

**Involvement of the RNA polymerase II-associated Paf1 Complex in Transcriptional
Regulation and 3'-End Formation of snoRNAs**

by

Kathryn Elizabeth Sheldon

B. S., San Francisco State University, 1999

Submitted to the Graduate Faculty of
of Arts and Sciences in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2005

UNIVERSITY OF PITTSBURGH

Faculty of Arts and Sciences

This dissertation was presented

by

Kathryn Elizabeth Sheldon

It was defended on

November 18, 2005

and approved by

Jeffrey Brodsky, Associate Professor, Department of Biological Sciences

Deborah Chapman, Associate Professor, Department of Biological Sciences

Paula Grabowski, Professor, Department of Biological Sciences

Martin Schmidt, Associate Professor, Department of Molecular Genetics and Biochemistry

Dissertation Advisor: Karen Arndt, Associate Professor, Department of Biological Sciences

Copyright © by Kathryn Elizabeth Sheldon

2005

INVOLVEMENT OF THE RNA POLYMERASE II-ASSOCIATED PAF1 COMPLEX IN TRANSCRIPTIONAL REGULATION AND 3'-END FORMATION OF snoRNAS

Kathryn Elizabeth Sheldon, PhD

University of Pittsburgh, 2005

Transcription elongation is an important regulatory step in the RNA polymerase II transcription cycle. Previous work from our laboratory and others strongly suggests that the Paf1 complex interacts with and regulates the activity of RNA polymerase II during transcription elongation. Affinity purification of Paf1 revealed that it exists in a complex in vivo with Ctr9, Rtf1, Cdc73 and Leo1 called the Paf1 complex. To further investigate the function of the Paf1 complex in vivo, we used a genetic approach to study the component Rtf1 and microarray analyses to identify Ctr9-regulated genes. Null mutations in *RTF1* confer two phenotypes associated with defective transcription elongation, sensitivity to base analogs and the Spt⁻ phenotype. To identify novel mutations in *RTF1*, we performed a genetic screen for *rtf1* mutations that confer conditional mutant phenotypes. We identified three new *rtf1* missense mutations. To identify proteins that functionally interact with the Paf1 complex, we performed a screen for multicopy suppressors of the *rtf1* mutations. This screen identified *NAB3* as a suppressor of *rtf1*. Nab3 interacts with the RNA polymerase II-associated hnRNP Nrd1. Together, Nrd1 and Nab3 are required for efficient 3'-end formation of certain nonpolyadenylated RNA polymerase II transcripts, including snoRNAs. Using assays to detect transcriptional readthrough of snoRNA genes, we have shown that deletion of certain Paf1 complex members causes strong defects in snoRNA 3'-end formation. By chromatin immunoprecipitation (ChIP) analysis, we have found that the Paf1 complex and Nab3 associate with snoRNA genes in vivo. In the second approach, expression analyses revealed a role for the Paf1 complex in directly regulating the expression of certain genes that are not transcribed during growth in rich media. ChIP experiments indicated that the Paf1 complex associates along the length of *ARG1* and with the promoter region of *SER3*. Together, our results reinforce the idea that the Paf1 complex is involved in the production of mRNAs and provide evidence that

the Paf1 complex also participates in snoRNA transcription and 3'-end formation during RNA polymerase II transcription.

TABLE OF CONTENTS

TABLE OF CONTENTS	VI
LIST OF TABLES	XIII
LIST OF FIGURES	XIV
ACKNOWLEDGEMENTS	XVI
1.0 CHAPTER 1: INTRODUCTION.....	1
1.1 THE CHROMATIN NATURE OF THE DNA TEMPLATE IN	
EUKARYOTES	1
1.1.1 Organization of DNA within the nucleus.....	1
1.1.2 Histone tail modifications.....	4
1.1.3 Histone variants	9
1.1.4 Chromatin remodeling factors.....	13
1.2 INITIATION OF TRANSCRIPTION BY RNA POLYMERASE II	14
1.2.1 Elements of the RNA polymerase II promoter.....	14
1.2.2 The RNA polymerase II machine	18
1.2.3 Regulation of transcription by phosphorylation of the carboxyl terminal	
domain (CTD) of the largest subunit of RNA polymerase II.....	22
1.2.4 Basal transcription: Assembling the preinitiation complex.....	25
1.2.5 Gene-specific transcriptional activators	28
1.2.5.1 Transcriptional activators.....	30
1.2.5.2 Transcriptional coactivators.....	32
1.2.5.3 General repressors of transcription	34
1.2.5.4 Gene specific transcriptional repressors	35
1.2.5.5 Transcriptional corepressors.....	37

1.3	MODULATION OF RNA POLYMERASE II TRANSCRIPT ELONGATION.....	39
1.3.1	Phosphorylation of RNA polymerase II during elongation	39
1.3.2	RNA polymerase II-associated factors.....	41
1.3.2.1	The co-transcriptional nature of RNA maturation	42
1.3.3	RNA polymerase II-associated factors involved in chromatin structure and function	45
1.3.3.1	Yeast FACT.....	46
1.3.3.2	Spt6.....	47
1.3.3.3	Spt4-Spt5	48
1.3.3.4	Elongator	49
1.3.3.5	The Paf1 Complex.....	51
1.3.4	Chromatin remodeling factors involved in transcription	54
1.3.4.1	The SWI/SNF chromatin remodeling complex.....	54
1.3.4.2	The RSC chromatin remodeling complex	56
1.3.4.3	The conserved "imitation switch" chromatin remodeling factors.	57
1.3.4.4	The Chd1 chromatin remodeling factor regulates transcription...	59
1.3.4.5	The INO80 chromatin remodeling complex.....	60
1.3.4.6	Swr1 is a Snf2 paralog that catalyzes the replacement of histone H2A with H2A.Z	61
1.3.5	Chromatin modifying proteins	62
1.3.5.1	Set1 and Set2 methylate histone H3 K4 and K36 respectively	63
1.3.5.2	Rad6-Bre1 ubiquitylate histone H2B on lysine 123 (K123)	65
1.3.6	Factors required for RNA polymerase II elongation processivity	66
1.4	REGULATION OF 3'-END PROCESSING AND TRANSCRIPTION TERMINATION.....	68
1.4.1	Transcript cleavage factors form the 3'-ends of polyadenylated and nonpolyadenylated transcripts.....	68
1.4.2	Transcript termination of RNA polymerase II	70

1.5	RATIONALE TO INVESTIGATE THE ROLE OF RTF1 AND MEMBERS OF THE PAF1 COMPLEX IN TRANSCRIPTION AND IDENTIFICATION OF NOVEL FUNCTION(S) OF THE PAF1 COMPLEX	71
2.0	CHAPTER 2: IDENTIFICATION OF MULTICOPY SUPPRESSORS OF A CONDITIONAL <i>RTF1</i> MUTATION	73
2.1	INTRODUCTION	73
2.2	METHODS AND MATERIALS	76
2.2.1	Yeast strains and methods.....	76
2.2.2	Plasmids	78
2.2.3	Isolation and characterization of <i>rtf1</i> alleles	78
2.2.3.1	Error-prone PCR.....	78
2.2.3.2	In vivo homologous recombination.	81
2.2.3.3	Screen for conditional <i>rtf1</i> missense mutations.....	81
2.2.3.4	Integration of <i>rtf1</i> point mutations.....	81
2.2.4	Immunoblotting analysis	86
2.2.4.1	Analysis of Rtf1 protein levels.	86
2.2.4.2	Analysis of histone H3 lysine 4 (K4) trimethylation levels.....	86
2.2.5	High copy number suppressor screen.....	86
2.2.6	Analysis of <i>nrd1-5</i> combined with deletions of Paf1 complex members	88
2.2.7	Analysis of strains lacking individual components of the Paf1 complex containing 2-micron <i>NAB3</i>	88
2.3	RESULTS	89
2.3.1	Isolation of mutations conferring conditional <i>rtf1</i> phenotypes.....	89
2.3.2	Characterization of missense mutations in <i>RTF1</i>	90
2.3.3	Genes in high copy number that suppress the phenotypes of <i>rtf1-107</i> ..	98
2.3.3.1	<i>NAB3</i> is a dosage suppressor of <i>rtf1-107</i>	110
2.3.3.2	<i>RTG3</i> partially suppresses the Spt ⁻ phenotype of <i>rtf1-107</i>	114
2.3.4	Additional genetic analyses support a functional link between <i>RTF1</i> and <i>NAB3</i>	117
2.3.4.1	Multicopy <i>NAB3</i> causes synthetic growth phenotypes in <i>ctr9Δ</i> and <i>paf1Δ</i> cells	117

2.3.4.2	<i>nrd1-5</i> cells require members of the Paf1 complex for viability at elevated temperatures.	121
2.4	CONCLUSIONS.....	124
2.4.1	Point mutations in <i>rtf1</i> confer conditional loss-of-function phenotypes	124
2.4.2	<i>NAB3</i> and <i>RTG3</i> were identified in a high-copy-number suppressor screen to identify genes that suppress the phenotypes of conditional <i>rtf1</i> alleles.....	124
3.0	CHAPTER 3: A NOVEL ROLE FOR THE PAF1 COMPLEX IN 3'-END FORMATION OF SNORNA TRANSCRIPTS.....	126
3.1	INTRODUCTION	126
3.2	METHODS AND MATERIALS.....	129
3.2.1	Yeast strains	129
3.2.2	RNA isolation and Northern analysis	132
3.2.2.1	RNA isolation.	132
3.2.2.2	Northern blotting analysis.	132
3.2.3	Plasmids	132
3.2.3.1	Construction of HA epitope-tagged version of Nab3.....	132
3.2.3.2	Subcloning of <i>SNR47</i> termination sequences into pG-CYC.ds. ...	135
3.2.4	In vivo reporter experiments	135
3.2.4.1	ACT1-CUP1 reporter assay for 3'-end formation.	135
3.2.4.2	<i>HIS3</i> reporter for transcriptional readthrough.....	136
3.2.5	Chromatin immunoprecipitation (ChIP) analysis	136
3.2.6	Co-IP and immunoblotting analysis.....	141
3.2.6.1	Analysis of Nrd1 protein levels.....	141
3.2.6.2	Co-immunoprecipitation (Co-IP) analysis.	141
3.2.7	Transcription run on (TRO) assays	142
3.2.7.1	Harvesting and permeabilizing cells.	142
3.2.7.2	Transcript labeling.	142
3.2.7.3	RNA isolation.	143
3.2.7.4	RNaseT1 digestion and denaturing gel electrophoresis.	143

3.2.7.5	5' end labeling of molecular weight standards.....	144
3.3	RESULTS	145
3.3.1	The Paf1 complex is required for 3'-end formation of <i>SNR13</i> transcripts	145
3.3.2	Is there a general requirement for transcription elongation factors in 3'-end formation of <i>snR13</i> ?	152
3.3.3	The Paf1 complex is required for 3'-end formation of <i>SNR47</i> transcripts	159
3.3.4	The Paf1 complex co-localizes with RNA polymerase II to <i>SNR47</i>	166
3.3.5	Nrd1 association with <i>SNR47</i> is dependent on the Paf1 complex.....	172
3.3.6	Is the Paf1 complex directly involved in RNA polymerase II transcript termination?	186
3.4	CONCLUSIONS	193
3.4.1	Novel role for the Paf1 complex in the transcription cycle	193
3.4.2	The Paf1 complex may couple transcription elongation to transcript cleavage	197
4.0	CHAPTER 4: IDENTIFICATION OF GENES REGULATED BY THE PAF1 COMPLEX THROUGH GLOBAL EXPRESSION ANALYSIS OF <i>CTR9</i> AND <i>RTF1</i> DELETION MUTANTS	199
4.1	INTRODUCTION	199
4.2	METHODS AND MATERIALS	201
4.2.1	Yeast strains	201
4.2.1.1	Analysis of <i>IMD2</i> induction	203
4.2.1.2	Northern blotting analysis.	203
4.2.2	Chromatin immunoprecipitation (ChIP) analysis	203
4.2.2.1	Harvesting yeast cultures for microarray analysis.....	206
4.2.3	RNA isolation for microarray analysis	207
4.2.4	Microarray analysis.....	208
4.2.4.1	cDNA preparation	208
4.2.4.2	Cy dye incorporation/probe clean up	209
4.2.4.3	Hybridization	210

4.2.4.4	Microarray scanning and image analysis	210
4.2.5	Statistical analysis	211
4.2.5.1	Dye normalization.....	211
4.2.5.2	Statistical analysis.....	212
4.3	RESULTS	213
4.3.1	The Paf1 complex and SBF may coordinately regulate transcription during the G ₁ /S phase of the cell cycle	213
4.3.2	<i>IMD2</i> transcription induction is impaired in Paf1 complex member mutants	217
4.3.3	Differential expression analysis to identify genes regulated by the Paf1 complex	221
4.4	CONCLUSIONS	236
4.4.1	Combined approaches of genetics and microarray analyses identify genes regulated by the Paf1 complex.....	236
5.0	CHAPTER 5: DISCUSSION	239
5.1	INTRODUCTION	239
5.2	WHAT HAVE WE LEARNED ABOUT RTF1 AND THE PAF1 COMPLEX BY ANALYZING THE <i>RTF1</i> POINT MUTANTS?.....	239
5.2.1	Can the <i>rtf1</i> missense mutations provide insight into Rtf1 activity?....	239
5.2.2	Multicopy suppressors of an <i>rtf1</i> point mutant suggest a role for the Paf1 complex in transcription termination	243
5.3	NOVEL GENETIC INTERACTIONS IMPLICATE THE PAF1 COMPLEX IN 3'-END FORMATION	245
5.3.1	Enhancement of <i>nrd1</i> phenotypes in Paf1 complex member deletion mutants	245
5.3.2	Overexpression of <i>NAB3</i> causes sickness in cells deleted for certain Paf1 complex components	246
5.4	IN VIVO AND IN VITRO ANALYSES SUPPORT THE ROLE OF THE PAF1 COMPLEX IN PROPER 3'-END FORMATION.....	248
5.4.1	<i>SNR13</i> transcripts are extended in Paf1 complex mutants.....	248

5.4.2	<i>SNR47</i> transcripts readthrough into downstream <i>YDR042c</i> in cells lacking individual Paf1 complex members	252
5.4.3	TRO analysis did not definitively determine whether the Paf1 complex is involved in RNA polymerase II transcript termination	260
5.5	WHAT GENES ARE REGULATED BY THE PAF1 COMPLEX?	264
5.5.1	Members of the Paf1 complex genetically interact with genes that encode certain transcriptional activators	264
5.5.1.1	Does the Paf1 complex regulate genes in the SBF pathway?	264
5.5.1.2	The Paf1 complex may regulate genes involved in the retrograde signaling pathway	265
5.5.2	<i>IMD2</i> requires the Paf1 complex for proper transcriptional induction in the presence of 6AU	267
5.5.3	Microarray analysis identifies genes regulated by Ctr9.....	268
5.6	WHAT ASPECTS OF THE PAF1 COMPLEX HAVE BEEN ELUCIDATED?	270
6.0	CHAPTER 6: FUTURE DIRECTIONS	271
6.1	INTRODUCTION	271
6.1.1	<i>rtf1</i> point mutations as tools for investigating the function of the Paf1 complex	272
6.1.2	Further characterization of multicopy suppressors of <i>rtf1-107</i> cells ...	273
6.1.3	How is the Paf1 complex loaded onto genes?	274
6.1.4	What other factors require the Paf1 complex for association with <i>SNR47</i> ?	275
6.1.5	Determine if deletion of individual Paf1 complex members result in transcription termination defects	276
6.1.6	Investigate interactions between the Paf1 complex and RNA metabolism	277
6.1.6.1	Is the Paf1 complex linked with exosome function?	278
6.2	SUMMARY	280
	BIBLIOGRAPHY	281

LIST OF TABLES

Table 1: Proteins and protein complexes important for transcriptional regulation with links to the Paf1 complex	29
Table 2: Yeast strains used in Chapter 2.....	77
Table 3: Oligonucleotide sequences used in Chapter 2	80
Table 4: Original <i>RTF1</i> mutants and plasmids	84
Table 5: Isolated <i>RTF1</i> point mutants.....	85
Table 6: Multicopy library plasmids that may confer suppression of <i>rtf1-107</i> through non-specific mechanisms	101
Table 7: Library plasmids capable of <i>rtf1-107</i> suppression that do not contain "obvious candidate genes" within their genomic inserts	103
Table 8: High-copy-number library plasmids that cause suppression of <i>rtf1-107</i> and contain genes within the genomic insert with connections to gene expression.....	106
Table 9: Phenotypic analysis of increased <i>NAB3</i> dosage in cells deleted for individual Paf1 complex members	119
Table 10: Yeast strains used in Chapter 3.....	130
Table 11: Oligonucleotide sequences used for plasmid and probe construction	134
Table 12: Oligonucleotide sequences used for ChIP in Chapter 3	138
Table 13: Yeast strains used in Chapter 4.....	202
Table 14: Oligonucleotide sequences used for ChIP in Chapter 4	205
Table 15: 2.5% of 6200 genes that require Ctr9 for their transcription.....	223
Table 16: 2.5% of 6200 genes that require Ctr9 for their repression.....	227

LIST OF FIGURES

Figure 1: Post-translational histone modifications	7
Figure 2: Histone H3 and Histone H2A variants	11
Figure 3: Idealized eukaryotic RNA polymerase II promoter	16
Figure 4: Two-dimensional representation of RNA polymerase II	20
Figure 5: Phosphorylation of the CTD of RNA polymerase II correlates with stages in the transcription cycle.....	23
Figure 6: Conditional <i>RTF1</i> alleles exhibit sensitivity to 6AU and the Spt ⁻ phenotype.....	91
Figure 7: Comparison of Rtf1 sequence to similar proteins in other eukaryotes.....	93
Figure 8: Rtf1 is expressed from the <i>rtf1</i> alleles at both the permissive and restrictive temperatures and histone H3 lysine 4 trimethylation is detected in the <i>rtf1</i> point mutants.....	96
Figure 9: High-copy-number <i>NAB3</i> suppresses the Spt ⁻ phenotype of conditional mutations in <i>RTF1</i>	112
Figure 10: Increased dosage of <i>RTG3</i> partially suppresses the Spt ⁻ phenotype of the <i>rtf1</i> temperature sensitive mutants.....	115
Figure 11: <i>nrd1-5</i> combined with deletions of individual Paf1 complex members confers synthetic phenotypes.....	122
Figure 12: Primer pair hybridization location relative to <i>SNR47</i> and <i>YDR042c</i>	139
Figure 13: Mutation of individual components of the Paf1 complex results in defective 3'-end formation of <i>SNR13</i> messages	146
Figure 14: Deletion of Paf1 complex members confers resistance to Cu ²⁺	150
Figure 15: Not all RNA polymerase II-associated elongation factors are important for 3'-end formation of <i>SNR13</i> transcripts.....	153

Figure 16: Several mutations in chromatin remodeling, histone modifying, and transcriptional activators do not exhibit transcriptional readthrough at the <i>SNR13</i> locus	157
Figure 17: Transcriptional readthrough from <i>SNR47</i> into <i>YDR042c</i> results in strains lacking constituents of the Paf1 complex	161
Figure 18: Strains deleted for members of the Paf1 complex exhibits transcriptional readthrough in a reporter assay	164
Figure 19: Epitope tagged Paf1 is enriched over <i>SNR13</i>	167
Figure 20: RNA polymerase II colocalizes with HA ₃ -Paf1 at the <i>SNR47</i> locus	170
Figure 21: Association of Nrd1 with <i>SNR47</i> DNA is dependent on a functional Paf1 complex	174
Figure 22: Association of Nab3 with <i>SNR47</i> DNA is dependent on a functional Paf1 complex	176
Figure 23: Nrd1 and Nab3 levels are not reduced in <i>paf1</i> Δ mutant cells	178
Figure 24: Nrd1 does not physically interact with members of the Paf1 complex.....	181
Figure 25: Nrd1 association with <i>SNR47</i> requires the function of the CTD kinase Ctk1	184
Figure 26: TRO analysis using <i>SNR13</i> and <i>SNR47</i> transcription termination sequences.....	189
Figure 27: Model for the role of the Paf1 complex in 3'-end formation of <i>SNR</i> transcripts.....	195
Figure 28: <i>RTF1</i> interacts genetically with <i>SWI4</i> and <i>SWI6</i> but not <i>MBP1</i>	215
Figure 29: <i>rtf1</i> Δ and <i>paf1</i> Δ cells are defective in <i>IMD2</i> induction.....	219
Figure 30: The Paf1 complex directly regulates the transcription of <i>ARG1</i>	230
Figure 31: The Paf1 complex directly regulates the transcription of <i>SER3</i>	234

ACKNOWLEDGEMENTS

First, I must acknowledge my advisor, Dr. Karen Arndt. I want to thank her for welcoming me into her lab. I also want to thank her for all of the opportunities she gave me. These include speaking at scientific meetings, sending me to UCSC to learn how to perform microarrays, and fostering my curiosity. She is also a great role model because she exhibits several qualities of exceptional teachers, scientists, and mentors.

Next, I must acknowledge past and present members of the Arndt lab. The former members of the Arndt lab that I must thank for paving the way for me with Rtf1 include Patrick Costa, David Mauger, and Kelli Roinick. Each contributed significantly to understanding more about Rtf1. I need to thank Peg Shirra, our post-doc in the lab, for tons of advice. I would also like to acknowledge their camaraderie in the lab. As for present members of the lab, I owe a lot of gratitude to the current graduate students, which include Maggie Braun, Marcie Warywoda, and Elia Crisucci. Not only are they a great source of information, they are a lot of fun to work with too. When experiments and science are looking gloomy, they are right there to make sure you see the positive side of the situation. The Arndt lab technician, Rajna Simic must also be acknowledged. She is very knowledgeable and fun to work with. I was very lucky to be a part of this lab.

I must also acknowledge our collaborators for their suggestions, advice and reagents. Dr. David Brow and Dr. Eric Steinmetz kindly provided Nrd1 antisera, *NRD1* plasmids, the nrd1-5 and isogenic wild-type strains, the ACT1-CUP1 reporter construct, and TRO plasmids. They also shared unpublished data and provided suggestions for experiments. Dr. Jeffry Corden provided plasmids for the *HIS3* reporter assay, shared unpublished information and advice. Dr. Judith Jaehning communicated unpublished results and advice. I must also thank Dr. Grant Hartzog and the members of his lab, in particular Todd Burckin. They taught me how to perform microarray

experiments. Dr. Jerry Nau provided critical advice on the microarray experiments I performed. Dr. Joseph Martens provided yeast strains, oligonucleotide information, and unpublished data regarding *SER3*. I would also like to take this opportunity to thank the labs of second floor Crawford Hall. The labs not only share their equipment and reagents but also are a source of information.

I must acknowledge the members of my thesis committee for their enthusiasm, support, and suggestions. They helped me to focus the scope of my thesis research, provided critical comments regarding my dissertation, and offered a wealth of advice.

I want to acknowledge the Department of Biological Sciences at the University of Pittsburgh. I need to thank the main office in the department. With special thanks to Cathy Barr, Natalie Marinzal, and Kathy Hoffman. I also want to thank the members of the fiscal office including Patty Henry, Lynn Mehaffey, and Pat Dean. I also need to acknowledge Patti Wells in the DNA sequencing facility. The members of the Teaching Club and my Teaching Minor Committee, Lydia Daniels and Valerie Oke, for many conversations about students and teaching.

I want to thank former members of the Department of Biological Sciences at the University of Pittsburgh. This list includes Bonnie Reinhart, Carina Endres Howell, Feng Ding, and Sarayu Ratnam (officially part of the Graduate School of Public Health). They were part of the Chaillet lab. I must also thank Annette Ahner and Li Liu, my former classmates. I also thank Stephanie Winter since I shared many fun and interesting teaching experiences with her! Thanks a lot everyone for your support!

Finally, I need to acknowledge my family and friends. I need to thank Jack and Carol, Lisa, and John for being a tremendous source of support. I must also thank Patrick for his support and understanding. I need to thank Cherie, Sonny, and Morgan. I would also like to extend my thanks to friends from home; Anne Johnson, Leia Hewitt, and Chris Kerksieck.

1.0 Chapter 1: Introduction

1.1 The chromatin nature of the DNA template in Eukaryotes

1.1.1 Organization of DNA within the nucleus

There are several impediments to transcription in cells but none as prevalent as the chromatin character of the eukaryotic DNA template. Genomic DNA does not exist in isolation within the nucleus, rather the heritable matter exists in complex with proteins to form chromatin. The basic unit of chromatin structure is the nucleosome where 146 bp of DNA is wound roughly two times around an octamer of four conserved histone proteins (Luger and Hansen, 2005). Histone octamers consist of two histone H3, histone H4, histone H2A and histone H2B proteins (Luger and Hansen, 2005). A fifth histone protein, histone H1, is known as the linker histone and associates with DNA in between nucleosomes (Luger and Hansen, 2005). Several studies suggest a twofold symmetry for the histone octamer where two histone H2A-H2B dimers associate and flank a histone H3-H4 tetramer (Luger and Hansen, 2005).

Unlike many non-histone chromosomal proteins, the histone octamer does not bind with sequence specificity to DNA. The nonspecific nature of the association between the components of the nucleosome allows for higher-order organization within the eukaryotic genome. The hierarchy of chromatin structural levels ranges from "beads on a string" or the 10nm fiber to higher levels of condensation that allows for compaction of large eukaryotic genomes (Loden

and van Steensel, 2005). A model for the compaction and condensation of genomic DNA is through the packing, stacking and/or looping of the chromatin fiber (Luger and Hansen, 2005). Histone H1 plays an important role in the higher order compaction of chromatin (Bustin et al., 2005). Importantly, regions of eukaryotic genomes can be differentially compacted; heterochromatin is densely compacted and contains transcriptionally silent genes and euchromatin is characterized by a more loosely compacted structure that is transcriptionally active (Fahrner and Baylin, 2003).

Histones are small, positively charged proteins that are lysine and arginine rich. The basic histone octamer associates with the negatively charged phosphate backbone of the DNA molecule through ionic interactions as indicated by crystallographic studies (Luger and Richmond, 1998; Luger and Richmond, 1998). Due to the proximity of the histone proteins to the template, it is not surprising that they are important for the proper regulation of transcription. The ordered chromatin structure surrounding the promoter region 5' of genes is a potent obstacle to initiation of transcription as well as transcription elongation (Boeger et al., 2005; Hartzog et al., 2002). Micrococcal nuclease and DNase I treated nuclei have provided information regarding the positions of nucleosomes within the promoters of genes (Yaniv and Cereghini, 1986). In one example, cells grown under non-inducing conditions for *PHO* gene regulation demonstrate ordered nucleosomes over the *PHO5* promoter region including the TATA box (Haswell and O'Shea, 1999; Steger et al., 2003). Upon induction of *PHO5*, a dramatic change in nucleosome organization occurs such that the UAS and TATA elements are exposed to transcription factors for initiation (Korber et al., 2004; Steger et al., 2003).

Eukaryotic histones contain a protein motif and important functional domains that are conserved in eukaryotes. The so-called histone fold was identified using X-ray crystallography

first in histones and later in the structures of other transcription factors. For example, several TATA binding protein (TBP)-associated factors or TAFs contain the histone fold motif (Luger and Richmond, 1998; Luger and Richmond, 1998). Certain TAFs heterodimerize through this interaction domain and these interactions are required for constituting the general transcription factor IID (Davidson et al., 2005). The basic structure of the histone fold motif is composed of three alpha helices where alpha helix 1 is linked to alpha helix 2 through loop 1 and helix 2 is connected to alpha helix 3 by loop 2 (Luger and Richmond, 1998; Luger and Richmond, 1998). Other important regions within the histone proteins are their amino terminal and carboxyl terminal domains. These regions of the histones are important sites for post-translational modifications that potentially affect histone-histone interactions within the nucleosome or histone-DNA associations (Peterson and Laniel, 2004). Interestingly, proteins in the prokaryotic domain archaea were discovered with a structure similar to the histone fold domain of eukaryotic histones (Sandman and Reeve, 2000). Unlike eukaryotic histones however, archaeal histone proteins do not possess amino terminal or carboxyl terminal extensions (Sandman and Reeve, 2000). Comparison of the primary amino acid sequences of archaeal histones and the sequences of the four core histone proteins in eukaryotes strongly suggests that archaeal histones are less diverged from the common archaeal-eukaryotic ancestral histone protein (Sandman and Reeve, 2000). Evidence suggests that archaeal histones form a tetrameric complex wrapped by approximately 80 bp of DNA rather than the octameric complex that is observed in eukaryotes (Sandman and Reeve, 2000). Together, analyses to date are consistent with the idea that histone proteins of eukaryotes and archaea bacteria shared a common precursor and one of the functions of this protein may have included DNA organization.

The packaging of DNA has vital roles in transcription, DNA repair, DNA replication and cell division for organisms in all domains of life. Machinery in the cell has evolved to allow access to the genetic material for transcription, DNA repair and replication. The three predominant mechanisms used by eukaryotes to achieve access to the template are histone modification, incorporation of histone variants within nucleosomes, and ATP-dependent chromatin remodeling. In the following sections, these mechanisms will be addressed.

1.1.2 Histone tail modifications

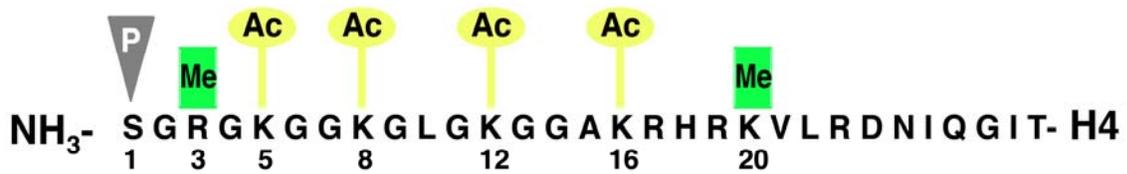
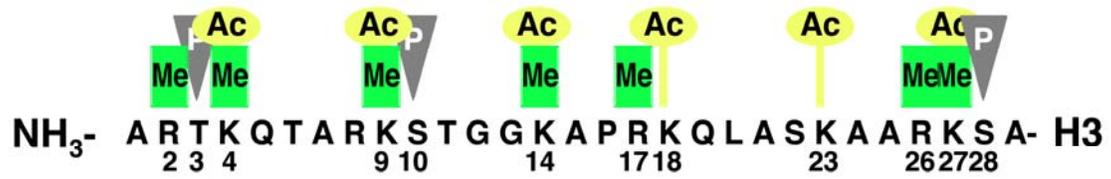
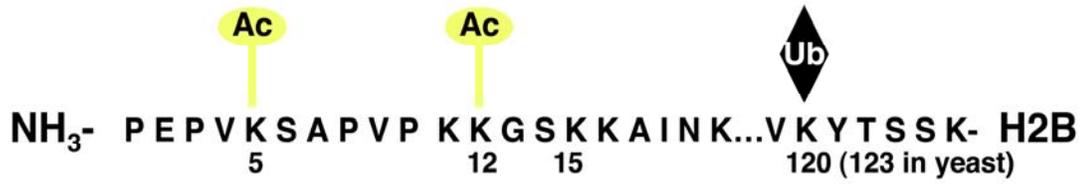
Upon closer inspection of the nucleosome structure, it was evident that the amino-terminal tails of the octamer subunits were solvent-exposed and disordered (Luger and Richmond, 1998; Luger and Richmond, 1998). A number of studies had revealed that the amino terminal tails of the individual histones could be post-translationally modified with a number of moieties including, acetyl, methyl, phosphate and ubiquitin (Wu and Grunstein, 2000). More recent evidence clearly demonstrated that these modifications do occur *in vivo* and that they are physiologically relevant for a number of DNA related processes, including transcription (Wu and Grunstein, 2000). The addition of a phosphate group to histones has implications for transcription and cell division (Nowak and Corces, 2000; Nowak and Corces, 2004). Specifically, histone H3 phosphorylation at serine 10 may be involved in transcriptional activation (Nowak and Corces, 2000). In addition, histone H3 serine 10 phosphorylation correlates with chromatin condensation that is required for proper segregation of chromosomes in mitosis and meiosis in metazoans (Nowak and Corces, 2000; Nowak and Corces, 2004). Acetylation of histones at promoters by histone acetyltransferases (HATs) often correlates with transcriptional activity (Kingston and Narlikar, 1999). In contrast, removal of the acetyl group

from histones by histone deacetylases (HDACs) at the promoter correlates with reduced levels of transcription (Kingston and Narlikar, 1999). HDACs are also involved in the formation of heterochromatin. For example, the *Saccharomyces cerevisiae* (*S. cerevisiae*) HDAC Hda1 is required for silencing chromatin near telomeres (Rundlett et al., 1996). The acetylation state of histones and therefore the transcriptional activity of genes, can be regulated by the activity of HATs and HDACs (Kingston and Narlikar, 1999). In addition, the post-translational addition of methyl groups to histone H3 at lysine 4 (K4) is associated with active or recently transcribed genes in *S. cerevisiae* (Briggs et al., 2001; Zheng et al., 2004). Histone H3 K4 is also methylated in human cells by the human counterpart of the Set1 histone methyltransferase in yeast (Tenney and Shilatifard, 2005). Interestingly, methylation of histone H3 in human cells is incompatible with histone H3 K9 methylation (Nishioka et al., 2002). In metazoans hypermethylated histone H3 K9 is associated with heterochromatin (Stewart et al., 2005). The methylation of histones, unlike the acetyl or phosphate modifications, was generally thought to be a stable modification since no histone demethylases had been identified and analyses suggested that once transcription ceased it took many generations to dilute away the epigenetically marked histones (Ng et al., 2003). However, a protein named LSD1 exhibited the long sought after histone demethylase activity and was found associated within transcriptional repressor complexes that also contained HDACs (Lee et al., 2005; Shi et al., 2005). Interestingly, LSD1 is conserved from *Schizosaccharomyces pombe* to humans but is not present in *S. cerevisiae* (Shi et al., 2004). Histone monoubiquitylation has also emerged as a histone modification associated with transcription, specifically the monoubiquitylation of histone H2B on K123 (Ng et al., 2003; Ng et al., 2002; Wood et al., 2003). Notably, ubiquitylation of histone H2B K123 is required for subsequent histone H3 K4 and H3 K79 methylation (Ng et al., 2002). Nakayama and colleagues

have proposed a histone code that is conserved in eukaryotes and establishes epigenetic inheritance through the sequential modification of histones (Nakayama et al., 2001). The histone code is constantly evolving as novel sites of histone modification are being identified and their functions in DNA metabolism investigated.

Figure 1: Post-translational histone modifications

Known or suspected histone modifications are mapped to the amino terminal domains of the four histones. This diagram was organized based on a similar figure from Jaskelioff and Peterson (2003). Histones H2A, H2B, H3, and H4 and their amino terminal (NH₃) histone tails are indicated. Acetylated, methylated, ubiquitylated and phosphorylated amino acid residues are indicated by yellow lollipops, green rectangles, black diamonds, and gray triangles, respectively.



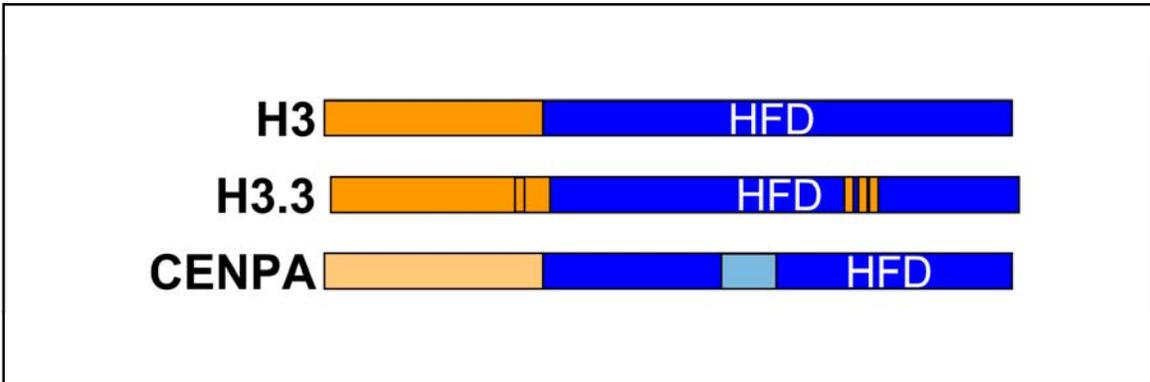
1.1.3 Histone variants

The many post-translational modifications on histones and their roles in the histone code are further complicated by the discovery of variant histone proteins that can replace the canonical histones within nucleosomes in certain regions of the genome (Palmer et al., 1987). Histone H3 variants include H3.3 and CENPA (Fretzin et al., 1991; Palmer et al., 1987). Histone H3 is replaced by H3.3 in *Drosophila* in a replication-independent manner at transcriptionally active genes (Ahmad and Henikoff, 2002). CENPA replaces histone H3 within the nucleosomes of the centromere (Palmer et al., 1987). There are several variants of histone H2A, which include H2AX, H2A.Z, macroH2A, and H2ABBD as reviewed in (Redon et al., 2002; Sarma and Reinberg, 2005). Yeast contains the H2AX and H2A.Z versions of H2A with H2AX as the major constituent of nucleosomes (Downs et al., 2000). The histone H2A variant H2A.Z or Htz1 in *S. cerevisiae* replaces H2A at transcriptionally active genes that border heterochromatin-like areas in the genome (Meneghini et al., 2003; Zhang et al., 2004). Recently, a genome-wide study determined Htz1-containing nucleosomes localize within the promoters of genes transcribed by RNA polymerase II (Zhang et al., 2005). In contrast to earlier analyses, which indicated Htz1 is required to establish heterochromatin-like border near the telomeres (Zhang et al., 2004), Htz1-containing nucleosomes are located throughout the entire genome (Zhang et al., 2005). Zhang and colleagues have demonstrated that Htz1-containing nucleosomes are less stably associated with chromatin than canonical nucleosomes and this property allows for Htz1-containing nucleosomes to be subject to transcription-induced removal or loss, which correlates with increased capacity for transcriptional activity (Zhang et al., 2005). Taken together, chromatin

modification and histone variant substitution contribute to chromatin structure and participate in regulating transcription.

Figure 2: Histone H3 and Histone H2A variants

Histone H3 and Histone H2A and their variants adapted from Sarma and Reinberg (2005). The conserved histone fold domain (HFD) and the amino and terminal tails are diagrammed in this cartoon. Top panel features histone H3 and its variants. The histone H3.3 variant has proposed roles in transcriptional activity while histone variant CENPA functions in kinetochore assembly (Ahmad and Henikoff, 2002; Van Hooser et al., 2001). H3.3 differs from the canonical H3 with the yellow highlighted residues, one in the amino terminal tail and three in the HFD. The amino terminal region of CENPA differs from the amino terminal tail of H3 and other core histone proteins and is diagrammed by a lighter color of orange. The light blue rectangle in the CENPA HFD directs CENPA to centromeric DNA. Bottom panel exhibits the histone H2A variants that differ greatly from H2A within their carboxyl terminal domains. H2AX participates in DNA repair and recombination, H2A.Z plays a role in gene expression and chromosome segregation, macroH2A takes part in X chromosome inactivation, and H2ABBD may be involved in transcriptional activation as reviewed in (Redon et al., 2002; Sarma and Reinberg, 2005). The H2AX variant of H2A is the predominant form of H2A in *S. cerevisiae* while the minor form is H2A.Z (Downs et al., 2000). MacroH2A contains a carboxyl terminal extension as compared to canonical H2A. H2ABBD contains an amino terminal tail that is distinctly different from H2A and lacks residues that are known sites of modification in H2A (Figure 1).



1.1.4 Chromatin remodeling factors

An evolutionarily conserved method to reveal transcriptional regulatory sequences within the chromatinized template is the utilization of ATP-dependent chromatin remodeling factors. As the name of this category implies, these factors use the energy of ATP hydrolysis to physically move or reposition nucleosomes to expose the promoter region to the general transcription machinery (Kingston and Narlikar, 1999; Lusser and Kadonaga, 2003; Studitsky et al., 2004). Nucleosome positioning has been well studied for the *PHO5* gene of *S. cerevisiae* (Haswell and O'Shea, 1999) and genome-wide approaches are being used to characterize the positions of nucleosomes in yeast (Yuan et al., 2005). These studies could provide insight into which chromatin remodeling complexes are important for the regulation of certain genes under different conditions. What will make these analyses difficult is the observed redundancy across these different remodeling complexes since not all chromatin remodeling complexes are essential for viability (Tsukiyama et al., 1999). Chromatin remodeling complexes relevant to transcription in yeast will be discussed in subsequent sections.

1.2 Initiation of transcription by RNA polymerase II

1.2.1 Elements of the RNA polymerase II promoter

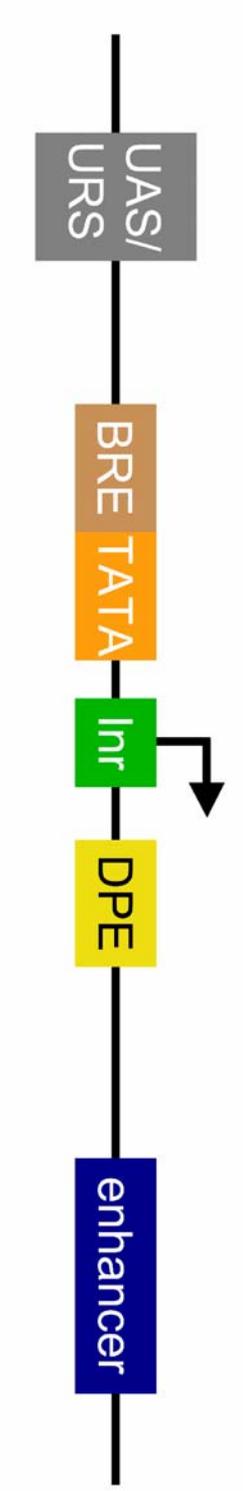
DNA sequence elements within promoters instruct the general transcription factors and RNA polymerase II where to initiate transcription. The transcript is initiated just 20 to 30 bp downstream of the TATA box in higher eukaryotes and viral protein coding genes while the TATA box lies 40 to 120 bp upstream of the initiation site in *S. cerevisiae* as reviewed in (Smale and Kadonaga, 2003; Struhl, 1989). This TATA box was named after its consensus sequence, which in yeast is TATA(A/T)A(A/T)N, where N is any nucleotide (Bucher, 1990). The TATA element is important since it is responsible for directing the polarity of transcription (Kim et al., 1993; Kim et al., 1993; O'Shea-Greenfield and Smale, 1992; Xu et al., 1991). The initiator (Inr) element of metazoans is another conserved sequence involved in transcription start site selection (Corden et al., 1980; Javahery et al., 1994; Smale and Baltimore, 1989). In fact, transcription initiates within the Inr element (Smale et al., 1990). Together, these core promoter elements direct the polarity of transcription (Emami et al., 1997; Smale et al., 1990). Another promoter element involved in basal transcription is the transcription factor IIB recognition element (BRE) (Lagrange et al., 1998). The BRE lies upstream of the TATA box at some eukaryotic promoters as reviewed in (Smale and Kadonaga, 2003). A core promoter element identified downstream of the transcription start site is the downstream promoter element (DPE) of higher eukaryotes (Kutach and Kadonaga, 2000). This element usually exists within TATA-less promoters and

functions together with the Inr to recruit transcription factors (Kutach and Kadonaga, 2000). Notably, not all RNA polymerase II transcribed genes will contain all four components of the core promoter mentioned here, rather these genes will contain different combinations of these elements as reviewed in (Smale and Kadonaga, 2003).

In addition to the core promoter other specific DNA sequences are required for regulating transcription as reviewed in (Smale and Kadonaga, 2003). Upstream activating sequences (UAS) and upstream repressor sequences (URS) often reside upstream of the core promoter elements. These proximal regulatory sequences are recognized by specific factors that activate or repress transcription of the adjacent gene. Furthermore, there are transcription enhancer elements that can be located kilobases upstream or downstream of the relevant gene. Interaction between either the UAS/URS and/or enhancer elements with core promoter elements is essential for the control of transcription. This interaction is mediated by the RNA polymerase II general transcription machinery, gene-specific regulatory proteins and accessory factors.

Figure 3: Idealized eukaryotic RNA polymerase II promoter

An idealized version of an RNA polymerase II promoter adapted from Smale and Kadonaga (2003). The elements required for transcription are depicted relative to the transcription start site (arrow) and are not drawn to scale. The core promoter elements, BRE, TATA box, Inr, and DPE sequence are indicated by rectangles. Surrounding the core promoter elements are sequences required for transcriptional regulation including the UAS/URS upstream of the core promoter and an enhancer element depicted downstream of the core promoter.



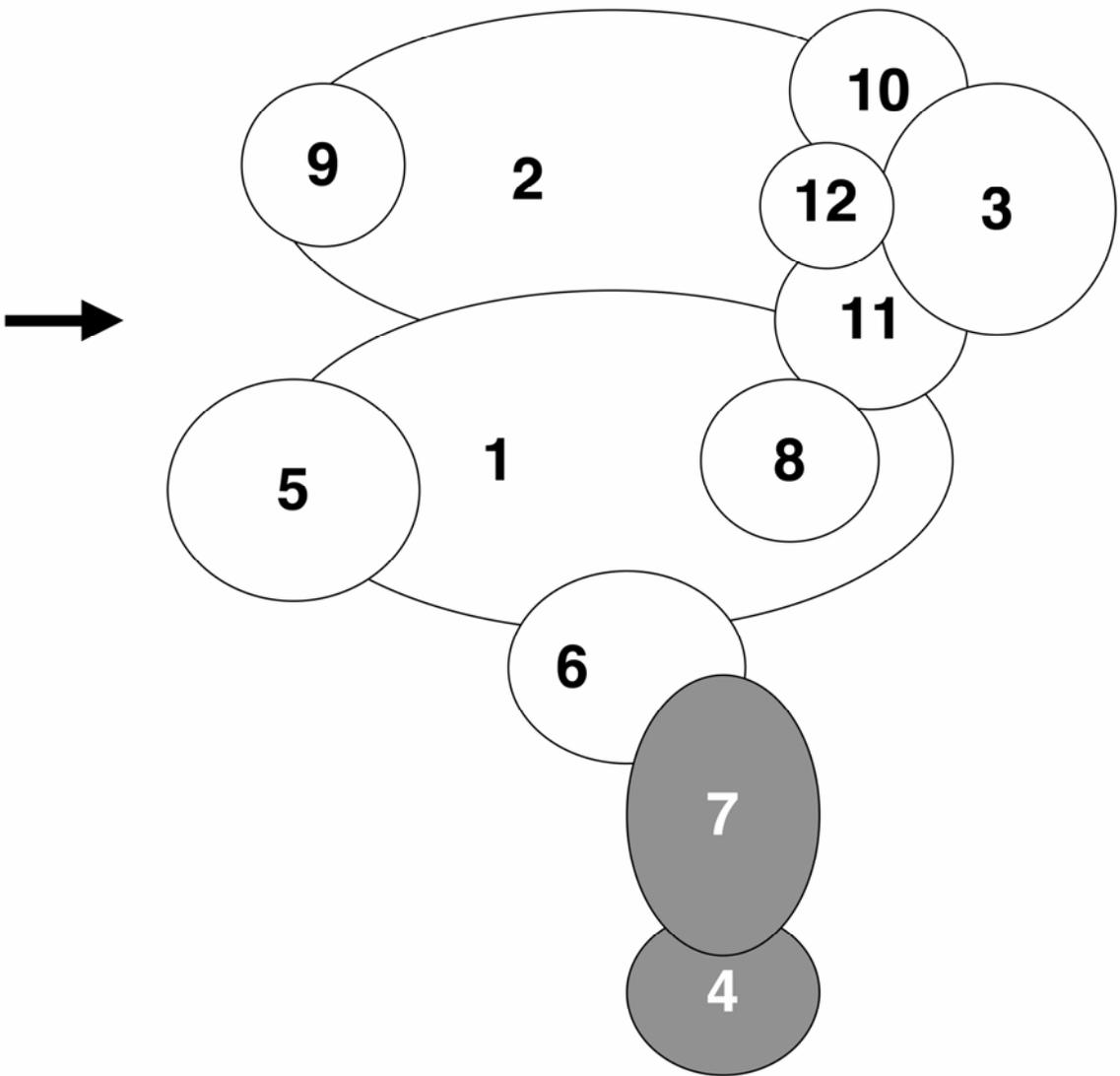
1.2.2 The RNA polymerase II machine

RNA polymerase II is intricately assembled and is subject to many levels of regulation important for proper transcription of protein-coding genes and to small nuclear and small nucleolar transcripts. The twelve subunits of RNA polymerase II comprise an enzyme complex of about 0.5MDa in yeast as reviewed in (Cramer, 2004). All subunits are absolutely essential for the transcription of almost all genes as reviewed in (Cramer, 2004). Together they form a large, multifunctional enzyme responsible for reading the DNA template and incorporating the appropriate incoming nucleotide into the growing RNA chain, backtracking along the template where necessary to allow for transcript cleavage using the intrinsic nuclease activity of the enzyme, to create proper RNA molecules (Zhang et al., 2003). Interestingly, RNA polymerase II shares three subunits, Rpb5, Rpb6 and Rpb8, with RNA polymerases I and III (Woychik et al., 1990). X-ray crystallographic studies indicate that Rpb5, Rpb6 and Rpb8 associate with the largest subunit of RNA polymerase II as shown in Figure 4. Together, Rbp1 and Rbp2 form the bulk of the enzyme. A carboxyl terminal extension on Rpb1, the largest subunit of RNA polymerase II, consists of heptapeptide repeats that are subject to differential phosphorylation by specific protein kinases during initiation and elongation phases of transcription (Komarnitsky et al., 2000). The post-translational phosphorylation modification and its significance will be discussed in further detail in subsequent sections. The second largest subunit of RNA polymerase II has been implicated in transcriptional efficiency and processivity during transcriptional elongation (Mason and Struhl, 2005; Shaw and Reines, 2000). In addition, in yeast the Rpb4-

Rpb7 submodule dissociates from the RNA polymerase II assembly (Tan et al., 2003). Rpb7 has been proposed to participate in transcript termination due to association with a transcription termination factor (Mitsuzawa et al., 2003). Interaction and communication within the constituents of RNA polymerase II is important for the function of the machine in transcription. Furthermore, the surfaces, pores and crevices of RNA polymerase II allow for interaction of RNA polymerase II with incoming nucleotides, the DNA template that exists in close association with nucleosomes, and transcription regulatory factors (Kettenberger et al., 2004).

Figure 4: Two-dimensional representation of RNA polymerase II

Cartoon of RNA polymerase II adapted from Cramer (2004). This diagram represents a view from the top of the enzyme and serves to provide a map for the physical associations among and between the twelve subunits of RNA polymerase II. The individual subunits are designated Rpb1-12. The arrow indicates upstream DNA entering the RNA polymerase II active site. The Rpb4-Rbp7 submodule that dissociates from RNA polymerase II under certain conditions is shaded gray.

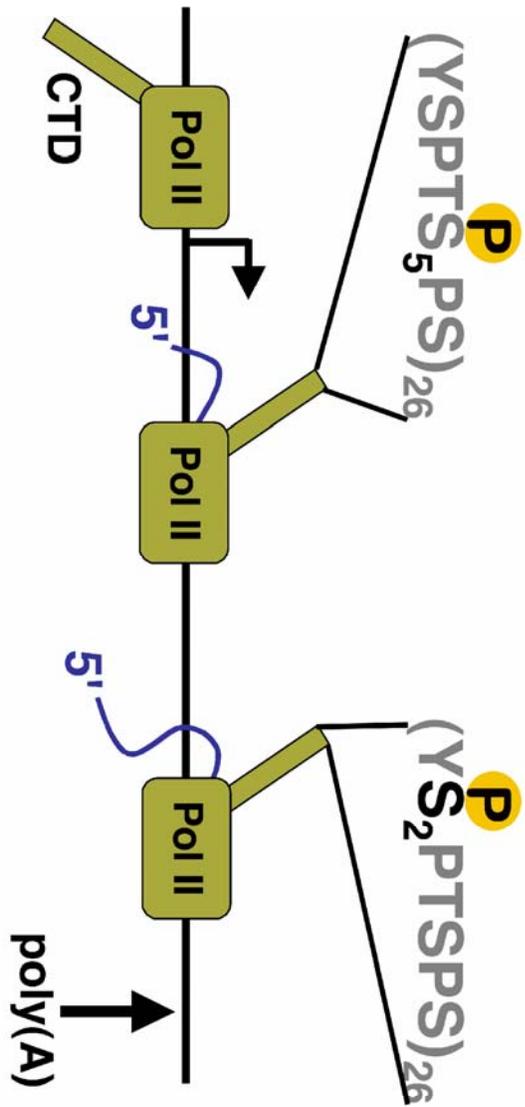


1.2.3 Regulation of transcription by phosphorylation of the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II

RNA polymerase II is unique among RNA polymerases involved in eukaryotic transcription. Specifically, the largest subunit of RNA polymerase II contains a consensus heptapeptide repeat sequence of YSPTSPS at the C-terminal domain (CTD) that becomes differentially phosphorylated during the transcription cycle (Cho et al., 2001; Komarnitsky et al., 2000). The number of repeats varies from organism to organism with 26 repeats in *Saccharomyces cerevisiae* and 52 repeats in humans (Palancade and Bensaude, 2003). Moreover, the nature of the CTD repeat sequence is degenerate with respect to the number of consensus versus nonconsensus repeats in the CTD across eukaryotes (Palancade and Bensaude, 2003). Studies on CTD phosphorylation appear to agree that Ser-5 of the heptad repeat is phosphorylated by a cyclin-dependent kinase associated with the general transcription machinery at the 5' region of the gene during initiation (Cho et al., 2001; Komarnitsky et al., 2000). Further, as RNA polymerase II travels toward the 3' end of the gene Ser-2 becomes preferentially phosphorylated (Cho et al., 2001; Komarnitsky et al., 2000). The correlation between CTD phosphorylation state and the phase of the transcription cycle suggests these modifications may regulate different stages of transcription (Palancade and Bensaude, 2003).

Figure 5: Phosphorylation of the CTD of RNA polymerase II correlates with stages in the transcription cycle

The phosphorylation state of RNA polymerase II changes depending on the phase of the transcription cycle. The bent arrow indicates the site of transcription initiation. RNA polymerase II is depicted by a yellow rectangle with a yellow CTD. The RNA transcript is shown in navy. The heptad repeat is indicated with the amino acid residues in gray. Serine -2 or -5 are predominantly phosphorylated when depicted in black with a phosphate group, depicted by an orange circle, attached. Initiation of transcription begins when hypophosphorylated RNA polymerase II is recruited to the promoter. The transition between initiation and elongation is coincident with phosphorylation of Ser-5 within the CTD. As elongation proceeds phosphorylation of Ser-5 becomes less prevalent while phosphorylation of Ser-2 becomes increasingly predominant.



1.2.4 Basal transcription: Assembling the preinitiation complex

The basal components of RNA polymerase II transcription include several factors conserved in eukaryotes. Prior to entering the transcription cycle, RNA polymerase II must be recruited to the promoter of the gene. RNA polymerase II molecules that are hypophosphorylated on their CTD are recruited to the transcription preinitiation complex (PIC) that is assembled at gene promoters (Bensaude et al., 1999; Lin et al., 2002; Lin et al., 2003). Components of the transcription machinery associate with the template in order to establish a "launch pad" for the RNA polymerase II holoenzyme as reviewed in (Hampsey, 1998). The founding member of the PIC, is TBP (Buratowski et al., 1989). TBP is a small protein that binds within the minor groove of DNA with high sequence specificity for the TATA element causing an 80° bend within the minimal promoter element just upstream of the gene to be transcribed (Lee et al., 1991; Wu et al., 2001). A unique feature of TBP is that it is essential for transcription by RNA polymerases I and III in addition to its role in RNA polymerase II transcription (Kim and Roeder, 1994). A protein with homology to TBP referred to as TBP-related factor (TRF) identified in metazoans is expressed in certain cells or tissue types and may be important for maintaining cell identity by controlling gene expression (Hansen et al., 1997). Moreover, TRF can replace TBP in basal transcription assays in vitro and TRF can replace TBP for activated transcription in vivo (Hansen et al., 1997). Archaeal systems also contain a TBP homolog that together with archaeal homologs of RNA polymerase II and transcription factor IIB (TFIIB) are required for transcription (Bell and Jackson, 2001; Bell et al., 2001). As observed for the conservation of

histone proteins in archaea and eukaryotes, the sequence similarity of TBP homologs and their conserved roles in transcription emphasize their importance in the cell.

The general transcription factors assemble around TBP at the promoter to form the PIC to recruit RNA polymerase (Qiu et al., 2004). Conserved TBP-associated factors (TAFs) in complex with TBP form the general transcription factor TFIID (Chen and Hampsey, 2002). TAFs are also associated with the coactivator complex SAGA (Spt-Ada-Gcn5 acetyltransferase) and participate in regulating transcription initiation (Lee et al., 2000). The transcription factor TFIIA associates with TBP bound at the TATA box (Biswas et al., 2004). TFIIA was originally identified as a general transcription factor (Matsui et al., 1980; Reinberg et al., 1987). However, more recent studies suggest the activity of TFIIA is more accurately described as a transcriptional coactivator that interacts with TFIID (Biswas et al., 2004). TFIIB binds DNA in association with TBP at the core promoter to form the TFIIB-TBP-DNA ternary complex (Nikolov et al., 1995). Order of addition experiments in in vitro reconstitution assays suggested that once TFIIA and TFIIB have associated with TFIID, TFIIB makes contact with RNA polymerase II and TFIIF (Buratowski et al., 1989; Fang and Burton, 1996; Ha et al., 1993; Lei et al., 1998) and reviewed in (Hampsey, 1998). RNA polymerase II enters the assembling PIC associated with TFIIF (Lei et al., 1998). The roles of TFIIF include suppressing nonspecific DNA binding and stabilization of the PIC (Flores et al., 1989; Killeen and Greenblatt, 1992). Another role for TFIIF includes participation in DNA melting at the PIC in association with TFIIE and TFIIH (Robert et al., 1998). TFIIE enters the PIC after RNA polymerase II and TFIIF (Buratowski et al., 1989). One possible role for TFIIE may be to recruit TFIIH (Flores et al., 1992). In addition, TFIIE is important for DNA melting (Holstege et al., 1995; Robert et al., 1996). Association of TFIIH with the PIC leads to an opening of the DNA at the promoter likely

through the DNA helicase activity of TFIIH (Kim et al., 2000). The Kin28 subunit of TFIIH also possesses ATPase and cyclin-dependent kinase activity that is regulated by the associated cyclin Ccl1 (Valay et al., 1993). Importantly, the target of the kinase activity is the Ser-5 residue within the heptad repeat of the CTD (Liu et al., 2004). Compared to other components of the PIC, TFIIH is unique since it is also involved in nucleotide excision repair as reviewed in (Lee and Young, 2000). The basal transcription machinery is a complex array of proteins that assemble into a protein scaffold to recruit RNA polymerase II and function in transcription initiation.

Following PIC assembly, DNA melting and promoter clearance are essential for productive transcription. DNA within the promoter must be opened in order for transcription initiation to occur since opening leads to the formation of a transcription bubble. TFIIF may play an important role in DNA melting since it is required to wrap DNA around the PIC potentially increasing torsional stress in the template (Robert et al., 1998). The zinc ribbon motif of TFIIE is implicated in contacting promoter DNA and may be required in the formation of or maintenance of the DNA complex (Qian et al., 1993). This TFIIE activity may be stimulated by TFIIH since TFIIH has helicase activity. Once DNA is open, transcription initiation may begin by incorporating nucleotides according to the DNA template. The growing RNA chain forms an RNA-DNA hybrid within the active site of RNA polymerase II (Kettenberger et al., 2004). In vitro, many transcripts initiated by RNA polymerase II are released before reaching full length. This process is referred to as abortive initiation. To overcome abortive initiation and arrest, initiating RNA polymerase II must become competent for promoter clearance. Competency for promoter clearance depends on associations between the template and RNA polymerase II, the length of the nascent transcript, and availability of necessary cofactors such as ATP, as reviewed in (Dvir, 2002). The more stable associations between RNA polymerase II and the template the

more likely RNA polymerase II is to proceed into productive transcription. The stability of RNA polymerase II with the template increases with increasing length of the transcript since the growing chain of nucleotides can make increasing contacts within RNA polymerase II as reviewed in (Dvir, 2002). Once promoter clearance occurs, initiating RNA polymerase II is competent to transition into the elongation phase of transcription.

1.2.5 Gene-specific transcriptional activators

Activated RNA polymerase II transcription requires association of RNA polymerase II with the mediator complex. Together, RNA polymerase II and the mediator complex form the RNA polymerase II holoenzyme (Li et al., 1995). Mediator is an evolutionarily conserved complex that aids in recruitment of RNA polymerase II through transcriptional activators (Wang et al., 2005). The role of mediator and other transcriptional coactivators will be described in section 1.2.5.2. in the context of transcriptional activators.

Table 1: Proteins and protein complexes important for transcriptional regulation

Yeast Complex/Protein	Proposed Function(s)
SBF	cell cycle specific transcriptional activator
MBF	cell cycle specific transcriptional activator
SAGA	coactivator; histone acetyltransferase activity
SLIK	coactivator; histone acetyltransferase activity
Ctk1	Ser-2 specific CTD kinase
Fcp1	Ser-2 specific CTD phosphatase
TREX	recombination; transcription elongation; couples elongation to mRNA export
FACT	transcription elongation; histone chaperone activity
Spt6	transcription elongation; chromatin assembly
Spt4-Spt5	transcription elongation
Elongator	transcription elongation; histone acetyltransferase activity
The Paf1 complex	transcription elongation; recruits histone modifiers; 3'-end formation
Dst1/Ppr2 (TFIIS)	transcription elongation; stimulates transcript cleavage
SWI/SNF	nucleosome remodeling activity; nucleosome sliding; octamer transfer
RSC	nucleosome remodeling activity; ATP-dependent DNA translocation
ISW1	nucleosome remodeling activity; nucleosome sliding
Chd1	nucleosome remodeling activity; chromatin assembly
INO80	nucleosome remodeling activity; nucleosome displacement; nucleosome sliding
SWR1	nucleosome remodeling activity; exchanges nucleosomal H2A with H2A.Z
SET1/COMPASS	methyltransferase; methylates histone H3 on K4
Set2	methyltransferase; methylates histone H3 on K36
Rad6-Bre1	ubiquitin conjugase and ubiquitin ligase; monoubiquitylates histone H2B on K123

See accompanying text for additional details.

1.2.5.1 Transcriptional activators

An important class of transcription factors is comprised of gene-specific transcriptional activators. Examples of these transcription factors exist across the three domains of life. These transcriptional activators are themselves activated under diverse physiological stimuli in order to execute a transcriptional plan required to meet the needs of the cell. The stimuli could be cell autonomous such as cell cycle regulation or cell non-autonomous for instance as a response to nutrient availability. Transcriptional activation occurs through a general mechanism where the following steps occur. For example, the transcriptional activator binds a specific DNA sequence within the promoter of the activator-dependent gene. Upon activator binding, components of the general transcription machinery are recruited to the promoter for high levels of activated transcription. TFIID and RNA polymerase II holoenzyme are recruited to promoters subsequent to activator binding at the UAS.

Regulation of the cell cycle is important for asexual reproduction in prokaryotic and eukaryotic organisms as well as for the proper growth and development of multicellular organisms. In *S. cerevisiae*, the transcriptional activator SBF is integrally involved in regulating the transcription of genes during the G₁ to S phase transition of the cell cycle as reviewed in (Wittenberg and Reed, 2005). SBF is a heterodimeric activator comprised of the DNA binding protein Swi4 and Swi6, which is believed to regulate the activity of Swi4 (Table 1) (Koch et al., 1993; Moll et al., 1993). A subset of the genes that contain consensus recognition sites, CACGAAA, in their promoter are involved in cell cycle progression and these include the G₁ cyclins *CLN1* and *CLN2* (Bean et al., 2005; Iyer et al., 2001). Importantly, Swi6 forms a complex with another DNA-binding protein Mbp1 to form MBF, a transcriptional activator complex that is distinct from SBF and also important for progression through the cell cycle

(Table 1) (Iyer et al., 2001; Koch et al., 1993; Moll et al., 1993). MBF recognizes and binds to the consensus site ACGCGT within the promoters of genes that must be transcribed for the S phase of the cell cycle including genes involved in DNA synthesis (Bean et al., 2005; Iyer et al., 2001). ChIP with antibodies specific towards SBF or MBF followed by microarray analysis revealed that some cell cycle regulated genes are regulated by SBF, MBF, or both transcriptional activators (Iyer et al., 2001). Therefore Swi4, Mbp1 and Swi6 comprise two transcriptional activator complexes with distinct and partially overlapping transcriptional targets important for progression through the cell cycle (Bean et al., 2005).

A transcriptional activator complex analogous to SBF exists in mammalian cells (Costanzo et al., 2004; de Bruin et al., 2004; Wittenberg and Reed, 2005). This family of transcription factors are known as E2Fs and were originally identified as proteins required for the transcriptional activation of early region 2 (E2) of the adenovirus genome (Cam and Dynlacht, 2003). The similarities between SBF and E2F family members are quite striking. Not only are the E2F family members involved in the transcriptional activation of DNA tumor viruses but they are also required for regulated progression through the cell cycle in mammalian cells through binding specific DNA sequences within the promoters of the genes they regulate (Cam and Dynlacht, 2003). The retinoblastoma protein (Rb) is responsible for regulating the transcriptional activity of E2F family members whereas Whi5 has been identified in the regulation of SBF function (Costanzo et al., 2004; de Bruin et al., 2004). Similar to Whi5 and SBF, impaired Rb function causes cells to enter S phase of the cell cycle in an E2F-dependent manner as reviewed in (Wittenberg and Reed, 2005). The study of DNA dependent transcriptional activation in yeast and mammalian systems provide a reference for learning more

about the regulation of these transcription factors and their involvement in properly regulating the cell cycle.

