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Publication Date

2005

Peer reviewed|Thesis/dissertation

Defining the Roles of Multiple Mechanisms in Tup1-Mediated Repression in *Saccharomyces cerevisiae*

by

Sarah R. Green

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Date .... University Librarian

Degree Conferred:..

Acknowledgements

If I had merely wanted to be called Dr. Green and become both overeducated and woefully underqualified for almost any job, I would have gone to medical school. Instead, I chose the interminable pursuit of minutiae that is a doctorate in biological sciences and, while only time will tell if that was a wise decision, I did enjoy my time as a graduate student at UCSF. That graduate school was not an entirely soul-crushing experience is in large part due to the involvement of the people I thank here, although I am sure I will be unable to truly capture the extent of my appreciation and the debt that is owed them.

First, I must thank my advisor Sandy Johnson. Sandy and I did not exactly have the easiest or most comfortable mentor-student interaction but, as dysfunctional as it might have been at times, I think it all turned out for the best. I have learned an incredible amount during my time in Sandy's lab about how to design precise, solid, productive experiments, without which there is no chance of success in this field. I think I have always been a fairly independent thinker, another important trait for a scientist, but by working with Sandy I learned that independence is only beneficial if it is tempered with a willingness to take sound advice when it is offered.

I also would like to thank the members of my Thesis Committee, Hiten Madhani and Carol Gross, for their advice and insightful comments about my project. I wish I had made more frequent use of their numerous strengths. Ira Herskowitz was on my original Thesis Committee and I want to acknowledge his influence on this work. It was immediately obvious upon talking to him that he was someone interested in Science with a capital S—in the big questions and the little details—and I know there was still so much more I could have learned from him.

Anita Sil was not an official member of my Thesis Committee but deserves special recognition in her own right. Despite being perpetually in demand, Anita made time to meet with me during the darkest hours of my graduate career. I cannot say enough nice things about Anita and she is, simply, an inspiration. I do not know anyone who works

The two people who most influenced my success are my parents, Michael Green and Ann Sartori. Biased by genetics, as all parents should be, they think everything I do is the best thing they have ever seen and their alternate reality is always a welcome retreat from the real world. My father is the first scientist I ever knew and he taught me to appreciate the promise of a lull between protocol steps. My mother has sustained an enthusiasm for entertaining herself that most people cannot manage after about third grade, and I say that with only profound envy. I am grateful that they are the people who taught me about the world and got me started on my way. The remaining member of my immediate family, excluding a distinguished line of much-adored golden retrievers, is my older brother, Josh Green. He has bossed me around for as long as I can remember and, as a consequence, toughened me up enough to survive unruly mutants, blank gels, and the myriad pitfalls of the confidence-obliterating experience that is graduate school. No matter how old I become, I have no doubt that Josh will always be ready to provide whatever I might need from him without hesitation. For that, I thank him.

Finally, I know that I would not have been able to finish graduate school without the love and understanding I have gotten from Jeff Ubersax. He was my first friend in San Francisco and has become my best friend anywhere. He is the exact opposite of me in all of the important ways—he is sweet and patient and trusting and confident and generous. His certainty that I would be successful kept me going even when I could not see an end in sight. He has disproved my belief that people cannot be trusted and I feel lucky to have the privilege to spend every day with him.

MOLECULAR BIOLOGY OF THE CELL

January 6, 2005

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Defining the Roles of Multiple Mechanisms in Tup1-Mediated Repression in *Saccharomyces cerevisiae*

by

Sarah R. Green

Abstract

Eukaryotic transcription is a highly regulated cellular process that represents the balance of positive and negative factors acting on the promoter of a given gene. In *Saccharomyces cerevisiae*, the Tup1-Sn6 repressor complex negatively influences the expression of approximately five percent of all genes. Functional homologs of this complex exist in other organisms, and a better understanding of the functions of Tup1 in yeast will provide us with insight into the broader questions of how transcriptional repression is regulated across species and the consequences of its misregulation.

Most of the targets of Tup1-mediated repression have been identified, but the means by which Tup1 inhibits transcription of these target genes is not entirely clear. To explore this question, we focused on two proteins know to be involved in Tup1-mediated repression—Hda1, a histone deacetylase, and Srb10, a cyclin-dependent kinase associated with the Mediator complex. We disrupted each of these genes separately and in combination and compared the effects of the disruptions on Tup1-regulated genes using a statistical analysis of microarray data. We saw a strong overlap between the genes derepressed in an *hda1Δ* strain and the set of Tup1-regulated genes and a smaller but still significant intersection between the mutant *srb10* and *tup1Δ* datasets. Tup1-regulated genes can be divided into subclasses based on their requirements for Hda1 and/or Srb10 function for full repression. However, the magnitudes of the derepression defect in these mechanistic disruptions are rarely as severe as that of a *tup1Δ* strain. We also showed that there was not a strict correlation between the loss of Hda1 deacetylation function and a loss transcriptional repression. These data imply that there are multiple and overlapping

mechanisms that contribute to full Tup1-mediated repression and that often several of these mechanisms are acting at a given promoter to repress transcription.

We also tried to better understand the mechanisms of Tup1-mediated repression by analyzing the functions of Tup1 itself. We mutagenized a surface of Tup1 conserved among metazoan homologs and measured the effects of the mutants on Tup1-mediated repression by microarray analysis. The mutant alleles represented a range of deficiencies in repression, with the strongest mutant affecting about half of Tup1-regulated genes. For one set of Tup1-regulated genes, some of the point mutants disrupted the recruitment of Tup1 to regulated promoters; however, for the majority of Tup1-repressed genes, the mutant proteins are properly recruited but cannot repress transcription. These point mutants of Tup1 demonstrate that the conserved surface of Tup1 is important for two different aspects of Tup1-mediated repression—recruitment to repressed promoters and the active repression of transcription.

Alexander D. Johnson

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Chapter 1

Introduction

Eukaryotic Transcriptional Regulation

Each cell in the human body is exquisitely designed to perform specific, vital functions, and yet nearly all of these cells contain the identical genetic information. Much of the structural and functional differences between a liver cell made to produce cholesterol and a nerve cell built to transmit electrical signals is the result of precisely controlled and developmentally timed transcriptional regulation of the genome, a process that is absolutely necessary for the viability of a multicellular organism. When the correct program of gene expression is disturbed, drastic consequences such as physical deformities, alterations of neural pathways, and unregulated cell proliferation can occur. While unicellular organisms do not need to coordinate the functions of multiple cell types, accurate transcriptional regulation is still indispensable for their ability to adapt to cellular damage or changing external conditions or to regulate cellular growth. The importance of transcriptional regulation to organisms both simple and complex is obvious, yet the various mechanisms that are responsible for it are more mysterious. The purpose of this work is to better understand how one type of transcriptional regulation--the active repression of the expression of genes--is achieved using *Saccharomyces cerevisiae* as a model system. We concentrated our analysis on the Tup1-Ssn6 repression complex because of the solid foundation of previous work on the basic mechanics of the complex and because functional homologs have been identified in several organisms, including mammals (Pflugrad, Meir et al. 1997; Levanon, Goldstein et al. 1998; Chen and Courey 2000; Smith and Johnson 2000).

The Tup1 Complex

The Tup1 protein in *S. cerevisiae* is part of a larger complex made up of four molecules of Tup1 and one molecule of Ssn6 (Varanasi, Klis et al. 1996; Redd, Arnaud et al. 1997). Tup1 contains seven degenerate WD repeats in the C-terminal

half of the protein. WD repeats contain a few well-conserved residues necessary for

the basic structure of the repeats interspersed with stretches of variable sequence,

giving each WD-containing protein a unique surface suitable for its particular function

(Smith, Gaitatzes et al. 1999). Typically, between four and eight WD repeats are

found in WD-containing yeast proteins and crystal structures of such proteins

demonstrate that the repeats form interlocking, four-stranded β sheet blades of a

larger propeller structure (Gaudet, Bohm et al. 1996; Sprague, Redd et al. 2000;

Pickles, Roe et al. 2002). This arrangement creates large surfaces that are available

for interactions with other proteins. WD repeats have been found in proteins of

varied functions, and it seems likely that, while there is no unifying function common

to WD repeat-containing proteins, they all share the ability to interact with multiple

proteins and participate in the formation of large, multisubunit complexes (Smith,

Gaitatzes et al. 1999). Such a characteristic is particularly critical for Tup1 as it is

known to repress the expression of a large set of disparately regulated genes and

would be expected to require multiple interacting partners to achieve regulation on

this scale.

Metazoan Homologs of Tup1

Studying Tup1 in order to learn more about general eukaryotic transcriptional

repression is an attractive approach because of the breadth of experimental methods

available in *S. cerevisiae* and the strong functional conservation of Tup1 homologs in

metazoans. Groucho in *Drosophila*, Unc-37 in *C. elegans*, and TLE proteins in

humans have been designated homologs of Tup1 based on sequence similarity and

their transcriptional repression functions (Pflugrad, Meir et al. 1997; Levanon,

Goldstein et al. 1998). Remarkably, these homologs can interact with Ssn6 and

repress transcription when expressed in yeast (Grbavec, Lo et al. 1999).

Furthermore, they, like Tup1, lack the ability to bind directly to DNA and depend on

the function of sequence-specific DNA-binding proteins to be brought to regulated promoters (Fisher and Caudy 1998). These findings suggest that, although the functions of the targets of repression vary greatly among organisms, Tup1 and its homologs share common repression mechanisms. It is reasonable to posit that the repression of transcription is likely to involve many components of other highly conserved machineries like those that package DNA into chromatin and those responsible for transcription itself. Therefore, determining the mechanisms of Tup1-mediated repression in yeast will surely provide novel avenues of investigation for these other homologous transcriptional repressors.

Functional Analysis of Tup1

While a substantial amount of work has been done studying the roles of the proteins that are recruited by Tup1 to establish repression, experiments directly analyzing the functions of Tup1 and its domains have been more limited. Many experiments focusing on defining Tup1 functions have used cells lacking the protein, restricting the types of experiments that can be done. As stated above, Tup1 is responsible for repressing the transcription of a large group of disparately regulated genes, and deleting *TUP1* derepresses all of these genes regardless of the downstream mechanisms responsible for their repression. Consequently, there is no way to dissect the contributions of overlapping repression mechanisms or to identify subsets of genes that maintain repression in the face of weakened Tup1 function, for instance. Also, completely eliminating Tup1 protein prohibits any biochemical analysis of the proteins interacting with the Tup1-Ssn6 complex. As schemes for genetic screens of Tup1 function become exhausted and as protein identification techniques improve and require smaller amounts of material, identifying Tup1 interactors will likely become the most fruitful method for determining components involved in unidentified repression mechanisms. One of the objectives of my studies

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was to take advantage of the extensive conservation of sequence and domain structure among Tup1 homologs to design mutant alleles of Tup1 defective for repression that can be used to investigate the functions of Tup1 itself.

Targets of Tup1-Mediated Repression

The Tup1-Ssn6 complex is a general repressor of transcription in *S. cerevisiae* that is responsible for the regulation of over three hundred genes involved in diverse cellular functions and responding to numerous signals (DeRisi, Iyer et al. 1997; Green and Johnson 2004). The targets of Tup1-mediated repression are typically repressed under standard laboratory growth conditions and seem to represent cellular responses to stressful or suboptimal environments. In essence, these genes represent emergency responses of the cell; however, although unwarranted expression of Tup1-repressed genes creates a drain on the resources of the cell, the deletion of *TUP1* (and subsequent loss of repression of its target genes) is not lethal to the cell. However, cells lacking *TUP1* do have a severe growth defect and would be at a considerable disadvantage if forced to compete with wild type cells for survival. In such a context, robust maintenance of Tup1-mediated repression would prove evolutionarily beneficial.

The Tup1-Ssn6 complex does not bind to the promoters of its regulated targets directly but is recruited through an interaction with sequence-specific DNA-binding proteins responsible for the control of subsets of similarly regulated genes. In this way, Tup1-mediated repression at a specific set of genes can be relieved in response to an appropriate signal via regulation of the sequence-specific DNA binding proteins without disrupting the repression of the majority of Tup1-repressed genes (Zitomer and Lowry 1992). Several of these sequence-specific DNA-binding proteins and the corresponding sets of genes under their control have been identified. Tup1 represses glucose-repressed genes necessary for the metabolism of alternative

sugars through recruitment by Mig1 and Mig2 (Treitel and Carlson 1995; Ozcan and Johnston 1996; Lutfiyya, Iyer et al. 1998). Hypoxia-induced genes that allow the cell to adapt to growth in low oxygen environments contain a site recognized by Rox1, which then recruits the Tup1-Ssn6 complex (Balasubramanian, Lowry et al. 1993; Zitomer, Limbach et al. 1997). One of the two natural haploid cell types of *S. cerevisiae*, the α-cell type, requires the repression of a-cell type specific genes by Tup1-Ssn6 via recruitment by Matα2 for its cell fate (Mukai, Harashima et al. 1991; Komachi, Redd et al. 1994). In diploid cells, Matα2 (in complex with Mata1) recruits Tup1 to a different set of genes to turn off expression of haploid specific genes (Mukai, Harashima et al. 1991; Keleher, Redd et al. 1992; Galgoczy, Cassidy-Stone et al. 2004). Until DNA damage is sensed, promoters of *RNR* genes are bound by Crt1 which recruits Tup1 to shut off transcription (Huang, Zhou et al. 1998). The sequence-specific DNA-binding proteins regulating the majority of the other groups of Tup1-regulated genes such as osmotic stress response genes and flocculation genes remain unidentified (Marquez, Pascual-Ahuir et al. 1998; Fleming and Pennings 2001).

Models of Tup1-Mediated Repression

Two models have emerged to explain the establishment of Tup1-mediated repression. The first model, referred to here as the Transcription Model, suggests that the Tup1 complex acts on a component of the Mediator (a large multisubunit complex that interacts with RNA polymerase II and modulates its activity in response to positive and negative signals) or a factor of the general transcriptional machinery to inhibit transcription. Alternatively, it has been hypothesized that Tup1 creates a repressive chromatin structure to turn off transcription, for instance by recruiting factors that modify nucleosomes directly (herein the Chromatin Model). There is evidence to support both of these models of Tup1-mediated repression and, given

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the complexity of the set of genes repressed by Tup1 and the importance of the maintenance of that repression to the survival of the organism, it is likely that multiple mechanisms would be required for full repression.

The Transcription Model

　　　　Much of the evidence supporting the Transcription Model of Tup1-mediated repression comes from genetic screens designed to measure a loss of repression by Tup1. One such screen fused the promoter of *MFA2*, a Tup1-repressed a-specific gene, to a reporter gene and looked for mutants that could no longer repress its expression (Wahi and Johnson 1995). Another screen identified mutations that bypassed a defect in the ability to relieve repression of glucose-repressed genes, targets of Tup1-mediated repression (Kuchin, Yeghiayan et al. 1995; Song, Treich et al. 1996). Other screens monitored the derepression of different subsets of Tup1-regulated genes like the hypoxic and meiotic genes (Strich, Slater et al. 1989; Rosenblum-Vos, Rhodes et al. 1991). Additional support for the conclusion that Tup1 acts on the general transcriptional machinery or the Mediator complex comes from an *in vitro* assay for Tup1-mediated repression that demonstrated significant repression activity in a partially purified cell extract (Herschbach, Arnaud et al. 1994; Redd, Arnaud et al. 1997). This assay measures the levels of transcriptional repression from a DNA template that contains sites only for Tup1 recruitment and RNA polymerase II holoenzyme binding, suggesting these were the only two complexes necessary to achieve repression. Furthermore, the template DNA was not packaged into nucleosomes, indicating that in this assay chromatin factors were not required to establish repression. Proteins considered part of the Mediator complex or the general transcriptional machinery and have been shown to play a role in Tup1-mediated repression are summarized below.

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Sin4

Sin4 was identified in screens for loss of Tup1-mediated repression at a-specific genes and glucose-repressed genes (Chen, West et al. 1993; Wahi and Johnson 1995). It has been shown to have both positive and negative effects on transcription and seems to act in basal as well as activated transcription (Carlson 1997). Sin4 is found in some Mediator preparations and has a possible role in nucleosome remodeling, suggesting it might be a link between the Transcription Model and the Chromatin Model of Tup1-mediated repression (Jiang and Stillman 1992; Gustafsson and Samuelsson 2001).

Rox3

A role for Rox3 in Tup1-mediated repression was also uncovered in several genetic screens (Rosenblum-Vos, Rhodes et al. 1991; Wahi and Johnson 1995; Song, Treich et al. 1996). Rox3 is an essential gene and is considered part of the core Mediator complex (Gustafsson and Samuelsson 2001). Rox3 has been shown to co-immunoprecipitate with a protein believed to be one of the sequence-specific DNA-binding proteins that recruit Tup1 (Song and Carlson 1998). Little is known about the function of Rox3 beyond its association with the Mediator complex. Recently, a mammalian homolog of Rox3 was identified by mass spectrometry in a purification of other Mediator subunits (Sato, Tomomori-Sato et al. 2003).

Rgr1

Rgr1 is another essential member of the core Mediator complex with mammalian homologs (Gu, Malik et al. 1999; Gustafsson and Samuelsson 2001). Rgr1 mutants were isolated in screens for resistance to glucose-repression, and recently Rgr1 (in addition to Sin4) was shown to be required for the repression of *MAL* genes (Sakai, Shimizu et al. 1988; Wang and Michels 2004). Also, like Sin4,

there is evidence that Rgr1 can influence chromatin structure (Jiang, Dohrmann et al. 1995)

Srb8-11

Srb8, Srb9, Srb10, and Srb11 form a distinct subcomplex of the Mediator and were originally identified in a screen for suppressors of a truncation of the CTD tail of RNA polymerase II (Nonet and Young 1989; Liao, Zhang et al. 1995). Mutations in each of these genes were found in several Tup1-related genetic screens (Strich, Slater et al. 1989; Wahi and Johnson 1995; Song, Treich et al. 1996). Srb8 and Srb9 are believed to be important for the association of Srb10–Srb11, a cyclin-dependent kinase and its associated cyclin, with the larger Mediator complex (Myer and Young 1998). Substrates of the Srb10-Srb11 complex include several transcription factors and the CTD of RNA polymerase II (Chi, Huddleston et al. 2001; Borggrefe, Davis et al. 2002). There is evidence that CTD phosphorylation by Srb10 before RNA polymerase II association with promoters can inhibit transcriptional activity (Hengartner, Myer et al. 1998). The phenotype of an *SRB10* deletion resembles that of a *TUP1* deletion in many significant ways—cells are flocculent, sporulation is inhibited, and cells have abnormal morphologies (Carlson 1997; Cooper and Strich 2002). Additionally, the expression pattern of an *srb10Δ* strain, as measured by microarrays, shares a significant overlap with that seen when Rox1, a sequence-specific DNA binding protein that recruits Tup1 to hypoxic genes, is deleted (Becerra, Lombardia-Ferreira et al. 2002). Srb10 has been shown to directly interact with Tup1 and appears to be a major point of contact between Tup1 and the RNA polymerase II holoenzyme (Zaman, Ansari et al. 2001).

Srb7 and Hrs1/Med3

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Two other proteins associated with the Mediator, Srb7 and Hrs1, have been shown to physically interact with Tup1 but little is known about their functions (Gromoller and Lehming 2000; Papamichos-Chronakis, Conlan et al. 2000). A mutation of *SRB7* that prevents its association with Tup1 results in the derepression of several Tup1-regulated genes (Gromoller and Lehming 2000).

The Chromatin Model

Two types of chromatin regulation have been implicated in Tup1-mediated repression--the positioning of nucleosomes at repressed promoters and the recruitment of histone deacetylases. The positioning of nucleosomes to inhibit transcription of Tup1-repressed genes has been best studied for the case of the a-specific genes. The chromatin at these seven specific genes has been shown to form a highly ordered structure under repressing conditions (MATα cells), which is lacking under derepressing conditions (MATa cells) (Shimizu, Roth et al. 1991; Ganter, Tan et al. 1993; Ducker and Simpson 2000; Gavin, Kladde et al. 2000). These positioned nucleosomes are disrupted upon deletion of *TUP1* or *SSN6* even when transcription is prevented, suggesting the destabilization of the positioned nucleosomes is not simply the result of active transcription (Cooper, Roth et al. 1994). Additionally, though Matα2 remains bound to a-specific promoters when *TUP1* is deleted, the ordered chromatin structure is disturbed, implying that Tup1, not Matα2, is blocking chromatin remodeling at repressed genes (Gavin, Kladde et al. 2000). More recently, positioned nucleosomes have been demonstrated at other Tup1-repressed genes as well (Kastaniotis, Mennella et al. 2000; Li and Reese 2001).

Supporting the role of positioned nucleosomes in Tup1-mediated transcription, recent work has shown that chromatin-remodeling factors contribute to

the inhibition of transcription at Tup1-regulate genes. The deletion of either

component of the Isw2-Itc1 chromatin remodeling complex results in a loss of

repression at a few Tup1-repressed genes (Ruiz, Escribano et al. 2003; Trachtulcova,

Frydlova et al. 2004; Zhang and Reese 2004; Zhang and Reese 2004). Also, a loss

of positioned nucleosomes at some of these promoters has been shown upon

deletion of either *TUP1* or *ISW2* (Zhang and Reese 2004). However, microarray

experiments analyzing the expression pattern of an *isw2Δ* strain suggest Isw2

function is not required for full repression of the majority of Tup1-regulated genes

(Hughes, Marton et al. 2000). While a role for chromatin remodeling, or perhaps the

prevention of chromatin remodeling, in Tup1-mediated repression is well supported,

the extent and mechanism of that contribution remains unclear.

In addition to the ordered arrangement of nucleosomes, transcriptional

repression can also be achieved through direct modifications of chromatin. Tup1

recruitment to promoters has been correlated with a decrease in histone tail

acetylation (in other words, an increase in the levels of deacetylated histones) at

those promoters (Bone and Roth 2001; Wu, Suka et al. 2001; Davie, Trumbly et al.

2002). Deacetylated histone tails are a hallmark of silent or repressed genes and

several histone deacetylases (HDACs) in *S. cerevisiae* have been linked to Tup1.

Class I HDACs

This class of HDACs in *S. cerevisiae* comprises Hos1, Hos2, Hos3, and Rpd3,

which share sequence similarities (Grozinger, Hassig et al. 1999). Rpd3 is thought

to globally deacetylate chromatin, rather than specifically targeting the chromatin at

promoters, and often has a modest effect on transcription (Kurdistani, Robyr et al.

2002). Rpd3 appears to specifically deacetylate the histone H4 protein of

nucleosomes (Wu, Suka et al. 2001). While a few Tup1-regulated genes appear to

be targets of Rpd3 deacetylation, a genome-wide analysis of deacetylation by Rpd3

fails to identify any strong correlation between the two sets of genes—the targets of Rpd3 deacetylation and the targets of repression by Tup1 (Kadosh and Struhl 1997; Kadosh and Struhl 1998; Kurdistani, Robyr et al. 2002). However, the *Drosophila* Tup1 homolog, Groucho, has been shown to interact with the fly Rpd3, so it is possible a stronger link between Tup1 and Rpd3 exists and remains to be investigated (Chen, Fernandez et al. 1999). In fact, a physical interaction has been shown between Ssn6 and Hos1, Hos2, and Rpd3 (Davie, Edmondson et al. 2003). Much less is known about the Hos1-3 deacetylases, and there is even some evidence that they may have a positive influence on transcription (Wang, Kurdistani et al. 2002).

Class II HDACs

The class II HDACs in *S. cerevisiae* are Hda1, Hda2, and Hda3, which form the Hda1 complex (Wu, Carmen et al. 2001). Hda1 has been shown to interact with Tup1 *in vitro* (Wu, Suka et al. 2001). A specific increase in acetylation of histone H3 was seen at targets of Tup1-mediated repression when either *HDA1* or *TUP1* was deleted, suggesting that Tup1 is recruiting Hda1 to regulated promoters to deacetylate chromatin (Wu, Suka et al. 2001). Both the recruitment of Tup1 and the deacetylation that is a consequence of *HDA1* deletion is focused at the promoter of the genes, rather than the more global specificity of the Rpd3 deacetylase (Wu, Suka et al. 2001). Several Tup1-repressed genes have been shown to be derepressed in an *hda1Δ* strain, suggesting that Hda1 is required for full Tup1-mediated repression of these genes (Wu, Suka et al. 2001; Green and Johnson 2004). The most compelling evidence of a link between Hda1 function and Tup1-mediated repression is the striking correlation between the set of genes deacetylated by Hda1 and targets of repression by Tup1 (Robyr, Suka et al. 2002).

Genome-wide Analysis of Tup1-Mediated Repression

The previous sections of this chapter outlined the considerable amount of information known about Tup1-mediated repression, but it is abundantly clear that many questions remain. In the early years of the study of Tup1, the available experimental tools restricted evaluation of disruptions in repression to only a few genes at a time. These stalwart markers of Tup1 function were selected for the ease of the detection of their transcripts or the availability of previously constructed strains or plasmids, not because they were representative of all of the genes targeted for repression by Tup1. In truth, these were likely the genes that exhibited the most drastic, easily discernable changes in a *tup1Δ* strain. As a result, many early conclusions about the mechanism of Tup1-mediated repression were probably skewed to explain the behavior of the most sensitive of the targeted genes

It has become clear that the "conflicting" data generated by early Tup1 mechanistic experiments represent evidence for multiple repression mechanisms contributing to the overall repression of individual Tup1-repressed genes rather than actual inconsistencies. Because of these gene-specific repression mechanism requirements, it is risky to base conclusions about the regulation of the all targets of Tup1 on experiments that only analyze a single Tup1-repressed gene. The most comprehensive approach to dissecting the network of repression mechanisms is to analyze the effects of disruptions on all Tup1-repressed genes at once. The advent of expression microarrays made such an approach to the study of Tup1 possible. Using this technology and the developing statistical tools facilitating the interpretation of the massive amounts of data generated by it, the primary objectives of this work were to definitively map the genome-wide contributions of the mechanisms of Tup1-mediated repression that have already been sketched out on a gene-by-gene basis and to identify new, candidate repression mechanisms.

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Chapter 2

Promoter-Dependent Roles for Srb10 and Hda1 in Tup1-Mediated Repression

Promoter-Dependent Roles for the Srb10 Cyclin-Dependent Kinase and the Hda1 Deacetylase in Tup1-Mediated Repression in *Saccharomyces cerevisiae*

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Abstract

The Ssn6-Tup1 complex has been well characterized as a *Saccharomyces cerevisiae* general transcriptional repressor with functionally conserved homologs in metazoans. These homologs are essential for cell differentiation and many other developmental processes. The mechanism of repression of all of these proteins remains poorly understood. Srb10 (a cyclin/CDK associated with the Mediator complex) and Hda1 (a class I histone deacetylase) have each been implicated in Tup1-mediated repression. We present a statistically based genome-wide analysis that reveals that Hda1 partially represses roughly 30% of Tup1-repressed genes, whereas Srb10 kinase activity contributes to the repression of about 15% of Tup1-repressed genes. These effects only partially overlap, suggesting that different Tup1-repression mechanisms predominate at different promoters. We also demonstrate a distinction between histone deacetylation and transcriptional repression. In an *HDA1* deletion, many Tup1-repressed genes are hyperacetylated at lysine 18 of histone H3, yet are not derepressed, indicating deacetylation alone is not sufficient to repress most Tup1-controlled genes. In a strain lacking both Srb10 and Hda1 functions, over half of the Tup1-repressed genes are still repressed, suggesting that Tup1-mediated repression occurs by multiple, partially overlapping mechanisms, at least one of which is unknown.

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Introduction

The Tup1-Ssn6 complex is a general transcriptional repressor in *Saccharomyces cerevisiae* that controls a diverse set of genes generally characterized as being important for adaptation to nonstandard growth. Homologs of Tup1 have been identified in several other organisms (for example *unc-37 in C. elegans*, Groucho in *Drosophila*, and TLE proteins in humans) and their repression functions are essential for embryonic development, cell differentiation, neurogenesis, and other developmental processes (Pflugrad, Meir et al. 1997; Fisher and Caudy 1998; Levanon, Goldstein et al. 1998; Grbavec, Lo et al. 1999). Consequently, a better understanding of the mechanism of Tup1-mediated repression in yeast should illuminate this same process and its wide-ranging downstream consequences in other organisms. The Tup1-Ssn6 complex does not itself bind DNA but is recruited to target promoters through an association with sequence-specific DNA binding proteins; however, the crucial question of how transcriptional repression is established once this event occurs has not been clearly answered.

Two models for Tup1-mediated repression are supported by a number of earlier observations. One proposes that Tup1 produces a transcriptionally repressed chromatin state by recruiting histone deacetylases (HDACs). Hda1, a class I HDAC, has emerged as the most likely deacetylase to be acting with Tup1. Hda1 binds to Tup1 *in vitro* and an *HDA1* deletion results in hyperacetylation of histones at several Tup1-controlled genes (Wu, Suka et al. 2001). Hyperacetylation of Tup1-repressed genes is also seen when Tup1 is deleted (Bone and Roth 2001; Davie, Trumbly et al. 2002). Recently, a genomic analysis of *hda1Δ*-dependent hyperacetylation and Tup1-controlled genes expanded this correlation to a larger set of genes (Robyr, Suka et al. 2002). However, a clear link between loss of Hda1-mediated

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deacetylation activity and loss of transcriptional repression has been more difficult to establish.

