; Physically associates with the ribosome
		YNL228w	Uncharacterized; physically interacts with Eds1 (unknown function)
		URE2	Nitrogen catabolite repression regulator; inhibits *GLN3* expression
4	9	*GPD1*	NAD-dependentglycerol-3-phosphate dehydrogenase; required for glycerol biosynthesis
		DIA3	Unknown function; “digs into agar”; involved in invasive and pseudohyphal growth
5	2	*TPO1*	Polyamine transporter; membrane protein
		ISA1	Mitochondrial matrix protein involved in the biogenesis of iron-sulfur clusters used as cofactors of many proteins
		HSP104	Heat shock protein; works to refold misfolded and aggregated proteins; cytoplasmic and nuclear localization
6	7	*AIR1*	Contains a C3HC4 type RING-domain; nuclear protein; Physically interacts with Hmt1 (arginine methyltransferase) to regulate the methylation of Npl3 (mRNA processing and export factor)
		THS1	Threonyl-tRNA synthetase

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