Another role for transcriptional activators is to activate the transcription of genes important for metabolism. There are several genes in *S. cerevisiae* that are repressed in rich medium and induced only when certain nutrients are limiting. One example is the induction of the *GAL* genes that are repressed when glucose is in the growth medium and induced when galactose is the sole carbon source available (Bhat and Murthy, 2001). The transcriptional activator responsible for *GAL* gene activation is Gal4 (Bhat and Murthy, 2001). Gal4 is a modular protein that is composed of a separable DNA binding domain containing a zinc cluster motif at the amino terminus that binds and recognizes a specific DNA sequence within *GAL* gene promoters (UAS_{GAL}) (Bhat and Murthy, 2001). Under noninducing conditions, Gal4 is bound to the UAS_{GAL} in association with Gal80, which blocks Gal4-mediated transcriptional (Bhat and Murthy, 2001). During inducing conditions Gal3 enters the nucleus and disrupts interactions between Gal4 and Gal80 (Bhat and Murthy, 2001). Gal3-Gal80 interaction at the UAS_{GAL} elements results in the activation of the *GAL* genes allowing recruitment of the general transcription machinery via the separable carboxyl terminal activation domain of Gal4 (Bhat and Murthy, 2001).

1.2.5.2 Transcriptional coactivators

A class of proteins called transcriptional coactivators is required to establish contacts between components of the general transcription machinery and activators. This class of transcription factors includes SAGA, the Srb/Mediator complex, TFIIA, and TAFs. As mentioned previously, TFIIA was initially identified as a general transcription factor. The TAFs associate closely with TBP and influence TBP-TATA box associations through interactions with

transcriptional activators (Dymlacht et al., 1991; Wu and Chiang, 2001). But the two most relevant coactivators for this discussion are the Srb/Mediator complex, now commonly referred to as Mediator, and SAGA/SLIK (Table 1).

The Mediator is functionally linked to activated transcription initiation by RNA polymerase II. A genetic screen using truncation mutations within the CTD of RNA polymerase II identified extragenic suppressors that reversed the phenotypes of the truncation mutants, which included cold and temperature sensitivity, and these genes were designated as *SRB* (suppressor of RNA polymerase B) genes (Nonet and Young, 1989). Complete deletion of the CTD from RNA polymerase II is lethal in yeast cells even though the CTD is not required for transcription in some in vitro systems (McCracken et al., 1997; Meininghaus et al., 2000). Shortly after the identification of SRB genes, a mediator complex was proposed as an activity within a partially purified yeast extract that reduced activator induced interference (squenching) in RNA polymerase II transcription assays (Kelleher et al., 1990). Purification of this mediator complex revealed the presence of the *SRB* gene products (Myers et al., 1998). Convergence of genetic and biochemical studies on Mediator resulted in the observations that Mediator is a multisubunit complex important for facilitating transcriptional activation by relaying information from transcriptional activators to RNA polymerase II and that Mediator is conserved in eukaryotes (Gustafsson and Samuelsson, 2001; Kim et al., 2002; Li et al., 1995; Spahr et al., 2001)

SAGA (Spt-Ada-Gcn5 acetyltransferase) is a multifunctional coactivator complex that is conserved in eukaryotes. Components of SAGA were isolated as suppressors of Ty insertions in the promoters of yeast genes as reviewed in (Winston, 1992). Mutations within *SPT* genes coding for subunits of SAGA exhibited Spt⁻ phenotypes similar to mutations in *SPT15*, which codes for TBP (Winston, 1992). Genome wide high density oligonucleotide array analyses

indicated that SAGA and the TAFs might function redundantly as coactivators at certain genes since a subset of the yeast genome was regulated by both coactivators (Lee et al., 2000). SAGA is recruited to the promoters of genes by transcriptional activators and this association facilitates the association of TBP at the promoter, which is a rate limiting step in the formation of the PIC (Dudley et al., 1999). A SAGA-like complex (SLIK or SALSA) was recognized as an alternative SAGA complex with roles in transcriptional activation. SLIK/SALSA contains a truncated version of the SAGA integral component Spt7 and lacks Spt8 (Sterner et al., 2002; Wu and Winston, 2002). Specific subunits of SAGA may be important for determining if SAGA will act either as an activator or repressor since certain components of SAGA are required for the repression of a subset of yeast genes (Belotserkovskaya et al., 2000). Two histone modifying enzymes are components of SAGA: the Gcn5 HAT which acetylates histone H3 K14 and the ubiquitin specific protease Ubp8, which deubiquitylates histone H2B K123 (Henry et al., 2003; Pray-Grant et al., 2005; Tse et al., 1998).

1.2.5.3 General repressors of transcription

General transcriptional repressors like Mot1 and the components of negative cofactor 2 (NC2) regulate transcription by binding to TBP and preventing TBP association with the promoter or dissociating TBP already bound to the promoter. Also of interest, transcriptional repressors like Mot1 of yeast have been implicated not only in transcriptional repression but in transcriptional activation (Andrau et al., 2002; Dasgupta et al., 2005; Dasgupta et al., 2004). Mot1 is a member of the SWI/SNF family and contains a DNA-dependent ATPase domain as reviewed in (Pugh, 2000). The proposed mechanism of Mot1 function uses the hydrolysis of ATP to drive the dissociation of TBP from the promoter (Pugh, 2000). Expression profiles of temperature sensitive *mot1* cells identified transcripts that were either up-regulated or down-

regulated in cells with impaired Mot1 function (Andrau et al., 2002). The potential Mot1 target genes were analyzed further by ChIP and indicated direct involvement of Mot1 in the activation of a subset of genes (Andrau et al., 2002). Another conserved transcriptional corepressor is negative cofactor 2 (NC2), which is composed of Bur6 and Ncb2. *BUR6* (bypass upstream activating sequence requirements) was isolated in a screen for mutations that bypass the requirement for upstream activating sequences in *SUC2* transcription, indicating a role for Bur6 in repressing transcription of *SUC2* (Prelich and Winston, 1993). Interestingly, another mutation that also caused a *bur* phenotype was uncovered in *MOT1* (allelic with *BUR3*) (Prelich, 1997). Subsequent characterization of Bur6 revealed amino acid sequence similarity with the mammalian transcriptional repressor DRAP1 or NC α (Prelich, 1997). NC2 binds to TBP at the promoter and prevents the assembly of the PIC (Pugh, 2000). However, like Mot1, a subset of genes require the activity of NC2 for their expression (Cang and Prelich, 2002). Taken together, this evidence indicates that the line between transcriptional activators and repressors is quite blurred. A rationalization for the observed positive effect of these transcriptional repressors on transcription may derive from their dependence on regulatory sequences within the promoters of these genes or on different complements of regulatory factor-associated proteins.

1.2.5.4 Gene specific transcriptional repressors

In contrast to transcriptional activators, the role of many transcriptional repressors is to antagonize the formation of the PIC. Similar to gene-specific transcriptional activators, transcriptional repressor proteins limit the transcription of certain genes based on current physiological requirements. The regulation of genes involved in various biosynthetic pathways is under tight transcriptional control and these genes are only expressed in response to depletion of certain nutrients in the growth medium. For example, the Mig1 transcriptional repressor binds

within the promoters of glucose-repressed genes when cells are grown in medium containing glucose (Lundin et al., 1994). The ArgR/Mcm1 repressor complex is involved in the repression of genes in the arginine biosynthesis pathway (Turner et al., 2002; Yoon et al., 2004). Transcriptional repressors act to block assembly of the PIC. Transcriptional repressor proteins, their roles in repression, and possible roles in activation will be discussed below.

The Mig1 transcriptional repressor exerts its influence through binding to specific DNA sequences within the promoters of genes that are glucose repressed. The function of Mig1 is regulated in response to limiting glucose. In the presence of glucose, Mig1 is bound to regulatory elements within genes and is responsible for recruiting the Ssn6-Tup1 corepressor complex (Schuller, 2003). Ssn6-Tup1 association may work through chromatin remodeling or histone deacetylation to interfere with the formation of the PIC (Edmondson et al., 1996; Watson et al., 2000; Zhang et al., 2004). Repression by Mig1 is relieved in response to glucose by a mechanism involving phosphorylation by the Snf1 protein kinase (Ostling and Ronne, 1998). Phosphorylation of Mig1 causes it to dissociate from its DNA regulatory sequence and translocate out of the nucleus as reviewed in (Schuller, 2003).

Expression of genes in the arginine biosynthetic pathway in *S. cerevisiae* requires transcriptional repressors to ensure that *ARG1* and other genes are properly regulated in response to arginine availability. The transcriptional repressor responsible for regulating *ARG1* expression is ArgR/Mcm1, which is composed Arg80, Arg81, Arg82 and Mcm1. ArgR/Mcm1 binds to two arginine control elements (ARC) in the promoter of *ARG1* (Ricci et al., 2002; Yoon et al., 2004). Genes in the arginine biosynthetic pathway are positively regulated by the transcriptional activator Gcn4 (Hinnebusch, 1985). Surprisingly, the Gcn4 activator participates in recruiting the ArgR/Mcm1 repressor to ARCs within *ARG1* for repressor activity (Yoon et al., 2004). *ARG1*

transcription requires other transcription factors for proper regulation. For example, components of the SAGA coactivator complex are important for the repression of *ARG1* (Ricci et al., 2002). ChIP analysis using antibodies against acetylated histone H3 revealed that histone H3 acetylation at *ARG1* was dependent on the HAT Gcn5 of SAGA (Ricci et al., 2002). These results are in agreement with Northern analyses that demonstrated *ARG1* transcripts were derepressed in the absence of SAGA components when cells were grown in rich medium (Ricci et al., 2002). These observations are consistent with the result that the Rad6 ubiquitin conjugase is also involved in repressing *ARG1* when cells are growing in rich medium (Turner et al., 2002). Rad6 and SAGA are required to prevent TBP binding at the TATA box of *ARG1* (Ricci et al., 2002; Turner et al., 2002). This is noteworthy for SAGA since it has been classified as a transcriptional coactivator and in this case it is acting more like a corepressor. Moreover, substitution of the Rad6 target histone H2B with a form that cannot be modified by Rad6, also results in derepression of *ARG1* (Turner et al., 2002). This is consistent with the result that the absence of Ubp8, the ubiquitin specific ligase component of SAGA, does not result in derepression of *ARG1* under normal growth conditions (Lee et al., 2005).

1.2.5.5 Transcriptional corepressors

Proteins that interfere with PIC formation by interaction with gene-specific repressors belong to a group of factors termed transcriptional corepressors. This group of transcriptional regulators includes the Sin3-Rpd3 histone deacetylase and Ssn6-Tup1. Sin3-Rpd3 is recruited to promoters by transcriptional repressor proteins bound to the upstream regulatory sequences, where the complex deacetylates histone H4 on lysine residues 5 and 12 (Kadosh and Struhl, 1997; Rundlett et al., 1996; Rundlett et al., 1998). The transcriptional repressor Ume6 is bound to promoter elements of genes to recruit Sin3-Rpd3 to maintain the repression of genes involved

in meiosis (Kadosh and Struhl, 1997; Kadosh and Struhl, 1998; Kadosh and Struhl, 1998). Ssn6-Tup1 represses transcription by positioning nucleosomes over promoters by recruiting the Isw1 chromatin remodeling factor (Zhang et al., 2004). The association of Tup1 with chromatin is enhanced when the histone amino tails are hypoacetylated, this is consistent with the observation that Ssn6-Tup1 associates with histone deacetylases in vivo including Rpd3 (Edmondson et al., 1996; Watson et al., 2000).

1.3 Modulation of RNA polymerase II transcript elongation

1.3.1 Phosphorylation of RNA polymerase II during elongation

As mentioned previously, the phosphorylation state of the RNA polymerase II CTD correlates with different stages in transcription, and the phosphorylation state that corresponds to transcript elongation is phosphorylation of Ser-2. The kinase responsible for this co-transcriptional modification of the CTD in yeast is the CTD kinase 1 (CTDK-1) composed of the three subunits Ctk1, Ctk2, and Ctk3 (Table 1) (Sterner et al., 1995). Based on sequence homology searches Ctk1 exhibits similarity to cyclin dependent kinases and Ctk2 exhibits limited sequence similarity with cyclin C, while Ctk3 did not exhibit sequence similarity with other known proteins at the time of this study (Sterner et al., 1995). The positive transcription elongation factor b (P-TEFb) complex exhibited RNA polymerase II kinase activity in human cells (Marshall and Price, 1992; Marshall and Price, 1995). Sequence analysis of the components of P-TEFb revealed that certain components showed sequence similarity to cyclin-dependent kinases and cyclin activating kinases (Grana et al., 1994). These subunits are reminiscent of members of CTDK-1 of yeast (Sterner et al., 1995). While it is not clear whether CTKD-1 is the bona-fide homolog of P-TEFb, it is clear that these factors share functional similarities (Murray et al., 2001). Both P-TEFb and Ctk1 have been implicated in positively regulating transcript elongation. P-TEFb has important implications for human diseases such as AIDS, since transcription of the HIV-1 genome is an important step in production of viral particles and P-TEFb is required for positive stimulation of HIV-1 transcript elongation in conjunction with viral

and host factors (Ping and Rana, 2001). DSIF, the human counterpart of the yeast Spt4-Spt5 complex, was identified based on a requirement for it in inhibiting transcription elongation upon exposure to the nucleoside analog 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (Wada et al., 1998). Interestingly, mutations in *SPT4* and *SPT5* exhibit genetic interactions with *ctk1 Δ* cells (Lindstrom and Hartzog, 2001). Like DSIF, the Spt4-Spt5 complex has been implicated in regulating RNA polymerase II elongation both positively and negatively (Hartzog et al., 1998; Wada et al., 1998).

CTD dephosphorylation is an important regulatory mechanism that is performed by two essential CTD phosphatases in *S. cerevisiae*. Ssu72 is required for catalyzing the removal of phosphate groups on Ser-5, while Fcp1 is essential for removing phosphates from Ser-2 of the CTD (Table 1) (Archambault et al., 1997; Archambault et al., 1998; Cho et al., 2001; Friedl et al., 2003; Kong et al., 2005; Mandal et al., 2002). Fcp1 associates with a subunit of TFIIF and the RNA polymerase II holoenzyme (Archambault et al., 1997; Archambault et al., 1998). As shown by CHIP, Fcp1 localizes to the open reading of genes (Cho et al., 1999). TFIIF stimulates the phosphatase activity of Fcp1 and dephosphorylation of the CTD is required for re-entry into the PIC at the promoter (Archambault et al., 1997; Cho et al., 1999). Additional support for a role of Fcp1 in transcription elongation came from the observation that mutations in *FCPI* genetically interact with mutations in genes encoding elongator, an RNA polymerase II-associated complex implicated in transcript elongation (Kong et al., 2005). The activity of Fcp1 is modulated by casein kinase II (protein kinase CK2 or CK2) and phosphorylation of Fcp1 by CK2 stimulates the association between TFIIF and Fcp1 (Abbott et al., 2005). Fcp1 is conserved in eukaryotes and interactions between human Fcp1 and the HIV-1 encoded Tat protein have been reported (Abbott et al., 2005; Marshall et al., 1998). In particular, Tat competes with TFIIF

for association with Fcp1 at the HIV-1 promoter. Tat interaction with Fcp1 may prevent dephosphorylation of the CTD to allow HIV-1 transcription (Abbott et al., 2005). While phosphorylation states of the CTD correlate with various phases of transcription, Fcp1 is also important for stimulating RNA polymerase II elongation by a mechanism distinct from its role as a CTD phosphatase (Cho et al., 1999). Investigation by the Buratowski group suggested another protein is responsible for the dephosphorylation of Ser-5 residues within the CTD (Cho et al., 1999). The Ser-5 phosphatase was recently identified as Ssu72, a protein that has been implicated in transcription initiation and 3'-end formation of mRNA and snoRNA (He et al., 2003; Krishnamurthy et al., 2004; Steinmetz and Brow, 2003). Whether Ssu72 is important for transcription elongation is not clear.

1.3.2 RNA polymerase II-associated factors

A recent study suggests that many proteins associated with RNA polymerase II are not involved in improving transcriptional efficiency or processivity (Mason and Struhl, 2005). However, genetic and biochemical analyses indicate that many proteins do functionally interact with RNA polymerase II (Krogan et al., 2002; Lindstrom et al., 2003; Mason and Struhl, 2005; Mueller and Jaehning, 2002; Squazzo et al., 2002). Since RNA polymerase II is a central player in the biogenesis of snRNA, snoRNA, and mRNA, it is not surprising that other factors important for RNA modification, formation of heterogeneous ribonucleoprotein (hnRNP) particles, and export of hnRNPs associate with RNA polymerase II. Other factors expected to associate with RNA polymerase II include proteins involved in nucleosome dynamics and chromatin structure since evidence suggests that nucleosomes are evicted from the template during transcription elongation. Interestingly, several observations indicate that the 19S and 26S proteasome associate with elongating RNA polymerase II (Ferdous et al., 2001; Ferdous et al.,

2002). The function of the proteasome in regulating transcription has been reviewed in (Kinyamu et al., 2005).

1.3.2.1 The co-transcriptional nature of RNA maturation

Several proteins involved in RNA processing associate with RNA polymerase II. These include the enzymes required for adding the 7-methyl guanosine cap to the 5'-end of the newly transcribed mRNA. In *S. cerevisiae*, the capping reaction is performed by a methyltransferase and a heterodimeric complex composed of a guanylyltransferase and an RNA 5'-triphosphatase, which are encoded by the *ABD1*, *CEG1*, and *CET1* genes respectively (Kim et al., 2004; McCracken et al., 1997; Rodriguez et al., 2000; Schroeder et al., 2000; Schroeder et al., 2004). The addition of a functional 7-methyl guanosine cap is required for translation of the mRNA (Gu and Lima, 2005). Together, Abd1 and Ceg1-Cet1 associate with the RNA polymerase II CTD in a manner dependent on Ser-5 phosphorylation (Komarnitsky et al., 2000; McCracken et al., 1997). Unlike Ceg1-Cet1, Abd1 associates along the entire open reading frame with RNA polymerase II indicating Abd1 may function in RNA polymerase II transcript elongation (Komarnitsky et al., 2000; Schroeder et al., 2000; Schroeder et al., 2004).

Splicing of the nascent transcript also occurs co-transcriptionally. Observations indicate that splicing factors associate with elongating RNA polymerase II in a CTD-dependent manner to facilitate splicing (Bird et al., 2004; McCracken et al., 1997). In particular, the phosphorylated form of RNA polymerase II stimulates splicing in in vitro splicing assays (Hirose et al., 1999). The CTD may mediate interactions between factors involved in transcription and splicing such as *S. cerevisiae* Spt5 and human TAT-SF1 (Kornblihtt et al., 2004). Both Spt5 and TAT-SF1 are involved in regulating elongation and are also linked to splicing. TAT-SF1 associates with spliceosomal U small nuclear ribonucleoproteins (snRNP) (Fong and Zhou, 2001). In a study of

TAT-SF1, the Zhou group revealed that the TAT-SF1 elongation factor and spliceosomal snRNPS exhibited reciprocal stimulation of transcriptional elongation and splicing (Fong and Zhou, 2001). Affinity purification of the transcription elongation factor Spt5 revealed that the protein associated with subunits of RNA polymerase II, RNA polymerase II elongation factors, RNA processing factors, 3'-end formation factors, and proteins involved in RNA export (Lindstrom et al., 2003). Moreover, mutations in *SPT5* confer defects in splicing such that detectable levels of unspliced yeast transcripts were observed by RT-PCR analysis (Lindstrom et al., 2003). These observations illustrate the mutual relationship between transcript elongation and co-transcriptional splicing.

Proteins important for proper shuttling of RNA from the nucleus to the cytoplasm are also associated with RNA polymerase II. A founding member of the THO/TREX nuclear RNA export complex called *HPRI* was originally identified in a screen for mutations associated with hyper-recombination (Table 1) (Aguilera and Klein, 1988). Several observations suggested that Hpr1 participated in transcription. Deletion of *HPRI* resulted in the derepression of *SUC2* a glucose-repressed gene and *hpr1Δ* cells exhibited reduced *GALI* transcription upon galactose induction (Zhu et al., 1995). Additionally, Hpr1 was identified as an RNA polymerase II-associated protein in association with Paf1, Cdc73 and Ccr4 (Chang et al., 1999). A role for Hpr1 in transcription elongation was inferred by the observation that *hpr1Δ* cells exhibit kinetic defects in the induction of a galactose-inducible *lacZ* gene in yeast and *hpr1Δ* cells exhibit defects in transcribing long genes or genes with high G+C content (Chavez and Aguilera, 1997; Chavez et al., 2001). In the latter case, long genes and genes with high G+C content are assumed to require elongation factors to improve elongation efficiency or processivity of transcription over the length of the open reading frame or to aid in overcoming defects in elongation due to DNA

sequences that may impair elongation. *THO2* was discovered in a screen to identify multicopy suppressors of the hyperrecombination phenotype of a deletion of *HPRI* (Piruat and Aguilera, 1998). Further biochemical characterization of Tho2 revealed it was a member of a tetrameric complex associated with Hpr1, Mft2, and Thp2 (Chavez et al., 2000). Individual components of the THO complex reside in the nucleus based on visualization of functional GFP fusion constructs and microscopy (Chavez et al., 2000). Furthermore, deletion of individual THO complex members exhibited similar phenotypes and defects in transcription elongation (Chavez et al., 2000). Additional insight into the role of the THO complex came from the affinity purification of the RNA export protein Sub2, which indicated that the THO complex associated stoichiometrically with Sub2 (Strasser et al., 2002). Other proteins that associated with Sub2 in addition to the members of the THO complex include Tex1 and Yra1, proteins involved in mRNA export (Strasser et al., 2002). The Sub2 containing complex is referred to as TREX for transcription/export (Strasser et al., 2002). TREX is recruited to actively transcribed genes and mutation of TREX components causes defects in export of poly(A)⁺ RNA from the nucleus, indicating that members of the TREX complex are functionally linked with transcription elongation and mRNA export (Strasser et al., 2002). The members of the TREX complex are conserved between yeast and humans (Masuda et al., 2005; Strasser et al., 2002). Therefore, it is likely that members of the THO complex are important for association of mRNA export proteins like Yra1 and Sub2 with transcribing RNA polymerase II.

1.3.3 RNA polymerase II-associated factors involved in chromatin structure and function

Several analyses have investigated the mechanism whereby RNA polymerase II transcribes nucleosome-containing templates since nucleosomes are a potent barrier to elongating RNA polymerase II (Bednar et al., 1999; Bednar and Woodcock, 1999). A possible mechanism for transcription of RNA polymerase II through the chromatin template is that the histones dissociate from DNA. Alternatively the histones remain associated with the template during RNA polymerase II transcription as postulated by the spooling or step-around model (Studitsky et al., 1994; Studitsky et al., 1995). Briefly, these models suggest that as RNA polymerase II approaches a nucleosome, histone-DNA contacts are partially disrupted and the exposed surface of the histone octamer is available to make contacts with DNA that has been newly transcribed (Bednar et al., 1999) (Studitsky et al., 1994; Studitsky et al., 1995). Transcriptional intermediates have also been analyzed using electron cryomicroscopy. Observations from this study provided support for the model of transcription through nucleosomes where histones remain intact (Bednar et al., 1999; Bednar and Woodcock, 1999). Importantly, the electron cryomicroscopy studies also provided support for the formation of nucleosomal DNA-RNA polymerase II intermediates that cause RNA polymerase II pausing (Bednar et al., 1999; Bednar and Woodcock, 1999; Studitsky et al., 1995). These biochemical studies demonstrate the need for proteins *in vivo* that are important for aiding RNA polymerase II in traversing the nucleosome-assembled template.

Many RNA polymerase II-associated factors have been implicated in assisting RNA polymerase II in overcoming nucleosome-induced pausing during elongation. One group of RNA polymerase II-associated factors was identified in a screen for genes that suppress the insertion

of δ elements, or long terminal repeats of yeast retrotransposons, between the core promoter and UAS sequences (*SPT* genes) (Clark-Adams et al., 1988; Fassler and Winston, 1988; Winston et al., 1984). The δ insertions alter the utilization of transcription start sites. This Spt⁻ screen uncovered several factors that restored transcription initiation to the normal transcriptional start site. Moreover, two different categories of *SPT* genes were revealed based in part on individual mutant phenotypes. The first category of *SPT* genes exhibited similar mutant phenotypes with mutations in *SPT15*, which codes for TBP. Mutations in genes within the second group of *SPT* genes conferred phenotypes similar to those of histone mutants. Further genetic characterization of the latter class of Spt factors, including Spt4, Spt5, Spt6, and Spt16, suggests that they may be important for chromatin structure (Compagnone-Post and Osley, 1996; Malone et al., 1991; Swanson et al., 1990; Swanson et al., 1991; Swanson and Winston, 1992).

1.3.3.1 Yeast FACT

The yeast protein Spt16 associates with Pob3 in a complex that is referred to as FACT (facilitates chromatin transcription) (Table 1) (Orphanides et al., 1998). The human homolog of FACT is comprised of the human Spt16 protein and SSRP1, a protein with sequence similarity to Pob3 (Brewster et al., 2001). What is unusual about the similarity between SSRP1 and Pob3 is that SSRP1 contains an extended carboxyl terminal domain (Brewster et al., 2001). The extended carboxyl terminal end of SSRP1 exhibits similarity to Nhp6, which may recruit FACT to histones (Formosa et al., 2001). As mentioned above, mutations in the essential gene *SPT16* confer Spt⁻ phenotypes similar to mutations in histone genes, indicating a role for Spt16 in chromatin structure (Malone et al., 1991). Affinity purification of FACT revealed that it associates with RNA polymerase II, and proteins that associate with elongating RNA polymerase

II such as the Paf1 complex, Spt4-Spt5, and Chd1 (Krogan et al., 2002). Interestingly, the association between FACT and Chd1 appears to be conserved since a human homolog of Chd1 associates with SSRP1 through two-hybrid interactions (Kelley et al., 1999). Yeast and *Drosophila* FACT have been shown to associate with actively transcribed regions of the genome by ChIP and immunofluorescence assays (Kim et al., 2004; Mason and Struhl, 2003; Saunders et al., 2003). Mutations in *SPT16* result in aberrant transcription initiation within the open reading frames of certain yeast genes, presumably due to defects in chromatin structure (Kaplan et al., 2003). In fact, recent analyses from the Reinberg lab indicate that FACT possesses histone chaperone activity where the acidic carboxyl terminal region of Spt16 is required for the disruption of a histone H2A-H2B dimer (Belotserkovskaya et al., 2003). In support of a role for FACT in histone chaperone activity, mutations in *SPT16* are synthetically lethal with defects in the *HIR/HPC* (histone regulatory/histone promoter control) (Formosa et al., 2002). The HIR/HPC proteins are involved in replication-independent nucleosome deposition (Formosa et al., 2002). The possibility that Spt16 may regulate RNA polymerase II transcription through chromatin structure is in agreement with the observation that FACT associates with a subunit of DNA polymerase α , such that FACT may also be important for stabilizing chromatin structure during DNA replication (Schlesinger and Formosa, 2000; Wittmeyer and Formosa, 1997; Wittmeyer et al., 1999).

1.3.3.2 Spt6

Another RNA polymerase II-associated factor that is important for regulation of transcription through maintaining chromatin structure is Spt6 (Table 1). Micrococcal nuclease experiments with *spt6* strains demonstrated defects in the chromatin structure of the *SUC2* gene similar to those caused by mutations in histone genes (Bortvin and Winston, 1996). In addition,

mutations in *SPT6* and genes encoding histones suppress the *SUC2* transcriptional phenotype of *snf2* and *snf5* strains (Bortvin and Winston, 1996). This study also provided the first evidence that Spt6 associated with tetramers of histone H3 and H4 and can assemble nucleosomes on plasmid DNA in vitro (Bortvin and Winston, 1996). Interestingly, *spt6* cells exhibit transcription initiation from cryptic TATA boxes within the open reading frames of genes, causing the production of both full length mRNAs and short mRNAs that result from transcription initiation within the gene (Kaplan et al., 2003). This observation suggests that Spt6 is critical for maintaining proper chromatin structure during transcription elongation. Affinity purification of Spt6 revealed that it associates with RNA polymerase II, the RNA polymerase II-associated elongation factor Spt5, and a novel RNA polymerase II-associated protein called Iws1 (interacts with Spt6) (Krogan et al., 2002). Association of Spt6 with RNA polymerase II appears to be a conserved function since *Drosophila* Spt6 co-localizes to open reading frames and polytene chromosomes with RNA polymerase II (Saunders et al., 2003). Spt6 physically associates with components of the nuclear exosome, a complex of 3' to 5' exonucleases important for mRNA surveillance and maturation of snRNA and snoRNA, and could act to couple the exosome with elongating RNA polymerase II (Andrulis et al., 2002). These observations point to conserved functions of Spt6 in regulating chromatin structure during transcription elongation and linking transcription with the nuclear exosome complex. In vitro transcription studies indicated that human Spt6 was also important for transcription of a naked DNA template (Endoh et al., 2004).

1.3.3.3 Spt4-Spt5

The conserved transcription elongation factors Spt4-Spt5 coordinate several events during transcript elongation by RNA polymerase II (Table 1). DSIF, the human counterpart of Spt4-Spt5, was identified based on a requirement for it in inhibiting transcription elongation upon

exposure to the nucleoside analog 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (Wada et al., 1998). In vitro transcription analyses using partially purified components suggested that DSIF affects elongation both positively and negatively (Wada et al., 1998; Wada et al., 1998). Like DSIF, the Spt4-Spt5 complex has been implicated in regulating RNA polymerase II elongation both positively and negatively (Hartzog et al., 1998; Wada et al., 1998). Interestingly, Spt5 exhibits homology with an essential bacterial transcription elongation and termination factor called NusG (Wada et al., 1998). Biochemical analysis of Spt4, Spt5, and Spt6 indicated that Spt4 and Spt5 form a complex in vivo and that a less abundant complex of Spt5-Spt6 also exists in vivo (Krogan et al., 2002; Swanson and Winston, 1992). Affinity purification of an epitope-tagged version of Spt5 revealed associations between Spt5 and RNA polymerase II, transcription initiation factors, RNA capping factors, RNA processing factors, and proteins involved in RNA polymerase II elongation (Lindstrom et al., 2003). Also, mutations in *SPT5* caused defects in the splicing of certain transcripts and deletion of *SPT4* caused defects in proper 3'-end formation at the *GAL10* locus (Kaplan et al., 2005; Lindstrom et al., 2003). Mutations in *SPT5*, *DST1* and *RPB2* cause defects in poly(A) site utilization such that poly(A) sites within the coding regions of genes are preferred to the canonical poly(A) sites of certain yeast genes (Cui and Denis, 2003). These observations suggest that the Spt4-Spt5 complex regulates different events during elongation by RNA polymerase II.

1.3.3.4 Elongator

An RNA polymerase II-associated protein complex that is important for transcription elongation is elongator (Table 1). This protein complex was isolated based on its interaction with hyperphosphorylated RNA polymerase II purified from a non-soluble chromatin preparation (Otero et al., 1999). Elongator interacted genetically with TFIIS, where deletion of an elongator

component in combination with deletion of the gene encoding TFIIS resulted in enhanced sensitivity to the base analog 6-azauracil (6AU) (Otero et al., 1999). Characterization of Elp3, a member of the elongator complex, demonstrated amino acid sequence similarity with histone acetyltransferases, and "in gel" HAT assays revealed that Elp3 was capable of acetylating the amino terminal tails of the histones H3, H4, H2A, and H2B (Wittschieben et al., 1999). Further characterization of the intact, six subunit elongator complex revealed specific acetylation of histone H3 K14 and histone H4 K8 (Winkler et al., 2002). Interestingly, the HAT component of SAGA also acetylates histone H3 K14 (Grant et al., 1997). Not only are acetylated histone levels reduced in *elp3Δ* lysates but genetic interactions among genes encoding certain components of the SAGA coactivator complex and genes encoding elongator indicate these HAT-containing complexes possess similar or overlapping functions (Winkler et al., 2002; Wittschieben et al., 2000). Together, the evidence indicates a role for elongator during transcription elongation and that the HAT activity of Elp3 is required for this function. However, ChIP assays using epitope-tagged forms of elongator provided contradictory evidence in that elongator did not associate with the open reading frames of transcribed genes and was localized to the cytoplasm (Pokholok et al., 2002). If elongator is not associated with DNA, then how could elongator have been identified as an RNA polymerase II-associated factor? This question is being addressed and recent analysis using RNA immunoprecipitation (RIP) indicates that elongator associates with the nascent, unspliced RNA of RNA polymerase II transcribed genes (Gilbert et al., 2004). This study raises questions about interpreting negative results of ChIP experiments. However, other questions regarding the function of elongator remain such as whether the true targets of Elp3 HAT activity are histone tails or proteins associated with RNA during elongation.

1.3.3.5 The Paf1 Complex

Another RNA polymerase II-associated complex is the Paf1 complex and it consists of five proteins including Paf1, Ctr9, Rtf1, Cdc73, and Leo1 (Table 1) (Mueller and Jaehning, 2002; Squazzo et al., 2002). Paf1 (RNA polymerase II-associated factor 1) and Cdc73 were originally identified by immunopurification of RNA polymerase II using antibodies directed toward the CTD (Shi et al., 1997; Wade et al., 1996). TFIIB, TFIIF, and TFIIS were also identified in the fraction that associated with RNA polymerase II as described in other biochemical analyses of RNA polymerase II (Kim et al., 1994; Shi et al., 1997; Wade et al., 1996). Compared to other schemes, the Jaehning lab method for purification of RNA polymerase II revealed a new complement of proteins; for example, the Srb/Mediator complex was not identified in the fraction of proteins that associate with RNA polymerase II unlike the preparation from the Kornberg lab (Kim et al., 1994; Shi et al., 1997; Wade et al., 1996). Association of the Paf1 complex with RNA polymerase II was supported by further biochemical studies, where individual members of the Paf1 complex were affinity purified and RNA polymerase II was present in the eluted fractions (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002). Further analysis of affinity purification of members of the Paf1 complex revealed association of the Paf1 complex with other RNA polymerase II-associated factors including Spt4-Spt5, Spt16-Pob3, and Chd1 (Krogan et al., 2002). The potential functional link indicated by biochemical analyses with the Paf1 complex, Spt4-Spt5 and Spt16-Pob3 was supported by genetic analyses (Krogan et al., 2002; Mueller and Jaehning, 2002; Simic et al., 2003; Squazzo et al., 2002). *RTF1* was identified as a mutational suppressor of a TBP mutant with altered DNA binding specificity, suggesting a role for Rtf1 in transcription initiation (Stolinski et al., 1997). Deletion of individual Paf1 complex members confers similar phenotypes like sensitivity to the

6AU and the Spt⁺ phenotype (Krogan et al., 2002; Squazzo et al., 2002). But *paf1Δ* and *ctr9Δ* mutants exhibit the most severe phenotypes including temperature sensitivity (in some strain backgrounds), sensitivity to caffeine, sensitivity to hydroxyurea and slow growth (Betz et al., 2002; Mueller and Jaehning, 2002). Interestingly, deletion of *leo1Δ* suppressed the hydroxyurea sensitivity of *ctr9Δ* strains and *rtf1Δ* suppressed the temperature sensitivity of *paf1Δ* cells (Mueller and Jaehning, 2002), indicating that Rtf1 and Leo1 may antagonize the function of Paf1 and Ctr9. Taken together, these observations suggest a role for the Paf1 complex in transcription elongation despite early indications that it may be involved in initiation.

Members of the Paf1 complex are required for histone modifications associated with actively transcribed genes. Absence of Rtf1, Paf1, and Ctr9 results in the reduction of methylated histone H3 K4 and histone H2B K123 ubiquitylation (Ng et al., 2003; Ng et al., 2003). Set1 is recruited to the 5' end actively transcribed genes and methylates histone H3 K4 (Ng et al., 2003). Set1 recruitment to the 5' region of genes is dependent on prior histone H2B K123 ubiquitylation by Rad6-Bre1 (Ng et al., 2003; Wood et al., 2003). Interestingly, Rad6 and Bre1 are required for subsequent trimethylation but not monomethylation of histone H3 K4 (Dehe et al., 2005). Association and activation of Rad6-Bre1 is dependent on the Paf1 complex (Laribee et al., 2005; Wood et al., 2003; Xiao et al., 2005). In particular, Rad6-Bre1 is targeted to promoters independently of the Paf1 complex but requires the Paf1 complex for association with elongating RNA polymerase II (Xiao et al., 2005). These observations reveal a requirement for the Paf1 in proper histone modifications that occur concurrently with transcript elongation by RNA polymerase II.

The Paf1 complex is conserved from yeast to humans and homologs have also been identified in plants (He et al., 2004; Oh et al., 2004; Rozenblatt-Rosen et al., 2005; Woodard et

al., 2005; Yart et al., 2005). Studies of the Paf1 complex in yeast have provided information regarding the functional interaction of the Paf1 complex with RNA polymerase II, transcription elongation factors, and proteins involved in 3'-end formation (Mueller and Jaehning, 2002; Rozenblatt-Rosen et al., 2005; Sheldon et al., 2005; Simic et al., 2003; Squazzo et al., 2002; Woodard et al., 2005; Yart et al., 2005). Functional analyses of the human Paf1 complex have drawn on and extended these analyses. The product of the hyperparathyroidism-jaw tumor gene (*HRPT2*), parafibromin, exhibits sequence similarity with Cdc73 (Rozenblatt-Rosen et al., 2005). The most compelling evidence that parafibromin was a component of the human Paf1 complex was the observation that affinity purified parafibromin eluted with the human homologs of Paf1, Ctr9, and Cdc73 (Rozenblatt-Rosen et al., 2005; Yart et al., 2005). Moreover, the human Paf1 complex associates with RNA polymerase II that is phosphorylated on Ser-2 and Ser-5 (Yart et al., 2005). Localization studies of parafibromin and hLeo1 indicated that they reside in the nucleus (Rozenblatt-Rosen et al., 2005; Woodard et al., 2005). Like the yeast Paf1 complex, the human Paf1 complex associates with the histone H3 K4 lysine methyltransferase and this association is diminished in cells expressing mutant forms of parafibromin (Rozenblatt-Rosen et al., 2005). The human Paf1 complex may be involved in tumor suppressor function since cell proliferation and increased cyclin D1 expression were observed in cells transfected with a construct expressing a mutation in parafibromin that is associated with parathyroid cancer (Woodard et al., 2005). These results are consistent with observations that the yeast Paf1 complex is required for regulating the transcription of cell-cycle regulated genes (Betz et al., 2002; Koch et al., 1999; Mueller and Jaehning, 2002; Porter et al., 2002). Interestingly, hRtf1 was not identified in association with the human Paf1 complex. This is in contrast to studies in yeast where Rtf1 is a component of the Paf1 complex (Krogan et al., 2002; Mueller and

Jaehning, 2002; Rozenblatt-Rosen et al., 2005; Squazzo et al., 2002; Yart et al., 2005). These results indicate that hRtf1 may not associate with the human Paf1 complex in vivo (Rozenblatt-Rosen et al., 2005; Yart et al., 2005). One possible explanation for these observations is that the function of Rtf1 has diverged from yeast to human. Alternatively, human Rtf1 may carry out a conserved function that does not require its association with the Paf1 complex.

1.3.4 Chromatin remodeling factors involved in transcription

1.3.4.1 The SWI/SNF chromatin remodeling complex

The prototypical chromatin remodeling complex is SWI/SNF, first identified in *S. cerevisiae* (Table 1) (Smith and Peterson, 2005). The catalytic component of SWI/SNF is Snf2 and contains the Swi2/Snf2 ATPase/helicase motif that is important for the catalytic activity of the remodeling complex (Martens and Winston, 2003; Smith and Peterson, 2005). The catalytic region is comprised of several domains including the DEAD-like helicase domain, helicase superfamily C-terminal domain, and a bromodomain. Importantly, the catalytic subunits of many chromatin remodeling complexes share amino acid sequence similarity with the Swi2/Snf2 ATPase/helicase motif of Snf2 (Smith and Peterson, 2005). Proteins containing DEAD-like helicase domains are involved in different aspects of RNA metabolism and function by unwinding dsRNA (Fuller-Pace, 1994). Factors possessing the helicase superfamily C-terminal domain or DEAD/H domain reportedly have helicase activity, ATP binding, and nucleic acid binding associated with them (Caruthers and McKay, 2002). The bromodomain of a Swi2 paralog has been shown to anchor RSC at acetylated histone H3 K14 in *S. cerevisiae* (Kasten et al., 2004; Smith and Peterson, 2005).

Several *S. cerevisiae* SWI/SNF complex components were originally identified in two different genetic screens to uncover transcriptional regulators of *HO* and *SUC2* (Haber, 1977; Neigeborn and Carlson, 1984; Stern et al., 1984), as reviewed in (Carlson and Laurent, 1994). Based on the genetic screen and the mutations isolated, components of SWI/SNF were categorized as transcriptional activators (Cote et al., 1994). Additional analyses provided compelling evidence for the role of SWI/SNF members as transcriptional activators from yeast to humans (Cote et al., 1994). Transcriptional defects caused by mutations in SWI/SNF were suppressed by deletion of one copy of the genes coding for H2A and H2B and the observed repression was corrected at the level of chromatin structure in the case of *SUC2* (Hirschhorn et al., 1992). Elimination of either Snf2 or Swi1 of SWI/SNF reduced Gal4 binding as observed in β -galactosidase assays (Cote et al., 1994). Genome-wide expression analyses in *SNF2* and *snf2 Δ* cells provided additional support for a role of SWI/SNF in transcriptional regulation of individual genes (Sudarsanam et al., 2000). Observations from this analysis also revealed that SWI/SNF is required for the repression of certain genes (Sudarsanam et al., 2000). Therefore, SWI/SNF provides an example of the potential for ATP-dependent chromatin remodeling factors to participate in both activation and repression.

SWI/SNF-like complexes have been identified in higher eukaryotes, where Brahma (Brm) is the SWI/SNF homolog in *Drosophila* and BRG1 and BRM1 have been identified as the SWI/SNF homologs in humans (Elfring et al., 1994; Wang et al., 1996). In an experiment where the Swi2/Snf2 ATPase/helicase motif of BRG1 is replaced with that of Snf2 in *S. cerevisiae*, the heterologous protein rescued transcriptional activation in *snf2* cells, suggesting functional conservation between yeast SWI/SNF and human SWI/SNF (Khavari et al., 1993).

1.3.4.2 The RSC chromatin remodeling complex

Individual components of the RSC chromatin remodeling complex were identified in part by sequence homology with components of SWI/SNF (Table 1) (Du et al., 1998). Sth1, the catalytic component of RSC shares extensive sequence similarity with Snf2 yet, Sth1 is essential (Du et al., 1998). Other essential components of the RSC complex, Rsc6, Rsc8, and Sfh1, were identified by the Kornberg lab based on their sequence similarity to Snf12, Swi3, and Snf5 respectively (Angus-Hill et al., 2001; Cairns et al., 1996; Cao et al., 1997; Du et al., 1998). Importantly, RSC complex purified from yeast extract exhibits DNA-dependent ATPase activity, the ability to remodel nucleosomes in vitro, and the ability to transfer a histone octamer to naked DNA in vitro (Kornberg and Lorch, 1999; Saha et al., 2002). Mutation of *SFHI* resulted in cell cycle arrest and yeast extracts from synchronized cells revealed that Sfh1 is controlled by phosphorylation in the S phase of mitosis (Cao et al., 1997). These observations suggested that the chromatin remodeling activity of RSC is regulated by the cell cycle in yeast (Cao et al., 1997; Du et al., 1998).

Evidence in support of a role for RSC at the level of chromatin remodeling came from both genetic and biochemical studies. For example, temperature sensitive mutations in *STH1* combined with deletion of histone genes cause enhanced lethality at increased temperatures (Du et al., 1998). Not only do members of RSC genetically interact with genes encoding the histone proteins but RSC physically associates with core histone particles (Saha et al., 2002; Saha et al., 2005). Further, using reconstituted nucleosomal arrays and in vitro assays the Peterson lab demonstrated that RSC and SWI/SNF both remodel nucleosomal arrays and the histone tails are required for this activity (Logie et al., 1999).

Due to the high abundance of RSC complex as compared to SWI/SNF in *S. cerevisiae*, it is possible that RSC provides essential chromatin remodeling activities while other chromatin remodeling complexes like SWI/SNF share some overlapping function with RSC. Interestingly, genetic and biochemical studies have provided evidence for two distinct RSC complexes that may have partially overlapping roles (Cairns et al., 1999; Ng et al., 2002). The two RSC complexes differ in that one RSC complex contains Rsc1 and the other contains Rsc2 (Cairns et al., 1999; Ng et al., 2002). Rsc1 and Rsc2 contain similar domains including two bromodomains, a bromo-associated homology domain, and an AT hook domain (Cairns et al., 1999). Identification of RSC component homologs in metazoans is complicated by the high sequence similarities shared across the subunits of RSC and SWI/SNF complexes (Muchardt and Yaniv, 2001). Mammalian cells contain the two proteins Brg and Brm that exhibit similarity with Snf2/Swi2 (Mohrmann et al., 2004; Wang et al., 1996). Brg and Brm containing complexes purify with proteins similar to the yeast SWI/SNF complex (SWI/SNF-A or BAF) (Mohrmann et al., 2004; Wang et al., 1996). While Brg copurifies with another set of proteins to form SWI/SNF-B or PBAF, this complex is distinct from Brg or Brm containing SWI/SNF-A complexes since it contains Polybromo, a component that contains six bromodomains and shares sequence similarity within and outside the bromodomains of Rsc1, Rsc2 and Rsc4 (Mohrmann et al., 2004). Further investigation must be performed if the mammalian RSC homolog is to be identified.

1.3.4.3 The conserved "imitation switch" chromatin remodeling factors

Imitation switch or ISWI was isolated in a screen to identify novel members of the SWI/SNF family in *Drosophila* (Table 1) (Elfring et al., 1994). Similar to SWI/SNF, ISWI proteins contain a domain with similarity to the DNA-dependent ATPase domain of SWI/SNF

(Elfring et al., 1994). ISWI is distinguished further from SWI/SNF in that ISWI contains a SANT domain. SANT domains are associated with chromatin remodeling and have been identified in Swi3, Ada2, N-COR, TFIIB B" (Tsukiyama et al., 1999). Not only is ISWI speculated to function as a chromatin remodeling protein based on its primary amino acid sequence, but another group identified *Drosophila* ISWI as a component necessary to remodel chromatin in vitro (Tsukiyama et al., 1999). Recombinant ISWI and ISWI associated with NuRF or CHRAC can mobilize nucleosomes by sliding by a mechanism in which nucleosomes remain intact (Hamiche et al., 1999; Langst et al., 1999). Two ISWI homologs were identified in *S. cerevisiae* using the primary amino acid sequence of *Drosophila* ISWI (Tsukiyama et al., 1999). Both Isw1 and Isw2 associate within distinct protein complexes in vivo and each complex is associated with ATP dependent nucleosome remodeling activity like their *Drosophila* homologs (Tsukiyama et al., 1995; Tsukiyama et al., 1999).

The nucleosome sliding ability of ISWI is important for transcriptional elongation and termination in *S. cerevisiae* (Morillon et al., 2003). In fact, microarray analysis identified genes that are both positively and negatively regulated by Isw1 and Isw2 (Fazzio et al., 2001; Vary et al., 2003). Isw1 localizes to the open reading frames of transcriptionally active genes (Morillon et al., 2003). Defects associated with proper Isw1 function include loss of signal for CTD Ser-2 and Ser-5 phosphorylation and histone H3 methylation along actively transcribed genes (Morillon et al., 2003). Interestingly, Isw1 associates with trimethylated histone H3 K4 (Santos-Rosa et al., 2003). Association of Isw1 with transcribed genes may therefore depend on interactions between methylated histone H3 and Isw1. Transcription run-on assays indicate that catalytic Isw1 mutants are defective for proper transcription termination (Morillon et al., 2003). Together, these observations indicate that interaction between Isw1 and trimethylated histone H3

K4 may be important to target Isw1 to actively transcribed genes and that the conserved ATPase domain of Isw1 is important for transcription termination.

1.3.4.4 The Chd1 chromatin remodeling factor regulates transcription

The conserved chromatin remodeling factor Chd1 is involved in remodeling chromatin and associates with the open reading frames of actively transcribed genes (Table 1) (Kelley et al., 1999; Simic et al., 2003; Stokes and Perry, 1995; Stokes et al., 1996; Tran et al., 2000). Chd1 possesses two chromodomains, a DNA binding domain and the SNF2/SWI2 ATPase/helicase domain (Delmas et al., 1993). Chromodomain-containing factors have been implicated in chromatin related processes including remodeling of chromatin (Brehm et al., 2004; Jones et al., 2000). Recent evidence indicates that a chromodomain of Chd1 in yeast is important for interaction between Chd1 and methylated histone H3 K4 (Pray-Grant et al., 2005). Chd1 biochemically associates with the yeast coactivator complexes SAGA and SLIK, which share similar protein components (Pray-Grant et al., 2005).

In addition to a role for Chd1 in coactivator function, Chd1 is also required for the transcription of a subset of genes. Microarray analyses indicated that Chd1 positively and negatively regulates the expression of certain genes (Tran et al., 2000). The requirement for Chd1 was shown to be direct for a sample of Chd1 regulated genes (Tran et al., 2000). The ATPase/helicase activity of Chd1 may be important for its role in transcriptional regulation. Evidence for this possibility came from a synthetic lethality screen performed with a *chd1Δ* strain, which indicated that *SNF2* became essential in the absence of *CHD1* (Tran et al., 2000). This genetic interaction suggests that Chd1 and Swi2 possess similar or overlapping functions in the cell. In support of this idea, purified yeast Chd1 was shown to alter interactions between DNA and nucleosomes in an ATP-dependent fashion (Tran et al., 2000). Importantly, the

nucleosome remodeling activity differed from SWI/SNF in in vitro assays, supporting the notion that these proteins are required for different aspects of nucleosome organization. The chromatin remodeling activity of Chd1 may be important for transcription elongation. This hypothesis was tested by performing ChIP (ChIP) with an epitope-tagged version of Chd1 and measuring the relative occupancy of Chd1 along actively transcribed genes (Simic et al., 2003). Importantly, Chd1 associated with transcribed open reading frames (Simic et al., 2003). The occupancy of Chd1 over the genes analyzed in this study indicated that Chd1 association was dependent in part on an RNA polymerase II-associated protein Rtf1 and that Rtf1 and Chd1 associated in vivo through Co-IP and two-hybrid analysis (Simic et al., 2003).

1.3.4.5 The INO80 chromatin remodeling complex

INO80 was identified in a genetic screen to identify mutants that exhibit inositol auxotrophy (Table 1) (Ebbert et al., 1999). The primary amino acid sequence of Ino80 revealed significant sequence similarity to the SWI/SNF catalytic subunit (Ebbert et al., 1999). Biochemical analysis of Ino80 indicated that it was a component of a high molecular weight complex (Ebbert et al., 1999; Shen et al., 2000). Mutations in the INO80 complex caused defects in the induction of the phosphate inducible gene *PHO5* that correlated with impaired nucleosome positioning over the *PHO5* promoter (Steger et al., 2003). Identification of INO80 subunits indicated that actin-related proteins are members of the INO80 complex (Shen et al., 2003). Interestingly, actin-related protein 8 (Arp8) associates with histones and exhibits a preference for histone H3 and H4 (Shen et al., 2003). These observations indicate that the Arp8 component of the INO80 complex may be important for INO80 to establish association with nucleosomes prior to nucleosome mobilization. Additional characterization of Ino80 revealed that Ino80 possessed ATPase activity as predicted based on sequence similarity to Snf2. Unlike Snf2, Ino80 associates

with Rvb1 and Rvb2 and *ino80Δ* mutations confer sensitivity to hydroxyurea, methyl methanesulphonate, as well as ionizing and ultraviolet radiation. Rvb1/Rvb2 are similar to the RuvB protein in bacteria that is involved in double strand break repair and recombination (Shen et al., 2000). Together, these observations indicate that INO80 is important for DNA repair as well as transcription.

1.3.4.6 Swr1 is a Snf2 paralog that catalyzes the replacement of histone H2A with H2A.Z

An attempt to identify the mechanism of histone variant substitution uncovered another Snf2-related protein, Swr1 (Table 1). Affinity purification of Swr1 followed by mass spectrometry identified Htz1, the histone H2A.Z variant, and several components of the Swr1 complex (Kobor et al., 2004; Mizuguchi et al., 2004). Members of the Swr1 complex included several proteins that possess domains associated with chromatin remodeling (Kobor et al., 2004). One interesting observation was that certain components of the Swr1 complex have been identified as members of the INO80 chromatin remodeling complex and the NuA4 HAT complex (Kobor et al., 2004; Mizuguchi et al., 2004). Unlike mutations in *INO80*, *swr1Δ* mutants do not exhibit sensitivity to hydroxyurea, ultraviolet radiation, or methanesulphonate (Mizuguchi et al., 2004). Microarray analyses provided evidence that Swr1 and Htz1 are required for the repression of an overlapping set of genes (Kobor et al., 2004; Krogan et al., 2003; Meneghini et al., 2003; Mizuguchi et al., 2004). The subset of genes co-regulated by Swr1 and Htz1 reside close to telomeres (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). ChIP localized Htz1 to the promoter or 5' region of Htz1-target genes. Htz1 association at these regions was reduced in the absence of Swr1 (Krogan et al., 2003; Mizuguchi et al., 2004; Raisner et al., 2005). Recent observations also point to the incorporation of H2A.Z within nucleosomes

positioned over the promoter region of both active and inactive genes (Raisner et al., 2005). However, Htz1 density mapping indicated that Htz1 is also incorporated within TATA-less promoters of repressed genes. These results in conjunction with localization analyses performed by the Greenblatt lab support the idea that incorporation of the H2A.Z variant may function like a boundary element to prevent the spreading of heterochromatin from the telomere (Krogan et al., 2003; Meneghini et al., 2003). Remarkably, the Wu lab demonstrated that the Swr1 complex catalyzes the ATP-dependent exchange of nucleosomal H2A with Htz1 in vitro (Mizuguchi et al., 2004). This group postulated that the exchange of other histone variants may also be catalyzed by other chromatin remodeling complexes that contain a Snf2-related protein (Mizuguchi et al., 2004).

1.3.5 Chromatin modifying proteins

Chromatin modifying proteins are an emerging class of post-translational modification enzymes that target specific residues within histone proteins. These modifications have been associated with long term epigenetic effects on transcriptional activity and transcriptional repression. Unlike chromatin remodeling factors, these enzymes do not require ATP hydrolysis for chromatin modification, but they do require certain co-factors; for example S-adenosylmethionine is a cofactor involved in methylation of certain histone residues by SET domain containing methyltransferases (Trievel et al., 2002; Trievel et al., 2003). As mentioned previously, a wide range of histone modifications occur including phosphorylation, methylation, acetylation, and ubiquitylation, which have important implications for transcription in yeast and metazoans.

1.3.5.1 Set1 and Set2 methylate histone H3 K4 and K36 respectively

The lysine and arginine residues within histone proteins are targets for methylation and this reaction is catalyzed by a class of protein that contains SET (Su(var)39, Enhancer of zeste, Trithorax) domains (Briggs et al., 2001). In particular, Set1 and Set2 are two SET domain containing proteins that are responsible for the methylation of histone H3 K4 and histone H3 K36, respectively (Table 1) (Hampsey and Reinberg, 2003; Strahl et al., 2002). Methylation of histone H3 K4 and K36 is not essential for viability, nor are the proteins that catalyze the methylation (Briggs et al., 2001). Set1 catalyzes the methylation of mono-, di-, or trimethylation on histone H3 K4 (Briggs et al., 2001). The Set1 protein contains motifs in addition to the SET domain, including an RNA recognition motif (RRM) and a central region that do not have functions ascribed to them. However, the RRM domain can exert positive effects on methylation by Set1 and the central domain can inhibit methylation by Set1 (Schlichter and Cairns, 2005). Importantly, histone H3 K4 trimethylation is enriched at the 5' region of actively transcribed genes as analyzed by ChIP experiments and recruitment of Set1 relies on the phosphorylation of the CTD by TFIIF (Ng et al., 2003). Not surprisingly, Set1 and members of the Set1-methyltransferase complex (COMPASS) also associate with this region of actively transcribed genes (Ng et al., 2003). Trimethylation of histone H3 is a stable epigenetic mark for recently transcribed genes (Ng et al., 2003). The histone methyltransferase activity of Set1 is implicated in the regulation of certain genes, including *PHO5* and the *GAL* genes, which are typically repressed during growth in rich medium (Carvin and Kladde, 2004). Together, Set1 recruitment and histone H3 K4 methylation are functionally linked to the regulation of transcription elongation.

Recruitment of Set2 to actively transcribed genes is also dependent upon the phosphorylation state of RNA polymerase II. However, unlike Set1, Set2 does not require prior histone H2B K123 ubiquitylation for methylating histone H3 K36 (Briggs et al., 2002; Strahl et al., 2002). Also unlike Set1, Set2 is required for histone H3 K36 methylation at the promoter and the open reading frames of actively transcribed genes (Krogan et al., 2003; Xiao et al., 2003). Affinity purification of Set2 and RNA polymerase II revealed reciprocal interactions between RNA polymerase II and Set2 (Li et al., 2002; Xiao et al., 2003). Several observations indicated that Set2 interacted with the phosphorylated form of RNA polymerase II. First, association of affinity purified Set2 with GST-CTD was enhanced when the GST-CTD had been incubated with HeLa nuclear extract and ATP (Li et al., 2002). Second, RNA polymerase II phosphorylated on Ser-2 and Ser-5 was identified in epitope-tagged Set2 immunoprecipitates by immunoblotting (Krogan et al., 2003; Xiao et al., 2003). Third, histone H3 K36 methylation was reduced in strains that contained truncation mutations within the CTD or cells that did not contain the Ser-2 specific CTD kinase (Xiao et al., 2003). Lastly, the domain required for the Set2 and Rpb1 interaction (SRI) was mapped to the carboxyl terminal region of Set2 (Kizer et al., 2005). Using unmodified, Ser-2 phosphorylated, Ser-5 phosphorylated, or Ser-2 and Ser-5 doubly phosphorylated synthetic CTD peptides, the Strahl lab demonstrated that the SRI bound with the greatest affinity to the doubly modified CTD peptides (Kizer et al., 2005). Interestingly, mutations in *SET2* confer sensitivity to the base analog 6AU similar to mutations in genes that code for elongation factors (Krogan et al., 2003; Li et al., 2002). Furthermore, using *set2Δ* cells in synthetic genetic analysis by crossing the strain to cells with deletions within genes involved in elongation uncovered genetic interactions with *SET2* and the genes coding for subunits of COMPASS, the Paf1 complex and the proteins required for histone H2B K123 ubiquitylation

(Krogan et al., 2003). These results provide strong support for the role of Set2 and histone H3 K36 methylation in participating in RNA polymerase II transcript elongation.

1.3.5.2 Rad6-Bre1 ubiquitylate histone H2B on lysine 123 (K123)

The activities of Rad6 and Bre1 are required for the ubiquitylation of histone H2B K123 and this histone modification is conserved in higher eukaryotes. Rad6 and Bre1 possess ubiquitin conjugating and ubiquitin ligase activity, respectively (Table 1). Histone H2B K123 monoubiquitylation does not target histone H2B for degradation via the proteasome degradation pathway, rather this modification is associated with actively transcribed genes. It is noteworthy that Rad6 is involved in other cellular processes that require its ubiquitin conjugating activity (Ulrich, 2002). Components of the Paf1 complex are required for normal levels of histone H2B K123 ubiquitylation (Ng et al., 2003; Wood et al., 2003; Xiao et al., 2005). Moreover, Rad6-Bre1 associate with actively transcribed genes and hyperphosphorylated RNA polymerase II (Xiao et al., 2005). Rad6 is recruited to the promoters of genes in a Paf1 complex-independent manner but occupancy of Rad6 and histone H2B K123 ubiquitylation over the coding regions of genes is dependent upon the Paf1 complex (Wood et al., 2003; Xiao et al., 2005). Interestingly, Rad6 activity at the promoter and within the coding regions of genes is dependent upon Bre1 and the Paf1 complex (Xiao et al., 2005). The activity of Rad6 is also dependent on the CTD and the Ser-2 and Ser-5 kinases, which is consistent with a role for Rad6 in the transition between transcription initiation and elongation (Xiao et al., 2005). The 6AU sensitivity of *rad6Δ* strains and strains where the only form of histone H2B is replaced with histone H2B K123R is also in agreement with a role for Rad6 and ubiquitylation of H2B K123 in elongation (Xiao et al., 2005). Histone H2B K123 ubiquitylation occurs transiently at the promoters of actively transcribed genes where ubiquitylation and deubiquitylation, by the ubiquitin specific protease

Ubp8 component of the SAGA/SLIK coactivator, is required for activation of transcription (Henry et al., 2003). Together, these observations indicate that Rad6 and Bre1 are required for transcription elongation.

1.3.6 Factors required for RNA polymerase II elongation processivity

The general term transcription elongation factor implies that the function of this class of proteins is important for the processivity and elongation rate of RNA polymerase II. Reference to processivity in this context describes the ability of RNA polymerase II to traverse the length of the coding region of a gene while elongation rate conveys information regarding the time it takes to transcribe the length of a given gene (Mason and Struhl, 2005). Recent analysis of a collection of RNA polymerase II-associated factors revealed that only members of the THO complex and Spt4 were required for processive elongation (Mason and Struhl, 2005). Treatment of cells with 6AU, a drug that lowers intracellular pool of UTP and GTP, revealed a decrease in processivity in wild-type cells as determined by the post-transcriptional density of RNA polymerase II on a model gene (Mason and Struhl, 2005). This processivity defect was aggravated in cells lacking transcription elongation factor IIS (TFIIS) or the Ser-2 CTD kinase (Table 1) (Mason and Struhl, 2005). Collectively, the observations from this study argue against a role for RNA polymerase II-associated factors in contributing to the elongation rate of transcribing polymerase (Mason and Struhl, 2005). Rather, protein complexes associated with RNA polymerase II are important for the processivity of the enzyme since the ability of RNA polymerase II to transcribe to the end of the gene was compromised when cells lacked Spt4 or members of the THO complex (Mason and Struhl, 2005). This study also reinforced the idea that RNA polymerase II-associated factors may be redundant with respect to their role in RNA polymerase II processivity because a need for

processivity factors was not required unless the cell was insulted with drugs that presumably reduce cellular levels of nucleotides (Mason and Struhl, 2005).

Components of RNA polymerase II and the transcription elongation factor TFIIS contribute to processivity of RNA polymerase II. The analysis by Mason and Struhl stressed the connection between elongation rate and processivity such that elongation rate of RNA polymerase II was dependent upon the enzyme's processivity (Mason and Struhl, 2005). They also suggested that elongation rate of RNA polymerase II is determined by the empirical enzymatic properties of RNA polymerase II (Mason and Struhl, 2005). Interestingly, RNA polymerase II and TFIIS are physically and functionally linked, where TFIIS stimulates the intrinsic nuclease activity of RNA polymerase II as reviewed in (Wind and Reines, 2000). The RNA cleavage activity is provoked in response to RNA polymerase II pausing or arrest that is induced by certain DNA templates (reviewed in (Wind and Reines, 2000)).

1.4 Regulation of 3'-end processing and transcription termination

1.4.1 Transcript cleavage factors form the 3'-ends of polyadenylated and nonpolyadenylated transcripts

RNA polymerase II generated transcripts must be cleaved at their 3'-ends prior to maturation. Factors important for transcript maturation were originally identified biochemically, based on their ability to either endonucleolytically cleave pre-mRNA or catalyze the cleavage and polyadenylation of pre-mRNA (Edmonds, 2002). Genetic studies in yeast have also contributed to the identification of additional components of the cleavage and polyadenylation machinery required for 3'-end formation (Ahn et al., 2004; Barilla et al., 2001; Cheng et al., 2004; Cui and Denis, 2003; Dichtl et al., 2004; Hirose and Manley, 1998; Kaplan et al., 2005; Kim et al., 2004; Komarnitsky et al., 2000; Krishnamurthy et al., 2004; Licatalosi et al., 2002; Meinhart and Cramer, 2004; Nedea et al., 2003). Recent studies have demonstrated that the cleavage and polyadenylation machinery associate with actively transcribing RNA polymerase II and their recruitment is dependent upon phosphorylation of Ser-2 of the CTD.

Two general modes of 3'-end formation take place in eukaryotes: poly(A)-dependent and poly(A)-independent. Both varieties utilize RNA elements, whether the elements recognized are poly(A) sites as in the polyadenylation pathway or the specific four nucleotide RNA motifs identified by either Nab3 or Nrd1 in the poly(A)-independent pathway (Carroll et al., 2004; Steinmetz and Brow, 1996). RNA sequence recognition is followed by endonucleolytic cleavage to produce 3'-ends that are subsequently processed. The pathways diverge such that

polyadenylated transcripts acquire poly(A) tails and nonpolyadenylated transcripts are processed by the exosome, a complex containing 3'-5' exonucleases important for producing mature 3' ends of sn/snoRNAs (Allmang et al., 1999; Allmang et al., 1999).