A second model invokes a direct effect of Tup1 on the general transcriptional machinery. Tup1-mediated repression has been observed in an *in vitro* system employing a naked DNA template (Herschbach, Arnaud et al. 1994; Redd, Arnaud et al. 1997). In addition, several components of the PolII transcriptional machinery (Rgr1, Sin4, Rox3, Hrs1, Srbs8-11) have been identified in genetic screens for loss of Tup1-mediated repression (Sakai, Shimizu et al. 1990; Kuchin, Yeghiayan et al. 1995; Wahi and Johnson 1995; Song, Treich et al. 1996; Carlson 1997). A few have also been shown to physically interact with the Ssn6-Tup1 complex (Gromoller and Lehming 2000; Papamichos-Chronakis, Conlan et al. 2000). Defining their roles in Tup1-mediated repression has been difficult as many of them are essential and have wide-ranging effects on transcription in general. One component, Srb10, a nonessential cyclin-dependent kinase, is part of a distinct Mediator-associated complex (the Srb8-11 complex) that interacts with Tup1 (Myer and Young 1998; Zaman, Ansari et al. 2001; Borggrefe, Davis et al. 2002). Srb10 has been shown to negatively affect transcription and its kinase function is necessary for full repression of a Tup1-controlled reporter construct (Holstege, Jennings et al. 1998; Kuchin and Carlson 1998; Song and Carlson 1998; Lee, Chatterjee et al. 2000). Furthermore, expression microarray experiments demonstrated some overlap between genes repressed by Srb10 and by Rox1, a DNA-binding protein that recruits the Tup1-Ssn6 complex and is responsible for repressing hypoxia-induced genes (Holstege, Jennings et al. 1998; Becerra, Lombardia-Ferreira et al. 2002).

Previous work designed to dissect the mechanism of Tup1-mediated repression has concentrated mainly on the analysis of a few Tup1-repressed genes and reporter constructs. This piecemeal approach makes it difficult to determine the relative importance of the various mechanisms of Tup1-mediated repression at all

Tup1-controlled genes. In particular, a genome-wide analysis that systematically investigates the contributions of both of the mechanisms represented by Hda1 and Srb10 functions has not been previously reported. This approach avoids the problems inherent in extrapolating a general mechanism of Tup1-mediated repression from the examination of only a few cases. Here, we describe the statistical analysis of gene expression microarrays of strains disrupted in all combinations of Tup1, Hda1, and Srb10 function. We have been able to divide the total set of Tup1-repressed genes into subclasses dependent on one, both, or neither of these mechanisms. As a result it is clear that Srb10 and Hda1 are only two aspects of a complex, multi-layer system for establishing Tup1-mediated gene repression.

Results

Deletion of Tup1 Derepresses A Large Group of Diverse Genes

One of the first papers describing the expression microarray technique included a set of data for a *tup1Δ* mutant (DeRisi, Iyer et al. 1997). The wild type control strain used in that study was later shown to have a duplicated chromosome XIII, which resulted in all the genes on chromosome XIII appearing to be slightly downregulated in a *tup1Δ* strain (Hughes, Roberts et al. 2000). To correct for this strain abnormality and because microarray techniques and analysis have advanced since that first publication, we present a new set of expression microarray data for a *tup1Δ* mutant. The mutant constructed for this study is derived directly from the wild type control strain. Our data represent seven duplicate experiments and have been analyzed using the Significance Analysis of Microarrays (SAM) methodology (Tusher, Tibshirani et al. 2001). SAM assigns each gene a *d*-score based on both its level of expression and reproducibility. All genes whose scores are higher than a selected threshold are then deemed significant. SAM also calculates a False

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Discovery Rate (FDR) for each threshold of significance. This value estimates the percentage of the genes with scores higher than a given threshold that are likely to be false positives. We decided to select a significance threshold that resulted in the lowest calculated FDR (based on the 90th percentile of d-scores) and, therefore, represented the highest-confidence set of differentially expressed genes in the *tup1Δ* strain.

Three hundred and thirty four genes passed this standard and are considered significantly derepressed in the *tup1Δ* mutant. Many of these genes were also reported in the initial published *tup1Δ* dataset (using a $\geq$2-fold increase in expression cut-off), but over half were not. These newly described Tup1-repressed genes share a similar distribution among broad functional categories as the previously known Tup1-controlled genes (Table 1). For example, Table 2 lists more information about the newly reported genes found in the membrane transport, metabolism, cell wall, and stress response categories. The complete set of Tup1-repressed genes responds to very different signals and represents strategies for the cell to adjust to everything from simple changes in sugar availability to noxious environments. The statistical analysis of our multiple *tup1Δ* microarray experiments allowed us to determine a more comprehensive set of Tup1-controlled genes, which is vital for exposing the overall impact of different Tup1-repression mechanisms at regulated promoters.

Overlap of *tup1Δ*, *srb10^{D304}*, and *hda1Δ* Expression Profiles

As described in the introduction, a number of previous studies have demonstrated a link between Tup1-mediated repression and the functions of Srb10 and Hda1. We have applied the same techniques we used to analyze the *tup1Δ* microarrays to determine the sets of genes derepressed when the functions of Srb10 and Hda1 are disrupted. We constructed an isogenic set of strains deleted for Tup1

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or *HDA1* or containing a mutant of Srb10 that lacks kinase activity (*srb10^{D304}*) (Liao, Zhang et al. 1995; Ansari, Koh et al. 2002). We also made a strain lacking both Hda1 and Srb10 functions to determine the combined effects of the two mutations on expression levels. Expression profiles of the mutant and wild type strains (all grown to mid-log phase) were then compared to identify differentially regulated genes. We analyzed data sets representing at least four duplicates for each strain using SAM and again adopted a significance threshold for each set of microarrays that corresponded to the lowest calculated FDR. The one exception was the *HDA1* SAM analysis. Because the deletion of *HDA1* affected many genes by only a small magnitude, we allowed a slightly higher FDR (see Methods).

We chose to analyze our data with SAM because the levels of expression changes we observed in the different mutant strains varied significantly and there was no reasonable way to apply a uniform requirement of a fold-change in expression to each set of data. For instance, a fairly standard cutoff of ≥2-fold change in expression worked well for identifying significant genes in the *tup1Δ* dataset, but was not a practical measure of significance for the *hda1Δ* strain in which the expression of many genes increased only ~1.5-fold. Rather than subjectively setting an expression threshold that was unique to each dataset, we chose the lowest FDR calculated by SAM as a universal standard for all datasets. Using SAM allowed us to apply a consistently stringent significance criterion that did not require all datasets to have similar ranges of expression changes.

Table 3 lists the total number of genes considered significant for each mutant and the FDR corresponding to that significance threshold. The largest portion of the significant genes for each disruption are upregulated (derepressed) compared to a wild type strain, consistent with the previously described roles of Tup1, Hda1, and Srb10 in transcriptional repression. To determine the roles Srb10 and Hda1 play in Tup1-mediated repression specifically, we compared the set of derepressed genes for

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the *tup1Δ* strain to those of each of the other three mutant strains. We focused only on derepressed genes because the downregulation of genes in the *tup1Δ* strain is likely to be due to indirect effects. A substantial fraction (73%) of the genes derepressed upon deletion of *HDA1* are also derepressed in the *tup1Δ* microarrays, suggesting that a primary transcriptional regulatory function of Hda1 is to repress Tup1-controlled genes (Figure 1A). However, less than a third of Tup1-controlled genes are significantly derepressed in the *hda1Δ* strain, indicating there must be at least one Hda1-independent mechanism of Tup1-mediated repression. The overlap between the sets of derepressed genes identified in the *srb10^{D304}* and *tup1Δ* microarrays was smaller than that observed between the *hda1Δ* and *tup1Δ* datasets but still significant. Thirty-three percent of the significantly derepressed genes in the *srb10^{D304}* strain overlap with those derepressed in the *tup1Δ* dataset (Figure 1B). Clearly, Srb10 participates in many other modes of transcriptional repression that are independent of Tup1. For example, Srb10 has been shown to directly downregulate the activity of several transcriptional activators, which could account for many of the genes derepressed in the *srb10^{D304}* microarrays (Chi, Huddleston et al. 2001). There is relatively little overlap between the *hda1Δ* and *srb10^{D304}* datasets (~16-20%), which suggests they are parts of two separate mechanisms of Tup1-repression, both of which are required at only a relatively small number of genes.

As expected the expression profile of the *srb10^{D304}hda1Δ* double mutant exhibits a degree of overlap with Tup1-repressed genes (47%) that falls between those of each of the single mutants (Figure 1C). Interestingly, there are 32 Tup1-controlled genes that are only significantly derepressed when both *SRB10* and *HDA1* are disrupted, demonstrating that each of these mechanisms can compensate for the loss of the other at some promoters. Furthermore, 22 genes are derepressed in either mutant strain, providing evidence that for some genes Hda1 and Srb10 are both required for full repression. Each mutation disrupted the repression of many

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Tup1-controlled genes, but rarely was the level of derepression in the mutant strains equal to the full derepression measured in a $tup1\Delta$ strain (Figure 1). This is consistent with the premise that Tup1-mediated repression occurs through several mechanisms including, but not limited to, those disrupted by the *HDA1* and *SRB10* mutations.

We performed the same microarray and SAM analysis described above on the double and triple mutants $hda1\Delta tup1\Delta$, $srb10^{D304}tup1\Delta$, and $srb10^{D304}hda1\Delta tup1\Delta$. The expression profiles of Tup1-repressed genes in these strains closely resemble that of the $tup1\Delta$ strain (our unpublished results). We conclude that both Hda1 and Srb10 are working through Tup1 to cause repression at Tup1-controlled genes and do not represent independent mechanisms of repression acting on Tup1-repressed genes. We also used microarrays to compare the effects on transcription of the $srb10^{D304}$ mutation and an *SRB10* deletion. We saw no significant difference in the expression patterns of these two mutants (our unpublished results), and conclude that the kinase activity of Srb10 accounts for the transcriptional effects observed in this study.

DNA-Binding Proteins Do Not Dictate Mechanism of Tup1 Repression

The microarray experiments presented above allow us to divide the larger set of Tup1-controlled genes into five subclasses based on the influence of the other mutations on gene expression. These subclasses are described as follows: (1) genes derepressed in the $tup1\Delta$ and $srb10^{D304}$ strains, (2) genes derepressed in the $tup1\Delta$ and $srb10^{D304}hda1\Delta$ strains but not the single $hda1\Delta$ and $srb10^{D304}$ mutant strains, (3) genes derepressed in the $tup1\Delta$, $hda1\Delta$, $srb10^{D304}$, and $srb10^{D304}hda1\Delta$ strains, (4) genes derepressed in the $tup1\Delta$ and $hda1\Delta$ strains, and (5) genes derepressed in the $tup1\Delta$ strain but none of the other mutant strains (Figure 2). These subclasses

of Tup1-controlled genes represent the first genome-wide evidence that there are different sets of repression mechanisms acting at different genes.

One possible characteristic of Tup1-repressed promoters that could dictate the repression mechanism(s) in use at that gene is the identity of the DNA-binding protein that recruits the Tup1-Ssn6 complex to the promoter. Tup1 does not itself bind to DNA, but instead is recruited to promoters by corepressors specific for the various classes of Tup1-repressed genes. Several of these sequence-specific DNA-binding proteins have been identified and, for a few, a set of direct target genes has been defined. Two well-characterized Tup1 corepressors are Mig1 and Rox1, the sequence-specific DNA-binding proteins controlling glucose-repressed and hypoxia-induced genes respectively (Lowry, Cerdan et al. 1990; Zitomer and Lowry 1992; Balasubramanian, Lowry et al. 1993; Amillet, Buisson et al. 1995; Treitel and Carlson 1995; Ozcan and Johnston 1996; Deckert, Torres et al. 1998; Lutfiyya, Iyer et al. 1998; Johnston 1999; Lee, Rinaldi et al. 2002). If the sequence-specific DNA-binding protein were responsible for determining which Tup1-repression mechanism(s) acts at a particular gene, then all of the genes controlled by that DNA-binding protein might be expected to fall into the same subclass of Tup1-repressed genes. To test this possibility, we mapped well-documented Mig1- and Rox1-controlled genes onto a cluster diagram of Tup1-repressed genes that reflects the five subclasses (Figure 2). Both the Mig1- and the Rox1-controlled genes are found throughout the cluster and across multiple subgroups. We conclude that genes repressed by the same DNA-binding protein can have different requirements for Hda1 and Srb10 and, therefore, the identity of the DNA-binding protein is not likely to determine the mechanism(s) of repression employed at a particular promoter.

Chromosomal Position Bias of Tup1-Controlled Genes

We wished to know if the mechanism(s) of Tup1-repression acting at a particular gene could be dictated by the position of that gene along its chromosome. For example, previous work has shown that the hyperacetylation seen upon deletion of *HDA1* is concentrated in regions of the genome within 25kb of a chromosome end (subtelomeric regions) (Robyr, Suka et al. 2002). Our expression microarrays show this same subtelomeric bias for the genes that are derepressed upon deletion of *HDA1* (Figure 3A). Approximately one third of all genes derepressed in the *hda1Δ* mutant lie within these subtelomeric regions, whereas only ~6% of all genes are contained within this same region. We see a similar bias for the set of genes derepressed in a *tup1Δ* strain. Thirty percent of Tup1-repressed genes identified in our microarray experiments are subtelomeric, five times higher than the 6% predicted for a random chromosomal distribution. The genes derepressed in an *srb10^{D304}* strain, however, do not exhibit this subtelomeric bias and, in fact, have the same positional distribution as the total genome (Figure 3A).

The overlaps we observed among our microarrays increase significantly when only considering the subtelomeric genes (Figure 3). For example, ~90% of the subtelomeric genes affected by Hda1 or Srb10 are also Tup1-repressed genes. In other words, the transcriptional functions of Hda1 and Srb10 in subtelomeric regions appear more dedicated to Tup1-mediated repression than they are at internal chromosome positions. However, this increase in the overlap between the gene sets probably reflects the density of Tup1-repressed genes in subtelomeric regions rather than any mechanistic bias. Each mutant strain showed roughly the same percentage of overlap with the total set of Tup1-repressed genes whether considering only subtelomeric genes or all genes (Figure 3C).

Loss of Deacetylation by Hda1 Is Not Sufficient for Loss of Tup1 Repression

Previous work described a correlation between the set of promoters that are hyperacetylated upon the deletion of *HDA1* and the set of genes repressed by Tup1. Hda1 preferentially deacetylates histones H3 and H2B (on positions K9, K14, K18, K23, K27 of H3 and positions K11 and K16 on H2B), and these same residues are hyperacetylated at a Tup1-repressed gene (*ENA1*) when Tup1 is deleted (Wu, Suka et al. 2001). Chromatin-IP (ChIP) microarrays have also demonstrated that the pattern of genes derepressed upon Tup1 deletion significantly overlaps with acetylation patterns resulting from an *HDA1* deletion (Robyr, Suka et al. 2002). These data, in addition to evidence of a physical interaction between Tup1 and Hda1, suggest that Tup1 recruits Hda1 to promoters in order to repress transcription (Wu, Suka et al. 2001). The substantial overlap between the *tup1Δ* and *hda1Δ* microarray datasets is also consistent with the idea that at least one function of Hda1 is to repress transcription at Tup1-controlled promoters.

To further examine the relationship between hyperacetylation and derepression, we compared the acetylation of lysine 18 of histone H3 (H3-K18) in mutant and wild type strains by ChIP. We selected a representative set of Tup1-repressed promoters, shown by ChIP to be directly controlled by Tup1 (our unpublished results), that included examples from each of the five subclasses defined by our microarrays. *MFA1* and *MAL12* represent genes derepressed upon deletion of Tup1, but whose expression is not significantly affected by either an *HDA1* or *SRB10* disruption. *HXT16* is derepressed in only the *tup1Δ* and *hda1Δ* strains, while *HSP12* is derepressed in only the *tup1Δ* and *srb10^{D304}* strains. Finally, *CYC7* and *SPI1* are derepressed in all three mutant strains (Figure 4B).

In the *tup1Δ* strain, all of the promoters we examined were transcriptionally derepressed and hyperacetylated at H3-K18 compared to a wild type strain (Figure 4A,C). We next examined the effect of an *HDA1* deletion on H3-K18 acetylation at Tup1-controlled genes. In the experiments shown in Figure 4C, all of the Tup1-

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controlled promoters we tested are hyperacetylated at H3-K18 in an *hda1Δ* strain compared to a wild type strain. However, this hyperacetylation does not correlate with the derepression of these genes in an *hda1Δ* strain (Figure 4B). The observation that some promoters (i.e. *MFA1*, *HSP12*, and *MAL12*) can be hyperacetylated at H3-K18 but still remain transcriptionally repressed by Tup1, suggests that an additional Tup1-mediated repression mechanism is at work at these promoters. It cannot be the mechanism defined by Srb10 because only a few of these genes are derepressed when its kinase function is disrupted. Thus, it appears that Hda1 is functioning at most (if not all) promoters directly repressed by Tup1 as part of a multi-component repression mechanism that can maintain significant transcriptional repression even when one arm of the machinery has been disrupted.

We tested whether Hda1 is the only deacetylase responsible for the hyperacetylation we observed at Tup1-repressed promoters. ChIP experiments measuring acetylated H3-K18 in a *tup1Δhda1Δ* double deletion strain showed no increase in the level of hyperacetylation at these promoters in the double mutant versus a single *tup1Δ* mutant strain. This confirms that the H3-K18 hyperacetylation resulting from a Tup1 deletion is dependent on Hda1 and that Hda1 is acting in concert with Tup1 at these promoters to produce repression.

Finally, we analyzed a gene that is indirectly controlled by Tup1, *FIG1*, to demonstrate that hyperacetylation at H3-K18 is a result of the loss of Tup1 (and consequently Hda1) rather than an increase in transcription (our unpublished results and (Erdman, Lin et al. 1998). The *FIG1* promoter is not hyperacetylated at H3-K18 in either the *tup1Δ* or *hda1Δ* strains compared to a wild type strain despite the fact that its expression is induced in the *tup1Δ* mutant (Figure 4A). Therefore, we conclude the hyperacetylation at H3-K18 that we observe at other genes is likely due to a specific loss of deacetylase activity rather than an indirect result of increased transcription.

Discussion

The Tup1-Ssn6 complex is a general repressor of transcription in *Saccharomyces cerevisiae* that is recruited to hundreds of promoters through association with sequence-specific DNA-binding proteins. Several repetitions of expression microarrays and statistical analysis of the results have allowed us to compile a more complete list of genes repressed by the Tup1-Ssn6 complex. This analysis takes advantage of the improvements in microarray technology and analysis since the original publication of the *tup1Δ* mutant study and has added nearly 200 genes (Table 1) to that earlier list of Tup1-controlled genes. The newly assigned Tup1-controlled genes fall into roughly the same functional categories as those of the previous list of Tup1-controlled genes and, therefore, we believe this represents an expansion of the set of Tup1-repressed genes rather than the identification of new networks of genes. While uncharacterized genes still represent the largest portion of Tup1-repressed genes, we also saw an abundance of genes involved in cellular metabolism, membrane transport, and cell wall organization. Within the category of transport, for instance, are transporters of everything from glycerol and sugars to water and ferrochromes. The fact that genes coding for proteins of such diverse specificities and sensitivities are all repressed by Tup1 reflects its role in mediating the cell's adaptive response to the external environment.

In this paper we address several aspects of the mechanism of Tup1-mediated repression subsequent to promoter recruitment by disrupting known components of the repression machinery. Careful, statistically based microarray analysis has also allowed us to compare the effects of a Tup1 deletion to those observed upon disruption of two previously described Tup1-repression mechanisms. First, the expression microarrays clearly reflect each protein's overall role in transcription. Tup1's role as a transcriptional repressor is made obvious by the fact that >94% of

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the genes whose expression is significantly altered in a *tup1Δ* strain are upregulated. Similarly, Hda1 seems to act primarily as a transcriptional repressor. In fact, no genes (save *HDA1* itself) are significantly downregulated in the *hda1Δ* strain (Table 3). In contrast to Hda1 and Tup1, Srb10 positively affects the expression of a substantial number of genes in addition to its role as a transcriptional repressor.

Second, we can examine the extent of the functional overlap between Tup1 and the two mechanisms represented by Hda1 and Srb10. The Hda1-repressed genes are almost entirely included within the set of genes repressed by Tup1. Conversely, deletion of *HDA1* significantly affects the expression of less than a third of Tup1-repressed genes. These data suggest that Hda1's main role in transcriptional regulation is to function with Tup1, while Tup1-mediated repression at most genes is not solely dependent on Hda1 function. Approximately 16% of Tup1-repressed genes are significantly derepressed by inactivation of the Srb10 kinase, and similarly most of the Srb10-repressed genes are not affected by a *TUP1* deletion. These results indicate Srb10 plays roles in both Tup1-dependent and Tup1-independent transcriptional repression. Our microarray comparisons identified five subclasses of Tup1-controlled genes defined by their dependence (or lack thereof) on Hda1 and Srb10 function (Figure 2). Some Tup1-repressed genes are responsive to either of the mechanisms involving Srb10 and Hda1, but there are also genes (~10% of the total set of Tup1-repressed genes) that are only derepressed when both mechanisms are disrupted, indicating both of these mechanisms are at work at these promoters.

The substantial overlap observed between the genes repressed by Hda1 and those repressed by Tup1 raises several issues about the role of deacetylation in repression. Two sets of Hda1-affected genes can be defined. Previous work identified a set of genes whose promoters become hyperacetylated at H3-K18 upon deletion of *HDA1* (Robyr, Suka et al. 2002). The experiments in our work describe a

set of genes that are derepressed upon deletion of *HDA1*, which constitutes only a subset of the hyperacetylated genes. Seventy-three percent of the Hda1-repressed genes are also repressed by Tup1, whereas only a minority of the genes deacetylated by Hda1 is also regulated by Tup1. This difference suggests that, while there could be other roles for Hda1-mediated H3-K18 deacetylation in addition to transcriptional repression, at promoters for which Hda1-mediated deacetylation is a requirement for repression, Tup1 is responsible for that repression.

This distinction between Hda1-mediated deacetylation and Hda1-mediated repression is further supported by our ChIP experiments. We saw no correlation between *hda1Δ*-dependent transcriptional derepression and *hda1Δ*-dependent H3-K18 hyperacetylation when examining the acetylation of H3-K18 at various genes in the five subclasses of Tup1-repressed genes. While deletion of *TUP1* always resulted in both hyperacetylation and increased expression, deletion of *HDA1* always caused hyperacetylation but did not always lead to increased transcription. It seems likely that Hda1-mediated deacetylation is one of several, complementary mechanisms working to repress transcription at Tup1-controlled promoters (Figure 5). The concept of multiple factors converging at Tup1 to produce repression has been proposed before and seems to fit well with the regulatory requirements of a repressor of diverse gene sets (Carlson 1997; Lee, Chatterjee et al. 2000; Papamichos-Chronakis, Conlan et al. 2000; Smith and Johnson 2000; Schreiber and Bernstein 2002).

We attempted to identify traits of Tup1-controlled genes that might dictate which mechanisms are important for transcriptional repression at that gene. First, we examined whether control by a certain sequence-specific DNA-binding protein correlated with one mechanism or another. Genes controlled by a common DNA-binding protein are found in all subclasses of Tup1-repressed genes, and therefore do not appear to be subject to a particular repression mechanism simply because of

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their shared regulation. The position of the genes in the genome also had little predictive value for placing a gene in a particular subclass. However, we did observe a general bias in the occurrence of Tup1-repressed genes in these subtelomeric regions. It is possible that proximity to a telomere somehow facilitates the establishment or maintenance of Tup1-repression; however, most Tup1-controlled genes are found at internal chromosomal positions and repression is maintained there as well. Recent work describing the synteny among closely related *Saccharomyces* species suggests an intriguing explanation for the propensity of Tup1-repressed genes to be found in subtelomeric regions. Kellis et al. (2003) note that the remarkably conserved synteny between these genomes breaks down close to the telomeres, which appear to be areas of rapid genomic evolution. The authors also point out the occurrence of several large gene families in these regions and even mention some that are repressed by Tup1 (the *HXT*, *FLO*, *PAU*, and *THI* families). It is possible that Tup1's subtelomeric bias began as a few Tup1-repressed genes found within this region of genomic flexibility that then expanded into evolutionarily advantageous gene families while maintaining their Tup1-conrolled gene expression.

Another possibility for a characteristic of Tup1-controlled promoters that could influence which repression mechanisms are important at a particular gene is the composition of the general transcriptional machinery regulating expression at that promoter. For example, Basehoar et al. and Huisinga et al. (2004; 2004) identified a set of genes (~10% of the genome) whose regulation is dominated by the SAGA complex rather than the TFIID complex and showed that Tup1-controlled genes disproportionately fall into this category. However, all five subclasses of Tup1-repressed genes we describe in this paper exhibit this same propensity for SAGA-dominated transcriptional regulation, so while inclusion in this group does seem to be a characteristic of Tup1-repressed genes, it does not appear to dictate the influence of a particular repression mechanism.

Another model, proposed by Edmundson et al. (1996), is that Tup1 interacts directly with histones to repress transcription. However, recent work has demonstrated effective repression by a Tup1 protein lacking most of the described histone-binding domain (Zhang, Varanasi et al. 2002). In addition, our own initial microarray analysis of a strain containing this internally deleted Tup1 allele (Δ129-282aa) revealed no affect on Tup1-mediated repression (our unpublished results). Similarly, microarrays of a strain with the histone H3 tail deleted (Δ1-28) show that the tails are important for the repression of a large set of genes, but that only a minority of these are repressed by Tup1 (Sabet, Tong et al. 2003).

Another recent report linked repression of a-specific genes, a Tup1-repressed set of genes, to a chromatin remodeling complex, Isw2-Itc1 (Ruiz, Escribano et al. 2003). However, derepression in an Itc1 deletion is not complete, and microarrays of an isw2Δ strain do not demonstrate this loss of repression for the larger set of Tup1-controlled genes (Hughes, Marton et al. 2000). It seems that this mechanism does not apply to the broader set of all Tup1-repressed genes. Finally, it is possible that the presence at a promoter of the Tup1-Ssn6 complex (which measures ~450kDa) could itself be sufficient to generate a significant degree of repression simply by interfering with transcriptional initiation conditions (Varanasi, Klis et al. 1996; Redd, Arnaud et al. 1997). This could be the mechanism responsible for the ability of the Tup1-Ssn6 complex to repress transcription initiation on a naked DNA template and could constitute the missing repression mechanism. However, recent work shows Tup1 remains bound to some promoters even under inducing conditions, seemingly discounting this idea, although it is not know from these studies whether the complex remains intact (Papamichos-Chronakis, Petrakis et al. 2002; Proft and Struhl 2002; Mennella, Klinkenberg et al. 2003).

We believe the work presented here substantially clarifies the picture of Tup1-mediated transcriptional repression. Previous work concentrated on the analysis of a

few carefully selected promoters and sometimes resulted in conflicting conclusions. Two basic mechanisms arose from this work, one based on the deacetylation of histones and the other involving the mediator, although the degree of intersection between the models was never established. We have provided a comprehensive, genome-wide picture of how these two mechanisms affect all Tup1-mediated repression. Much like the emerging picture of transcriptional activation, transcriptional repression appears to be more complicated than previously appreciated. Of the 334 Tup1-repressed genes identified in this study, few were fully derepressed (as measured by microarray) by the simultaneous disruptions of *HDA1* and *SRB10*. Moreover, again by microarray analysis, full levels of repression for more than half of Tup1-controlled genes are maintained even when these two repression mechanisms are disrupted. Our experiments point to a model of Tup1-mediated repression that is the result of several, functionally overlapping mechanisms whose relative importance for overall repression varies at different genes.

Materials and Methods

Yeast Strains. The *Saccharomyces cerevisiae* strains used in this paper were all generated from a parental strain of genotype MATα *ura3-52, lys2-801^amb, ade2-101^och, leu2-Δ1, his3-Δ200, trp1-Δ1,* which was a descendent of the original S288c strain. SGY201 (*srb10^D304*) was made by transforming a full-length ORF fragment containing the mutation into a strain in which the *SRB10* locus has been replaced with *URA3*, leaving about 200bp of ORF homology on either side. Growth on 5-FOA selected for a strain in which the mutated *SRB10* ORF had been integrated at the *SRB10* genomic locus. SGY160 (*hda1Δ*), SGY84 (*tup1Δ*), and SGY203 (*srb10^D304 hda1Δ*) were constructed by transforming the parental strains with PCR products of the *TRP1* gene flanked by homologous sequences of the appropriate

target gene. SGY167 (*tup1Δhda1Δ*) was made by crossing SGY160 and SGY83

(MATa *tup1Δ*) and SGY205 (*srb10^{P304}tup1Δhda1Δ*) were made by repeating the

protocol for mutating *SRB10* described above but using SGY167 as the starting

strain. The gene knock outs we made resulted in *TRP1* strains so we replaced the

trp1-Δ1 mutation with *TRP1* in SGY201 and the wild type strain used in the

microarrays (SGY92) so that all strains were matched for auxotrophies.

Microarrays and SAM Analysis. Microarrays of cDNA ORFs (~6100 spots) were

performed as previously described

(http://derisilab.ucsf.edu/microarray/protocols.html). Briefly, mRNA was prepared

from each strain and either labeled with Cy5 dye or mixed with the other mRNA

samples and labeled with Cy3 dye to make a reference sample. Each microarray was

hybridized with a mixture of a Cy5-labeled mRNA sample and a Cy3-labeled

reference sample. After scanning on a GenePix4000A scanner, arrays were analyzed

with GenePix 3.0 software. The data were normalized and filtered (sum of the

median signal intensity $\geq$1000) using NOMAD (http://derisilab5.ucsf.edu/NOMAD),

and each spot's signal ratio (ratio of the median signal intensities) in the mutant

strains was divided by its signal ratio in the wild type control. Each of the

microarrays was done four times (from independently grown cultures), except for the

tup1Δ microarrays, which were done seven times (data available as Supplemental

Tables 1 and 2). The set of repeats for each strain was then analyzed by SAM using

the One-Class Response and Row Average settings and the default Random Number

Seed (1234567). Twenty-four permutations (the complete set for four repeats) were

done for all mutant datasets, except *tup1Δ* upon which 5000 permutations were

performed. The delta values for all datasets except *hda1Δ* were selected as the

value that resulted in the lowest FDR calculated for the 90th percentile *d*-scores. The

FDRs for the *hda1Δ* data were higher in general than those of the other mutants

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most likely because of the relatively low levels of changes in expression in the dataset as a whole. The plot of the SAM analysis for the *hda1Δ* data suggested an alternative delta value selection. *HDA1* was the only gene substantially downregulated in the SAM Plot of the *hda1Δ* data, so we used its sole inclusion in the set of significantly downregulated genes as the criterion for selecting the delta value. We chose the smallest delta value that still excluded any other gene from the set of downregulated genes. This added 33 genes to the set of significant genes for the *hda1Δ* strain (and only increased the FDR by four percentage points) compared to the set of genes resulting from the delta value corresponding to the lowest FDR. The degree of overlap between the significant genes for the *tup1Δ* strain and the sets of significant genes for the *hda1Δ* strain using these two delta values was essentially unchanged, so we elected to allow for a slightly higher FDR and to use the larger *hda1Δ* significant gene set.

Chromatin Immunoprecipitation and Quantitative PCR. Antibodies against acetylated lysine 18 of histone H3 were purchased from Upstate Cell Signaling (Cat. No. 07-354). Cultures were cross-linked with formaldehyde for five minutes and ChIPs were performed with slight modifications as previously described (Strahl-Bolsinger, Hecht et al. 1997). Extract from 50-100mls of culture at OD_{600} ~1 was used for each IP. Extracts were sonicated ten times for twelve seconds using a Branson Sonifier 450 at 50% output power. ChIPs were quantitated by PCR (Q-PCR) in a DNA Engine Opticon machine (MJ Research). PCR products were between 200-400bp. Input ratios were calculated for each mutant strain versus wild type α-cells to normalize the amount of total DNA added to each IP. The amount of immunoprecipitated DNA in each IP was normalized for input and then divided by the amount measured in an IP of the wild type α-strain to produce a relative level of enrichment for each mutant. Q-PCR reactions were done at least twice for each gene

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from a particular ChIP experiment. Enrichment levels for each analyzed gene are averages of data from 2-4 independent ChIPs. Enrichment for a-specific genes was calculated separately for the two cell types, but for all other non-cell type controlled genes data for both a- and α-cells were averaged.

Supplemental Tables can be found on the Mollecular Biology of the Cell website (http://www.molbiolcell.org)

Table 1

GO classifications for Tup1-Regulated Genes

Category	Not Found in Derisi et al. set		Included in Derisi et al. set	
	#	%	#	%
Unknown	79	41	56	39
Metabolism	34	18	21	15
Transport	23	12	25	18
Mating/Meiosis	16	8	13	9
Cell Wall	5	3	7	5
Stress Response	9	5	9	6
Transcription	10	5	1	1
Kinase	3	2	2	1
Signal Transduction	3	2	1	1
Miscellaneous	10	5	7	5
Total	192		142	

Table 2

Some Previously Unassigned Tup1-Regulated Genes

ORF	Gene Name	Molecular Function	
YOR348C	PUT4	amino acid permease activity / neutral amino acid transporter activity	
YDL181W	INH1	enzyme inhibitor activity	
YNR060W	FRE4	ferric-chelate reductase activity	
YOL156W	HXT11	fructose transporter activity / galactose transporter activity / glucose transporter activity / mannose transporter activity	
YMR011W	HXT2	fructose transporter activity / glucose transporter activity / mannose transporter activity	
YKR039W	GAP1	general amino acid permease activity	
YFL054C		glycerol transporter activity / transporter activity / water channel activity	
YPL134C	ODC1	intracellular transporter activity / organic acid transporter activity	
YKL217W	JEN1	lactate transporter activity	
YDR534C	FIT1	molecular_function unknown	**Transport**
YOR382W	FIT2	molecular_function unknown	
YOR306C	MCH5	monocarboxylic acid transporter activity / transporter activity	
YKL221W	MCH2	monocarboxylic acid transporter activity / transporter activity	
YCR098c	GIT1	phospholipid transporter activity	
YHL040C	ARN1	siderochrome-iron transporter activity	
YGL121C	GPG1	signal transducer activity	
YLR237W	THI7	thiamin transporter activity	
YOR192C		transporter activity	
YDR536W	STL1	transporter activity	
YOL162W		transporter activity	
YGR289C	MAL11	trehalose transporter activity	
YIL107C	PFK26	6-phosphofructo-2-kinase activity	**Metabolism**
YIL160C	POT1	acetyl-CoA C-acyltransferase activity	
YNR058W	BIO3	adenosylmethionine-8-amino-7-oxononanoate aminotransferase activity	
YOR374W	ALD4	aldehyde dehydrogenase (NAD+) activity	
YGR292W	MAL12	alpha-glucosidase activity	
YFL056C	AAD6	aryl-alcohol dehydrogenase activity	
YIR039C	YPS6	aspartic-type endopeptidase activity	
YNL015W	PBI2	endopeptidase inhibitor activity	
YPL276W	FDH2	formate dehydrogenase activity	
YCL040w	GLK1	glucokinase activity	
YIL172C		glucosidase activity	
YIR038C	GTT1	glutathione transferase activity	
YLL060C	GTT2	glutathione transferase activity	

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YJL052W	TDH1	glyceraldehyde 3-phosphate dehydrogenase (phosphorylating) activity	
YLR258W	GSY2	glycogen (starch) synthase activity	
YFR053C	HXK1	hexokinase activity	
YMR095C	SNO1	imidazoleglycerol phosphate synthase activity / protein binding	
YNL334C	SNO2	imidazoleglycerol phosphate synthase activity / protein binding	
YFL060C	SNO3	imidazoleglycerol phosphate synthase activity / protein binding	
YMR120C	ADE17	IMP cyclohydrolase activity / phosphoribosylaminoimidazole-carboxamide formyltransferase activity	
YLR178C	TFS1	lipid binding / protease inhibitor activity	
YMR135C	GID8	molecular_function unknown	
YFL058W	THI5	molecular_function unknown	
YDL244w	THI13	molecular_function unknown	
YMR096W	SNZ1	molecular_function unknown / protein binding	
YNL274C		Oxidoreduct ase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	
YMR105C	PGM2	phosphoglucomutase activity	
YDL021W	GPM2	phosphoglycerate mutase activity	
YPR121W	THI22	phosphomethylpyrimidine kinase activity	
YNL333W	SNZ2	protein binding	
YFL059W	SNZ3	protein binding	
YJR156C	THI11	protein binding	
YKR053C	YSR3	sphingosine-1-phosphate phosphatase activity	
YGR032W	GSC2	1,3-beta-glucan synthase activity	
YDL049C	KNH1	molecular_function unknown	
YKR076W	ECM4	molecular_function unknown	**Cell Wall**
YPR030W	CSR2	molecular_function unknown	
YDR077W	SED1	structural constituent of cell wall	
YGR088W	CTT1	catalase activity	
YDL022w	GPD1	glycerol-3-phosphate dehydrogenase (NAD+) activity	
YFL014W	HSP12	heat shock protein activity	
YCR021c	HSP30	heat shock protein activity	
YMR173W	DDR48	molecular_function unknown	
YOL053CA	DDR2	molecular_function unknown	**Stress Response**
YMR175W	SIP18	phospholipid binding	
YEL060c	PRB1	serine-type endopeptidase activity	
YIL101C	XBP1	transcription factor activity	
YML054C	CYB2	L-lactate dehydrogenase (cytochrome) activity	
YEL039c	CYC7	electron carrier activity	

Table 3

Genes Deemed Significant by SAM

	# of Significant Genes			FDR [*]
	Total	Upregulated	Downregulated	%
tup1Δ	354	334	20	0.12
hda1Δ	133	132	1	11.4
srb10^{D304}	217	166	51	0.24
srb10^{D304}hda1Δ	327	277	50	0.13

[*] False Discovery Rate calculated for 90th percentile d-scores, expressed as a percentage of the total number of significant genes

Figure 1

A

tup1Δ
334 genes

hda1Δ
132 genes

238 96 36

$hda1Δ$ $srb10^{D304}$ $srb10^{D304}hda1Δ$ $tup1Δ$

B

tup1Δ
334 genes

$srb10^{D304}$
166 genes

280 54 112

$hda1Δ$ $srb10^{D304}$ $srb10^{D304}hda1Δ$ $tup1Δ$

C

tup1Δ
334 genes

$srb10^{D304}hda1Δ$
277 genes

205 129 148

$hda1Δ$ $srb10^{D304}$ $srb10^{D304}hda1Δ$ $tup1Δ$

-64 -32 -16 -8 -4 -2 1 2 4 8 16 32 64

Fold Change:
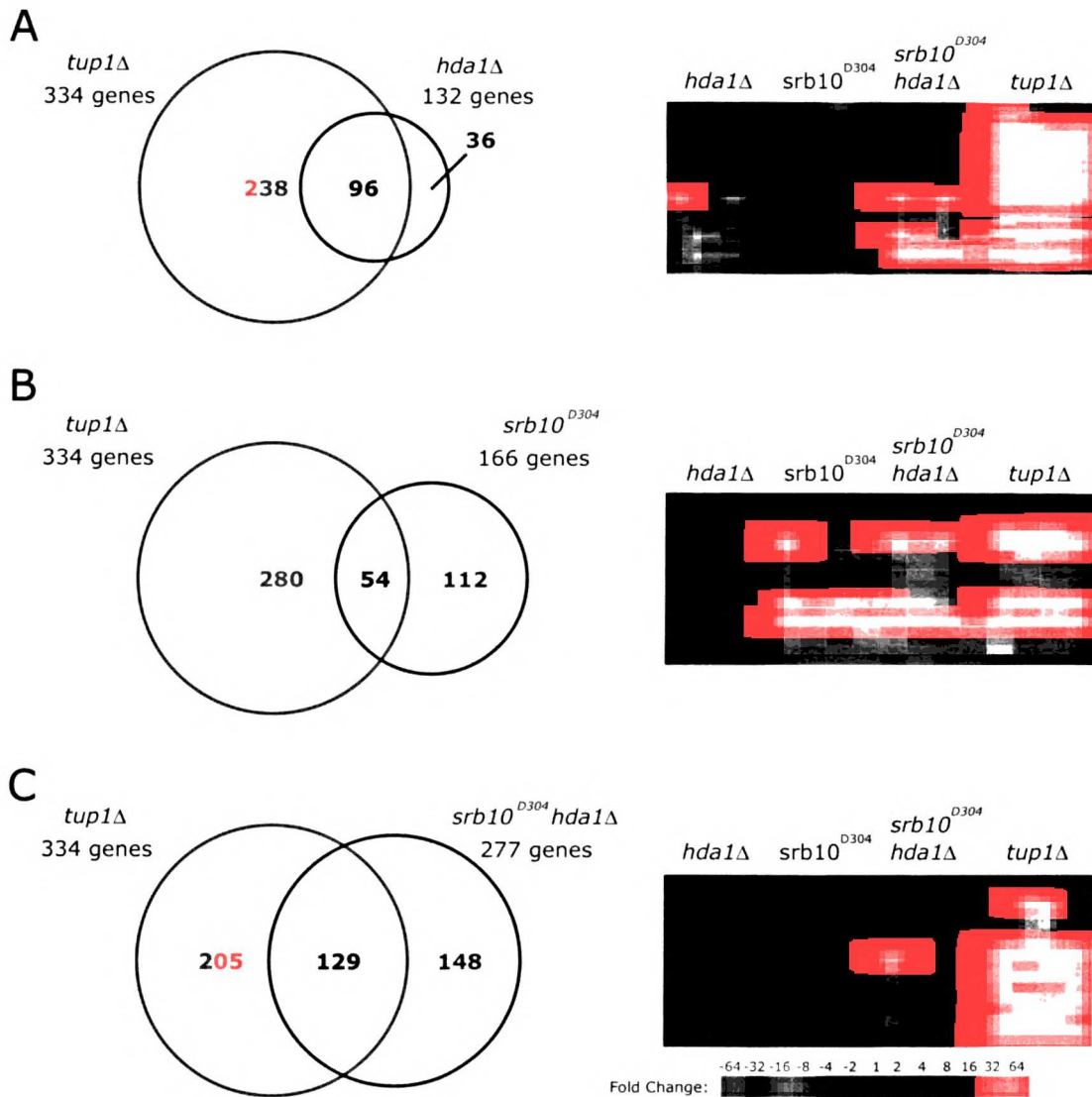
Figure 1. *Pairwise Comparison of Derepressed Genes in tup1Δ, hda1Δ, srb10^{D304}, and srb10^{D304}hda1Δ strains.*

The area of the circles in the Venn diagrams are proportional to the number of significantly derepressed genes for each strain and depict the overlap of the genes for the corresponding pairs of strains: *tup1Δ* vs. (A) *hda1Δ*, (B) *srb10^{D304}*, and (C)

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srb10^{D304}hda1Δ. Numbers below strain names are the total number of significantly derepressed genes in that strain, and numbers within the Venn diagram reflect the number of genes that fall into that category. Cluster diagrams show representative genes (selected for those with data for each replicate of each strain) falling into the corresponding overlaps of datasets. Red represents an increase in gene expression and green represents a decrease in gene expression compared to a wild type strain. Lists of the significantly derepressed genes in each mutant strain are available as Supplementary Table 3.

Figure 2
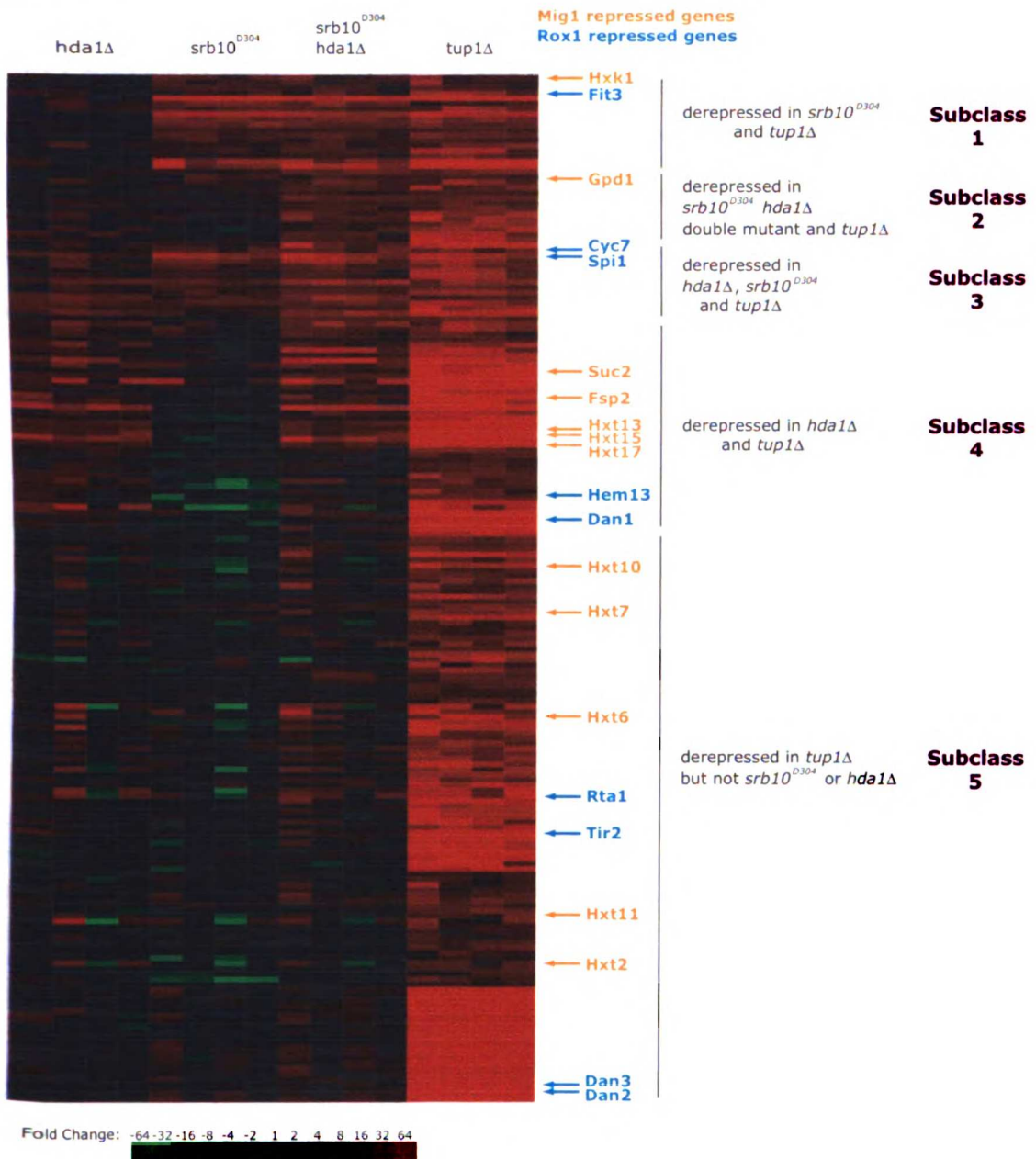
Figure 2. *Distribution of Mig1- and Rox1-Repressed Genes Across Subclasses of Tup1-Repressed Genes*

Four independent microarrays are shown for each strain; displayed are all significantly upregulated genes in the *tup1Δ* microarray that had data for all sixteen

arrays represented; genes were clustered by their d-scores calculated by SAM. Five subclasses of Tup1-repressed genes are displayed representing genes derepressed in: (1) $tup1\Delta$ and $srb10^{D304}$, (2) $tup1\Delta$ and $srb10^{D304}hda1\Delta$, (3) $tup1\Delta$, $hda1\Delta$, $srb10^{D304}$, and $srb10^{D304}hda1\Delta$, (4) $tup1\Delta$ and $hda1\Delta$, and (5) $tup1\Delta$ only. Mig1- and Rox1-controlled genes for which there is some evidence of direct regulation are identified by their gene names. Red and green colors represent an increase and a decrease in expression respectively compared to a wild type strain.

Figure 3

A

	Total genes at telomeres	Total genes outside of telomeres	% at telomeres
tup1Δ	99	235	30
hda1Δ	40	92	30
srb10^{D4}	10	156	6
srb10^{D4} hda1Δ	52	225	19
Whole Genome	340	5374	6

*genes at telomeres defined as ORFs < 25 kb from a chromosome end

C

	% of Tup1-controlled genes:	
	at telomeres	all genes
hda1Δ	35	29
srb10^{D4}	9	16
srb10^{D4} hda1Δ	47	39

B

tup1Δ 99 genes — hda1Δ 40 genes: 65 | 34 | 6

tup1Δ 99 genes — srb10^{D304} 10 genes: 90 | 9 | 1

tup1Δ 99 genes — srb10^{D304} hda1Δ 52 genes: 55 | 44 | 8

Figure 3. *Overlap of Repressed Subtelomeric Genes*

(A) Table shows the total number of significantly derepressed genes both within and outside of subtelomeric regions (≤ 25kb from a chromosome end) for each mutant strain.

(B) Venn diagrams depicting the overlap between only the subtelomeric genes that are significantly derepressed in *tup1Δ* and each mutant strain.

(C) Table shows the percentage of the total set of Tup1-repressed genes either within or outside of subtelomeric regions that are shared by the corresponding mutant strain.

Figure 4

B — Derepressed in:

Promoter	tup1Δ	hda1Δ	srb10^{D304}	srb10^{D304} hda1Δ
ACT1	no	no	no	no
FIG1	yes	no	no	no
MFA1	yes	no	no	no
MAL12	yes	no	no	no
HXT16	yes	yes	no	no
SPI1	yes	yes	yes	yes
CYC7	yes	yes	yes	yes
HSP12	yes	no	yes	yes
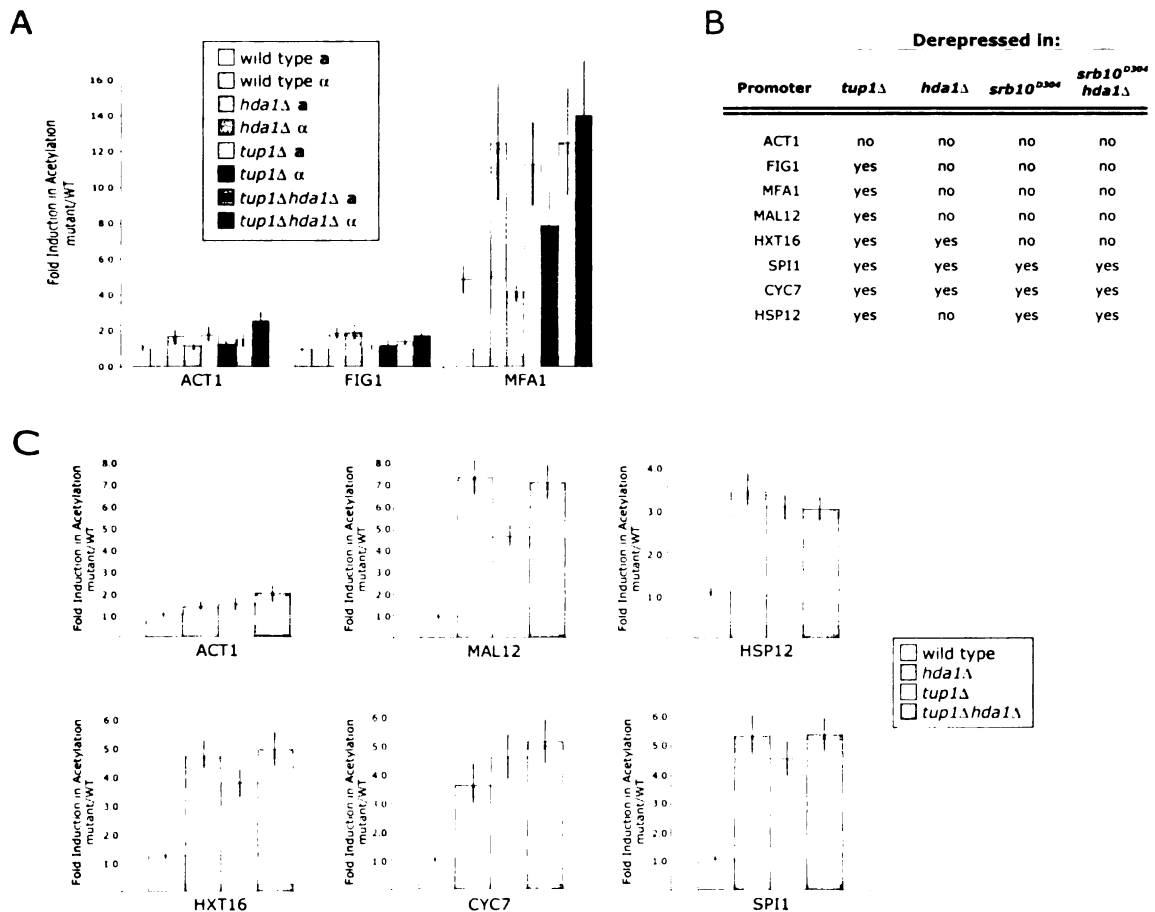
Figure 4. *Acetylation of Histone H3 at Tup1-Repressed Genes*

Chromatin IPs were carried out for acetylated K18 of histone H3. The *y*-axis represents the fold increases in acetylation in a mutant strain compared to a wild type strain. Bars represent the average of at least six repeats in (A) and 12 repeats in (C); data for (C) includes measurements for both mating types. Error bars reflect the standard error calculation for the averaged data.

Table in (B) describes the effect of each of the mutations on the expression of the genes tested by ChIP.

Figure 5

54 genes, i.e. Spi1, Cyc7:
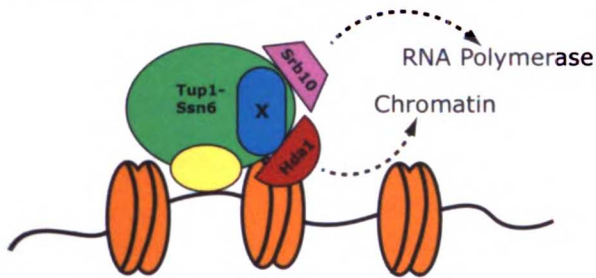
32 genes, i.e. Hsp12:
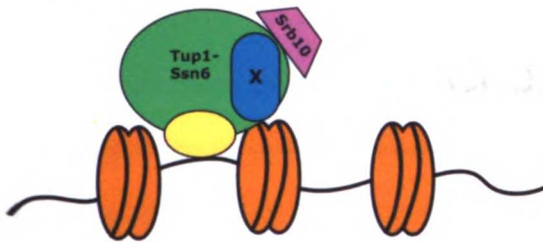
74 genes, i.e. Hxt16:
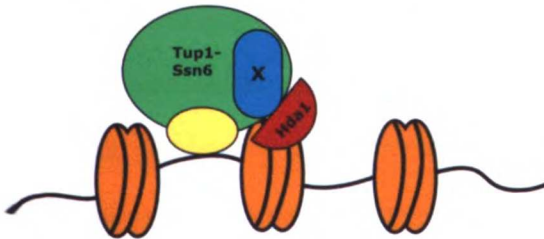
205 genes, i.e. Mal12, Mfa1:
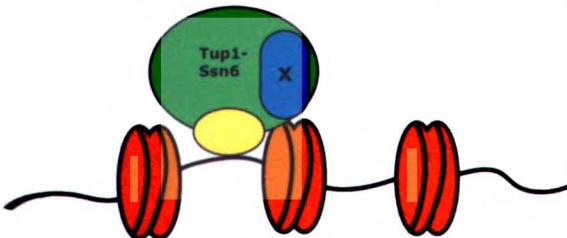
Figure 5. *Model of Multiple Tup1-Mediated Repression Mechanisms*

This model depicts only the dependency of repression at a particular gene on Hda1 and Srb10 function, and does not imply anything about the presence of either factor at Tup1-repressed promoters. It is possible that Hda1 and Srb10 are still present at genes that are not significantly derepressed when either Hda1 or Srb10 function is disrupted. It remains to be proven whether Factor X is indeed one or more distinct components or an as yet undocumented role of Tup1 itself.

Chapter 3

Functions of a Conserved Surface of Tup1

Genome-wide Analysis of the Functions of a Conserved Surface on the Co-repressor Tup1

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Abstract

The general transcriptional repressor Tup1 is responsible for the regulation of a large, diverse set of genes in *Saccharomyces cerevisiae*, and functional homologs of Tup1 have been identified in many metazoans. The crystal structure for the C-terminal portion of Tup1 has been solved and, when sequences of Tup1 homologues from fungi and metazoans were compared, a highly conserved surface was revealed. In this paper, we analyze five point mutations that lie on this conserved surface. A statistical analysis of expression microarrays demonstrates that the mutant alleles are deficient in the repression of different subsets of Tup1-regulated genes. We were able to rank the mutant alleles of *TUP1* based on the severity of their repression defects measured both by the number of genes derepressed and the magnitude of that derepression. For one particular class of genes, the mutations on the conserved surface disrupted recruitment of Tup1 to the repressed promoters. However, for the majority of the genes derepressed by the Tup1 point mutants, recruitment of Tup1 to the regulated promoters is largely unaffected. These mutations affect the mechanism of repression subsequent to recruitment of the complex and likely represent a disruption of a mechanism that is conserved in fungi and metazoans. This work demonstrates that the evolutionarily conserved surface of Tup1 interacts with two separate types of proteins—sequence-specific DNA-binding

proteins responsible for recruiting Tup1 to promoters as well as components that are likely to function in a conserved repression mechanism.

Introduction

The Tup1-Ssn6 complex in *Saccharomyces cerevisiae* represses the transcription of over three hundred genes under standard growth conditions (DeRisi, Iyer et al. 1997; Green and Johnson 2004). In other organisms, repression by Tup1-Ssn6 homologues is essential for cellular differentiation-- specifically neurogenesis, hematopoiesis and embryonic development, and a deeper understanding of the function of Tup1-Ssn6 in *S. cerevisiae* will likely shed light on the regulation of these diverse processes in other organisms (Pflugrad, Meir et al. 1997; Fisher and Caudy 1998; Levanon, Goldstein et al. 1998; Chen and Courey 2000). Tup1 is recruited to the genes it represses through an interaction with a sequence-specific DNA-binding protein responsible for the regulation of subsets of Tup1-repressed genes. Whereas the targets of the repressor complexes in fungi and metazoans depend on the specific needs of the organisms, it is believed the mechanism of Tup1-mediated transcriptional repression is conserved (Grbavec, Lo et al. 1999; Zhang and Emmons 2002). Recent work suggests that Tup1-mediated repression is the result of the integrated contributions of distinct mechanisms (Lee, Chatterjee et al. 2000; Bone and Roth 2001; Wu, Suka et al. 2001; Zaman, Ansari et al. 2001; Robyr, Suka et al. 2002; Green and Johnson 2004; Zhang and Reese 2004).