Some proteins have been demonstrated to participate in both types of 3'-end formation while others do not appear to have overlapping roles in 3'-end formation (Dheur et al., 2003; Morlando et al., 2002; Nedea et al., 2003; Steinmetz and Brow, 2003). A complex important for proper 3'-end formation of both types of transcripts is termed the holo-CPF in yeast (Nedea et al., 2003). The APT (Associated with the Pta1 subunit of CPF) subcomplex of CPF bridges CPF with CFIA (He et al., 2003; Nedea et al., 2003). Importantly, mutations within the components of CFIA and APT cause transcriptional read through of certain snoRNAs. The role of the holo-CPF is most likely direct because ChIP results indicate the presence of CPF components at these loci (Nedea et al., 2003). The Pti1 and Ref2 subunits of the APT complex are proposed to uncouple cleavage from polyadenylation during 3'-end formation of snoRNA messages (Dheur et al., 2003; Nedea et al., 2003).

The phosphorylation state of the CTD and its implications in RNA processing is more established than that of elongation factors in 3'-end formation. While the Paf1 complex associates with RNA polymerase II over protein coding genes, work from the Buratowski laboratory has shown that the Paf1 complex dissociates from RNA polymerase II near the poly(A) site (Kim et al., 2004). In addition, deletion of Paf1 complex members causes bulk poly(A) tail length of mRNAs to be shorter than poly(A) tails in wild-type cells (Mueller et al., 2004). Therefore, it is possible that the Paf1 complex collaborates in pathways to correctly couple cleavage and polyadenylation for mRNAs and to uncouple cleavage and polyadenylation for *SNR* transcripts with the APT module of holo-CPF. Recent observations from the Jaehning

lab support this idea because absence of Paf1 results in 3'-extended mRNAs (Penheiter et al., 2005).

1.4.2 Transcript termination of RNA polymerase II

RNA polymerase II must complete the transcription cycle by dissociating from the DNA template thus allowing RNA polymerase II re-entry within the transcription cycle at the stage of initiation. The CTD itself is important for mediating transcription termination (Kim et al., 2004). Interactions between the CTD and poly(A) sites within the mRNA transcript allow for termination through a mechanism that is initiated by cleavage at the poly(A) site and subsequent degradation of the 5'-cleaved transcript (Dye and Proudfoot, 2001; Kaneko and Manley, 2005; Kim et al., 2004; West et al., 2004). The yeast RNA polymerase II-associated transcription termination factor Rat1 is a 5'-3' exonuclease that recognizes the 5'-cleaved ends of mRNAs and degrades them (Kim et al., 2004). Once Rat1 reaches the RNA polymerase II it is possible that Rat1 destabilizes RNA polymerase II-DNA associations causing dissociation of the enzyme in a manner reminiscent to rho-dependent transcription termination in bacteria. There rho hydrolyzes ATP to destabilize interactions between RNA polymerase and the template (Richardson, 2002).

1.5 Rationale to Investigate the role of Rtf1 and members of the Paf1 complex in transcription and identification of novel function(s) of the Paf1 complex

Evidence from our lab implicated a functional role for Rtf1 in transcription elongation by RNA polymerase II. Mutations in genes whose protein products are important for the transition from transcription initiation to elongation exhibited severe synthetic defects when combined with a deletion of *RTF1* (Costa and Arndt, 2000). Subsequent biochemical experiments performed in our lab and others placed Rtf1 in the context of a multiprotein complex that also contains Paf1, Ctr9, Cdc73, and Leo1, known as the Paf1 complex (Chang et al., 1999; Krogan et al., 2002; Mueller et al., 2004; Squazzo et al., 2002). Significantly, the Paf1 complex associates with RNA polymerase II along the open reading frames of actively transcribed genes. This evidence strongly indicated the Paf1 complex may regulate events that occur during elongation. However, we could not completely eliminate the possibility that the Paf1 complex functioned in transcription initiation since the Paf1 complex was identified based on its association with RNA polymerase II and general transcription factors and because mutations within *RTF1* suppressed the altered DNA binding specificity of a TBP mutant (Chang et al., 1999; Shi et al., 1997; Stolinski et al., 1997; Wade et al., 1996).

The role of the Paf1 complex in transcription was somewhat controversial when I began my thesis research. We developed a research plan that would center our efforts on identifying missense mutations within *RTF1* that could be used in downstream genetic and expression analyses. Ideally, the point mutants we recovered could be used to identify genes that in high

dosage could suppress their mutant phenotypes. Through the identification and characterization of multicopy suppressors of *rtf1*, we speculated that the function ascribed to these suppressors would clarify the function of the Paf1 complex in transcription or uncover new roles for the Paf1 in transcription or other processes. During the isolation of *rtf1* mutants and characterization of multicopy suppressors, a complementary study was ongoing. We sought Paf1-dependent target genes using microarrays. Identification of genes that require the Paf1 complex for their transcriptional regulation could be valuable tools for further evaluating the role of the Paf1 complex in transcription initiation and elongation. These two approaches were successful in that they led to the discovery that the Paf1 complex is involved in forming 3'-ends of certain RNA polymerase II transcripts and the Paf1 complex physically associates with RNA polymerase II transcribed genes for their repression.

2.0 Chapter 2: Identification of multicopy suppressors of a conditional *rtf1* mutation

2.1 Introduction

The affinity purification of Rtf1 revealed that Rtf1 is a component of the Paf1 complex. This observation offers a framework for the function of Rtf1 as well as other members of the Paf1 complex in the cell. While previous studies implicated Rtf1 and the Paf1 complex in RNA polymerase II transcription, the roles of the Paf1 complex during transcription are not well understood.

Several attributes have been assigned to the Paf1 complex based on numerous biochemical and genetic studies. For example, RNA polymerase II, elongation factors involved in modulating histone octamer integrity during transcription, and proteins known to remodel nucleosomes during active transcription interact physically with the Paf1 complex (Krogan et al., 2002; Lindstrom et al., 2003; Mueller and Jaehning, 2002; Simic et al., 2003; Squazzo et al., 2002). Additionally, ChIP experiments indicate that the Paf1 complex, RNA polymerase II and other known elongation factors co-localize to transcribed genes. Genetic interactions have been reported for deletions of individual members of the Paf1 complex as well as studies that revealed deletion of members of the Paf1 complex affect elongation and histone modification (Ng et al., 2003; Ng et al., 2003; Rondon et al., 2004; Xiao et al., 2005). Together, these observations strongly support a role for the Paf1 complex in transcription elongation through an effect on chromatin structure. However, recent work from the Struhl lab suggests that the Paf1 complex

and other reported elongation factors may not have a role in RNA polymerase II elongation efficiency or processivity in vivo (Mason and Struhl, 2005). Alternatively, in vitro evidence indicates that efficient transcription elongation on naked DNA requires certain members of the Paf1 complex (Rondon et al., 2004; Rondon et al., 2003). The results from the Struhl lab suggests that while the Paf1 complex may be associated with actively transcribing RNA polymerase II the precise function of the Paf1 complex remains to be resolved. While it is odd that transcription elongation factors may not directly affect RNA polymerase II transcription efficiency or processivity, results from the Jaehning lab offered some insight into this conundrum by uncovering evidence that an important role of the Paf1 complex may occur off the DNA template (Mueller et al., 2004; Penheiter et al., 2005; Yart et al., 2005). Specifically, deletion of *paf1Δ* resulted in RNA polymerase II transcripts that exhibited shorter than average poly(A) tails, altered poly(A) site utilizations and changes in rRNA processing . The intriguing possibility is that the function of the Paf1 complex involves the bridging or coordination of events occurring on the template with those occurring off the template, however this remains to be elucidated. To better define the role of Rtf1 and the Paf1 complex in transcription we sought additional proteins that functionally interact with Rtf1.

To identify proteins that functionally or physically interact with Rtf1 we chose to perform a high copy number genetic screen with specific *rtf1* missense mutations. First, we generated temperature sensitive *RTF1* alleles using error-prone PCR. These mutants were screened for conditional 6-azauracil (6AU) sensitivity and subsequently for conditional Spt⁻ phenotypes. Three novel *rtf1* missense mutations were identified and characterized. We chose one *rtf1* mutation for the dosage suppression screen, *rtf1-107*. This mutation results in the substitution of methionine 289 for lysine. The *rtf1-107* mutation confers a conditional 6AU phenotype as well

as a weak Spt⁻ phenotype at the permissive temperature. A high copy number library was transformed into this strain to identify multicopy suppressors of the *rif1-107* mutant. Many suppressor candidates were identified, however, we focused on one candidate, *NAB3*. Nab3 was of particular interest since it is functionally linked to RNA polymerase II transcript termination (Steinmetz et al., 2001).

2.2 Methods and materials

2.2.1 Yeast strains and methods

Rich (YPD), synthetic complete (SC), synthetic dextrose (SD), and 5-fluoroorotic acid (5FOA) media were prepared as described previously (Rose et al., 1990). Media containing 50µg/ml of the base analog 6AU were prepared essentially as described (Squazzo et al., 2002). Unless otherwise indicated, the yeast strains used in this study are isogenic with FY2 (Winston et al., 1995), a *GAL2*⁺ derivative of S288C (Table 2). Strains were constructed by tetrad dissection or transformation (Rose et al., 1990). All deletion strains were created using a PCR-based method (Ausubel et al., 1988) with the exception of the *nrd1*Δ strain, KKY100, which was constructed by using the disruption plasmid pRS316-*nrd1*Δ::*HIS3* (Steinmetz and Brow, 1996) to replace the essential *NRD1* gene in the diploid strain KKY97.

Table 2: Yeast strains used in Chapter 2

STRAIN	GENOTYPE	ORIGIN
AY5	<i>MATa rtf1Δ102::ARG4 his4-912δ lys2-173R2 leu2Δ1 trp1Δ63 arg4-12</i>	Arndt lab
AY6	<i>MATα rtf1Δ102::ARG4 his4-912δ lys2-173R2 trp1Δ63 ade8 arg4-12</i>	"
KY406	<i>MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8</i>	"
KY410	<i>MATα rtf1Δ101::LEU2 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63</i>	"
KY562	<i>MATa rtf1Δ102::ARG4 his3Δ200 lys2-173R2 ura3-52 trp1Δ63 arg4-12</i>	"
KY656	<i>MATa rtf1Δ100::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52</i>	"
KY664	<i>MATα his3Δ200 his4-912δ leu2Δ1 ura3-52 trp1Δ63</i>	"
KY767	<i>MATa his4-912δ leu2Δ1 trp1Δ63 ade8</i>	"
KKY37	<i>MATα rtf1-105 his4-912δ lys2-128δ leu2Δ1 ura3-52 ade8</i>	"
KKY40	<i>MATα rtf1-100 his4-912δ lys2-128δ leu2Δ1 ura3-52 ade8</i>	"
KKY45	<i>MATα rtf1-107 his4-912δ lys2-128δ leu2Δ1 ura3-52 ade8</i>	"
KKY54	<i>MATa rtf1-100 his4-912δ leu2Δ1 trp1Δ63</i>	"
KKY56	<i>MATα rtf1-100 his4-912δ leu2Δ1 trp1Δ63</i>	"
KKY58	<i>MATα rtf1-107 his4-912δ leu2Δ1 trp1Δ63</i>	"
KKY60	<i>MATα rtf1-107 his4-912δ leu2Δ1 trp1Δ63 ade8</i>	"
KKY64	<i>MATα rtf1-105 his4-912δ leu2Δ1 trp1Δ63</i>	"
KKY97	<i>MATa/MATα rtf1Δ102::ARG4/RTF1 his3Δ200/" HIS4/his4-912δ lys2-173R2/LYS2 LEU2/ leu2Δ1 ura3-52/" trp1Δ63/" arg4-12/ARG4</i>	"
KKY100	<i>MATa/MATα rtf1Δ102::ARG4/RTF1 NRD1/nrd1Δ::HIS3 his3Δ200/" HIS4/his4-912δ lys2-173R2/LYS2 LEU2/leu2Δ1 ura3-52/" trp1Δ63/" arg4-12/ARG4</i>	"
KKY128	<i>MATα rtf1Δ102::ARG4 nrd1Δ::HIS3 arg4-12 or ARG4 his3Δ200 his4-912δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63 pRS316-NRD1</i>	"
KKY129	<i>MATa nrd1Δ::HIS3 arg4-12 or ARG4 his3Δ200 lys2-173R2 ura3-52 trp1Δ63 pRS316-NRD1</i>	"
KKY143	<i>MATa nrd1Δ::HIS3 cdc73Δ::KANMX4 his3Δ200 lys2-173R2 ura3-52 trp1Δ63 pRS316-NRD1</i>	"
nrd1-5	<i>MATα nrd1-5 cup1Δ his3 lys2 leu2 ura3 trp1 ade2</i>	Brow lab

2.2.2 Plasmids

Standard techniques were used for plasmid construction, isolation, and transformation of *S. cerevisiae* and *Escherichia coli* (*E. coli*) strains (Ausubel et al., 1988; Rose et al., 1990). *RTF1* was subcloned into a pRS314 vector backbone and subsequently amino terminally tagged using a triple HA1 epitope (Stolinski et al., 1997).

Library plasmid 305 contains a genomic fragment that encodes the essential gene *NAB3*. *NAB3* was subcloned from 305 into pRS425 by ligating a 3.8 kb *SwaI-NcoI* end-filled fragment into the *SmaI* digested pRS425 vector resulting in pKK5. This plasmid was transformed into KKY58 to confirm that *NAB3* suppressed the *Spt⁻* phenotype of *rtf1-M289K* in high copy number.

Library plasmid 309 contains the *RTG3* gene within the genomic fragment. This construct was digested with *NcoI* and *SwaI* releasing a fragment of 2.1 kb in length containing *RTG3*. The *NcoI* site was treated with Klenow to end-fill the resultant 5' overhang to form a blunt end. This fragment was ligated into the *SmaI* digested pRS425 vector and candidates were identified using restriction analysis.

2.2.3 Isolation and characterization of *rtf1* alleles

2.2.3.1 Error-prone PCR.

A modified PCR protocol was used to incorporate mutations into and amplify *RTF1*. The final reaction conditions included 1X PCR buffer (Promega), 2.5mM MgCl₂ (Promega), 50μM each dNTP, 2 fmole pLS21-5 (*HA3-RTF1* in the pRS314 vector (Stolinski et al., 1997)), 30 pmole LQ30, 30 pmole LQ31, and 1 unit of Taq polymerase (Promega) (Table 3). The PCR cycling conditions utilized were: 1 cycle-95°C for 3 minutes; 35 cycles-94°C for 1 minute, 54°C

for 2 minutes, and 72°C for 3 minutes; 1 cycle-72°C for 10 minutes; and 1 cycle-4°C hold. The 3.6 kb PCR fragments were separated by 1.0% TAE-low melt agarose gel electrophoresis and were gel purified (Wizard PCR Prep, Promega).

Table 3: Oligonucleotide sequences used in Chapter 2

Oligonucleotide Name	5'-Sequence	Purpose
LQ30	GGGAGCCCCCGATTTAGAG	Error-prone PCR <i>RTF1</i>
LQ31	CTGTGGATAACCGTATTACC	Error-prone PCR <i>RTF1</i>
KA109	GCTCGCTTCGCTACTTGGAGCC	Sequence YEp13
KA110	GGCGCCAGCAACCGCACCTGTG	Sequence YEp13

2.2.3.2 In vivo homologous recombination.

pLS21-5 was digested with *EcoRI* and *SpeI* to release a 2.5 kb fragment containing *RTF1* and a 5.0 kb fragment containing vector backbone. The vector backbone was gel purified. Together, the PCR fragments and the pLS21-5 backbone were transformed into AY6 an *rtf1* Δ strain, where in vivo homologous recombination and plasmid selection on SC-Trp introduced *rtf1* fragments into pLS21-5 (Muhlrad et al., 1992).

2.2.3.3 Screen for conditional *rtf1* missense mutations.

Approximately 1800 transformants were screened for the 6AU^S phenotype at both 30°C and 37°C. Those candidates exhibiting sensitivity to 6AU at the elevated temperature were screened for the Spt⁻ phenotype. Plasmids from initial candidates were recovered from yeast and transformed into *E. coli*. Upon re-transformation of the candidate plasmids into KY618, we recovered eight candidates that exhibited conditional 6AU^S and or Spt⁻ phenotypes. Sequence analysis of the original candidates revealed that all contained multiple mutations (Table 4). For a subset of these candidates, mutations were separated by subcloning or site-directed mutagenesis (QuikChange, Stratagene) to obtain single missense mutations. These plasmids were analyzed for the ability to complement the 6AU^S and Spt⁻ phenotypes of the *rtf1* Δ strain. Plasmids that conferred 6AU^S and/or Spt⁻ phenotypes were characterized further (Table 5).

2.2.3.4 Integration of *rtf1* point mutations.

The N-terminal HA1 epitope tag was removed from Rtf1 prior to integration of the *rtf1* alleles into the yeast genome to eliminate any possible influence of the tag on the Rtf1 mutant protein. Digestion of the candidate plasmids was performed with *NdeI* (New England Biolabs

(NEB)) to release the 99 bp fragment encoding the triple HA1 tag. The cohesive *NdeI* ends of the candidate plasmids were ligated using T4 DNA ligase (Roche) using varying concentrations of linearized plasmid to promote intramolecular ligation. Ligation reactions were transformed into *E. coli*, single colonies were purified, and plasmids were isolated from standard overnight cultures. Diagnostic restriction enzyme digests using *BamHI* (NEB) were performed with pLS21-5 and plasmids isolated from the intramolecular ligation reactions to identify plasmids lacking the 99 bp fragment encoding the HA1 epitopes.

The *rtf1-100* (Q172R), *rtf1-105* (V274D), and *rtf1-107* (M289K) mutations were integrated at the endogenous *RTF1* locus by the two-step gene replacement method in KY410 using the *URA3*-marked integrating vector pRS306 (Scherer and Davis, 1979). Plasmids containing the three point mutations were digested sequentially with *SpeI* (NEB) followed by *SalI* to release a 2.7 kb product. pRS306 was also sequentially digested with *SpeI* and *SalI* to linearize a 4.3 kb fragment. The 2.7 kb *SpeI-SalI* fragments containing the *rtf1* missense mutations were ligated with the *SpeI-SalI* linearized pRS306 backbone with T4 DNA ligase. Diagnostic digests were performed to identify plasmids containing the *rtf1* mutation inserts. Integrating vectors containing the proper DNA inserts were digested with *EcoRI*, which linearized the plasmids 5' to the coding region of *RTF1*. DNA fragments were gel purified and transformed into KY410 log phase cultures allowing in vivo recombination to occur between the fragment and the *rtf1Δ* locus using SC-Ura media to maintain the integrating DNA under selection. Single stably, transformed candidates were purified. Transformants were grown on YPD in small patches, scraped into 1ml H₂O with sterile toothpicks, and 100μl of suspension was spread onto solid 5FOA containing media to select for cells that have "looped out" the region of DNA containing *URA3* and *rtf1Δ101::LEU2*. Individual colonies from the 5FOA plate were patched onto YPD and replica

printed to the following medium: YPG, YPD, SC-Ura, SC-Leu and SC complete. Candidates that grew on glycerol containing media and did not grow on medium lacking uracil or leucine were analyzed by Southern analysis to confirm integration at the *RTF1* locus (data not shown).

To ensure that the HA1 epitope tag was not influencing the phenotypes of the original candidates, the phenotypes conferred by the *rtf1* mutations lacking the tag were analyzed using dilution analysis. In brief, saturated overnight cultures were pelleted, washed two times with H₂O, and ten fold serial dilutions were spotted onto media to analyze 6AU^S and Spt⁻ phenotypes at both the permissive and restrictive temperatures.

Table 4: Original *RTF1* mutants and plasmids

ISOLATE	AMINO ACID SUBSTITUTIONS ENCODED BY THE <i>RTF1</i> MUTANT GENE	AMINO ACID SUBSTITUTIONS ANALYZED
pDM1	E264G, V274D	E264G, V274D
pDM2	E186G, V403I, L409STOP	E186G
pDM3	Q172R, K246E, Y341F	Q172R, K246E, Y341F
pDM4	R223G, T282P, deletion at T282	R223G, T282P
pDM5	K41R, D243G, Q409L, K473R, Q502L	None
pDM6	M289K, K495STOP	M289K
pDM7	K246STOP, F253L, F312L, Y327C, Y338F, K391R	None
pDM8	D218G, D236G, Y301H, L345S, K390I	None

Table 5: Isolated *RTF1* point mutants

AMINO ACID SUBSTITUTION	ALLELE DESIGNATION	PHENOTYPE
Q172R	<i>rtf1-100</i>	Conditional Spt⁻
V274D	<i>rtf1-105</i>	Weak Spt⁻, conditional 6AU^S
M289K	<i>rtf1-107</i>	Weak Spt⁻, conditional 6AU^S

2.2.4 Immunoblotting analysis

2.2.4.1 Analysis of Rtf1 protein levels.

Whole cell lysates were prepared as described in (Squazzo et al., 2002; Stolinski et al., 1997) and samples were resolved on 7.5% SDS-polyacrylamide gels. Nitrocellulose filters were probed with anti-Rtf1 antisera diluted to 1:4000 in Blotto (5% w/v dry nonfat milk in TBST; TBST = 50mM Tris-HCl pH 7.4, 150mM NaCl, 0.05% Tween-20) (Squazzo et al., 2002) or anti-L3 antisera diluted to 1:5000 in Blocko (TBST plus 3% w/v BSA) (Villardell and Warner, 1997), which served as the loading control. Immunoreactive proteins were detected by chemiluminescence (Pierce) of HRP-coupled secondary antibodies (Amersham Biosciences) and visualized using BioMax film.

2.2.4.2 Analysis of histone H3 lysine 4 (K4) trimethylation levels.

Protein extracts were prepared essentially as described in (Ng et al., 2003). The extracts were separated on 15% SDS-polyacrylamide gels. Nitrocellulose filters were probed with a 1:2000 dilution of ab8580 (Abcam) in Blotto overnight at 4°C as described in (Ng et al., 2003). This antibody detects the trimethylated K4 residue of histone H3. Filters were stripped and re-probed with anti-L3 antisera and developed as described above.

2.2.5 High copy number suppressor screen

The average size of genomic inserts in the *LEU2*-marked 2-micron library used in this study is approximately 10 kb. We determined that the number of colonies to screen with 99% probability of covering the complete *S. cerevisiae* genome for multicopy suppressors of *rtf1-107* would be approximately 6000 (Ausubel et al., 1988). A pilot transformation was performed to empirically determine the amount of library DNA to transform, the volume and dilution of

transformation mixes to spread, and the number of selective plates required for use in the screen. The actual screen utilized three library pools, where 2 μ g of each library pool were used in the transformation. Three transformation mixes were prepared for each library pool, three plates were spread per transformation mix for a total of 27 plates to screen. To estimate the number of colonies screened, the colonies from three randomly chosen plates were counted and averaged to determine that roughly 13,000 colonies were analyzed in this screen.

The transformants were screened for their ability to suppress the 6AU^S phenotype of *rtf1-107* at 37°C on SC-Leu-Ura medium supplemented with 50 μ g/ml 6AU as compared to SC-Leu-Ura medium alone. Transformants were also screened for the ability to restore the phenotype of the *rtf1* mutants to Spt⁺ at 30°C by comparing growth on SD-Leu-His and SD-Leu plates. In order to identify potential candidates, 388 transformants that conferred suppression of either or both phenotypes were patched onto SC-Leu and re-replica printed to media as described above. Transformants were purified and 79 remained after replica printing. Library plasmids were recovered from yeast and transformed into *E. coli*. To eliminate the possibility that *RTF1* was present within the genomic fragments, candidates were placed into groups based on the appearance of *EcoRV* restriction digest patterns when compared to pLS21-5 or YEp13, the plasmid library vector backbone. Representative library plasmids were retransformed into *rtf1-107* cells. Library plasmids that conferred suppression upon retransformation were sequenced using oligonucleotides KA109 and KA110 that hybridized to YEp13 sequences flanking the insert (Table 3). The *Saccharomyces* Genome Database (SGD) <http://www.yeastgenome.org/> (Balakrishnan et al.), in combination with sequence data of the candidates provided detailed information on each genomic insert.

2.2.6 Analysis of *nrd1-5* combined with deletions of Paf1 complex members

Double deletion strains containing pRS316-*NRD1* were transformed with pRS314, pRS314-*NRD1* or pRS314-*nrd1-5*. Purified *rtf1Δ nrd1Δ* and *cdc73Δ nrd1Δ* transformants were passaged over 5FOA containing medium to evict the pRS316-*NRD1* plasmid. The indicated strains were subjected to dilution analysis on the following types of media and incubated at the designated temperatures: YPG at 30°C and YPD at 30° or 37°C. *paf1Δ nrd1Δ* and *ctr9Δ nrd1Δ* transformants were purified on SC-Trp selective media. Dilution analysis was performed with these cells as in section 2.2.3.4., using the specified media and plates and incubated at the temperatures listed: YPG at 30°C and 5FOA at 30° or 37°C.

2.2.7 Analysis of strains lacking individual components of the Paf1 complex containing 2-micron *NAB3*

Empty vector and high copy number *NAB3* were transformed into wild-type cells and cells deleted for members of the Paf1 complex. Transformants were selected and purified on medium lacking leucine. Dilution analysis was performed as described in section 2.2.3.4., with the indicated media and the plates were incubated at 30°C.

2.3 Results

2.3.1 Isolation of mutations conferring conditional *rtfl* phenotypes

Mutations in *RTF1* and genes encoding other members of the yeast Paf1 complex confer sensitivity to 6AU (6AU) and suppression of transposable element insertion mutations within promoters (Spt phenotype = suppression of Ty) (Krogan et al., 2002; Riles et al., 2004; Squazzo et al., 2002). The Spt⁻ phenotype is associated with defects in both transcription initiation and elongation (Hartzog et al., 2002; Winston and Sudarsanam, 1998). To elucidate the functions of the Paf1 complex, we performed a genetic screen to identify novel missense mutations in *RTF1*. We specifically screened for *RTF1* mutations that confer stronger 6AU^S and Spt⁻ phenotypes at higher growth temperatures (37°C) than at lower growth temperatures (30°C). By screening for conditional mutations we hoped to avoid the isolation of complete loss-of-function alleles. Approximately 1800 transformants were screened for conditional sensitivity to the base analog 6AU; after purification and retransformation eight *rtfl* candidate plasmids conferred temperature sensitivity to 6AU (D. M. Mauger and K. M. Arndt, unpublished observations). Cells containing these *rtfl* alleles were replica printed to media lacking histidine to assay for a temperature sensitive Spt⁻ phenotype. Each plasmid conferred a weak to severe Spt⁻ phenotype comparable to that of an *rtfl*Δ mutant (D. M. Mauger, K. E. Sheldon and K. M. Arndt, unpublished observations). Sequence analysis of the eight *rtfl* alleles suggested that the phenotypes might be due to multiple mutations (Table 4). Therefore, subcloning and site-directed mutagenesis were used to isolate individual missense mutations from candidate plasmids pDM1,

pDM2, pDM3, pDM4 and pDM6 for further characterization. Plasmids containing the missense mutations were transformed into *rtf1* Δ cells for complementation analysis. We sought *rtf1* mutations that could complement the phenotypes of the *rtf1* null strain at the permissive temperature but not at the restrictive temperature.

2.3.2 Characterization of missense mutations in *RTF1*

Three new missense mutations encoding single amino acid substitutions in Rtf1 were identified (Table 5). The *rtf1-100* mutation, which encodes a glutamine 172 to arginine (Q172R) substitution, confers a conditional Spt⁻ phenotype, as indicated by suppression of the *his4-912 δ* insertion mutation and cell growth on medium lacking histidine at 37°C but not at 30°C (Figure 6). The *rtf1-105* (V274D) and *rtf1-107* (M289K) alleles cause conditional sensitivity to 6AU; however, these mutations also cause weak Spt⁻ phenotypes at 30°C (Figure 6). The amino acids altered by the mutations lie within a highly conserved region of Rtf1 but are not themselves invariant across species (Figure 7). Interestingly, the region in which the point mutations reside has been shown to be essential for Rtf1 function based on the phenotypic analysis of a series of internal deletions within *RTF1* (K. L. Roinick and K. M. Arndt, unpublished observations).

Figure 6: Conditional *RTF1* alleles exhibit sensitivity to 6AU and the Spt⁻ phenotype

Characterization of *rtf1* point mutants. Equivalent numbers of cells from *RTF1* (KY767), *rtf1-105* (KKY64), *rtf1-100* (KKY56), *rtf1-107* (KKY58) and *rtf1*Δ (KY656) strains were spotted in ten-fold serial dilutions onto the indicated media. Sensitivity to 6AU was examined on SC-Ura medium supplemented with 50μg/ml 6AU (5 days of incubation). The Spt phenotype was analyzed on medium lacking histidine (3 days of incubation).

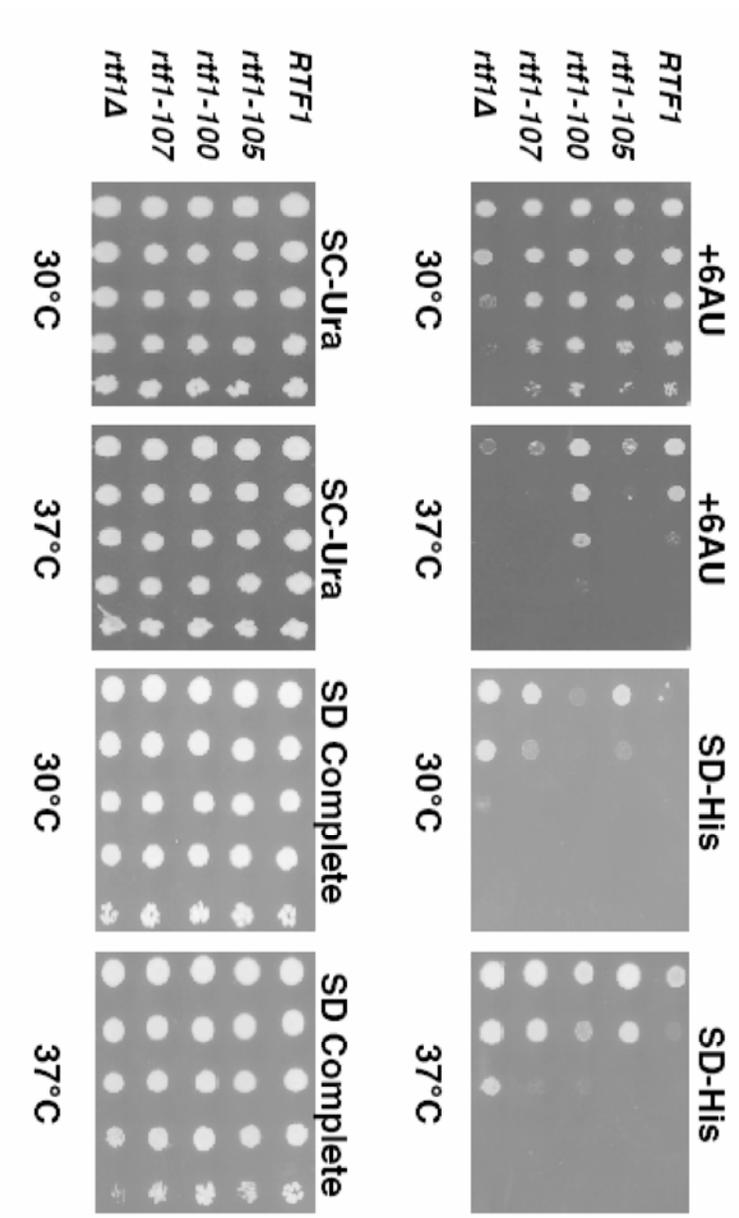


Figure 7: Comparison of Rtf1 sequence to similar proteins in other eukaryotes

Primary amino acid sequence alignment using Rtf1 proteins from *S. cerevisiae*, *C. elegans*, and *H. sapiens*. Rtf1 (accession number P53064) amino acid sequence from *Saccharomyces* Genome Database was used for a BLAST search that identified proteins with similar amino acid sequences in *C. elegans* (accession number CAB03200) and *H. sapiens* (accession number AAH15052). ClustalW was used to align the amino acids sequences. Conserved amino acid identity is indicated by red colored letters and amino acid similarity is indicated by blue letters. Yellow rectangles indicate the residues altered by the three *rtf1* point mutations.

C.elegans MSSSESASDDEETKRRAPATSDSDSDSDAGPKPGKPLSTDSSASDSDAEKPAKPAKKKT 60
H.sapiens -----MKKQANKTASSGSSDKDSSAESSAPEEGEVSDSDSNSSS-----S 60
S.cerevisiae -----MSDLDELLALAGADESEEDQVLTTSAKRAKNDQSLSKKR-----RIEVG 48
consensus *

C.elegans LTKRKRATGSSDDQVDDDLFADKEDKAR---WKKLTELEKEQEIFERMEARENAIARE 117
H.sapiens SSSDSSSEDEFHDGYGEDLMGDEEDRAR---LEQMTEKEREQELFNRIEKREVLKRRF 97
S.cerevisiae SVEDDDEEDDYNPYSVGNADYGSEEEEEANPFLEGGYKDESDREHLESLEPEMERETLLF 108
consensus * * * *

C.elegans EIAQQLAKKAKKSSEKGVKTEKRRKMNSGGSDAGSPKRKASSDSDSEMDAAFHRPSDINR 117
H.sapiens EIKKKLKTAKKKE-----KKEKKKKQEEQEKKLTQIQESQVTSHNKERRSKRDEKLDK 152
S.cerevisiae ERSQIMQKYQERKLFRRGRDMKEQQORAKNDEDSRKTRASTRTHATGHSDIKASKLSQ 168
consensus * *

C.elegans KHFEKNAMDALKNKRKE-IEKKNAKNEALSIDAVFGANSGSSSSSSSSSESSRSSSSSSRES 236
H.sapiens KSCAMEELKAEREKRRNRTAELLAKKQPLKTSSEVYSDDEEEEDDKSSEKSDRSSRTSSS 212
S.cerevisiae LKHQR-----ARKNRHYSDNEDEDEEDYREEDYKDEGESEYGDDEEYNPFDRRRTYDK 222
consensus *

C.elegans SPERVSEKDKIVKKDVDGLSELRRARLSRHKLSLMIHAPFFDSTVVGCVYRIQGQMSG 296
H.sapiens DEEEKEEIPPKSQVSLPEELNRVRLSRHKLERWCHMPFFAKTVTGCFFVRIIGIGNHN-S 271
S.cerevisiae REEVWAEEDDEQDREPEISDFNKLRIGRSFAKFCFYPGFEDAVKGCYGVVNVGTDKRT 282
consensus . * * * * *

C.elegans GSKYRIWKIVGVEESNKVYLEGKKTNKIKCQNGGSERPFRMQFVSNADFEQIEFDEWL 356
H.sapiens KPVYRVAEITGVVETAKVYQLGGTRTNKGLQLRHGNDQRFVRLFEFVSNQEFTESEFMKWK 331
S.cerevisiae GKTSYEMVRIERVFLQKPYNMGKFYTNQYFGVTQGGDRKVFQMNYSDFLFAEDEYQRYL 342
consensus * * * * *

C.elegans LACKRHGN-LPTVDIMDKKKQDIEKAINHKYSKDEVDLMIKEKSKYQTVPR--NFAMTKA 413
H.sapiens EAMFSAQMPLPTLDEINKKELSIKEALNYKFNQDIEEIVKEKERFRKAPP--NYAMKKT 389
S.cerevisiae RALDNSQMIKPSLHSLSNKTKVEVMDFVNTPLTDKTTDEVVRHRMQFNKLSGTNAVLEKT 402
consensus * * * * *

C.elegans NWSKQKELAQQRGDIREAEQIQTKIDEIERQADELEKERSKSISAIAFINHRNRSK---I 470
H.sapiens QLLKEKAMAEDLGDQDKAQIQDLNELEERAEALDRQRTKNISAIYSINQRNREWNIVE 449
S.cerevisiae VLREKLOYAKETNNEKDIKYSQALRNFEKRMVYKHHENDOSDIKKLGELTSKNR--K 460
consensus * * * *

C.elegans KDQVLSGQLKIEENSQDDPFTRKKGMRVVSQSK-----SRLDGTLSASSSTNLSGDK 525
H.sapiens SEKALVAESHNMKNQMDPFTRRQCKPTIVSNSRDPVQAAILAQLNAKYGGVLPDAPK 509
S.cerevisiae LNMSNIRNAEHVKKEDSNFDSKSDPFSRLKTRTKVYQEIQKEENAKAKEIAQQEKLQE 520
consensus *

C.elegans DKSSSLAKPTQPPSTQIKKTDISSLHDFDLIDLGLKDFSTPESSGNKRPSISS--- 582
H.sapiens EMSKGQKDKDLNSKASDLSDELKVFHDFDVKIDLQVPSSSEKALAITSKAPPAKDGAP 569
S.cerevisiae DKDAKDKREKELLVAQFRRLGGLERMVGLDIFDLKF----- 558
consensus * * *

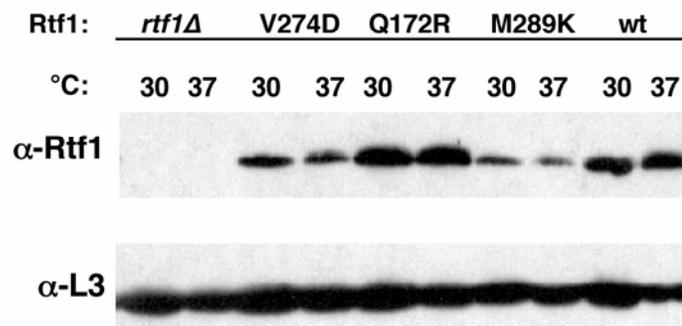
C.elegans SKGVLSDYRMRRSGGGDAGSSTSAAPSSAV 613
H.sapiens RRSLNLEDYKRRGLI----- 585
S.cerevisiae -----
consensus

The conditional phenotypes of the *rtf1* point mutants suggest that Rtf1 retains partial function at the permissive temperature. We performed immunoblot analysis to measure expression of Rtf1 from the conditional alleles. For each of the mutant strains, similar levels of Rtf1 were detected when cells were grown at 30°C or incubated for 2 hr at 37°C (Figure 8A). However, the *rtf1-105* and *-107* mutants express Rtf1 to lower levels than the wild-type control strain (Figure 8A). Steady state levels of histone H3 K4 trimethylation in the mutant strains correlate with Rtf1 protein levels, indicating that the mutations do not cause a specific defect in this modification (Figure 8B).

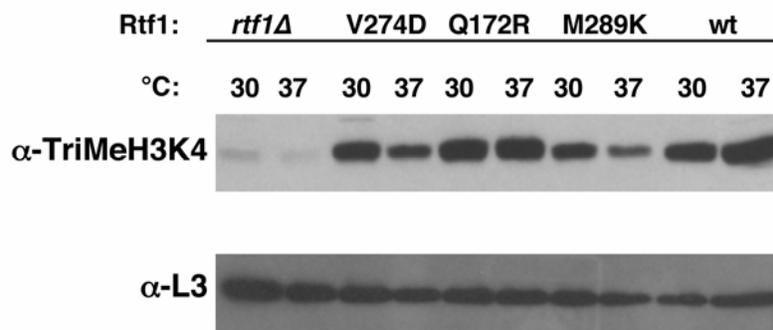
Figure 8: Rtf1 is expressed from the *rtf1* alleles at both the permissive and restrictive temperatures and histone H3 K4 trimethylation is detected in the *rtf1* point mutants

(A) Immunoblot analysis was performed on wild-type (WT) (KY406), *rtf1-105* (V274D; KKY37), *rtf1-100* (Q172R; KKY40), *rtf1-107* (M289K; KKY45) and *rtf1*Δ (KY410) cells. (B) Cultures were grown to early log phase, divided and further incubated at 30° or 37°C for 2 hrs. Extract from 1×10^7 cells per sample was analyzed by immunoblotting with anti-Rtf1 and, as a loading control, anti-L3 antisera. Histone H3 K4 trimethylation was analyzed by immunoblotting of whole cell extracts prepared from strains grown as in (A).

A



B



2.3.3 Genes in high copy number that suppress the phenotypes of *rtf1-107*

Isolating and determining the identity of the dosage suppressors of *rtf1* could uncover new as well as confirm established roles for the Paf1 complex. The phenotypes of *rtf1-107* strongly indicated that defects in Rtf1 function contribute to impaired transcription elongation. To further clarify the role of Rtf1 and the Paf1 complex in vivo, we sought to identify multicopy suppressors that could reverse the 6AU^S and/or Spt⁻ phenotypes of *rtf1-107*. Since many lines of evidence indicate a role for Rtf1 in transcription, the possibility exists that high-copy-number suppressors of *rtf1-107* could encode factors involved in different aspects of RNA polymerase II transcription or factors involved in coupling RNA capping, splicing and processing to transcription. Other likely multicopy suppressors include genes encoding mRNA transport proteins, such as components of THO/TREX that are intimately linked with elongation and hnRNP formation (Fischer et al., 2002; Rondon et al., 2003; Strasser et al., 2002). However, not all candidates will be bona-fide suppressors. For example, *URA3* or genes encoding drug resistant membrane transporters or protein chaperones could confer phenotypic suppression of *rtf1-107*.

Sequence analysis of the recovered library plasmids revealed inserts with multiple open reading frames consistent with the approximate insert size of the library that ranges from 5 Kb to 10 Kb. Information available from the *Saccharomyces* Genome Database was used to determine the gene most likely responsible for suppression on each library plasmid (Balakrishnan et al.). As anticipated, we identified multicopy suppressors that contained *RTF1* within the genomic insert. For example, plasmid isolate 172 suppressed both the Spt⁻ and 6AU^S phenotypes and *RTF1* was identified within the genomic insert of this plasmid. Additionally, we identified

dosage suppressors likely to suppress the 6AU^S phenotype of *rtf1-107* nonspecifically. For example, candidate 192 was isolated as a multicopy suppressor of 6AU^S and its genomic insert contained *URA3*. Overexpression of *URA3* could suppress the 6AU^S phenotype because Ura3 catalyzes a reaction important for de novo synthesis of pyrimidines, in turn restoring cellular pools of UTP. These candidate plasmids and the genes within their genomic inserts are displayed in Table 6 with genes likely to cause suppression in bold type. We discovered that two library plasmids, 7 and 176, exhibited different restriction digest patterns and suppressed the 6AU^S phenotype of *rtf1-107* strains (Table 6). Upon sequence analysis these candidates contained overlapping genomic inserts. The one gene that they both shared in its entirety was *TPO1*, suggesting that *TPO1* was the gene responsible for conferring 6AU^R in *rtf1-107* cells. Recent reports indicate that Tpo1 is a polyamine transporter localized to the vacuolar membrane (Uemura et al., 2005). Interestingly, in response to 6AU and mycophenolic acid (MPA), another drug that reduces intracellular pools of GTP, *TPO1* is transcriptionally induced similarly to *IMD2* (*IMD2/PUR5*) a gene required for pyrimidine biosynthesis (Hedstrom, 1999). The basis for the observed 6AU^S suppression caused by the overexpression of *TPO1* is not clear, but it likely does not involve a direct interaction between Tpo1 and the Paf1 complex.

Library plasmid 91 was also identified on the basis of its ability to confer suppression of the 6AU^S phenotype of the *rtf1-107* cells (Table 6). The genomic insert of this candidate contained *SDT1* among other genes. *SDT1* encodes a protein with pyrimidine nuclease activity and is known to suppress the 6AU^S phenotype of a *dst1Δ* deletion mutant (Shimoaraiso et al., 2000). *DST1* encodes the transcription elongation factor TFIIIS. As an elongation factor, TFIIIS associates with RNA polymerase II and promotes intrinsic transcript cleavage by RNA polymerase II to overcome obstacles to elongation (Kettenberger et al., 2003; Kettenberger et al.,

2004). Sdt1 involvement in pyrimidine metabolism suggests that suppression of *dst1Δ* occurs indirectly (Nakanishi and Sekimizu, 2002), and therefore, it is likely multicopy *SDT1* could suppress the 6AU^S phenotype of *rtf1-107* in a nonspecific fashion.

Plasmid number 304 was isolated in the dosage suppression screen because it suppressed the Spt⁻ phenotype of *rtf1-107* (Table 6 and 8). Sequence analysis of the genomic insert revealed several likely candidate genes that in multicopy could be responsible for the observed suppression. *YDJI* stands out in particular because it encodes a cytosolic HSP40 chaperone protein that could indirectly suppress the Spt⁻ phenotype of *rtf1-107* (Caplan et al., 1993). Overexpression of *YDJI* in *rtf1-107* cells could help mutant Rtf1 protein fold properly thereby suppressing phenotypes associated with *rtf1-107*. If this is the case, why does high-copy-number *YDJI* not confer resistance to 6AU in *rtf1-107* mutants? High-copy-number plasmids containing *YDJI* were not identified in this screen as suppressors of the 6AU^S phenotype of *rtf1-107*. One explanation for not recovering *YDJI* containing plasmids as multicopy suppressors of the conditional 6AU^S phenotype of *rtf1-107* is that increased dosage of *YDJI* does help mutant Rtf1 fold properly only at the permissive temperature. Suppressors of the Spt⁻ phenotype of *rtf1-107* cells were screened for at the permissive temperature (30°C) and suppressors of 6AU^S were screened for at the non-permissive temperature (37°C). An alternative explanation is that overexpression of *YDJI* does not cause suppression of the phenotypes of *rtf1-107* strains. Rather, another candidate on the genomic insert may directly or indirectly suppress the Spt⁻ phenotype of *rtf1-107*. This possibility will be addressed below.

Table 6: Multicopy library plasmids that may confer suppression of *rtf1-107* through non-specific mechanisms

Library isolate	Genes within genomic insert	Phenotype (s) suppressed
7	<i>TPO1, ISA1, HSP104</i>	6AU ^R
91	<i>VRG4, SDT1, COD3, EDC1, NIF3, YGL220W, YGL218W, MMM2, KIP3</i>	6AU ^R
176	<i>GPII3, YLL030C, YLL029W, TPO1</i>	6AU ^R
192	<i>URA3, TIM9, RPR1, YELCTAU1, MMS21</i>	weak 6AU ^R
304	<i>YDJI, MTQ1, GCD10, NOP2, partial ARP5</i>	Spt ⁺

As expected, library plasmids that suppressed the 6AU^S and/or the Spt⁻ phenotypes of *rtf1-107* were identified that contained no obvious candidate genes. Table 7 contains a subset of these types of dosage suppressors of *rtf1-107*. In addition, most of these plasmids do not confer strong suppression. The two plasmids in Table 7 that do confer strong suppression contain genes that are not directly involved in transcription by RNA polymerase II. The observation that these candidates may not confer strong suppression or that genomic inserts conferring strong suppression do not have known roles in RNA polymerase II transcription does not mean that these candidates should be ignored. Understanding the mechanism of suppression caused by these candidates could provide insight into roles that Rtf1 may perform outside of transcription. Regardless, while these candidates are considered a low priority, they should be revisited as more information about the genes contained within their inserts becomes available.

Table 7: Library plasmids capable of *rtf1-107* suppression that do not contain "obvious candidate genes" within their genomic inserts

Library isolate	Genes within genomic insert	Phenotype(s) suppressed
47	<i>CCA1, RPH1, ADK2</i>	weak 6AU ^R , weak Spt ⁺
169	<i>PHM8, KRE29, HVG1, YER039C-A</i>	weak 6AU ^R , weak Spt ⁺
197	<i>NOPI2, RPS15, RPP2A, PRE6, YOL036W, YOL037C, YOL035C, SNR50</i>	weak 6AU ^R , weak Spt ⁺
280	<i>NCE101, RCY1, PRP21, YJL202C, ECM25, YJL200C, TT(AUG)J, YJLWDELTA1, YJL199C</i>	weak Spt ⁺
312	<i>KTR4, BEM1, DER1</i>	6AU ^R
317	<i>SEC31, SNF3</i>	Spt ⁺

The purpose of identifying dosage suppressors of phenotypes conferred by *rtf1-107* was to identify genes that functionally interact with *RTF1*. The function of such genes could provide further insight into the function of Rtf1 and the other members of the Paf1 complex in transcription as well as extend the type and number of roles for the Paf1 complex in the cell. Table 8 contains the most exciting library plasmids isolated from the high-copy-number genetic screen performed with *rtf1-107*. The genomic inserts of the library plasmids in Table 8 contain at least one gene with links to RNA polymerase II transcription, RNA metabolism, splicing or export, or some other nuclear function. The genes most likely responsible for conferring suppression are in bold type. As expected, library plasmids containing *RTF1* within the genomic insert were capable of suppressing the Spt⁻ phenotype of *rtf1-107*. Candidate 172 contained *RTF1* and suppressed both phenotypes caused by the *rtf1-107* mutation.

Six different library plasmids that conferred suppression of the Spt⁻ phenotype of *rtf1-107* were candidates 281, 304, 305, 309, 326, and 352 (Table 8). Candidates 305 and 309 will be discussed further in sections 2.3.3.1. and 2.3.3.2., respectively. Suppressor candidate 281 contained *SSDI* within the genomic insert. A reported function for Ssd1 includes RNA binding and nuclease activity (Luukkonen and Seraphin, 1999). While Ssd1 is potentially an exciting candidate, the fact that *SSDI* had been identified in several other high-copy-number suppressor screens suggested it may not have a direct role in Paf1 complex function (Kosodo et al., 2001; Lorenz and Heitman, 1998; Stettler et al., 1993), and our focus was directed toward other candidates. To date, we cannot rule out the possibility of a genetic interaction between *RTF1* and *SSDI*. However, it is worth noting here that recent reports on Ssd1 indicate it could be a worthwhile candidate for further study. For example Ssd1 has been reported to associate with phosphorylated CTD and Set2 (Jones et al., 2004), and be involved in maintaining cell wall

integrity possibly through the target-of-rapamycin (TOR) signaling pathway in yeast (Reinke et al., 2004).

Table 8: High-copy-number library plasmids that cause suppression of *rtf1-107* and contain genes within the genomic insert with connections to gene expression

Library isolate	Genes within genomic insert	Phenotype (s) suppressed
172	<i>PDE1, BRR6, RAI1, YGL244W, RTF1, TAD1, YGL242C</i>	6AU ^R , Spt ⁺
281	<i>SRP101, SSD1, DPL1</i>	Spt ⁺
304	<i>YDJ1, MTQ1, GCD10, NOP2, partial ARP5</i>	Spt ⁺
305	<i>RSA1, PRM3, YPL191C, NAB3^a</i>	Spt ⁺
309	<i>RTG3^a, SFT2, ECM21</i>	Spt ⁺
326	<i>YMR046W-A, YMRCTY1-3, YMRDELTA8, TH(GUG)M, NUP116, CSM3</i>	Spt ⁺
352	<i>YNL200C, GCR2, YNL198C, WHI3</i>	Spt ⁺

^a designates genes tested for their ability to confer suppression in the *rtf1-107* strain.

Bold terms indicate genes that are candidates for suppressing *rtf1-107* phenotypes as indicate.

Candidate 304 contains a number of genes of interest. The name *MTQ1* has been reserved on the *Saccharomyces* Genome Database (Balakrishnan et al.) and is the uncharacterized open reading frame, *YNL063W*. Mtq1 exhibits amino acid sequence similarity with HemK, a protein in *E. coli*, which is a glutamine methyltransferase that modifies peptide release factors involved in translation termination (Nakahigashi et al., 2002). Interestingly, the HemK family of methyltransferases exhibit amino acid sequence similarity with proteins involved in DNA methylation (Nakahigashi et al., 2002). While DNA base methylation is an important modification that serves to regulate gene expression in higher eukaryotes, there is no evidence that this modification is involved in regulating transcription in *S. cerevisiae* (Feng et al., 2004). Therefore, *MTQ1* is likely not the gene responsible for the observed dosage suppression of *rtf1-107*.

Another interesting gene on plasmid 304 is *GCD10*. Gcd10 forms a heterodimeric complex with Gcd14 that is involved in methylating (N)1 the adenine 58 residue within eukaryotic tRNAs (Anderson et al., 1998; Anderson et al., 2000; Ozanick et al., 2005). Interestingly, Gcd10 interacts with TFIIB, a component of the general transcription machinery via two-hybrid analysis (Ito et al., 2001). This result was reported in a genome-wide two-hybrid study and has not been confirmed independently. In the future, overexpression of *GCD10* should be assayed in *rtf1-107* cells to determine whether *GCD10* and *RTF1* do genetically interact.

The last full length gene of note on candidate 304 is *NOP2*. The Nop2 protein is important for the maturation of the 27S pre-rRNA presumably through the protein's S-adenosylmethionine-dependent methyltransferase activity that is responsible for the methylation at position 5 of cytosine (Hong et al., 1997; King and Redman, 2002). However, the function of *NOP2* like the

function of the other genes of interest on plasmid 304, do not directly point to a probable mechanism of suppression of the *rtf1-107* mutation.

The partial open reading frame of *ARP5* is also worth mentioning. Unfortunately, the position of *ARP5* on the genomic insert results in the removal of the first 133 nucleotides that correspond to the start of the coding region and promoter sequences. With this stated, it is possible, although unlikely, that sequences within the vector could contribute to the transcription of the remaining coding sequence of *ARP5*. Further, this candidate would be most likely to suppress the Spt⁻ phenotype of *rtf1-107* since Arp5 is a component of the INO80 chromatin remodeling complex and the Spt⁻ phenotype is associated with defects in chromatin structure (Ebbert et al., 1999; Jonsson et al., 2004; Shen et al., 2003). Theoretically, it is possible that increased dosage of even a truncated form of Arp5 could reverse the Spt⁻ phenotype in *rtf1-107* cells. Previous evidence from our lab independently verified a functional interaction between INO80 and the Paf1 complex. Specifically, genes encoding members of the INO80 complex interact genetically with genes encoding the Paf1 complex (M. H. Warywoda and K. M. Arndt, unpublished observations).

NUP116 of library plasmid 326 is the most reasonable candidate to confer suppression of the Spt⁻ phenotype in *rtf1-107* cells. First, Nup116 is a component of the nuclear pore complex (NPC) that interacts with the mRNA export protein Mex67 a protein closely associated with the yeast TREX complex (Strasser et al., 2002). Like TREX, the Paf1 complex associates with actively transcribing RNA polymerase II and could possibly function in mRNA export (Strasser et al., 2002). Second, Nup116 is implicated in snRNA and snRNP particle export (Strawn et al., 2001). *RTF1* functionally interacts with *NAB3* and *NRD1* as described in section 2.3.3.1., section 2.3.4.1., and in chapter 3; these genes encode factors that are important for proper transcription

termination of snRNAs and snoRNAs (Steinmetz et al., 2001). Third, a two-hybrid analysis conducted in our lab with Rtf1 uncovered an interaction between a component of the nuclear pore complex and Rtf1 (K. L. Roinick and K. M. Arndt, unpublished observations). However, high-copy-number *NUP116* may not directly suppress the Spt⁻ phenotype of *rtf1-107* at the level of transcription. This mechanism of suppression could occur if extra copies of Nup116 improve the kinetics of RNA export out of the nucleus. Since cells containing the mutant form of Rtf1 are viable, it is inferred that transcription does proceed albeit with consequences we may not fully appreciate. In this manner, RNA polymerase II associated with the mutant form of Rtf1 produces transcripts that may be more efficiently exported when *NUP116* is overexpressed, perhaps compensating for a kinetic defect in RNA synthesis.

The last library plasmid of interest in Table 7 is 352. This plasmid contains two annotated genes that could confer suppression of the Spt⁻ phenotype of *rtf1-107* cells. The *GCR2* gene encodes a transcriptional activator involved in regulating the expression of genes involved in glycolysis (Henry et al., 1994; Turkel and Bisson, 1999; Uemura et al., 1997). A second reason for our interest in *GCR2* is that it was identified in a two-hybrid screen in our lab where the sequence encoding the N-terminal region of *YMR247C* was used as bait (R. Gonda, M. A. Braun, and K. M. Arndt, unpublished observation). *YMR247C*, which is required for viability in cells lacking *RTF1* (P. J. Costa, M. A. Braun, K. M. Arndt, unpublished observations), has been implicated in chromatin dynamics (M. A. Braun and K. M. Arndt, unpublished observations; D. Hess and F. Winston, personal communications). Gcr2 could potentially suppress the Spt⁻ phenotype of *rtf1-107* at the level of transcription since Gcr2 functions in a complex with Gcr1 to activate transcription of a subset of genes (Henry et al., 1994; Turkel and Bisson, 1999; Uemura et al., 1997). Alternatively, multicopy *WHI3* could reverse the Spt⁻ phenotype in *rtf1-*

107 strains. The Whi3 protein binds RNA and is implicated in the sequestration of transcripts from the G₁ cyclin genes *CLN2* and *CLN3* (Gari et al., 2001; Wang et al., 2004). Overexpression of *WHI3* leads to a large cell phenotype and ultimately in a lethal arrest in G₁ (Gari et al., 2001; Nash et al., 2001; Wang et al., 2004). Observations from the Jaehning lab indicate that members of the Paf1 complex as well as the G₁ specific transcriptional activator SBF are required for the transcription of *CLN1* and *CLN2* and other cell cycle regulated genes (Wittenberg and Reed, 2005). Further, deletion of either individual subunit of the activator complex SBF, *SWI4* or *SWI6*, together with deletion of either *RTF1* or *PAF1* causes synthetic lethality (Figure 28) (Betz et al., 2002; Koch et al., 1999; Porter et al., 2002). Hence, the possibility remains that a functional connection between *WHI3* and *RTF1* results in suppression of the Spt⁻ phenotype of *rtf1-107* by overexpression of *WHI3*.

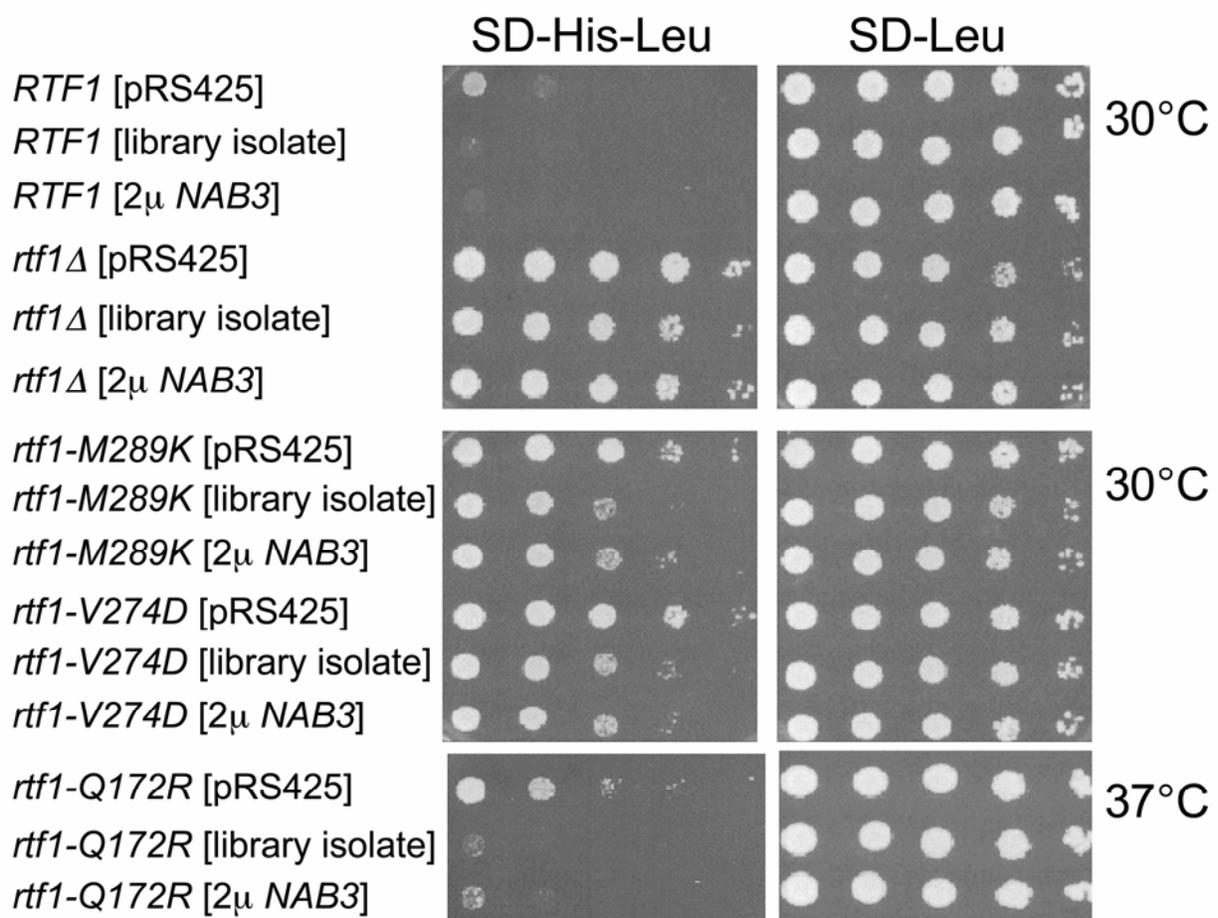
2.3.3.1 *NAB3* is a dosage suppressor of *rtf1-107*

Library plasmid 305 was of particular interest since it suppressed the Spt⁻ phenotype of *rtf1-107* strains and contained the *NAB3* gene within a large genomic fragment. Nab3 functions in the Nrd1 pathway for 3'-end formation of RNA polymerase II transcripts that are not polyadenylated (Conrad et al., 2000; Steinmetz et al., 2001). Together, the essential Nab3 and Nrd1 proteins recognize and bind to specific sequences within snRNA and snoRNA transcripts (Carroll et al., 2004) to promote transcript termination and 3'-end formation in a reaction that also requires the Sen1 helicase (Steinmetz et al., 2001) and components of the APT and CFI cleavage/polyadenylation factor complexes (Cheng et al., 2004; Dheur et al., 2003; Dichtl et al., 2004; Fatica et al., 2000; Morlando et al., 2002; Nedeá et al., 2003; Steinmetz and Brow, 2003). Final maturation of snoRNA 3' ends also requires the 3'-to-5' exonuclease activity of the nuclear exosome complex (Allmang et al., 1999; Allmang et al., 1999; van Hoof et al., 2000). To

determine whether *NAB3* overexpression was responsible for the suppression, the *NAB3* gene was subcloned into a 2-micron vector and transformed into the *rtf1* point mutants, *rtf1* Δ , and *RTF1*⁺ strains (Figure 9). While *NAB3* overexpression suppressed the Spt⁻ phenotypes caused by the three *rtf1* point mutations (Figure 9), it did not suppress the phenotypes associated with deletion of *RTF1* (Figure 9). Therefore, *NAB3* is not a bypass suppressor of *rtf1*.

Figure 9: High-copy-number *NAB3* suppresses the Spt⁻ phenotype of conditional mutations in *RTF1*

Genetic interaction between *RTF1* and *NAB3*. Vector (pRS425), candidate 305 (library isolate), high-copy-number *NAB3* (2 μ *NAB3*) were transformed into *RTF1* (KY767), *rtf1* Δ (KY656), *rtf1-107* (M289K; KKY60), *rtf1-105* (V274D; KKY64), *rtf1-100* (Q172R; KKY54) cells. Transformants were used to inoculate saturated overnight cultures. Equivalent numbers of cells were spotted in ten-fold serial dilutions onto the indicated media. The Spt phenotype was analyzed on medium lacking histidine and leucine. Plates were incubated at either 30°C or 37°C as indicated.



2.3.3.2 *RTG3* partially suppresses the Spt⁻ phenotype of *rtf1-107*

Candidate 309 was also of interest since this plasmid caused the suppression of the Spt⁻ phenotype of *rtf1-107*. The genomic insert contained *RTG3*, which encodes a basic helix-loop-helix leucine zipper factor that complexes with Rtg1, another basic helix-loop-helix leucine zipper protein (Jia et al., 1997; Rothermel et al., 1997). Together, Rtg1-Rtg3 activate RNA polymerase II transcription of genes involved in the retrograde (RTG) and/or TOR signaling pathways (Dilova et al., 2004; Dilova et al., 2002). RTG signaling is important for communication between the mitochondrion and the nucleus and is induced in the event that the activity of the tricarboxylic acid cycle is impaired (Jazwinski, 2005). This results in the expression of metabolic and stress-related genes that provide alternate sources of biosynthetic precursors (Strawn et al., 2001). Preliminary dilution analyses where *RTG3* was subcloned into a 2-micron plasmid and transformed into *RTF1*, *rtf1Δ* and the *rtf1* point mutants, indicates overexpression of *RTG3* is only partially responsible for suppression of the Spt⁻ phenotype of the *rtf1* missense mutations (Figure 10). The possible relationship between Rtg3 and the Paf1 complex should remain a research interest since *ACO1*, a gene regulated by Rtg1-Rtg3 in the RTG response, is induced in *ctr9Δ* cells when analyzed by microarray analysis (Table 16).

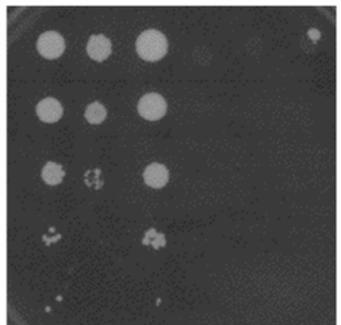
Figure 10: Increased dosage of *RTG3* partially suppresses the Spt⁻ phenotype of the *rtf1* temperature sensitive mutants

Genetic interaction between *RTF1* and *RTG3*. Vector (pRS425), candidate 309 (library isolate), high-copy-number *RTG3* (2 μ *RTG3*) were transformed into *RTF1* (KY406), *rtf1* Δ (KY410), *rtf1-107* (M289K; KKY60), and *rtf1-105* (V274D; KKY64). Transformants were used to inoculate saturated overnight cultures. Equivalent numbers of cells were spotted in ten-fold serial dilutions onto the indicated media. The Spt phenotype was analyzed on medium lacking histidine and leucine (6 days of incubation at 30°C).