Several domains of Tup1 have been identified and are known to have varying effects on transcriptional repression (Komachi, Redd et al. 1994; Tzamarias and Struhl 1994; Edmondson, Smith et al. 1996; Varanasi, Klis et al. 1996; Zhang, Varanasi et al. 2002). The highly conserved WD domain of Tup1 in particular has been shown to be sufficient for partial transcriptional repression. The well-defined sequences of the degenerate WD repeat are found in many proteins of diverse

functions and are typically sites of protein-protein interactions (Neer, Schmidt et al. 1994; Garcia-Higuera, Fenoglio et al. 1996; Smith, Gaitatzes et al. 1999). The structural residues of the WD repeat necessary for folding are conserved, but much of the additional sequence in the repeat is varied, giving each WD protein a unique surface (Neer, Schmidt et al. 1994). Sprague *et al.* solved the crystal structure of the Tup1 WD domain (282-713aa of Tup1) and showed that this fragment assumed the propeller-like ring-shape typical of WD domains in other proteins (Figure 1A). In addition, these authors also described a high degree of amino acid conservation among fungi on one surface of the structure. This level of conservation suggested a vital, evolutionarily maintained function common to all fungal Tup1 homologues.

In this paper, we examine the function of the highly conserved surface of Tup1 by analyzing five point mutations made within this region. We analyzed the genome-wide effects of these point mutations using a statistical analysis of transcriptional microarray data and show that the mutations affect different subsets of Tup1-repressed genes and represent a spectrum of repression deficiencies, none of which are as severe as a *TUP1* deletion. Further analysis, including chromatin IP (ChIP) experiments, demonstrates that this conserved surface participates in both the interaction of Tup1 with sequence-specific DNA-binding partners and with the proteins participating in Tup1-mediated repression mechanisms.

Results

Constructing Mutant Alleles of *TUP1*

The C-terminal portion of Tup1 contains seven WD repeats that form a characteristic propeller-like structure (Figure 1A). As described by Sprague *et al.*(2000), the mapping of other fungal Tup1 homologues onto this structure revealed a strikingly conserved surface on one side of the propeller. When sequences of Tup1 homologues from more divergent organisms (*Drosophila, Xenopus, C. elegans*, and

mice) were added to this alignment much of the conservation at this surface was maintained (data not shown). It seems likely that this surface of Tup1 is involved in a highly conserved set of interactions, and in this study we tested the functions of this surface in *S. cerevisiae*. We selected amino acids in the conserved surface of *S. cerevisiae* for mutation based on three criteria: (1) they are conserved in our expanded alignment of metazoan homologues of Tup1, (2) they are solvent exposed and, as judged by the crystal structure, are not required for maintaining the structure of the protein, and (3) they are amino acids that are overrepresented at sites of protein-protein interactions, as determined in a study of published mutational analyses (Bogan and Thorn 1998). We chose five residues of Tup1 for mutagenesis to alanine, shown for convenience on a single molecule in Figure 1A. We constructed isogenic strains that had the individual mutant alleles of *TUP1* integrated at the genomic *TUP1* locus and verified that each mutant protein was stable and expressed at levels comparable to that of the wild type Tup1 protein (Figure 1B). A region of Tup1 necessary for interaction with Ssn6 and complex formation has been localized outside of the domain used in the crystallization experiments that informed our selection of candidate residues for mutagenesis (Tzamarias and Struhl 1994; Varanasi, Klis et al. 1996). From this observation and preliminary biochemical experiments that show our mutants interacting with Ssn6, we conclude that these five point mutants do not disrupt Tup1-Ssn6 complex formation (data not shown).

Microarray Analysis of Tup1 Point Mutants

To understand the impact of these point mutants on Tup1-mediated repression, we analyzed globally each of the mutant strains using expression microarray analysis. For each point mutant and an isogenic wild type strain, we carried out six independent microarray hybridizations and analyzed the data using the Significance Analysis of Microarrays (SAM) methodology (Tusher, Tibshirani et al.

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2001). In brief, SAM ranks each gene based on the magnitude of the change in expression and the reproducibility of that measurement in duplicate experiments. SAM also estimates the number of false positives (the false discovery rate or FDR) in a set of genes corresponding to a selected threshold of significance. We chose a threshold of significance for each of our datasets that yielded the lowest FDR for that dataset, thus representing the most stringently selected set of regulated genes. Figure 2A lists the total numbers of significantly regulated genes in each of the Tup1 point mutant datasets. Each set typically has less than one predicted false positive and, as expected for mutations affecting transcriptional repression, the overwhelming majority (>93%) of the genes in each set are upregulated, implying a loss of negative regulation. The few downregulated genes generally have small magnitudes of changes in expression, and we believe this downregulation represents mostly indirect effects of Tup1-mediated repression.

We compared the significantly derepressed genes identified for each Tup1 point mutant dataset to determine the degree of overlap between them. We focused our analysis on genes that earlier microarray experiments identified as targets of regulation by Tup1 (Green and Johnson 2004). $tup1^{R447A}$ showed the strongest effects (the largest set of derepressed genes and the highest levels of derepression) and we saw significant overlap of all the other point mutant datasets with the set of genes derepressed by $tup1^{R447A}$ (Figure 2B). Moreover, there was considerable overlap between the datasets from any two of the other mutants (data not shown). The weakest effects overall in the magnitude of derepression are seen in the $tup1^{D443A}$ mutant, consistent with the small number of genes affected overall in this mutant. These findings suggest the five mutations can be arranged in a hierarchy and that they share at least one common defect but to varying degrees.

The set of Tup1-repressed genes can be divided into four categories based on the effects of the mutants on their expression (Figure 2C). About half of the Tup1-

repressed genes (53%) maintain wild type levels of repression in each of the point mutant strains, as measured by microarray analysis. A smaller subset of genes is derepressed to some degree in all of the point mutants. A third set of genes is derepressed in the $tup1^{R447A}$, $tup1^{Y489A}$, and $tup1^{E463A}$ mutant strains but is unaffected by the other two point mutants. Finally, a fourth group of Tup1-repressed genes is significantly derepressed only in the $tup1^{R447A}$ mutant. In general, the magnitudes of changes in expression caused by the point mutants are less than that observed when *TUP1* is deleted, suggesting that even at affected promoters the Tup1 point mutants are partially functional. However, one set of genes, the a-specific genes (and their downstream targets), is fully derepressed in the $tup1^{R447A}$, $tup1^{Y489A}$, and $tup1^{E463A}$ mutant strains (Figure 2C).

Tup1 Point Mutants Are Properly Recruited to Derepressed Genes

To accurately regulate the full set of its target genes, Tup1 must be recruited to the proper promoters. Point mutants that have a defect in Tup1-mediated repression may reflect (1) the inability of the Tup1 mutant to interact with its sequence-specific DNA-binding partners (a defect in recruitment to promoters) or (2) an inability to interact with the repression machinery once recruited to a regulated promoter. To test whether the repression defects of the Tup1 point mutants are due to a defect in recruitment, we used ChIP experiments to monitor the presence of Tup1 at regulated promoters in a wild type strain and in the Tup1 mutant strains. We selected genes that are derepressed to varying degrees by the point mutants (Figure 3A). *SUC2* is a previously known direct target of Tup1-mediated repression that was identified by SAM as significantly derepressed in only the *tup1Δ* and $tup1^{R447A}$ datasets. *HSP12* and *SPI1* are newly identified Tup1-controlled genes that were identified as derepressed by all five of the point mutants in our SAM analysis.

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Using antibodies to Tup1, we immunoprecipitated DNA from a wild type strain, a *tup1Δ* strain, and the five Tup1 point mutants and measured the amount of precipitated DNA by quantitative PCR (QPCR). The ChIP from the *tup1Δ* strain, in which no Tup1 protein is expressed, measures the background (non-specific) precipitation of DNA by the Tup1 antibodies and serves as our baseline against which to compare the amount of DNA precipitation in the other Tup1 strains. As expected, wild type Tup1 occupies the three promoters (*SUC2*, *HSP12*, and *SPI1*) well above the background levels determined in the *tup1Δ* strain (Figure 3B). Because *HSP12* and *SPI1* had not been previously shown to be direct targets of Tup1-mediated repression, we measured the amount of Tup1 bound up- and downstream of the beginnings of the open reading frames. The enrichment of Tup1 occupancy at *HSP12* and *SPI1* is indeed focused at their promoters, and we conclude that these two genes are direct targets of Tup1-mediated repression. When analyzing the ChIP data for the Tup1 point mutants, we found that all five of the Tup1 point mutants also significantly occupied the three promoters and that overall the amounts of precipitated DNA were similar to that measured by ChIP in a wild type Tup1 strain (Figure 3B). The enrichment of *tup1^{R447A}* at *SUC2* appears to be greater than that of wild type Tup1, but we do not believe this is biologically significant. In any case, this result shows that the defect in repression of *tup1^{R447A}* at *SUC2* is not due to a failure of the mutant protein to be recruited to the promoter.

As described above, most of the point mutants cause only partial derepression of affected genes. If this partial derepression was caused by small defects in Tup1 recruitment, it is possible that ChIP experiments are not sensitive enough to distinguish these defects. To address this concern, we determined whether the Tup1 point mutants were still recruited to the promoter of a gene that was fully derepressed in a Tup1 mutant strain. To find an appropriate gene for this analysis, we compared the median levels of derepression for all of the Tup1-

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regulated genes in a *tup1Δ* strain to those measured in our strongest mutant,

tup1^{R447A} (Figure 3C). For the most part, *tup1^{R447A}* either had no effect on repression

or only partially derepressed genes, the exception being the a-specific genes

discussed below. However, there were a few fully derepressed genes that were not

a-specific genes. *ZRT1*, a zinc transporter, was identified by SAM as a gene

derepressed by *tup1^{R447A}* and was derepressed to the same extent as that measured

in a *tup1Δ* strain (median increases in expression over a wild type strain of 2.4 fold

and 2.9 fold respectively). ChIP experiments measuring Tup1 recruitment to *ZRT1*

established that all of the Tup1 mutants showed the same levels of enrichment over

background at the promoter as wild type Tup1 (Figure 3D). Like *HSP12* and *SPI1*,

ZRT1 has not been shown to be a direct target of Tup1-mediated repression, so we

confirmed that the ChIP signal we measured was concentrated at the *ZRT1* promoter

specifically (Figure 3D). This concentration at the promoter of *ZRT1* is subtler than

that seen for other genes we analyzed, but as the data displayed in Figure 3D

comprises several independent repetitions of the experiments, we feel it is highly

reproducible and represents a real phenomenon.

Tup1 Point Mutants Exhibit Repression Defect Even When Artificially
Recruited to Promoters

The presence of the Tup1 point mutants at a fully derepressed gene implies

that the mutations in Tup1 do not affect recruitment. To confirm that these mutant

proteins exhibit a defect in repression independent of recruitment, we constructed

LexA fusions of the Tup1 point mutants. In these strains, Tup1 is bound directly to a

promoter containing a LexA operator via the fused LexA domain, bypassing the

requirement for recruitment by a sequence-specific DNA-binding protein. We

confirmed that the Tup1 mutant fusions could not repress a *lacZ* reporter that is

under the control of LexA operators as efficiently as a wild type Tup1-LexA fusion (Figure 4A). We then carried out ChIP experiments using antibodies directed against LexA to establish the presence of the fusion proteins at the promoter of the *lacZ* reporter. All of the mutant Tup1 fusions were indeed bound to the reporter promoter, despite their inability to fully repress expression of *lacZ* (Figure 4B). As determined by QPCR, the amounts of DNA precipitated in the ChIP experiments were the same for the wild type and mutant Tup1-LexA fusions. We performed ChIP experiments on these strains using antibodies against Tup1 itself and obtained the same result (data not shown). Thus, although the LexA fusion proteins are efficiently bound to DNA, they are deficient in Tup1-mediated repression.

Some Mutations Can Disrupt Recruitment of Tup1 to the a-Specific Genes

We saw the strongest effects of the Tup1 point mutants on gene expression for one class of genes, the a-specific genes and their downstream targets. These genes were significantly derepressed only in the $tup1^{R447A}$, $tup1^{Y489A}$, and $tup1^{E463A}$ strains. Unlike the other subsets of Tup1-repressed genes, this group was typically fully derepressed compared to a deletion of *TUP1*. We confirmed this complete loss of repression when we quantitatively measured the level of repression in the $tup1^{R447A}$ mutant at a reporter construct repressed by Matα2, the sequence-specific DNA-binding protein regulating the a-specific genes. The a-specific genes are normally on in a cells, in which Matα2 is not present, and off in α cells, which express Matα2. By comparing the levels of expression in α and a cells (repressed vs. derepressed conditions) of a gene controlled by Matα2, we can determine the level of repression of that gene (see Figure 5A figure legend). Figure 5A shows that $tup1^{R447A}$ is as defective in repressing an a-specific operator as a strain in which Tup1 is deleted (*tup1Δ*). ChIP experiments showed that at this a-specific reporter, the

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Tup1^{R447A} mutant was not properly recruited to the promoter in α cells expressing Matα2 (Figure 5B). In contrast, Matα2, the sequence-specific DNA-binding protein responsible for recruiting Tup1, was properly bound to the a-specific reporter (ChIP data not shown), so we conclude that the absence of Tup1^{R447A} from the reporter results from the inability of the mutant protein to interact with Matα2.

Discussion

The Tup1-Ssn6 complex is a conserved transcriptional repressor that is recruited to promoters by sequence-specific DNA-binding proteins. Mapping sequences of homologues of Tup1 ranging from fungi to metazoans onto the crystal structure of *S. cerevisiae* Tup1 revealed a conserved protein surface on one side of a WD repeat propeller structure. To address the function of this highly conserved surface, we targeted five surface residues in this conserved area for mutation and confirmed that all of the mutant proteins were expressed at wild type levels. All five Tup1 mutants had repression defects, but they were less severe than that of *tup1Δ*, demonstrating that all of the mutant proteins are partially functional. Statistical analysis of expression microarray data for each mutant allowed us to rank the mutations based on the severity of their repression defects. *tup1^{R447A}* emerged as the strongest mutant of Tup1 both in terms of the number of significantly derepressed genes and in the magnitude of the derepression at affected genes. The sets of significantly derepressed Tup1-regulated genes for the other mutants were almost entirely contained within the set of genes derepressed by *tup1^{R447A}*. We conclude these mutations are disrupting a common aspect of Tup1-mediated repression.

Our global analysis of the repression defects proved much more informative than the use of a single Tup1-repressed gene or a reporter as the indicator of a loss of Tup1-mediated repression. By observing the effects of the Tup1 point mutants

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simultaneously on the complete set of Tup1-regulated genes, we were able to divide the larger group of genes into subsets based on their sensitivity to particular mutations of Tup1. A large portion (53%) of Tup1-repressed genes maintains full levels of repression, as determined by microarray analysis, in all of our mutant strains. The Tup1-regulated genes that are derepressed to some degree by one or more mutants can be placed into three subsets. One subset of genes is partially derepressed in all of the mutant strains. The genes in this subset do not appear to share any common functions or to be regulated by the same sequence-specific DNA-binding protein. A second subset of genes is derepressed by $tup1^{R447A}$, $tup1^{Y489A}$, and $tup1^{E463A}$, but not by the other two mutants. Unlike the other subsets of Tup1-regulated genes, the subset affected by these three alleles is fully derepressed and is either directly or indirectly regulated by the sequence-specific DNA-binding protein Matα2. Finally, a third subset of Tup1-regulated genes is derepressed by only $tup1^{R447A}$. These genes are only slightly derepressed compared to the magnitudes of changes in expression seen in a $tup1\Delta$ strain but are considered significant by SAM analysis. The genes in this subset do not appear to share a common functional or regulatory pattern; however, many are uncharacterized genes, and it is possible that a common theme will emerge in the future.

Further characterization of the point mutants revealed two distinct defects in Tup1-mediated repression. In the majority of the cases we tested, the point mutations disrupted the repression function of Tup1 but not its recruitment to DNA, as measured by ChIP experiments. This trend held for genes that were both partially and fully derepressed in the Tup1 mutant strains. Consistent with this interpretation, the Tup1 point mutants showed repression defects even when artificially recruited to promoters through fusion to LexA. ChIP experiments indicated that all of the mutant protein fusions occupied the LexA binding site of a LacZ reporter, even though the

mutant Tup1 fusion proteins could not repress transcription as effectively as a wild type Tup1-LexA fusion.

For a small group of Tup1-repressed genes, the a-specific genes, some Tup1 mutations ($tup1^{R447A}$, $tup1^{Y489A}$, and $tup1^{E463A}$) failed to repress transcription simply because the mutant proteins were not recruited to the regulated promoters. For example, our ChIP experiments show that Tup1^{R447A} is not present at an a-specific operator even though Matα2, the sequence-specific DNA-binding protein that normally recruits Tup1, is clearly bound. We did not analyze $tup1^{Y489A}$ and $tup1^{E463A}$ in our ChIP experiments, but these two residues (Y489 and E463) were identified in a prior study of *TUP1* designed to isolate mutations specifically disrupting the interaction between Tup1 and Matα2 (Komachi and Johnson 1997). This earlier result, combined with our ChIP data and the observation that the a-specific genes are fully derepressed in the three mutants as compared to levels seen in a *tup1Δ* strain, support the conclusion that these three residues of Tup1 are critical for the interaction between Tup1 and the sequence-specific DNA-binding protein Matα2. However, our microarray analyses of $tup1^{R447A}$, $tup1^{Y489A}$, and $tup1^{E463A}$ demonstrate that these three mutations also fail to fully repress Tup1-regulated genes outside of the group of a-specific genes, confirming that the mutations cause defects in repression beyond their inability to bind Matα2. These data indicate that two functions of Tup1—recruitment to a-specific gene promoters and transcriptional repression—both lie on this conserved surface of Tup1 and that the surfaces of the protein necessary for the two functions appear to overlap (Figure 6A). A mutant allele of the *C. elegans* homologue of Tup1 (*unc-37*) corresponds to a mutation of the E463 residue in the *S. cerevisiae* Tup1 and results in severe, pleiotropic effects, suggesting a severe loss of repression function (Pickles, Roe et al. 2002). The *S. cerevisiae* Tup1 crystal structure predicts a hydrogen bond interaction between the E463 and R447, the mutation of which results in the broadest, most severe defects

discussed in this work. Thus, this surface of Tup1 is likely to carry out the same basic functions in diverse organisms.

Each Tup1-Ssn6 complex consists of four molecules of Tup1 and one molecule of Ssn6, so in the single Tup1 point mutant strains there would actually be four mutations per Tup1-Ssn6 complex formed (Varanasi, Klis et al. 1996; Redd, Arnaud et al. 1997). Therefore, a single point mutation could potentially disrupt four different interactions at a Tup1-repressed promoter. Based on this idea, it is not surprising that we see defects in multiple functions of Tup1 in our point mutant strains. For example, the same mutation could prevent the association of Tup1 with a DNA-binding protein, could disrupt interactions with components of the general transcriptional machinery, and could disrupt an interaction with a chromatin-modifying component (Figure 6B). Recent work has demonstrated that the full levels of repression at many Tup1-regulated genes requires contributions from several independent mechanisms, including mechanisms involving components of the transcriptional machinery as well as chromatin modifying factors (Smith and Johnson 2000; Green and Johnson 2004; Zhang and Reese 2004). The relative contributions of these mechanisms to full transcriptional repression vary among Tup1-regulated genes, and these gene-specific requirements could explain the gradients of repression defects we observe. We believe that this surface of Tup1, which is conserved in Tup1 homologues from distantly related species, is critical for the orchestration of these multiple mechanisms of repression acting at a single promoter. The mutations described in this work will be valuable in working out additional details of the different mechanisms of Tup1-mediated repression and in understanding how those mechanisms work together to efficiently repress transcription in *S. cerevisiae* and other organisms.

Materials and Methods

Plasmids. Plasmid pKK602 was described previously (Komachi and Johnson 1997). Plasmid pAJ201 contains two LexA operators at the SmaI site in pLGΔ312S (Guarente and Mason 1983). The a-specific gene reporter, pKK78, contains three Matα2 operator sites inserted into the SmaI site of pLGΔ312S with the selectable marker switched to *ADE2* and the 2μ sequences deleted to allow for integration. All LexA-fusion plasmids were made by inserting the full length Tup1-LexA sequence (wild type or mutated) and 800bp of sequence upstream of the *TUP1* ORF into the NotI/XhoI site of pRS424 (Sikorski and Hieter 1989). They are: pSG82 (wild typ1 Tup1-LexA), pSG72 (Tup1^{F632A}-LexA), pSG68 (Tup1^{R447A}-LexA), pSG71 (Tup1^{D443A}-LexA), pSG76 (Tup1^{Y489A}-LexA), and pSG93 (Tup1^{E463A}-LexA). Plasmid pSG96 has the 800bp *TUP1* upstream sequence fused directly to the LexA protein sequence.

Yeast Strains. The *Saccharomyces cerevisiae* strains used in this paper were all generated from a parental strain of genotype MATα *ura3-52, lys2-801^{amb}, ade2-101^{och}, leu2-Δ1, his3-Δ200, trp1-Δ1*, which was descended from the original S288c strain. SGY84 (*tup1Δ*) was constructed by transforming the parental S288c strain with a PCR product of the *TRP1* gene flanked by homologous sequences of the *TUP1* locus. SGY145 (*tup1^{F632A}*), SGY146 (*tup1^{R447A}*), SGY147 (*tup1^{D443A}*), SGY148 (*tup1^{Y489A}*), and SGY128 (*tup1^{E463A}*) were made by transforming a full-length ORF fragment containing the mutation into a strain in which the *TUP1* locus has been replaced with *URA3*, leaving about 200bp of ORF homology on either side. Growth on 5-fluorootic acid selected for a strain in which the mutated *TUP1* ORF had been integrated at the *TUP1* genomic locus. These strains and SGY84 were then crossed to the MATa strain matching the parental S288c strain (SGY69) and sporulated to generate MATa versions of each mutant (SGY141, SGY142, SGY143, SGY144, and SGY140, respectively). For some of the microarrays, SGY200 was used for *tup1^{R447A}*,

which differs from SGY146 only in having had the *trp1-Δ1* deletion restored. The wild type strain used in the microarrays was either SGY70 (the parental MATα strain described above) or SGY92 in which the *trp1-Δ1* deletion was restored.

SGY212, SGY220, and SGY226 are MATa strains with pKK78 integrated at the *ADE2* locus and wild type *TUP1*, *tup1^{R447}*, and *tup1Δ* at the genomic *TUP1* locus respectively. SGY213, SGY221, and SGY227 are the matching MATα strains. SGY215 is SGY70 with pKK602 integrated at the *ADE2* locus. SGY219 was made by selecting for the replacement of a *tup1Δ* with a full-length TUP1-LexA sequence as described above and then integrating pKK602. The mutant Tup1-LexA fusion strains used in the ChIP experiments (SGY282 (*tup1^{F632A}*-LexA), SGY253 (*tup1^{R447A}*-LexA), SGY283 (*tup1^{D443A}*-LexA), SGY284 (*tup1^{Y489A}*-LexA), SGY285 (*tup1^{E463A}*-LexA)) were derived from SGY219. A wild type MATα strain was transformed with pAJ201 and then this strain was transformed with the Tup1-lexA fusion plasmids to make the strains used in the liquid β-galactosidase assays: SGY286 (pSG96), SGY287 (pSG82), SGY288 (pSG72), SGY289 (pSG68), SGY290 (pSG71), SGY291 (pSG76), and SGY292 (pSG93).

Microarrays and SAM Analysis. Microarrays of cDNA ORFs (~6100 spots) were performed as previously described (http://derisilab.ucsf.edu/microarray/protocols.html and (Green and Johnson 2004). Each of the microarrays was done six times (from independently grown cultures), except for the *tup1Δ* and *tup1^{R447A}* microarrays, which were done seven times (data available as Supplementary Material). The set of repeats for each strain was then analyzed by SAM using the One-Class Response and Row Average settings and the default Random Number Seed (1234567). Seven hundred and twenty permutations (the complete set for six repeats) were done for all mutant datasets, except *tup1Δ* and *tup1^{R447A}* upon which 5000 permutations were performed. The delta values for
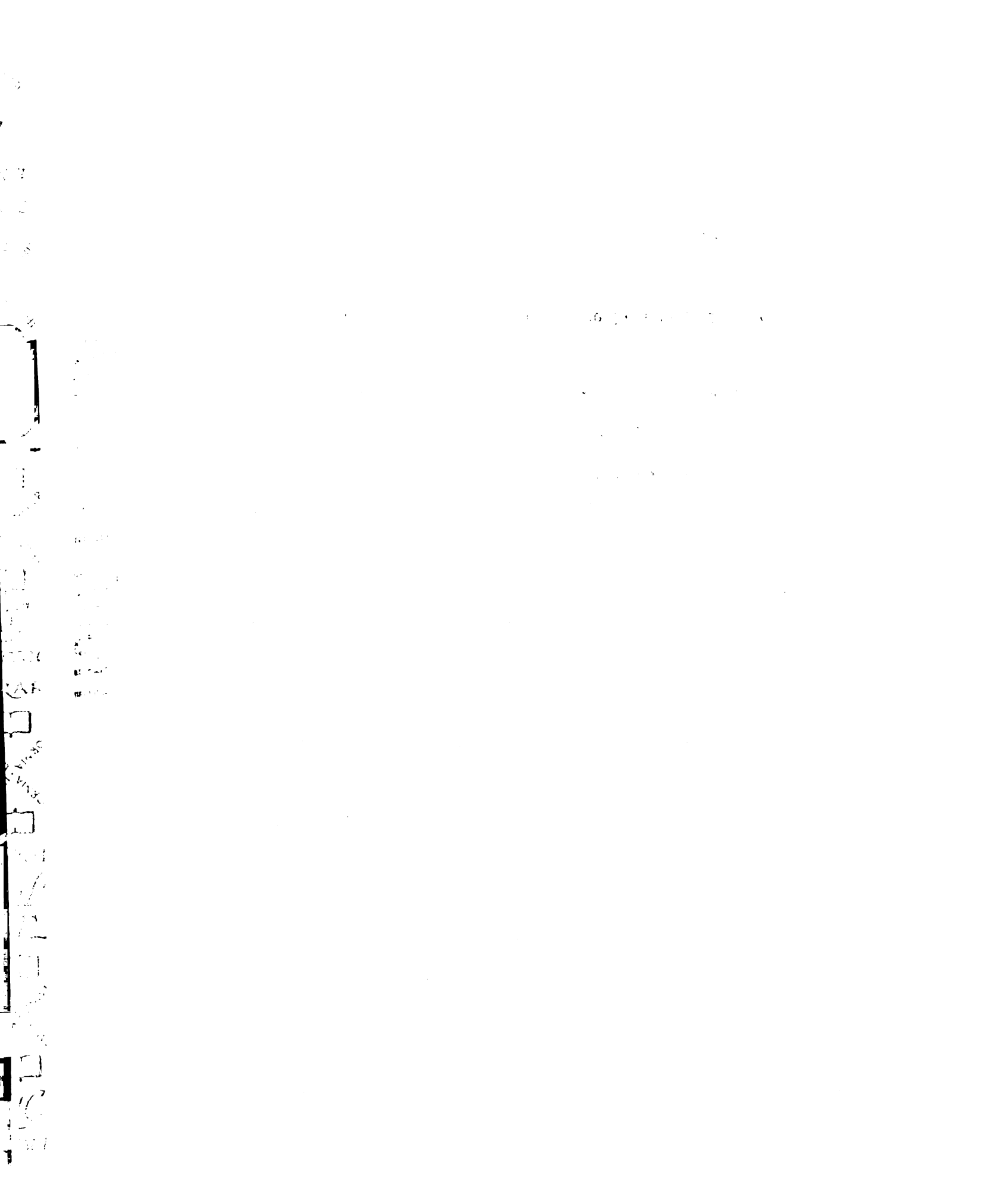
all datasets were selected as the value that resulted in the lowest FDR calculated for the 90th percentile *d*-scores. Microarray and SAM analysis data for each Tup1 point mutant are included in Supplemental Tables 1 and 2.

Liquid β-galactosidase Assays. Quantitative assays were performed as described in Current Protocols in Molecular Biology (pp 13.6.2-13.6.5, Editor F.M. Ausubel). Activities are reported as Miller Units and represent the average of measurements from three independently grown cultures for each strain.

Chromatin Immunoprecipitation and Quantitative PCR (QPCR). Antibodies against LexA were purchased from Upstate Cell Signaling (Cat. No. 06-719). Antibodies against Tup1 were generated using a bacterially expressed full length Tup1-GST fusion. Cultures were cross-linked with formaldehyde for five minutes and ChIPs were performed with slight modifications as previously described (Strahl-Bolsinger, Hecht et al. 1997). Extract from 50-100mls of culture at OD_{600} ~1 was used for each immunoprecipitation. Extracts were sonicated 7 times for 12s using a Branson sonifier 450 at 50% output power. ChIPs were analyzed by QPCR in a DNA Engine Opticon machine (MJ Research). PCR products were between 200-400bp. For a given Tup1 ChIP experiment, a median input ratio was calculated for each mutant strain versus wild type α-cells to normalize the amount of total DNA added to each IP. The amount of immunoprecipitated DNA in each IP was normalized for input and the measurements from a and α strains for each mutant were averaged. This average was then divided by the average of the measurements for tup1Δ a and α strains to produce a relative level of enrichment for each mutant. QPCR reactions were done 1-3 times for each monitored genomic location from an individual ChIP experiment. Enrichment levels for each site are averages of data from three

independent Tup1 ChIPs. For the Tup1 ChIP experiments analyzing the a-specific

reporter, the ratios of QPCR measurements for the a and α cells for the wild type and

$tup1^{R447A}$ strains (rather than the averages) were determined after normalization for

input. The displayed data represents the average of these ratios from two

independent ChIPs with three QPCRs performed for each ChIP.

The LexA ChIP experiments were performed as described above, with a median input

ratio calculated for each strain compared to SGY215 (no LexA strain). The displayed

data represents the average of the ratios of each strain to SGY215 from two

independent ChIPs with three QPCRs performed for each ChIP.

Supplemental Tables have not yet been published.

Figure 1

A
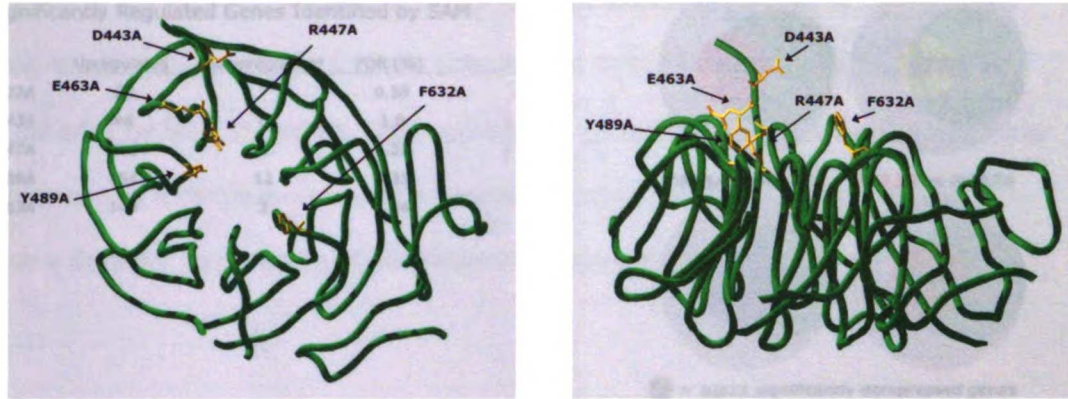
B
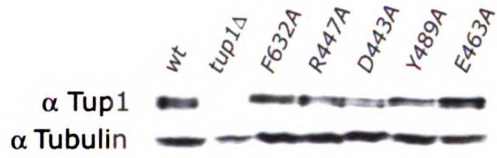
Figure 1. *Point Mutants of Tup1*

(A) Ribbon diagrams of the WD domain of Tup1 (282-713aa) depicting the five amino acids selected for mutation to alanine. (B) Western blots showing expression levels of mutant Tup1 proteins.

Figure 2

A

Significantly Regulated Genes Identified by SAM

	Upregulated	Downregulated	FDR (%)
F632A	89	7	0.58
D443A	46	0	1.8
R447A	240	1	0.22
Y489A	154	12	0.33
E463A	149	2	0.34

B

F632A vs **R447A**   **D443A** vs **R447A**

75%   92%

Y489A vs **R447A**   **E463A** vs **R447A**

87%   85%

= *tup1Δ* significantly derepressed genes
(334 genes)

C

F632A   D443A   R447A   Y489A   E463A   *tup1Δ*

Derepressed by *tup1Δ* and *R447A*

Derepressed by *tup1Δ* and all mutants

Derepressed by *tup1Δ* and
R447A, *Y489A*, and *E463A*
(direct and indirect
targets of Matα2)

Fold Change: -64 -32 -16 -8 -4 -2 1 2 4 8 16 32 64

Figure 2. *Microarray Analysis of Tup1 Point Mutants*

(A) Table of significantly regulated gene sets identified by SAM analyses of Tup1
point mutants. Complete lists of genes identified as significantly upregulated in each
mutant dataset are available in Supplemental Table 1. (B) Venn diagrams depicting
the overlap between sets of significantly derepressed Tup1-regulated genes in the

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point mutants. Percentages displayed are the percent of the individual mutant datasets that overlaps with the $tup1^{R447A}$ dataset. (C) Cluster diagram of six independent microarrays for each strain; displayed are all significantly upregulated Tup1-repressed genes for which there was data in 90% of the experiments; genes were clustered by SAM d-scores. Red represents an increase in gene expression and green represents a decrease in gene expression compared to a wild type strain. Microarray data for each strain is available in Supplemental Table 2.

Figure 3

A

Median fold derepression
(vs wild type)

	tup1Δ	F632A	R447A	D443A	Y489A	E463A
SUC2	9.8	1.2	1.8	1.3	1.1	1.5
SPI1	6.5	5.2	3.6	2.5	3.9	1.5
HSP12	40.0	16.0	5.6	1.9	12.8	2.8

B

C

D

Figure 3. *Recruitment of Tup1 Point Mutants to Regulated Promoters*

(A) Table of expression microarray data for Tup1 point mutants compared to a wild type strain.
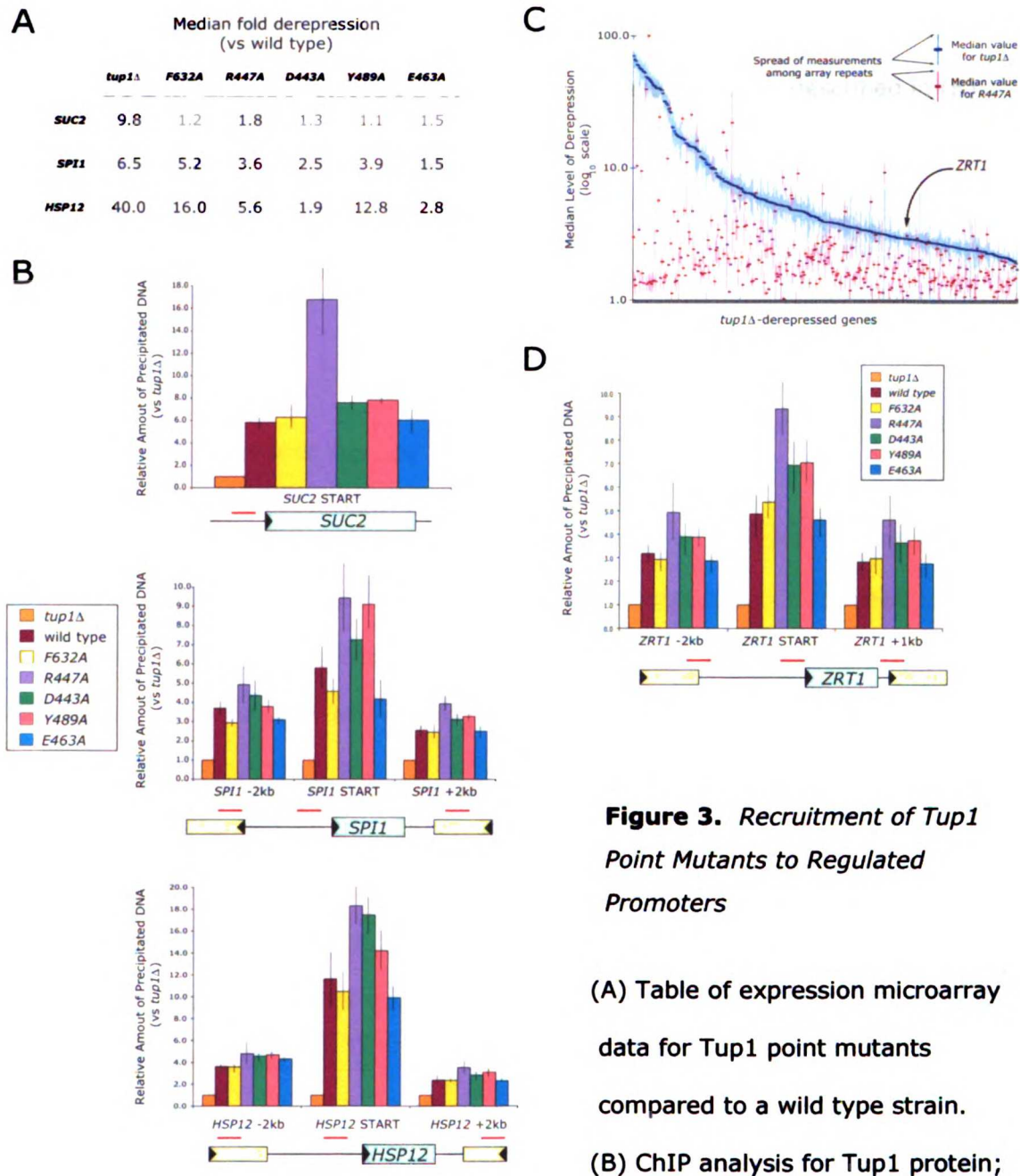
(B) ChIP analysis for Tup1 protein; bars represent the average of 1-3 QPCRs each on material from three independent ChIP experiments; data was collected for both cell (**a** and α) types for each strain. Error bars represent the Standard Error (SE) calculation for the averaged data. The diagrams below each chart represent the genomic locus of the corresponding gene

(not to scale) with the red lines indicating the approximate location of the sites

amplified in the QPCR. (C) Log$_{10}$-based graph of median change in expression by

microarray for *tup1Δ* (blue) and *tup1*^{R447A} (pink). The arrow indicates the location of

ZRT1. (D) ChIP analysis for Tup1 protein; bars represent data as described in (B).

Figure 4

A

	Miller Units (average)	SD	% Repression
LexA	27	±0.9	9
wt Tup1-LexA	2	±0.2	100
F632A-LexA	4	±0.8	64
R447A-LexA	4	±0.4	57
D443A-LexA	4	±0.2	65
Y489A-LexA	5	±2.5	53
E463A-LexA	3	±0.2	79

B
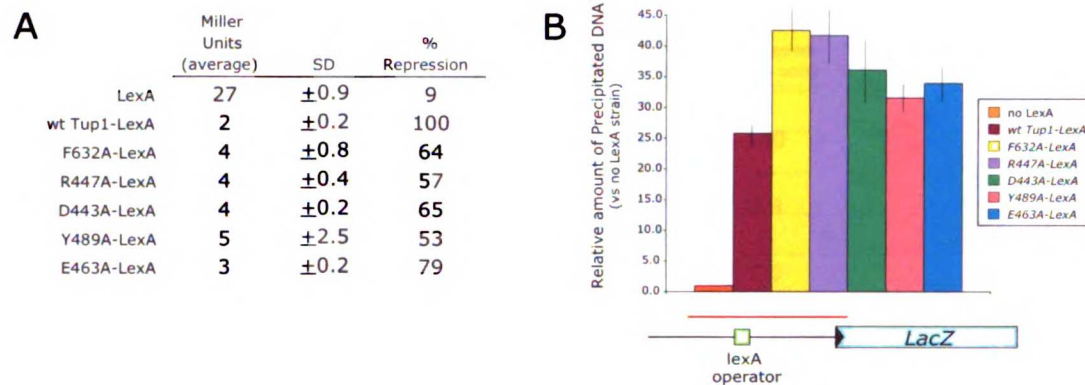
Figure 4. *Recruitment of Tup1 by LexA Domain*

(A) Miller Unit measurements for β-galactosidase levels of the construct depicted in (B) determined for each mutant strain. Experiments were done in triplicate. 100% Repression is considered the ratio of the average units for wild type Tup1-LexA to the average units for LexA alone. Strains used in this experiment are SGY286, SGY287, SGY288, SGY289, SGY290, SGY291, and SGY292. (B) ChIP analysis for LexA protein; bars represent the average of three QPCRs on material from two independent ChIP experiments (six total) with error bars depicting the SE. Data is shown for SGY215, SGY219, SGY282, SGY253, SGY283, SGY284, and SGY285. The red line above the diagram of the reporter gene indicates the approximate region amplified by the QPCR.

Figure 5

A   Repression of α2op-*LacZ* Reporter Construct

	Miller Units (average)	Standard Deviation	% Repression (**a** vs α cells)
wt **a**	129	± 9.1	--
*wt*α	0.3	±0.05	100
*tup1Δ***a**	46	± 6.1	--
*tup1Δ*α	12	± 2.3	0.8
*R447A***a**	79	± 8.4	--
R447A α	17	± 1.2	1.0

B

Relative Amount of Precipitated DNA (α vs **a** cells)

3 asg operators
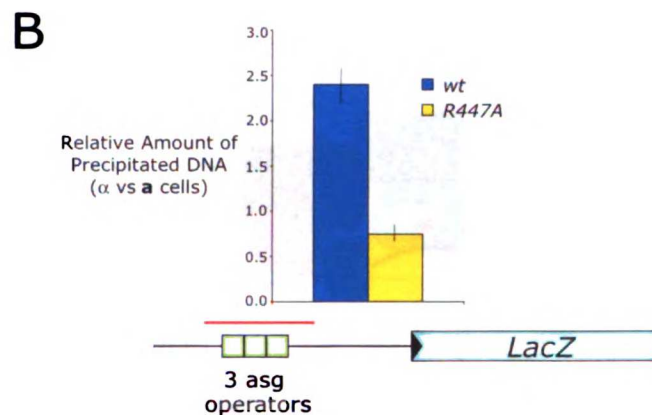
Figure 5. *Recruitment of Tup1 Point Mutants to **a**-Specific Reporter*

(A) Repression by Tup1 point mutants of a reporter construct controlled by an **a**-specific gene operator depicted in (B); 100% repression is defined as the ratio of wild type **a** and α cells (435X) and the percents repression in *tup1Δ* and *tup1^{R447A}* strain are the ratios of **a** and α cells in these strains divided by that for the wild type strains (4/435 and 5/435 respectively). SGY212, SGY213, SGY220, SGY221, SGY226, and SGY227 were assayed (B) ChIP analysis for Tup1 protein occupying the reporter construct used in (A); bars represent the average of three QPCR experiments each on two ChIPs. Data is shown for strains SGY212, SGY213, SGY226, and SGY227. The red line above the diagram of the reporter gene indicates the approximate region amplified by the QPCR.

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Figure 6

A

B

Mutation Disrupts Recruitment	**Mutation Disrupts Repression Function**
Example: α2-repressed genes	Example: *HSP12*, *SPI1*, *SUC2*, *ZRT1*, etc.

Figure 6. *Functions of a Conserved Surface of Tup1*

(A) Surface structure of the Tup1 WD domain. The structure on the left shows the conserved residues described in Sprague *et al.* (2000) in purple. The structure on the right shows the same conserved surface with the five mutants described in this work shown in red and residues identified in Komachi and Johnson (1997) as being

important for the interaction with Matα2 shown in blue. (B) Model depicts the two situations created by the mutants described in this paper. For one set of Tup1-regulated genes (the **a**-specific genes), these mutations disrupted an interaction with a DNA-binding protein (Matα2). However, those same mutations, in addition to others in the conserved surface, also disrupt full repression at many genes to which the mutant Tup1 protein is efficiently recruited. In this instance, the mutations could be disrupting an interaction with a component of the conserved repression mechanism.

(B) Model depicts the two situations created by the mutants described in this paper. For a minority of Tup1-regulated genes, these mutations disrupted an interaction with a DNA-binding protein. Those same mutations and two others, however, disrupt full repression at many genes even when properly brought to the promoters.

Appendix A

Unpublished Microarray Analyses of Tup1 Point Mutants and
hda1Δ and srb10[D304] Strains

Disruptions Tup1 Repression Mechanisms and the tup1^{R447A} Mutant

Of the five point mutants described in Chapter 3, *tup1^{R447A}* had the most

severe defects in transcriptional repression and affected the largest set of Tup1-

regulated genes. Many of these genes were only partially derepressed compared to

levels seen in a *tup1Δ* strain, suggesting that some aspects of the mechanisms of

Tup1-mediated repression were still functional in this mutant. Similarly, our

experiments investigating the disruption of two mechanisms of Tup1-mediated

repression, a chromatin-based mechanism disrupted by an *HDA1* deletion and a

Mediator-based mechanism disrupted by the *srb10^{D304}* mutation, revealed partial

defects when either mechanism was disabled (Chapter 2). To determine the

cumulative effects on Tup1-mediated repression of these different types of

mutations, we made strains coupling the *tup1^{R447A}* mutant with each of our

mechanistic disruptions, alone and in combination. If the *tup1^{R447A}* mutation were

disrupting the interaction of Tup1 with either Hda1 or Srb10, then the expression

pattern of the dual mutants would look the same as that of each of the individual

mutants; however, if *tup1^{R447A}* disrupted a third repression mechanism, we would

see greater numbers of genes being derepressed and an increase in the magnitude

of changes in expression at genes whose full repression requires the contributions of

more than one mechanism.

As with the *hda1Δ*, *srb10^{D304}*, and *srb10^{D304}hda1Δ* strains, we performed four

repetitions of expression microarrays on *tup1^{R447A}*, *tup1^{R447A}hda1Δ*, *tup1^{R447A}*

srb10^{D304}, and *tup1^{R447A}srb10^{D304}hda1Δ* and analyzed the data using SAM (Tusher,

Tibshirani et al. 2001). We did not see any global pattern emerge when we clustered

the data from all of the strains by their SAM scores (Figure A1). In general, the

genes that were sensitive to either or both of the disruptions of the Hda1 and Srb10

functions were also identified as significantly derepressed by $tup1^{R447A}$. Only about 40% of Tup1-repressed genes depend on either of these mechanisms for full repression and a similar proportion is derepressed in the $tup1^{R447A}$ mutant. It is interesting to note that, rather than a random overlap of these two sets of genes resulting in roughly 25% of Tup1-repressed genes being derepressed in both, we see a strong correlation between the two groups. However, the expression pattern of $tup1^{R447A}$ does not phenocopy either one of the two mechanistic disruptions but rather resembles a combination of the two like both. It is possible that the surface of Tup1 that contains the R447A mutation is a nexus for interactions with downstream proteins necessary to generate repression and that both Hda1 and Srb10 are recruited by this surface. The bulk of the Tup1-repressed genes that are only derepressed in the $tup1^{R447A}$ mutant and not either of the mechanistic mutants are the a-specific genes, which fail to be repressed because of an inability of the Tup1 point mutant to be recruited to the promoters rather than a loss of a repression-specific mechanism. Nevertheless, there are some other genes derepressed by $tup1^{R447A}$ that are unaffected by either $hda1\Delta$ or $srb10^{D304}$ (and are not a-specific) and it is possible that the loss of repression at these genes represents the disruption of a previously unidentified mechanism of repression making use of this same surface of Tup1.

We also compared the data from our mechanistic disruptions ($hda1\Delta$ and $srb10^{D304}$) to the data from all five of the point mutants we made on the conserved surface of Tup1 (Chapter 3). Not surprisingly the relationships between the datasets of the other four point mutants and the mechanistic disruptions are the same as that for $tup1^{R447A}$, the strongest of the point mutants (Figure A2). For the most part, genes derepressed in the point mutants are also derepressed in the mechanistic disruptions. However, in Figure A1 a small subset of genes emerges that could represent a third mechanism of Tup1-mediated repression that is effected by

mutations on the conserved surface of Tup1. These genes are derepressed in many of the point mutants but unaffected by the $hda1\Delta$ and $srb10^{D304}$ mutations. Many of the genes in this set are uncharacterized, and it is not clear if this subcluster represents a relevant, reproducible pattern of regulation. Regardless of the caveats, they present an interesting focus for future analysis of the point mutants.

Figure A1
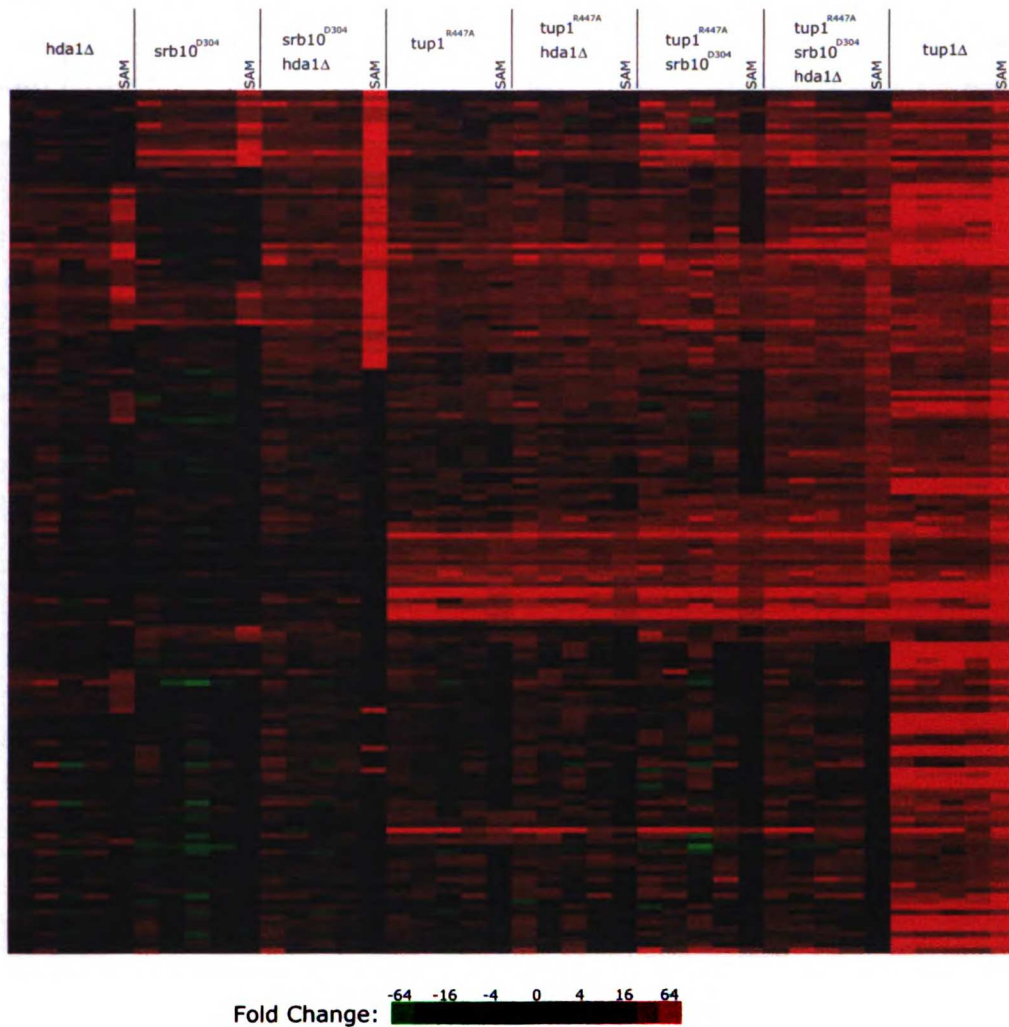
Figure A1. *The* tup1[R447A] *Mutant in Combination with Disruptions of Mechanisms of Tup1-Mediated Repression*

Displayed are four repetitions of microarrays measuring the changes in expression of Tup1-repressed genes in the mutant strains *hda1Δ*, *srb10[D304]* and *tup1[R447A]* (alone and in all combinations) versus a wild type strain. Every fifth column shows the SAM score assigned to that gene for the corresponding dataset. Raw microarray data was filtered and transformed as described in Chapter 2 (see Materials and Methods). Genes in the Cluster diagram (Eisen, Spellman et al. 1998) were required to have data in 100% of the repetitions and were clustered by their SAM scores.
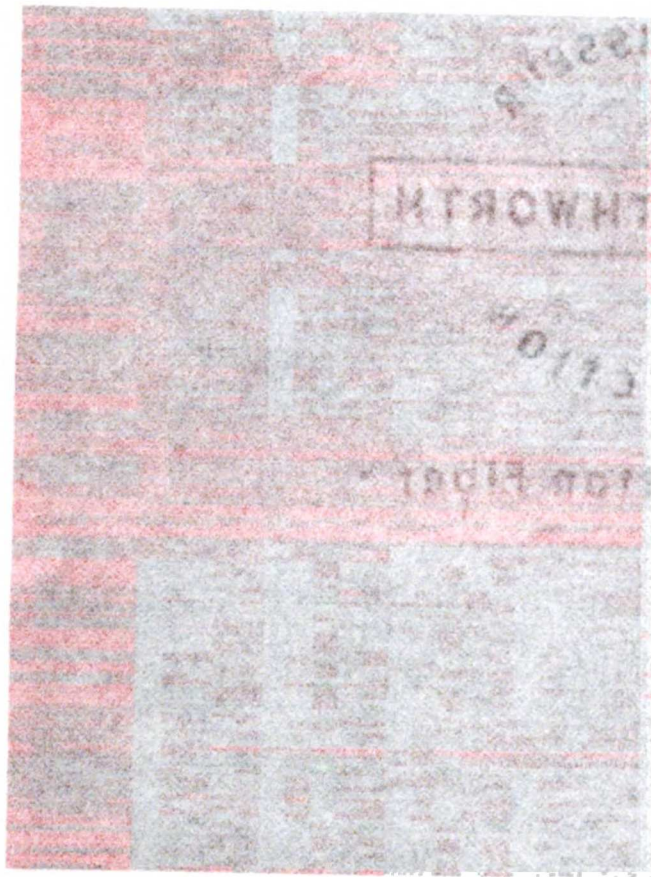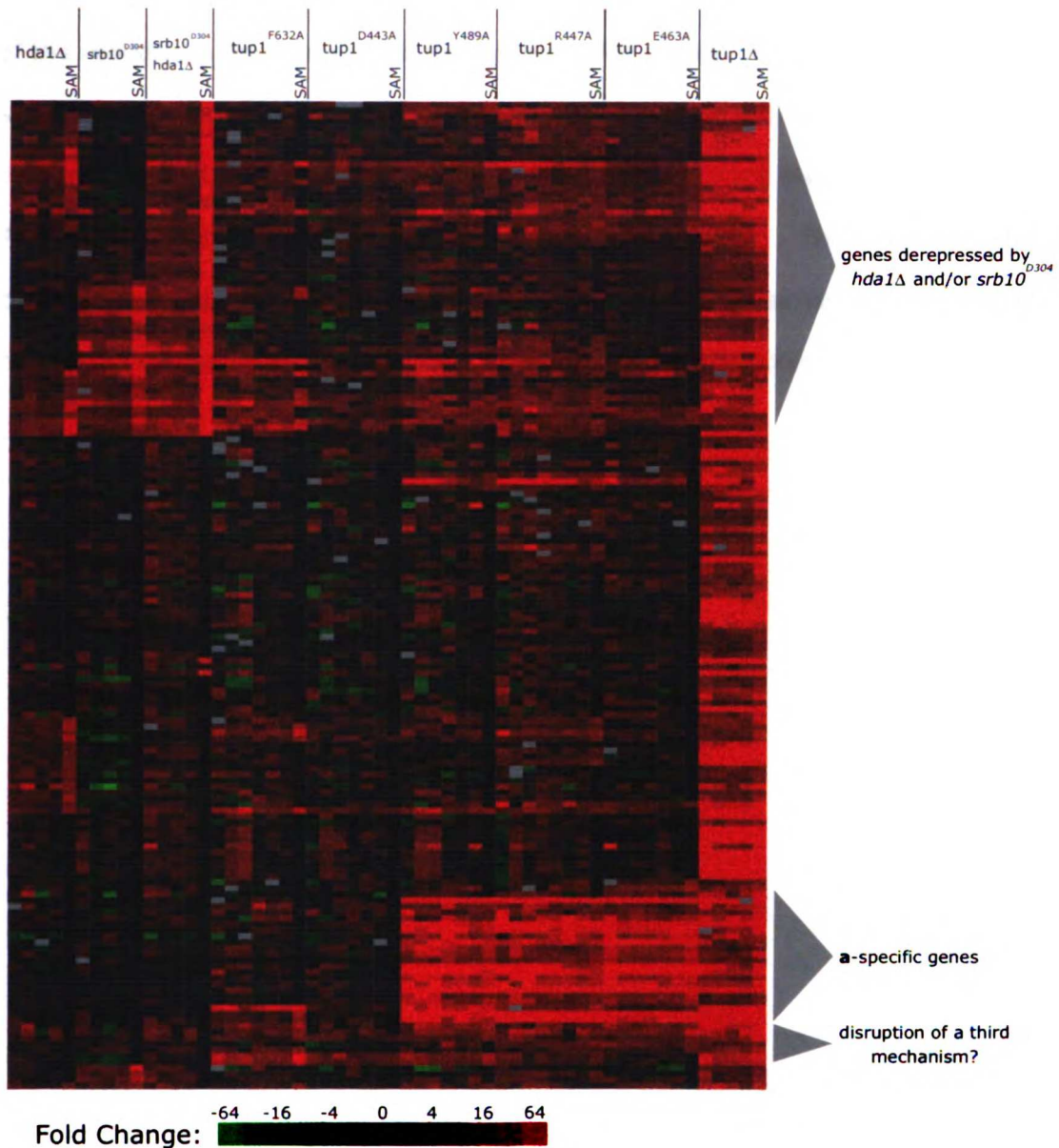
Figure A2

Figure A2. *Cluster of All Tup1 Point Mutants and Mechanistic Disruption Strains*

The microarray data gathered from five Tup1 point mutants and three repression

mechanism mutants (*hda1Δ, srb10^{D304}*, and *srb10^{D304}hda1Δ*) are shown for the

Tup1-repressed genes identified in the experiments of Chapter 2. The columns

labeled "SAM" represent the SAM score assigned to each gene in the corresponding

dataset. Raw microarray data was filtered and transformed as described in Chapter 2 (see Materials and Methods). Displayed genes were selected to have data in 95% of the repetitions and were clustered by their SAM scores. The locations of three subclusters are indicated on the diagram. One subcluster indicated is the genes derepressed by the various mechanistic disruptions and were discussed in Chapter 2. A second subcluster contains the a-specific genes (Chapter3). The third subcluster contains genes derepressed by the point mutants on the conserved surface of Tup1 but not by disruption of either of the two mechanisms studied in this work.