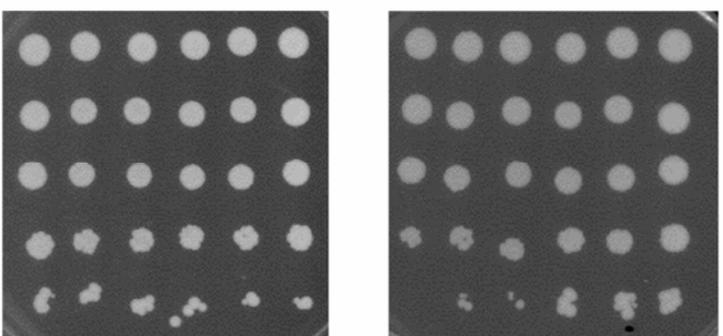
RTF1 [pRS425]
RTF1 [library isolate]
RTF1 [2 μ NAB3]
rff1 Δ [pRS425]
rff1 Δ [library isolate]
rff1 Δ [2 μ NAB3]

rff1-M289K [pRS425]
rff1-M289K [library isolate]
rff1-M289K [2 μ NAB3]
rff1-V274D [pRS425]
rff1-V274D [library isolate]
rff1-V274D [2 μ NAB3]

SD-His-Leu



SD-Leu



2.3.4 Additional genetic analyses support a functional link between *RTF1* and *NAB3*

2.3.4.1 Multicopy *NAB3* causes synthetic growth phenotypes in *ctr9Δ* and *paf1Δ* cells

Since *NAB3* is a multicopy suppressor of the *rtf1* missense mutants, we were curious to determine if *NAB3* genetically interacted with the genes encoding other members of the Paf1 complex. To this end, 2-micron *NAB3* was transformed into strains lacking individual members of the Paf1 complex. Phenotypic analysis was performed and the data from two separate experiments are summarized in Table 9. Interestingly, overexpression of *NAB3* in the absence of either *PAF1* or *CTR9* caused synthetic growth defects when compared to empty vector (Table 9). The growth defect was so severe in the *paf1Δ* mutant that liquid cultures did not grow to saturation and were not analyzed by dilution analysis (K. E. Sheldon and K. M. Arndt, unpublished observations). For *ctr9Δ* mutants, growth on synthetic dextrose (SD) medium at 30°C and synthetic complete (SC) medium at 37°C was impaired when transformed with empty vector (pRS425) as compared to the growth of wild-type cells on SC media at 30°C. Solid SD and SC growth media are prepared essentially the same, however SC medium contains all amino acids, uracil and adenine bases but lack the amino acid or base used for selection while SD medium contains only the amino acids or bases required to sustain the auxotrophies of the cells, lacking the amino acids and bases required for maintaining selection. Healthy cells that are supplemented with the appropriate amino acids or bases are capable of growing on either type of medium, yet some mutant strains are unable to grow on these synthetic media since they are either sick even on rich media or they are defective for inducing biosynthetic pathways.

Importantly, *ctr9Δ* cells with 2-micron *NAB3* are even further impaired for growth on SD plates at 30°C or SC plates at 37°C as compared to *ctr9Δ* cells with vector only.

Table 9: Phenotypic analysis of increased *NAB3* dosage in cells deleted for individual Paf1 complex members

YEAST STRAIN	pRS425 SD-LEU 30°C	pRS425 SC-LEU 37°C	pRS425 SC-LEU 30°C	2μ <i>NAB3</i> SD-LEU 30°C	2μ <i>NAB3</i> SC-LEU 37°C	2μ <i>NAB3</i> SC-LEU 30°C
wild-type	+++ ^a	+++++ ^a	+++++	+++ ^a	+++++ ^a	+++++
<i>rtf1Δ</i>	++ ^a	+++++ ^a	+++++	++ ^a	+++++ ^a	+++++
<i>paf1Δ</i>	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
<i>ctr9Δ</i>	++ ^a	++ ^a	++++	+ ^a	- ^a	++++
<i>cdc73Δ</i>	++++ ^a	+++++ ^a	+++++	+++ ^a	+++ ^a	+++++
<i>leo1Δ</i>	+++ ^a	+++++ ^a	+++++	+++ ^a	+++++ ^a	+++++

^a The experiment was performed one time only; unless otherwise indicated all other experiments were performed two times.

N.D. Not Determined.

A modest defect in growth was observed for *cdc73Δ* mutants when transformed with 2-micron *NAB3* (Table 9). *cdc73Δ* cells containing vector alone did not exhibit severe growth defects. However, *cdc73Δ* strains containing multiple copies of *NAB3* grew poorly on SD medium at 30°C or SC medium at 37°C as compared to vector-containing cells. Perhaps of some significance is the result that the synthetic growth phenotype observed in *cdc73Δ* cells with multiple copies of *NAB3* is not as severe as those observed for *ctr9Δ* mutants with 2-micron *NAB3*.

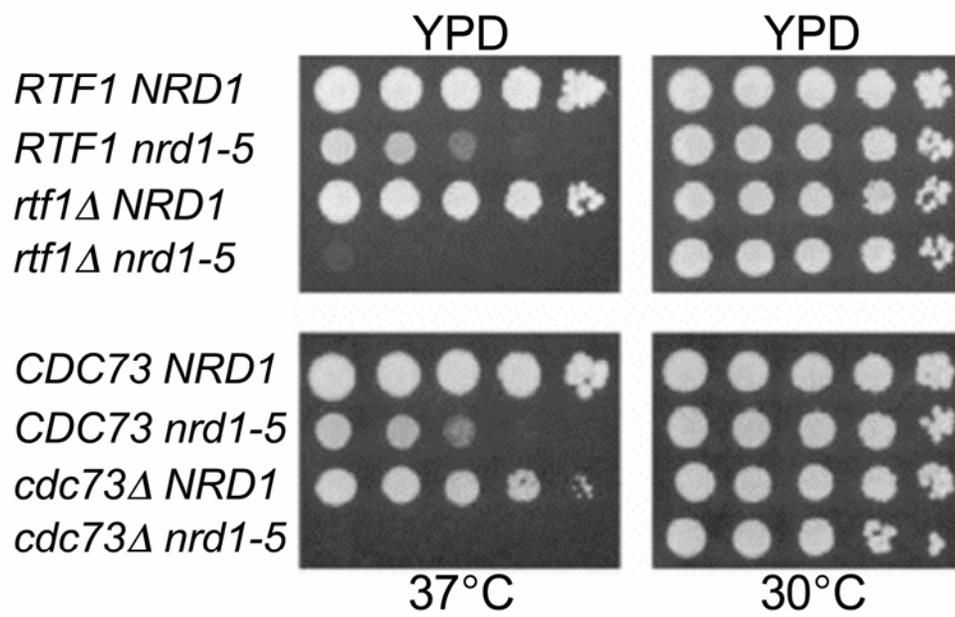
Increased dosage of *NAB3* displayed no discernable defect in *rtf1Δ* or *leol1Δ* mutants as compared to empty vector (Table 9). When transformed with either empty vector or 2-micron *NAB3*, these mutants grew similar to wild-type on SC media at either 30°C or 37°C. However, *rtf1Δ* and *leol1Δ* mutants transformed with either empty vector or high-copy-number *NAB3* did exhibit poor growth only on SD medium at 30°C as compared to the wild-type control cells. These results contrast with those for the *ctr9Δ* and *paf1Δ* mutants. While *ctr9Δ* and *paf1Δ* mutants containing vector or 2-micron *NAB3* grew poorly in selective SC media, this could simply be due to the poor growth phenotypes of these strains alone. Conversely, the synthetic phenotype could be caused by the overexpression of *NAB3* in the *ctr9Δ* cells. This analysis also provides additional evidence in support of functional differences between individual members of the Paf1 complex. Importantly, this study does strengthen the basis for a functional interaction between *RTF1* and *NAB3*.

2.3.4.2 *nrd1-5* cells require members of the Paf1 complex for viability at elevated temperatures.

In another approach to test for functional interactions between Nrd1 and the Paf1 complex, we generated double mutant strains containing the *nrd1-5* mutation and deletions of individual components of the Paf1 complex. The *nrd1-5* allele alters a single amino acid in the RNA recognition motif (RRM) of Nrd1 (Steinmetz and Brow, 1996) and causes a moderate temperature sensitive growth phenotype (Figure 11). However, strains that contain *nrd1-5* in combination with a deletion of *RTF1*, *CDC73*, *PAF1*, or *CTR9* exhibit strongly enhanced temperature sensitivity (Figure 11). These synthetic interactions between a mutation in *nrd1* and defects in the Paf1 complex are consistent with our discovery of *NAB3* as a dosage suppressor of an *rtf1* mutation. Taken together, the genetic results demonstrate a functional connection between the Paf1 complex and factors involved in poly(A)-independent 3'-end formation.

Figure 11: *nrd1-5* combined with deletions of individual Paf1 complex members confers synthetic phenotypes

Paf1 complex members genetically interact with *NRD1*. A plasmid shuffle experiment was performed by transforming pRS314, pRS314-*NRD1*, or pRS314-*nrd1-5* into *nrd1Δ RTF1* (KKY129), *nrd1Δ rtf1Δ* (KKY128), or *nrd1Δ cdc73Δ* (KKY143) cells that contained pRS316-*NRD1*. Transformants were passaged on 5FOA to select against pRS316-*NRD1* plasmid. Equivalent numbers of cells were spotted in ten-fold serial dilutions onto YPD (4 days incubation).



2.4 Conclusions

2.4.1 Point mutations in *rtf1* confer conditional loss-of-function phenotypes

Mutagenic PCR of *RTF1* followed by homologous recombination in vivo was used to identify new mutations in *RTF1* that confer conditional $6AU^S$ and Spt^- phenotypes. From eight candidates initially identified, three mutations causing individual amino acid substitutions were recovered that conferred conditional mutant phenotypes. The substitutions encoded by the mutations were Q172R, V274D, and M289K. These residues reside in the central, conserved region of Rtf1 (Figure 7). The *rtf1* allele that causes the Q172R substitution is *rtf1-100* and confers a conditional Spt^- phenotype (Figure 6). *rtf1-105* that encodes the V274D and *rtf1-107* encoding M289K cause conditional $6AU^S$ and weak Spt^- phenotypes (Figure 6). Importantly, the phenotypes of the three alleles are not caused by the absence of Rtf1 in the cell as observed by immunoblot analysis (Figure 8A).

2.4.2 *NAB3* and *RTG3* were identified in a high-copy-number suppressor screen to identify genes that suppress the phenotypes of conditional *rtf1* alleles

The *rtf1-107* strain was chosen for use in a high-copy-number genetic screen. The goal of the screen was to identify library plasmids that suppressed the conditional $6AU^S$ and/or the weak Spt^- phenotype exhibited by this mutant. Genes responsible for the suppression of the *rtf1-107* phenotypes could be the result of authentic genetic interactions or indirect mechanisms. To

eliminate the latter possibility, the genomic inserts of candidate plasmids were identified. The individual genes contained within the inserts were analyzed in detail using the *Saccharomyces* Genome Database (Balakrishnan et al.), literature searches, and web-based protein motif searches. These approaches helped to filter out the genes least likely to confer suppression in most cases. However, candidate plasmids also contained genes that have no known roles in transcription or RNA metabolism and/or open reading frames without functions ascribed to them. Fortunately, a few strong candidate genes likely to be responsible for the observed suppression were identified. These candidates included *NAB3* and *RTG3*, as well as a number of genes in Table 8. For *NAB3* and *RTG3*, several lines of evidence indicate that they were responsible for suppression, including their connection to transcription by RNA polymerase II and the observation that library plasmids that contained them suppressed the Spt⁻ phenotype of *rtf1-107*, a phenotype indicative of defects in transcription initiation and elongation. *NAB3* and *RTG3* were subjected to further analysis.

Both *NAB3* and *RTG3* were subcloned and tested directly for suppression of *rtf1* mutations (Figure 9 and Figure 10). Increased dosage of *NAB3* suppressed the Spt⁻ phenotype of the *rtf1* point mutants and did not suppress the Spt⁻ phenotype of an *rtf1Δ* strain. In a preliminary experiment, high-copy-number *RTG3* partially suppressed the Spt⁻ phenotype of the *rtf1* point mutants and not the Spt⁻ phenotype of *rtf1Δ* cells. Additionally, overexpression of *NAB3* results in synthetic sickness in *ctr9Δ* and *paf1Δ* cells (Table 9). The suppression of *rtf1-107* caused by increased *NAB3* is also supported by the observation that *nrd1-5* in combination with deletion of individual Paf1 complex members resulted in enhanced synthetic phenotypes (Figure 11).

3.0 Chapter 3: A novel role for the Paf1 complex in 3'-end formation of snoRNA transcripts

3.1 Introduction

RNA polymerase II is required for the transcription of both polyadenylated and non-polyadenylated transcripts. The transcripts of protein-coding genes must be polyadenylated within the nucleus, otherwise these transcripts will be recognized and degraded via RNA surveillance pathways (Penheiter et al., 2005; Vanacova et al., 2005; Wyers et al., 2005; Yart et al., 2005). On the other hand, snRNAs and snoRNAs also transcribed by RNA polymerase II are not normally polyadenylated and are destabilized by the aberrant addition of poly(A) sequences (Vanacova et al., 2005; Wyers et al., 2005). The 3'-ends of polyadenylated and non-polyadenylated messages are formed similarly and perhaps unexpectedly with a small subset of factors that participate in 3'-end formation of both types of transcripts (Dheur et al., 2003; Dichtl et al., 2004; Nedea et al., 2003; Steinmetz et al., 2001). Further, RNA polymerase II transcription termination of RNA synthesis is tightly coupled with 3'-end formation of the nascent transcript (Bentley, 2005; Proudfoot, 2004). Together these processes ensure that mature RNAs are properly formed.

Among factors that associate with RNA polymerase II, Nab3 and Nrd1 are known to function in terminating transcription of non-polyadenylated messages. Two-hybrid analysis uncovered an interaction between RNA polymerase II and Nrd1 (Conrad et al., 2000). This

interaction may be facilitated through the N-terminal domain of Nrd1, which exhibits sequence similarity with proteins known to interact with the C-terminal domain (CTD) of the largest subunit of RNA polymerase II through their CTD interaction domain (CID) (Meinhart and Cramer, 2004). The CID of the *S. cerevisiae* Pcf11 protein, a factor involved in terminating the transcription of polyadenylated messages, directly contacts the CTD (Hollingworth et al., 2005; Meinhart and Cramer, 2004; Noble et al., 2005). Alternatively, the association between Nrd1 and RNA polymerase II may be established or maintained through contacts involving the RRM of Nrd1 and/or Nab3 and their known binding sequences within the nascent transcript.

A high-copy-number suppressor screen identified *NAB3* as a dosage suppressor of *rtf1-107*. This genetic relationship was further supported upon additional analyses, where overexpression of *NAB3* in strains lacking individual Paf1 complex members caused synthetic sickness and deletion of Paf1 complex members enhanced phenotypes of a mutation in *NRD1*. These observations indicated that Nab3 and Nrd1 do not bypass the function of Rtf1 or the Paf1 complex. Rather, we propose that a functional link exists between Nab3 and the Paf1 complex. The underlying genetic relationships among members of the Paf1 complex and Nrd1 and Nab3 suggested that Rtf1 and the Paf1 complex could be involved in the 3'-end formation and/or transcription termination of snRNAs and snoRNAs.

To establish a functional relationship between the Paf1 complex and the transcription termination factors Nab3 and Nrd1, the processes of 3'-end formation and transcription termination needed to be examined in Paf1 complex mutants. Co-IP experiments could be used to determine physical interaction among Paf1 complex members and Nrd1 and/or Nab3. Further, ChIP could provide detailed information regarding the occupancy and interrelationships of the Paf1 complex, Nrd1 and Nab3, and RNA polymerase II on actively transcribed genes. Results

from the above experiments may contribute to ascertain whether the Paf1 complex is functionally connected to Nrd1 and Nab3 and provide insight into additional roles performed by the Paf1 complex in transcription.

3.2 Methods and materials

3.2.1 Yeast strains

SC medium was prepared with or without the addition of 100mM CuSO₄ as described (Steinmetz and Brow, 1996). As described in section 2.2.1, yeast strains used in this study are isogenic with FY2 (Table 10). However, two strains outside of the S288c genetic background were utilized for an in vivo reporter analysis that required the absence of the *CUP1* gene. Outside haploid strains 46a and 46 α were mated to construct a homozygous *cup1* Δ strain. In this diploid strain, genes encoding the members of the Paf1 complex were singly deleted using a PCR-based method (Ausubel et al., 1988) as described in section 2.2.1. and haploid strains were recovered using tetrad analysis (Rose et al., 1990). These strains were utilized in the ACT1-CUP1 reporter analysis described in sections 3.2.4.1. and 3.3.1.

Table 10: Yeast strains used in Chapter 3

STRAIN	GENOTYPE	ORIGIN
46α	<i>MATα cup1Δ his3 lys2 leu2 ura3 trp1 ade2</i>	Brow lab
46a	<i>MATa cup1Δ his3 lys2 leu2 ura3 trp1 ade2</i>	"
46α/46a	<i>MATα/ MAT a cup1Δ/" his3/" lys2/" leu2/" ura3/" trp1/" ade2/"</i>	Arndt lab
FY118	<i>MATa his4-912δ lys2-128δ leu2Δ1ura3-52 trp1Δ63</i>	Winston lab
FY348	<i>MATa spt16-197 his4-912δ lys2-128δ leu2Δ1ura3-52</i>	"
FY1635	<i>MATα spt5-242 his4-912δ lys2-128δ leu2Δ1ura3-52</i>	"
GHY166	<i>MATa spt4Δ2::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63</i>	Hartzog lab
GHY250	<i>MATα leo1Δ::URA3 his4-912δ lys2-128δ leu2Δ1ura3-52</i>	"
GHY972	<i>MATα HA3::PAF1 his3Δ200 lys2-128δ ura3Δ0</i>	"
GHY1094	<i>MATα ctr9Δ::KANMX4 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
GHY1177	<i>MATα his4-912δ lys2-128δ leu2Δ0 ura3Δ0 trp1Δ63 CTR9-6XMYC</i>	
KY508	<i>MATa snf2Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	Arndt lab
KY560	<i>MATα rtf1Δ102::ARG4 arg4-12his3Δ200 lys2-173R2 leu2Δ1 ura3-52</i>	"
KY586	<i>MATα ctk1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
KY591	<i>MATa leu2Δ1ura3-52 trp1Δ63</i>	"
KY632	<i>MATα chd1::URA3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
KY656	<i>MATa rtf1Δ100::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52</i>	"
KY661	<i>MATa his3Δ200 his4-912δ lys2-128δ leu2Δ1 ura3-52</i>	"
KY669	<i>MATa his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63</i>	"
KY693	<i>MATa ctr9Δ::KANMX4 leu2Δ1ura3-52 trp1Δ63</i>	"
KY671	<i>MATα/ MAT a his3Δ200/" lys2-128δ/" leu2Δ1/" ura3-52/" trp1Δ63/TRP1 ADE8/ade8</i>	"
KY685	<i>MATa paf1Δ::URA3 his4-912δ lys2-128δ leu2Δ(0 or 1) ura3(Δ0 or -52)</i>	"
KY689	<i>MATα cdc73Δ::KANMX4 lys2Δ0 leu2Δ0</i>	"
KY718	<i>MATa spt5-194 his3Δ200 lys2-128δ ura3-52</i>	"
KY802	<i>MATa paf1Δ::URA3 his3Δ200 lys2-173R2 ura3(Δ0 or -52)</i>	"
KY817	<i>MATα ctr9Δ::KANMX4 his4-912δ lys2-128δ leu2Δ1 ura3-52</i>	"
KY884	<i>MATa isw2::HIS3 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63</i>	"

Table 10 (continued)		
KY907	<i>MATa set1::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
KY912	<i>MATa set2::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
KY927	<i>MATa gcn5Δ::HIS3 his3Δ200 leu2Δ1</i>	"
KY930	<i>MATα rad6Δ::URA3 his3Δ200 ura3-52</i>	"
KY935	<i>MATα dot1Δ::HIS3 his3Δ200 ura3-52</i>	"
KY968	<i>MATa bre1Δ::KANMX4 his3Δ200 ura3-52</i>	"
KY972	<i>MATa swr1Δ::KANMX4 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
MHY57	<i>MATa ino80Δ::HIS3 his3Δ200 his4-912δ lys2-173R2 leu2Δ1 ura3-52</i>	"
MHY63	<i>MATα arg82Δ::URA3 his3Δ200 leu2Δ(0 or 1) ura3(Δ0 or -52)</i>	"
MHY75	<i>MATa isw1::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
MHY88	<i>MATa arp8Δ::HIS3 his3Δ200 lys2-128δ leu2Δ(0 or 1) ura3(0Δ or -52)</i>	"
KKY93	<i>MATα/ MAT a nab3Δ::HIS3/NAB3 his3Δ200/" lys2-128δ/" leu2Δ1/" ura3-52/" trp1Δ63/TRP1 ADE8/ade8</i>	""
KKY113	<i>MATα cup1Δ ctr9Δ::KANMX4 his3 lys2 leu2 ura3 trp1 ade2</i>	"
KKY119	<i>MATα cup1Δ paf1Δ::KANMX4 his3 lys2 leu2 ura3 trp1 ade2</i>	"
KKY120	<i>MATa nab3Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8 pKK10[HA₃NAB3, URA3, CEN/ARS]</i>	"
KKY122	<i>MATα paf1Δ::KANMX4 nab3Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 pKK10[HA₃NAB3, URA3, CEN/ARS]</i>	"
KKY125	<i>MATα nab3Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8 pKK11[NAB3, URA3, CEN/ARS]</i>	"
KKY126	<i>MATa paf1Δ::KANMX4 nab3Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63 ade8 pKK11[NAB3, URA3, CEN/ARS]</i>	"
PCY48	<i>MATα fcp1-110 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
nrd1-5	<i>MATα nrd1-5 cup1Δ his3 lys2 leu2 ura3 trp1 ade2</i>	Brow lab
YSB1560	<i>MATa ctk1Δ::HIS3 his3Δ200 lys2Δ202 leu2Δ1 ura3-52 trp1Δ63</i>	Buratowski lab

3.2.2 RNA isolation and Northern analysis

3.2.2.1 RNA isolation.

Cells were grown in YPD and harvested at a density of $1-2 \times 10^7$ cells/ml (Squazzo et al., 2002). Total RNA was isolated from collected cells using acid-washed glass beads, RNA buffer (500mM NaCl; 200mM Tris-HCl, pH 7.6; 10mM EDTA, 1% w/v SDS), and 25:24:1 PCI (phenol equilibrated with RNA buffer without SDS; CHCl₃; ddH₂O equilibrated isoamyl alcohol). The aqueous supernatant was precipitated with 100% EtOH, washed with 70% EtOH, and resuspended in 100µl ddH₂O. Total RNA was subjected to spectrophotometric analysis to determine concentration and visualized using TBE-agarose gel electrophoresis.

3.2.2.2 Northern blotting analysis.

The indicated RNAs were resuspended in RNA sample buffer (50% deionized formamide; 1X MOPS; 2.12M formaldehyde; 0.5mg/ml ethidium bromide), electrophoresed through a 1.5% denaturing agarose gel, and photographed with the 440CF digital imaging station (Kodak). Genescreen filters (PerkinElmer Life Sciences Inc.) were hybridized with random-prime labeled probes containing [α -³²P]dATP (Dupont NEN) and/or [α -³²P]dCTP (Dupont NEN), using PCR-amplified coding sequences of *TRS31*, *SNR13*, *YDRO42c*, *SNR47* and *SCRI* (Table 11) (Squazzo et al., 2002). The blots were exposed to both phosphorimaging screen and film (Kodak).

3.2.3 Plasmids

3.2.3.1 Construction of HA epitope-tagged version of Nab3.

Nab3 was N-terminally epitope-tagged using PCR primers KKO92 and KK093 that encode *Bgl*II restriction sites engineered at their 5' ends to amplify the triple HA1 epitope as in (Table

11) (Stolinski et al., 1997). Site-directed mutagenesis was performed on pKK5 (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene) with oligonucleotides KKO90 and KKO91 to engineer a single *Bgl*III site adjacent to the ATG of *NAB3* generating plasmid pKK5 (Table 11). pKK6-3 was created by subcloning a sequenced *Nhe*I-*Bsg*I fragment that encompassed the engineered *Bgl*III restriction site of pKK5. A *Bgl*III flanked (HA)₃ 108 bp PCR fragment was ligated into the *Bgl*III site of pKK6-3 and transformed into *E. coli*. Candidate plasmids were confirmed by sequencing over the 5' end of *HA₃NAB3* candidates and immunoblotting to identify *HA₃NAB3* gene fusions, resulting in pKK9-3. Plasmids pKK9-3 and pKK6-3 were digested with *Xho*I and *Spe*I to subclone *NAB3* and *HA₃NAB3* containing inserts directionally into pRS316 yielding pKK10 and pKK11. Dissection of *nab3Δ::HIS3* heterozygote KY93 resulted in haploid spores sorting 2:0 for viability and 2:0 for His⁻ phenotypes. The *NAB3* and *HA₃NAB3* containing plasmids fully complemented a *nab3Δ* strain for viability at 30°C and 37°C (K. E. Sheldon and K. M. Arndt, unpublished observations).

Table 11: Oligonucleotide sequences used for plasmid and probe construction

Oligonucleotide Name	5'-Sequence	Purpose
KKO80	GCGCATCTGACCAACAATTCCC	<i>TRS31</i> Northern probe
KKO81	GGAATCCTTTGATAATGCCGC	<i>TRS31</i> Northern probe
KKO82	GATTTTTTAAAGGGTCAGG	<i>SNR13</i> Northern probe
KKO83	CATTATATATGCAGCGCTACG	<i>SNR13</i> Northern probe
KKO90	CCATCTGCAAGCCATG <u>AGATCT</u> GATGAAA ACCATAACAGTG	Amplify HA1 cassette with <i>Bg</i> III site
KKO91	CACTGTTATGGTTTTTCATC <u>AGATCT</u> CATG GCTTGCAGATGG	Amplify HA1 cassette with <i>Bg</i> III site
KKO92	TGTATAATGA <u>AGATCT</u> TACCCATACGATG TTCCTGAC	Engineer <i>Bg</i> III site within <i>NAB3</i>
KKO93	TGTATATAGA <u>AGATCT</u> GCCGCACTGAGCA GCGTAATC	Engineer <i>Bg</i> III site within <i>NAB3</i>
KKO142	GGCGGTAACGTAAATCAGAGTAGC	<i>YDR042c</i> Northern probe
KKO143	GCATACCGCTTAATATTGTTATCGC	<i>YDR042c</i> Northern probe
KKO144	GGCTTCAGCTCCATATCTTTTG	<i>SNR47</i> Northern probe
KKO145	CCACCTATAAAGGATTCGGA	<i>SNR47</i> Northern probe
KKO171	GTGGGATGGGATACGTTGAG	<i>SCR1</i> Northern probe
KKO172	TAGCCGGGACACTTCAGAAC	<i>SCR1</i> Northern probe
KKO182	CCTAG <u>CTAGCCGCGTCAT</u> TCTTGCTGATT TTC	Clone <i>SNR47(70)</i> into p.G-CYC.ds
KKO183	CCGG <u>CTCGAGCAGAAATAAAGAAA</u> TGA AAGCTAC	Clone <i>SNR47(70)</i> into p.G-CYC.ds

3.2.3.2 Subcloning of *SNR47* termination sequences into pG-CYC.ds.

The 70 bp required for normal transcription termination of the *SNR47* transcript (*SNR47(70)*) were PCR amplified using the Expand high fidelity PCR system (Roche) and oligonucleotides engineered with restriction sites for directional subcloning. KKO182 contains an *NheI* restriction site and amplifies the 5' end of *SNR47(70)* (Table 11). KKO183 contains a *XhoI* site and amplifies the 3' end of *SNR47(70)* (Table 11). Restriction digests with *NheI* and *XhoI* were performed on gel-purified PCR products and pG-CYC.ds (Steinmetz and Brow, 2003). The products of digestion were subject to ligation, *E. coli* transformation, and plasmids were isolated from individual colonies. Candidate plasmids were subjected to restriction digest and sequencing analyses to identify constructs containing the *SNR47(70)* insert, pGCYC-*SNR47(70)*.ds.

3.2.4 In vivo reporter experiments

3.2.4.1 ACT1-CUP1 reporter assay for 3'-end formation.

The constructs for the ACT1-CUP1 reporter assay were a generous gift of E. J. Steinmetz and D. A. Brow (Steinmetz and Brow, 1996). The ACT1-CUP1 reporter contained a *TDH3* promoter driving the expression of the *ACT1* gene. Further, the intron of *ACT1* was replaced with the transcription termination sequence of *SNR13* 125-232. The *CUP1* gene was subcloned downstream of the *SNR13* termination sequences. Cup1 confers resistance to Cu²⁺. The indicated cells were transformed with the ACT1-CUP1 constructs and selection was maintained on medium lacking leucine. Cultures were subjected to dilution analysis essentially as described in section 2.2.3.4., with transformants grown in selective medium overnight and spotted in ten-fold

serial dilutions onto SC-Leu medium or SC-Leu medium supplemented with 100mM CuSO₄. The plates were incubated at 30°C and the images were recorded.

3.2.4.2 *HIS3* reporter for transcriptional readthrough.

The reporter assay for transcriptional readthrough developed by Carroll *et al.* utilizes two reporter plasmids (Carroll et al., 2004). The reporter constructs were a generous gift of J. L. Corden. Transcription of the control reporter construct is driven by the *ADHI* promoter with the *CYCI* terminator placed downstream of the *HIS3* gene to ensure proper transcription of *HIS3* and histidine prototrophy in *his3⁻* cells. The second reporter plasmid contains the same promoter, *HIS3* reporter gene and terminator, but also contains the SNR47(70) termination sequences inserted between the *ADHI* promoter and *HIS3*. The SNR47(70) cassette is recognized by the 3'-end formation machinery in wild-type cells and the *HIS3* reporter gene is not transcribed; mutant cells defective for 3'-end formation express *HIS3* and exhibit growth in the absence of histidine. The two constructs were transformed into *RTF1 his3Δ200* (KY669) and *rtf1Δ his3Δ200* (KY560) strains and maintained under selection on SC-Leu medium. Cultures were grown overnight in selective medium. The cells were washed two times with sterile ddH₂O and the cell density of each culture was determined using a hemacytometer. Serial ten-fold dilutions were prepared and spotted onto SC-Leu and SC-His-Leu media. The plates were incubated at 30°C and the images were recorded.

3.2.5 Chromatin immunoprecipitation (ChIP) analysis

Cells were grown in YPD to 1-2x10⁷ cells/ml. ChIP assays and immunoprecipitations with 8WG16 antibody (Covance) or anti-HA antibody conjugated to agarose beads (Santa Cruz Biotechnology) were performed as described (Komarnitsky et al., 2000; Simic et al., 2003). Nrd1 ChIPs were performed by incubating chromatin extracts overnight at 4°C with a 1:100 final

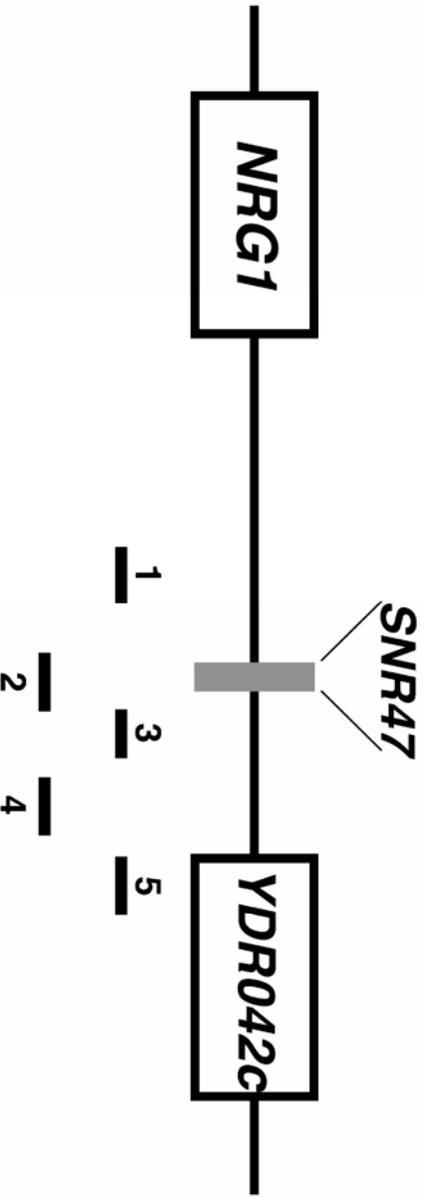
dilution of Nrd1 antisera (a gift from E. Steinmetz and D. Brow (Steinmetz and Brow, 1998)), followed by incubation with protein A-coupled Sepharose beads (Amersham Biosciences). To confirm signal linearity, quantitative PCR was performed on two dilutions of input and immunoprecipitated DNA using [α - 32 P]dATP, and relative ChIP signals were calculated as described in the figure legends. Table 12 lists the oligonucleotides used for ChIP at the *SNR13* and *SNR47-YDR042c* loci. Oligonucleotides KKO146 and KKO147 amplified the 3' end of *SNR13* from +117 to +333 relative to +1 of the *SNR13* gene, which is 124 bp in length (per *Saccharomyces* Genome Database (Balakrishnan et al.)). KKO147 annealed 70 bp upstream of the translational start of *TRS31*. PCR primers amplified the sequences relative to +1 of the *SNR47* genomic sequence (per *Saccharomyces* Genome Database (Balakrishnan et al.)). Oligonucleotides KKO156 and KKO157 amplified downstream of *SNR47* relative to ATG=+1 of *YDR042c*, from -11 to +206 (+531 to +749 relative to *SNR47*).

Table 12: Oligonucleotide sequences used for ChIP in Chapter 3

Gene Amplified Relative to +1 of the Indicated Gene	5'-Oligonucleotide Sequence	Primer Pairs
<i>SNR13</i> +117 to +333	KKO146-TCTGACCTTTTAACTTCCCCGTAG KKO147- CAATCTTAGCATGAACTTCGTCAC	NA
<i>SNR47</i> -350 to -150	KKO149- AATGGTAACAGCTCATTGAAGAAG KKO150- TAGAGTGGATGAAAATTTTGTGAA	1
<i>SNR47</i> -51 to +168	KKO151- GTACTGAGCAAAGTAGGAGAAAGGA KKO152-GCAAGAATGACGCGAAAATA	2
<i>SNR47</i> +99 to +303	KKO153- TTATAGGTGGAAACAACTTTGACA KKO154- GCCATTAGTAAGTACGCTTTGTTATC	3
<i>SNR47</i> +298 to +484	KKO155- AATGGCTATTATTTTTGCTAATGACAC KKO156- GATTCGCTATTTTCAATAGTAAAGTTTC	4
<i>YDR042c</i> -11 to +206	KKO156- CTTTTCACAACATGGAGCAAATA KKO157- AAAGTTTCGCTCAGTACTCCAATA	5

Figure 12: Primer pair hybridization location relative to *SNR47* and *YDR042c*

The oligonucleotides used to amplify DNA surrounding the *SNR47* locus are listed below. The primer pairs are labeled 1-5 and hybridize relative to *SNR47* and *YDR042c* as indicated in Table 12 above. The *SNR47* gene is indicated by a gray box and is drawn to scale.



3.2.6 Co-IP and immunoblotting analysis

3.2.6.1 Analysis of Nrd1 protein levels.

To analyze Nrd1 levels, whole cell extracts (Squazzo et al., 2002) were resolved on 10% SDS-polyacrylamide gels and nitrocellulose filters were probed with a 1:3000 dilution of anti-Nrd1 antisera (Steinmetz and Brow, 1998). L3 levels were determined as described in section 2.2.4. Immunoreactive proteins were detected by chemiluminescence of HRP-coupled secondary antibodies and visualized the 440CF digital imaging station (Kodak).

3.2.6.2 Co-immunoprecipitation (Co-IP) analysis.

Cells were grown to a cellular density of $2-5 \times 10^7$ cells/ml in YPD. The cells were harvested and protein extract preparation was performed as described in (Squazzo et al., 2002). Co-IPs were performed as in (Squazzo et al., 2002) using 2mg of protein extract and the following dilutions of primary antibodies to isolate the indicated proteins and associated immune complexes: 1) no antibody, 2) 1:150 anti-Myc to precipitate Ctr9-6XMyC, 3) 1:1000 anti-Nrd1, and 1:3000 anti-HA to precipitate either 4) HA₃-Rtf1 or 5) HA₃-Paf1. Extracts and primary antibodies were rotated overnight at 4°C. Secondary antibodies conjugated to agarose beads (Sigma) were incubated with the primary immune complexes for 1-2 hours at 4°C on a rotator. Unbound fractions were collected and bound fractions were washed 5 times with 1X lysis buffer (1X lysis buffer=20mM HEPES-KOH, pH7.4; 10% glycerol; 2mM Mg(OAc)₂, 10mM EDTA, pH7.4; and 0.05% Tween-20) containing 1X protease inhibitors (25ml 100X protease inhibitors in ethanol= 0.0039g Pepstatin A, 0.0008g Leupeptin, 0.005g Chymostatin, 0.85g Benzamidine, and 0.43g PMSF) without 1mM DTT and either 100 or 150mM NaOAc prior to elution in SDS

sample buffer at 100°C. The bound and unbound fractions were resolved on 10% SDS-polyacrylamide gels. Nitrocellulose filters were probed with the following dilutions of primary antibody 1:1000 anti-Myc in Blotto, 1:3000 anti-HA in Blotto, and 1:1000 anti-Nrd1 in Blocko. The filters were probed with the primary antibody used in the immunoprecipitation to qualitatively estimate the immunoprecipitation efficiency of the reaction.

3.2.7 Transcription run on (TRO) assays

3.2.7.1 Harvesting and permeabilizing cells.

TRO analysis was performed essentially as described in (Steinmetz and Brow, 2003) and constructs were provided generously provided by E. J. Steinmetz and D. A. Brow. Briefly, strains transformed with pG-CYC.ds, pG-SNR13-125-232-CYC.ds, or pG-SNR47(70).CYC.ds were grown at 30°C, unless otherwise indicated, to an OD₆₀₀ between 0.12-0.2 in selective media. Cells were harvested by centrifugation in the GS-15R centrifuge (Beckman) at 4°C, washed in ice-cold ddH₂O, and transferred to a pre-chilled eppendorf tube. The cells were resuspended in 950µl of ice-cold ddH₂O and were permeabilized by adding 50µl of 10% w/v sarkosyl and incubating the suspension on ice for 20 minutes. The permeabilized cells were microfuged at 4°C for 1 minute at maximum rpm, supernatant was removed with a pipet tip, and this procedure was repeated one more time to remove as much Sarkosyl as possible.

3.2.7.2 Transcript labeling.

The permeabilized cells were resuspended in 71µl of transcription mix: 60µl 2.5X transcription buffer (50mM Tris-HCl, pH7.7; 500mM KCl; 80mM MgCl₂), 8µl NTP mix (10mM each ATP and CTP), 3µl 100mM DTT. Transcripts were labeled by adding 100µCi of [α -³²P]-UTP (Amersham Pharmacia) and incubating the suspension at 30°C for 5 minutes. The labeling

reaction was followed by a 5 minute "chase" at 30°C by adding 10µl 25mM UTP/0.25mM GTP. 900µl AE buffer (50mM NaOAc, pH 5.2; 10mM EDTA) was added to the suspension to stop the labeling reaction.

3.2.7.3 RNA isolation.

An equal volume of 40:8:1 aPCI (acid-phenol (Sigma); CHCl₃; ddH₂O-equilibrated isoamyl alcohol) pre-warmed to 65°C was combined with cells resuspended in 400µl TES (10mM Tris-HCl, pH 7.5; 10mM EDTA; 0.5% SDS) in 1.5ml screw cap tubes (Sarstedt) and incubated for 1 hour, vortexing for 10 seconds every 15 minutes. The lysates were incubated on ice for 5 minutes followed by a 5 minute spin at 4°C at 14K rpm in a microfuge. Supernatants were extracted again with 40:8:1 aPCI. RNA was EtOH precipitated and resuspended in 40µl ddH₂O.

3.2.7.4 RNaseT1 digestion and denaturing gel electrophoresis.

RNaseT1 digests containing 20µl of total RNA, 5µl RNaseT1 (Ambion, catalog number 2280) in 100µl TE (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0) were incubated at 37°C for 2 hours. The digests were treated with 2.5mg/ml Proteinase K (Roche) and 0.5% SDS, final concentration, for 20 minutes at 37°C. Labeled transcripts were EtOH-precipitated and resuspended in 15µl formamide loading buffer (80% deionized formamide; 1mM EDTA, pH 8.0; 0.1% xylene cyanol; 0.1% bromophenol blue). Samples were heated to 95°C for 3 minutes and 7.5µl of each were subjected to 6%/8.3M urea PAGE analysis. Gels were dried and exposed to both phosphorimaging screens and film.

3.2.7.5 5' end labeling of molecular weight standards.

Approximately 5µg each of BstNI-digested pBR322 (NEB) and 100 bp ladder (Promega) were treated with calf intestinal phosphatase (NEB), 25:24:1 PCI extracted, and EtOH precipitated. Resuspended DNA markers were 5' end labeled with 20µCi of [γ -³²P] ATP (Amersham Biosciences) and T4 polynucleotide kinase (NEB) in freshly prepared 1X T4 polynucleotide kinase buffer (70mM Tris-HCl, pH 7.6; 10mM MgCl₂; 5mM DTT). Labeled DNA standards were purified over G-50 columns (Roche). Dilutions of labeled markers were loaded with RNaseT1 digested RNAs and electrophoresed on denaturing polyacrylamide gels.

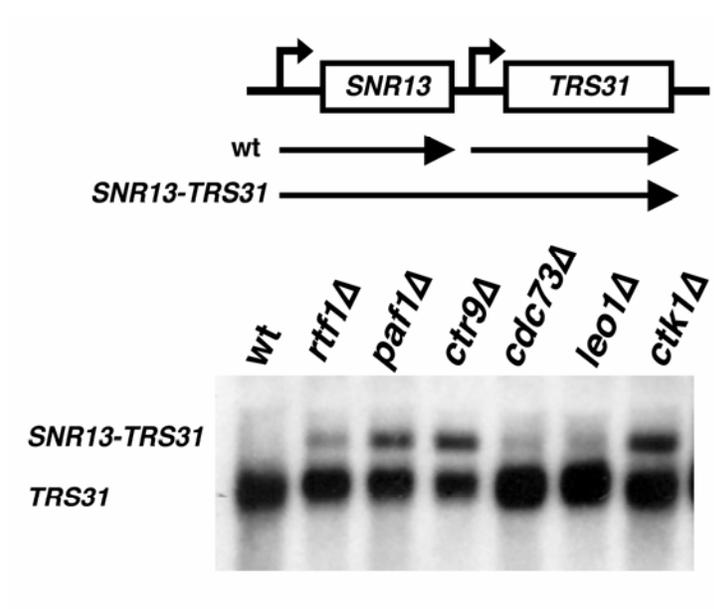
3.3 Results

3.3.1 The Paf1 complex is required for 3'-end formation of *SNR13* transcripts

To investigate a possible role for the Paf1 complex in the 3'-end formation of snoRNAs, we used Northern analysis to detect transcripts produced from the snoRNA gene *SNR13* and the downstream protein-coding gene *TRS31*. For strains defective in 3'-end formation, such as *nrd1* and *nab3* mutants, a hybridization probe specific for *TRS31* detects an *SNR13-TRS31* fusion RNA arising from readthrough of the *SNR13* termination signals (Figure 13). Northern analysis of RNAs expressed from the *SNR13-TRS31* locus was performed on strains lacking members of the Paf1 complex. While the normal *TRS31* transcript was detected in all strains (Figure 13), *rtf1Δ*, *paf1Δ*, and *ctr9Δ* mutants express an additional, lower mobility RNA that is also recognized by the *TRS31* probe and corresponds to the product of transcriptional readthrough. The migration of this longer transcript is similar to that seen in strains lacking Ctk1 (Steinmetz et al., 2001). When compared to the Paf1 complex mutants, *nrd1* and *nab3* mutants accumulate higher levels of this 3'-extended RNA, as might be expected for central components of the termination machinery (Steinmetz et al., 2001). These results indicate that the Paf1 complex plays an important role in the 3'-end formation of snoRNA transcripts.

Figure 13: Mutation of individual components of the Paf1 complex results in defective 3'-end formation of *SNR13* messages

SNR13 readthrough transcripts accumulate in Paf1 complex mutant strains. Top, *SNR13-TRS31* genomic locus and transcription products. The transcriptional readthrough product is indicated as *SNR13-TRS31*. Northern analysis using a *TRS31* probe and RNA isolated from the following yeast strains: wild-type (KY661), *rtf1* Δ (KY656), *paf1* Δ (KY685), *ctr9* Δ (GHY1094), *cdc73* Δ (KY689), *leo1* Δ (GHY250), and *ctk1* Δ (KY586).

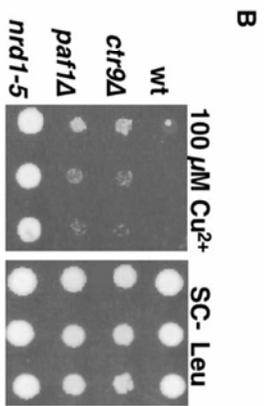
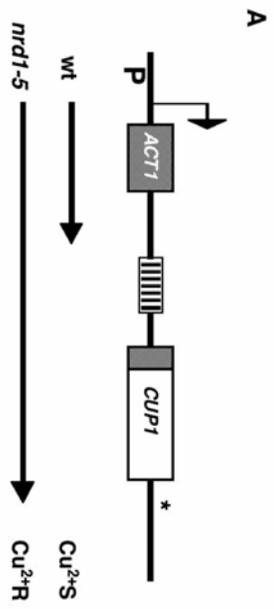


Transcriptional readthrough at the *SNR13* locus was also analyzed using the ACT1-CUP1 reporter construct (Steinmetz and Brow, 1996). As described in the Methods and Materials section 3.2.4.1. and in Figure 10A, the ACT1-CUP1 reporter construct contains a promoter that drives the transcription of the *ACT1* gene. The intron in the *ACT1* gene is replaced with sequences required for proper 3'-end formation of *SNR13* transcripts. The *CUP1* gene resides downstream of the 3'-end formation sequences and confers resistance to Cu^{2+} only in strains where transcription readthrough of the *SNR13* termination sequences occurs. ACT1-CUP1 was transformed into *cup1Δ*, *paf1Δ cup1Δ*, or *ctr9Δ cup1Δ* strains and dilution analysis was performed to determine if these cells were resistant to Cu^{2+} in the medium. The *paf1Δ cup1Δ* and *ctr9Δ cup1Δ* cells exhibited resistance to Cu^{2+} in the growth medium as compared to the wild-type control cells that lack only the *CUP1* gene (Figure 14). This analysis was partially complicated by the observation that the *paf1Δ cup1Δ* and *ctr9Δ cup1Δ* strains were very slow growing or sick when compared to the wild-type cells lacking *CUP1*. The *paf1Δ* and *ctr9Δ* deletion strains were constructed in a genetic background with the *cup1Δ* allele that is different from the S288c background normally used in the lab. This strain background was utilized because the single *CUP1* gene had been disrupted in it (Steinmetz and Brow, 1996) as compared to the S288c genetic background, which contains two copies of the *CUP1* gene (Balakrishnan et al.). Disrupting both copies of *CUP1* in the S288c background would be technically challenging due to the homology-based method used for gene disruption in *S. cerevisiae*. In addition, there may be additional *CUP1* genes in the S288c genetic background (Balakrishnan et al.). Since, *PAF1* and *CTR9* were disrupted in a genetic background other than S288c, it is possible that unknown genetic elements in this background in combination with *paf1Δ* or *ctr9Δ* mutations

result in severe sickness not observed in the S288c genetic background. In any event, results of the ACT1-CUP1 reporter assay in *paf1Δ* and *ctr9Δ* mutants indicates that RNA polymerase II reads through the sequences required for normal 3'-end formation of the *SNR13* message. Taken together, results from the ACT1-CUP1 assay and the *TRS31* Northern analysis support a role for the Paf1 complex in 3'-end formation of *SNR13* transcripts.

Figure 14: Deletion of Paf1 complex members causes defects in snR13 3'-end formation in an in vivo reporter assay

Deletion of *PAF1* and *CTR9* confers resistance to Cu^{2+} in the ACT1-CUP1 reporter assay. (A) Top panel, diagram of the ACT1-CUP1 construct. The *TDH3* promoter (indicated by P) drives the transcription of *ACT1* (gray box). The striped box represents sequences required for termination of *SNR13* within the *ACT1* intron. *CUP1* (white box) is fused to the 3'-end of *ACT1*. The asterisk indicates the relative position of the poly(A) site. Bottom panel, product of transcription in wild-type (wt) or *nrd1-5* strains. Transcription readthrough of *SNR13* termination sequences results in Cu^{2+} resistance. (B) Wild-type (wt) (46 α), *paf1* Δ (KKY119), *ctr9* Δ (KKY113), and *nrd1-5* (*nrd1-5*) were transformed with the ACT1-CUP1 reporter construct. Transformants were spotted in ten-fold serial dilutions onto SC-Leu media either supplemented with or without 100 μM Cu^{2+} . α

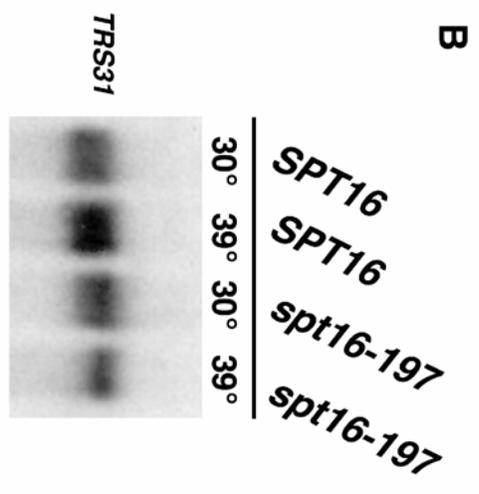
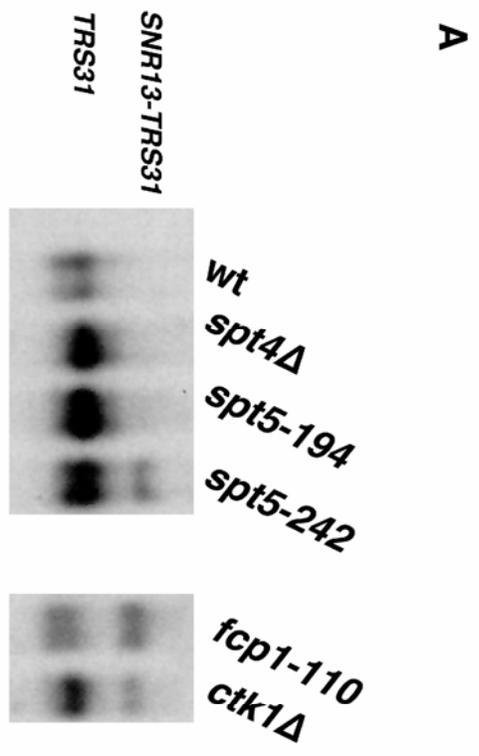


3.3.2 Is there a general requirement for transcription elongation factors in 3'-end formation of snR13?

The Paf1 complex physically and genetically interacts with Spt4-Spt5 and yFACT (Costa and Arndt, 2000; Krogan et al., 2002; Lindstrom et al., 2003; Mueller and Jaehning, 2002; Squazzo et al., 2002). To determine whether an effect on snR13 3'-end formation is specific to the Paf1 complex, we performed Northern analysis on strains containing a null mutation in *SPT4* or specific point mutations in *SPT5* and *SPT16*. While *spt4* Δ and *spt5-194* cells produce only the normal *TRS31* transcript, *spt5-242* cells accumulate the *SNR13-TRS31* fusion transcript (Figure 15A). Interestingly, the *spt5-242* mutation but not the *spt5-194* mutation can be suppressed by defects in the Paf1 complex (Squazzo et al., 2002). The temperature sensitive *spt16-197* strain did not accumulate the 3'-extended transcript at either the permissive or restrictive growth temperatures (Figure 15B). Therefore, an involvement in 3'-end formation is not necessarily a property shared by all RNA polymerase II elongation factors.

Figure 15: Not all RNA polymerase II-associated elongation factors are important for 3'-end formation of *SNR13* transcripts

SNR13 readthrough transcripts accumulate in Paf1 complex mutant strains. (A-B) Northern analysis using a *TRS31* probe and RNA isolated from the following yeast strains: (A) wild-type (wt) (FY118), *spt4* Δ (GHY166), *spt5-194* (KY718), *spt5-242* (FY1635), *fcp1-110* (PCY448), and *ctk1* Δ (KY586); (B) *SPT16* (FY118) and *spt16-197* (FY348).



We also failed to observe a significant effect of fourteen other RNA polymerase II transcription or chromatin factors on snR13 3'-end formation (Figure 16). These factors include those involved in histone modification (Set1, Set2, Dot1, Bre1 and Gcn5), chromatin remodeling (Isw1, Isw2, Snf2, Chd1, Swr1 and Arg82) or transcriptional activation (Swi4, Swi6, and Mbp1). The absence of the *SNR13* transcriptional readthrough product in *set1Δ*, *set2Δ* and *dot1Δ* strains (Figure 16) suggests that the function of the Paf1 complex in RNA 3'-end formation is independent of its role in histone H3 K4, K36 and K79 methylation. Similar conclusions were drawn for Swd2, which is a member of both the Set1 histone methyltransferase complex and the APT cleavage/polyadenylation factor complex (Cheng et al., 2004; Dichtl et al., 2004).

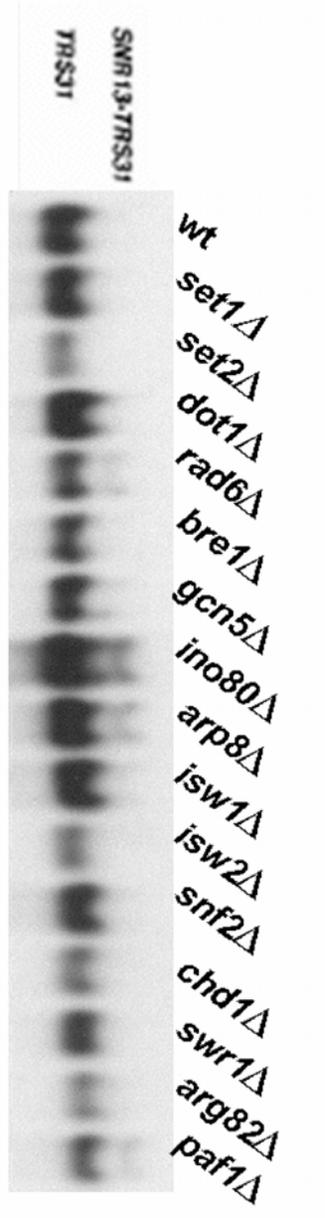
Mutations that prevent the methylation of lysine residues in histone H3 do not result in the extension of *SNR13* transcripts (Figure 16). However, deletion of *RAD6*, which encodes a factor involved in the ubiquitylation of lysine 123 (K123) of histone H2B does result in extended *SNR13* messages (Figure 16). This observation is of interest since the ubiquitylation of K123 on histone H2B, is required prior to lysine methylation of either residues 4 or 79 on histone H3 (Ng et al., 2003; Ng et al., 2002; Wood et al., 2003; Wood et al., 2003). *SNR13* 3'-extended transcripts observed in *rad6Δ* strains could arise indirectly. For example, Rad6 may target proteins involved in transcription or 3'-end formation of *SNR* genes for degradation via the ubiquitin-proteasome pathway.

Interestingly, mutations in *ARP8* and *INO80* caused defects in 3'-end formation of snR13 (Figure 16). Arp8 is integral for assembly of the INO80 chromatin remodeling complex while Ino80 is the catalytic component of the remodeling complex (Shen et al., 2003). Preliminary observations indicate that these factors may not be generally required for 3'-end formation of snoRNAs since proper 3'-end formation of snR47 occurred in the absence of Ino80 and Arp8 (K.

E. Sheldon and K. M. Arndt, unpublished observations). The 3'-extended form of snR13 in INO80 complex mutants is intriguing since library plasmid 304 from the high-copy-number suppressor screen described in section 2.3.3. contained a truncated form of *ARP5*, another member of the INO80 complex.

Figure 16: Several mutations in genes that encode components of chromatin remodeling complexes, histone modification proteins, and transcriptional activators do not cause transcriptional readthrough at the *SNR13* locus

SNR13 readthrough transcripts accumulate in Paf1 complex mutant strains. Northern analysis using a *TRS31* probe and RNA isolated from the following yeast strains: wild-type (KY669), *set1Δ* (KY907), *set2Δ* (KY912), *dot1Δ* (KY935), *rad6Δ* (KY930), *bre1Δ* (KY968), *gcn5Δ* (KY927), *ino80Δ* (MHY57), *arp8Δ* (MHY88), *isw1Δ* (MHY75), *isw2* (KY884), *snf2Δ* (KY508), *chd1Δ* (KY632), *swr1Δ* (KY972), *arg82Δ* (MHY63) and *paf1Δ* (KY802). *TRS31* or *SNR13-TRS31* transcripts are indicated.



Ctk1 and the Ser5-directed CTD phosphatase Ssu72 are required for efficient snoRNA 3'-end formation (Steinmetz and Brow, 2003; Steinmetz et al., 2001). We asked whether a mutation in *FCP1*, which encodes a Ser2-directed CTD phosphatase, also causes defects in snR13 synthesis. The *fcp1-110* mutation was isolated in a synthetic lethal screen with an *rtf1Δ* mutation and is predicted to truncate the two TFIIF binding domains of Fcp1 (Costa and Arndt, 2000). The predicted *fcp1-110* protein retains the phosphatase and BRCT domains, and strains expressing this protein exhibit nearly wild-type levels of Ser-2 phosphorylation (M. Shirra and K. Arndt, unpublished observations). However, like defects in other CTD-modifying enzymes, the *fcp1-110* mutation causes significant accumulation of the *SNR13* readthrough product (Figure 15A).

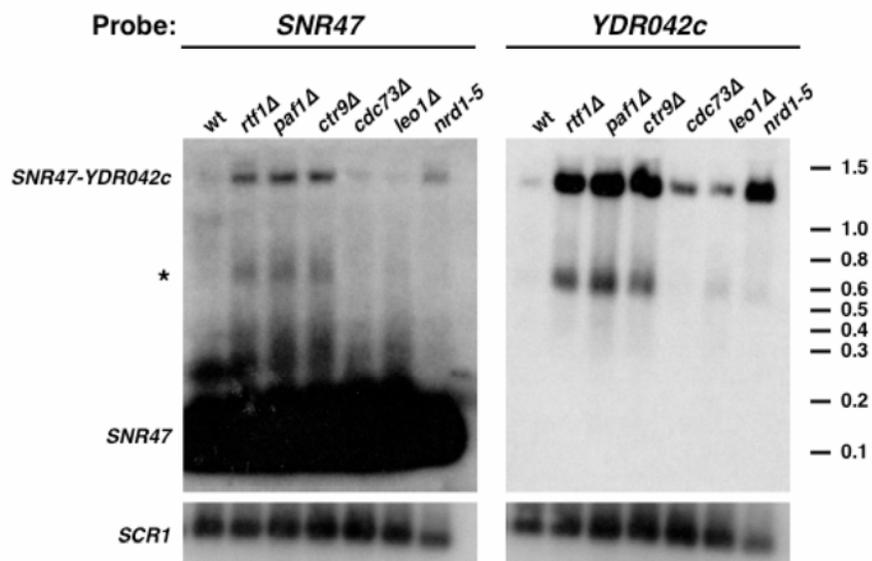
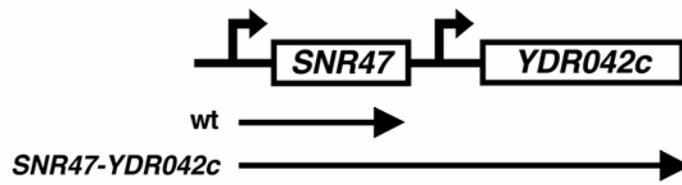
3.3.3 The Paf1 complex is required for 3'-end formation of *SNR47* transcripts

To ask if there is a general requirement for the Paf1 complex in the proper 3'-end formation of snoRNAs, we assayed expression of the *SNR47* gene. Northern hybridization probes were designed against *SNR47* and the uncharacterized ORF *YDR042c*, which resides downstream of *SNR47* in the genome (Figure 17). A prominent ~1400 nt transcript was detected by the *YDR042c* probe in *rtf1Δ*, *paf1Δ*, *ctr9Δ*, and *nrd1-5* strains (Figure 17). This transcript, which is present in greatly reduced levels in wild-type strains, has the approximate size of the predicted *SNR47-YDR042c* chimeric transcript (~1150 nt + poly(A) tail). In addition, a transcript with the same mobility was detected by the *SNR47* probe (Figure 17). A shorter transcript (marked by asterisk in Figure 17), which hybridized to both probes, may represent a degradation product of this readthrough transcript. Apparently, wild-type cells do not detectably express *YDR042c* under the growth conditions used in our experiments, in agreement with ChIP results in the absence of

the Paf1 complex correlates with increased RNA polymerase II density over the 5' region of *YDR042c* (Figure 21C). Accumulation of 3'-extended RNAs at both the *SNR13* and *SNR47* loci suggests that the Paf1 complex may be generally required for proper snoRNA synthesis.

Figure 17: Transcriptional readthrough from *SNR47* into *YDR042c* occurs in strains lacking components of the Paf1 complex

Top, the *SNR47* genomic locus and transcripts detected in wild-type or mutants defective in snR47 3'-end formation (*SNR47-YDR042c*). Transcription of *YDR042c* is not detected in wild-type cells. Bottom, Northern analysis on RNA from wild-type (FY118), *rtf1Δ* (KY656), *paf1Δ* (KY685), *ctr9Δ* (GHY1094), *cdc73Δ* (KY689), *leo1Δ* (GHY250), and *nrd1-5* cells. RNA samples were loaded in duplicate on the same gel, one-half of which was subjected to Northern analysis with a *YDR042c* probe and the other half with an *SNR47* probe. The positions of the ethidium bromide stained RNA molecular weight markers are shown. Asterisks denote a hybridization signal that is not specific to either the *YDR042c* or the *SNR47* probe. This signal may arise from a breakdown product of the chimeric *SNR47-YDR042c* transcript. Filters were reprobated for *SCR1* transcript levels as a loading control.



To determine whether the Paf1-mediated effect on *SNR47* expression maps to the known *SNR47* 3'-end formation element or to some other aspect of the *SNR47-YDR042c* transcript, we used a plasmid-based reporter assay in which the *SNR47* 3'-end formation element is placed in a heterologous context, between a promoter and a reporter gene (Carroll et al., 2004). In the control plasmid for this assay, AHC, the *ADHI* promoter drives expression of the yeast *HIS3* gene and confers histidine prototrophy to *his3Δ* strains. The ASHC reporter plasmid contains 70 bp of *SNR47* sequence required for 3'-end formation inserted between the *ADHI* promoter and *HIS3* (Figure 18). *RTF1* and *rtf1Δ* strains containing AHC grow in the absence of histidine, as expected (Figure 18). However, while *RTF1*⁺ strains containing ASHC grow poorly in the absence of histidine, *rtf1Δ* strains containing this plasmid grow similarly to *rtf1Δ* strains containing the control plasmid that lacks the *SNR47* sequence (Figure 18). The results from this reporter assay suggest that the Paf1 complex is required for efficient recognition of the *SNR47* 3'-end formation signal.

Figure 18: Strains deleted for members of the Paf1 complex exhibit transcriptional readthrough in a reporter assay

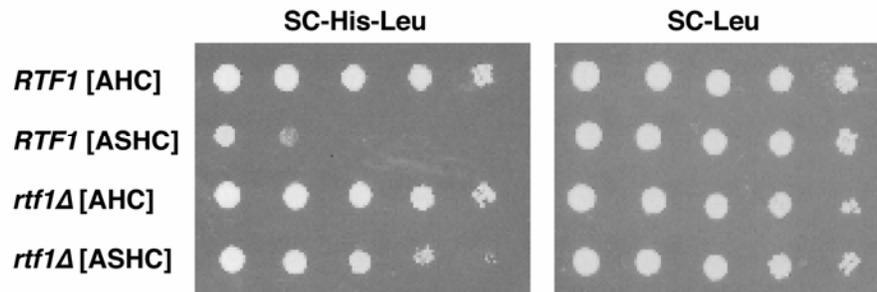
Top, plasmids used in the reporter assay to monitor snR47 3'-end formation. Bottom, *RTF1* (KY669) and *rtf1* Δ (KY560) cells transformed with the *LEU2*-marked plasmids AHC and ASHC were plated in ten-fold serial dilutions onto SC-His-Leu and SC-Leu media (2 days incubation at 30°C).

pADH1-HIS3-CYC1 (AHC)

<i>ADH1</i>	<i>HIS3</i>	<i>CYC1</i>
-------------	-------------	-------------

pADH1-SNR47(70)-HIS3-CYC1 (ASHC)

<i>ADH1</i>	<i>SNR47 (70)</i>	<i>HIS3</i>	<i>CYC1</i>
-------------	-------------------	-------------	-------------

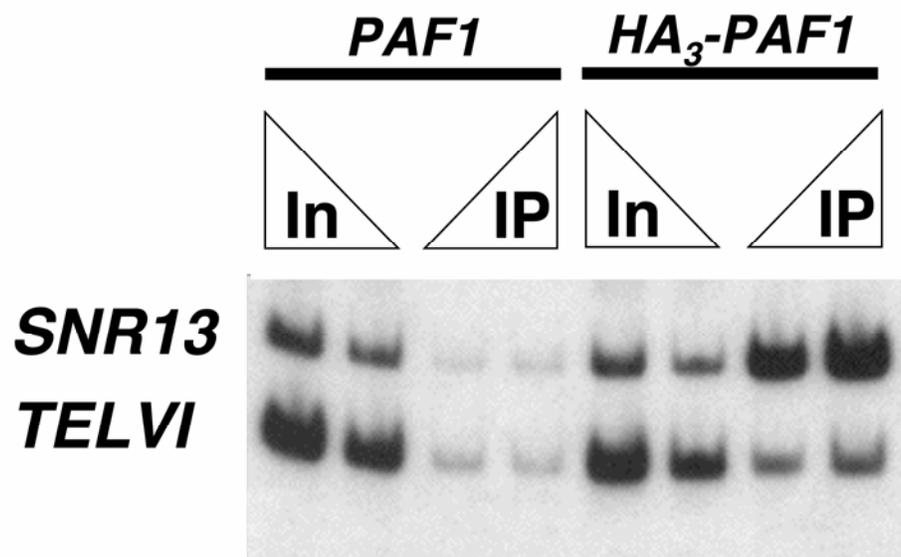


3.3.4 The Paf1 complex co-localizes with RNA polymerase II to *SNR47*

To begin to elucidate the mechanism by which the Paf1 complex affects the synthesis of snoRNA transcripts, we performed CHIP analysis. Using antibodies toward an epitope-tagged form of Paf1 followed by quantitative PCR, we determined the Paf1 complex directly associates with the 3'-end of *SNR13* (Figure 19). This observation is in agreement with results documented in (Nedea et al., 2003), where Nrd1 and Nab3 levels are enriched on DNA downstream of *SNR13*. These studies are complicated by the fact that the distance between *SNR13* and *TRS31* is only 280 bp. Since sonication of chromatin typically results in DNA fragment sizes ranging from 250 to 500 bp, the separation of these two genes is not within the resolution of sonication. Therefore, association of Nrd1, Nab3 or members of the Paf1 complex downstream of *SNR13* could be affected by the recruitment of these factors to the promoter and the 5' region of *TRS31* (Nedea et al., 2003).