Appendix B

Mass Spectrometry Analysis of the Tup1-Ssn6 Complex

Tandem Affinity Purification of the Tup1-Ssn6 Complex

It has become clear that the repression of transcription at a given Tup1-regulated gene typically reflects the contributions of overlapping, partially redundant mechanisms, some of which are still unknown. To identify components involved in these as yet uncharacterized repression mechanisms, we attempted to identify proteins that interact with the Tup1-Ssn6 complex. We tagged Ssn6 with the Tandem Affinity Purification (TAP) tag, a double tag consisting of two IgG-binding domains of Protein A, an intervening TEV protease cleavage site, and a calmodulin binding peptide (CBP). Microarrays of the Ssn6-TAP strain confirmed that the tag did not effect the repression function of the Tup1-Ssn6 complex (Figure B1). We also generated a version of the Ssn6-TAP strain that had $tup1^{R447A}$ at the *TUP1* locus, enabling us to identify interacting proteins whose associations with Tup1 had been disrupted by the mutation.

The TAP purification scheme takes advantage of the extremely strong interaction between Protein A and IgG, followed by a precise elution using TEV protease cleavage, to efficiently recover tagged proteins. The CBP portion of the tag allows for a second, mild purification of the desired complex using a calmodulin matrix, maximizing removal of nonspecific contaminating proteins from the preparation. In fact, we saw a considerable increase in the purity of the Tup1-Ssn6 complex between the first elution with TEV protease and the final elution from the calmodulin column (Figure B2).

We had the purified samples from both tagged strains (wild type and $tup1^{R447A}$) analyzed by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) at the Yeast Resource Center (YRC, http://depts.washington.edu/~yeastrc/). We submitted two independently purified samples for each strain in addition to

matched control samples purified from an untagged, wild type strain. In brief, MALDI-TOF is a procedure in which proteins are trypsinized into peptides that are subsequently ionized by a laser. The mass-to-charge ratio for each peptide can then be measured and the sequence of the peptide predicted based on the expected peptides generated by a theoretical trypsin digest of all yeast proteins. Table B1 lists the proteins for which at least two nonredundant peptides were detected in the MALDI-TOF analysis of any of the preparations from the tagged wild type or $tup1^{R447A}$ strains. Many of the proteins identified in both samples were common contaminants of proteins purifications (Gavin, Bosche et al. 2002). These contaminants are typically highly expressed proteins such as the ribosomal proteins or heat shock proteins. Other proteins in our samples were disregarded because they were also detected in the matched untagged sample and were considered to be nonspecific. Many nonspecific proteins likely remain in this selected set of identified proteins, particularly the ribosomal proteins (*RPS16B*, *RPL23A*, *RPP1A*, etc.). Three of the remaining identified proteins do have a role in transcription and might be interesting potential Tup1-interactors to pursue. *TAF47* encodes a TFIID subunit, *TRA1* encodes a component found in several histone acetyltransferase complexes (SAGA, SLIK, and NuA4), and *GAL11* is a component of the Mediator complex (Sakurai, Ohishi et al. 1994; Grant, Schieltz et al. 1998; Myer and Young 1998; Nishizawa 2001; Pray-Grant, Schieltz et al. 2002). However, as each protein was identified in only one sample, the significance of their detection in the analyzed samples is unclear and repeated mass spectrometry analyses on optimized Tup1-complex preparations are needed.

Purification of the Tup1-Ssn6 complex also presented an opportunity to determine the sites of phosphorylation in each of these proteins. Both proteins had previously been shown to be phosphoproteins, although the implication of this regulation in their functions is unknown (Schultz, Marshall-Carlson et al. 1990;

Keleher, Redd et al. 1992; Redd, Arnaud et al. 1997). The YRC analyzed the sample

by tandem mass spectrometry (also known as ESI, Electrospray Ionization). In ESI,

one mass-to-charge ratio (peptide) at a time is isolated and then further fragmented

so that, when the data for each initially selected peptide is taken as a whole, the

actual sequence of the peptide is revealed. This differs from MALDI-TOF in which the

peptide sequence is inferred by comparing the mass-to-charge ratios to predictions

for all possible peptides. Figure B3 shows representative peptides corresponding to

each potential phosphorylation site identified in the two yeast samples of the Tup1-

Ssn6 complex and an independently purified complex that was expressed in insect

cells (unpublished data from J. Penko). The putative phosphorylation sites are

colored based on the number of times the phosphorylation of that site was detected

in nonredundant peptides. Several of the sites were only detected in a single peptide

from one or two preparations, so it is unclear if these are legitimate phosphorylation

sites (Table B2). However, others were represented by many peptides from

multiple sample preparations and would appear to be more promising. For instance,

S741 in Ssn6 was found in several peptides from four of the five analyzed samples.

Residues S805 and T715 in Ssn6 also seem like strong candidates for possible sites

of phosphorylation (Table B2).

The phosphopeptide data collected for Tup1 was much less reliable than that

for Ssn6. No sites were represented by more than two peptides and none were

detected more than once in a given sample (Table B2). The overall coverage of both

Ssn6 and Tup1--that is, the percentage of the full protein represented by all of the

peptides detected--was comparable (~75-85%), so it is unclear why the Tup1

phosphopeptide data was not as convincing as the Ssn6 data. Despite the ambiguity

of the Tup1 phosphopeptide data, we mutated some of the putative phosphorylation

sites and monitored the migration of the protein on a gel to determine if we had

eliminated the ability of Tup1 to act as a kinase substrate. We made five single

mutations (T245 and S246 were considered one site and both changed to alanine in the mutant named T245. Similarly, S520 and T522 were simultaneously mutated and the resultant mutant is referred to as S520) and various combinations of all of the mutants. However, even the sextuply mutated Tup1 protein migrated as a doublet on a gel, indicating it remained phosphorylated, and appeared to be indistinguishable from wild type Tup1 (Figure B4). No phosphatase inhibitors were used in the preparations of these samples, so it is possible that some or all of these mutations have altered the phosphorylation state of Tup1 but that this distinction was lost in the processing of the samples. It is also possible that these sites are phosphorylated in response to a specific condition and, while enough of those species of phosphorylated Tup1 were present in the analyzed samples to be detected by ESI, a Western blot is not sensitive enough to verify such a circumstance. We cannot confirm the validity of the putative phosphorylation sites we mutated, but it is clear that Tup1 is still stably phosphorylated in all of the mutants we constructed and, therefore, other sites of phosphorylation remain unmapped. Future preparations, perhaps incorporating the use of phosphatase inhibitors or other modifications of the protocol to stabilize phosphorylation, are needed to resolve these questions.

Figure B1
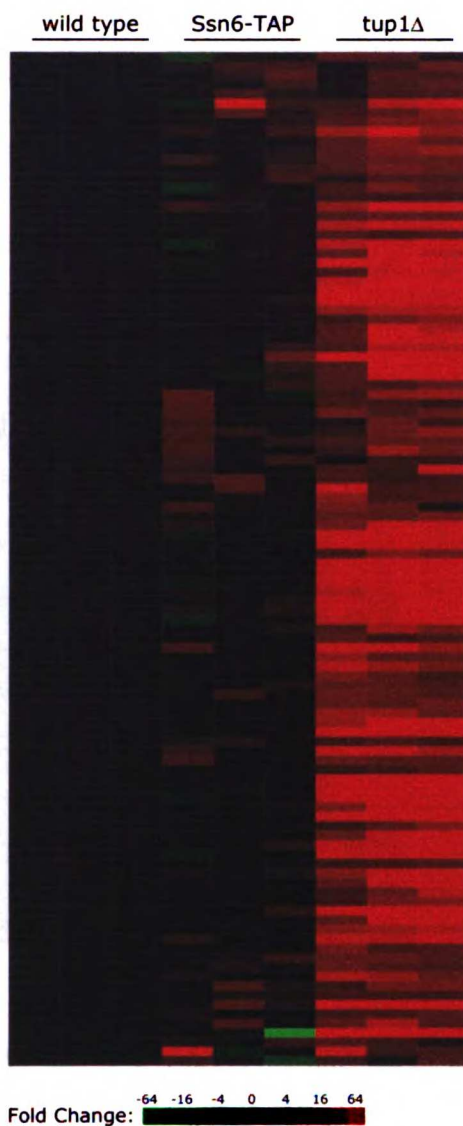
Figure B1. *Cluster Diagram of TAP-tagged Strain*

Three independent repetitions of microarrays were done for a matched set of strains: wild type, the tagged strain Ssn6-TAP, and *tup1Δ*. Raw microarray data was filtered and transformed as described in Chapter 2 (see Materials and Methods). The Cluster diagram (Eisen, Spellman et al. 1998) contains genes for which there was data in 90% of the experiments and for which at least two experiments showed a ≥2.5-fold increase in expression compared to a wild type strain.
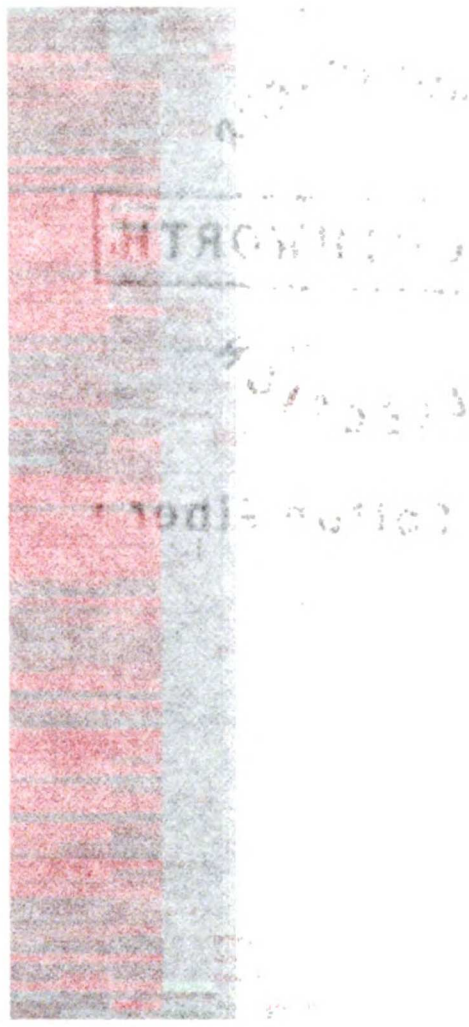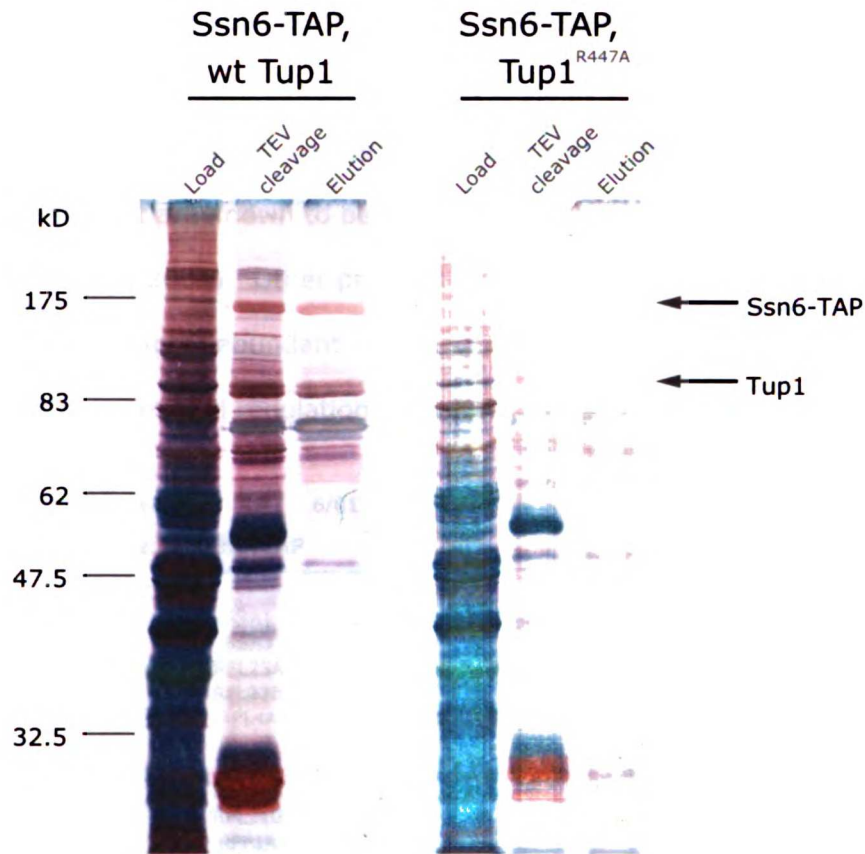
Figure B2

Figure B2. *Purification of the Tup1-Ssn6 Complex from a TAP-tagged strain*

A tagged version of Ssn6 (Ssn6-TAP) was used to purify the Tup1-Ssn6 complex from *S. cerevisiae* expressing either Tup1 or Tup1^{R447A}. Six liters of late log phase cultures (OD_{600} ~1) were used in each purification. Samples of the whole cell extract (Load), the first elution (TEV cleavage), and the final elution (Elution) were run on a denaturing gel and silver stained. The positions of Ssn6-TAP and Tup1 are indicated.

Table B1. *Tup1 Interacting Proteins Identified by Mass Spectrometry*

Two yeast samples of the Tup1-Ssn6 complex were examined for interacting proteins. The proteins listed were identified by at least two nonredundant peptides in each sample. Proteins listed in grey were either also identified in a control (untagged) sample or are known to be common contaminants of TAP purifications (Gavin, Bosche et al. 2002). Other proteins listed, such as the ribosomal proteins, are also likely to be highly abundant, nonspecific contaminants. Three proteins that have roles in transcriptional regulation are shown in blue.

Analysis Date:	6/01		7/02	
Sample:	**Ssn6-TAP**	**Ssn6-TAP tup1^{R447A}**	**Ssn6-TAP**	**Ssn6-TAP tup1^{R447A}**
Proteins Identified:				
	SSA1	SSA1	**SSN6**	**SSN6**
	SSA3	**SSA3**	**TUP1**	**TUP1**
	RPL23A	**SSA4**	ILV2	GPM1
	RPL23B	RPL4A	GPM1	ILV2
	RPL4A	RPL4B	SSA2	SSA1
	RPL4B	**SSN6**	**ENO1**	TEF2
	SSN6	**TUP1**	**ENO2**	TEF1
	RPL31A	TDH3	SAM1	SSA2
	RPL31B	RPS5	**SAR1**	TDH3
	RPP1A	SSA2	CCT2	SSB2
	RPS16B	**RPP2A**	KAR2	CCT9
	RPS16A	**GAL11**	TEF2	**LEU2**
	SSB1	RPS7A	TEF1	**TDH1**
	RPP2B	DED1	**TAF47**	DED1
	NPL3		CLU1	SRO9
	SSA4		SSB1	SSE1
	RPL7A		SSB2	**PDR13**
	RPL7B		**RDH54**	HSP1
	MLC1		HSP1	**SSA3**
	ENO2			KAR2
	TDH3			**TIF4**
	RPS14A			**CFT1**
	RPS16B			PFK1
	RPL4B			CCT2
	RPS14A			**PSP1**
	SSC1			**TRA1**
	STE24			
	RPS5			
	SSA2			
	RPL10			
	RPL10			
	RPS8B			
	SSB2			
	RPS19B			
	RPS19A			
	RPS15			
	RPL25			
	RPS7A			
	DED1			

Figure B3

Insect Cell Expressed Tup1-Ssn6 4/01

Ssn6

S708, S710	S.STHTENNTkSPRQPTHAIPT.Q
T909, T911, T912, T925	K.QQDETAAITTITVIKPTLETMEIV.K
S943	M.ETVKEEAKMREEEQTSQEK S PQENTLPREN.V
T825	NJEPESSSVQPTVSSESSTTK.A
S288	Q.KALDYLLK SLEADPSDATTWYHLGR.V
S485	Q.QQHPAQQTPINSSATMYS NGASP.Q

9/01

Ssn6

S741	Q.KLN SPNSNINKLVNTA.T
S790	A.N SIPSVIGAQEPPQEA.S

Tup1

S567	W.DSETGFLVERLD S ENESGTGHKDSVYSVVFTR.D
T245, S246, T251, S253	T.EPENNN T SKINDT G SATTATTTTATETEIKPK.E

Ssn6-TAP, tup1^{R447A} 6/01

Ssn6

S741, S744	Q.KLN SPNSNINKLVNTATSIEENAK.S
S710	T.LVDAAVSSSTHTENNTK SPR.Q
S866	L.STATVPAEA SPVEDEVRQH.S
S817	A.EEATKAASV SPSTKPL.N

Ssn6-TAP 4/01

Ssn6

S700, S709, S710, T715, T720	S.S STHTENN T k SPRQP T HAIP T QAPATGITNAEPQVKKQ.K
S741, S?, S?	K.KQKLN S PNSNINKLVNTATSIEENAKSEVSNQ S PAVVESNTNNT S QEE
S943	K.MREEEQTSQEK S PQENTLPR.E
S866	V.PAEA SPVEDEVR.Q

Tup1

S393	N.NHRN SITENNTTTSTDNNTMTTT.T

6/01

Ssn6

S866	E.EAEPAA S RDAEKQQDETAATTITVIKPTLETMETV.K
S485	V.QQQHPAQQTPINSSATMYS NGASPQLQAQAQAQ.A
T919	K.QQDETAATTITVIKP T LETMETVKEEA.K
T815	P.LNTEPESSSVQP T V.S
S836	I.EL STATVPAEASPVEDEVRQH.S
S790, S?, S805	K.AN SIP SVIGAQEPPQEA S PAEEATK.A
S741, S744	Q.KLN S PN SNINKLVNTA.T
S943	K.MREEEQTSQEK S PQENTLPR.E

Tup1

T?, S716	T.EPENNN T SKINDTGSATTATTTTATETEIKPK.E
S567	W.DSETGFLVERLD SENESGTGHKDSVYSVVFTR.D
S570, T522	G.QC S L TLSIEDGVTTVAVSPGDGK.Y
S390	K.IVMILQGHEQDIY SLDYFPSGDKLVSGSGDR.T
T318	R.ANHSKPIPPFLLDLDSQSVPDALKKQ T NDYYI.L

■ = 1-2 peptides

■ = 3-4 peptides

■ = ≥5 peptides

Figure B3. *Lists of Possible Sites of Phosphorylation on Tup1 and Ssn6*

Three samples of the Tup1-Ssn6 complex were prepared for phosphopeptide mapping: two from *S. cerevisiae* (Ssn6-TAP and Ssn6-Tap, tup1^{R447A}) and one from an insect cell expression system. Two preparations each of Ssn6-TAP and insect cell expressed Tup1-Ssn6 and a single Ssn6-Tap, tup1^{R447A} sample were analyzed. The potential phosphopeptides identified by tandem mass spectrometry are listen with the possible sites of phosphorylation shown in color. Residues in light pink were only

identified in one or two peptides from that sample preparation.   Large print residues in dark pink were found to be phosphorylated in three or four peptides. Large print residues in red were found in over five peptides in a particular sample preparation.

Table B2. *Summary of Phosphorylation Analysis*

Summarized below are all of the residues identified by mass spectrometry as possible sites of phosphorylation in Tup1 or Ssn6. Analyzed samples were purified either from *S. cerevisiae* (Ssn6-Tap and Ssn6-Tap tup1^{R447A}) or an insect cell expression system (Baculovirus). Numbers indicate the incidences of phosphorylated peptides corresponding to that site that were detected in each sample.

Ssn6

Date:	4/01		6/01		9/01	
Sample:	Ssn6-TAP	Baculo-virus	Ssn6-TAP	Ssn6-TAP tup1^{R447A}	Baculo-virus	Total
S288		1				1
S493		1	1			2
S708	1	1				2
S741	3		7	3	3	16
S768	1					1
S780	2					2
S790					1	1
S805			4			4
S817				1		1
S836			1			1
S866	1		1	2		4
S943		1	1			2
T715	5					5
T720	1					1
T825		1				1
T835			1			1
T908		1				1
T912		1				1
T919			1			1
T925		1				1

Tup1

Date:	4/01		6/01		9/01	
Sample:	Ssn6-TAP	Baculo-virus	Ssn6-TAP	Ssn6-TAP tup1^{R447A}	Baculo-virus	Total
T245			1		1	2
S246			1		1	2
S567			1		1	2
T318			1			1
S393	1					1
S490			1			1
S520			1			1
T522			1			1
T251					1	1
S253					1	1

91
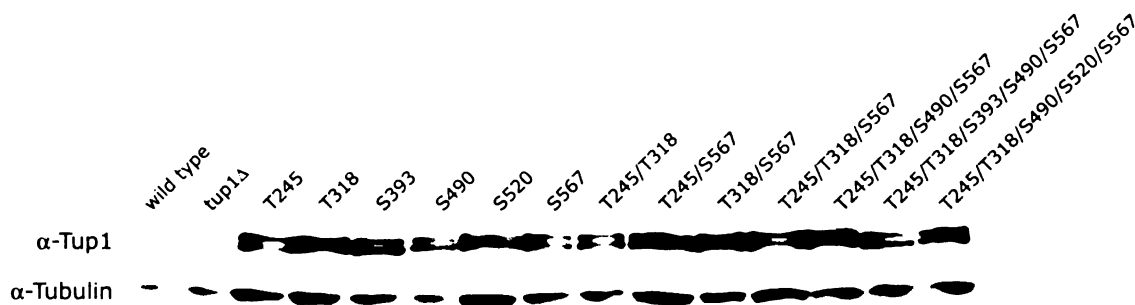
Figure B4

Figure B4. *Western of Putative Tup1 Phosphorylation Mutants*

Potential sites of phosphorylation were identified in Tup1 by mass spectrometry and mutated to alanine. The single and multiple mutant sequences were then integrated at the *TUP1* genomic locus. A Western blot against Tup1 protein was done on 15µg of total protein from the parental wild type strain and Tup1 phosphorylation mutants. Extracts were made in buffers lacking phosphatase inhibitors. The α-tubulin Western Blot demonstrates that equal amounts of total protein were loaded for each sample.

Appendix C

Characterization of Additional Mutants of Tup1

Point mutants of Tup1

     The residues of Tup1 mutated in the work described in Chapter 3 were

selected based on their conservation in an alignment of homologs of Tup1 from four

fungi and *Dictyostelium discoideum, Caenorhabditis elegans, Drosophila*

melanogaster, Xenopus laevis, and *Mus musculus* (Figure C1). These mutations

were then analyzed in depth for their influence on Tup1-mediated repression. Three

additional mutant Tup1 proteins that we constructed (*tup1*^{L547A}, *tup1*^{R652A}, and

tup1^{D548A}) were expressed at levels comparable to those of wild type Tup1 and the

better-characterized mutants (Figure C2). Regardless, we chose not to pursue

analysis of *tup1*^{L547A}, *tup1*^{R652A}, and *tup1*^{D548A} because they appeared to have weak

defects in repression based on initial tests of the mutants' abilities to repress a *lacZ*

reporter (Chapter 3). However, we did complete a single expression microarray for

each mutant and compared the data to the full set of Tup1-repressed genes (Figure

C3A). Considering the mild defects of these point mutants measured in the reporter

assay, it is not surprising to see that very few genes appear to be derepressed by

any of the mutants. Interestingly, one small cluster of genes was strongly

derepressed in the *tup1*^{L547A} and *tup1*^{D548A} strains and this cluster comprised four of

the seven a-specific genes (*BAR1, AGA2, MFA2,* and *STE2*) (Galgoczy, Cassidy-Stone

et al. 2004). Three of the seven known Matα2-controlled genes do not cluster with

these other genes because they did not meet our data-filtering criteria (there was

valid data for less than 90% of the experiments); however, they do appear to share

the same regulatory pattern as the four genes listed in Figure C3A. There was no

data for *MFA1* in any of the point mutant datasets, but *STE6* was 13-fold more highly

expressed in *tup1*^{L547A} and 12-fold more highly expressed in *tup1*^{D548A} than in a wild

type strain (Figure C3B). In a *tup1Δ* strain, the median fold change in expression of

STE6 is 5.5. *STE6* expression in *tup1*[R652A] was unchanged compared to the wild type strain. *ASG7* was 6-fold more highly expressed in *tup1*[L547A] than in a wild type strain compared to a median value of 5-fold measured in the *tup1Δ* microarrays. There was no data for *ASG7* in the *tup1*[D548A] dataset (Figure C3B). Oddly, the genes that are derepressed subsequent to derepression of the a-specific genes (indirect targets of Tup1-mediated repression) are not derepressed in either the *tup1*[L547A] or the *tup1*[D548A] mutant. Several are indicated in Figure C3A with asterisks and are clearly expressed at similar levels as in a wild type strain. It is unclear if this is a biologically relevant result or an artifact that would be resolved after several repetitions of the microarrays. While it is difficult to imagine how these indirect targets of Tup1 repression could remain turned off given what we know of their regulation, it is intriguing that this same phenomenon is seen in two of the three mutants, suggesting it is not a quirk of a single array. This expression pattern of the a-specific genes also seems unlikely to be an artifact of experimental or growth conditions given that it is not seen in all three of the mutant microarrays that were performed as a set. Several more repetitions of these microarray experiments are needed before the implication of this result becomes clear.

Microarray Analysis of an Internal Deletion Mutant of Tup1 and a Tup1-LexA Fusion Strain

Experiments examining the repression function of truncations and internal deletions of Tup1 have identified several domains necessary and/or sufficient to repress transcription with varying efficiencies (Tzamarias and Struhl 1994; Zhang, Varanasi et al. 2002). Other experiments identified a region of Tup1 that can bind to

histones and this region overlaps with some of the recognized repression domains (Edmondson, Smith et al. 1996). We considered including deletions of some of these domains in our microarray analysis, but several have been found to result in unstable proteins (Zhang, Varanasi et al. 2002). One internal deletion mutant that removes part of the histone-binding domain and one of the repression domains was expressed at wild type levels. This mutant ($TUP1^{\Delta 129-282}$) has been shown to have gene-specific effects on repression by Tup1, suggesting this domain might be involved in one or more of the repression mechanisms used by Tup1. We replaced the genomic copy of $TUP1$ with this internal deletion and monitored its effect on genome-wide Tup1-mediated repression by microarray (Figure C4). The data gathered from the $TUP1^{\Delta 129-282}$ microarray were divided by the data from a wild type microarray and the relative changes in expression were compared to those seen in a $tup1\Delta$ strain. The $TUP1^{\Delta 129-282}$ strain appears to repress transcription of Tup1-regulated genes as efficiently as a wild type strain. We saw no signs of the systematic defect in repression that would have been expected if this domain of Tup1 were necessary for its repression function and, contrary to the results of earlier work, we conclude this domain does not play a significant role in transcriptional repression.