Figure 19: Epitope-tagged Paf1 is enriched over *SNR13*

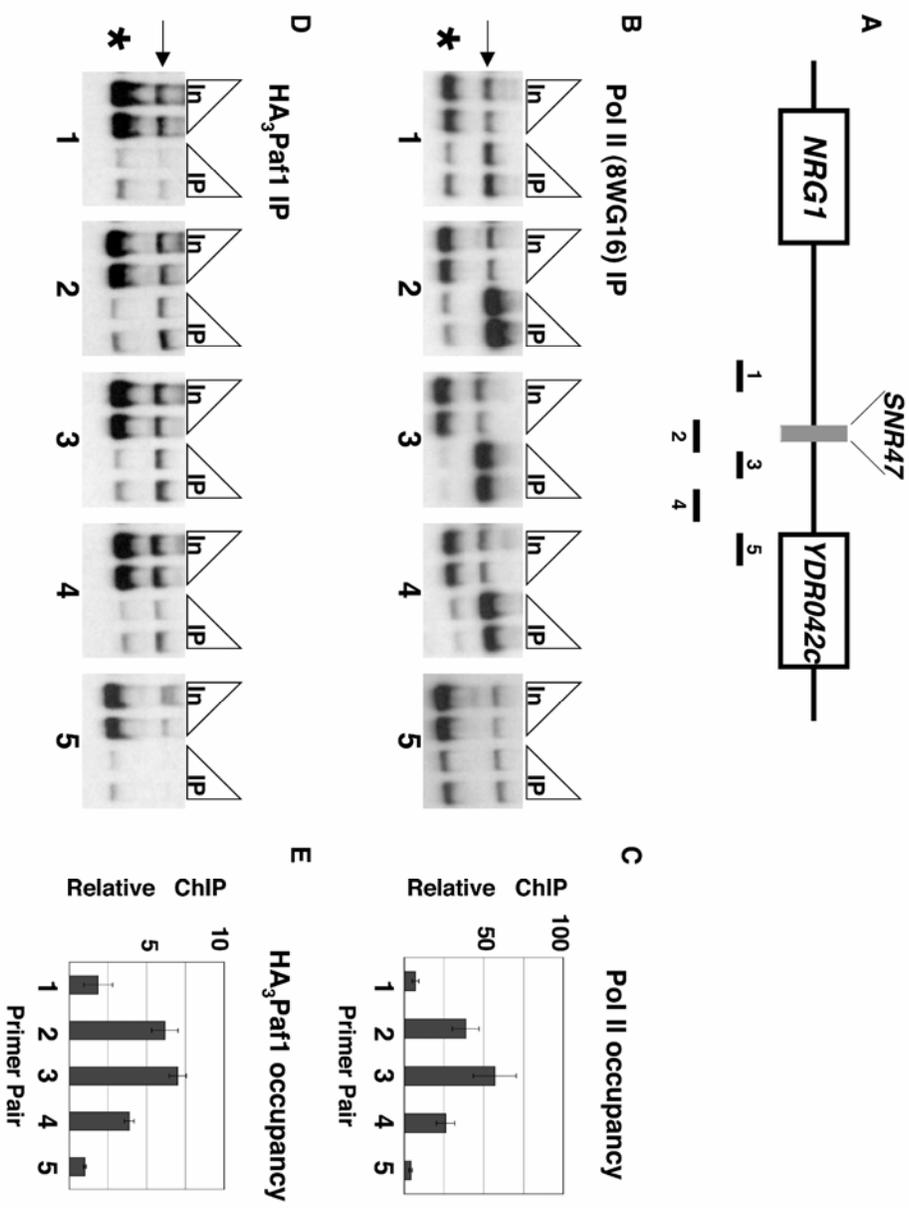
ChIP analysis of Paf1 occupancy over *SNR13*. Sonicated chromatin from *PAF1* (KY669) and *HA₃-PAF1* (KY783) cells was immunoprecipitated with anti-HA antibody. Two amounts of immunoprecipitated (IP) DNA (2 μ l and 4 μ l) and input DNA (4 μ l of 1:125 and 1:250 dilutions) were analyzed by PCR. *SNR13* ChIP signals were normalized to *TELVI* signals (Simic et al., 2003).



The Paf1 complex is important for 3'-end formation of snR13 and ChIP revealed that the Paf1 complex associates with *SNR13*. For these reasons, the question of whether the Paf1 complex associates with *SNR47* DNA arose. Therefore, we designed five sets of primer pairs to amplify the region spanning the *SNR47* gene and extending downstream into the *YDR042c* ORF (Figure 20A). We chose *SNR47* for our ChIP assays because the adjacent *YDR042c* gene appears to be poorly expressed from its own promoter (Figure 17). Therefore, the interpretation of our ChIP results should not be complicated by the association of transcription factors with the downstream gene. The occupancy of RNA polymerase II was mapped over the *SNR47* locus by using the CTD-specific antibody 8WG16. As expected, RNA polymerase II associates with the *SNR47* locus including sequences, amplified by primer pair 3, known to contain Nrd1 and Nab3 binding motifs (Figure 19B and 19C) (Carroll et al., 2004). In a similar experiment we assayed for the presence of the Paf1 complex using strains that express a triple HA-tagged form of Paf1 (Figure 19D and 19E). The profile of HA₃-Paf1 association over *SNR47* closely matches that of RNA polymerase II. These data are consistent with a direct involvement of the Paf1 complex in the synthesis of snR47.

Figure 20: RNA polymerase II colocalizes with HA₃-Paf1 at the *SNR47* locus

RNA polymerase II and Paf1 co localize to the *SNR47* gene. (A) Diagram of the *SNR47* locus with PCR products amplified in ChIP experiments. (B) Chromatin from wild-type (KY669) cells was immunoprecipitated with 8WG16 antibody. Two amounts of immunoprecipitated (IP) DNA (2 μ l and 4 μ l) and input DNA (4 μ l of 1:125 and 1:250 dilutions) were analyzed by PCR. The reactions included the *SNR47* primer pair specified below each panel (arrow denotes PCR product) and a primer pair to an intergenic region of chromosome VIII ((Ng et al., 2003); asterisk denotes PCR product). (C) *SNR47* ChIP signals were normalized to chromosome VIII signals. Normalized IP values were divided by normalized input values, and the ratios from three independent ChIP experiments were averaged. (D) ChIP was performed on chromatin prepared from *HA₃-PAF1* (GHY972) cells with anti-HA antibody. (E) Quantitation of three HA₃-Paf1 ChIP experiments.



3.3.5 Nrd1 association with *SNR47* is dependent on the Paf1 complex

To test whether association of Nrd1 with *SNR47* requires a functional Paf1 complex, we performed ChIP analysis on Nrd1. These experiments demonstrate that Nrd1 associates with *SNR47* in a pattern similar to that of RNA polymerase II and the Paf1 complex (Figure 21A and 21B). Importantly, Nrd1 occupancy over the *SNR47* locus is significantly reduced in strains lacking Paf1 (Figure 21A and 21B). This reduction in the Nrd1 ChIP signal could be due to masked Nrd1 epitopes in chromatin prepared from *paf1Δ* cells, reduced levels of Nrd1 protein in *paf1Δ* strains, reduced RNA polymerase II occupancy in *paf1Δ* strains causing a concomitant decrease in Nrd1 occupancy, or a requirement for the Paf1 complex in the recruitment or stable association of Nrd1 with *SNR47*. To address the first possibility, we mapped the association of Nab3 to *SNR47* using antibodies toward an HA₃-tagged form of Nab3. Our experiments revealed a reduction in HA₃-Nab3 occupancy at *SNR47* in *paf1Δ* strains similar to that observed for Nrd1, suggesting that a decrease in epitope availability is not responsible for the lower Nrd1 ChIP signal at *SNR47* (Figure 22A,B). By immunoblotting, we found that Nrd1 levels are increased, not reduced, in *paf1Δ* extracts when compared to *PAF1* extracts (Figure 23A). Nab3 levels are also similar in *PAF1* and *paf1Δ* cells (Figure 23B). This finding is consistent with a role for the Paf1 complex in Nrd1-dependent 3'-end formation, since *NRD1* expression is auto-regulated by a termination sequence present within its 5' untranslated region (Steinmetz et al., 2001). Finally, ChIP analysis using an epitope-tagged form of Rpb3 indicated approximately equivalent association of RNA polymerase II with *SNR47* in *PAF1* and *paf1Δ* strains, eliminating the possibility that reduced Nrd1 occupancy in *paf1Δ* strains is simply due to reduced levels of RNA polymerase II on the gene (Figure 21C). Of interest is the increase in HA-Rpb3 signal detected in

paf1 Δ cells downstream of *SNR47* over the region spanned by primer pairs 3-5 (Figure 20A and 21C). While small, this increased signal correlates with the production of the *SNR47-YDR042c* transcriptional readthrough product, which accumulates over time to readily detectable levels. These results suggest that the Paf1 complex facilitates 3'-end formation of snR47, at least in part, by regulating the recruitment or stable association of Nrd1 and Nab3 with the RNA polymerase II elongation apparatus.

Figure 21: Association of Nrd1 with *SNR47* DNA is dependent on a functional Paf1 complex

Paf1 is required for normal Nrd1 association with *SNR47*. (A) ChIP was performed on chromatin prepared from *PAF1* (KKY120) and *paf1* Δ (KKY122) cells with anti-Nrd1 antibody (IP) or preimmune sera (Pi). Two amounts of immunoprecipitated (IP) DNA (2 μ l and 4 μ l) and input (In) DNA (4 μ l of 1:125 and 1:250 dilutions) were analyzed by PCR. The reactions included the *SNR47* primer pair specified below each panel from figure (arrow denotes PCR product) and a primer pair to an intergenic region of chromosome VIII ((Ng et al., 2003); asterisk denotes PCR product). (B) Quantitation of three ChIP experiments as in (A). *SNR47* ChIP signals were normalized to chromosome VIII signals. Normalized IP values were divided by normalized input values, and the ratios from three independent ChIP experiments were averaged. Relative ChIP signals are shown for Nrd1 in the presence (black bars) or the absence (white bars) of Paf1. (C) Quantitation of three ChIP experiments as in (A), except quantitative PCR was performed using DNA immunoprecipitated using anti-HA antibody, which recognizes epitope-tagged versions of Rpb3. *SNR47* ChIP signals were normalized to chromosome VIII signals. Normalized IP values were divided by normalized input values, and the ratios from three independent ChIP experiments were averaged. Relative ChIP signals are shown for Nrd1 in the presence (black bars) or the absence (white bars) of Paf1.

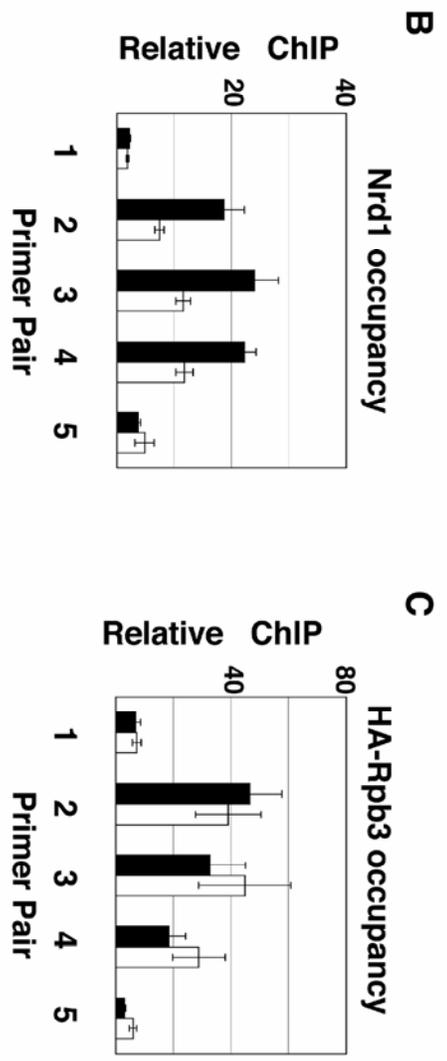
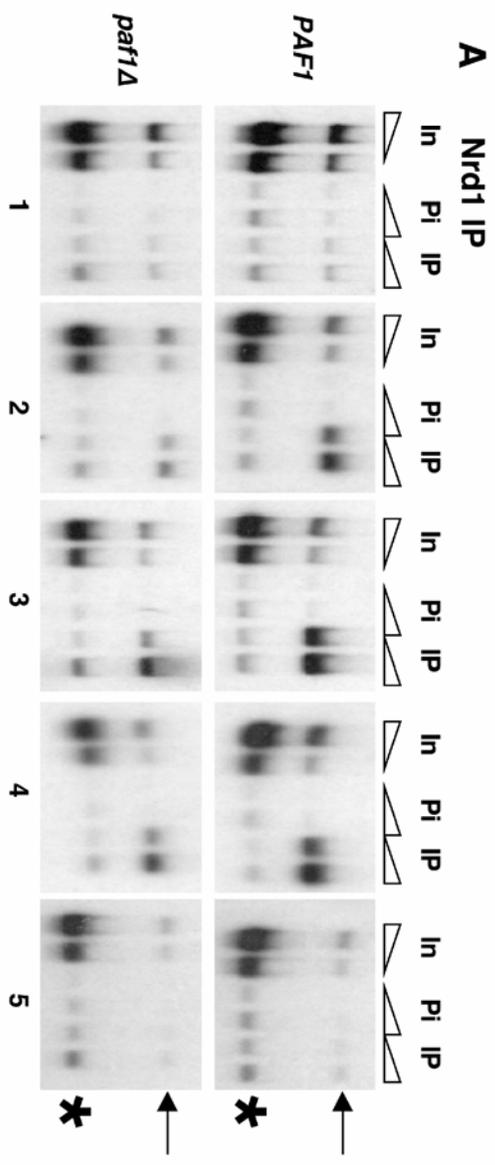


Figure 22: Association of Nab3 with *SNR47* DNA is dependent on a functional Paf1 complex

Paf1 is required for normal Nab3 association with *SNR47*. (A) ChIP was performed on chromatin prepared from *PAF1* (KKY120) and *paf1Δ* (KKY122) cells with anti-HA antibody (IP) or preimmune sera (Pi). Two amounts of immunoprecipitated (IP) DNA (2μl and 4μl) and input (In) DNA (4μl of 1:125 and 1:250 dilutions) were analyzed by PCR. The reactions included the *SNR47* primer pair specified adjacent to the representative experiment and a primer pair to an intergenic region of chromosome VIII ((Ng et al., 2003); asterisk denotes PCR product). (B) Quantitation of three ChIP experiments as in (A). *SNR47* ChIP signals were normalized to chromosome VIII signals. Normalized IP values were divided by normalized input values, and the ratios from three independent ChIP experiments were averaged. Relative ChIP signals are shown for Nrd1 in the presence (black bars) or the absence (gray bars) of Paf1.

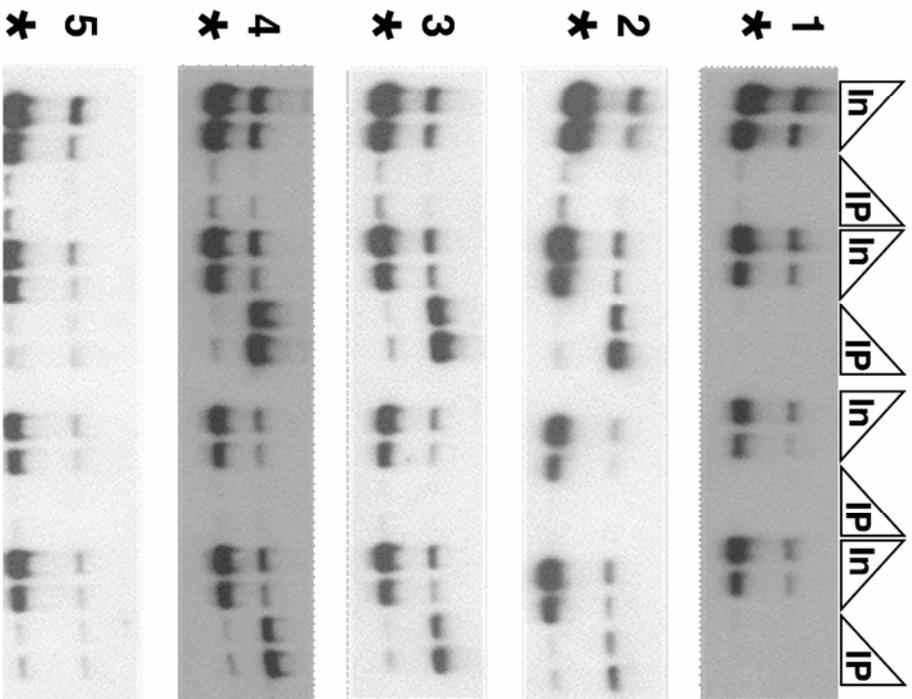
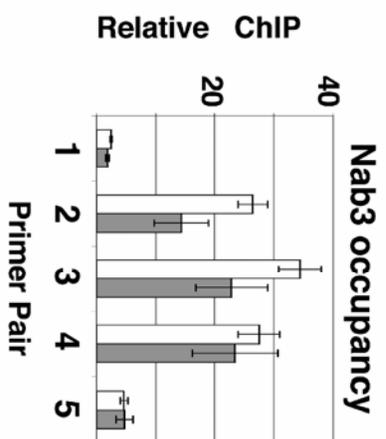
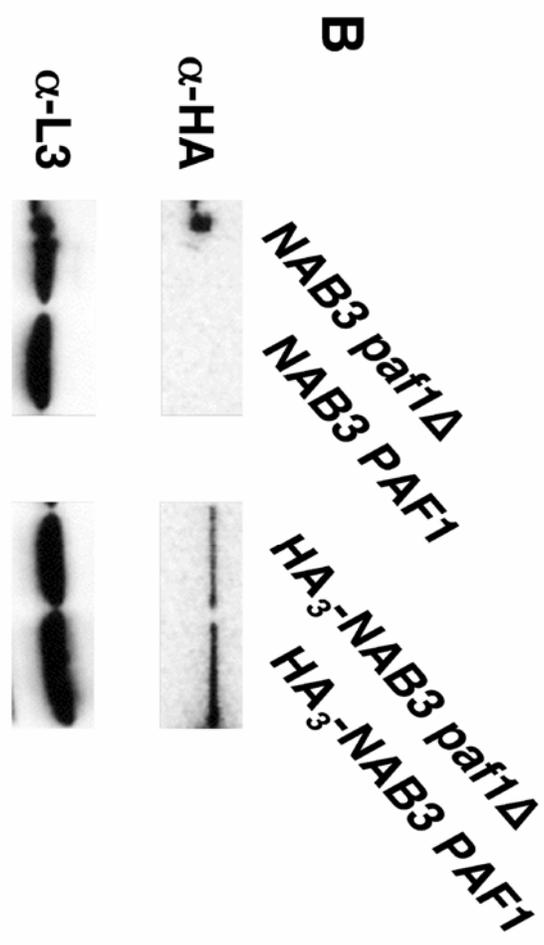
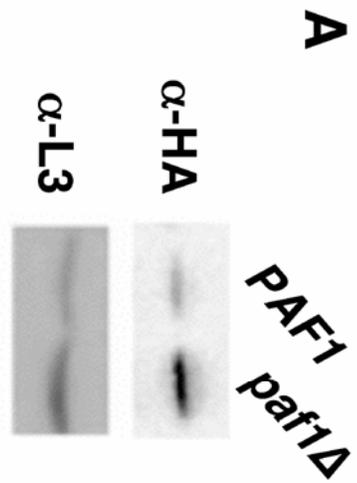
A**B**

Figure 23: Nrd1 and Nab3 levels are not reduced in *paf1Δ* cells

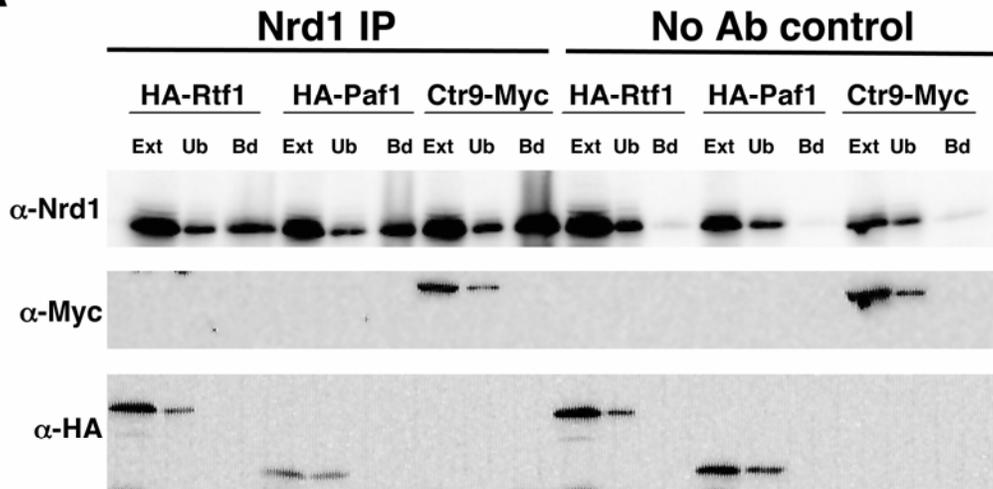
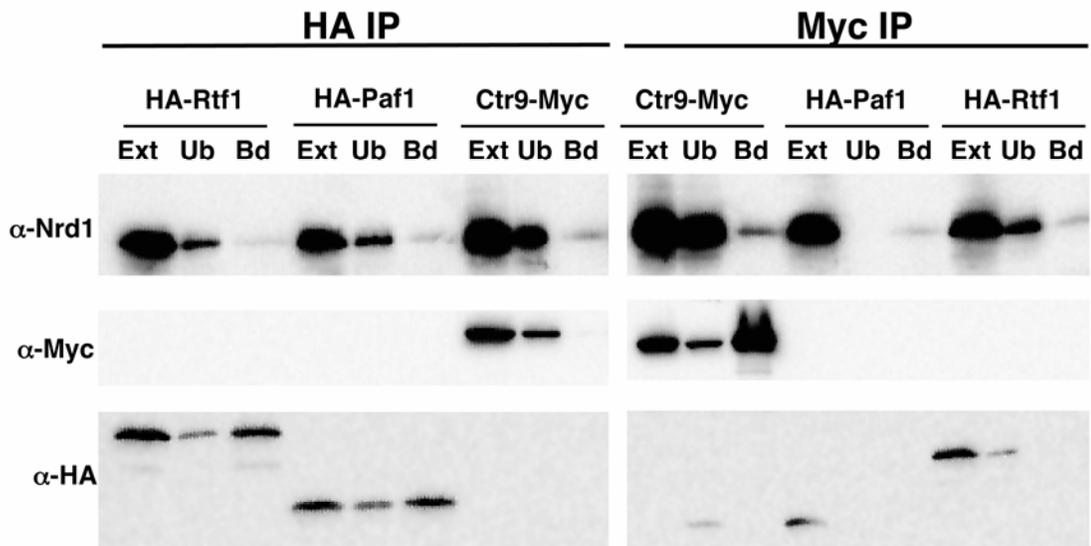
Nrd1 and Nab3 levels are similar in *PAF1* and *paf1Δ* cells. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with either anti-HA, anti-Nrd1, or anti-L3 to serve as a loading control. Protein extracts were prepared from cells grown for ChIP as indicated: (A) *PAF1* (KKY120) and *paf1Δ* (KKY122). (B) *NAB3 PAF1* (KKY125), *NAB3 paf1Δ* (KKY126), *HA₃-NAB3 PAF1* (KKY120), *HA₃-NAB3 paf1Δ* (KKY22).



Since the Paf1 complex may participate in recruiting Nrd1-Nab3 to *SNR47*, we tested the hypothesis that Nrd1-Nab3 physically associates with the Paf1 complex in vivo. We performed Co-IP experiments using epitope-tagged versions of Rtf1 (HA₃-Rtf1), Paf1 (HA₃-Paf1), and Ctr9 (Ctr9-6XMyc). Protein extracts were precipitated with antibodies against Nrd1, HA, or Myc. In addition, we performed a mock immunoprecipitation using no primary antibody for a control. We did not detect association between Nrd1 or members of the Paf1 complex using low stringency immunoprecipitation washes with either 100 or 150mM of sodium acetate (Figure 24). Immunoprecipitation efficiencies were tested by immunoblotting with antibodies that were used in the corresponding immunoprecipitation reactions. Using HA or Myc to immunoprecipitate HA₃-Rtf1, HA₃-Paf1, or Ctr9-6XMyc and associated proteins, immunoblotting with Nrd1 antibodies exhibited near background levels of Nrd1 (Figure 24). The most intriguing results were obtained with Ctr9-Myc immunoprecipitation, where the level of Nrd1 in the bound fraction was slightly elevated above background (compare to no antibody control reaction). These results suggest that members of the Paf1 complex may not be tightly associated with Nrd1-Nab3 in vivo and any possible interaction may be transient or mediated through another protein. Future co-immunoprecipitation experiments should include antibodies specific to RNA polymerase II. This way, probing filters for Nrd1 or members of the Paf1 complex could serve as a positive control. In fact, a possible candidate for mediating association of the Paf1 complex and Nrd1-Nab3 would be RNA polymerase II because Nrd1 associates with RNA polymerase II and affinity purification of individual Paf1 complex members revealed the presence of RNA polymerase II in eluted fractions (Conrad et al., 2000; Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002).

Figure 24: Nrd1 does not co-immunoprecipitate with members of the Paf1 complex

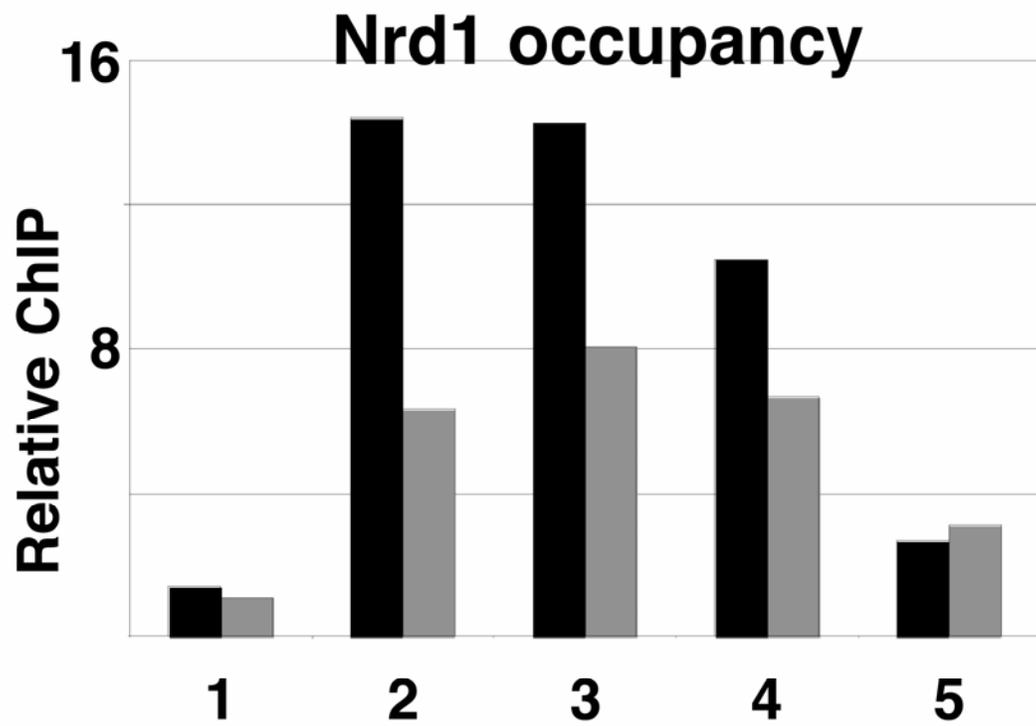
Co-immunoprecipitation followed by immunoblot analysis indicates Nrd1 does not stably associate with members of the Paf1 complex. Protein extracts were prepared from *HA₃-RTF1* (KY980), *HA₃-PAF1*(KY783), and *CTR9-6XMYC* (GHY1177) cells. The protein lysate (L), unbound fraction (U) or bound (B) fraction were subjected to SDS-PAGE. Resolved proteins were electotransferred to nitrocellulose and antibodies against Nrd1, HA, or Myc were used to detect Nrd1, HA₃-Rtf1, HA₃-Paf1, or Ctr9-Myc, respectively. (A) Antibodies against Nrd1 or no antibody were used in immunoprecipitation experiments. (B) Antibodies toward HA or Myc were used in immunoprecipitation experiments.

A**B**

Nrd1 and Nab3 recruitment or association with RNA polymerase II along *SNR47* is partially dependent on the Paf1 complex (Figure 21A and 21B; Figure 22A and 22B). Since Nrd1 and Nab3 association is not completely dependent on the Paf1 complex for recruitment to *SNR47*, the possibility exists that other RNA polymerase II-associated proteins are required for stabilizing interactions of Nrd1-Nab3 to elongating RNA polymerase II at *SNR47*. The amino acid sequence of Nrd1 indicates it contains a CID motif similar to Pcf11, a factor involved in polyadenylated transcript 3'-end formation. The structure of Pcf11 binding to the CTD has been solved and revealed Pcf11 directly interacts with Ser-2 phosphorylated CTD repeats through its CID domain (Meinhart and Cramer, 2004). The CTD kinase responsible for phosphorylating Ser-2 is Ctk1. Thus, we tested chromatin extracts prepared from *CTK1* and *ctk1* Δ cells for Nrd1 occupancy along *SNR47*. Preliminary ChIP experiments demonstrated reduced Nrd1 signal over *SNR47* in a pattern reminiscent of that observed in the absence of Paf1 (Figure 25; Figure 21A and 21B). Reduced Nrd1 occupancy over *SNR47* in cells that lack the Ser-2 CTD kinase supports the idea that Nrd1 may also interact with Ser-2 phosphorylated CTD through the CID motif of Nrd1. The observation that Ser-2 phosphorylation and the Paf1 complex are required for occupancy of Nrd1 across *SNR47* is consistent with the previously reported synthetic genetic interactions between *ctk1* Δ and *rtf1* Δ (Costa and Arndt, 2000).

Figure 25: Nrd1 association with *SNR47* requires the function of the CTD kinase Ctk1

Nrd1 occupancy at *SNR47* is dependent on Ctk1. Chromatin from wild-type (FY118) and *ctk1Δ* (YSB1560) cells was immunoprecipitated with anti-Nrd1 antibody. Two amounts of immunoprecipitated (IP) DNA (2μl and 4μl) and input DNA (4μl of 1:125 and 1:250 dilutions) were analyzed by PCR. *SNR47* ChIP signals were normalized to *TELVI* signals. Normalized IP values were divided by normalized input values, and the ratios from two independent ChIP experiments were averaged. Relative ChIP signals are shown for Nrd1 in the presence (black bars) or the absence (gray bars) of Ctk1.



3.3.6 Is the Paf1 complex directly involved in RNA polymerase II transcript termination?

Cumulative evidence strongly indicates the Paf1 complex is required for 3'-end formation of snoRNAs. However, since transcript cleavage is intimately linked with transcription termination we cannot distinguish between a role for the Paf1 complex in termination and a role in post-transcriptional processing. To directly investigate a role for the Paf1 complex in termination, we performed transcription run on (TRO) analysis. TRO maps the density of RNA polymerase II along the transcription unit. In the assay, one of three G-less cassette constructs was transformed into wild-type, *paf1* Δ and *nrd1-5* cells (Figure 26A). In each of these cassettes, the 5' G-less cassette is 262 nucleotides in length while the 3' G-less cassette is 132 nt long. The construct pG-CYC.ds contains inert spacer DNA sequence that is flanked by the two G-less cassettes. pG-SNR13-125-232-CYC.ds contains the sequences required for 3'-end formation of *SNR13*. The third plasmid is pG-SNR47(70).CYC.ds and contains the region of *SNR47* required for transcription termination. In these cells, RNA polymerase II molecules are actively transcribing the G-less cassettes. However, transcription was halted upon the addition of the detergent sarkosyl, an inhibitor of TFIIS (Wind and Reines, 2000). RNA polymerase II transcription resumed upon the addition of ATP, CTP, and radiolabeled UTP. This allowed labeled UTP to be incorporated within the transcript near the site where RNA polymerase II was paused with sarkosyl. Excess GTP and unlabeled UTP were incorporated into the transcript in a chase reaction, allowing RNA polymerase II to transcribe through the spacer DNA. Total RNA was isolated from these cells for RNaseT1 digestion. Treatment of the RNA with this enzyme

results in endonucleolytic cleavage of RNA adjacent to G residues. The digested RNA was resolved on denaturing polyacrylamide gels.

The hypothesis tested in this experiment is that only cells defective for transcription termination containing either pG-SNR13-125-232.CYC.ds or pG-SNR47(70).CYC.ds will result in the production of the 132 nt transcript from the downstream G-less cassette (Figure 26A). Cells, either wild-type or mutant, containing pG-CYC.ds give rise to two transcripts of 262 nt and 132 nt since the spacer DNA contains no termination signals (Figure 26A). However, only strains with defects in transcription termination will produce both G-less transcripts when containing pG-SNR13-125-232.CYC.ds or pG-SNR47(70).CYC.ds. In the case of *nrd1* mutants, the cells have lost the ability to recognize the sequences required for termination (Carroll et al., 2004; Steinmetz et al., 2001).

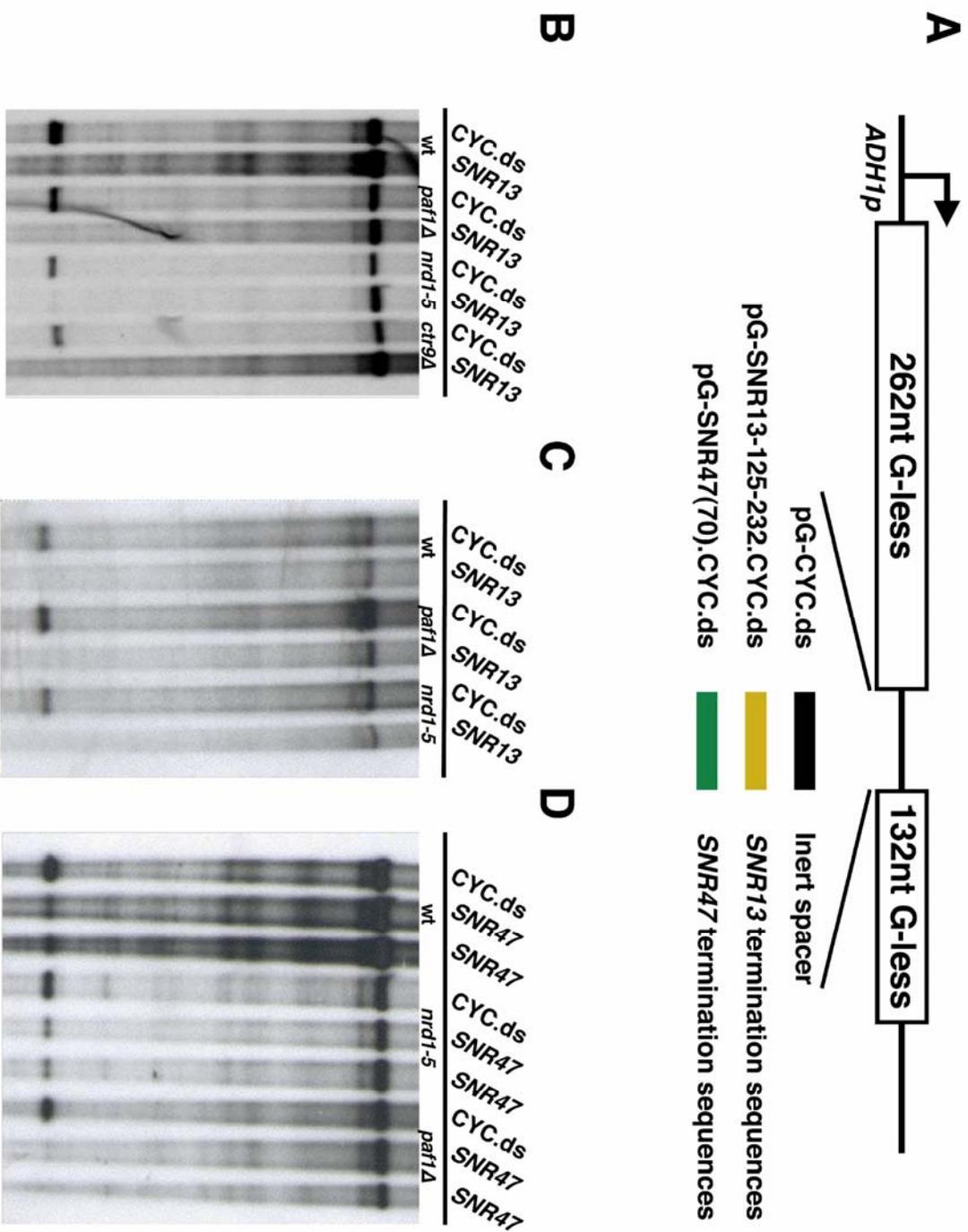
Using the G-less cassettes, three experiments were performed in order to determine a role for the Paf1 complex in either 3'-end formation or transcription termination. In the first experiment pG-CYC.ds or pG-SNR13-125-232.CYC.ds was transformed into the indicated cells and TRO analysis was performed. Cells transformed with pG-CYC.ds displayed G-less cassette transcripts of 262 nt and 132 nt as predicted (Figure 26B). However, mutant strains defective for transcription termination containing the pG-SNR13-125-232.CYC.ds plasmid did not contain the 132nt transcript (Figure 26B). This result was perplexing since *nrd1-5*, the positive control for transcription termination, did not exhibit a termination defect (Figure 26B). This observation could be explained either due to low sensitivity levels of the experiment or absence of a transcription termination defect in *nrd1-5* cells. However, evidence from our lab and others indicates that the latter explanation may not be correct. First, TRO analyses have previously been performed with other *nrd1* alleles and defects in transcription termination reported (Steinmetz et

al., 2001). Second, while *nrd1-5* is the only *nrd1* allele in our lab we have demonstrated that this mutation confers strong transcriptional readthrough defects of both snR13 and snR47. Finally, the *nrd1-5* mutation results in a single amino acid substitution of V368G within the single RRM of Nrd1 (Steinmetz and Brow, 1996). Thus, this missense mutation may not confer as strong a transcription termination defect as other *nrd1* alleles with more severe phenotypes.

The data obtained from the TRO experiment outlined in Figure 26A-B do not provide conclusive evidence to indicate a role for the Paf1 complex in transcription termination of *SNR13* (Figure 26A and 26B). As described above, the positive control for this TRO experiment did not exhibit anticipated results according to (Steinmetz et al., 2001). Therefore, we decided to challenge cells transformed with the TRO constructs from Figure 26A with a temperature shift, which is described below.

Figure 26: TRO analysis using *SNR13* and *SNR47* transcription termination sequences

Results of the TRO analysis are not definitive. Wild-type (FY118), *paf1Δ* (KY685), *ctr9Δ* (KY817), and *nrd1-5* (*nrd1-5*) strains were transformed with the indicated plasmids as described in the text. Transcription using radiolabeled UTP was reinitiated after Sarkosyl addition to induce pausing of elongated RNA polymerase II. Total RNA was isolated, digested with RNaseT1 and electrophoresed using denaturing-PAGE.



The second TRO experiment was performed similarly to the first experiment except cells were shifted from 30°C to 37°C for 90 minutes prior to transcript labeling. We postulated that stressing the cells with increased temperature would exacerbate defects in transcription termination. As predicted, cells containing pG-CYC.ds displayed G-less cassette transcripts of 262 nt or 132 nt (Figure 26C). The 132 nt transcript was not present in cells containing the pG-SNR13-125-232.CYC.ds plasmid (Figure 26C). However, either the duration of the temperature shift was not long enough or the level of sensitivity of the TRO analysis with the *SNR13* termination sequences was again too low because we observed no defect in termination for any of the strains tested (Figure 26C). Again, this result was reminiscent of the results from Figure 26B, where *nrd1-5* mutants did not exhibit transcription termination defects with *SNR13* termination sequences at the permissive temperature. The results obtained from this TRO experiment are not consistent with published observations that suggest Nrd1 and Nab3 are involved in transcription termination (Steinmetz et al., 2001). Again, using the TRO assay described in Figure 26A and 26C did not provide evidence in support of a role for the Paf1 complex in transcription termination of SNR13 transcripts (Figure 26A and 26C).

For experiment three, pG-CYC.ds or pG-SNR47(70).ds was transformed into the indicated cells and TRO analysis was performed. Cells transformed with pG-CYC.ds displayed G-less cassette transcripts of 262 nt and 132 nt as predicted (Figure 26D). Interestingly, mutant strains defective for transcription termination containing the pG-SNR47(70).ds plasmid contained the 132 nt transcript (Figure 26D). However, wild-type cells also contained a transcript of 132 nt. In this preliminary experiment the ratio of 132/262 transcripts from wild-type cells was lower than the 132/262 ratio from *nrd1-5* cells. *paf1*Δ cells transformed with the pG-SNR47(70).ds plasmid

did not exhibit the 132 nt transcript (Figure 26D). This observation was unexpected due to ChIP experiments performed in *PAF1* and *paf1Δ* cells (Figure 21A-B). These experiments indicated that the absence of the Paf1 complex resulted in increased occupancy of RNA polymerase II over the 5' region of *YDR042c* (Figure 21C). A possible explanation for the discrepancy between TRO and ChIP experiments is that the TRO experiment is a read out of transcription termination defects using a heterologous template. Experiments using transcription termination sequences from two genes that require the Paf1 complex for proper 3'-end formation in heterologous templates, pG-SNR13-125-232.CYC.ds or pG-SNR47(70).ds, were either inconclusive or not supported by ChIP experiments.

3.4 Conclusions

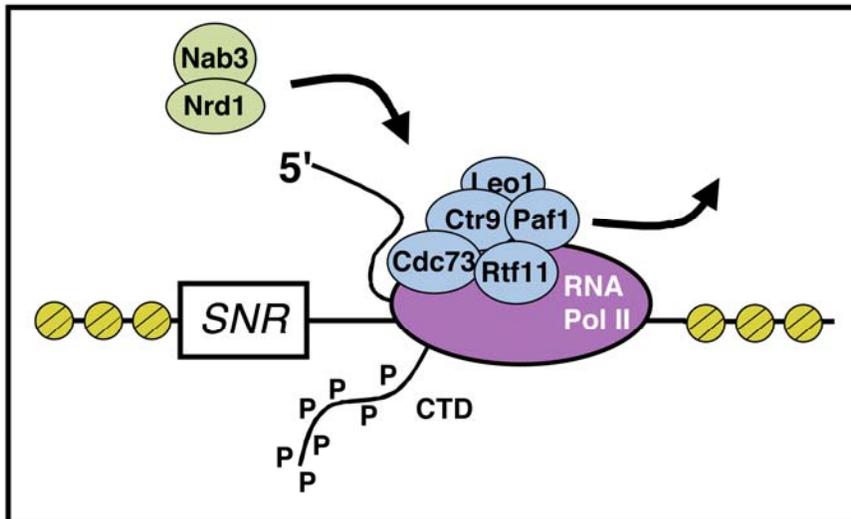
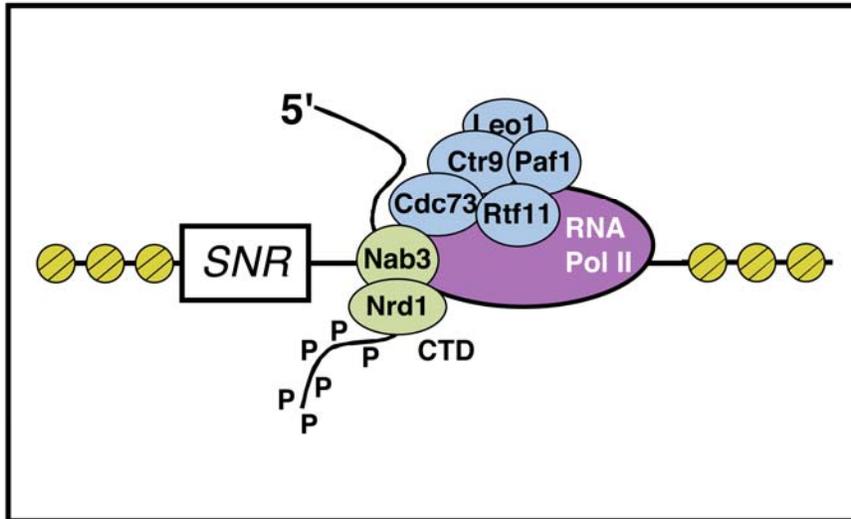
3.4.1 Novel role for the Paf1 complex in the transcription cycle

Investigation of a possible functional link between Nrd1-Nab3 and the Paf1 complex revealed a novel role for the Paf1 complex in 3'-end formation of nonpolyadenylated transcripts. High-copy-number suppression of point mutations in *rtf1* by *NAB3* lead to additional genetic analyses. Genetic interactions of deletions within the Paf1 complex with high-copy-number *NAB3* or mutations in *NRD1* hinted toward the possibility that the Paf1 complex may participate in forming 3'-ends of RNA polymerase II transcripts. Northern analysis indicated that in the absence of certain Paf1 complex members, the 3'-ends of *SNR13* and *SNR47* transcripts were extended (Figure 13 and Figure 17). Observations from in vivo reporter assays also provided evidence that the Paf1 complex was required for 3'-end formation of *SNR13* and *SNR47* transcripts since *paf1Δ* and *ctr9Δ* cells conferred Cu²⁺ resistance in the ACT1-CUP1 reporter assay, and *rtf1Δ* cells conferred histidine prototrophy when transformed with the ASHC reporter construct (Figure 18). Preliminary TRO analyses were difficult to interpret due to sensitivity levels of the experiment or results that were contradictory with those from repeated ChIP experiments (Figure 26A-26D compare to Figure 21A and 21B). Therefore, we were unable to determine whether 3'-extended snR13 or snR47 was a result of defective 3'-end formation or transcription termination. The most compelling evidence in support of a role for the Paf1 complex in 3'-end formation of nonpolyadenylated transcripts came from the use of ChIP experiments. This method demonstrated that the members of the Paf1 complex directly associate

with *SNR13* and *SNR47* and the pattern of Paf1 complex localization correlated with the density of RNA polymerase II (Figure 19 and Figure 20). Moreover, ChIP analysis revealed that Nrd1 and Nab3 association with *SNR47* was dependent upon the Paf1 complex (Figure 21 and Figure 22). The dependence of Nrd1-Nab3 association with *SNR47* on the Paf1 complex may therefore manifest in defects in 3'-extended transcripts, because cells lacking Paf1 complex members exhibit reduced levels of Nrd1 and Nab3 with *SNR47* by ChIP analysis. Two models address the possible function of the Paf1 complex in 3'-end formation of nonpolyadenylated transcripts (Figure 27A and 27B). In the first model, the genetic, Northern analysis, and ChIP data described support a role for the Paf1 complex in 3'-end formation of *SNR* transcripts (Figure 27A). The role of the Paf1 complex in this model is to recruit Nrd1-Nab3 to RNA polymerase II or stabilize the association of Nrd1-Nab3 with elongating RNA polymerase II. The second model (Figure 27B) is based on ideas and work from the Buratowski lab (Kim et al., 2004). In their study, they analyzed the association and dissociation of elongation factors along the length of genes that produce polyadenylated transcripts (Kim et al., 2004). This analysis revealed that the Paf1 complex dissociates from open reading frames near the poly(A) site, similar to the association mapped for Ctk1 (Ahn et al., 2004; Kim et al., 2004). This "exchange" of RNA polymerase II-associated factors may hold for *SNR* transcripts where association of Nrd1-Nab3 requires dissociation of the Paf1 complex before they can stably associate with RNA polymerase II. However, the short length of *SNR* genes, typically 100 bp, combined with the resolution of ChIP experiments make distinguishing between these two models technically difficult.

Figure 27: Models for the role of the Paf1 complex in 3'-end formation of *SNR* transcripts

Two models that might explain the role of the Paf1 complex in the formation of 3'-ends of *SNR* transcripts. Each diagram depicts RNA polymerase II transcribing downstream of *SNR13*. Top panel, the Paf1 complex may recruit or stabilize associations between Nrd1-Nab3 and RNA polymerase II during *SNR* gene transcription. Bottom panel, dissociation of the Paf1 complex may be a prerequisite for the association of Nrd1-Nab3 with transcribing RNA polymerase II. This model was originally proposed by the Buratowski lab based on the results of experiments with polyadenylated transcripts (Ahn et al., 2004; Kim et al., 2004).



3.4.2 The Paf1 complex may couple transcription elongation to transcript cleavage

Dependence of Nrd1-Nab3 association with *SNR* genes on the Paf1 complex and the Ser-2 CTD kinase has uncovered a role for the Paf1 complex in linking transcription elongation and *SNR* transcript cleavage. This role of the Paf1 complex is not associated with previously ascribed functions of the Paf1 complex. For example, the Paf1 complex is important for methylation of certain histone H3 lysine residues. The role of these modifications for association of Nrd1-Nab3 with RNA polymerase II may not be important since mutations in genes that encode these methyltransferases do not cause 3'-extended *SNR13* transcripts. However, *rad6Δ* cells, which are defective in histone H2B K123 ubiquitylation did exhibit transcription readthrough of snR13 (Figure 16). Rad6 is an ubiquitin conjugase that has other targets for ubiquitylation besides histone H2B K123 that may be relevant for proper 3'-end formation of *SNR* transcripts. Also, other RNA polymerase II-associated factors did not exhibit 3'-extended *SNR13* transcripts, including proteins with characterized roles in RNA polymerase II elongation or termination (Figure 16). A subset of transcription factor mutants did exhibit defects in 3'-end formation of snR13 (Figure 16). Deletion of *INO80* and *ARP8*, which code for integral components of the INO80 complex resulted in defective 3'-end formation of *SNR13* transcripts, but did not exhibit 3'-extended *SNR47* transcripts (Figure 16 and data not shown). Collectively, these observations support the idea that the Paf1 complex may be specifically required for Nrd1-Nab3 association with *SNR* genes. The observation that the Paf1 complex is also required for normal levels of CTD phosphorylation on Ser-2 over open reading frames of protein-coding genes suggests that the Paf1 complex may also recruit or stabilize association of Ctk1 with elongating RNA

polymerase II (Mueller et al., 2004). This idea is consistent with observations that indicate Ctk1 is required for Nrd1 association with *SNR47* (Figure 21A and 21B).

4.0 Chapter 4: Identification of genes regulated by the Paf1 complex through global expression analysis of *CTR9* and *RTF1* deletion mutants

4.1 Introduction

Factors involved in modulating transcription can be classified in part by the genes they regulate. RNA polymerase II is essential for the transcription of all protein coding genes, snRNA genes, and snoRNA genes. However, not all transcription factors are involved in the transcription of all RNA polymerase II transcribed genes (Holstege et al., 1998). In fact, global transcription profiling studies have demonstrated that different genes have differing requirements for general transcription factors, coactivators, and chromatin modification (Holstege et al., 1998). Added to this complexity is the observation that several genes require gene-specific activators and/or repressors for proper transcriptional activation in response to physiological requirements of the cell.

Imagine the possibility where not only particular genes require a subset of general transcription factors and gene-specific activators or repressors for proper initiation of transcription, but also require additional signaling through transcription elongation factors. Importantly, recent studies indicate that the Ras/PKA signaling pathway targets the transcription elongation factor Spt5 in response to control yeast cell growth (Howard et al., 2003). Since the Paf1 complex has emerged as an RNA polymerase II-associated factor important for coordinating histone modification events during transcription, the possibility exists that some

genes will require the Paf1 complex for proper transcription. Further, deletion of individual Paf1 complex members results in a range of phenotypes from moderate to severe (Betz et al., 2002; Porter et al., 2002). These results lead to the hypothesis that individual Paf1 complex members regulate the transcription of different sets of genes. Alternatively, the members of the Paf1 complex regulate the transcription of the same set of genes. These two hypotheses need not be mutually exclusive in that particular genes may require the entire Paf1 complex and different sets of genes may require a subset of the Paf1 complex components.

We chose two complementary approaches to identify genes regulated by the Paf1 complex. The first method used genetic interactions between components of the Paf1 complex and transcriptional activators identified in a synthetic lethality screen (Costa and Arndt, 2000). The rationale was that the Paf1 and SBF were required for the expression of overlapping essential transcripts; hence when the function of both complexes are missing cells, will die (Bean et al., 2005). The second approach was to use microarray analyses to obtain transcriptional profiles for each component of the Paf1 complex. Using this approach, the transcriptional profiles could be evaluated by computer algorithms to determine which particular subset of the yeast genome requires the Paf1 complex for transcription. For example, the use of these algorithms could indicate if genes required for cell wall maintenance, sporulation, or other processes with known components require the Paf1 complex for regulating their expression. Analysis in this fashion could be used to identify genes that require different members of the Paf1 complex for their expression.

4.2 Methods and materials

4.2.1 Yeast strains

As described in section 2.2.1, yeast strains used in this study are isogenic with FY2 (Table 13). *SWI4*, *SWI6*, and *MBP1* were replaced with *HIS3* using a PCR-based method (Ausubel et al., 1988). Strains to be compared in microarrays analyses were isogenic at all loci except the deletion strain, where *CTR9* was replaced using a PCR-based method with *KANMX*, which confers resistance to the antibiotic G418 (Ausubel et al., 1988).

Table 13: Yeast strains used in Chapter 4

STRAIN	GENOTYPE	ORIGIN
FY118	<i>MATa his4-912δ lys2-128δ leu2Δura3-52 trp1Δ63</i>	Winston lab
GHY250	<i>MATα leo1Δ::URA3 his4-912δ lys2-128δ leu2Δura3-52</i>	Hartzog lab
GHY972	<i>MATα his3Δ200 lys2-128δ ura3Δ0 HA₃-PAF1</i>	"
GHY1094	<i>MATα ctr9Δ::KANMX4 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
GHY1177	<i>MATα his3Δ200 lys2-128δ leu2Δ0 ura3Δ0 trp1Δ63 CTR9-6XMYC::LEU2</i>	"
KY399	<i>MATa rtf1Δ100::URA3 leu2Δ1 ura3-52 trp1Δ63 pLS21-5[HA₃RTF1, TRP1, CEN/ARS]</i>	Arndt lab
KY452	<i>MATa rtf1Δ100::URA3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63</i>	"
KY453	<i>MATα rtf1Δ100::URA3 his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63</i>	"
KY508	<i>MATa snf2Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52</i>	"
KY589	<i>MATα leu2Δ1 trp1Δ63</i>	"
KY656	<i>MATa rtf1Δ100::URA3 his4-912δ lys2-128δ leu2Δ1 ura3-52</i>	"
KY661	<i>MATa his3Δ200 his4-912δ lys2-128δ leu2Δ1 ura3-52</i>	"
KY685	<i>MATα paf1Δ::URA3 his4-912δ lys2-128δ leu2Δ(0 or 1) ura3(Δ0 or -52)</i>	"
KY687	<i>MATα paf1Δ::URA3 lys2-173R2 leu2Δ(0 or 1) ura3(Δ0 or -52)</i>	"
KY689	<i>MATα cdc73Δ::KANMX4 lys2Δ0 leu2Δ0</i>	"
KY980	<i>MATα his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63 HA₃-RTF1</i>	"
KKY5	<i>MATα mbp1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63</i>	"
KKY11	<i>MATα swi4Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63</i>	"
KKY13	<i>MATα swi6Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 ura3-52 adr8</i>	"
KRY2	<i>MATa rtf1Δ100::URA3 his3Δ200 his4-912δ lys2-173R2 leu2Δ1 ura3-52</i>	"
PCY431	<i>MATa rtf1Δ101::LEU2 his4-912δ lys2-128δ leu2Δ1 ura3-52 trp1Δ63 leu2Δ1 ura3-52 trp1Δ63</i>	"

4.2.1.1 Analysis of *IMD2* induction

Cells were grown to approximately 1×10^7 cells/ml in SC-Ura media and divided. 6AU was added to a final concentration of $75 \mu\text{g/ml}$ to one half of the culture and both cultures were grown at 30°C for the indicated times in hours prior to RNA isolation. Cells were harvested, RNA was isolated and Northern analysis was performed as described previously (Stolinski et al., 1997). Hybridization probes were prepared by random-prime labeling of PCR-generated DNA fragments containing *IMD2* and *SEDI*. Oligonucleotides used for the PCR were as described by Shaw and Reines (2000).

4.2.1.2 Northern blotting analysis.

Aliquots of $10 \mu\text{g}$ of the indicated RNAs were resuspended in RNA sample buffer (50% deionized formamide; 1X MOPS; 2.12M formaldehyde; 0.5mg/ml ethidium bromide), electrophoresed through a 1.5% denaturing agarose gel, and photographed with the 440CF digital imaging station (Kodak). Genescreen filters (PerkinElmer Life Sciences Inc.) were hybridized with random-prime labeled probes containing $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (Dupont NEN) and/or $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (Dupont NEN), using PCR-amplified coding sequences of *ARG1*, *SER3* and *SEDI* (Squazzo et al., 2002). The blots were exposed to both phosphorimaging screen and film (Kodak).

4.2.2 Chromatin immunoprecipitation (ChIP) analysis

Cells were grown in YPD to $1\text{-}2 \times 10^7$ cells/ml. ChIP assays and immunoprecipitations with anti-Myc antibody or anti-HA antibody conjugated to agarose beads (Santa Cruz Biotechnology) were performed as described (Komarnitsky et al., 2000; Simic et al., 2003). To confirm signal linearity, quantitative PCR was performed on two dilutions of input and immunoprecipitated DNA using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$, and relative ChIP signals were calculated as described in the figure legends. Table 14 lists the oligonucleotides used for ChIP at the *ARG1* and *SRG1-SER3* loci.

Oligonucleotides amplified sequences relative to +1 of either *ARG1* or *SER3* (per *Saccharomyces* Genome Database).

Table 14: Oligonucleotide sequences used for ChIP in Chapter 4

Oligonucleotide Name	5'-Oligonucleotide Sequence	Hybridizes Relative to +1
KKO84	TGGTTAAGCAGTTAGGCT	-424 <i>SER3</i>
KKO85	TTTCCTTATCCTCTGCTCCC	-123 <i>SER3</i>
KKO86	CGTTCCACAGCGCTTGAATGCTG	-149 <i>SER3</i>
KKO87	GCTGGTAGCGTAGTCTAAGTCAAC	+143 <i>SER3</i>
KKO88	GTGACACTACATGTACCAGC	+760 <i>SER3</i>
KKO89	GCCACCAATGTGTGGTGTCAAG	+1050 <i>SER3</i>
KKO125	CCCATTGTGCAGCGATAAGG	-75 <i>ARG1</i>
KKO126	GGCCTTTTCCTTGGCGGC	+150 <i>ARG1</i>
KKO127	CCCCACCAAAGGACATGTGG	+599 <i>ARG1</i>
KKO128	CCTTGCTAAGTTGGATGCGG	+787 <i>ARG1</i>

4.2.2.1 Harvesting yeast cultures for microarray analysis

Growth of cultures for use in microarray analysis differed as described below from standard techniques. Liquid microarray YPD (maYPD) was prepared in glass flasks and graduated cylinders that had been well washed with double distilled water, at least four times to remove residual detergents left over from cleansing. Standard 5ml saturated overnight cultures were inoculated in maYPD with strains that were freshly struck out from the freezer with verified markers, in 15ml sterile conical tubes (Falcon) and incubated at 30°C. Dilution of overnight cultures was performed as usual except sterile, plastic pipets (Sarstedt) were used to transfer overnight culture to 1.5ml centrifuge tubes. Log phase overnight cultures were inoculated into 250ml of maYPD with a dilution of overnight culture using sterile micropipettor tips and incubated at 30°C overnight. Log phase cultures were harvested between $1-2 \times 10^7$ cells/ml. The cells were harvested in pre-washed GS3 bottles in the RC-5B centrifuge for 10 minutes at 4K rpm, washed one time with water, transferred to pre-washed Oakridge tubes, and pelleted in the SA-600 centrifuge for 5 minutes at 4K rpm. After the water was decanted from the cells, they were quick frozen in liquid nitrogen and stored at -70°C.

4.2.3 RNA isolation for microarray analysis

After cell pellets were harvested as described in section 4.2.1., RNA was isolated using the hot-acid phenol extraction method as in (A. M. Dudley and G. M. Church, personal communication). Briefly, the thawed cell pellets were resuspended in TES solution (10mM Tris-HCl, pH 7.5; 10mM EDTA, pH 8.0; 0.5% SDS w/v). An equal volume of acid phenol pre-warmed to 65°C was added to the suspension, which was subsequently agitated in a vortex mixer at maximum speed for 10 seconds. The suspension was incubated for 1 hour at 65°C, with vortexing every ten minutes. Importantly, the neutralizing component shipped with the phenol (Sigma, # P-4557) was not used, leaving the pH of the phenol to be acidic. The permeabilized cells were incubated on ice for 5 minutes prior to centrifugation in the SA-600 rotor at 10K rpm in the RC-5B centrifuge for 5 minutes at 4°C. The aqueous layer was extracted again with room temperature acid phenol followed by a chloroform extraction using 12ml of chloroform, agitated in a vortex mixer and centrifugation. The aqueous layer was subjected to ethanol precipitation using 1.2ml 3M NaOAc, pH5.2, and 32.5ml 100% ethanol, inversion of the suspension followed by centrifugation at 4°C for 20 minutes at 10K rpm. Precipitated RNAs were washed two times with 25ml 70% ethanol. RNA was air-dried on the bench prior to suspension in 4ml water. Total RNA was column purified and subjected to DNase I digestion using RNeasy maxi preps and DNase I (Qiagen) according to the total RNA cleanup procedure developed by Qiagen. Column purified total RNA was subjected UV analysis to determine RNA concentration, agarose gel electrophoresis to analyze rRNA integrity, and in some cases RT-PCR to determine the relative level of genomic DNA contamination. Aliquots of 20µg of RNA from either *CTR9* or *ctr9Δ* cells

were pipeted into pre-labeled 1.5ml microcentrifuge tubes and stored at -70°C for downstream applications.

4.2.4 Microarray analysis

4.2.4.1 cDNA preparation

The cDNA preparation protocol was followed according to the Atlas Glass Fluorescent Labeling kit (<http://www.clontech.com/clontech/techinfo/manuals/PDF/PT3452-1.pdf>, BD Biosciences). Briefly, equivalent amounts of total RNA derived from *CTR9* and *ctr9Δ* strains was thawed at 70°C for 1-2 minutes and cooled on ice for 1-2 minutes. Contents were collected by centrifugation in a microcentrifuge and returned to ice. The annealing reaction was performed in a total volume of 25μl with 7μg of oligonucleotide dT_(N=18) and 20μg of total RNA. Samples were incubated at 70°C for 5 minutes and transferred to 48°C for 5 minutes. While the oligonucleotides were annealing, 2X reverse transcription master mix was prepared at room temperature. 2X master mix contained the following reagents supplied with the labeling kit: 10μl 5X cDNA synthesis buffer, 5μl 10X dNTP mix that includes aminoallyl-dUTP, 7.5μl water, and 80 units of MMLV. Annealing reactions were cooled on ice for 2 minutes, microcentrifuged to collect contents, added to 25μl of 2X RT-PCR master mix and pipeted gently to mix reagents. RT-PCR proceeded for 30 minutes at 48°C, incubated at 70°C for 5 minutes, followed by incubation at 37°C with 5 units RNaseH for 15 minutes to degrade RNA hybridized to cDNA.

cDNAs were treated with Quickclean Purification Resin as directed by the manufacturer (Clontech), where 5μl of resin was vortexed with the products of the RT-PCR reactions. This step removes proteins and is a substitute for phenol/chloroform extraction. Resin was separated

from the cDNAs using 0.45µm spin filters and collection tube provided by BD Biosciences, where the suspension was transferred to the filter column and microfuged at maximum rpm for 1 minute. cDNAs were precipitated using manufacturer provided 3M sodium acetate (Clontech) and 137.5µl 100% ethanol, inverted, and incubated at -20°C for a minimum of 1 hour.

4.2.4.2 Cy dye incorporation/probe clean up

Precipitated cDNAs from section 4.2.4.2. were microcentrifuged at maximum speed for 20 minutes at 4°C. The pellets were washed with 70% ethanol and centrifuged for 5 minutes at maximum speed at 4°C. The remove supernatant was removed. The cDNA pellet was resuspended in 10µl of 2X Fluor-labeling buffer and 10µl of either Cy3 or Cy5 dye suspension was added; the Cy dye suspension was created by mixing 45µl of manufacturer supplied DMSO with a vial of Cy3 Mono-reactive dye (Amersham Pharmacia #PA23001) or Cy5 Mono-reactive dye (Amersham Pharmacia #PA25001). Dye coupling proceeded in the dark for 30 minutes. Labeled cDNA was precipitated with manufacturer provided 3M sodium acetate and 30µl 100% ethanol, inverted, and incubated at -20°C for a minimum of 1.5 hours. Precipitates were washed with 70% ethanol prior to resuspension in 100µl water. Unincorporated dye was removed using the Atlas NucleoSpin column extraction kit according to the manufacturer's instructions supplied with the Atlas Glass Fluorescent Labeling Kit (Clontech). Samples were combined and concentrated to 36µl using Microcon 30 filters with microcentrifugation for 6-8 minutes at 10K rpm. Filters were inverted and contents were collected by centrifugation for 2 minutes at 10K rpm (Amicon).

4.2.4.3 Hybridization

In preparation for competitive hybridization of labeled cDNA, 2X hybridization buffer (250 μ l formamide (F-5786, Sigma Ultra), 250 μ l 20XSSC, and 10 μ l 10% SDS) was prepared and prewarmed to 42°C. To the concentrated samples, 2 μ l 10mg/ml sonicated salmon sperm DNA and 2 μ l 10mg/ml polyadenylic acid were added to reduce nonspecific hybridization. Samples were incubated at 90°C for 2 minutes to denature labeled cDNAs. An equal volume of 2X hybridization buffer was mixed with denatured cDNAs and applied to microarray slides (Microarray Centre (<http://www.microarrays.ca>), University Health Network, Toronto, Canada) covered with Lifterslips (Erie Scientific Company). Microarray slides were sealed in either microarray chambers with a drop of 3XSSC buffer for humidity or within P-1000 tip boxes that contained paper towels and water for humidity for 16 hours at 42°C.

4.2.4.4 Microarray scanning and image analysis

Following hybridization, three microarray wash solutions were prepared. All washes were performed at room temperature for 5 minutes in volumes large enough to fill washed P-1000 tip boxes (500ml) with gentle shaking. Microarrays were quickly removed from the hybridization chambers, placed in slide racks, and submerged in wash buffer one composed of 0.2XSSC/0.1% SDS. Slides were subsequently transferred to wash buffer two 0.2XSSC and then to wash buffer three 0.1XSSC. Finally, microarray slides were quickly plunged into 0.01XSSC and dried by centrifugation for 5 minutes at 800rpm.

Hybridized and washed microarray slides were stored in microscope slide boxes to prevent exposure to light prior to array scanning. Microarrays were scanned using the Affymetrix Genetic Microsystem 418 array scanner (Department of Biological Sciences, University of Pittsburgh). Slides were scanned individually first with a laser set to 635nm to excite the Cy5

fluor within the cDNA. Emissions were collected by the photomultiplier tube (PMT) in order to produce an image. Once data acquisition for the Cy5 labeled cDNA was complete, a laser set to 532nm excited the Cy3 fluor and the emissions were collected. The images were assigned a false color based on the wavelength of the scanner and were superimposed using GenepixPro 4.0 image analysis software (Axon Instruments).

4.2.5 Statistical analysis

4.2.5.1 Dye normalization

Identities of the printed genes or features were identified by applying the array list settings provided by the Microarray Centre to the merged features. The settings are locked onto the image and GenepixPro 4.0 determined the intensities of individual pixels within the features and the background intensity of the pixels for each channel, either 532nm for Cy3 or 635nm for Cy5. With this information the Genepix Pro 4.0 software calculated several values that were used to determine the median intensity in each channel for each feature and the $\log_2(635/532)$ ratio for each feature. The median intensity in each channel was summed. Ideally, the sums for Cy3 and Cy5 intensity should be equal since 20 μ g of total RNA was used for preparing labeled cDNA and equivalent amounts of Cy3 and Cy5 dye were used in the dye coupling reaction. However, variation in total intensity was used to normalize the signals. The ratio of Cy3 to Cy5 was used to configure the wavelength-based normalization feature within GenepixPro 4.0 and the normalization was applied to every feature, resulting in a dye-normalized $\log_2(635/532)$ ratio.

4.2.5.2 Statistical analysis

The dye normalized \log_2 (635/532) ratios were exported into Excel (Microsoft) along with gene name, ORF designation, and unique feature indicator number. \log_2 (635/532) ratios from individual replicates can be extracted from GenepixPro 4.0 and cataloged by their microarray slide number side by side with the data from other replicates. For the *CTR9* versus *ctr9Δ* microarray experiments, data from four total biological replicates and data from four reciprocal dye labeling experiments were ordered into Excel. Preliminary statistical analysis was performed by calculating the average and standard deviation of the \log_2 (635/532) for each feature across all eight experiments.

4.3 Results

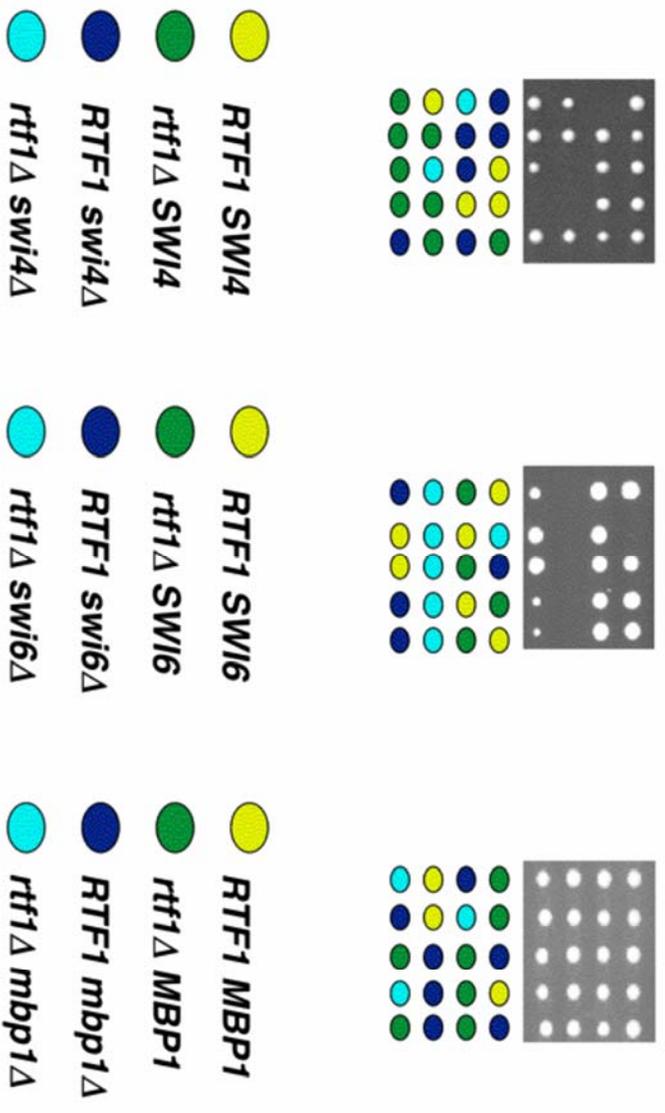
4.3.1 The Paf1 complex and SBF may coordinately regulate transcription during the G₁/S phase of the cell cycle

Initial attempts to identify genes regulated by the Paf1 complex relied on genetic interactions among components of SBF and MBF and members of the Paf1 complex. In particular, mutations in *SWI4* and *SWI6* were isolated in a synthetic lethal screen using an *rtf1Δ* strain (Costa and Arndt, 2000). Mutations in *mbp1* were not recovered in this genetic screen. We verified the synthetic lethal interactions by producing deletions in *SWI4*, *SWI6*, and *MBP1* and crossing them by an *rtf1Δ* strain (Figure 28). Double mutant combinations were recovered for *mbp1Δ rtf1Δ*, but not for *swi4Δ rtf1Δ* and *swi6Δ rtf1Δ* (Figure 28). The lethality observed for *swi4Δ rtf1Δ* and *swi6Δ rtf1Δ* could manifest from the absence of transcriptional activation combined with a defect in transcription elongation. The Paf1 complex together with SBF were implicated in co-regulating the transcription of gene products required for the G₁/S phase transition in the cell cycle. Therefore, we attempted to identify Rtf1-regulated genes with Northern analysis using total RNA isolated from wild-type, *rtf1Δ*, *swi4Δ*, *swi6Δ*, *mbp1Δ* strains. Transcript abundance levels for *CLN1*, *CLN2*, and *CLN3* were analyzed; however the results from these experiments did not conclusively establish a role for the Paf1 complex in regulating G₁ cyclins or other targets of SBF (data not shown). These results could be due to the possibility that Rtf1 does not have a significant role in G₁ cyclin expression because the cultures were grown

asynchronously rather than synchronously thereby reducing the opportunity to observe the lowered transcript levels in *rtf1Δ* cells. However, two groups did demonstrate a role for the Ctr9 and Paf1 members of the Paf1 complex in regulating the transcription of a subset of genes expressed during the G₁/S phase of the cell cycle. First, in a screen to identify mutations that failed to activate the transcription of G₁ cyclins, a mutation in *CTR9* was recovered (Koch et al., 1999). Interestingly, *ctr9Δ* and *paf1Δ* cells but not *cdc73Δ* cells were defective in the transcription of a G₁ cyclin *CLN2* (Koch et al., 1999). Second, microarray analyses using Affymetrix oligonucleotide arrays and differential display identified genes regulated by Paf1 and Ctr9 complex Jaehning lab (Penheiter et al., 2005; Porter et al., 2002). These studies revealed that Paf1 and Ctr9 were important for the transcription of cell cycle-regulated genes.

Figure 28: *RTF1* interacts genetically with *SWI4* and *SWI6* but not *MBP1*

Genetic interactions between *RTF1* and components of SBF. Crosses of *rtf1* Δ (KY452) X *swi4* Δ (KKY11), *rtf1* Δ (KY452) X *swi6* Δ (KKY13), and *rtf1* Δ (KRY2) X *mbp1* Δ (KKY5) cells were performed followed by tetrad dissection. The genotypes were inferred from phenotypic analysis and identified wild-type, single and double mutants as indicated by color-coded spores.



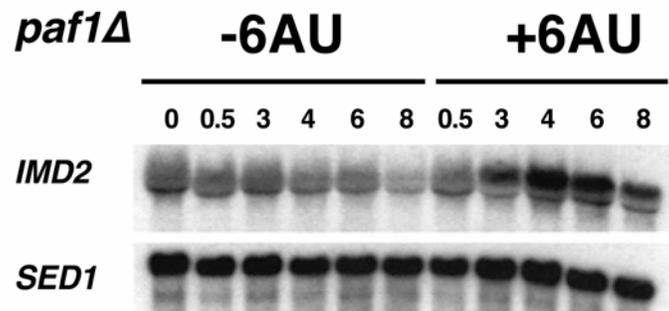
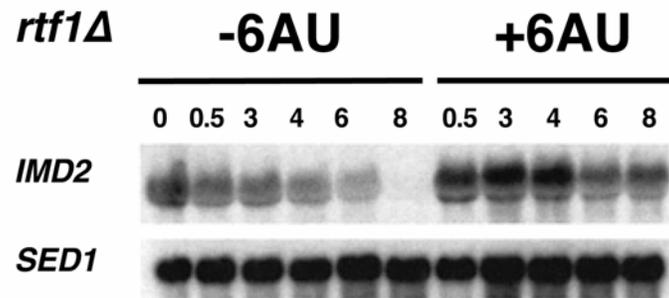
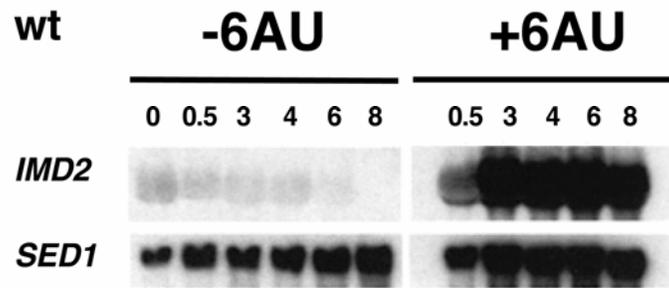
4.3.2 *IMD2* transcription induction is impaired in Paf1 complex member mutants

As previously described mutations within the Paf1 complex and other elongation factors often results in sensitivity to the base analog 6AU (Exinger and Lacroute, 1992; Shaw and Reines, 2000). However, sensitivity of 6AU is frequently but not always indicative of defects in transcription elongation (Exinger and Lacroute, 1992; Shaw and Reines, 2000). Shaw and Reines demonstrated that *IMD2/PUR5*, which encodes inosine monophosphate dehydrogenase, is transcriptionally induced by exposure of yeast cells to 6AU (Squazzo et al., 2002). Mutations that cause transcription elongation defects and 6AU sensitivity also prevent the induction of *IMD2* transcription in response to 6AU treatment. In contrast, mutations that cause 6AU sensitivity but do not affect transcription elongation do not interfere with *IMD2* induction. Thus, a 6AU-sensitive phenotype coupled with the inability to induce *IMD2* in response to 6AU is suggestive of a defect in transcription elongation. We therefore isolated RNA from wild type, *rtf1Δ*, and *paf1Δ* cells before and after 6AU treatment and performed a Northern analysis of *IMD2* levels (Figure 29). This analysis showed that deletion of *PAF1* or *RTF1* significantly reduces *IMD2* induction, and therefore suggests that the Paf1 complex may play a role in transcription elongation. The expression analysis also indicated that under non-inducing conditions the absence of either Paf1 or Rtf1 resulted in increased transcription of *IMD2* message. This result suggests that the Paf1 complex may regulate the expression of *IMD2* under normal growth conditions (Figure 29). One of the caveats of this experiment is that any phase of the transcription cycle including initiation or elongation could result in impaired *IMD2* expression in the presence of 6AU. Therefore, impaired ability of *rtf1Δ* and *paf1Δ* cells to

induce *IMD2* expression cannot serve as the only evidence implicating members of the Paf1 complex in transcription elongation. Rather, impaired *IMD2* induction together with genetic, physical, and chromatin interaction data implicating the Paf1 complex in elongation should be considered when attempting to describe the function of the Paf1 complex in transcription.

Figure 29: *rtf1*Δ and *paf1*Δ cells are defective in *IMD2* induction

*rtf1*Δ and *paf1*Δ strains are defective in *IMD2* induction. Northern analysis of *IMD2* transcription was performed on total RNA samples prepared from wild-type (KY589), *rtf1*Δ (KY453), and *paf1*Δ (KY687) cells. The strains were grown to $\sim 1 \times 10^7$ cells/ml in SC-Ura media and then divided. 6AU was added to a final concentration of 75μg/ml to one half of the culture and both cultures were grown at 30°C for the indicated times, in hours, prior to RNA isolation. Each lane contained 10μg of total RNA. The same filters that were probed for *IMD2* transcript were stripped and reprobbed for *SEDI* mRNA to serve as a loading control.



4.3.3 Differential expression analysis to identify genes regulated by the Paf1 complex

In an effort to identify genes regulated by the Paf1 complex, microarray analysis was performed. Total RNA was isolated from *CTR9* and *ctr9Δ* cells and cDNA was prepared using a two-step labeling protocol in which reverse transcription was followed by Cy dye incorporation. Identification of differentially expressed genes involved competitive hybridization to glass microarrays, image analysis and preliminary statistical analyses. The microarrays were obtained from the Microarray Centre. Microarray slides contained approximately 6,000 cDNAs representative of the transcriptome of *S. cerevisiae*, spotted in duplicate. Northern analyses were used to verify changes observed between the transcription profiles of *CTR9* and *ctr9Δ* cells for genes exhibiting differential expression in strains deleted for individual Paf1 complex members. Finally, ChIP (ChIP) analysis was performed to determine whether components of the Paf1 complex are directly or indirectly involved in regulating the genes identified using microarray analysis.

Four biological replicate microarray experiments were performed using RNA isolated from *CTR9* and *ctr9Δ* cells. In these experiments, cDNA prepared from *CTR9* total RNA was labeled with Cy3 dye while cDNA prepared from *ctr9Δ* total RNA was labeled with Cy5 dye. To account for potential differences in labeling efficiency and differential oxidation of the Cy fluors, four reciprocal labeling experiments were also performed. Upon image analysis, dye incorporation normalization and data extraction, the eight sets of microarray data were used to calculate the average \log_2 ratio and standard deviation for each representative gene. Table 15 and Table 16 contain Ctr9-regulated genes; Table 15 contains 2.5% of the genes in *S. cerevisiae*

genome that require Ctr9 for transcription while Table 16 contains 2.5% of the genes in *S. cerevisiae* genome that require Ctr9 for their repression. Out of the many genes that were potentially regulated by Ctr9, we focused on *SER3* and *ARG1*, these genes are highlighted in yellow in Table 16. *ACO1* is highlighted in pink (Table 16). Evidence from microarrays performed in our laboratory indicate that the Paf1 complex may negatively regulate *ACO1* transcription (Table 16).

Table 15: 2.5% of 6200 genes that require Ctr9 for their transcription

Open Reading frame	Gene name	Average log₂ ratio	Standard deviation
<i>YJL196C</i>	<i>ELO1</i>	-2.50	1.65
<i>YDR139C</i>	<i>RUB1</i>	-2.49	5.16
<i>YGL117W</i>	NA	-2.41	1.16
<i>YGR049W</i>	<i>SCM4</i>	-2.30	2.57
<i>YJR147W</i>	<i>HMS2</i>	-2.24	1.62
<i>YDR525W</i>	<i>API2</i>	-2.21	2.04
<i>YER091C</i>	<i>MET6</i>	-2.09	1.10
<i>YKL069W</i>	NA	-2.05	1.07
<i>YAR071W</i>	<i>PHO11</i>	-2.03	0.69
<i>YDR469W</i>	<i>SDC1</i>	-2.02	4.04
<i>YMR195W</i>	<i>ICY1</i>	-2.00	0.58
<i>YJR025C</i>	<i>BNA1</i>	-1.99	1.39
<i>YCR071C</i>	<i>IMG2</i>	-1.98	4.92
<i>YFR047C</i>	<i>BNA6</i>	-1.95	1.40
<i>YGR177C</i>	<i>ATF2</i>	-1.93	1.35
<i>YGR018C</i>	NA	-1.92	4.73
<i>YKL091C</i>	NA	-1.92	1.06
<i>YEL059W</i>	NA	-1.89	4.87
<i>YNL129W</i>	NA	-1.86	0.70
<i>YLR156W</i>	NA	-1.86	6.14
<i>YPL250C</i>	<i>ICY2</i>	-1.84	0.78
<i>YLR364W</i>	NA	-1.83	2.61
<i>YGR163W</i>	<i>GTR2</i>	-1.83	0.84
<i>YGL039W</i>	NA	-1.83	0.69
<i>YOR236W</i>	<i>DFR1</i>	-1.81	0.70
<i>YER062C</i>	<i>HOR2</i>	-1.77	0.96
<i>YML113W</i>	<i>DAT1</i>	-1.75	4.10
<i>YOL014W</i>	NA	-1.74	0.30
<i>YLR118C</i>	NA	-1.74	2.64

Table 15 (continued)			
<i>YDL198C</i>	<i>YHMI</i>	-1.72	2.33
<i>YGL155W</i>	<i>CDC43</i>	-1.72	4.96
<i>YLR348C</i>	<i>DIC1</i>	-1.72	0.95
<i>YDL241W</i>	NA	-1.70	0.59
<i>YDL022W</i>	<i>GPD1</i>	-1.69	0.83
<i>YHL041W</i>	NA	-1.68	4.46
<i>YIL119C</i>	<i>RPI1</i>	-1.68	0.79
<i>YPL014W</i>	NA	-1.67	1.80
<i>YGR131W</i>	NA	-1.65	0.82
<i>YJR024C</i>	NA	-1.65	2.27
<i>YER032W</i>	<i>FIR1</i>	-1.64	0.42
<i>YPL099C</i>	NA	-1.63	0.36
<i>YDR270W</i>	<i>CCC2</i>	-1.63	4.91
<i>YKR074W</i>	NA	-1.60	0.35
<i>YLR268W</i>	<i>SEC22</i>	-1.59	0.81
<i>YGL159W</i>	NA	-1.59	2.04
<i>YIL086C</i>	NA	-1.59	1.47
<i>YER067W</i>	NA	-1.58	0.84
<i>YDR367W</i>	NA	-1.57	0.44
<i>YER053C</i>	NA	-1.56	2.83
<i>YOR344C</i>	<i>TYE7</i>	-1.54	0.68
<i>YGL080W</i>	NA	-1.54	1.59
<i>YMR141C</i>	NA	-1.54	2.26
<i>YLR349W</i>	NA	-1.54	1.82
<i>YMR022W</i>	<i>QRI8</i>	-1.53	4.99
<i>YDR491C</i>	NA	-1.52	4.53
<i>YMR317W</i>	NA	-1.51	1.54
<i>YPR116W</i>	NA	-1.51	2.47
<i>YKL096W</i>	<i>CWP1</i>	-1.51	0.28
<i>YCL018W</i>	<i>LEU2</i>	-1.51	0.71
<i>YER093C-A</i>	NA	-1.50	1.28
<i>YOR163W</i>	<i>DDP1</i>	-1.50	0.45
<i>YMR284W</i>	<i>YKU70</i>	-1.49	4.18
<i>YDR126W</i>	<i>PSL10</i>	-1.48	1.27

Table 15 (continued)			
<i>YMR030W</i>	NA	-1.48	4.84
<i>YLR228C</i>	<i>ECM22</i>	-1.48	4.76
<i>YHR049C-A</i>	NA	-1.46	4.80
<i>YDL049C</i>	<i>KNH1</i>	-1.46	1.17
<i>YML075C</i>	<i>HMG1</i>	-1.46	2.73
<i>YNL288W</i>	<i>CAF40</i>	-1.46	4.87
<i>YER037W</i>	<i>PHM8</i>	-1.46	1.28
<i>YOR095C</i>	<i>RKII</i>	-1.45	0.46
<i>YCR090C</i>	NA	-1.45	0.51
<i>YGR033C</i>	NA	-1.45	1.72
<i>YBR022W</i>	NA	-1.44	1.78
<i>YCLX09W</i>	NA	-1.44	2.42
<i>YHL009C</i>	<i>YAP3</i>	-1.44	1.17
<i>YJL161W</i>	NA	-1.43	0.77
<i>YMR157C</i>	NA	-1.43	5.06

Open reading frames with NA indicate these genes are unannotated as per SGD.

ARG1 and *SER3* are involved in the biosynthesis of arginine and serine, respectively. Interestingly, our microarray data indicated that Ctr9 is required for repression of *ARG1* and *SER3* in rich medium (Table 16), which indicates that members of the Paf1 complex may negatively regulate the expression of certain genes. Northern analysis revealed that Paf1 complex members were required for repression of *ARG1* transcription (Figure 30A and 30; Crissuci and K. M. Arndt, unpublished observations). ChIP analysis using antibodies directed against epitope-tagged forms of Rtf1 and Ctr9 revealed that both proteins associate with the 5' and 3' regions of *ARG1* when cells are grown in rich medium (Figure 30B-30D). The data presented in Figure 30 is consistent with results from the Hinnebusch lab where they show *ARG1* transcription is increased in *paf1* Δ cells and that Paf1 localizes to the *ARG1* UAS. These results are of interest since some transcription factors are known to positively and negatively regulate the transcription of certain genes. The *ARG1* locus is desirable for studying the mechanism of possible repression through the Paf1 complex for many reasons. First, manipulation of *ARG1* transcription is dependent on the presence of arginine in the cell. For example, *ARG1* transcription is induced in the absence of arginine in the growth medium and transcription is repressed when arginine is present. Second, many transcription factors involved in regulating *ARG1* expression in response to limiting levels of arginine are known. Therefore, the use of *ARG1* as a model gene could provide insight into the mechanism by which the RNA polymerase II-associated Paf1 complex may repress *ARG1* transcription.

Table 16: 2.5% of 6200 genes that require Ctr9 for their repression

Open reading frame	Gene name	Average log₂ ratio	Standard deviation
YER081W	SER3	3.07	0.54
YMR096W	SNZ1	2.68	1.03
YOL058W	ARG1	2.58	1.33
YKR039W	GAP1	2.58	1.05
YLR259C	HSP60	2.53	1.87
YLR237W	THI7	2.47	1.81
YLL034C	NA	2.46	4.55
YGL255W	ZRT1	2.37	1.17
YLR007W	NA	2.22	4.75
YMR026C	PEX12	2.17	5.01
YPL278C	NA	2.11	4.73
YOR192C	NA	2.04	1.48
YDR473C	PRP3	2.03	2.91
YNR039C	ZRG17	1.95	4.44
YPL154C	PEP4	1.78	0.89
YGL222C	EDC1	1.77	5.58
YIL082W-A	NA	1.75	4.81
YPL274W	SAM3	1.74	2.15
YLR382C	NAM2	1.74	4.34
YPL227C	ALG5	1.74	4.58
YDR443C	SSN2	1.73	4.57
YGR223C	NA	1.72	4.97
YKL155C	RSM22	1.71	4.58
YLL004W	ORC3	1.69	4.66
YLR330W	CHS5	1.66	4.45
YDL109C	NA	1.65	4.60
YJR121W	ATP2	1.62	1.49
YNL061W	NOP2	1.61	2.40
YJR045C	SSC1	1.61	1.59
YOR375C	GDH1	1.58	0.69

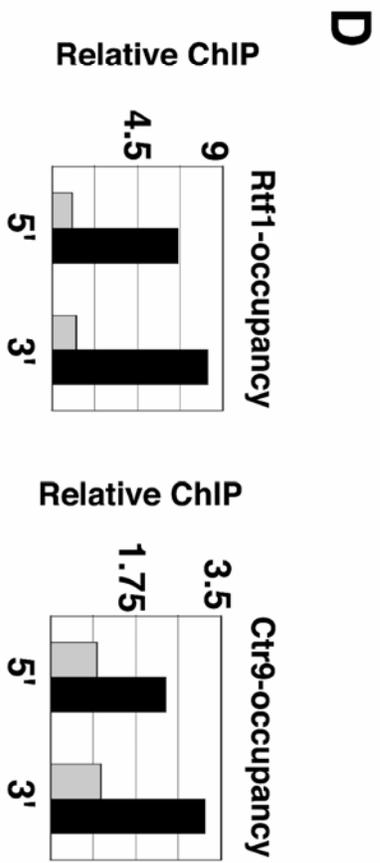
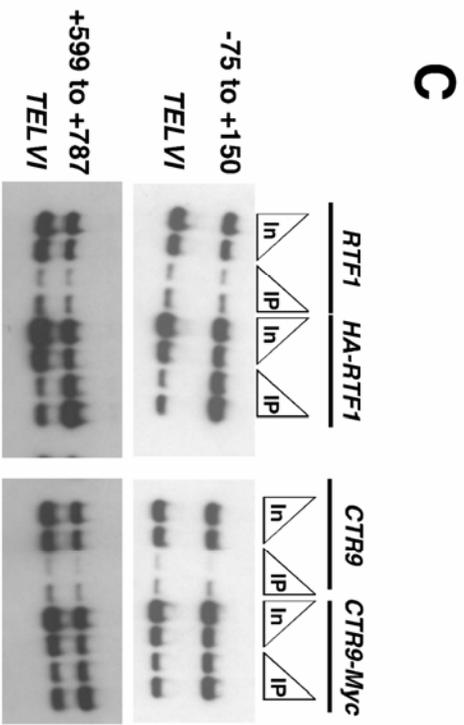
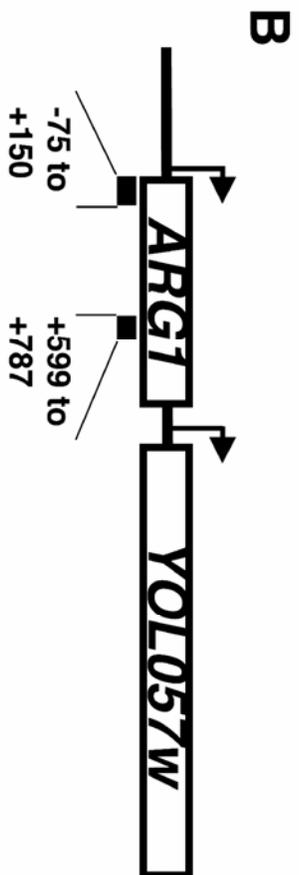
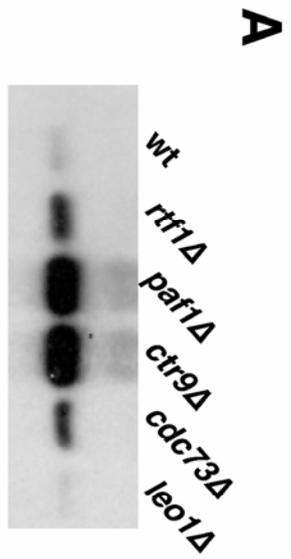
Table 16 (continued)			
<i>YPL038W</i>	<i>MET31</i>	1.58	3.30
<i>YBR131W</i>	<i>CCZ1</i>	1.57	4.57
<i>YDR019C</i>	<i>GCV1</i>	1.56	1.48
<i>YNR068C</i>	NA	1.55	5.12
<i>YGL113W</i>	<i>SLD3</i>	1.51	4.82
<i>YLL060C</i>	<i>GTT2</i>	1.51	0.75
<i>YLL022C</i>	<i>HIF1</i>	1.50	3.24
<i>YIR034C</i>	<i>LYS1</i>	1.49	0.85
<i>YGR157W</i>	<i>CHO2</i>	1.48	0.91
<i>YMR017W</i>	<i>SPO20</i>	1.48	4.81
<i>YOR379C</i>	NA	1.47	4.99
YLR304C	ACO1	1.44	0.82
<i>YMR173W-A</i>	NA	1.43	0.54
<i>YKR080W</i>	<i>MTD1</i>	1.43	1.15
<i>YOR027W</i>	<i>STI1</i>	1.43	1.84
<i>YER099C</i>	<i>PRS2</i>	1.43	4.85
<i>YHR105W</i>	NA	1.42	2.81
<i>YNL033W</i>	NA	1.42	2.13
<i>YHR064C</i>	<i>SSZ1</i>	1.41	1.34
<i>YOR389W</i>	NA	1.40	1.17
<i>YMR173W</i>	<i>DDR48</i>	1.40	0.53
<i>YLL051C</i>	<i>FRE6</i>	1.40	5.06
<i>YPL003W</i>	<i>ULA1</i>	1.39	5.04
<i>YGL079W</i>	NA	1.39	2.75
<i>YKR048C</i>	<i>NAP1</i>	1.37	1.82
<i>YOL036W</i>	NA	1.37	0.95
<i>YJL034W</i>	<i>KAR2</i>	1.37	0.46
<i>YOR023C</i>	<i>AHC1</i>	1.35	1.70
<i>YGR110W</i>	NA	1.35	0.69
<i>YKL145W</i>	<i>RPT1</i>	1.35	1.11
<i>YER176W</i>	<i>ECM32</i>	1.33	1.16
<i>YOR048C</i>	<i>RAT1</i>	1.33	3.22
<i>YDL090C</i>	<i>RAM1</i>	1.33	4.78
<i>YMR265C</i>	NA	1.32	1.88

Table 16 (continued)			
<i>YJR065C</i>	<i>ARP3</i>	1.32	1.16
<i>YNL158W</i>	NA	1.31	1.63
<i>YGL230C</i>	NA	1.30	2.85
<i>YHL017W</i>	NA	1.30	1.60
<i>YKR079C</i>	NA	1.30	1.82
<i>YDR438W</i>	NA	1.30	4.57
<i>YNL202W</i>	<i>SPS19</i>	1.29	1.85
<i>YLR224W</i>	NA	1.29	0.82
<i>YPR191W</i>	<i>QCR2</i>	1.28	0.53
<i>YDR311W</i>	<i>TFB1</i>	1.28	4.51
<i>YIL138C</i>	<i>TPM2</i>	1.28	0.52
<i>YNL165W</i>	NA	1.27	1.17
<i>YJR062C</i>	<i>NTA1</i>	1.27	1.35
<i>YJL002C</i>	<i>OST1</i>	1.27	0.93

Open reading frames with NA indicate these genes are unannotated as per SGD.

Figure 30: The Paf1 complex directly regulates the transcription of *ARG1*

Expression analysis of *ARG1*. (A) Northern analysis was performed with 10 μ g of total RNA isolated from the indicated strains: wild-type (KY661), *rtf1* Δ (KY656), *paf1* Δ (KY685), *ctr9* Δ (GHY1094), *cdc73* Δ (KY689), *leo1* Δ (GHY250), *snf2* Δ (KY508). *ARG1* cDNA random-prime labeled was used to probe filters for *ARG1* transcripts. (B) Diagram of the *ARG1* locus. Primer pairs amplifying *ARG1p* (-75 to +150) and *ARG1c* (+599 to +787) and their positions relative to the ATG +1 are indicated. (C) ChIP analysis over the *ARG1* locus was performed with *RTF1* (FY118), *HA₃-RTF1* (KY399 or PCY431 grown in SC-Trp), *CTR9* (FY118) and *CTR9-6XMYC* (GHY1177) strains. Chromatin was precipitated with antibodies against the HA or Myc epitope. Two amounts of immunoprecipitated (IP) DNA (2 μ l and 4 μ l) and input (In) DNA (4 μ l of 1:125 and 1:250 dilutions) were analyzed by PCR. The reactions included the *ARG1* primer pair specified adjacent to each panel as in (B) and primer pairs that amplifies a subtelomeric region of chromosome VI (*TELVI*), which does not contain genes transcribed by RNA polymerase II. (D) The relative occupancy of tagged and untagged forms of Rtf1 and Ctr9 was determined by calculating the ratio of IP to In for primer pairs spanning *ARG1* and normalizing to the IP to In ratio for the telomere signal. Relative ChIP was plotted graphically with the untagged strain represented by gray bars and the tagged strain represented by black bars.

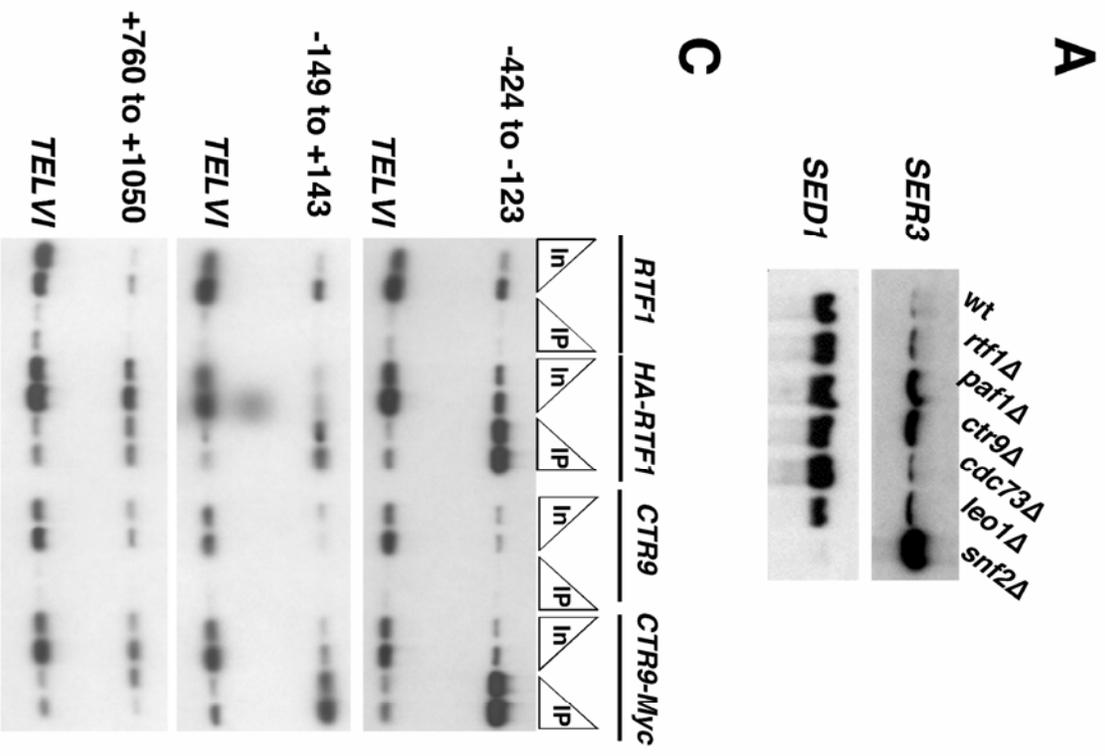


Microarray analysis also indicated that *SER3* is repressed by the Paf1 complex when serine was not limiting. Northern analysis was performed with total RNA isolated from strains lacking individual members of the Paf1 complex (Figure 31A). Included in this Northern analysis, was RNA isolated from a *snf2Δ* strain since SWI/SNF was necessary for mediating *SER3* repression in rich medium (Martens and Winston, 2002). ChIP experiments were performed on the *SER3* gene using primer pairs that amplified the promoter region, the 5' open reading frame, or 3' end of *SER3* (Figure 31B-31D). Using epitope-tagged forms of Rtf1 and Ctr9 we observed enrichment of the Paf1 complex over the promoter and 5' region of *SER3* (Figure 31B-31D). Density of both Ctr9 and Rtf1 was severely reduced to near background levels toward the 3' end of *SER3* (Figure 31C and 31D). The pattern of Paf1 complex localization over *SER3* was different from what we had observed for *ARG1*, therefore it was possible that the mechanism by which the Paf1 complex acted to repress *SER3* was different from the mechanism the Paf1 used to repress *ARG1*. However, the preliminary *ARG1* ChIP experiments must be repeated because we have observed variation in *ARG1* derepression dependent upon the auxotrophic markers of the particular strain (Crissuci and K. M. Arndt, unpublished observations). The Winston lab communicated to us that they had identified a gene within the promoter of *SER3* that had been unannotated in the yeast genome (Martens et al., 2004). The gene within the *SER3* promoter was designated *SER3* regulatory gene 1 or *SRG1* (Martens et al., 2004). Transcription from *SRG1* results in the expression of a noncoding RNA (Martens et al., 2004). In fact, expression of *SRG1* serves to repress transcription of *SER3* by a transcriptional interference mechanism (Martens et al., 2004). *SRG1* produces a transcript of approximately 551 nt in length according to SGD, however the 3'-end of the *SRG1* transcript has not been mapped. Therefore, the 3' region of the *SRG1* gene could potentially overlap with the 5' region of *SER3* (Figure 31B). Our ChIP results

support the idea that the Paf1 complex may be important for the transcription of *SRG1* and serve to regulate *SER3* transcription through the transcriptional interference mechanism.

Figure 31: The Paf1 complex directly regulates the transcription of *SER3*

Expression analysis of *SER3*. (A) Northern analysis was performed with 10 μ g of total RNA isolated from the indicated strains: wild-type (KY661), *rtf1* Δ (KY656), *paf1* Δ (KY685), *ctr9* Δ (GHY1094), *cdc73* Δ (KY689), *leo1* Δ (GHY250), *snf2* Δ (KY508). RNA isolated from *snf2* Δ cells served as a positive control for derepression of *SER3* (Martens and Winston, 2002). A random-prime labeled *SER3* probe was used to probe filters for *SER3* transcripts. The blots were stripped and reprobated with labeled *SEDI* cDNA as an internal control (B) Diagram of the *SRG1-SER3* locus. Primer pairs amplifying *SRG1* (-424 to -123), the *SER3* promoter (*SER3p*; -149 to +143), and the *SER3* coding region (*SER3c*; + 760 to +1050) and their positions relative to the ATG (+1) are indicated. (C) ChIP analysis over the *SER3* locus was performed with *RTF1* (FY118), *HA₃-RTF1* (KY399 or PCY431 grown in SC-Trp), *CTR9* (FY118) and *CTR9-6XMYC* (GHY1177) strains. Chromatin was precipitated with antibodies against the HA or Myc epitope. Two amounts of immunoprecipitated (IP) DNA (2 μ l and 4 μ l) and input (In) DNA (4 μ l of 1:125 and 1:250 dilutions) were analyzed by PCR. The reactions included the *SER3* primer pair specified adjacent to each panel as in (B) and a primer pair that amplifies a subtelomeric region of chromosome VI (*TELVI*), which does not contain genes transcribed by RNA polymerase II. (D) The relative occupancy of tagged and untagged forms of Rtf1 and Ctr9 were determined by calculating the ratio of IP to In for primer pairs spanning *SER3* and normalizing to the IP to In ratio for the telomere signal. Relative ChIP was plotted graphically with the untagged strain represented by gray bars and the tagged strain represented by black bars.



4.4 Conclusions

4.4.1 Combined approaches of genetics and microarray analyses identify genes regulated by the Paf1 complex

Deletion of individual members of the Paf1 complex causes defects in the transcription of genes. Genetic interactions between members of the Paf1 complex and SBF provided support to the idea that the Paf1 complex may be involved in regulating a subset of SBF-regulated genes. While Northern analysis with total RNA isolated from *rtf1* Δ strains did not exhibit defects in the transcription of SBF-regulated target genes, only a small subset of SBF target genes was analyzed (data not shown). Differential display and microarray analysis identified transcriptional targets of Paf1 and Ctr9 in the Jaehning lab and revealed a role for the Paf1 complex in regulating the transcription of genes that are expressed during the G₁/S phase of the cell cycle. Inspection of the genes that require SBF, Ctr9 and Paf1 for proper transcription combined with Northern analysis using total RNA isolated from strains deleted for individual members of the Paf1 complex could reveal whether Rtf1, Cdc73 or Leo1 are also important for the transcription of the genes regulated by SBF, Ctr9, and Paf1.

Microarray studies performed to identify Ctr9 target genes revealed a direct role for the Paf1 complex in regulating genes in the arginine and serine biosynthetic pathways. Expression analyses of *ARG1* indicated that individual Paf1 complex members are required for repression of *ARG1* under noninducing conditions (Figure 30A-30D). Moreover, Paf1 and Ctr9 associated

directly with the length of the *ARG1* open reading frame suggesting that the Paf1 complex is actively involved in repressing *ARG1* transcription in the presence of arginine (Figure 30A-30D). Again, observations from our laboratory and communication with the Hinnebusch laboratory suggest that strains with certain auxotrophic markers may derepress *ARG1* through general amino acid control (Hinnebusch, 2005). Therefore, the *ARG1* Northern and ChIP experiments must be repeated with strains that do not cause the induction of general amino acid control. The mechanism through which Paf1 acts to repress transcription of *ARG1* is not clear. However, the mechanism of Paf1 complex-dependent repression at *ARG1* is probably distinct from the repressive mechanism occurring at *SER3*. *SER3* transcription is regulated by transcriptional interference caused by the transcription of *SRG1* (Martens et al., 2004). A role for the Paf1 complex in regulating *SRG1* transcription has not been directly tested, however, ChIP analysis using epitope-tagged versions of Rtf1 and Ctr9 over the *SRG1-SER3* region suggests the Paf1 complex associates with *SRG1* while association of the Paf1 complex is reduced over the 3' region of *SER3* (Figure 31B-31D). This pattern of Paf1 complex occupancy is consistent with the Paf1 complex regulating *SRG1* expression and indirectly regulating *SER3* expression via transcriptional interference.

Impaired *IMD2* induction is a hallmark of elongation factor mutants. Deletion of *DST1* the gene that codes for the well-characterized elongation factor TFIIS results in impaired *IMD2* induction in the presence of 6AU (Shaw and Reines, 2000). Analysis of *IMD2* induction in *paf1Δ* and *rtf1Δ* cells revealed a defect in *IMD2* induction similar to *dst1Δ* cells (Figure 28). Interestingly, deletion of *DST1* suppresses the Spt⁻ phenotype of *rtf1Δ* cells and triple mutant cells deleted for *DST1*, *SPT4*, and *RTF1* are inviable (Costa and Arndt, 2000). Overall, these

observations suggest that TFIIIS, Spt4, and the Paf1 complex may be important for regulating transcription elongation.

5.0 Chapter 5: Discussion

5.1 Introduction

To address possible functions of Rtf1 and members of the Paf1 complex in cells I combined conventional genetic approaches with genome-wide expression analyses for my thesis research. The two approaches are complementary to each other. For instance, discovery of multicopy suppressors of point mutations within *RTF1* could identify novel genetic interactions with implications for regulating RNA polymerase II transcription. On the other hand, the use of DNA microarrays to identify genes directly and differentially regulated by the Paf1 complex could be used to uncover and/or further dissect transcription regulatory networks. A discussion of the success of these approaches follows.

5.2 What have we learned about Rtf1 and the Paf1 complex by analyzing the *rtf1* point mutants?

5.2.1 Can the *rtf1* missense mutations provide insight into Rtf1 activity?

Multiple genetic tools to clarify the function of *RTF1* in the cell were constructed in the Arndt lab after the initial isolation of *rtf1-1*, a mutation that restored the Spt⁺ phenotype of a TBP mutant (Stolinski et al., 1997). The *rtf1*Δ mutant and a series of small deletions within *RTF1* have contributed greatly to our understanding of Rtf1 in transcription. For example, a genetic

screen with *rtf1* Δ cells identified genes that become essential in the absence of Rtf1 (Costa and Arndt, 2000). The identity of these mutations revealed the first link between Rtf1 and factors involved in the transition from transcription initiation to elongation (Costa and Arndt, 2000). The *RTF1* deletion series in combination with primary amino acid alignments among Rtf1 homologs provided information regarding the function of different regions within Rtf1. For example, deletions within the central region of Rtf1 confer 6AU^S and Spt⁻ phenotypes. Interestingly, the central region of the protein is among the most conserved across eukaryotes, suggesting strongly that this portion of Rtf1 is critical for its function.

Additional analysis on the internal *RTF1* deletion series has provided information regarding the function of Rtf1 and which regions of Rtf1 are important for known physical, biochemical, and genetic relationships. Not surprisingly, different regions are responsible for the diverse roles ascribed to Rtf1. For example, the N-terminal region encompassing amino acid residues 3-30 of Rtf1 is important for association with the chromatin remodeling factor Chd1 (K. L. Roinick and K. M. Arndt, unpublished observations). Deletion of residues 62-109 and 111-152 and to a lesser extent deletion of the conserved region of Rtf1 result in a significant reduction in global levels of histone H3 trimethylation that is associated with transcriptionally active genes (M. H. Warywoda and K. M. Arndt, unpublished observations). The *rtf1* point mutants do not cause defects in histone H3 K4 trimethylation even at elevated temperatures. Taken together, the above observations strongly suggest the conserved, central region of Rtf1 is associated with chromatin dynamics.

In an effort to learn more about Rtf1, we sought conditional mutations within *RTF1*. Mutagenic PCR and screening for 6AU^S and Spt⁻ phenotypes at elevated temperatures lead to the identification of several conditional *RTF1* mutations. These alleles contained multiple mutations,

increasing the likelihood that these phenotypes arose from a combination of one or more mutations within the gene. To isolate single missense mutations conferring conditional phenotypes and to determine the contribution of each mutation, individual mutations were separated by subcloning or singly introduced using site-directed mutagenesis. We uncovered three point mutants that exhibited conditional phenotypes. The *rtf1* allele within parent plasmid pDM1 contained two mutations, the first resulted in an E264D substitution and the second resulted in a V274D substitution. The valine to aspartic acid substitution is a striking change as compared to the more conservative glutamic acid to aspartic acid substitution. Both of these point mutations reside in the central conserved region of Rtf1 that is essential for function since deletion of residues 251-300 results in both 6AU^S and Spt⁻ phenotypes. The phenotypes of the individual point mutants were analyzed and indicated that the conditional 6AU^S and weak Spt⁻ phenotypes were the result of the V274D substitution in *rtf1-105*. The E264D substitution within the central conserved region of Rtf1 did not greatly affect the function of Rtf1 at either the permissive or restrictive temperatures indicating that this position could be occupied by a less bulky, but acidic residue. If the change had been more severe, it is likely that mutant phenotypes would have been observed. As for V274D, valine is a small, nonpolar amino acid that likely resides within the hydrophobic core of the protein, replacing valine with aspartic acid, a charged amino acid, could impact the folding of Rtf1. The substitution of a nonpolar amino acid with a charged amino acid could force a residue that is normally within the protein into a more solvent exposed position resulting in reduced protein activity at higher temperatures.

Two mutations were observed in *RTF1* within parent plasmid pDM6. The first caused the substitution of methionine 289 to lysine, which replaces a nonpolar amino acid with a basic charged residue. The second mutation introduces a stop codon at amino acid 495. Since deletion

of either 491-535 or 536-558 of Rtf1 does not result in either 6AU^S or Spt⁻ phenotypes, we reasoned that the missense mutation contributed more to the phenotype than the deletion at the C-terminus. *rtf1-107* (M289K) was analyzed for conditional phenotypes. This mutant exhibited a conditional 6AU^S phenotype and a weak Spt⁻ phenotype at the permissive temperature but mutation did not severely affect function of Rtf1 at the permissive temperature. Replacing a nonpolar residue with lysine could disrupt nonpolar interactions within the protein that are important for Rtf1 stability, while potentially changing the path of the amino acid backbone to accommodate a charged side chain.

Identification of the point mutation(s) that caused the phenotype of the *rtf1* allele on parent plasmid pDM3 was more challenging. Sequence analysis uncovered 3 mutations that resulted in the following amino acid substitutions: Q172R, K246E and Y341F. The latter two mutations reside in the conserved region of Rtf1 important for its function, however the mutation that causes the Q172R substitution resides within a region of *RTF1* that when deleted confers a Spt⁻ phenotype. Interestingly, the three mutations appear to have contributed to the Spt⁻ phenotype of pDM3 because this plasmid conferred a strong Spt⁻ phenotype at 30° and 37°C. At the permissive temperature the separated mutations did not cause a Spt⁻ phenotype, however at elevated temperatures the Q172R substitution resulted in a strong Spt⁻ phenotype while the other substitutions caused substantially weaker phenotypes. The error-prone PCR screen for conditional *RTF1* mutations identified pDM3 in a selection for mutants that at 30°C were resistant to 6AU but at 37°C exhibited sensitivity to 6AU. Surprisingly, strains containing the individual point mutations were just as resistant as wild-type strains to 6AU at the elevated temperature.

5.2.2 Multicopy suppressors of an *rtf1* point mutant suggest a role for the Paf1 complex in transcription termination

The goal of the high-copy-number suppressor screen was to identify new *RTF1* interacting genes that in increased dosage suppressed the phenotypes of an *rtf1* mutant. This strategy could provide additional evidence for the previously recognized genetic and/or biochemical interactions between *RTF1* and genes involved in transcription. However, it was the possibility of identifying novel *RTF1*-interacting genes that stimulated this study. The dosage suppression screen was successful in that several candidates that suppressed the phenotypes of *rtf1-107* were identified. For example, we identified library plasmids that contained *RTF1*, genes known to reverse sensitivity to 6AU and genes that could be functionally linked to *RTF1*. In particular, the genomic insert of library plasmid 305 contained *NAB3* and could suppress the Spt⁻ phenotype of *rtf1-107*. *NAB3* subcloned into a 2-micron vector alone suppressed the Spt⁻ phenotype of all *rtf1* point mutants, but not an *rtf1Δ* strain. This result was further supported by the enhancement of the *nrd1-5* temperature sensitivity phenotype that occurred when the *nrd1-5* allele was combined with deletions of individual Paf1 complex members.

What are the functional implications for Rtf1 and the Paf1 complex in the case of dosage suppression by *NAB3*? In order for *NAB3* to be a bypass suppressor of *RTF1*, overexpression of *NAB3* in *rtf1Δ* cells must reverse the *rtf1Δ* mutant phenotypes. This is not the case because multicopy *NAB3* did not suppress the phenotypes of the *rtf1Δ* strain (Figure 9). Since *NAB3* is not a bypass suppressor of *rtf1*, it is likely Nab3 does not activate a parallel pathway required in the absence of Rtf1 or the Paf1 complex. Moreover, the combined genetic observations reported here more strongly support a model where Rtf1 and the Paf1 complex are required in order for Nab3 and Nrd1 to perform their function in the cell. Nab3 and Nrd1 participate in terminating

snRNAs and snoRNAs that are transcribed by RNA polymerase II (Carroll et al., 2004; Conrad et al., 2000; Steinmetz et al., 2001). Therefore, Rtf1 and the Paf1 complex could be involved in transcription termination of RNA polymerase II messages.

5.3 Novel genetic interactions implicate the Paf1 complex in 3'-end formation

5.3.1 Enhancement of *nrd1* phenotypes in Paf1 complex member deletion mutants

The observation that deletion of individual members of the Paf1 complex: *PAF1*, *CTR9*, *CDC73*, or *RTF1* in combination with *nrd1-5* enhanced the temperature sensitivity of the *nrd1-5* cells provided additional support for non-bypass, multicopy suppression of point mutations in *RTF1* by *NAB3* (Figure 11 and data not shown). Nrd1 and Nab3 are reported to associate by Co-IP experiments (Conrad et al., 2000). Importantly, the interaction between Nrd1 and Nab3 is functionally significant since they are required for proper transcription termination of sn/snoRNAs transcribed by RNA polymerase II (Carroll et al., 2004; Sheldon et al., 2005; Steinmetz et al., 2001). The involvement of the Paf1 complex in 3'-end formation of certain snoRNA transcripts taken together with previous observations strongly suggest a functional genetic link among members of the Paf1 complex with Nrd1 and Nab3.

While the *leo1Δ nrd1-5* double mutant was not analyzed for enhanced temperature sensitivity the *cdc73 nrd1-5* strain was tested and did exhibit synthetic enhancement of the *nrd1-5* temperature sensitive phenotype (Figure 11). The *LEO1* and *CDC73* deletion strains did not exhibit 3'-extended *SNR13* or *SNR47* transcripts, in contrast to cells with deletions in *PAF1*, *CTR9*, or *RTF1* (Figure 13 and Figure 17). Therefore, the possibility remains that *leo1 nrd1-5* cells would exhibit enhanced temperature sensitivity that is greater than the sensitivity of *nrd1-5* alone. However, one explanation for the enhanced temperature sensitivity of *cdc73Δ nrd1-5* mutant is that Cdc73, and perhaps Leo1, is responsible for the 3'-end formation of snRNAs or

snoRNAs other than *SNR13* or *SNR47*. Another explanation for the synthetic interactions between *cdc73Δ* and *nrd1-5* is that Cdc73, perhaps Leo1 too, could participate in the 3'-end formation of polyadenylated transcripts and not snRNAs or snoRNAs. Alternatively, Cdc73 and Leo1 may not have a direct role in forming 3'-ends of any transcripts. In this situation the genetic interactions between *cdc73Δ* and *nrd1-5* could be caused by compromised Paf1 complex function since deletion of *CDC73* reduces the association of the Paf1 complex with chromatin (Mueller et al., 2004).

5.3.2 Overexpression of *NAB3* causes sickness in cells deleted for certain Paf1 complex components

Further genetic analyses were performed with deletions of individual Paf1 complex members and *NAB3*. Overexpression of *NAB3* in cells lacking components of the Paf1 complex revealed different levels of synthetic phenotypes that ranged from mild to severe. *ctr9Δ* cells transformed with a high-copy-number *NAB3* plasmid grew poorly as compared to wild-type control cells or *ctr9Δ* strains with vector alone (Table 9). *paf1Δ* cells containing high-copy-number *NAB3* were too sick to analyze as compared to *paf1Δ* cells with vector alone, which is consistent with the idea that multicopy *NAB3* exacerbates the growth phenotypes of *paf1Δ* strains. In contrast, increased dosage of *NAB3* did not aggravate the growth defects of *leo1Δ*, *cdc73Δ*, or *rtf1Δ* to the level of *paf1Δ* or *ctr9Δ* cells (Table 9). However, the growth phenotypes of *leo1Δ*, *cdc73Δ*, or *rtf1Δ* cells with multicopy *NAB3* were increased as compared to isogenic strains with vector or wild-type strains with high-copy-number *NAB3* (Table 9). These results provide additional support that high-copy-number *NAB3* is not a bypass suppressor of point mutations within *RTF1*. Moreover, these observations suggest that the Paf1 complex may be functionally linked to Nab3.

The phenotypes conferred by overexpression of *NAB3* in Paf1 complex deletion mutants mirror closely the severity of phenotypes previously reported for deletion of individual Paf1 complex components. For example, the most severe phenotypes are observed in *paf1Δ* and *ctr9Δ* strains and include sensitivity to hydroxyurea and caffeine while *rtf1Δ*, *cdc73Δ*, and *leo1Δ* are resistant to these chemicals (Betz et al., 2002; Porter et al., 2002). Deletion of individual Paf1 complex members do have two phenotypes in common, sensitivity to the base analog 6AU and the Spt⁻ phenotype (Squazzo et al., 2002). Both phenotypes are associated with defects in transcription elongation, which suggests all members of the Paf1 complex are important for event(s) occurring during elongation.

5.4 In vivo and in vitro analyses support the role of the Paf1 complex in proper 3'-end formation

The genetic interactions that were observed between members of the Paf1 complex and Nrd1-Nab3 indicated these proteins may functionally interact. Nab3 and Nrd1 are required for 3'-end formation of snRNAs and snoRNAs, which are nonpolyadenylated RNA polymerase II transcripts (Steinmetz and Brow, 2003; Steinmetz et al., 2001). Therefore, the hypothesis that the Paf1 complex was required for proper 3'-end formation of snRNAs and snoRNAs was tested.

5.4.1 *SNR13* transcripts are extended in Paf1 complex mutants

Since the Paf1 complex was postulated to functionally interact with Nrd1 and Nab3 in 3'-end formation, the possibility that the Paf1 complex was involved in forming 3'-ends of certain RNA polymerase II transcripts was tested. Denaturing gel electrophoresis was performed on total RNA isolated from strains lacking individual members of the Paf1 complex and the resolved RNAs were probed with labeled *TRS31* DNA to identify 3'-extended *SNR13* transcripts (Figure 13). Consistent with the hypothesis that the Paf1 complex is important for 3'-end formation of snoRNAs, extended *SNR13* transcripts were identified in samples isolated from strains lacking *PAF1*, *CTR9*, and *RTF1* (Figure 13). Transcriptional readthrough of *SNR13* transcripts in these Paf1 complex mutants was observed, however the level of readthrough in these cells was less than readthrough levels attained in cells with mutations in *nrd1* and *nab3* (Steinmetz et al., 2001).

In a complementary approach to determine the participation of the Paf1 complex in 3'-end formation of *SNR13*, an in vivo reporter assay was performed. The ACT1-CUP1 reporter construct consists of the *TDH3* promoter driving the expression of *ACT1* where the *ACT1* intron is replaced with the sequences sufficient for 3'-end formation of *SNR13* (Steinmetz and Brow, 2003; Steinmetz et al., 2001). Downstream of the modified *ACT1* intron is the *CUP1* gene that when expressed in *cup1Δ* cells confers resistance to Cu^{2+} (Steinmetz and Brow, 2003; Steinmetz et al., 2001). When the ACT1-CUP1 plasmid is transformed into wild-type cells lacking an endogenous copy of *CUP1*, the cells will not grow in the presence of Cu^{2+} due to the sequence that directs 3'-end formation of *SNR13* within the *ACT1* intron. Alternatively, we predicted that *paf1Δ* or *ctr9Δ* cells transformed with the ACT1-CUP1 reporter construct would be resistant to Cu^{2+} since these cells would not respond normally to the termination sequences that replaced the *ACT1* intron. As was predicted, wild-type cells were sensitive to Cu^{2+} while cells lacking *PAF1* or *CTR9* exhibited increased resistance to Cu^{2+} (Figure 14). The *nrđ1-5* cells transformed with the ACT1-CUP1 reporter exhibited the greatest Cu^{2+} resistance and this result correlates well with the *TRS31* Northern analyses (Figure 13 and Figure 14), where increased Cu^{2+} resistance of *nrđ1-5* cells is in agreement with the high ratio of 3'-extended *SNR13* to normal *SNR13* transcripts. The moderate Cu^{2+} resistance of either *paf1Δ* or *ctr9Δ* cells in this assay correlates well with the intermediate levels of 3'-extended *SNR13* transcripts in these cells (Figure 13 and Figure 14).

One explanation for the greater ratio of 3'-extended *SNR13* to normal *SNR13* transcripts in *nrđ1* and *nab3* cells as compared to strains lacking individual Paf1 complex components is that Nab3 and Nrd1 play a more integral role in 3'-end formation. In support of this idea it should be noted that *NAB3* and *NRD1* are essential genes while *PAF1*, *CTR9*, and *RTF1* are not in *S.*

cerevisiae. Also consistent with this notion is that evidence implicates both Nrd1 and Nab3 in transcription termination of snRNA and snoRNA transcript (Conrad et al., 2000; Steinmetz and Brow, 1996; Steinmetz and Brow, 2003; Steinmetz et al., 2001). Alternatively, use of the TRO assay with cells lacking *PAF1* did not provide evidence to indicate that the Paf1 complex is clearly involved in transcription termination (Figure 26A-26D). Since 3'-end formation is intimately linked with transcription termination it is possible that cells with defects in transcription termination, have more severe phenotypes than cells with defects in 3'-end formation and this causes the difference in the ratios of 3'-extended *SNR13* to normal *SNR13* transcripts observed for *nab3* and *nrd1* mutants as compared to Paf1 complex mutants. While these two explanations are not necessarily mutually exclusive it is noteworthy that most genes encoding known termination factors in *S. cerevisiae* are essential while the genes encoding 3'-end formation factors can be either essential or nonessential (Cheng et al., 2004; Dheur et al., 2003).

The Paf1 complex has been classified as an elongation complex due to its association with actively transcribed genes through ChIP analyses and affinity purification experiments where RNA polymerase II is immunoprecipitated in complex with epitope-tagged versions of different Paf1 complex members (Krogan et al., 2002; Mueller and Jaehning, 2002; Pokholok et al., 2002; Squazzo et al., 2002). However, there is no evidence to indicate whether the Paf1 complex is required to improve RNA polymerase II processivity or enhance the efficiency of the enzyme (Mason and Struhl, 2005). The Paf1 complex is not the only RNA polymerase II-associated complex implicated in elongation without direct evidence in support of improved processivity or efficiency of RNA polymerase II on the template (Mason and Struhl, 2005). In fact, several RNA polymerase II-associated factors are involved in different aspects of transcription including CTD

phosphorylation, histone modification, nucleosome remodeling, nucleosome disruption and nucleosome assembly (Gilbert et al., 2004; Krogan et al., 2002; Lindstrom et al., 2003; Mueller and Jaehning, 2002; Otero et al., 1999; Pokholok et al., 2002; Squazzo et al., 2002). However, many of these elongation factors also localize to active genes by ChIP experiments and/or co-precipitate with RNA polymerase II. The hypothesis that RNA polymerase II-associated proteins and/or factors involved in chromatin structure are required for 3'-end formation of nonpolyadenylated transcripts is testable using the *TRS31* Northern as a readout for improper 3'-end formation of *SNR13*. Strains lacking many genes involved in histone modification (*SET1*, *SET2*, *DOT1*, *BRE1* and *GCN5*), chromatin remodeling (*ISW1*, *ISW2*, *SNF2*, *SWR1* and *ARG82*), transcriptional activation (*SWI4*, *SWI6*, and *MBP1*) did not exhibit 3'-extended *SNR13* transcripts in this assay (Figure 16 and data not shown). However, improper 3'-end formation of *SNR13* transcripts was observed in strains lacking *RAD6*, *INO80* and *ARP8* (Figure 16). Rad6 is an E2-ubiquitin conjugase enzyme that is required for histone H2B ubiquitylation at lysine residue 123 (Ng et al., 2002; Wood et al., 2003; Wood et al., 2003). Histone H2B ubiquitylation is a prerequisite for histone H3 trimethylation on lysine 4 that is associated with active transcription on genes transcribed by RNA polymerase II (Ng et al., 2003; Ng et al., 2003). Ino80 and Arp8 are components of the INO80 chromatin remodeling complex (Ebbert et al., 1999; Jin et al., 2005; Shen et al., 2000; Shen et al., 2003). Arp8 is an integral component of INO80 and Ino80 is a Swi/Snf-related protein that catalyzes ATP-dependent chromatin remodeling (Shen et al., 2003). Taken together, the Paf1 complex, Rad6, and components of INO80 are required for proper 3'-end formation of *SNR13*; however not all RNA polymerase II-associated proteins are involved in regulating 3'-end formation of this nonpolyadenylated transcript.

The requirement for the Paf1 complex in 3'-end formation of *SNR13* transcripts could either be direct or indirect. Since the Paf1 complex is implicated in transcription it is formally possible that the Paf1 complex is required for the transcription of a gene whose protein product is involved in 3'-end formation of *snR13* and not the Paf1 complex. Evidence in support of direct involvement of transcription factors has been obtained using inducible genes. For example, ChIP experiments were used to demonstrate that occupancy of Chd1 is greatly increased upon *GAL* gene induction (Simic et al., 2003). In order to test if the Paf1 complex is directly involved in 3'-end formation, ChIP experiments with epitope-tagged versions of Paf1 were performed (Figure 19). Initial experiments provided support for a direct role of the Paf1 complex in forming normal 3'-ends of *SNR13*. These analyses were complicated due to the close proximity of the *TRS31* genes adjacent to the *SNR13* gene. Interfering in the analysis is the possibility that epitope-tagged Paf1 may associate with both *SNR13* and *TRS31*, since both are RNA polymerase II transcribed genes. Immunoprecipitated chromatin is sonicated to an average size of 500 bp and the distance between *SNR13* and *TRS31* is just under 300 bp. Therefore, distinguishing between the occupancy signal contributed by epitope-tagged Paf1 at either gene would be nearly impossible.