We also used expression microarrays to confirm that a Tup1-LexA fusion protein could effectively repress transcription. The conclusions in Chapter 3 relied heavily upon the use of a fusion of Tup1 to LexA, a bacterial protein that binds to a specific DNA sequence, and an integral assumption of those conclusions is that the repression function of the fusion protein is indistinguishable from that of the wild type protein. Figure C4 compares the changes in expression seen in a Tup1-LexA strain to those seen upon deletion of $TUP1$. As expected, the Tup1-lexA protein has no significant repression defect, although it is possible that a minority of Tup1-

Figure C1

```
            |---------+---------+---------+---------+---------+---------+---------+---------|
ScTup1                                           MTASVSNTONFLNELLDAIPOEFLOVSO ANTYPL NOK
KlTup1                                           MSSVAASONI INDLLEAIPOEFANVSO ANSYPL NOK
CaTup1                                     MSMYFOPTOHOOPLTELLDAIF TEFDVASN ASSFK-KVOE
DdTup1                                     MYGPTAPASELLDN---LFGEIDALNH LSLYKH FDD
NcTup1                                     MSMYPHPFIAGAPIONNGRLNELLDGIPM FESOAP VET
SpTup1                                 MITVPOFTFTIFFFOFMATSNVSSPVNELLEAVF EFEDICOKTFTVEA FDD
Unc37                              MKASVLETLDPIFDEHAEMSFHVNOOPSDIEFVALEFENMNPSYMTYAEVSNT
DmGro     MYPSPVPHPAAGGFP--------POGPIFFTIADTLEPIFEEFNFLOAOVHSIFLECEFLSNEFTEMOPHYVMYYEMSYG
XlGro     MFPON-PPPTHLOASVATGAASGFPOSLKLTYPETLDPIFEEFOFLOSOVHSLFLECEFLATEFTEIOPHYVMYYEMSYG
MmGro     MYPOT-PHPAPHO----------PAOPFFFTISESCDPIFEEFOFLOAOVHSLFLECEFLASEFTEMOPHYVMYYEMSYG
Consensus                                         #      E         %  .#
```

```
            |---------+---------+---------+---------+---------+---------+---------+---------|
ScTup1     -DVDFFMNOOL  MOOIPN  YEL LTHP M DA  E IFH KLG  OPDHOI S---------------------LTV
KlTup1     -DVDFFINOOL  MOOUPN  YEL LTHP M DA  E ISP KLE  OFDPOL S---------------------IA-
CaTup1     -DVDSFVOOOA  MOOIPO  YDL LAHP I EA  E ILR KNE  TPDFOMF N----------------------
DdTup1     YERF--FONOL  LNTIOO LVDL PGON M IH  E IPO KPO  OONINASO--------------------PDL
NcTup1     COHD--LATOLO LOVIPEF FOM OHOMNV OF  D IAL PPO  APG--G P---------------------GNM
SpTup1     FEYFAMISAOIN MALMFO  MDL MOOS V DP  E ITS KAO  APPFEI S---------------------GVV
Unc37      LPSDLPFAEEINTPLOEFIMOSLAPOLSODNOANCLAALEAFKTASPPENGNG --------------------------
DmGro      LNVEMHFOTEIFPLNTLINO-LLPFLOADHOOOVLOAVEPAKOVTMOELNLIIG---------OOIHAOOVPGG----
XlGro      LNIEMHFOTEIFPLNVICAO-LVPFLSOEHOOOVVOAVEPAKOVTMAELNAAIGVPFIPPOLTVHOLOAOHLSH-HAPP
MmGro      LNIEMHFOAEIVFPLNAICAO-VIPFLSOEHOOOVVOAVEPAKOVTMAELNAIIG---------OOLOAOHLSHGHGLP
Consensus  # #      #              $       #   #!  K
```

```
            |---------+---------+---------+---------+---------+---------+---------+---------|
ScTup1     OOOPOOOOOOOOVOOHLOOOOOOLAAASASVPVAOOPPATTSATATPAANTTTGSPSAFPVOASPPNLVGSOLPTTTLPVV
KlTup1     HGSTVGNVPGOV  OLSPNSGAOGNANIAPPNIFO--PMVSOTVGTGMAPOMAPLNTOHPTOOTFSN-AGEOAAANLAPVI
CaTup1     --------------------------------------------------------------------------------
DdTup1     NSPSTFPSNSPA PAOHNNNNNNIINNNN-------------------------------------------NNNN--
NcTup1     NFPPOHPGOOOP AIGLOSNVFSAIMAGO------------------------------------------GGOALV
SpTup1     POSSFTFHGPNSVSFGFVGNA-GPFNSDN------------------------------------------SSKPLI
Unc37      ---PALPPGFPPGAAGNLGNNFNNMPFGMS---------------------PAMSOLFNOFASPHV--NGGDGAGGSSGG
DmGro      --PPOFMGALN -FGALGATMGLPHGFOGLLNFPPEHHPPDIFPTGLEGPAAAEEPLPNSVSPAD--PEFYRTPSPLDI
XlGro      IPLTPHPSSLOH GLAAATSASSLLGYLVFLAPPHNLL---OFPAADAEH-PEPDPGPSCLTLPNG-----EPVPTLSDY
MmGro      VPLTPHPSGLOP AIPPIGSSAGLLALSSALGGOSHLPIFDEFKHHDNDHOPDPDSIFSSSVSPSASFPGSEFHPNSTDY
Consensus
```

```
            |---------+---------+---------+---------+---------+---------+---------+---------|
ScTup1     SSNAOOOLPO-----------OOLOOOOLOOOOPP--------POVSVAPLSNTAINGSPTSFETTTLPSVKAPE TLK
KlTup1     Q---OOOOPO-----------OOLPPOOOOOOOO--------OOSNIPVTTHAPVOPAGGNLDOTVPNSISPOOOPTE
CaTup1     ----GFOOOO-----------OOOOOOOOOOOOOO--------OOIVAPPA--------------------------
DdTup1     ----NNNNNO-----------PNTPTPTIPD-FGO--------PFOPOPSGSGEFYOOPGLGPOOLNFOOLNLOOOOOOO
NcTup1     PPPPPPOOOE-----------OPAHMPAPPGLOGP--------PPPPPPPSOOPPFOOOVOGPOGPGNFPPOPPO TAS
SpTup1     LNNGSSGGTP-----------FNLPSPAIDS-DGT--------VLAPIOTSNVDLGSOYVSSPH------VPPAVGATM
Unc37      ASEAFFAFLE-----------DFDIDGELEIDVTND--------DHPSTASNG----------GAANFNGRDSTN VAS
DmGro      ENDSFPPFDEFLO-----EDEGEFSDOOLVVDVANE-------MESHSPPPNG-EHVSMEVPDPESLNGEPLEFPS SGI
XlGro      LNSNTFPFTEEFDFGTDVGSDADFSEDNLVVD---EDPASPHSVHSVSSPENGVDFPTLOPFDPPPASPNSMTSSS VSP
MmGro      SSESFFOFTEEFEIAAPYVDSDGEFSDDNLVVDVSNEDPSSPPGSPAHSPPENGLDFTRLLKFDAP-ISPASVASSS TPS
Consensus               #                #          #
```

```
         246
         245
            |---------+---------+---------+---------+---------+---------+---------+---------|
ScTup1     ETEFENNN(TS)INDTGSATTATTTTATETEIFPFEEDATPASLHODHYLVPYNOPANHSFPIPPFLLDL SO--------
KlTup1     OOOP-----------ASTATTAPATASTAPPTS--APSDOVGODHYLVPADOPAVHAFPIPPFLLDL SO--------
CaTup1     ----------------APPAPPTPVTSLSV----------IDFSOYIVHPTOPANHVAEIPPFLODL IA--------
DdTup1     LOOOOOOOGSGO--SFFPSLSPLDSNPHPFEMGNNMSGNSMSMNNNNNNL----NFKPDMEEVFEEDPPPH TE--------
NcTup1     PGPAGFPGIGRPPAGGPATPOINTPIPVNGGPAOSPOVPTHPTPDHTPMAOHHOPPPPPPSOTNALSEL PD--------
SpTup1     AGSAMP-------TFFSNLPLGHPFPPPSDSANSSVTPIAAPLVVNGFVS---GNPFPYPAEI-------IPTS--------
Unc37      SG---------------ASTPSIASNSPAPOOOOPLAGLOGLEONNFLAGFNP--------NLLPOASAAGGF ----------
DmGro      FOEPPPS-----PSGSSSSPSTPSLFTFDMEFPGTFGAFARTPTPNAAAPAPOVNPFONNFPOGPPPAGYPGA--------
XlGro      SPSFDIPSOOVEFAGTPGLFSSTPNSOSDILNTPGPSGTSASOFPSIATFPAIDSLALGLRTPLGGOGGYPAAFSIA-HPS
MmGro      SFSFELSLN--EFSTTPVSFSNTPTPRTDAPTPGSNSTPGLR-PVPGFPPGVDPLASSLPTPMAVPCPYPTPFGIVPHAG
Consensus
```

98

```
                                                              318
        |----------+---------+---------+---------+---------+---------+---------+---------|
ScTup1  -----------------------------------SV DAL  QTNDYYILY - ALPREIDVE HK  --LD T V
KlTup1  -----------------------------------LV SHL  QNNDYYVLH - ALPTDLDVE HK  --LE S V
CaTup1  -----------------------------------KANPEF  QHLEYYVLY - AFSKDLDID VH  --LD S V
DdTup1  --------------------------------MSEENG EKGTDNLVGY - SVQTNLNID LHN--LQ N V
NcTup1  -----------------------------------RL NHI  MKDNJJVIF -AAVPRVLDVE VHT--LQ E V
SpTup1  -----------------------------------NV NREE ---DNTVTS V NKEPPISVQ LHT--LE T V  Y
Unc37   --------------------------------FLNDPHAQAPLAAA----IGQIGSPPAY FKIVDGGVPTP
DmGro   -------PYQRPADPYQRPPSDPA----------YGRP PMPYDPHAHVRTNGIPHP-SALTGGKPAY FHMNGEGSLQP
XlGro   VNAD---GAGAYAGLHLMSPOINGATAAGT----YGPS LVSYDPHAHMR--GLGSAIPGSASGKPAY FHVSADGQMOP
MmGro   MNGELTSPGAHYAGLHNISPOMSAAAAAAAAAAAYGPS VVGFDFHHHMRVPAIP NLTGIPGGKPAY FHVSADGQMOP
Consensus                                       #  !                    $#   V

                                                              393
        |----------+---------+---------+---------+---------+---------+---------+---------|
ScTup1  VKF ND EYL    KTTQVYR SDGSLVAR S D AANNHKNSITENNTTTSDNNTMTTTTTTTTTTTAMTSARELAK
KlTup1  VRF SD EFL    KTTQVYK S G LVAR S D ASQPOPOPONO------------TVTAETSTSNSNGSSAEDGT
CaTup1  VRF RD KFI    KTTQVFN T G LVAK I E S--------------------------------------
DdTup1  VNF ND KYL    RSAOIYD D GKKVHAFV E ---------------------------------------
NcTup1  VRF MD KYV    RSAOIYD E G KLCI O EN-------------------------------------
SpTup1  VRF AD KFL    RAAMVFN E GKLITL O E ---------------------------------------
Unc37   TSFPPDAOKGPGIPTGLKKKMELNHG VVCAATISPDNSRVVYTGGKG--------------CVKIWDVKESDISGATVV
DmGro   VPFPPDALVGVGIPFHAPQINTLSHG VVCAVTISNPTKYVYTGGKG--------------CVKVWDISOPG-------
XlGro   VPFPPDALIGSGIPFHAPOLHVLNHG VVCAVTISNSTRHVYTGGKG--------------CVKVWDVGOPG-------
MmGro   VPFPPDALIGPGIPFHAPOINTLNHG VVCAVTISNPTRHVYTGGKG--------------CVKVWDISHPG-------
Consensus V F D         Q!      G  V

               443  447                463              490
                                                          489
        |----------+---------+---------+---------+---------+---------+---------+---------|
ScTup1  DVENLNTSSSPSSD YIRSV  PDG FL  G E RLI IWDIE--NRKIVMILQ     YSL YF S DK   S   D
KlTup1  GNON-SAASTASSD YIRSV  PDG FL  G E KLI IWD E--  KIVMTLK     Y L YF S NK   S   D
CaTup1  -NENKDDNTTASGD YIRSV  PDG LL  G E KLI IWD S--  RIIKILP     Y L FF D DR   S   D
DdTup1  ---------EKDGD YIRSV  PDGNYL  G E KTVKWUDIH--  KIOHTFY  L  Y L Y SD RFI S   DK
NcTup1  --------IDLTGD YIRSV  PDG YL  G E KLI VWDIO--SRTIRNTFH     Y L F PD RTIAS     D
SpTup1  --------SKREGD YWRSVA PDG YL  GVE QQI IWDIA--Q RVYRLLT     Y L F KD KT   S   D
Unc37   NPPPIASLDCL-KENYIPSCKLFEDGNTLLIGGEASTVALWD TTE  TL--DLETDS ACYALAM   DEKL FACLADG
DmGro   NKNFVSOLDCLORDNYIRSVKLLPDGRTLIVGGEASNLSIWD ASP PPIKAELTSSAPACYALAI   IDSKVCFSCCSDG
XlGro   TKTPVAOLDCLNRDNYIRSCKLLPDGRSLIVGGEASTLSIWD ASP PPIKAELTSSAPACYALAI   DAKVCFSCCSDG
MmGro   NKSPVSOLDCLNRDNYIRSCRLLPDGRTLIVGGEASTLSIWD AAP PPIKAELTSSAPACYALAI   IDSKVCFSCCSDG
Consensus            D YIPSV   PDG  L  G E  ! IWD    I   L       YL    D   S   D

               522                     548               567
               520                     547
        |----------+---------+---------+---------+---------+---------+---------+---------|
ScTup1  TVRIWD RT OCS_TLSI D-----G  V VSPGDGKYI  G LDPAVPVWDSETGF  E  SEN SGTG K  VYS
KlTup1  TVRIWD TT TCSL LSI D-----G  V VSPGEGKFI  G LDRTVRVWDSDTGF  E   SEN LGTG R  VYS
CaTup1  SVRIWD RTSOCSL LSI D-----G  V VSP-DGKLI  G LDRTVPVWDSTTGF  E   SGN NGNG E  VYS
DdTup1  KAKIWDIEK KCAF LGN EVGPKNG  SV MSP-DGPLV  G LDNIVRLWDAOTGYFLE Y---- G--- L  VYS
NcTup1  TVRLWDIET ONTSVLSI D-----G  V ISP-DKOFV  G LDKSVRVWDMR-GY AE  ---- GPDG K  VYS
SpTup1  TVCLWDWEA EOKLILHT D-----G  V VMFSP-DGOFI  G LDKVIPIWTSS-GT EO ----HG--- E  VYS
Unc37   NILIVDIHNKVKVIG LPGHO----DGASCLDLSK-DGTKLWSGGLDNSVRCWDLAOPKE AKH --------FASOVFSL
DmGro   NIAVUD HNEILVPOFOGHT----DGASCIDISP-DGSRLWSGGLDNTVRSWDLPEGROLOOH --------FSSOIFSL
XlGro   NIVVUD ONOTLVPOFOGHT----DGASCIDISH-DGTKLWTGGLDNTVRCWDLPEGROLOOH --------FNSOIFSL
MmGro   NIAVUD HNOTLVPOFOGHT----DGASCIDISN-DGTKLWTGGLDNTVRSWDLPEGROLOOH --------FTSOIFSL
Consensus  ! IWD        L  #    G   !  SP DG   G LD VR WD   G   #                VZS

                                                    632             652
        |----------+---------+---------+---------+---------+---------+---------+---------|
ScTup1  VFTPDGOSVV G  DPSV  N ONANNKSDSKTPNSGT EV YI H  FVLS ATTONDEYILS  KDRG L W KKSG
KlTup1  VFTRDGKGVV G  DPSV  N NGLSGOK-----SHAE EV YT H  VLS ATTONDEYILS  KD G L W TKSG
CaTup1  AFSNNGEDI G  DPTV  H EGKSDKK-------ST EV YI H  VLS CCTPDNEYILS  KD G I W OASG
DdTup1  AFS DGKS  G  DKSL  D SGSRS-------RSK RA FN H  VLS AFSPD S LIS  KD S O W PRNG
NcTup1  AFS DGRN V G  DKTI  E SAPRGIPSSAPPKGGR IK FE HR  VLS ALTPDSO VLS  KD G O W PPTG
SpTup1  AFS DGKY V G  DNTI  E OCVSNVAPSNYKEGGI KO FT H  ILS TVSPD K IIS  KD T O WSPDSP
Unc37   GCC NDEVV VGMENNYVEVLSTTGKE-----------KYOLTOHESCVLSLKFAHS KFFISTGKDNALNAVRTPYG
DmGro   GYC TGDV  VGMENSHVEVLHASKPD-----------KYOLHLHESCVLSLRFPAAC K FVSTGKDNLLNAVRTPYG
XlGro   GYC TGEV  VGMESSNIEVLHVSKPD-----------KYOLHLHESCVLSLOFASC K FVSTGKDNLLNAVRTPYG
MmGro   GYC TGEV  VGMENSHVEVLHVTKPD-----------KYOLHLHESCVLSLKFAHC K FVSTGKDNLLNAVRTPYG
Consensus  Z  #G    G  #  !              H    VLS       S  KD     W    G
```