5.4.2 *SNR47* transcripts readthrough into downstream *YDR042c* in cells lacking individual Paf1 complex members

The Paf1 complex was postulated to functionally interact with Nrd1 and Nab3 do to the amassed genetic observations described herein, and because of the role for the Paf1 complex in forming 3'-ends of a particular RNA polymerase II transcript (Figure 13 and Figure 14). The next hypothesis to test was whether the Paf1 complex is involved in forming proper 3'-ends of other

snoRNAs. To test this hypothesis we performed Northern analyses on total RNA isolated from individual Paf1 complex member mutants and probed filters with labeled *YDR042c* DNA (Figure 17). The *YDR042c* gene lies downstream of the snoRNA gene *SNR47* and therefore measures transcriptional readthrough of RNA polymerase II molecules from *SNR47* to *YDR042c*. Northern analysis using *YDR042c* revealed that the *YDR042c* transcript is not produced under the growth conditions used. Instead a transcript of the size estimated for transcriptional readthrough occurring at *SNR47*, *SNR47-YDR042c*, was identified in strains lacking *PAF1*, *CTR9*, *RTF1* or cells containing the *nrd1-5* or *ctk1Δ* mutations (Conrad et al., 2000). Consistent with this result Northern analyses performed with labeled *SNR47* DNA detected a transcript of the same size as that detected with the labeled *YDR042c* probe (Figure 17). Together, Northern analyses using *SNR47* or *YDR042c* revealed a chimeric transcript that is formed by transcriptional readthrough indicative of improper 3'-end formation of snR47.

To verify the 3'-extended *SNR47* transcripts in another context, in vivo reporter analyses were performed. For this experiment, a control and experimental plasmid construct were used. The control construct consisted of the *HIS3* gene driven by the *ADHI* promoter with the *CYCI* terminator downstream of *HIS3* for proper 3'-end formation and polyadenylation (Carroll et al., 2004). When this construct is transformed into *his3Δ* strains, cells grown under selection for the plasmid but in the absence of exogenous histidine will grow. The experimental construct is identical to the control plasmid but the sequences sufficient for 3'-end formation of *SNR47* are inserted between the *ADHI* promoter and the *HIS3* gene (Carroll et al., 2004). When this construct is transformed into *RTF1* cells, the transcription machinery is predicted to engage the sequences for 3'-end formation and terminate transcription prior to producing the *HIS3* transcript (Figure 18). Alternatively, when transformed into *rtf1Δ* cells, it is predicted that if Rtf1 is

involved in 3'-end formation of *SNR47*, then the transcription machinery will not engage the sequences required for 3'-end formation and the cells will grow in the absence of histidine (Figure 18). As predicted, wild-type and *rtf1* Δ cells transformed with the control construct grew on medium lacking histidine (Figure 18). Also, *rtf1* Δ but not wild-type cells grew in the absence of histidine when transformed with the experimental reporter plasmid (Figure 18). The *SNR47* termination sequence reporter assays taken together with the *SNR47* and *YDR042c* Northern analyses provides evidence that Rtf1 and other members of the Paf1 complex are required for proper 3'-end formation of snR47.

To this point, observations in support of the Paf1 complex in 3'-end formation of certain RNA polymerase II transcripts have come from two different snoRNA genes. Importantly, 3'-extended *SNR47* transcripts were identified in *paf1* Δ , *ctr9* Δ , and *rtf1* Δ cells, similar to *nrd1-5* mutant strains (Figure 17). Again, it is possible that the Paf1 complex is participating indirectly in 3'-end formation of *SNR47* transcripts. To begin to establish if the Paf1 complex directly participates in 3'-end formation of *SNR47*, ChIP analysis was performed. Therefore, a set of primer pairs was designed to amplify DNA along the *SNR47* locus and within the open reading frame of *YDR042c* to test for the occupancy of Paf1 complex members (Figure 12 and Figure 20). The *SNR47* locus differs from the *SNR13* locus in that DNA surrounding *SNR47* is less gene-dense than that of *SNR13*. Furthermore, the nearest upstream gene of *SNR47* is *NRG1* and it is 930 bp upstream of *SNR47* while *YDR042c* is 444 bp downstream of *SNR47* so the transcription of genes surrounding *SNR47* are less likely to complicate ChIP analyses. Using the 8WG16 antibody that is specific for the CTD of RNA polymerase II, DNA that was associated with RNA polymerase II was immunoprecipitated. The result of this ChIP analysis indicated that RNA polymerase II associated strongly with *SNR47* and regions downstream of *SNR47* required

for transcription termination of the snoRNA message (Carroll et al., 2004) (Figure 20). Inspection of the 5' open reading frame of *YDR042c* indicated that RNA polymerase II density was reduced in agreement with Northern assay experiments that provided evidence that *YDR042c* was not transcribed to detectable levels under the growth conditions used (Figure 20B and 20C). Moreover, ChIP assays revealed that the occupancy of Paf1 over the *SNR47* locus mirrored that of RNA polymerase II; the Paf1 signal was strongest over *SNR47* and the downstream DNA sequences required for 3'-end formation of snR47 (Figure 20B and 20C compare to 20D and 20E). Additionally, Paf1 density was greatly reduced over the 5' region of *YDR042c* as was observed for RNA polymerase II (Figure 20B-20E). The observation for epitope-tagged Paf1 can be extrapolated to the other members of the Paf1 complex, since Paf1 has been shown to be an integral member of the complex (Mueller et al., 2004). The co-localization pattern of RNA polymerase II and Paf1 at *SNR47* strongly indicates that the Paf1 complex is directly involved in the transcription and 3'-end formation of *SNR47* (Figure 20A-20E).

The Greenblatt lab demonstrated that both Nrd1 and Nab3 associated with *SNR13* by ChIP analysis (Morlando et al., 2004). Given the transcriptional context of *SNR13* relative to *TRS31* and since the 3'-extended *SNR13* and *SNR47* transcripts were identified in *paf1Δ*, *ctr9Δ*, and *rtf1Δ* as well as strains with mutations in either *NRD1* or *NAB3*, the association of these transcription termination factors along the *SNR47* locus was important to map. Using ChIP analysis, the occupancy of Nrd1 and epitope-tagged Nab3 was mapped to the *SNR47* locus (Figure 12; Figure 21A and 21B; Figure 22A and 22B). The density of Nrd1 and Nab3 overlapped with the previously determined densities of RNA polymerase II and Paf1 (Figure 20A-20E; Figure 21A and 21B; Figure 22A and 22B). The *SNR47* association data for Nrd1 and

Nab3 are also in agreement with TRO analyses where Nrd1 and Nab3 were required for proper transcription termination (Steinmetz et al., 2001). Taken together, RNA polymerase II, Paf1, Nrd1, and Nab3 associate with *SNR47* and the DNA elements associated with normal 3'-end formation of snR47 and signal intensities drop significantly over the 5' open reading frame of *YDR042c* (Figure 12; Figure 20A-20E; Figure 21A-21C; Figure 22A and 22B).

A possible explanation for the observed 3'-end formation defect of snoRNAs in Paf1 complex member mutants is that the Paf1 complex is required for the association of Nrd1 and/or Nab3 with transcribing RNA polymerase II. To test this hypothesis, chromatin isolated from *PAF1* and *paf1Δ* cells was immunoprecipitated with antibodies toward Nrd1 or epitope-tagged Nab3. Quantitative PCR across the *SNR47* locus indicated a modest decrease in Nab3 density over *SNR47* with primer pair 2 in *paf1Δ* cells as compared to *PAF1* cells (Figure 22A and 22B). Analysis of Nrd1 occupancy over *SNR47* revealed a more dramatic decrease in Nrd1 occupancy over *SNR47* at primer pair 2 in *paf1Δ* as compared to *PAF1* cells (Figure 21A and 21B). Significantly, Nrd1 and Nab3 did associate with *SNR47* DNA in *paf1Δ* cells but to lower levels as compared to signal intensity in *PAF1* cells. Together, these results indicated the Paf1 complex is required in part for the association of Nrd1 and Nab3 with RNA polymerase II at the *SNR47* locus.

Normal levels of Nab3 and Nrd1 association with the *SNR47* gene may be partially dependent on the Paf1 complex (Figure 21A and 21B; Figure 22A and 22B). However, reduction in Nrd1 and Nab3 signal observed using ChIP experiments could be the result of epitope masking within the triple HA-tagged Nab3 and Nrd1 proteins during the immunoprecipitation. Furthermore, the level of Nrd1 and Nab3 association with *SNR47* in *PAF1* chromatin extracts closely matches peak association with RNA polymerase II and Paf1 (Figure 20A-20D; Figure

21A and 21B; Figure 22A and 22B). The low signal levels of Nrd1 and Nab3 over the *SNR47* gene could be a result of reduced RNA polymerase II across *SNR47* since Nrd1 associates with RNA polymerase II via two-hybrid interaction. To rule out this possibility, ChIP experiments were performed with antibodies toward an HA-tagged version of Rbp3, a subunit of RNA polymerase II with chromatin isolated from *paf1Δ* and *PAF1* cells (Figure 21C). The results of these experiments suggested that RNA polymerase II association with *SNR47* is independent of the Paf1 complex. Another possibility regarding the lower levels of Nrd1 and Nab3 on chromatin in *paf1Δ* is that the Paf1 complex is required for normal levels of Nrd1 and Nab3 proteins. However, immunoblot analysis of extracts prepared from *PAF1* and *paf1Δ* cells revealed no difference in expression of Nrd1 or Nab3 (Figure 23A and 23B). The most likely explanation for reduced association of Nrd1 and Nab3 with *SNR47* in *paf1Δ* cells is that the Paf1 complex recruits or stabilizes association of these transcription termination factors with RNA polymerase II on snoRNA genes.

The reduced Nrd1 and or Nab3 association with RNA polymerase II at snoRNA genes in *paf1Δ* cells may be directly responsible for the 3'-end formation defect observed in the Paf1 complex mutant strains. The prediction that the Paf1 complex is the only RNA polymerase II-associated complex required for the stable association of Nrd1 and Nab3 at snoRNA genes is unlikely since Nrd1 and Nab3 are essential proteins and Paf1 complex members are not. Consistent with this argument the possibility exists that the Paf1 complex may act redundantly with other RNA polymerase II-associated proteins in stabilizing associations of Nrd1 and Nab3 with transcribing RNA polymerase II. Based on preliminary *TRS31* Northern analyses with RNA isolated from transcription-associated mutants, Spt5, Ino80 and Arp8, as well as Rad6 may

function redundantly in 3'-end formation at *SNR13* with the Paf1 complex in recruitment and stabilization of Nrd1 and Nab3 with elongating RNA polymerase II.

Several lines of evidence indicate the Paf1 complex associates with Nrd1 and Nab3 in vivo. First, Nab3 and Nrd1 co-localize to *SNR47* with RNA polymerase II and the Paf1 complex. Second, Nrd1 and Nab3 depend on a functional Paf1 complex for proper recruitment to *SNR47*. Finally, both Nrd1 and the Paf1 complex are RNA polymerase II-associated proteins (Conrad et al., 2000; Sheldon et al., 2005). Therefore, Co-IP experiments addressed the hypothesis that Nrd1 and/or Nab3 physically associate with members of the Paf1 complex in vivo. Protein extracts were prepared from strains that contained either *CTR9-Myc9 HA3-RTF1* or *CTR9-Myc9 HA3-PAF1* and immunoprecipitation studies were performed (Figure 24). While immunoprecipitation efficiency was optimal for Ctr9, Rtf1, Paf1, and Nrd1, there was no evidence of strong association between members of the Paf1 complex and Nrd1 (Figure 24). Low salt concentrations were utilized in the immunoprecipitation reactions to address the possibility of weak associations existed between Nrd1 and the Paf1 complex. The possibility remains that associations occur between Nrd1 and the Paf1 complex since they co-localize to actively transcribed region of snoRNA genes. Only the soluble protein extracts and not insoluble fraction, were tested for the Nrd1-Paf1 complex interaction so it is formally possible that Nrd1-Paf1 complex interactions occur only in the presence of DNA or RNA.

How did the observation that multicopy *NAB3* suppression of the Spt⁻ phenotype of *rtf1-107* lead to a role for the Paf1 complex in 3'-end formation of nonpolyadenylated RNA polymerase II transcripts? The genetic evidence obtained from the high-copy-number genetic screen revealed a second novel genetic interaction between deletion of individual Paf1 complex members and the Nab3 associated factor Nrd1. 3'-extended snoRNA transcripts discovered in

paf1Δ, *ctr9Δ*, and *rtf1Δ* by Northern blot analyses is a defect that occurs in *nrd1* and *nab3* mutant strains (Carroll et al., 2004; Sheldon et al., 2005; Steinmetz et al., 2001). Further, ChIP experiments indicated the Paf1 complex is necessary for the recruitment or stabilization of Nrd1 and Nab3 with transcribing RNA polymerase II. Therefore, how can we reconcile the genetic observations and co-localization to *SNR47* without the physical association of Nrd1 and Nab3 with the Paf1 complex? The explanation may require two answers that are not mutually exclusive. First, one plausible explanation is that the Nrd1-Nab3 module associates with one region of RNA polymerase II and the Paf1 complex associates with another region. In support of this idea, the primary amino acid sequence analysis of Nrd1 indicates that this protein may directly interact with the CTD of RNA polymerase II (Meinhart and Cramer, 2004). Further, the CTD is adjacent to the RNA exit channel and since Nrd1 and Nab3 each contain an RRM that specifically recognizes a different four nucleotide motif within the nascent RNA, the likelihood these factors are localized to this region of RNA polymerase II is high (Carroll et al., 2004). The largest subunit of RNA polymerase II co-purifies with individual members of the Paf1 complex, however the precise subunit or domain of RNA polymerase II required for this interaction is not known. The possibility remains that RNA polymerase II does not directly interact with individual members of the Paf1 complex rather another RNA polymerase II-associated factor is required for the co-precipitation of the Paf1 complex with RNA polymerase II. Perhaps RNA mediates the association of the Nrd1-Nab3 module with the Paf1 complex and an RNA of a particular length is required for the interaction. Once the message attains the appropriate length it facilitates the exchange of the Paf1 complex for Nrd1 and Nab3. Alternatively, the phosphorylation state of the CTD may mediate an exchange of factors associated with RNA polymerase II as first postulated by the Buratowski lab in the case of polyadenylated transcripts (Kim et al., 2004).

Second, as far as reconciling the genetic observations with the association of transcription factors to *SNR47* in the absence of physical interactions the effect of *NAB3* overexpression on individual Paf1 complex deletion mutants was quite striking. The observation that multicopy *NAB3* enhanced the growth defects of *paf1Δ* and *ctr9Δ* suggests the idea that the Paf1 complex regulates an inappropriate function of Nab3 (Table 9). The Spt⁻ phenotype of *rtf1-107* cells is more pronounced than any defect these cells may have in growth. We also observed that high-copy-number *NAB3* was not a bypass suppressor of the Spt⁻ phenotype conferred by the *rtf1* point mutants (Figure 9).

5.4.3 TRO analysis did not definitively determine whether the Paf1 complex is involved in RNA polymerase II transcript termination

Tight coupling of 3'-end formation and transcription termination exists in the production of RNA polymerase II transcripts (Bentley, 2005; Proudfoot, 2004). The observation that Paf1 complex member mutants produce 3'-extended snoRNA messages suggests that 1) the Paf1 complex is required for 3'-end formation or 2) the Paf1 complex is required for snoRNA transcription termination. Mutations of *nab3* and *nrd1* cause the production of 3'-extended snoRNA transcripts (Carroll et al., 2004; Sheldon et al., 2005; Steinmetz et al., 2001). However, this defect on its own is not enough to ascribe a role for Nrd1 and Nab3 let alone the Paf1 complex in transcription termination since termination is coupled with 3'-end formation (Bentley, 2005; Proudfoot, 2004). TRO assays where the density of RNA polymerase II molecules were compared in wild-type, *nrd1* or *nab3* cells revealed increased density of RNA polymerase II molecules beyond sequences required for proper 3'-end formation in the *nrd1* and *nab3* strains (Steinmetz et al., 2001). TRO experiments conducted in wild-type, *paf1Δ* or *nrd1-5*

cells tested the hypothesis that the Paf1 complex participates in RNA polymerase II transcription termination.

Wild-type, *paf1Δ*, and *nrd1-5* cells were transformed with the G-less cassette pG-SNR13-125-232.CYC.ds and pG-CYC.ds constructs for TRO analysis (Figure 26A-26D). The pG-CYC.ds construct contains the 262 bp G-less cassette separated from the 132 bp G-less by inert spacer sequence from the *CYC1* gene (Steinmetz and Brow, 2003). The pG-SNR13-125-232.CYC.ds is similar to the first G-less cassette construct except that the inert spacer DNA is replaced with the sequences necessary for transcription termination of the *SNR13* transcript (Steinmetz and Brow, 2003). All three strains transcribed the inert spacer sequence between the G-less cassettes of pG-CYC.ds as indicated by the two G-less cassette transcripts of 262 nt and 132 nt in length (Figure 26). Alternatively, in strains harboring the pG-SNR13-125-232.CYC.ds construct only the 262 nt G-less cassette was observed indicative of no defect in transcription termination (Figure 26A and 26B). Even *nrd1-5* cells, the positive control for this TRO experiment, did not cause an observable defect in transcription termination using this construct (Figure 26A and 26B). This result was odd since Nrd1 is involved in 3'-end formation and transcription termination of *SNR13*. In fact the Corden lab argues that Nrd1 is primarily responsible for termination at *SNR13* and not Nab3 (Carroll et al., 2004). To increase the sensitivity of this experiment, cells underwent a temperature shift from 30°C to 37°C for two hours prior to TRO analysis. Surprisingly the increased temperature did not contribute to an increase in downstream G-less cassette transcription (Figure 26A-26C). This result is also in contrast with the moderate temperature sensitivity observed for *nrd1-5* mutants (Figure 11). However, this observation came from the plasmid shuffle experiment in our strain background using a plasmid borne version of *nrd1-5* to cover the *nrd1Δ* mutation, which is lethal (Steinmetz

and Brow, 1996). The TRO experiment utilized a *nrd1-5* strain that was not isogenic to the S288c background used in our laboratory (Steinmetz and Brow, 1996). The most likely reason we did not observe transcription termination defects using the *nrd1-5* strain is because this allele was not the *nrd1* mutant used in TRO experiments (Steinmetz et al., 2001)

In another effort to determine if the Paf1 complex is involved in transcription termination, TRO assays using the sequences required for 3'-end formation of snR47 were performed (Carroll et al., 2004; Steinmetz et al., 2001). This experiment involved the subcloning of the seventy base pairs required for optimal 3'-end formation of *SNR47* into the region between the 262 nt G-less and 132 nt G-less cassettes. The resulting construct was pG-SNR47(70)-CYC.ds. pG-CYC.ds and pG-SNR47(70).CYC.ds were transformed into wild-type, *paf1Δ*, *ctr9Δ* and *nrd1-5* cells and preliminary experiments were performed (Figure 26A and 26D). Again, the 262 nt and 132 nt G-less cassettes were observed in RNaseT1 digested RNA construct that contained the inert spacer sequence (Figure 26A and 26D). Interestingly, the *nrd1-5* cells transformed with the pG-SNR47(70).CYC.ds construct exhibited the presence of both 262 nt and 132 nt G-less cassettes as predicted if Nrd1 participates in transcription termination (Figure 26A and 26D). Surprisingly, wild-type cells containing the pG-SNR47(70).CYC.ds construct also exhibited both the 262 nt and 132 nt G-less cassettes but the 132 nt band was not as strong as the 132 nt band from the *nrd1-5* cells (Figure 26D). Most unusually, the *paf1Δ* cells by virtue of this particular TRO assay exhibited the least amount of 132 nt G-less cassette (Figure 26D) and this result would be consistent with the idea that the Paf1 complex antagonizes transcription termination. While preliminary, two biological replicates were examined in the TRO experiment.

The results of the TRO analysis together with the observations from the ChIP experiment appear to be at odds. The ChIP results indicate, in the absence of the Paf1 complex, almost two

times as much RNA polymerase II signal is associated with the promoter/5' region of *YDR042c* as compared to *PAF1* cells (Figure 21C). Meanwhile the TRO experiment indicated that *paf1Δ* cells terminated transcription better than wild-type or *nrd1-5* cells using the sequences required for 3'-end formation of *SNR47* transcripts (Figure 26D). The TRO experiment performed above utilized a heterologous template, which may introduce artifacts of the system that cannot be accounted for in these experiments. The fact that the positive control for transcription termination defects using the pG-SNR13-125-232.CYC.ds construct did not result in the predicted manner may indicate that any results of the TRO experiment performed with the pG-SNR47(70).CYC.ds construct should be suspect. Alternatively, if the model that Nab3 function is in part responsible for antagonizing the task of the Paf1 complex in transcription holds, it is conceivable that absence of the Paf1 complex results in better transcription termination.

5.5 What genes are regulated by the Paf1 complex?

In one approach to identify Paf1 complex target genes, DNA microarray analysis was implemented. By analyzing the expression profiles of individual Paf1 complex member mutants, genes that are differentially regulated by different Paf1 complex components may be identified. A second complementary approach to identify genes differentially regulated by the Paf1 complex relied on genetic interactions with known transcriptional activators and elongation factors.

5.5.1 Members of the Paf1 complex genetically interact with genes that encode certain transcriptional activators

5.5.1.1 Does the Paf1 complex regulate genes in the SBF pathway?

The components of the heterodimeric transcriptional activator complex SBF were identified in a synthetic lethality screen using an *rtf1* Δ strain (Costa and Arndt, 2000). SBF regulates the transcription of genes required for the G₁/S transition of the cell cycle and is composed of Swi4 and Swi6 (Bean et al., 2005; Ho et al., 1999). Mutations in *MBP1*, which encodes the DNA-binding component of a heterodimeric transcriptional activator together with Swi6 (MBF) that is analogous to SBF, were not recovered in the synthetic lethal screen with *rtf1* Δ cells. This result was somewhat surprising because the function of SBF and MBF are somewhat redundant (Bean et al., 2005). In an effort to confirm that the mutations identified in the synthetic lethality screen were caused by recessive mutations in *swi4* and *swi6*, *SWI4* and *SWI6* were individually deleted in our strain background and crossed by *rtf1* Δ cells. Tetrad dissection and double mutant analysis

revealed that both *rtf1Δ swi4Δ* and *rtf1Δ swi6Δ* combinations resulted in lethality (Figure 28). Intriguingly, overexpression of either *SWI4* or *MBP1* in *paf1Δ* cells partially suppressed the temperature sensitivity, caffeine or hydroxyurea phenotypes (Betz et al., 2002; Porter et al., 2002). A logical explanation for this result is that SBF and members of the Paf1 complex are required for the transcription of a set of overlapping genes. In support of this idea, evidence from the Jaehning lab indicates that certain members of the Paf1 complex are required for transcription of genes expressed during the G₁/S phase of the cell cycle (Penheiter et al., 2005; Porter et al., 2002). We had previously tried to identify genes regulated by Rtf1 using Northern analysis with hybridization probes designed transcripts of genes regulated by SBF. We did not observe significant reduction of the transcripts analyzed using total RNA isolated from *rtf1Δ* strains (K. E. Sheldon and K. M. Arndt, unpublished observations). One possible reason for not observing a significant decrease in expression of SBF target genes in *rtf1Δ* cells is that *rtf1* mutant phenotypes are not as severe as *paf1Δ* or *ctr9Δ* phenotypes (Betz et al., 2002; Porter et al., 2002). The severity of the *ctr9Δ* and *paf1Δ* phenotypes could indicate that the SBF-target genes are more dependent on the function of Paf1 and Ctr9 as compared to Rtf1.

5.5.1.2 The Paf1 complex may regulate genes involved in the retrograde signaling pathway

One high-copy-number library plasmid that suppressed the Spt⁻ phenotype of *rtf1-107* contained *RTG3* (Table 8). Rtg3 was a very interesting high-copy-number suppressor candidate of the point mutation in *RTF1* because it participates in the retrograde (RTG) signaling pathway that mediates communication between the mitochondria and nucleus (Epstein et al., 2001; Sekito et al., 2000). In particular, Rtg3 forms a heterodimeric transcriptional activator in association with Rtg1 and regulates the expression genes that contain R boxes (GGTCAC) within their

upstream activating sequences (Jia et al., 1997; Rothermel et al., 1997). Trans-activation domains of Rtg3 map to the amino and carboxyl terminal regions of the protein. When these domains are fused in frame with the Gal4 DNA binding domain, are sufficient for the activation of UAS_G-*lacZ* reporter constructs in yeast (Jia et al., 1997; Rothermel et al., 1997). Moreover, Rtg2 a potential master regulator of RTG signaling that positively regulates Rtg1-Rtg3 is a component of the SAGA-like or SLIK coactivator complex, which is important for transcription at a subset of genes (Pray-Grant et al., 2002). Analysis of SAGA and the compositionally similar SLIK coactivator complex revealed that the Rtg2 component may contribute a potential role for SLIK and not SAGA in the regulation of genes in the RTG response pathway by mediating activation in trans by the Rtg1-Rtg3 heterodimer (Liu et al., 2003; Pray-Grant et al., 2002; Rothermel et al., 1997; Sekito et al., 2000). To determine if high-copy-number *RTG3* was responsible for the suppression of the Spt⁻ phenotype of the *rtf1* point mutant, *RTG3* was subcloned into a high-copy-number vector and retransformed into *rtf1* strains. Multicopy *RTG3* partially suppressed the Spt⁻ phenotype of *rtf1-107* in addition to *rtf1-105* and *rtf1*Δ cells (Figure 10). The observation that increased dosage of *RTG3* partially suppressed the Spt⁻ phenotype of the *rtf1* point mutants could be explained either in the context of chromatin structure or overlapping target genes. Like defects in members of the Paf1 complex mutation of certain components of SAGA and SLIK also confer Spt⁻ phenotypes (Winston and Sudarsanam, 1998). Therefore, the likelihood exists that increased copies of Rtg3 may compensate for any possible defects in chromatin structure attributable to mutations in *RTF1*, since mutations conferring Spt⁻ phenotypes are associated with defects in chromatin structure (Kaplan et al., 2003). Alternatively, overexpression of Rtg3 may partially reverse the Spt⁻ phenotype of mutations in *rtf1* by increasing the recruitment of additional SLIK complexes to the promoters of genes

coordinately regulated by Rtg1-Rtg3 and the Paf1 complex. Support for this explanation comes from microarray analyses where a downstream target of RTG, *ACO1*, was identified as a gene that requires Ctr9 for its regulated expression (Table 16). While *ACO1* is one example of a gene regulated by both Rtg1-Rtg3 (Epstein et al., 2001; Liu et al., 2003; Rothermel et al., 1997) and the Paf1 complex, the possibility remains that there are other genes regulated by both complexes. However, the *ACO1* gene could be a particularly interesting model to study the regulation of transcription since RTG signaling regulates its expression in response to carbon and nitrogen sources (Pierce et al., 2001) and the Paf1 complex is required for its repression (K. E. Sheldon and K. M. Arndt, unpublished observations).

5.5.2 *IMD2* requires the Paf1 complex for proper transcriptional induction in the presence of 6AU

Mutations in genes coding for transcription elongation factors exhibit defects in *IMD2* induction. The *IMD2* gene is required for de novo synthesis of purine nucleotides and *IMD2* transcription is induced in the presence of 6AU (Exinger and Lacroute, 1992). Intracellular pools of GTP and UTP are reduced in cells treated with 6AU potentially resulting in increased RNA polymerase II pausing (Exinger and Lacroute, 1992). Cells lacking transcription elongation factors are thought to be more susceptible to RNA polymerase II pausing in response to lowered levels of GTP and UTP in cells exposed to 6AU resulting in 6AU sensitivity (Exinger and Lacroute, 1992). *rpb2-10* and *dst1Δ* cells exhibited defects in inducing *IMD2* in the presence of 6AU consistent with the idea that Rpb2 and Dst1 act positively to regulate transcription elongation (Shaw and Reines, 2000). Since *RTF1* interacts with *DST1* and other genes encoding transcription elongation factors, we asked if Rtf1 and Paf1 were required for *IMD2* induction (Squazzo et al., 2002). Deletion of *RTF1* or *PAF1* resulted in impaired *IMD2* induction in the

presence of 6AU (Figure 29 and (Squazzo et al., 2002)). This observation alone was not sufficient to connect the Paf1 complex to transcription elongation, since impaired *IMD2* induction could result from a defect in transcription initiation. Impaired *IMD2* induction in *paf1Δ* and *rtf1Δ* cells taken together with genetic and biochemical data strongly suggest a role for the Paf1 complex in regulating events during transcriptional elongation.

5.5.3 Microarray analysis identifies genes regulated by Ctr9

Two color microarray analysis utilizing cDNA prepared from total RNA isolated from *CTR9* and *ctr9Δ* cells was performed to identify differentially regulated genes. Using this approach, we identified genes that are positively and negatively regulated by Ctr9. *SER3* and *ARG1* both required the Paf1 complex for their repression under noninducing conditions (Figure 30A-30D; Figure 31A-31D). Consistent with a role for the Paf1 complex in repression of *SER3* and *ARG1*, microarray analysis performed in the Jaehning lab revealed that absence of Paf1 caused an increase in *SER3* and *ARG1* expression (Penheiter et al., 2005). Mechanistically, the Paf1 complex may function differently in the repression of *SER3* and *ARG1*. The Paf1 complex may indirectly act to repress transcription from *SER3* in the presence of serine. The promoter of *SER3* harbors the *SRG1* gene that is transcribed in the presence of serine to repress transcription of *SER3* via transcriptional interference. Transcription of *SRG1* occludes transcriptional activators from binding to their cognate sites within the *SER3* promoter, thus preventing the formation of the PIC and recruitment of RNA polymerase II (Martens et al., 2004). ChIP experiments revealed the highest Paf1 complex density over the *SRG1* locus with occupancy decreasing across the *SER3* gene (Figure 31B-30D). Unlike the *SRG1-SER3* region, the occupancy of the Paf1 complex across *ARG1* remains constant (Figure 30B-30D), indicating direct association between *ARG1* and the Paf1 complex may be required for regulating

transcription of *ARG1*. To further understand the mechanism by which the Paf1 complex regulates *ARG1*, the next question to address is what happens to the *ARG1*-Paf1 complex association under inducing conditions. Decreased occupancy across *ARG1* would indicate a role for the Paf1 complex in forming repressive chromatin structure. This is a possibility since Rtf1 associates with the chromatin remodeling factor Chd1, which is implicated in regulating the transcription of certain genes both positively and negatively (Tran et al., 2000).

The transcription profiles of *paf1Δ* and *ctr9Δ* strains were analyzed by the Jaehning lab and their studies suggest a role for the Paf1 complex in regulating the transcription of genes required for cell cycle progression through the G₁/S phase of the cell cycle (Penheiter et al., 2005; Porter et al., 2002). Comparison of transcriptional profiles for each member of the Paf1 complex should address whether members of the Paf1 complex perform functions outside of the complex or if they function together to regulate the transcription of genes. Since the mutant phenotypes of the Paf1 complex do vary in severity, it is possible Paf1 and Ctr9 may be required for the transcription of genes outside of the Paf1 complex. However, immunoblotting analysis revealed deletion of individual members of the Paf1 complex caused reduction of Paf1 complex component levels (Mueller et al., 2004). Further, when individual components of the Paf1 complex are absent, association of the remaining Paf1 complex members with chromatin drops to background levels (Mueller et al., 2004).

5.6 What aspects of the Paf1 complex have been elucidated?

The goal of my research project was to further define the function of Rtf1 and the Paf1 complex in coordinating events during transcription elongation. Research from several labs indicate the Paf1 complex is important for directing specific histone modifications during elongation (Krogan et al., 2002; Krogan et al., 2003; Larabee et al., 2005; Ng et al., 2002). However our work is consistent with the Paf1 complex linking transcriptional elongation to 3'-end formation of the nascent transcript (Sheldon et al., 2005). Observations that the Paf1 complex is required for regulating the length of the poly(A) tail of polyadenylated transcripts, poly(A) site utilization, and 3'-end formation of certain snoRNAs and polyadenylated RNAs supports the idea that the Paf1 complex may couple transcription with RNA processing (Mueller et al., 2004; Penheiter et al., 2005; Sheldon et al., 2005).

6.0 Chapter 6: Future Directions

6.1 Introduction

This study defined new functions for the Paf1 complex and provides the groundwork for future investigations. Identification of Ctr9-regulated genes using microarray analysis provided an initial list of genes that require Ctr9 for transcriptional regulation. This study provided a basis to initiate comparisons between transcription profiles across the individual members of the Paf1 complex. Once the transcription profiling studies are complete, testing for the direct involvement of Paf1 complex members in regulating target genes must be addressed, since the possibility exists that only certain members of the Paf1 complex may be essential for transcriptional regulation of a subset of yeast genes. Microarray analysis used in my research consisted of cDNAs representative of the protein-coding genes in yeast. Therefore, the involvement of the Paf1 complex in forming the 3'-ends of *SNR13* and *SNR47* may not have been discovered using conventional microarrays. The high-copy-number genetic screen with *rtf1-107* cells identified many library plasmids with interesting candidates for further study. A major focus of my research came from this multicopy suppressor screen, which revealed a role for the Paf1 complex in forming 3'-ends of certain transcripts. Moreover, the possibility remains that the Paf1 complex is important for proper 3'-end formation of other snRNA or snoRNA transcripts. To add to the understanding of how the Paf1 complex participates in 3'-end formation, new lines of inquiry should be directed toward investigating the link between 3'-end formation and snoRNA

processing mediated by the exosome. This section will focus on addressing the important questions that remain regarding the function of the Paf1 complex.

6.1.1 *rtf1* point mutations as tools for investigating the function of the Paf1 complex

Three *rtf1* point mutants were isolated and characterized in this study. The resulting amino acid substitutions occurred within the central conserved region of Rtf1 (Figure 7). Moreover, the three mutants could be categorized by their phenotypes. The *rtf1-100* allele (Rtf1-Q17R) conferred a conditional Spt⁻ phenotype, while the *rtf1-105* and *rtf1-107* alleles (Rtf1-V274D and Rtf1-M289K, respectively) caused weak Spt⁻ phenotypes and conditional sensitivity to 6AU (Figure 6). In addition, immunoblotting analysis revealed that levels of Rtf1 protein were not significantly altered when expressed from the point mutations at either 30°C or 37°C (Figure 8A). Nor did these mutations cause defects in histone H3 K4 trimethylation (Figure 8B). Analysis of the *rtf1* mutant strains indicated these particular mutations did not result in complete loss-of-function of Rtf1 at 30°C (Figure 8A). However, these mutations do result in the impairment of certain Rtf1 functions since they confer *rtf1*Δ mutant phenotypes. What function of Rtf1 is it that these missense mutations alter? One possibility is that incorporation of mutant Rtf1 protein into the Paf1 complex is impaired. To address this possibility, affinity purification using *RTF1* and *rtf1* mutant strains could be used to determine if less Rtf1 protein is associated with the Paf1 complex. CHIP analyses could be implemented to determine whether the Paf1 complex remains associated with Paf1 complex-regulated genes in *RTF1* and *rtf1* cells. This study could be compared to the situation where cells lacking *RTF1* caused impaired Paf1 complex association with Paf1 complex-regulated genes (Mueller et al., 2004).

The high-copy-number genetic screen using *rtf1-107* cells revealed several interesting candidates capable of suppressing the Spt⁻ of *rtf1-107*. Performing another multicopy

suppression screen using the *rtf1-100* strain could identify similar and non-overlapping dosage suppressors capable of suppressing the conditional Spt⁻ phenotype of this strain. The candidate multicopy suppressors could provide additional insight into the possible loss of Rtf1 function that correlates with the Spt⁻ phenotype.

6.1.2 Further characterization of multicopy suppressors of *rtf1-107* cells

Investigation of the *NAB3* dosage suppressor of *rtf1-107* revealed a functional connection between an RNA polymerase II-associated elongation factor with proteins involved in transcription termination. Library plasmids containing *RTG3* and *SSD1* may also reveal novel functions of the Paf1 complex. These two candidate genes are particularly interesting because they have reported functions relevant to RNA polymerase II transcription. Rtg3 is a transcriptional activator that regulates the transcription of genes involved in the RTG pathway (Velot et al., 1996). One of these genes induced in response to signals between the nucleus and mitochondria is *ACO1*. Microarray analysis indicates that Paf1 and Ctr9 are both responsible for negatively regulating *ACO1* transcription (Table 16) (Penheiter et al., 2005). Over expression of *RTG3* partially suppresses the Spt⁻ phenotype of *rtf1* point mutants and *rtf1Δ* cells (Figure 10), indicating that *RTF1* and *RTG3* genetically interact. To begin to investigate the role of the Paf1 complex in regulating RTG-target genes, Northern analysis with hybridization probes toward *ACO1* and other RTG-target genes should be used to analyze their transcript levels in strains lacking members of the Paf1 complex and *rtg3Δ* strains. If transcript levels are altered by deletion of individual members of the Paf1 complex, then ChIP analysis should be performed to determine whether the Paf1 complex participates directly in the regulation of RTG-target genes.

A library plasmid that contained *SSD1* suppressed the Spt⁻ phenotype of *rtf1-107* cells. Evidence suggests Ssd1 is important for RNA polymerase II transcription including the

observation that Ssd1 associates with phosphorylated CTD repeats (Kizer et al., 2005), and RNA. Furthermore, increased dosage of *SSDI* suppresses mutations within *SPT16* (A, F, O'Donnell and R. A. Singer, personal communication) and genes coding for subunits of RNA polymerase I, II, and III (Stettler et al., 1993). The connection between Ssd1 and RNA polymerase II transcription indicates *SSDI* overexpression is the most likely candidate on library isolate 281 important for suppressing the Spt⁻ phenotype of *rtf1-107* cells. The first step to characterize potential genetic interactions between *SSDI* and *RTF1* would include subcloning *SSDI* into a high-copy-number vector to determine if it was capable of suppressing the Spt⁻ phenotypes of mutations within *RTF1*. If multicopy number *SSDI* is required for suppressing the Spt⁻ phenotype of *rtf1* point mutants, then future investigation should focus on the mechanism of the observed suppression. For example, does increased dosage of *SSDI* bypass the requirement for Rtf1? If *SSDI* does not bypass the requirement for *RTF1*, then it is possible that Ssd1 and Rtf1 could coordinate events occurring during transcription elongation. In this situation, the Paf1 complex may be required for the recruitment of Ssd1 to the phosphorylated CTD during elongation. ChIP experiments could be used to determine if Ssd1 colocalizes to actively transcribed genes and whether this association is dependent upon the Paf1 complex. Alternatively, high-copy-number *SSDI* could bypass the requirement for the Paf1 complex altogether. In this instance, Ssd1 may compensate for Paf1 complex function during transcript elongation or other processes that require the Paf1 complex.

6.1.3 How is the Paf1 complex loaded onto genes?

Genome-wide expression analysis provided a list of genes that require Ctr9 for proper expression. Genes that require Ctr9 for their expression or repression were identified. The Paf1 complex may be directly involved in repressing transcription from *ARG1*. Yeast cultures grown

in the absence of arginine could be washed into medium containing arginine and ChIP experiments could be used to monitor the association kinetics of factors involved in *ARG1* repression. Replication of this experiment in strains lacking members of the Paf1 complex could indicate which *ARG1* repressors require the Paf1 complex for their association. Alternatively, the *ARG1* repressors could be important for recruiting the Paf1 complex to *ARG1* for repression during growth in medium containing arginine. The Paf1 complex may also be directly involved in repressing transcription from the *ACO1* locus (Table 16). Therefore, Northern analyses should be performed to investigate the transcription of *ACO1* in strains lacking individual members of the Paf1 complex to verify the results of the *Ctr9* microarrays. Activated transcription of *ACO1* is induced in response to the RTG signaling pathway through the transcriptional activator complex Rtg1-Rtg3 (Velot et al., 1996). Transcriptional activation of *ACO1* by Rtg1-Rtg3 may be transduced through SLIK in order to initiate PIC assembly because SLIK is implicated in regulating transcription of genes activated by the RTG signaling pathway (Pray-Grant et al., 2005; Pray-Grant et al., 2002). SLIK contains the chromatin remodeling protein Chd1 and Rtg2 a protein that functions upstream of Rtg1-Rtg3 (Pray-Grant et al., 2005). Rtf1 associates with Chd1 via two-hybrid interaction (Simic et al., 2003), therefore, performing ChIP analysis in *CTR9* and *ctr9Δ* strains may reveal requirements for the Paf1 complex in regulating *ACO1* transcription.

6.1.4 What other factors require the Paf1 complex for association with *SNR47*?

The Paf1 complex and CTD Ser-2 phosphorylation are important for the association of Nrd1 with *SNR47* (Figure 25). Nrd1 association with RNA polymerase II may be mediated

through Ser-2 phosphorylation of the CTD based on the structure of Pcf11 and the similarity between the CID of Pcf11 and Nrd1 (Hollingworth et al., 2005; Meinhart and Cramer, 2004; Noble et al., 2005). Evidence from the Jaehning lab suggests that association of the Paf1 complex is required for normal levels of CTD Ser-2 phosphorylation (Mueller et al., 2004). ChIP experiments could be performed in *PAF1* and *paf1Δ* cells to determine if the Paf1 complex is required for the association of Ctk1 with *SNR47*. I anticipate that absence of Paf1 would result in reduced occupancy of Ctk1 on the *SNR47* gene since *ctk1Δ rtf1Δ* cells are lethal (Costa and Arndt, 2000). To test this possibility, ChIP experiments could be performed using an inducible *RTF1* degron in a *ctk1Δ* background to determine whether Nrd1 association with *SNR47* is reduced to background levels upon Rtf1 degradation.

6.1.5 Determine if deletion of individual Paf1 complex members result in transcription termination defects

Initial attempts to investigate the requirement for the Paf1 complex in transcription termination using TRO analysis were inconclusive. ChIP evidence indicates that the level of RNA polymerase II is increased over the 5' region of the gene downstream of *SNR47*, indicating a possible role for the Paf1 complex in transcription termination. However, the sensitivity of the TRO assays used in this study was too low to determine if there was increased RNA polymerase II density downstream of *SNR13* termination sequences in the absence of the Paf1 complex or even in a *nrd1* strain (Figure 26). To further investigate the requirement for the Paf1 complex in transcription termination we could implement another version of the TRO assay in *PAF1* or *paf1Δ* cells. This assay is similar to the one we used in the current study but differs in that

radiolabeled transcripts are hybridized to specific, immobilized DNA sequences corresponding to regions within and downstream of the *SNR* genes of interest. The benefit of this TRO method is that the investigator chooses the loci to analyze and TRO does not depend on heterologous sequences.

Results from the Jaehning lab indicated that the Paf1 complex is involved in the 3'-end formation of certain polyadenylated transcripts and that 3'-extended mRNAs produced in strains lacking Paf1 were targets for nonsense-mediated decay (Penheiter et al., 2005). These observations are consistent with a role for the Paf1 complex in linking RNA processing factors with transcription. Our work revealed the requirement for the Paf1 complex in recruiting termination factors to snoRNAs. However, due to the limit of resolution of ChIP analysis and short length of snoRNAs (approximately 100 bp) less information is known about recruitment of such factors to these genes. Analysis of recruitment of these factors to a heterologous gene containing a snoRNA promoter and snoRNA termination sequences flanking a protein coding gene could reveal a more detailed description of how these factors are recruited, what proteins are required for association, and perhaps whether the Paf1 complex is important for transcription termination.

6.1.6 Investigate interactions between the Paf1 complex and RNA metabolism

If the Paf1 complex and other RNA polymerase II-associated factors are not important for elongation rate or processivity of RNA polymerase II, then exactly what function are these proteins responsible for? Emerging evidence suggests these proteins may be involved in coupling RNA polymerase II transcription with RNA processing. In support of this idea, we have presented compelling evidence that the Paf1 complex is required for the association of the Nrd1-

Nab3 transcription termination factors with regions of *SNR47* important for transcript termination (Figure 21 and Figure 22). Is the Paf1 complex involved in coordinating the association of other factors important for RNA processing during RNA polymerase II transcription? Addressing this question requires knowledge of RNA processing events occurring during transcription and what proteins are involved in regulating them. Future studies including reconstituting 3'-end formation in vitro may be utilized to analyze factors involved in the process and to identify new factors that are important for 3'-end formation. Including different templates in reconstituted 3'-end formation could also provide information on what genes require the Paf1 complex for 3'-end formation and could provide information regarding specific sequence requirements for 3'-end formation of this transcript.

6.1.6.1 Is the Paf1 complex linked with exosome function?

A complex that associates with the open reading frames of RNA polymerase II transcribed genes is the nuclear exosome complex (Andrulis et al., 2002; Haile et al., 2003). The exosome possesses 3'-5' exonuclease activity involved in maturation of snRNA and snoRNA transcripts in addition to polyadenylated transcripts (Estevez et al., 2003; LaCava et al., 2005; Mitchell et al., 2003; Mitchell and Tollervey, 2003). The exosome and the Ski complex, another multisubunit complex with RNA helicase function, have been implicated in the degradation of cytoplasmic mRNAs (Mitchell and Tollervey, 2003; Roth et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). Moreover, subunits of the exosome have been shown to physically interact with members of the Ski complex indicating both complexes are important for regulated RNA decay. Components of the human Paf1 complex have been shown to associate in vivo with components of the Ski complex (Yart et al., 2005). However, the question remains whether the Paf1 complex is involved in linking the Ski complex to actively transcribed genes through interactions with the

exosome. CHIP experiments could be used to determine whether association of the exosome and Ski complex depends on the Paf1 complex. In this model, the exosome and the Ski complex could participate in maturation of the nascent transcript or in RNA surveillance within the nucleus during transcript elongation.

6.2 Summary

In conclusion, my research has extended the function of the Paf1 complex to a role in linking RNA processing factors to elongating RNA polymerase II. My data supports the idea that the Paf1 complex is important for recruiting transcription termination factors. This function of the Paf1 complex appears to be distinct from its role in coordinating histone modifications with actively transcribed genes. However, it remains a possibility that certain histone modifications are important for events that occur during transcript elongation since a component of the COMPASS histone methyltransferase, Swd2, is also involved in proper 3'-end formation of snoRNAs (Cheng et al., 2004; Dichtl et al., 2004). The future directions outlined above propose experiments that could provide insight into how the Paf1 complex could coordinate such events. Further downstream genetic and biochemical studies could reveal how the Paf1 complex fits within the bigger picture of co-transcriptional RNA processing events and their regulation.

BIBLIOGRAPHY

- Abbott, K. L., Archambault, J., Xiao, H., Nguyen, B. D., Roeder, R. G., Greenblatt, J., Omichinski, J. G., and Legault, P. (2005). Interactions of the HIV-1 Tat and RAP74 proteins with the RNA polymerase II CTD phosphatase FCP1. *Biochemistry* *44*, 2716-2731.
- Abbott, K. L., Renfrow, M. B., Chalmers, M. J., Nguyen, B. D., Marshall, A. G., Legault, P., and Omichinski, J. G. (2005). Enhanced binding of RNAP II CTD phosphatase FCP1 to RAP74 following CK2 phosphorylation. *Biochemistry* *44*, 2732-2745.
- Aguilera, A., and Klein, H. L. (1988). Genetic control of intrachromosomal recombination in *Saccharomyces cerevisiae*. I. Isolation and genetic characterization of hyper-recombination mutations. *Genetics* *119*, 779-790.
- Ahmad, K., and Henikoff, S. (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol. Cell* *9*, 1191-1200.
- Ahn, S. H., Kim, M., and Buratowski, S. (2004). Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. *Mol. Cell* *13*, 67-76.
- Allmang, C., Kufel, J., Chanfreau, G., Mitchell, P., Petfalski, E., and Tollervey, D. (1999). Functions of the exosome in rRNA, snoRNA and snRNA synthesis. *EMBO J.* *18*, 5399-5410.
- Allmang, C., Petfalski, E., Podtelejnikov, A., Mann, M., Tollervey, D., and Mitchell, P. (1999). The yeast exosome and human PM-Scl are related complexes of 3' → 5' exonucleases. *Genes Dev.* *13*, 2148-2158.
- Anderson, J., Phan, L., Cuesta, R., Carlson, B. A., Pak, M., Asano, K., Bjork, G. R., Tamame, M., and Hinnebusch, A. G. (1998). The essential Gcd10p-Gcd14p nuclear complex is required

for 1-methyladenosine modification and maturation of initiator methionyl-tRNA. *Genes. Dev.* *12*, 3650-3662.

Anderson, J., Phan, L., and Hinnebusch, A. G. (2000). The Gcd10p/Gcd14p complex is the essential two-subunit tRNA(1-methyladenosine) methyltransferase of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U S A* *97*, 5173-5178.

Andrau, J. C., Van Oevelen, C. J., Van Teeffelen, H. A., Weil, P. A., Holstege, F. C., and Timmers, H. T. (2002). Mot1p is essential for TBP recruitment to selected promoters during in vivo gene activation. *EMBO. J.* *21*, 5173-5183.

Andrulis, E. D., Werner, J., Nazarian, A., Erdjument-Bromage, H., Tempst, P., and Lis, J. T. (2002). The RNA processing exosome is linked to elongating RNA polymerase II in *Drosophila*. *Nature* *420*, 837-841.

Angus-Hill, M. L., Schlichter, A., Roberts, D., Erdjument-Bromage, H., Tempst, P., and Cairns, B. R. (2001). A Rsc3/Rsc30 zinc cluster dimer reveals novel roles for the chromatin remodeler RSC in gene expression and cell cycle control. *Mol. Cell.* *7*, 741-751.

Archambault, J., Chambers, R. S., Kobor, M. S., Ho, Y., Cartier, M., Bolotin, D., Andrews, B., Kane, C. M., and Greenblatt, J. (1997). An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U S A* *94*, 14300-14305.

Archambault, J., Pan, G., Dahmus, G. K., Cartier, M., Marshall, N., Zhang, S., Dahmus, M. E., and Greenblatt, J. (1998). FCP1, the RAP74-interacting subunit of a human protein phosphatase that dephosphorylates the carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.* *273*, 27593-27601.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., and Struhl, K. (1988). *Current Protocols in Molecular Biology*.

Balakrishnan, R., Christie, K. R., Costanzo, M. C., Dolinski, K., Dwight, S. S., Engel, S. R., Fisk, D. G., Hirschman, J. E., Hong, E. L., Nash, R., *et al.* *Saccharomyces Genome Database*.

Barilla, D., Lee, B. A., and Proudfoot, N. J. (2001). Cleavage/polyadenylation factor IA associates with the carboxyl-terminal domain of RNA polymerase II in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U S A* *98*, 445-450.

- Bean, J. M., Siggia, E. D., and Cross, F. R. (2005). High Functional Overlap Between MluI Cell-Cycle Box Binding Factor and Swi4/6 Cell-Cycle Box Binding Factor in the G1/S Transcriptional Program in *Saccharomyces cerevisiae*. *Genetics* *171*, 49-61.
- Bednar, J., Studitsky, V. M., Grigoryev, S. A., Felsenfeld, G., and Woodcock, C. L. (1999). The nature of the nucleosomal barrier to transcription: direct observation of paused intermediates by electron cryomicroscopy. *Mol. Cell* *4*, 377-386.
- Bednar, J., and Woodcock, C. L. (1999). Cryoelectron microscopic analysis of nucleosomes and chromatin. *Methods Enzymol.* *304*, 191-213.
- Bell, S. D., and Jackson, S. P. (2001). Mechanism and regulation of transcription in archaea. *Curr. Opin. Microbiol.* *4*, 208-213.
- Bell, S. D., Magill, C. P., and Jackson, S. P. (2001). Basal and regulated transcription in Archaea. *Biochem. Soc. Trans.* *29*, 392-395.
- Belotserkovskaya, R., Oh, S., Bondarenko, V. A., Orphanides, G., Studitsky, V. M., and Reinberg, D. (2003). FACT facilitates transcription-dependent nucleosome alteration. *Science* *301*, 1090-1093.
- Belotserkovskaya, R., Sterner, D. E., Deng, M., Sayre, M. H., Lieberman, P. M., and Berger, S. L. (2000). Inhibition of TATA-binding protein function by SAGA subunits Spt3 and Spt8 at Gcn4-activated promoters. *Mol. Cell. Biol.* *20*, 634-647.
- Bensaude, O., Bonnet, F., Casse, C., Dubois, M. F., Nguyen, V. T., and Palancade, B. (1999). Regulated phosphorylation of the RNA polymerase II C-terminal domain (CTD). *Biochem. Cell. Biol.* *77*, 249-255.
- Bentley, D. L. (2005). Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. *Curr. Opin. Cell Biol.* *17*, 251-256.
- Betz, J. L., Chang, M., Washburn, T. M., Porter, S. E., Mueller, C. L., and Jaehning, J. A. (2002). Phenotypic analysis of Paf1/RNA polymerase II complex mutations reveals connections to cell cycle regulation, protein synthesis, and lipid and nucleic acid metabolism. *Mol. Genet. Genomics* *268*, 272-285.
- Bhat, P. J., and Murthy, T. V. (2001). Transcriptional control of the GAL/MEL regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediated signal transduction. *Mol. Microbiol.* *40*, 1059-1066.

Bird, G., Zorio, D. A., and Bentley, D. L. (2004). RNA polymerase II carboxy-terminal domain phosphorylation is required for cotranscriptional pre-mRNA splicing and 3'-end formation. *Mol. Cell. Biol.* *24*, 8963-8969.

Biswas, D., Imbalzano, A. N., Eriksson, P., Yu, Y., and Stillman, D. J. (2004). Role for Nhp6, Gcn5, and the Swi/Snf complex in stimulating formation of the TATA-binding protein-TFIIA-DNA complex. *Mol. Cell. Biol.* *24*, 8312-8321.

Boeger, H., Bushnell, D. A., Davis, R., Griesenbeck, J., Lorch, Y., Strattan, J. S., Westover, K. D., and Kornberg, R. D. (2005). Structural basis of eukaryotic gene transcription. *FEBS Lett.* *579*, 899-903.

Bortvin, A., and Winston, F. (1996). Evidence that Spt6p controls chromatin structure by a direct interaction with histones. *Science* *272*, 1473-1476.

Brehm, A., Tufteland, K. R., Aasland, R., and Becker, P. B. (2004). The many colours of chromodomains. *Bioessays* *26*, 133-140.

Brewster, N. K., Johnston, G. C., and Singer, R. A. (2001). A bipartite yeast SSRP1 analog comprised of Pob3 and Nhp6 proteins modulates transcription. *Mol. Cell. Biol.* *21*, 3491-3502.

Briggs, S. D., Bryk, M., Strahl, B. D., Cheung, W. L., Davie, J. K., Dent, S. Y., Winston, F., and Allis, C. D. (2001). Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes. Dev.* *15*, 3286-3295.

Briggs, S. D., Xiao, T., Sun, Z. W., Caldwell, J. A., Shabanowitz, J., Hunt, D. F., Allis, C. D., and Strahl, B. D. (2002). Gene silencing: trans-histone regulatory pathway in chromatin. *Nature* *418*, 498.

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

Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* *56*, 549-561.

Bustin, M., Catez, F., and Lim, J. H. (2005). The dynamics of histone H1 function in chromatin. *Mol. Cell* *17*, 617-620.

Cairns, B. R., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B., and Kornberg, R. D. (1996). RSC, an essential, abundant chromatin-remodeling complex. *Cell* *87*, 1249-1260.

Cairns, B. R., Schlichter, A., Erdjument-Bromage, H., Tempst, P., Kornberg, R. D., and Winston, F. (1999). Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. *Mol. Cell* 4, 715-723.

Cam, H., and Dynlacht, B. D. (2003). Emerging roles for E2F: beyond the G1/S transition and DNA replication. *Cancer Cell* 3, 311-316.

Cang, Y., and Prelich, G. (2002). Direct stimulation of transcription by negative cofactor 2 (NC2) through TATA-binding protein (TBP). *Proc. Natl. Acad. Sci. U S A* 99, 12727-12732.

Cao, Y., Cairns, B. R., Kornberg, R. D., and Laurent, B. C. (1997). Sfh1p, a component of a novel chromatin-remodeling complex, is required for cell cycle progression. *Mol. Cell. Biol.* 17, 3323-3334.

Caplan, A. J., Cyr, D. M., and Douglas, M. G. (1993). Eukaryotic homologues of *Escherichia coli* dnaJ: a diverse protein family that functions with hsp70 stress proteins. *Mol. Biol. Cell* 4, 555-563.

Carlson, M., and Laurent, B. C. (1994). The SNF/SWI family of global transcriptional activators. *Curr. Opin. Cell Biol.* 6, 396-402.

Carroll, K. L., Pradhan, D. A., Granek, J. A., Clarke, N. D., and Corden, J. L. (2004). Identification of cis elements directing termination of yeast nonpolyadenylated snoRNA transcripts. *Mol. Cell. Biol.* 24, 6241-6252.

Caruthers, J. M., and McKay, D. B. (2002). Helicase structure and mechanism. *Curr. Opin. Struct. Biol.* 12, 123-133.

Carvin, C. D., and Kladde, M. P. (2004). Effectors of lysine 4 methylation of histone H3 in *Saccharomyces cerevisiae* are negative regulators of PHO5 and GAL1-10. *J. Biol. Chem.* 279, 33057-33062.

Chang, M., French-Cornay, D., Fan, H. Y., Klein, H., Denis, C. L., and Jaehning, J. A. (1999). A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling. *Mol. Cell. Biol.* 19, 1056-1067.

Chavez, S., and Aguilera, A. (1997). The yeast HPR1 gene has a functional role in transcriptional elongation that uncovers a novel source of genome instability. *Genes. Dev.* 11, 3459-3470.

Chavez, S., Beilharz, T., Rondon, A. G., Erdjument-Bromage, H., Tempst, P., Svejstrup, J. Q., Lithgow, T., and Aguilera, A. (2000). A protein complex containing Tho2, Hpr1, Mft1 and a

novel protein, Thp2, connects transcription elongation with mitotic recombination in *Saccharomyces cerevisiae*. *EMBO J.* *19*, 5824-5834.

Chavez, S., Garcia-Rubio, M., Prado, F., and Aguilera, A. (2001). Hpr1 is preferentially required for transcription of either long or G+C-rich DNA sequences in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *21*, 7054-7064.

Chen, B. S., and Hampsey, M. (2002). Transcription activation: unveiling the essential nature of TFIID. *Curr. Biol.* *12*, R620-622.

Cheng, H., He, X., and Moore, C. (2004). The essential WD repeat protein Swd2 has dual functions in RNA polymerase II transcription termination and lysine 4 methylation of histone H3. *Mol. Cell. Biol.* *24*, 2932-2943.

Cho, E. J., Kobor, M. S., Kim, M., Greenblatt, J., and Buratowski, S. (2001). Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. *Genes. Dev.* *15*, 3319-3329.

Cho, H., Kim, T. K., Mancebo, H., Lane, W. S., Flores, O., and Reinberg, D. (1999). A protein phosphatase functions to recycle RNA polymerase II. *Genes. Dev.* *13*, 1540-1552.

Clark-Adams, C. D., Norris, D., Osley, M. A., Fassler, J. S., and Winston, F. (1988). Changes in histone gene dosage alter transcription in yeast. *Genes. Dev.* *2*, 150-159.

Compagnone-Post, P. A., and Osley, M. A. (1996). Mutations in the SPT4, SPT5, and SPT6 genes alter transcription of a subset of histone genes in *Saccharomyces cerevisiae*. *Genetics* *143*, 1543-1554.

Conrad, N. K., Wilson, S. M., Steinmetz, E. J., Patturajan, M., Brow, D. A., Swanson, M. S., and Corden, J. L. (2000). A yeast heterogeneous nuclear ribonucleoprotein complex associated with RNA polymerase II. *Genetics* *154*, 557-571.

Corden, J., Wasylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C., and Chambon, P. (1980). Promoter sequences of eukaryotic protein-coding genes. *Science* *209*, 1406-1414.

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

Costanzo, M., Nishikawa, J. L., Tang, X., Millman, J. S., Schub, O., Breitkreuz, K., Dewar, D., Rupes, I., Andrews, B., and Tyers, M. (2004). CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast. *Cell* *117*, 899-913.

Cote, J., Quinn, J., Workman, J. L., and Peterson, C. L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science* 265, 53-60.

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

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

Dasgupta, A., Juedes, S. A., Sprouse, R. O., and Auble, D. T. (2005). Mot1-mediated control of transcription complex assembly and activity. *EMBO J.* 24, 1717-1729.

Dasgupta, A., Ramsey, K. L., Smith, J. S., and Auble, D. T. (2004). Sir Antagonist 1 (San1) is a ubiquitin ligase. *J. Biol. Chem.* 279, 26830-26838.

Davidson, I., Kobi, D., Fadloun, A., and Mengus, G. (2005). New Insights into TAFs as Regulators of Cell Cycle and Signaling Pathways. *Cell Cycle* 4.

de Bruin, R. A., McDonald, W. H., Kalashnikova, T. I., Yates, J., 3rd, and Wittenberg, C. (2004). Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5. *Cell* 117, 887-898.

Dehe, P. M., Pamblanco, M., Luciano, P., Lebrun, R., Moinier, D., Sendra, R., Verreault, A., Tordera, V., and Geli, V. (2005). Histone H3 Lysine 4 Mono-methylation does not Require Ubiquitination of Histone H2B. *J. Mol. Biol.* 353, 477-484.

Delmas, V., Stokes, D. G., and Perry, R. P. (1993). A mammalian DNA-binding protein that contains a chromodomain and an SNF2/SWI2-like helicase domain. *Proc. Natl. Acad. Sci. U S A* 90, 2414-2418.

Dheur, S., Voile, T. A., Voisinet-Hakil, F., Minet, M., Schmitter, J. M., Lacroute, F., Wyers, F., and Minvielle-Sebastia, L. (2003). Pti1p and Ref2p found in association with the mRNA 3' end formation complex direct snoRNA maturation. *EMBO J.* 22, 2831-2840.

Dichtl, B., Aasland, R., and Keller, W. (2004). Functions for *S. cerevisiae* Swd2p in 3' end formation of specific mRNAs and snoRNAs and global histone 3 lysine 4 methylation. *RNA* 10, 965-977.

Dilova, I., Aronova, S., Chen, J. C., and Powers, T. (2004). Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1.Rtg3p-dependent target genes. *J. Biol. Chem.* 279, 46527-46535.

Dilova, I., Chen, C. Y., and Powers, T. (2002). Mks1 in concert with TOR signaling negatively regulates RTG target gene expression in *S. cerevisiae*. *Curr. Biol.* *12*, 389-395.

Downs, J. A., Lowndes, N. F., and Jackson, S. P. (2000). A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature* *408*, 1001-1004.

Du, J., Nasir, I., Benton, B. K., Kladde, M. P., and Laurent, B. C. (1998). Sth1p, a *Saccharomyces cerevisiae* Snf2p/Swi2p homolog, is an essential ATPase in RSC and differs from Snf/Swi in its interactions with histones and chromatin-associated proteins. *Genetics* *150*, 987-1005.

Dudley, A. M., Rougeulle, C., and Winston, F. (1999). The Spt components of SAGA facilitate TBP binding to a promoter at a post-activator-binding step in vivo. *Genes. Dev.* *13*, 2940-2945.

Dvir, A. (2002). Promoter escape by RNA polymerase II. *Biochim. Biophys. Acta.* *1577*, 208-223.

Dye, M. J., and Proudfoot, N. J. (2001). Multiple transcript cleavage precedes polymerase release in termination by RNA polymerase II. *Cell* *105*, 669-681.

Dynlacht, B. D., Hoey, T., and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* *66*, 563-576.

Ebbert, R., Birkmann, A., and Schuller, H. J. (1999). The product of the SNF2/SWI2 paralogue INO80 of *Saccharomyces cerevisiae* required for efficient expression of various yeast structural genes is part of a high-molecular-weight protein complex. *Mol. Microbiol.* *32*, 741-751.

Edmonds, M. (2002). A history of poly A sequences: from formation to factors to function. *Prog. Nucleic Acid Res. Mol. Biol.* *71*, 285-389.

Edmondson, D. G., Smith, M. M., and Roth, S. Y. (1996). Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. *Genes. Dev.* *10*, 1247-1259.

Elfring, L. K., Deuring, R., McCallum, C. M., Peterson, C. L., and Tamkun, J. W. (1994). Identification and characterization of *Drosophila* relatives of the yeast transcriptional activator SNF2/SWI2. *Mol. Cell. Biol.* *14*, 2225-2234.

Emami, K. H., Jain, A., and Smale, S. T. (1997). Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization. *Genes. Dev.* *11*, 3007-3019.

Endoh, M., Zhu, W., Hasegawa, J., Watanabe, H., Kim, D. K., Aida, M., Inukai, N., Narita, T., Yamada, T., Furuya, A., *et al.* (2004). Human Spt6 stimulates transcription elongation by RNA polymerase II in vitro. *Mol. Cell. Biol.* *24*, 3324-3336.

Epstein, C. B., Waddle, J. A., Hale, W. t., Dave, V., Thornton, J., Macatee, T. L., Garner, H. R., and Butow, R. A. (2001). Genome-wide responses to mitochondrial dysfunction. *Mol. Biol. Cell* *12*, 297-308.

Estevez, A. M., Lehner, B., Sanderson, C. M., Ruppert, T., and Clayton, C. (2003). The roles of intersubunit interactions in exosome stability. *J. Biol. Chem.* *278*, 34943-34951.

Exinger, F., and Lacroute, F. (1992). 6-Azauracil inhibition of GTP biosynthesis in *Saccharomyces cerevisiae*. *Curr. Genet.* *22*, 9-11.

Fahrner, J. A., and Baylin, S. B. (2003). Heterochromatin: stable and unstable invasions at home and abroad. *Genes. Dev.* *17*, 1805-1812.

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

Fassler, J. S., and Winston, F. (1988). Isolation and analysis of a novel class of suppressor of Ty insertion mutations in *Saccharomyces cerevisiae*. *Genetics* *118*, 203-212.

Fatica, A., Morlando, M., and Bozzoni, I. (2000). Yeast snoRNA accumulation relies on a cleavage-dependent/polyadenylation-independent 3'-processing apparatus. *EMBO J.* *19*, 6218-6229.

Fazio, T. G., Kooperberg, C., Goldmark, J. P., Neal, C., Basom, R., Delrow, J., and Tsukiyama, T. (2001). Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol. Cell. Biol.* *21*, 6450-6460.

Feng, S. Y., Ota, K., Yamada, Y., Sawabu, N., and Ito, T. (2004). A yeast one-hybrid system to detect methylation-dependent DNA-protein interactions. *Biochem. Biophys. Res. Commun.* *313*, 922-925.

Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S. A. (2001). The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. *Mol. Cell* *7*, 981-991.

Ferdous, A., Kodadek, T., and Johnston, S. A. (2002). A nonproteolytic function of the 19S regulatory subunit of the 26S proteasome is required for efficient activated transcription by human RNA polymerase II. *Biochemistry* *41*, 12798-12805.

Fischer, T., Strasser, K., Racz, A., Rodriguez-Navarro, S., Oppizzi, M., Ihrig, P., Lechner, J., and Hurt, E. (2002). The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. *EMBO J.* *21*, 5843-5852.

Flores, O., Lu, H., and Reinberg, D. (1992). Factors involved in specific transcription by mammalian RNA polymerase II. Identification and characterization of factor IIIH. *J. Biol. Chem.* *267*, 2786-2793.

Flores, O., Maldonado, E., and Reinberg, D. (1989). Factors involved in specific transcription by mammalian RNA polymerase II. Factors IIE and IIF independently interact with RNA polymerase II. *J. Biol. Chem.* *264*, 8913-8921.

Fong, Y. W., and Zhou, Q. (2001). Stimulatory effect of splicing factors on transcriptional elongation. *Nature* *414*, 929-933.

Formosa, T., Eriksson, P., Wittmeyer, J., Ginn, J., Yu, Y., and Stillman, D. J. (2001). Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN. *EMBO J.* *20*, 3506-3517.

Formosa, T., Ruone, S., Adams, M. D., Olsen, A. E., Eriksson, P., Yu, Y., Rhoades, A. R., Kaufman, P. D., and Stillman, D. J. (2002). Defects in SPT16 or POB3 (yFACT) in *Saccharomyces cerevisiae* cause dependence on the Hir/Hpc pathway: polymerase passage may degrade chromatin structure. *Genetics* *162*, 1557-1571.

Fretzin, S., Allan, B. D., van Daal, A., and Elgin, S. C. (1991). A *Drosophila melanogaster* H3.3 cDNA encodes a histone variant identical with the vertebrate H3.3. *Gene* *107*, 341-342.

Friedl, E. M., Lane, W. S., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2003). The C-terminal domain phosphatase and transcription elongation activities of FCP1 are regulated by phosphorylation. *Proc. Natl. Acad. Sci. U S A* *100*, 2328-2333.

Fuller-Pace, F. V. (1994). RNA helicases: modulators of RNA structure. *Trends Cell Biol.* *4*, 271-274.

Gari, E., Volpe, T., Wang, H., Gallego, C., Futcher, B., and Aldea, M. (2001). Whi3 binds the mRNA of the G1 cyclin CLN3 to modulate cell fate in budding yeast. *Genes. Dev.* *15*, 2803-2808.

Gilbert, C., Kristjuhan, A., Winkler, G. S., and Svejstrup, J. Q. (2004). Elongator interactions with nascent mRNA revealed by RNA immunoprecipitation. *Mol. Cell* *14*, 457-464.

Grana, X., De Luca, A., Sang, N., Fu, Y., Claudio, P. P., Rosenblatt, J., Morgan, D. O., and Giordano, A. (1994). PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein in vitro. *Proc. Natl. Acad. Sci. U S A* *91*, 3834-3838.

Grant, P. A., Duggan, L., Cote, J., Roberts, S. M., Brownell, J. E., Candau, R., Ohba, R., Owen-Hughes, T., Allis, C. D., Winston, F., *et al.* (1997). Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes. Dev.* *11*, 1640-1650.

Gu, M., and Lima, C. D. (2005). Processing the message: structural insights into capping and decapping mRNA. *Curr. Opin. Struct. Biol.* *15*, 99-106.

Gustafsson, C. M., and Samuelsson, T. (2001). Mediator--a universal complex in transcriptional regulation. *Mol. Microbiol.* *41*, 1-8.

Ha, I., Roberts, S., Maldonado, E., Sun, X., Kim, L. U., Green, M., and Reinberg, D. (1993). Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II. *Genes. Dev.* *7*, 1021-1032.

Haber, J. E. a. G., B. (1977). A new gene affecting the efficiency of mating type interconversions in homothallic strains of *S. cerevisiae*. *Genetics* *87*, 33-50.

Haile, S., Estevez, A. M., and Clayton, C. (2003). A role for the exosome in the in vivo degradation of unstable mRNAs. *RNA* *9*, 1491-1501.

Hamiche, A., Sandaltzopoulos, R., Gdula, D. A., and Wu, C. (1999). ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF. *Cell* *97*, 833-842.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. *Microbiol. Mol. Biol. Rev.* *62*, 465-503.

Hampsey, M., and Reinberg, D. (2003). Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation. *Cell* *113*, 429-432.

Hansen, S. K., Takada, S., Jacobson, R. H., Lis, J. T., and Tjian, R. (1997). Transcription properties of a cell type-specific TATA-binding protein, TRF. *Cell* *91*, 71-83.

Hartzog, G. A., Speer, J. L., and Lindstrom, D. L. (2002). Transcript elongation on a nucleoprotein template. *Biochim. Biophys. Acta.* *1577*, 276-286.

- Hartzog, G. A., Wada, T., Handa, H., and Winston, F. (1998). Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in *Saccharomyces cerevisiae*. *Genes Dev.* *12*, 357-369.
- Haswell, E. S., and O'Shea, E. K. (1999). An in vitro system recapitulates chromatin remodeling at the PHO5 promoter. *Mol. Cell. Biol.* *19*, 2817-2827.
- He, X., Khan, A. U., Cheng, H., Pappas, D. L., Jr., Hampsey, M., and Moore, C. L. (2003). Functional interactions between the transcription and mRNA 3' end processing machineries mediated by Ssu72 and Sub1. *Genes Dev.* *17*, 1030-1042.
- He, Y., Doyle, M. R., and Amasino, R. M. (2004). PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in *Arabidopsis*. *Genes Dev.* *18*, 2774-2784.
- Hedstrom, L. (1999). IMP dehydrogenase: mechanism of action and inhibition. *Curr. Med. Chem.* *6*, 545-560.
- Henry, K. W., Wyce, A., Lo, W. S., Duggan, L. J., Emre, N. C., Kao, C. F., Pillus, L., Shilatifard, A., Osley, M. A., and Berger, S. L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev.* *17*, 2648-2663.
- Henry, Y. A., Lopez, M. C., Gibbs, J. M., Chambers, A., Kingsman, S. M., Baker, H. V., and Stanway, C. A. (1994). The yeast protein Gcr1p binds to the PGK UAS and contributes to the activation of transcription of the PGK gene. *Mol. Gen. Genet.* *245*, 506-511.
- Hinnebusch, A. G. (1985). A hierarchy of trans-acting factors modulates translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *5*, 2349-2360.
- Hinnebusch, A. G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu. Rev. Microbiol.* *59*, 407-450.
- Hirose, Y., and Manley, J. L. (1998). RNA polymerase II is an essential mRNA polyadenylation factor. *Nature* *395*, 93-96.
- Hirose, Y., Tacke, R., and Manley, J. L. (1999). Phosphorylated RNA polymerase II stimulates pre-mRNA splicing. *Genes Dev.* *13*, 1234-1239.

Hirschhorn, J. N., Brown, S. A., Clark, C. D., and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes. Dev.* *6*, 2288-2298.

Ho, Y., Costanzo, M., Moore, L., Kobayashi, R., and Andrews, B. J. (1999). Regulation of transcription at the *Saccharomyces cerevisiae* start transition by Stb1, a Swi6-binding protein. *Mol. Cell. Biol.* *19*, 5267-5278.

Hollingworth, D., Kelly, G., Frenkiel, T. A., Noble, C. G., Taylor, I. A., and Ramos, A. (2005). Backbone assignment of PCF11 CTD binding domain. *J. Biomol. NMR* *31*, 363.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* *95*, 717-728.

Holstege, F. C., Tantin, D., Carey, M., van der Vliet, P. C., and Timmers, H. T. (1995). The requirement for the basal transcription factor IIE is determined by the helical stability of promoter DNA. *EMBO J.* *14*, 810-819.

Hong, B., Brockenbrough, J. S., Wu, P., and Aris, J. P. (1997). Nop2p is required for pre-rRNA processing and 60S ribosome subunit synthesis in yeast. *Mol. Cell. Biol.* *17*, 378-388.

Howard, S. C., Hester, A., and Herman, P. K. (2003). The Ras/PKA signaling pathway may control RNA polymerase II elongation via the Spt4p/Spt5p complex in *Saccharomyces cerevisiae*. *Genetics* *165*, 1059-1070.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl. Acad. Sci. U S A* *98*, 4569-4574.

Iyer, V. R., Horak, C. E., Scafe, C. S., Botstein, D., Snyder, M., and Brown, P. O. (2001). Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature* *409*, 533-538.

Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B., and Smale, S. T. (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell. Biol.* *14*, 116-127.

Jazwinski, S. M. (2005). The retrograde response links metabolism with stress responses, chromatin-dependent gene activation, and genome stability in yeast aging. *Gene* *354*, 22-27.

Jia, Y., Rothermel, B., Thornton, J., and Butow, R. A. (1997). A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus. *Mol. Cell. Biol.* *17*, 1110-1117.

Jin, J., Cai, Y., Yao, T., Gottschalk, A. J., Florens, L., Swanson, S. K., Gutierrez, J. L., Coleman, M. K., Workman, J. L., Mushegian, A., *et al.* (2005). A mammalian chromatin remodeling complex with similarities to the yeast INO80 complex. *J. Biol. Chem.*

Jones, D. O., Cowell, I. G., and Singh, P. B. (2000). Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* *22*, 124-137.

Jones, J. C., Phatnani, H. P., Haystead, T. A., MacDonald, J. A., Alam, S. M., and Greenleaf, A. L. (2004). C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats. *J. Biol. Chem.* *279*, 24957-24964.

Jonsson, Z. O., Jha, S., Wohlschlegel, J. A., and Dutta, A. (2004). Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex. *Mol. Cell* *16*, 465-477.

Kadosh, D., and Struhl, K. (1997). Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell* *89*, 365-371.

Kadosh, D., and Struhl, K. (1998). Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. *Genes. Dev.* *12*, 797-805.

Kadosh, D., and Struhl, K. (1998). Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. *Mol. Cell. Biol.* *18*, 5121-5127.

Kaneko, S., and Manley, J. L. (2005). The Mammalian RNA Polymerase II C-Terminal Domain Interacts with RNA to Suppress Transcription-Coupled 3' End Formation. *Mol. Cell* *20*, 91-103.

Kaplan, C. D., Holland, M. J., and Winston, F. (2005). Interaction between transcription elongation factors and mRNA 3'-end formation at the *Saccharomyces cerevisiae* GAL10-GAL7 locus. *J. Biol. Chem.* *280*, 913-922.

Kaplan, C. D., Laprade, L., and Winston, F. (2003). Transcription elongation factors repress transcription initiation from cryptic sites. *Science* *301*, 1096-1099.

Kasten, M., Szerlong, H., Erdjument-Bromage, H., Tempst, P., Werner, M., and Cairns, B. R. (2004). Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. *EMBO J.* *23*, 1348-1359.

Kelleher, R. J., 3rd, Flanagan, P. M., and Kornberg, R. D. (1990). A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell* *61*, 1209-1215.

Kelley, D. E., Stokes, D. G., and Perry, R. P. (1999). CHD1 interacts with SSRP1 and depends on both its chromodomain and its ATPase/helicase-like domain for proper association with chromatin. *Chromosoma* *108*, 10-25.

Kettenberger, H., Armache, K. J., and Cramer, P. (2003). Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage. *Cell* *114*, 347-357.

Kettenberger, H., Armache, K. J., and Cramer, P. (2004). Complete RNA polymerase II elongation complex structure and its interactions with NTP and TFIIS. *Mol. Cell* *16*, 955-965.

Khavari, P. A., Peterson, C. L., Tamkun, J. W., Mendel, D. B., and Crabtree, G. R. (1993). BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature* *366*, 170-174.

Killeen, M. T., and Greenblatt, J. F. (1992). The general transcription factor RAP30 binds to RNA polymerase II and prevents it from binding nonspecifically to DNA. *Mol. Cell. Biol.* *12*, 30-37.

Kim, H. J., Jeong, S. H., Heo, J. H., Jeong, S. J., Kim, S. T., Youn, H. D., Han, J. W., Lee, H. W., and Cho, E. J. (2004). mRNA capping enzyme activity is coupled to an early transcription elongation. *Mol. Cell. Biol.* *24*, 6184-6193.

Kim, J. L., Nikolov, D. B., and Burley, S. K. (1993). Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* *365*, 520-527.

Kim, M., Ahn, S. H., Krogan, N. J., Greenblatt, J. F., and Buratowski, S. (2004). Transitions in RNA polymerase II elongation complexes at the 3' ends of genes. *EMBO J.* *23*, 354-364.

Kim, M., Krogan, N. J., Vasiljeva, L., Rando, O. J., Nedeia, E., Greenblatt, J. F., and Buratowski, S. (2004). The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. *Nature* *432*, 517-522.

Kim, T. K., Ebright, R. H., and Reinberg, D. (2000). Mechanism of ATP-dependent promoter melting by transcription factor IIIH. *Science* *288*, 1418-1422.

Kim, T. K., and Roeder, R. G. (1994). Involvement of the basic repeat domain of TATA-binding protein (TBP) in transcription by RNA polymerases I, II, and III. *J. Biol. Chem.* *269*, 4891-4894.

Kim, Y., Geiger, J. H., Hahn, S., and Sigler, P. B. (1993). Crystal structure of a yeast TBP/TATA-box complex. *Nature* *365*, 512-520.

Kim, Y. J., Bjorklund, S., Li, Y., Sayre, M. H., and Kornberg, R. D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* 77, 599-608.

Kim, Y. K., Bourgeois, C. F., Isel, C., Churcher, M. J., and Karn, J. (2002). Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. *Mol. Cell. Biol.* 22, 4622-4637.

King, M. Y., and Redman, K. L. (2002). RNA methyltransferases utilize two cysteine residues in the formation of 5-methylcytosine. *Biochemistry* 41, 11218-11225.

Kingston, R. E., and Narlikar, G. J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes. Dev.* 13, 2339-2352.

Kinyamu, H. K., Chen, J., and Archer, T. K. (2005). Linking the ubiquitin-proteasome pathway to chromatin remodeling/modification by nuclear receptors. *J. Mol. Endocrinol.* 34, 281-297.

Kizer, K. O., Phatnani, H. P., Shibata, Y., Hall, H., Greenleaf, A. L., and Strahl, B. D. (2005). A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. *Mol. Cell. Biol.* 25, 3305-3316.

Kobor, M. S., Venkatasubrahmanyam, S., Meneghini, M. D., Gin, J. W., Jennings, J. L., Link, A. J., Madhani, H. D., and Rine, J. (2004). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLoS Biol.* 2, E131.

Koch, C., Moll, T., Neuberg, M., Ahorn, H., and Nasmyth, K. (1993). A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. *Science* 261, 1551-1557.

Koch, C., Wollmann, P., Dahl, M., and Lottspeich, F. (1999). A role for Ctr9p and Paf1p in the regulation G1 cyclin expression in yeast. *Nucleic Acids Res.* 27, 2126-2134.

Komarnitsky, P., Cho, E. J., and Buratowski, S. (2000). Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. *Genes. Dev.* 14, 2452-2460.

Kong, S. E., Kobor, M. S., Krogan, N. J., Somesh, B. P., Sogaard, T. M., Greenblatt, J. F., and Svejstrup, J. Q. (2005). Interaction of Fcp1 phosphatase with elongating RNA polymerase II holoenzyme, enzymatic mechanism of action, and genetic interaction with elongator. *J. Biol. Chem.* 280, 4299-4306.

Korber, P., Luckenbach, T., Blaschke, D., and Horz, W. (2004). Evidence for histone eviction in trans upon induction of the yeast PHO5 promoter. *Mol. Cell. Biol.* 24, 10965-10974.

Kornberg, R. D., and Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* 98, 285-294.

Kornblihtt, A. R., de la Mata, M., Fededa, J. P., Munoz, M. J., and Nogues, G. (2004). Multiple links between transcription and splicing. *RNA* 10, 1489-1498.

Kosodo, Y., Imai, K., Hirata, A., Noda, Y., Takatsuki, A., Adachi, H., and Yoda, K. (2001). Multicopy suppressors of the sly1 temperature-sensitive mutation in the ER-Golgi vesicular transport in *Saccharomyces cerevisiae*. *Yeast* 18, 1003-1014.

Krishnamurthy, S., He, X., Reyes-Reyes, M., Moore, C., and Hampsey, M. (2004). Ssu72 Is an RNA polymerase II CTD phosphatase. *Mol. Cell* 14, 387-394.

Krogan, N. J., Dover, J., Khorrami, S., Greenblatt, J. F., Schneider, J., Johnston, M., and Shilatifard, A. (2002). COMPASS, a histone H3 (Lysine 4) methyltransferase required for telomeric silencing of gene expression. *J. Biol. Chem.* 277, 10753-10755.

Krogan, N. J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Ryan, O. W., Golshani, A., Johnston, M., *et al.* (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Mol. Cell* 11, 721-729.

Krogan, N. J., Keogh, M. C., Datta, N., Sawa, C., Ryan, O. W., Ding, H., Haw, R. A., Pootoolal, J., Tong, A., Canadien, V., *et al.* (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. *Mol. Cell* 12, 1565-1576.

Krogan, N. J., Kim, M., Ahn, S. H., Zhong, G., Kobor, M. S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J. F. (2002). RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. *Mol. Cell. Biol.* 22, 6979-6992.

Krogan, N. J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien, V., Richards, D. P., Beattie, B. K., Emili, A., Boone, C., *et al.* (2003). Methylation of histone H3 by Set2 in *Saccharomyces cerevisiae* is linked to transcriptional elongation by RNA polymerase II. *Mol. Cell. Biol.* 23, 4207-4218.

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

LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., and Tollervey, D. (2005). RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* *121*, 713-724.

Lagrange, T., Kapanidis, A. N., Tang, H., Reinberg, D., and Ebright, R. H. (1998). New core promoter element in RNA polymerase II-dependent transcription: sequence-specific DNA binding by transcription factor IIB. *Genes. Dev.* *12*, 34-44.

Langst, G., Bonte, E. J., Corona, D. F., and Becker, P. B. (1999). Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer. *Cell* *97*, 843-852.

Larabee, R. N., Krogan, N. J., Xiao, T., Shibata, Y., Hughes, T. R., Greenblatt, J. F., and Strahl, B. D. (2005). BUR kinase selectively regulates H3 K4 trimethylation and H2B ubiquitylation through recruitment of the PAF elongation complex. *Curr. Biol.* *15*, 1487-1493.

Lee, D. K., Horikoshi, M., and Roeder, R. G. (1991). Interaction of TFIID in the minor groove of the TATA element. *Cell* *67*, 1241-1250.

Lee, K. K., Florens, L., Swanson, S. K., Washburn, M. P., and Workman, J. L. (2005). The deubiquitylation activity of Ubp8 is dependent upon Sgf11 and its association with the SAGA complex. *Mol. Cell. Biol.* *25*, 1173-1182.

Lee, M. G., Wynder, C., Cooch, N., and Shiekhattar, R. (2005). An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. *Nature* *437*, 432-435.

Lee, T. I., Causton, H. C., Holstege, F. C., Shen, W. C., Hannett, N., Jennings, E. G., Winston, F., Green, M. R., and Young, R. A. (2000). Redundant roles for the TFIID and SAGA complexes in global transcription. *Nature* *405*, 701-704.

Lee, T. I., and Young, R. A. (2000). Transcription of eukaryotic protein-coding genes. *Annu. Rev. Genet.* *34*, 77-137.

Lei, L., Ren, D., Finkelstein, A., and Burton, Z. F. (1998). Functions of the N- and C-terminal domains of human RAP74 in transcriptional initiation, elongation, and recycling of RNA polymerase II. *Mol. Cell. Biol.* *18*, 2130-2142.

Li, J., Moazed, D., and Gygi, S. P. (2002). Association of the histone methyltransferase Set2 with RNA polymerase II plays a role in transcription elongation. *J. Biol. Chem.* *277*, 49383-49388.

Li, Y., Bjorklund, S., Jiang, Y. W., Kim, Y. J., Lane, W. S., Stillman, D. J., and Kornberg, R. D. (1995). Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex/RNA polymerase II holoenzyme. *Proc. Natl. Acad. Sci. U S A* *92*, 10864-10868.

Licatalosi, D. D., Geiger, G., Minet, M., Schroeder, S., Cilli, K., McNeil, J. B., and Bentley, D. L. (2002). Functional interaction of yeast pre-mRNA 3' end processing factors with RNA polymerase II. *Mol. Cell* *9*, 1101-1111.

Lin, P. S., Marshall, N. F., and Dahmus, M. E. (2002). CTD phosphatase: role in RNA polymerase II cycling and the regulation of transcript elongation. *Prog. Nucleic Acid Res. Mol. Biol.* *72*, 333-365.

Lin, P. S., Tremeau-Bravard, A., and Dahmus, M. E. (2003). The repetitive C-terminal domain of RNA polymerase II: multiple conformational states drive the transcription cycle. *Chem. Rec.* *3*, 235-245.

Lindstrom, D. L., and Hartzog, G. A. (2001). Genetic interactions of Spt4-Spt5 and TFIIIS with the RNA polymerase II CTD and CTD modifying enzymes in *Saccharomyces cerevisiae*. *Genetics* *159*, 487-497.

Lindstrom, D. L., Squazzo, S. L., Muster, N., Burckin, T. A., Wachter, K. C., Emigh, C. A., McCleery, J. A., Yates, J. R., 3rd, and Hartzog, G. A. (2003). Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. *Mol. Cell. Biol.* *23*, 1368-1378.

Liu, Y., Kung, C., Fishburn, J., Ansari, A. Z., Shokat, K. M., and Hahn, S. (2004). Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. *Mol. Cell. Biol.* *24*, 1721-1735.

Liu, Z., Sekito, T., Spirek, M., Thornton, J., and Butow, R. A. (2003). Retrograde signaling is regulated by the dynamic interaction between Rtg2p and Mks1p. *Mol. Cell* *12*, 401-411.

Loden, M., and van Steensel, B. (2005). Whole-genome views of chromatin structure. *Chromosome Res.* *13*, 289-298.

Logie, C., Tse, C., Hansen, J. C., and Peterson, C. L. (1999). The core histone N-terminal domains are required for multiple rounds of catalytic chromatin remodeling by the SWI/SNF and RSC complexes. *Biochemistry* *38*, 2514-2522.

- Lorenz, M. C., and Heitman, J. (1998). Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. *Genetics* *150*, 1443-1457.
- Luger, K., and Hansen, J. C. (2005). Nucleosome and chromatin fiber dynamics. *Curr. Opin. Struct. Biol.* *15*, 188-196.
- Luger, K., and Richmond, T. J. (1998). DNA binding within the nucleosome core. *Curr. Opin. Struct. Biol.* *8*, 33-40.
- Luger, K., and Richmond, T. J. (1998). The histone tails of the nucleosome. *Curr. Opin. Genet. Dev.* *8*, 140-146.
- Lundin, M., Nehlin, J. O., and Ronne, H. (1994). Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. *Mol. Cell. Biol.* *14*, 1979-1985.
- Lusser, A., and Kadonaga, J. T. (2003). Chromatin remodeling by ATP-dependent molecular machines. *Bioessays* *25*, 1192-1200.
- Luukkonen, B. G., and Seraphin, B. (1999). A conditional U5 snRNA mutation affecting pre-mRNA splicing and nuclear pre-mRNA retention identifies SSD1/SRK1 as a general splicing mutant suppressor. *Nucleic Acids Res.* *27*, 3455-3465.
- Malone, E. A., Clark, C. D., Chiang, A., and Winston, F. (1991). Mutations in SPT16/CDC68 suppress cis- and trans-acting mutations that affect promoter function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *11*, 5710-5717.
- Mandal, S. S., Cho, H., Kim, S., Cabane, K., and Reinberg, D. (2002). FCP1, a phosphatase specific for the heptapeptide repeat of the largest subunit of RNA polymerase II, stimulates transcription elongation. *Mol. Cell. Biol.* *22*, 7543-7552.
- Marshall, N. F., Dahmus, G. K., and Dahmus, M. E. (1998). Regulation of carboxyl-terminal domain phosphatase by HIV-1 tat protein. *J. Biol. Chem.* *273*, 31726-31730.
- Marshall, N. F., and Price, D. H. (1992). Control of formation of two distinct classes of RNA polymerase II elongation complexes. *Mol. Cell. Biol.* *12*, 2078-2090.
- Marshall, N. F., and Price, D. H. (1995). Purification of P-TEFb, a transcription factor required for the transition into productive elongation. *J. Biol. Chem.* *270*, 12335-12338.
- Martens, J. A., Laprade, L., and Winston, F. (2004). Intergenic transcription is required to repress the *Saccharomyces cerevisiae* SER3 gene. *Nature* *429*, 571-574.

Martens, J. A., and Winston, F. (2002). Evidence that Swi/Snf directly represses transcription in *S. cerevisiae*. *Genes. Dev.* *16*, 2231-2236.

Martens, J. A., and Winston, F. (2003). Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr. Opin. Genet. Dev.* *13*, 136-142.

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

Mason, P. B., and Struhl, K. (2005). Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. *Mol. Cell* *17*, 831-840.

Masuda, S., Das, R., Cheng, H., Hurt, E., Dorman, N., and Reed, R. (2005). Recruitment of the human TREX complex to mRNA during splicing. *Genes. Dev.* *19*, 1512-1517.

Matsui, T., Segall, J., Weil, P. A., and Roeder, R. G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. *J. Biol. Chem.* *255*, 11992-11996.

McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., and Bentley, D. L. (1997). 5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. *Genes. Dev.* *11*, 3306-3318.

Meinhart, A., and Cramer, P. (2004). Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors. *Nature* *430*, 223-226.

Meininghaus, M., Chapman, R. D., Horndasch, M., and Eick, D. (2000). Conditional expression of RNA polymerase II in mammalian cells. Deletion of the carboxyl-terminal domain of the large subunit affects early steps in transcription. *J. Biol. Chem.* *275*, 24375-24382.

Meneghini, M. D., Wu, M., and Madhani, H. D. (2003). Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell* *112*, 725-736.

Mitchell, P., Petfalski, E., Houalla, R., Podtelejnikov, A., Mann, M., and Tollervey, D. (2003). Rrp47p is an exosome-associated protein required for the 3' processing of stable RNAs. *Mol. Cell. Biol.* *23*, 6982-6992.

Mitchell, P., and Tollervey, D. (2003). An NMD pathway in yeast involving accelerated deadenylation and exosome-mediated 3'→5' degradation. *Mol. Cell* *11*, 1405-1413.

Mitsuzawa, H., Kanda, E., and Ishihama, A. (2003). Rpb7 subunit of RNA polymerase II interacts with an RNA-binding protein involved in processing of transcripts. *Nucleic Acids Res.* *31*, 4696-4701.

Mizuguchi, G., Shen, X., Landry, J., Wu, W. H., Sen, S., and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* *303*, 343-348.

Mohrmann, L., Langenberg, K., Krijgsveld, J., Kal, A. J., Heck, A. J., and Verrijzer, C. P. (2004). Differential targeting of two distinct SWI/SNF-related *Drosophila* chromatin-remodeling complexes. *Mol. Cell. Biol.* *24*, 3077-3088.

Moll, T., Schwob, E., Koch, C., Moore, A., Auer, H., and Nasmyth, K. (1993). Transcription factors important for starting the cell cycle in yeast. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* *340*, 351-360.

Morillon, A., Karabetsou, N., O'Sullivan, J., Kent, N., Proudfoot, N., and Mellor, J. (2003). Isw1 chromatin remodeling ATPase coordinates transcription elongation and termination by RNA polymerase II. *Cell* *115*, 425-435.

Morlando, M., Ballarino, M., Greco, P., Caffarelli, E., Dichtl, B., and Bozzoni, I. (2004). Coupling between snoRNP assembly and 3' processing controls box C/D snoRNA biosynthesis in yeast. *EMBO J.* *23*, 2392-2401.

Morlando, M., Greco, P., Dichtl, B., Fatica, A., Keller, W., and Bozzoni, I. (2002). Functional analysis of yeast snoRNA and snRNA 3'-end formation mediated by uncoupling of cleavage and polyadenylation. *Mol. Cell. Biol.* *22*, 1379-1389.

Muchardt, C., and Yaniv, M. (2001). When the SWI/SNF complex remodels the cell cycle. *Oncogene* *20*, 3067-3075.

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

Mueller, C. L., Porter, S. E., Hoffman, M. G., and Jaehning, J. A. (2004). The Paf1 complex has functions independent of actively transcribing RNA polymerase II. *Mol. Cell* *14*, 447-456.

Muhlrad, D., Hunter, R., and Parker, R. (1992). A rapid method for localized mutagenesis of yeast genes. *Yeast* *8*, 79-82.

Murray, S., Udupa, R., Yao, S., Hartzog, G., and Prelich, G. (2001). Phosphorylation of the RNA polymerase II carboxy-terminal domain by the Bur1 cyclin-dependent kinase. *Mol. Cell. Biol.* *21*, 4089-4096.

Myers, L. C., Gustafsson, C. M., Bushnell, D. A., Lui, M., Erdjument-Bromage, H., Tempst, P., and Kornberg, R. D. (1998). The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. *Genes. Dev.* *12*, 45-54.

Nakahigashi, K., Kubo, N., Narita, S., Shimaoka, T., Goto, S., Oshima, T., Mori, H., Maeda, M., Wada, C., and Inokuchi, H. (2002). HemK, a class of protein methyl transferase with similarity to DNA methyl transferases, methylates polypeptide chain release factors, and hemK knockout induces defects in translational termination. *Proc. Natl. Acad. Sci. U S A* *99*, 1473-1478.

Nakanishi, T., and Sekimizu, K. (2002). SDT1/SSM1, a multicopy suppressor of S-II null mutant, encodes a novel pyrimidine 5'-nucleotidase. *J. Biol. Chem.* *277*, 22103-22106.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* *292*, 110-113.

Nash, R. S., Volpe, T., and Futcher, B. (2001). Isolation and characterization of WHI3, a size-control gene of *Saccharomyces cerevisiae*. *Genetics* *157*, 1469-1480.

Nedea, E., He, X., Kim, M., Pootoolal, J., Zhong, G., Canadien, V., Hughes, T., Buratowski, S., Moore, C. L., and Greenblatt, J. (2003). Organization and function of APT, a subcomplex of the yeast cleavage and polyadenylation factor involved in the formation of mRNA and small nucleolar RNA 3'-ends. *J. Biol. Chem.* *278*, 33000-33010.

Neugeborn, L., and Carlson, M. (1984). Genes affecting the regulation of SUC2 gene expression by glucose repression in *Saccharomyces cerevisiae*. *Genetics* *108*, 845-858.

Ng, H. H., Dole, S., and Struhl, K. (2003). The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. *J. Biol. Chem.* *278*, 33625-33628.

Ng, H. H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y., and Struhl, K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. *Genes. Dev.* *16*, 1518-1527.

Ng, H. H., Robert, F., Young, R. A., and Struhl, K. (2002). Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes. Dev.* *16*, 806-819.

Ng, H. H., Robert, F., Young, R. A., and Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* *11*, 709-719.

Ng, H. H., Xu, R. M., Zhang, Y., and Struhl, K. (2002). Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. *J. Biol. Chem.* *277*, 34655-34657.

Nikolov, D. B., Chen, H., Halay, E. D., Usheva, A. A., Hisatake, K., Lee, D. K., Roeder, R. G., and Burley, S. K. (1995). Crystal structure of a TFIIB-TBP-TATA-element ternary complex. *Nature* *377*, 119-128.

Nishioka, K., Chuikov, S., Sarma, K., Erdjument-Bromage, H., Allis, C. D., Tempst, P., and Reinberg, D. (2002). Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. *Genes. Dev.* *16*, 479-489.

Noble, C. G., Hollingworth, D., Martin, S. R., Ennis-Adeniran, V., Smerdon, S. J., Kelly, G., Taylor, I. A., and Ramos, A. (2005). Key features of the interaction between Pcf11 CID and RNA polymerase II CTD. *Nat. Struct. Mol. Biol.* *12*, 144-151.

Nonet, M. L., and Young, R. A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* *123*, 715-724.

Nowak, S. J., and Corces, V. G. (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci. *Genes. Dev.* *14*, 3003-3013.

Nowak, S. J., and Corces, V. G. (2004). Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet.* *20*, 214-220.

O'Shea-Greenfield, A., and Smale, S. T. (1992). Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription. *J. Biol. Chem.* *267*, 1391-1402.

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004). A mechanism related to the yeast transcriptional regulator Paf1c is required for expression of the Arabidopsis FLC/MAF MADS box gene family. *Plant Cell* *16*, 2940-2953.

Orphanides, G., LeRoy, G., Chang, C. H., Luse, D. S., and Reinberg, D. (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell* *92*, 105-116.

Ostling, J., and Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. *Eur. J. Biochem.* 252, 162-168.

Otero, G., Fellows, J., Li, Y., de Bizemont, T., Dirac, A. M., Gustafsson, C. M., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (1999). Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. *Mol. Cell* 3, 109-118.

Ozanick, S., Krecic, A., Andersland, J., and Anderson, J. T. (2005). The bipartite structure of the tRNA m1A58 methyltransferase from *S. cerevisiae* is conserved in humans. *RNA* 11, 1281-1290.

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

Palmer, D. K., O'Day, K., Wener, M. H., Andrews, B. S., and Margolis, R. L. (1987). A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell. Biol.* 104, 805-815.

Penheiter, K. L., Washburn, T. M., Porter, S. E., Hoffman, M. G., and Jaehning, J. A. (2005). A Posttranscriptional Role for the Yeast Paf1-RNA Polymerase II Complex Is Revealed by Identification of Primary Targets. *Mol. Cell* 20, 213-223.

Peterson, C. L., and Laniel, M. A. (2004). Histones and histone modifications. *Curr. Biol.* 14, R546-551.

Pierce, M. M., Maddelein, M. L., Roberts, B. T., and Wickner, R. B. (2001). A novel Rtg2p activity regulates nitrogen catabolism in yeast. *Proc. Natl. Acad. Sci. U S A* 98, 13213-13218.

Ping, Y. H., and Rana, T. M. (2001). DSIF and NELF interact with RNA polymerase II elongation complex and HIV-1 Tat stimulates P-TEFb-mediated phosphorylation of RNA polymerase II and DSIF during transcription elongation. *J. Biol. Chem.* 276, 12951-12958.

Piruat, J. I., and Aguilera, A. (1998). A novel yeast gene, THO2, is involved in RNA pol II transcription and provides new evidence for transcriptional elongation-associated recombination. *EMBO J.* 17, 4859-4872.

Pokholok, D. K., Hannett, N. M., and Young, R. A. (2002). Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo. *Mol. Cell* 9, 799-809.

Porter, S. E., Washburn, T. M., Chang, M., and Jaehning, J. A. (2002). The yeast paf1-RNA polymerase II complex is required for full expression of a subset of cell cycle-regulated genes. *Eukaryot. Cell* 1, 830-842.

Pray-Grant, M. G., Daniel, J. A., Schieltz, D., Yates, J. R., 3rd, and Grant, P. A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature* 433, 434-438.

Pray-Grant, M. G., Schieltz, D., McMahon, S. J., Wood, J. M., Kennedy, E. L., Cook, R. G., Workman, J. L., Yates, J. R., 3rd, and Grant, P. A. (2002). The novel SLIK histone acetyltransferase complex functions in the yeast retrograde response pathway. *Mol. Cell. Biol.* 22, 8774-8786.

Prelich, G. (1997). *Saccharomyces cerevisiae* BUR6 encodes a DRAP1/NC2alpha homolog that has both positive and negative roles in transcription in vivo. *Mol. Cell. Biol.* 17, 2057-2065.

Prelich, G., and Winston, F. (1993). Mutations that suppress the deletion of an upstream activating sequence in yeast: involvement of a protein kinase and histone H3 in repressing transcription in vivo. *Genetics* 135, 665-676.

Proudfoot, N. (2004). New perspectives on connecting messenger RNA 3' end formation to transcription. *Curr. Opin. Cell Biol.* 16, 272-278.

Pugh, B. F. (2000). Control of gene expression through regulation of the TATA-binding protein. *Gene* 255, 1-14.

Qian, X., Gozani, S. N., Yoon, H., Jeon, C. J., Agarwal, K., and Weiss, M. A. (1993). Novel zinc finger motif in the basal transcriptional machinery: three-dimensional NMR studies of the nucleic acid binding domain of transcriptional elongation factor TFIIS. *Biochemistry* 32, 9944-9959.

Qiu, H., Hu, C., Yoon, S., Natarajan, K., Swanson, M. J., and Hinnebusch, A. G. (2004). An array of coactivators is required for optimal recruitment of TATA binding protein and RNA polymerase II by promoter-bound Gcn4p. *Mol. Cell. Biol.* 24, 4104-4117.

Raisner, R. M., Hartley, P. D., Meneghini, M. D., Bao, M. Z., Liu, C. L., Schreiber, S. L., Rando, O. J., and Madhani, H. D. (2005). Histone Variant H2A.Z Marks the 5' Ends of Both Active and Inactive Genes in Euchromatin. *Cell* 123, 233-248.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. *Curr. Opin. Genet. Dev.* 12, 162-169.

Reinberg, D., Horikoshi, M., and Roeder, R. G. (1987). Factors involved in specific transcription in mammalian RNA polymerase II. Functional analysis of initiation factors IIA and IID and

identification of a new factor operating at sequences downstream of the initiation site. *J. Biol. Chem.* *262*, 3322-3330.

Reinke, A., Anderson, S., McCaffery, J. M., Yates, J., 3rd, Aronova, S., Chu, S., Fairclough, S., Iverson, C., Wedaman, K. P., and Powers, T. (2004). TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in *Saccharomyces cerevisiae*. *J. Biol. Chem.* *279*, 14752-14762.

Ricci, A. R., Genereaux, J., and Brandl, C. J. (2002). Components of the SAGA histone acetyltransferase complex are required for repressed transcription of ARG1 in rich medium. *Mol. Cell. Biol.* *22*, 4033-4042.

Richardson, J. P. (2002). Rho-dependent termination and ATPases in transcript termination. *Biochim. Biophys. Acta.* *1577*, 251-260.

Riles, L., Shaw, R. J., Johnston, M., and Reines, D. (2004). Large-scale screening of yeast mutants for sensitivity to the IMP dehydrogenase inhibitor 6-azauracil. *Yeast* *21*, 241-248.

Robert, F., Douziech, M., Forget, D., Egly, J. M., Greenblatt, J., Burton, Z. F., and Coulombe, B. (1998). Wrapping of promoter DNA around the RNA polymerase II initiation complex induced by TFIIF. *Mol. Cell* *2*, 341-351.

Robert, F., Forget, D., Li, J., Greenblatt, J., and Coulombe, B. (1996). Localization of subunits of transcription factors IIE and IIF immediately upstream of the transcriptional initiation site of the adenovirus major late promoter. *J. Biol. Chem.* *271*, 8517-8520.

Rodriguez, C. R., Cho, E. J., Keogh, M. C., Moore, C. L., Greenleaf, A. L., and Buratowski, S. (2000). Kin28, the TFIIF-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II. *Mol. Cell. Biol.* *20*, 104-112.

Rondon, A. G., Gallardo, M., Garcia-Rubio, M., and Aguilera, A. (2004). Molecular evidence indicating that the yeast PAF complex is required for transcription elongation. *EMBO Rep.* *5*, 47-53.

Rondon, A. G., Garcia-Rubio, M., Gonzalez-Barrera, S., and Aguilera, A. (2003). Molecular evidence for a positive role of Spt4 in transcription elongation. *EMBO J.* *22*, 612-620.

Rondon, A. G., Jimeno, S., Garcia-Rubio, M., and Aguilera, A. (2003). Molecular evidence that the eukaryotic THO/TREX complex is required for efficient transcription elongation. *J. Biol. Chem.* *278*, 39037-39043.

Rose, M. D., Winston, F., and Hieter, P. (1990). *Methods in Yeast Genetics: a laboratory course manual*.

Roth, K. M., Wolf, M. K., Rossi, M., and Butler, J. S. (2005). The nuclear exosome contributes to autogenous control of NAB2 mRNA levels. *Mol. Cell. Biol.* *25*, 1577-1585.

Rothermel, B. A., Thornton, J. L., and Butow, R. A. (1997). Rtg3p, a basic helix-loop-helix/leucine zipper protein that functions in mitochondrial-induced changes in gene expression, contains independent activation domains. *J. Biol. Chem.* *272*, 19801-19807.

Rozenblatt-Rosen, O., Hughes, C. M., Nannepaga, S. J., Shanmugam, K. S., Copeland, T. D., Guszczynski, T., Resau, J. H., and Meyerson, M. (2005). The parafibromin tumor suppressor protein is part of a human Paf1 complex. *Mol. Cell. Biol.* *25*, 612-620.

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. *Proc. Natl. Acad. Sci. U S A* *93*, 14503-14508.

Rundlett, S. E., Carmen, A. A., Suka, N., Turner, B. M., and Grunstein, M. (1998). Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. *Nature* *392*, 831-835.

Saha, A., Wittmeyer, J., and Cairns, B. R. (2002). Chromatin remodeling by RSC involves ATP-dependent DNA translocation. *Genes. Dev.* *16*, 2120-2134.

Saha, A., Wittmeyer, J., and Cairns, B. R. (2005). Chromatin remodeling through directional DNA translocation from an internal nucleosomal site. *Nat. Struct. Mol. Biol.* *12*, 747-755.

Sandman, K., and Reeve, J. N. (2000). Structure and functional relationships of archaeal and eukaryal histones and nucleosomes. *Arch. Microbiol.* *173*, 165-169.

Santos-Rosa, H., Schneider, R., Bernstein, B. E., Karabetsou, N., Morillon, A., Weise, C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2003). Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. *Mol. Cell* *12*, 1325-1332.

Sarma, K., and Reinberg, D. (2005). Histone variants meet their match. *Nat. Rev. Mol. Cell. Biol.* *6*, 139-149.

Saunders, A., Werner, J., Andrulis, E. D., Nakayama, T., Hirose, S., Reinberg, D., and Lis, J. T. (2003). Tracking FACT and the RNA polymerase II elongation complex through chromatin in vivo. *Science* *301*, 1094-1096.

Scherer, S., and Davis, R. W. (1979). Replacement of chromosome segments with altered DNA sequences constructed in vitro. *Proc. Natl. Acad. Sci. U S A* *76*, 4951-4955.

Schlesinger, M. B., and Formosa, T. (2000). POB3 is required for both transcription and replication in the yeast *Saccharomyces cerevisiae*. *Genetics* *155*, 1593-1606.

Schlichter, A., and Cairns, B. R. (2005). Histone trimethylation by Set1 is coordinated by the RRM, autoinhibitory, and catalytic domains. *EMBO J.* *24*, 1222-1231.

Schroeder, S. C., Schwer, B., Shuman, S., and Bentley, D. (2000). Dynamic association of capping enzymes with transcribing RNA polymerase II. *Genes. Dev.* *14*, 2435-2440.

Schroeder, S. C., Zorio, D. A., Schwer, B., Shuman, S., and Bentley, D. (2004). A function of yeast mRNA cap methyltransferase, Abd1, in transcription by RNA polymerase II. *Mol. Cell* *13*, 377-387.

Schuller, H. J. (2003). Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* *43*, 139-160.

Sekito, T., Thornton, J., and Butow, R. A. (2000). Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p. *Mol. Biol. Cell* *11*, 2103-2115.

Shaw, R. J., and Reines, D. (2000). *Saccharomyces cerevisiae* transcription elongation mutants are defective in PUR5 induction in response to nucleotide depletion. *Mol. Cell. Biol.* *20*, 7427-7437.

Sheldon, K. E., Mauger, D. M., and Arndt, K. M. (2005). A Requirement for the *Saccharomyces cerevisiae* Paf1 Complex in snoRNA 3' End Formation. *Mol. Cell* *20*, 225-236.

Shen, X., Mizuguchi, G., Hamiche, A., and Wu, C. (2000). A chromatin remodelling complex involved in transcription and DNA processing. *Nature* *406*, 541-544.

Shen, X., Ranallo, R., Choi, E., and Wu, C. (2003). Involvement of actin-related proteins in ATP-dependent chromatin remodeling. *Mol. Cell* *12*, 147-155.

Shi, X., Chang, M., Wolf, A. J., Chang, C. H., Frazer-Abel, A. A., Wade, P. A., Burton, Z. F., and Jaehning, J. A. (1997). Cdc73p and Paf1p are found in a novel RNA polymerase II-containing complex distinct from the Srbp-containing holoenzyme. *Mol. Cell. Biol.* *17*, 1160-1169.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941-953.

Shi, Y. J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. *Mol. Cell* 19, 857-864.

Shimoaraiso, M., Nakanishi, T., Kubo, T., and Natori, S. (2000). Transcription elongation factor S-II confers yeast resistance to 6-azauracil by enhancing expression of the SSM1 gene. *J. Biol. Chem.* 275, 29623-29627.

Simic, R., Lindstrom, D. L., Tran, H. G., Roinick, K. L., Costa, P. J., Johnson, A. D., Hartzog, G. A., and Arndt, K. M. (2003). Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. *EMBO J.* 22, 1846-1856.

Smale, S. T., and Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell* 57, 103-113.

Smale, S. T., and Kadonaga, J. T. (2003). The RNA polymerase II core promoter. *Annu. Rev. Biochem.* 72, 449-479.

Smale, S. T., Schmidt, M. C., Berk, A. J., and Baltimore, D. (1990). Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. *Proc. Natl. Acad. Sci. U S A* 87, 4509-4513.

Smith, C. L., and Peterson, C. L. (2005). A conserved Swi2/Snf2 ATPase motif couples ATP hydrolysis to chromatin remodeling. *Mol. Cell. Biol.* 25, 5880-5892.

Spahr, H., Samuelsen, C. O., Baraznenok, V., Ernest, I., Huylebroeck, D., Remacle, J. E., Samuelsson, T., Kieselbach, T., Holmberg, S., and Gustafsson, C. M. (2001). Analysis of *Schizosaccharomyces pombe* mediator reveals a set of essential subunits conserved between yeast and metazoan cells. *Proc. Natl. Acad. Sci. U S A* 98, 11985-11990.

Squazzo, S. L., Costa, P. J., Lindstrom, D. L., Kumer, K. E., Simic, R., Jennings, J. L., Link, A. J., Arndt, K. M., and Hartzog, G. A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. *EMBO J.* 21, 1764-1774.

Steger, D. J., Haswell, E. S., Miller, A. L., Wenthe, S. R., and O'Shea, E. K. (2003). Regulation of chromatin remodeling by inositol polyphosphates. *Science* 299, 114-116.

Steinmetz, E. J., and Brow, D. A. (1996). Repression of gene expression by an exogenous sequence element acting in concert with a heterogeneous nuclear ribonucleoprotein-like protein, Nrd1, and the putative helicase Sen1. *Mol. Cell. Biol.* *16*, 6993-7003.

Steinmetz, E. J., and Brow, D. A. (1998). Control of pre-mRNA accumulation by the essential yeast protein Nrd1 requires high-affinity transcript binding and a domain implicated in RNA polymerase II association. *Proc. Natl. Acad. Sci. U S A* *95*, 6699-6704.

Steinmetz, E. J., and Brow, D. A. (2003). Ssu72 protein mediates both poly(A)-coupled and poly(A)-independent termination of RNA polymerase II transcription. *Mol. Cell. Biol.* *23*, 6339-6349.

Steinmetz, E. J., Conrad, N. K., Brow, D. A., and Corden, J. L. (2001). RNA-binding protein Nrd1 directs poly(A)-independent 3'-end formation of RNA polymerase II transcripts. *Nature* *413*, 327-331.

Stern, M., Jensen, R., and Herskowitz, I. (1984). Five SWI genes are required for expression of the HO gene in yeast. *J. Mol. Biol.* *178*, 853-868.

Sterner, D. E., Belotserkovskaya, R., and Berger, S. L. (2002). SALSA, a variant of yeast SAGA, contains truncated Spt7, which correlates with activated transcription. *Proc. Natl. Acad. Sci. U S A* *99*, 11622-11627.

Sterner, D. E., Lee, J. M., Hardin, S. E., and Greenleaf, A. L. (1995). The yeast carboxyl-terminal repeat domain kinase CTDK-I is a divergent cyclin-cyclin-dependent kinase complex. *Mol. Cell. Biol.* *15*, 5716-5724.

Stettler, S., Chiannikulchai, N., Hermann-Le Denmat, S., Lalo, D., Lacroute, F., Sentenac, A., and Thuriaux, P. (1993). A general suppressor of RNA polymerase I, II and III mutations in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* *239*, 169-176.

Stewart, M. D., Li, J., and Wong, J. (2005). Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol. Cell. Biol.* *25*, 2525-2538.

Stokes, D. G., and Perry, R. P. (1995). DNA-binding and chromatin localization properties of CHD1. *Mol. Cell. Biol.* *15*, 2745-2753.

Stokes, D. G., Tartof, K. D., and Perry, R. P. (1996). CHD1 is concentrated in interbands and puffed regions of *Drosophila* polytene chromosomes. *Proc. Natl. Acad. Sci. U S A* *93*, 7137-7142.

Stolinski, L. A., Eisenmann, D. M., and Arndt, K. M. (1997). Identification of RTF1, a novel gene important for TATA site selection by TATA box-binding protein in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *17*, 4490-4500.

Strahl, B. D., Grant, P. A., Briggs, S. D., Sun, Z. W., Bone, J. R., Caldwell, J. A., Mollah, S., Cook, R. G., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2002). Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. *Mol. Cell. Biol.* *22*, 1298-1306.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A. G., Aguilera, A., Struhl, K., Reed, R., and Hurt, E. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* *417*, 304-308.

Strawn, L. A., Shen, T., and Wentz, S. R. (2001). The GLFG regions of Nup116p and Nup100p serve as binding sites for both Kap95p and Mex67p at the nuclear pore complex. *J. Biol. Chem.* *276*, 6445-6452.

Struhl, K. (1989). Molecular mechanisms of transcriptional regulation in yeast. *Annu. Rev. Biochem.* *58*, 1051-1077.

Studitsky, V. M., Clark, D. J., and Felsenfeld, G. (1994). A histone octamer can step around a transcribing polymerase without leaving the template. *Cell* *76*, 371-382.

Studitsky, V. M., Clark, D. J., and Felsenfeld, G. (1995). Overcoming a nucleosomal barrier to transcription. *Cell* *83*, 19-27.

Studitsky, V. M., Walter, W., Kireeva, M., Kashlev, M., and Felsenfeld, G. (2004). Chromatin remodeling by RNA polymerases. *Trends Biochem. Sci.* *29*, 127-135.

Sudarsanam, P., Iyer, V. R., Brown, P. O., and Winston, F. (2000). Whole-genome expression analysis of *snf/swi* mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U S A* *97*, 3364-3369.

Swanson, M. S., Carlson, M., and Winston, F. (1990). SPT6, an essential gene that affects transcription in *Saccharomyces cerevisiae*, encodes a nuclear protein with an extremely acidic amino terminus. *Mol. Cell. Biol.* *10*, 4935-4941.

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

Swanson, M. S., and Winston, F. (1992). SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in *Saccharomyces cerevisiae*. *Genetics* *132*, 325-336.

Tan, Q., Prysak, M. H., and Woychik, N. A. (2003). Loss of the Rpb4/Rpb7 subcomplex in a mutant form of the Rpb6 subunit shared by RNA polymerases I, II, and III. *Mol. Cell. Biol.* *23*, 3329-3338.

Tenney, K., and Shilatifard, A. (2005). A COMPASS in the voyage of defining the role of trithorax/MLL-containing complexes: linking leukemogenesis to covalent modifications of chromatin. *J Cell Biochem* *95*, 429-436.

Tran, H. G., Steger, D. J., Iyer, V. R., and Johnson, A. D. (2000). The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. *EMBO J.* *19*, 2323-2331.

Trievel, R. C., Beach, B. M., Dirk, L. M., Houtz, R. L., and Hurley, J. H. (2002). Structure and catalytic mechanism of a SET domain protein methyltransferase. *Cell* *111*, 91-103.

Trievel, R. C., Flynn, E. M., Houtz, R. L., and Hurley, J. H. (2003). Mechanism of multiple lysine methylation by the SET domain enzyme Rubisco LSM1. *Nat Struct Biol* *10*, 545-552.

Tse, C., Georgieva, E. I., Ruiz-Garcia, A. B., Sendra, R., and Hansen, J. C. (1998). Gcn5p, a transcription-related histone acetyltransferase, acetylates nucleosomes and folded nucleosomal arrays in the absence of other protein subunits. *J. Biol. Chem.* *273*, 32388-32392.

Tsukiyama, T., Daniel, C., Tamkun, J., and Wu, C. (1995). ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell* *83*, 1021-1026.

Tsukiyama, T., Palmer, J., Landel, C. C., Shiloach, J., and Wu, C. (1999). Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in *Saccharomyces cerevisiae*. *Genes. Dev.* *13*, 686-697.

Turkel, S., and Bisson, L. F. (1999). Transcription of the HXT4 gene is regulated by Gcr1p and Gcr2p in the yeast *S. cerevisiae*. *Yeast* *15*, 1045-1057.

Turner, S. D., Ricci, A. R., Petropoulos, H., Genereaux, J., Skerjanc, I. S., and Brandl, C. J. (2002). The E2 ubiquitin conjugase Rad6 is required for the ArgR/Mcm1 repression of ARG1 transcription. *Mol. Cell. Biol.* *22*, 4011-4019.

Uemura, H., Koshio, M., Inoue, Y., Lopez, M. C., and Baker, H. V. (1997). The role of Gcr1p in the transcriptional activation of glycolytic genes in yeast *Saccharomyces cerevisiae*. *Genetics* *147*, 521-532.

Uemura, T., Tachihara, K., Tomitori, H., Kashiwagi, K., and Igarashi, K. (2005). Characteristics of the polyamine transporter TPO1 and regulation of its activity and cellular localization by phosphorylation. *J. Biol. Chem.* *280*, 9646-9652.

Ulrich, H. D. (2002). Degradation or maintenance: actions of the ubiquitin system on eukaryotic chromatin. *Eukaryot. Cell* *1*, 1-10.

Valay, J. G., Simon, M., and Faye, G. (1993). The kin28 protein kinase is associated with a cyclin in *Saccharomyces cerevisiae*. *J. Mol. Biol.* *234*, 307-310.

van Hoof, A., Lennertz, P., and Parker, R. (2000). Yeast exosome mutants accumulate 3'-extended polyadenylated forms of U4 small nuclear RNA and small nucleolar RNAs. *Mol. Cell. Biol.* *20*, 441-452.

Van Hooser, A. A., Ouspenski, II, Gregson, H. C., Starr, D. A., Yen, T. J., Goldberg, M. L., Yokomori, K., Earnshaw, W. C., Sullivan, K. F., and Brinkley, B. R. (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J. Cell Sci.* *114*, 3529-3542.

Vanacova, S., Wolf, J., Martin, G., Blank, D., Dettwiler, S., Friedlein, A., Langen, H., Keith, G., and Keller, W. (2005). A new yeast poly(A) polymerase complex involved in RNA quality control. *PLoS Biol.* *3*, e189.

Vary, J. C., Jr., Gangaraju, V. K., Qin, J., Landel, C. C., Kooperberg, C., Bartholomew, B., and Tsukiyama, T. (2003). Yeast Isw1p forms two separable complexes in vivo. *Mol. Cell. Biol.* *23*, 80-91.

Velot, C., Haviernik, P., and Lauquin, G. J. (1996). The *Saccharomyces cerevisiae* RTG2 gene is a regulator of aconitase expression under catabolite repression conditions. *Genetics* *144*, 893-903.

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

Wada, T., Takagi, T., Yamaguchi, Y., Ferdous, A., Imai, T., Hirose, S., Sugimoto, S., Yano, K., Hartzog, G. A., Winston, F., *et al.* (1998). DSIF, a novel transcription elongation factor that

regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. *Genes. Dev.* *12*, 343-356.

Wada, T., Takagi, T., Yamaguchi, Y., Watanabe, D., and Handa, H. (1998). Evidence that P-TEFb alleviates the negative effect of DSIF on RNA polymerase II-dependent transcription in vitro. *EMBO J.* *17*, 7395-7403.

Wade, P. A., Werel, W., Fentzke, R. C., Thompson, N. E., Leykam, J. F., Burgess, R. R., Jaehning, J. A., and Burton, Z. F. (1996). A novel collection of accessory factors associated with yeast RNA polymerase II. *Protein Expr. Purif.* *8*, 85-90.

Wang, G., Balamotis, M. A., Stevens, J. L., Yamaguchi, Y., Handa, H., and Berk, A. J. (2005). Mediator requirement for both recruitment and postrecruitment steps in transcription initiation. *Mol. Cell* *17*, 683-694.

Wang, H., Gari, E., Verges, E., Gallego, C., and Aldea, M. (2004). Recruitment of Cdc28 by Whi3 restricts nuclear accumulation of the G1 cyclin-Cdk complex to late G1. *EMBO J.* *23*, 180-190.

Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M., *et al.* (1996). Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *EMBO J.* *15*, 5370-5382.

Watson, J., Matsui, G. Y., Leaphart, A., Wiegel, J., Rainey, F. A., and Lovell, C. R. (2000). Reductively debrominating strains of *Propionigenium maris* from burrows of bromophenol-producing marine infauna. *Int. J. Syst. Evol. Microbiol.* *50 Pt 3*, 1035-1042.

West, S., Gromak, N., and Proudfoot, N. J. (2004). Human 5' → 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. *Nature* *432*, 522-525.

Wind, M., and Reines, D. (2000). Transcription elongation factor SII. *Bioessays* *22*, 327-336.

Winkler, G. S., Kristjuhan, A., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (2002). Elongator is a histone H3 and H4 acetyltransferase important for normal histone acetylation levels in vivo. *Proc. Natl. Acad. Sci. U S A* *99*, 3517-3522.

Winston, F. (1992). Analysis of SPT genes: A Genetic Approach toward Analysis of TFIID, Histones, and Other Transcription Factors of Yeast. *Transcriptional Regulation*, 1271-1293.

Winston, F., Chaleff, D. T., Valent, B., and Fink, G. R. (1984). Mutations affecting Ty-mediated expression of the HIS4 gene of *Saccharomyces cerevisiae*. *Genetics* *107*, 179-197.

Winston, F., Dollard, C., and Ricupero-Hovasse, S. L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* 11, 53-55.

Winston, F., and Sudarsanam, P. (1998). The SAGA of Spt proteins and transcriptional analysis in yeast: past, present, and future. *Cold Spring Harb. Symp. Quant. Biol.* 63, 553-561.

Wittenberg, C., and Reed, S. I. (2005). Cell cycle-dependent transcription in yeast: promoters, transcription factors, and transcriptomes. *Oncogene* 24, 2746-2755.

Wittmeyer, J., and Formosa, T. (1997). The *Saccharomyces cerevisiae* DNA polymerase alpha catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein. *Mol. Cell. Biol.* 17, 4178-4190.

Wittmeyer, J., Joss, L., and Formosa, T. (1999). Spt16 and Pob3 of *Saccharomyces cerevisiae* form an essential, abundant heterodimer that is nuclear, chromatin-associated, and copurifies with DNA polymerase alpha. *Biochemistry* 38, 8961-8971.

Wittschieben, B. O., Fellows, J., Du, W., Stillman, D. J., and Svejstrup, J. Q. (2000). Overlapping roles for the histone acetyltransferase activities of SAGA and elongator in vivo. *EMBO J.* 19, 3060-3068.

Wittschieben, B. O., Otero, G., de Bizemont, T., Fellows, J., Erdjument-Bromage, H., Ohba, R., Li, Y., Allis, C. D., Tempst, P., and Svejstrup, J. Q. (1999). A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. *Mol. Cell* 4, 123-128.

Wood, A., Krogan, N. J., Dover, J., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Golshani, A., Zhang, Y., Greenblatt, J. F., *et al.* (2003). Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. *Mol. Cell* 11, 267-274.

Wood, A., Schneider, J., Dover, J., Johnston, M., and Shilatifard, A. (2003). The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. *J. Biol. Chem.* 278, 34739-34742.

Woodard, G. E., Lin, L., Zhang, J. H., Agarwal, S. K., Marx, S. J., and Simonds, W. F. (2005). Parafibromin, product of the hyperparathyroidism-jaw tumor syndrome gene HRPT2, regulates cyclin D1/PRAD1 expression. *Oncogene* 24, 1272-1276.

Woychik, N. A., Liao, S. M., Kolodziej, P. A., and Young, R. A. (1990). Subunits shared by eukaryotic nuclear RNA polymerases. *Genes. Dev.* 4, 313-323.

Wu, J., and Grunstein, M. (2000). 25 years after the nucleosome model: chromatin modifications. *Trends Biochem. Sci.* 25, 619-623.

Wu, J., Parkhurst, K. M., Powell, R. M., Brenowitz, M., and Parkhurst, L. J. (2001). DNA bends in TATA-binding protein-TATA complexes in solution are DNA sequence-dependent. *J. Biol. Chem.* *276*, 14614-14622.

Wu, P. Y., and Winston, F. (2002). Analysis of Spt7 function in the *Saccharomyces cerevisiae* SAGA coactivator complex. *Mol. Cell. Biol.* *22*, 5367-5379.

Wu, S. Y., and Chiang, C. M. (2001). TATA-binding protein-associated factors enhance the recruitment of RNA polymerase II by transcriptional activators. *J. Biol. Chem.* *276*, 34235-34243.

Wyers, F., Rougemaille, M., Badis, G., Rousselle, J. C., Dufour, M. E., Boulay, J., Regnault, B., Devaux, F., Namane, A., Seraphin, B., *et al.* (2005). Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell* *121*, 725-737.

Xiao, T., Hall, H., Kizer, K. O., Shibata, Y., Hall, M. C., Borchers, C. H., and Strahl, B. D. (2003). Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. *Genes. Dev.* *17*, 654-663.

Xiao, T., Kao, C. F., Krogan, N. J., Sun, Z. W., Greenblatt, J. F., Osley, M. A., and Strahl, B. D. (2005). Histone H2B ubiquitylation is associated with elongating RNA polymerase II. *Mol. Cell. Biol.* *25*, 637-651.

Xu, L. C., Thali, M., and Schaffner, W. (1991). Upstream box/TATA box order is the major determinant of the direction of transcription. *Nucleic Acids Res.* *19*, 6699-6704.

Yaniv, M., and Cereghini, S. (1986). Structure of transcriptionally active chromatin. *CRC Crit. Rev. Biochem.* *21*, 1-26.

Yart, A., Gstaiger, M., Wirbelauer, C., Pecnik, M., Anastasiou, D., Hess, D., and Krek, W. (2005). The HRPT2 tumor suppressor gene product parafibromin associates with human PAF1 and RNA polymerase II. *Mol. Cell. Biol.* *25*, 5052-5060.

Yoon, S., Govind, C. K., Qiu, H., Kim, S. J., Dong, J., and Hinnebusch, A. G. (2004). Recruitment of the ArgR/Mcm1p repressor is stimulated by the activator Gcn4p: a self-checking activation mechanism. *Proc. Natl. Acad. Sci. U S A* *101*, 11713-11718.

Yuan, G. C., Liu, Y. J., Dion, M. F., Slack, M. D., Wu, L. F., Altschuler, S. J., and Rando, O. J. (2005). Genome-scale identification of nucleosome positions in *S. cerevisiae*. *Science* *309*, 626-630.

- Zhang, C., Yan, H., and Burton, Z. F. (2003). Combinatorial control of human RNA polymerase II (RNAP II) pausing and transcript cleavage by transcription factor IIF, hepatitis delta antigen, and stimulatory factor II. *J. Biol. Chem.* *278*, 50101-50111.
- Zhang, H., Richardson, D. O., Roberts, D. N., Utley, R., Erdjument-Bromage, H., Tempst, P., Cote, J., and Cairns, B. R. (2004). The Yaf9 component of the SWR1 and NuA4 complexes is required for proper gene expression, histone H4 acetylation, and Htz1 replacement near telomeres. *Mol. Cell. Biol.* *24*, 9424-9436.
- Zhang, H., Roberts, D. N., and Cairns, B. R. (2005). Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell* *123*, 219-231.
- Zheng, L., Hoeflich, K. P., Elsby, L. M., Ghosh, M., Roberts, S. G., and Ikura, M. (2004). FRET evidence for a conformational change in TFIIB upon TBP-DNA binding. *Eur. J. Biochem.* *271*, 792-800.
- Zhu, Y., Peterson, C. L., and Christman, M. F. (1995). HPR1 encodes a global positive regulator of transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *15*, 1698-1708.