99

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          |----------+----------+----------+----------+----------+----------+-|
ScTup1    NPL  L    P SW S   VANGSPLGPEYNW   TGSGDC A I K KKIAPN
KlTup1    NPL  L    P SW Y   TVANGHPLGPEYGW  TGSGDC A I K SKKNGOONSTOIKEIKE
CaTup1    NPL  L    P SW S   VSLNSK-GTE-GI   TGSGDC A I HWTKK
DdTup1    TTH  L    K SW S   LSPKNN-SHGW--   TGSGDFPS L K DS
NcTup1    HTQ  L    K SW S   PSPVTG-SNGWGY   TGSGDMPA I S SPI
SpTup1    HSQ  TL   N SW S   VSPNGH-C-----   TGSGDLPA I S EDL
Unc37     ASLFOLK-ENSSVLSCDISFDDS------LIVTGSGEK ATLYAWEY
DmGro     ASIFOSK-ETSSVLSCDISTDDK------YIVTGSGDK ATVYEVIY
XlGro     ASIFOSK-ESSSVLSCDVSTDDQ------FIVTGSGDK ATVYEVIY
MmGro     ASIFOSK-ESSSVLSCDISVDDK------YIVTGSGDK ATVYEVIY
Consensus      L      SW S  -!S            TGSGD  A !
```

Figure C1. *Alignment of Protein Sequences of Tup1 Homologs*

Tup1 homologs from *Kluveromyces lactis, Candida albicans, Neurospora crassa,* and

Schizosaccharomyces pombe, Dictyostelium discoideum, Caenorhabditis elegans,

Drosophila melanogaster, Xenopus laevis, and *Mus musculus* were aligned using

Multalign (http://www.sacs.ucsf.edu/Resources/sequenceweb.html). Amino acids that are

identical in at least seven of the sequences (70%) are shown in red and amino acids

that are identical in five or six sequences are shown in yellow. Residues that were

chosen for mutagenesis and analyzed for repression function (Chapter 3 and this

chapter) are shown in blue with the number above indicating its location in the *S.*

cerevisiae sequence. Residues (and their *S. cerevisiae* sequence number) that were

identified as possible sites of phosphorylation by mass spectrometry are shown in
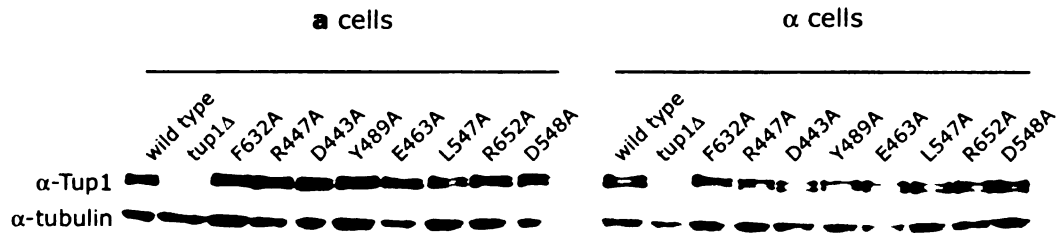
green.

Figure C2

Figure C2. *Expression of Tup1 Point Mutants*

α-Tup1 and α-tubulin Western blots were done on 15μg of total protein from the

parental wild type strain and the *tup1Δ* and point mutant strains. Mutant MATa

strains were derived from crosses with the mutant MATα strains in which the

genomic copy of *TUP1* had been replaced by the corresponding mutation.
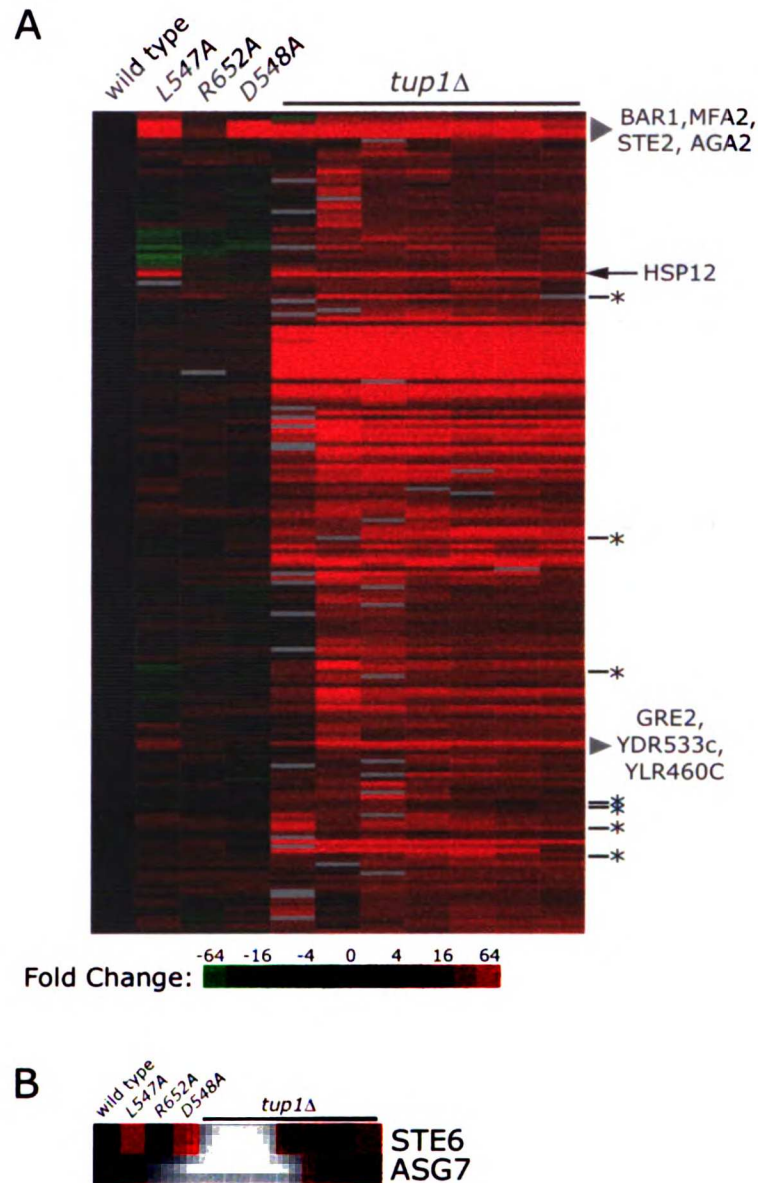
Figure C3

A

Fold Change: -64 -16 -4 0 4 16 64

B

STE6
ASG7

Figure C3. *Cluster Diagram of Microarrays on* tup1^{L547A}*,* tup1^{R652A}*, and* tup1^{D548A}*.*

(A) A single microarray was done for each point mutant and the displayed results were transformed by the corresponding wild type strain. Raw microarray data was filtered and transformed as described in Chapter 2 (see Materials and Methods). In Cluster (Eisen, Spellman et al. 1998), we selected for genes that had data in 90% of

the experiments and required that at least five experiments exhibit a $\geq$2-fold change over wild type expression. One set of genes, direct targets of Matα2 regulation, appears to be derepressed in the *tup1^L547A^* and *tup1^D548A^* mutants. Asterisks indicate downstream targets of a-specific gene regulation that are not derepressed in the point mutant strains. Other genes that show obvious derepression in one or more point mutants (typically *tup1^L547A^*) are also indicated on the Cluster diagram, but because the data represents only one microarray experiment for each strain it would be imprudent to make any conclusions about their regulation. (B) Two a-specific genes (*STE6* and *ASG7*) were excluded from the Cluster diagram in (A) because of insufficient data. Gray squares indicate unusable data for a gene in the corresponding microarray experiment.
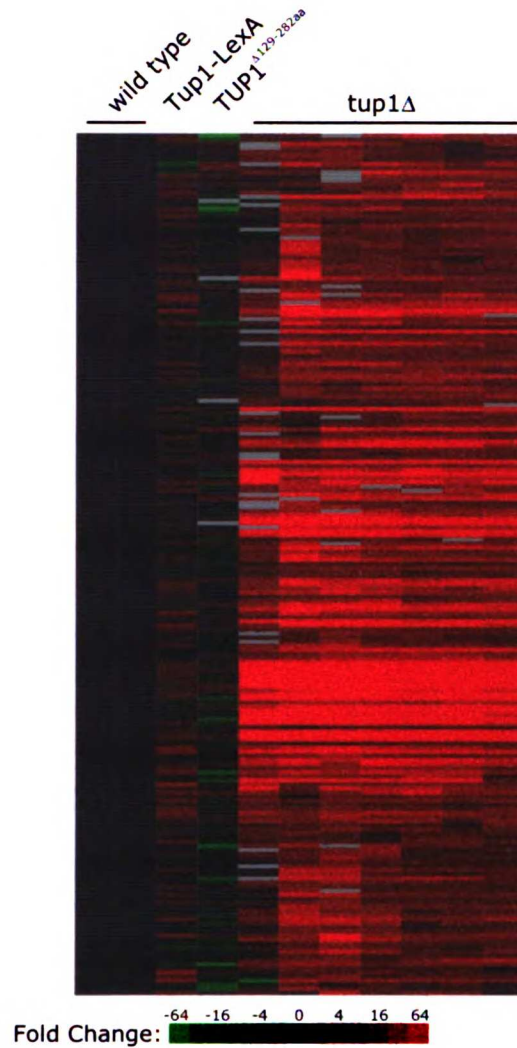
Figure C4

Figure C4. *Cluster Diagram of Tup1-lexA and TUP1$^{\Delta129-282}$*

A single microarray was done for both the Tup1-LexA strain and an internal deletion of *TUP1* (Δ129-282aa). Data was filtered as described in Chapter 2, divided by the data from a wild type sample done on the same day as each mutant, and then compared to the dataset for a *tup1Δ* strain. The Cluster diagram (Eisen, Spellman et al. 1998) contains genes for which there was data in 90% of the experiments and for which at least four experiments showed a $\geq$2-fold increase in expression compared to a wild type strain.

Appendix D

Unpublished Direct Targets of Tup1-Mediated Repression

Identification of Direct Targets of Tup1-Mediated Repression

A rigorously selected set of genes regulated by Tup1 has been compiled from the use of the invaluable technique of expression microarray experiments and the statistical analysis of that data (Galgoczy, Cassidy-Stone et al. 2004; Green and Johnson 2004). Before the inception of microarray technology, targets of Tup1-mediated repression were identified in isolation and a thorough synthesis of this dispersed data was difficult. While we can now use microarrays to identify the full complement of Tup1-repressed genes, this dataset contains both direct and indirect targets of repression by Tup1. Select Tup1-repressed genes have been shown to be direct targets in chromatin immunoprecipitation (ChIP) experiments (Wu, Suka et al. 2001; Davie, Trumbly et al. 2002; Zhang and Reese 2004; Zhang and Reese 2004) and future experiments using ChIPs hybridized to microarrays will eventually identify all of the direct targets of Tup1. Until that time, the list of genes proven to be directly regulated by Tup1 will grow in gene by gene as more ChIP experiments analyzing Tup1 recruitment to individual promoters are published. Figure D1 shows evidence for several novel direct targets of Tup1-mediated repression. Tup1 appears to be recruited to ten of the thirteen genes tested. We analyzed the relative occupancy of Tup1 at these promoters in a wild type strain, a $tup1\Delta$ strain, and two point mutants with varying repression deficiencies, $tup1^{R447A}$ and $tup1^{D443A}$ (Data for *HXT16* was only obtained from wild type and $tup1\Delta$ strains). DNA corresponding to the promoters of all ten of these genes was enriched in ChIP experiments in wild type and mutant Tup1 strains compared to background levels measured in the $tup1\Delta$ strain. Some of these genes (*MAL12, CYC7, CTT1, HAL1*) were already considered to be directly repressed by Tup1, although it had not been definitively shown, because they contained putative binding sites for sequence-specific DNA-binding proteins that

can recruit Tup1. Others were not previously characterized as targets of Tup1 either directly or indirectly. Three genes that were derepressed upon deletion of *TUP1* (*SNZ1*, *FIG1*, and *TIR3*) are shown by these experiments to be indirectly regulated by Tup1. Because the data displayed in Figure 1 is preliminary (typically measurements for the genes are from a single ChIP experiment and QPCR), further work is needed before these genes can conclusively be labeled direct targets of Tup1-mediated repression. The levels of enrichment of Tup1 over background at *DAN2* are particularly subtle and need to be more closely examined.
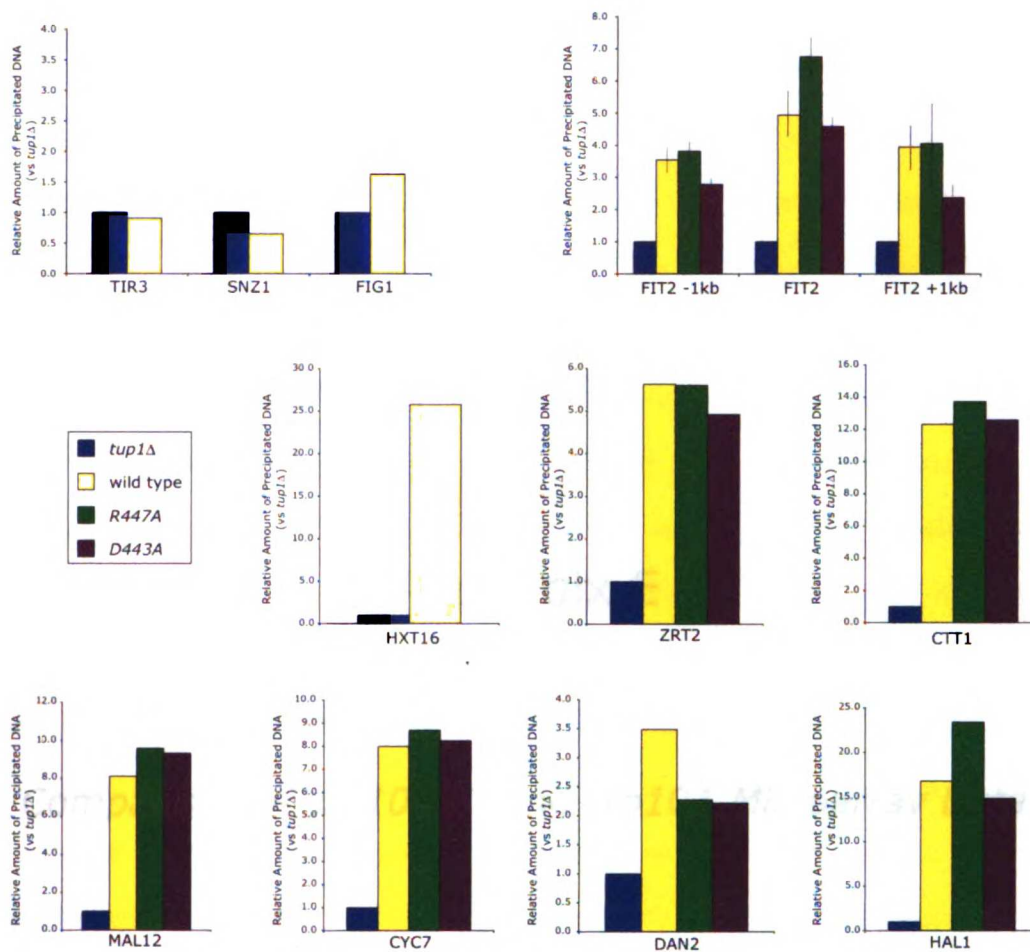
Figure D1

Figure 1. *Recruitment of Tup1 to Regulated Promoters*

ChIP experiments against Tup1 were performed and analyzed by QPCR as described in Chapter 3. The displayed results are typically the data from a single QPCR for each gene. The one exception is the *FIT2* data, which represents the average of data from 3-6 QPCRs from 2-3 independent ChIP experiments (SE measurements are displayed as error bars for these data). The first chart shows the results for genes (*TIR3*, *SNZ1*, and *FIG1*) derepressed in a *tup1Δ* strain but found to be indirect targets of Tup1-mediated repression. The remaining charts are the results for genes not previously shown to be direct targets of Tup1-mediated repression.

Appendix E

Comparison of srb10^{D304A} and srb10Δ Microarray Data

Disruption of Srb10 Function

To dissect the relative contributions of the mechanisms of Tup1-mediated repression to full repression levels, we disrupted the functions of two proteins known to play roles, Hda1 and Srb10 (Chapter 2). Although the cyclin-dependent kinase *SRB10* is not an essential gene, we opted to study the effects on repression of a kinase-dead allele of *SRB10* rather than deleting the entire gene. Srb10 has been shown to be a component of the Mediator complex and to be associated with a subcomplex consisting of Srbs 8-11 (Myer and Young 1998; Gustafsson and Samuelsson 2001; Borggrefe, Davis et al. 2002). It was unclear if a complete loss of Srb10 protein would affect Mediator-complex formation overall and cause disruptions of general, transcriptional regulation that might complicate the analysis of Srb10's specific role in Tup1-mediated repression. Precisely disabling the kinase function of Srb10 allows for the proper formation of complexes containing Srb10 while eliminating it chief documented function. A single mutation of the catalytic residue in the ATP-binding pocket of Srb10 (D304) completely inactivates its enzymatic function without disrupting its association with the Mediator (Liao, Zhang et al. 1995; Ansari, Koh et al. 2002). Our $srb10^{D304}$ mutant had many of the same phenotypes as an $srb10\Delta$, but we wanted to confirm that this kinase mutant recapitulated the global transcriptional regulation defects of the full deletion (Cooper and Strich 2002). We compared our microarray analysis of an $srb10^{D304}$ strain with a set of microarray data for an isogenic $srb10\Delta$ strain (Figure E1). The datasets from the two strains appear virtually identical for genes either positively or negatively regulated by Srb10. We conclude that the kinase function of Srb10 is wholly responsible for its role in transcriptional regulation and, therefore, is a viable alternative in our analysis of Tup1-mediated repression to a full gene deletion.
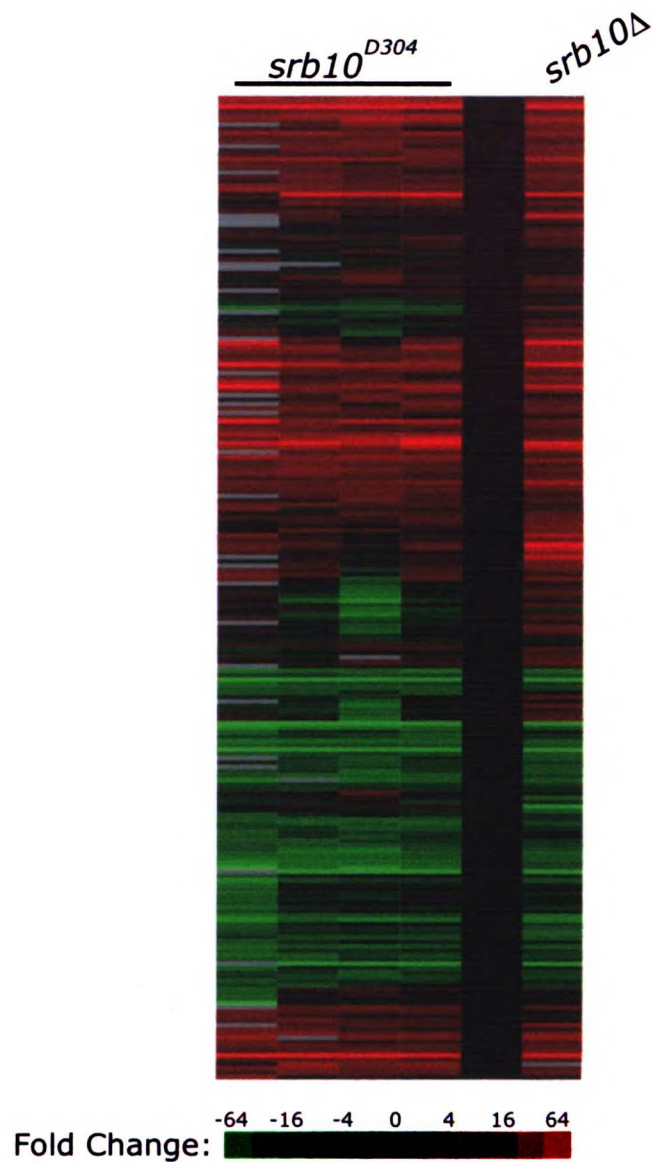
Figure E1

Figure E1. *Changes in Expression in an* srb10[D304] *strain and an* srb10Δ *strain.*

The results of four microarrays for the *srb10[D304]* strain and one microarray for the *srb10Δ* strain are displayed in the diagram. The data represent the ratio of the median pixel intensities for each array spot in the mutant strain versus a matched wild type strain. The data was then filtered in Cluster (Eisen, Spellman et al. 1998) to select for genes with data in 80% of the experiments and a ratio of mutant to wild

type expression levels of $\geq$2.5-fold in at least one experiment. The data from $srb10^{D304}$ does not appear to differ significantly from the results of the $srb10\Delta$ microarray, suggesting that disrupting the kinase function of Srb10 eliminates all of its transcriptional regulatory functions.

Appendix F

Relevant Strains and Plasmids

Table F1. List of Strains described in this text or necessary for the construction of those strains. All strains are ultimately derived from either SGY69 or SGY70, which are descended from the original S288c strain and were obtained through the generosity of Joachim Li (SGY69 and SGY70 are the same as YJL17 and YJL18).

SGY Number	Cell Type		Description
SGY69	a	haploid	wild type from Li Lab (Yeast 14, 1998, Philip Heiter) used as parent for any strains made in S288c a background
SGY70	α	haploid	wild type from Li Lab (Yeast 14, 1998, Philip Heiter) used as parent for any strains made in S288c α background
SGY71	α	haploid	ssn6::TRP1 KO by PCR off pRS304
SGY72	α	haploid	ssn6::SSN6-TAP tag (His+)
SGY74	α	haploid	cdc16::CDC16-TAP (His+) bar- from Topher Carroll (Morgan Lab) control strain for TAP purifications
SGY82	a	haploid	ssn6::TRP1 KO by PCR off pRS304
SGY83	a	haploid	tup1::TRP1 KO by PCR off pRS304
SGY84	α	haploid	tup1::TRP1 KO by PCR off pRS304
SGY91	α	haploid	tup1::URA3 leaves ~100bp of either end of Tup1, for counterselection when replacing TUP1 locus KO by PCR off pRS306
SGY92	α	haploid	wild type (SGY70) with trp1-Δ1 deletion restored MICROARRAY STRAIN also restores Gal3 to wild type levels (part of promoter is included in trp1-Δ1 deletion)
SGY97	α	haploid	ssn6::SSN6-TAP (His+) tup1::TUP1^F632A
SGY98	α	haploid	ssn6::SSN6-TAP (His+) tup1::TUP1^R447A
SGY99	α	haploid	ssn6::SSN6-TAP (His+) tup1::TUP1^D443A
SGY100	α	haploid	ssn6::SSN6-TAP (His+) tup1::TUP1^Y489A
SGY101	α	haploid	SGY93 (F632) transformed with pRS423 for mating tests
SGY102	α	haploid	SGY94 (R447) transformed with pRS423 for mating tests
SGY103	α	haploid	SGY95 (D443) transformed with pRS423 for mating tests
SGY104	α	haploid	SGY96 (Y489) transformed with pRS423 for mating tests
SGY105	α	haploid	SGY84 (tup1Δ) transformed with pRS423 for mating tests
SGY110	α	haploid	wild type with pRS424-F632A

SGY111	α	haploid	wild type with pRS424-R447A
SGY112	α	haploid	wild type with pRS424-D443A
SGY113	α	haploid	wild type with pRS424-Y489A
SGY125	α	haploid	*tup1::TUP1^{L547A}* mutation cuts with EagI
SGY126	α	haploid	*tup1::TUP1^{R652A}* mutation cuts with BsaHI
SGY127	α	haploid	*tup1::TUP1^{D548A}* mutation cuts with Sma1 @25°C
SGY128	α	haploid	*tup1::TUP1^{E463A}* mutation cuts with SacII
SGY133	α	haploid	SGY125 (L547A) transformed with pRS424 for mating
SGY134	α	haploid	SGY126 (R652A) transformed with pRS424 for mating
SGY135	α	haploid	SGY127 (D548A) transformed with pRS424 for mating
SGY136	α	haploid	SGY128 (E463A) transformed with pRS424 for mating
SGY137	a	haploid	*tup1::TUP1^{L547A}* from cross SGY133 x SGY80
SGY138	a	haploid	*tup1::TUP1^{R652A}* from cross SGY134 x SGY80
SGY139	a	haploid	*tup1::TUP1^{D548A}* from cross SGY135 x SGY80
SGY140	a	haploid	*tup1::TUP1^{E463A}* from cross SGY136 x SGY80
SGY141	a	haploid	*tup1::TUP1^{F632A}* Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY142	a	haploid	*tup1::TUP1^{R447A}* Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY143	a	haploid	*tup1::TUP1^{D443A}* Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY144	a	haploid	*tup1::TUP1^{Y489A}* Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.

SGY145	α	haploid	*tup1::TUP1*^{F632A} Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY146	α	haploid	*tup1::TUP1*^{R447A} Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY147	α	haploid	*tup1::TUP1*^{D443A} Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY148	α	haploid	*tup1::TUP1*^{Y489A} Backcrossed original mutants to wt a strain because streaks from original freezer strains (SGY93-96) had some colony heterogeneity. Froze two independent a and alpha strains (from same tetrad) for each mutant.
SGY160	α	haploid	*hda1::TRP1* KO by PCR off pRS304 a and alpha made in separate transformations
SGY161	a	haploid	*rpd3::TRP1* KO by PCR off pRS304 a and alpha made in separate transformations
SGY162	α	haploid	*rpd3::TRP1* KO by PCR off pRS304 a and alpha made in separate transformations
SGY163	α	haploid	*tup1::TRP1* transformed with pRS425 (Leu+) for matings
SGY164	α	haploid	*tup1::TUP1*^{R447A} transformed with pRS425 (Leu+) for matings
SGY165	α	haploid	*srb10::URA3* KO by PCR off pRS306 leaves ~100aa on either end of ORF for counterselection replacement
SGY166	a	haploid	*tup1::TRP1 hda1::TRP1* SGY163 x SGY159
SGY167	α	haploid	*tup1::TRP1 hda1::TRP1* SGY163 x SGY159
SGY168	a	haploid	*tup1::TUP1*^{R447A} *hda1::TRP1* SGY164 x SGY159
SGY169	α	haploid	*tup1::TUP1*^{R447A} *hda1::TRP1* SGY164 x SGY159
SGY170	α	haploid	tup1::Tup1Δ72-129aa poorly expressed see Trumbly, 2002
SGY171	α	haploid	tup1::Tup1Δ129-282aa ~full expression see Trumbly, 2002

SGY172	α	haploid	srb10::srb10^{D304A} (kinase dead)
SGY173	a	haploid	trp1-Δ1 mutation restored from cross of SGY92 x SGY80 (wt with HIS+)
SGY175	α	haploid	SGY160 (hda1Δ) transformed with pRS423 for matings
SGY176	a	haploid	SGY83 (tup1Δ) transformed with pRS423 for matings
SGY177	α	haploid	SGY84 (tup1Δ) transformed with pRS423 for matings
SGY178	a	haploid	SGY142 (R447) transformed with pRS423 for matings
SGY179	α	haploid	SGY142 (R447) transformed with pRS423 for matings
SGY180	α	haploid	SGY172 (srb10^{D304}) transformed with pRS423 for matings
SGY181	α	haploid	wild type with pRS424-Tup1
SGY182	α	haploid	wild type with pRS314-Tup1 R447
SGY183	α	haploid	wild type with pRS424-Tup1 R447
SGY184	α	haploid	wild type with pRS424-Tup1-lexA
SGY185	α	haploid	wild type with pRS424-wtTup1
SGY186	α	haploid	wild type with pRS424-Tup1^{R447A}-lexA
SGY187	α	haploid	wild type with pRS314-Tup1promoter-lexA
SGY188	α	haploid	tup1::URA3 with pRS314-wtTup1
SGY189	α	haploid	tup1::URA3 with pRS424-wtTup1
SGY190	α	haploid	tup1::URA3 with pRS314-Tup1 R447
SGY191	α	haploid	tup1::URA3 with pRS424-Tup1 R447
SGY192	α	haploid	tup1::URA3 with pRS424-Tup1 R447-lexA
SGY193	α	haploid	tup1::URA3 with pRS424-wtTup1-lexA
SGY194	α	haploid	tup1::URA3 with pRS314-Tup1promoter-lexA
SGY195	α	haploid	tup1::URA3 with Kelly's Tup1-lexA fusion plasmid
SGY196	α	haploid	Kelly's tup1 KO with pRS424-wtTup1-lexA
SGY197	α	haploid	Kelly's tup1 KO with pRS314-Tup1promoter-lexA
SGY198	α	haploid	Kelly's tup1 KO with Kelly's Tup1-lexA plasmid
SGY199	a	haploid	tup1^{R447} with trp1-Δ1 restored Trp+ GAL3+ From cross of SGY172 (R447) x SGY92
SGY200	α	haploid	tup1^{R447} with trp1-Δ1 restored Trp+ GAL3+ From cross of SGY172 (R447) x SGY92
SGY201	α	haploid	srb10^{D304A} with trp1-Δ1 restored Trp+ GAL3+ made by transformation into srb10^{D304A} strain
SGY202	α	haploid	srb10^{D304A} tup1::TRP1 made by transformation into srb10^{D304A} strain

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SGY203	α	haploid	$srb10^{D304A}$ $hda1::TRP1$ made by transformation into $srb10^{D304A}$ strain
SGY204	α	haploid	$srb10^{D304A}$ $tup1::tup1^{R447A}$ with $trp1$-$\Delta1$ restored Trp+ GAL3+ made by transformation into $srb10^{D304A}$ strain (SGY 201)
SGY205	α	haploid	$srb10^{D304A}$ $hda1::TRP1$ $tup1::TRP1$ made by transformation into $srb10^{D304A}$ tup1 strain (SGY202)
SGY206	α	haploid	Srb10^{D304} hda1::TRP1 tup1::Tup1^{R447A} made by transformation into Srb10^{D304} R447 strain (SGY204)
SGY207	α	haploid	tup1::Tup1 TS 245/246 AA at Tup1 locus creates Ava1 site
SGY209	a	haploid	tup1::Tup1-lexA transformation replacement from pSG82 Not1/Xho1 fragment
SGY210	α	haploid	tup1::Tup1-lexA transformation replacement from pSG82 Not1/Xho1 fragment
SGY212	a	haploid	integrated pKK78 (alpha2op-LacZ) at URA3 locus using Stu1 site
SGY213	α	haploid	integrated pKK78 (alpha2op-LacZ) at URA3 locus using Stu1 site
SGY214	a	haploid	integrated pKK602 (lexAop-LacZ) at ADE2 locus using Stu1 site
SGY215	α	haploid	integrated pKK602 (lexAop-LacZ) at ADE2 locus using Stu1 site
SGY218	a	haploid	SGY209 (Tup1-lexA) with pKK602 (lexAop-LacZ) integrated at Ade2 locus using Stu1 site
SGY219	α	haploid	SGY210 (Tup1-lexA) with pKK602 (lexAop-LacZ) integrated at ADE2 locus using Stu1 site
SGY220	a	haploid	SGY83 (tup1Δ) with pKK78 (alpha2op-LacZ) integrated at URA3 locus using Stu1 site
SGY221	α	haploid	SGY84 (tup1Δ) with pKK78 (alpha2op-LacZ) integrated at URA3 locus using Stu1 site
SGY222	a	haploid	SGY83 (tup1Δ) with pKK602 (lexAop-LacZ) integrated at ADE2 locus using Stu1 site
SGY223	α	haploid	SGY84 (tup1Δ) with pKK602 (lexAop-LacZ) integrated at ADE2 locus using Stu1 site
SGY224	α	haploid	KKY135 with pKK78 integrated at URA3 @ Stu1 Remake of strain from Kelly's paper
SGY225	α	haploid	KKY135 with pKK602 integrated at ADE2 @ Stu1 Remake of strain from Kelly's paper (Remake of SGY47, not in freezer stock?)
SGY226	a	haploid	SGY142 (R447) with pKK78 (alpha2op-LacZ) integrated at URA3 locus using Stu1 site

SGY227	α	haploid	SGY146 (R447) with pKK78 (alpha2op-LacZ) integrated at *URA3* locus using Stu1 site
SGY228	**a**	haploid	SGY214 (lexAop-LacZ) with pSG57 (high copy Tup1pro-Tup1)
SGY229	α	haploid	SGY215 (lexAop-LacZ) with pSG57 (high copy Tup1pro-Tup1)
SGY230	**a**	haploid	SGY214 (lexAop-LacZ) with pSG96 (high copy Tup1pro-LexA)
SGY231	α	haploid	SGY215 (lexAop-LacZ) with pSG96 (high copy Tup1pro-LexA)
SGY232	α	haploid	SGY215 (lexAop-LacZ) with pSG82 (high copy Tup1pro-Tup1-LexA)
SGY233	**a**	haploid	SGY214 (lexAop-LacZ) with pSG68 (high copy Tup1pro-Tup1R447-LexA)
SGY234	α	haploid	SGY215 (lexAop-LacZ) with pSG68 (high copy Tup1pro-Tup1R447-LexA)
SGY235	α	haploid	*tup1::Tup1^{R447}-lexA* integrated at tup1 locus transformation of Not1/Xho1 fragment of pSG68 STRAIN WAS BAD AFTER RESTREAKING...REMADE AND STOCK REPLACED
SGY236	**a**	haploid	*hda1::TRP1* REPLACES SGY159 from cross of SGY160 x SGY80 (same tetrad as SGY237), see SGY238 also used for H3 ChIP experiments checked for ORF and KO PCR
SGY239	α	haploid	*tup1*::Tup1 T318A creates BsrG1 site
SGY240	α	haploid	*tup1*::Tup1 TS 245/246 AA, T318A creates Ava1, BsrG1 sites
SGY241	α	haploid	*tup1*::Tup1 S567A creates Kpn1 site
SGY242	α	haploid	*tup1*::Tup1 TS 245/246 AA, S567A creates Ava1, Kpn1 sites
SGY243	α	haploid	*tup1*:: Tup1 T318A, S567A creates BsrG1, Kpn1 sites
SGY244	α	haploid	*tup1*:: Tup1 TS 245/246 AA, T318A, S567A creates Ava1, BsrG1, Kpn1 sites REPLACES SGY211
SGY251	α	haploid	*tup1*:: Tup1 S490A creates EcoRV site
SGY252	α	haploid	*tup1*:: Tup1T/S245,T318,S490,S567 creates Ava1,BsrG1, Kpn1, EcoRV sites
SGY253	α	haploid	*tup1*::Tup1R447-lexA with pKK602(lexAop-LacZ) integrated with Stu1 site
SGY258	α	haploid	SGY215 (wtα with pKK602 reporter) with pRS424-F632-lexA
SGY259	α	haploid	SGY215 (wtα with pKK602 reporter) with pRS424-D443-lexA
SGY260	α	haploid	SGY215 (wtα with pKK602 reporter) with pRS424-Y489-lexA
SGY261	α	haploid	SGY215 (wtα with pKK602 reporter) with pRS424-E463-lexA
SGY266	α	haploid	*tup1*::Tup1 S393A creates Bsm1 site

SGY267	α	haploid	*tup1*::Tup1 T/S245, T318, S393, S490, S567 creates Ava1, BsrG1, Kpn1, Bsm1, and EcoRV sites
SGY268	α	haploid	*tup1*::Tup1 S/T520 creates BssH1 site
SGY269	α	haploid	*tup1*::Tup1 T/S245, T318, S490, S/T520, S567 creates Ava1, BsrG1, Kpn1, BssH1, and EcoRV sites
SGY270	α	haploid	SGY215 (wt with integrated pKK602) with *tup1::URA3* leaving ~300bp on either end of ORF for integrating mutant Tup1-lexAs in reporter strain
SGY271	α	haploid	SGY219 (wtTup1-lexA with integrated pKK602) with *tup1-lexA::URA3* leaving ~300bp on either end of ORF for integrating mutant Tup1-lexAs in reporter strain
SGY282	α	haploid	SGY215 *tup1::F632-lexA*
SGY283	α	haploid	SGY215 *tup1::D443-lexA*
SGY284	α	haploid	SGY215 *tup1::Y489-lexA*
SGY285	α	haploid	SGY215 *tup1::E463-lexA*
SGY286	α	haploid	SGY281 transformed with pSG96 (Tup1prom-lexA)
SGY287	α	haploid	SGY281 transformed with pSG82 (Tup1-lexA)
SGY288	α	haploid	SGY281 transformed with pSG72 (F632-lexA)
SGY289	α	haploid	SGY281 transformed with pSG68 (R447-lexA)
SGY290	α	haploid	SGY281 transformed with pSG71 (D443-lexA)
SGY291	α	haploid	SGY281 transformed with pSG76 (Y489-lexA)
SGY292	α	haploid	SGY281 transformed with pSG93 (E463-lexA)

Table F2. List of plasmids used for the creation of yeast strains or experiments described in this thesis. The restriction sites listed in regular type are those used to clone into the vector and sites in bold type are those created by the mutation of the cloned gene. The selectable marker listed is the appropriate selection for growth of a yeast strain containing the corresponding plasmid.

pSG Number	Vector	Restriction Sites	Description	Selectable Marker
pSG47	pRS314	NotI/XhoI	Full length Tup1 with upstream 1000bp (promoter) SEQUENCED	TRP1
pSG57	pRS424	NotI/XhoI	Tup1 plus 1000bp upstream in 2μ vector	TRP1
pSG62	pRS314	NotI/XhoI	TUP1 R447 plus 1000bp upstream (promoter) mutant cuts with NruI SEQUENCED	TRP1
pSG63	pRS424	NotI/XhoI	TUP1 R447 plus 1000bp upstream (promoter) cut from pSG62	TRP1
pSG65	pRS314	NotI/XhoI	TUP1 Y489 plus 1000bp upstream (promoter) PARTIALLY SEQ	TRP1
pSG66	pRS314	NotI/XhoI	TUP1 F632 plus 1000bp upstream (promoter) PARTIALLY SEQ	TRP1
pSG67	pRS314	NotI/XhoI	TUP1 D443 plus 1000bp upstream (promoter) PARTIALLY SEQ	TRP1
pSG68	pRS424	NotI/XhoI	TUP1 R447-lexA plus 1000bp upstream (promoter) inserted (mutant containing) BsrGI/EcoRI fragment from pSG62 to remove seq errors introduced from pKK631 (template for making mutant fusion) PARTIALLY SEQ	TRP1
pSG71	pRS424	NotI/XhoI	TUP1 D443-lexA plus 1000bp upstream (promoter) inserted (mutant containing) BsrGI/EcoRI fragment from pSG67 to remove seq errors introduced from pKK631 (template for making mutant fusion) PARTIALLY SEQ	TRP1
pSG72	pRS424	NotI/XhoI	TUP1 F632-lexA plus 1000bp upstream (promoter) inserted (mutant containing) BsrGI/EcoRI fragment from pSG66 to remove seq errors introduced from pKK631 (template for making mutant fusion) PARTIALLY SEQ	TRP1

pSG74	pRS306	NotI/XhoI	TUP1 F632-lexA plus 1000bp upstream (promoter) cut from pSG72	URA3
pSG75	pRS424	NotI/XhoI	TUP1-lexA plus 1000bp upstream (promoter) Remade, pSG82 DO NOT USE	TRP1
pSG76	pRS424	NotI/XhoI	TUP1 Y489-lexA plus 1000bp upstream (promoter) inserted (mutant containing) BsrGI/EcoRI fragment from pSG65 to remove seq errors introduced from pKK631 (template for making mutant fusion) PARTIALLY SEQ	TRP1
pSG82	pRS424	NotI/XhoI	TUP1-lexA plus 1000bp upstream (promoter) use instead of pSG75 SEQUENCED	TRP1
pSG83	pRS424	NotI/XhoI	TUP1 F632 plus 1000bp upstream (promoter)	TRP1
pSG84	pRS424	NotI/XhoI	TUP1 D443 plus 1000bp upstream (promoter)	TRP1
pSG85	pRS424	NotI/XhoI	TUP1 Y489 plus 1000bp upstream (promoter)	TRP1
pSG86	pRS314	NotI/XhoI	TUP1 L547 plus 1000bp upstream (promoter) ligated XcmI/EcoRI fragment from original Quik Change plasmid (seq) into pSG47 to remove all extra sequence errors	TRP1
pSG87	pRS314	NotI/XhoI	TUP1 R652 plus 1000bp upstream (promoter) ligated XcmI/EcoRI fragment from origial Quik Change plasmid (seq) into pSG47 to remove all extra sequence errors	TRP1
pSG88	pRS314	NotI/XhoI	TUP1 D548 plus 1000bp upstream (promoter) ligated XcmI/EcoRI fragment from origial Quik Change plasmid (seq) into pSG47 to remove all extra sequence errors	TRP1
pSG89	pRS314	NotI/XhoI	TUP1 E463 plus 1000bp upstream (promoter) SEQUENCED	TRP1
pSG90	pRS424	NotI/XhoI	TUP1 L547-lexA plus 1000bp upstream (promoter) ligated BsrGI/EcoRI fragment from pSG86 into pSG82 to remove all extra seq errors	TRP1
pSG91	pRS424	NotI/XhoI	TUP1 R652-lexA plus 1000bp upstream (promoter) ligated BsrGI/EcoRI fragment from pSG87 into pSG82 to remove all extra seq errors	TRP1

pSG92	pRS424	NotI/XhoI	TUP1 D548-lexA plus 1000bp upstream (promoter) ligated BsrGI/EcoRI fragment from pSG88 into pSG82 to remove all extra seq errors	TRP1
pSG93	pRS424	NotI/XhoI	TUP1 E463-lexA plus 1000bp upstream (promoter) ligated BsrGI/EcoRI fragment from pSG89 into pSG82 to remove all extra seq errors	TRP1
pSG94	pRS314	NotI/XhoI	Tup1 Δ72-129aa plus 1000bp upstream (promoter) SEQUENCED	TRP1
pSG95	pRS314	NotI/XhoI	Tup1 Δ129-282aa plus 1000bp upstream (promoter) SEQUENCED	TRP1
pSG96	pRS314	NotI/XhoI	LexA under the control of Tup1 promoter (1000bp upstream of ORF start) LexA SEQUENCED	TRP1
pSG97	pRS314	NotI/XhoI	SRB10 plus 500bp upstream (promoter) SEQUENCED	TRP1
pSG98	pRS314	NotI/XhoI	SRB10^{D304} plus 500bp upstream (promoter) SEQUENCED	TRP1
pSG99	pRS314	Not1/Xho1/**Ava1**	Tup1 TS 245/246 AA under Tup1 promoter creates Ava1 site SEQUENCED	TRP1
pSG100	pRS314	Not1/Xho1/**Ava1/ BsrG1/ Kpn1**	Tup1 TS 245/246 AA, T318, S567A under Tup1 promoter creates Ava1, BsrG1, Kpn1 sites SEQUENCED	TRP1
pSG101	pRS314	Not1/Xho1/**BsrG1**	Tup1 T318 under Tup1 promoter creates BsrG1 sites SEQUENCED	TRP1
pSG102	pRS314	Not1/Xho1/**Ava1/ BsrG1**	Tup1 TS 245/246 AA, T318 under Tup1 promoter creates Ava1, BsrG1 sites SEQUENCED	TRP1
pSG103	pRS314	Not1/Xho1/**Kpn1**	Tup1 S567A under Tup1 promoter creates Kpn1 site SEQUENCED	TRP1
pSG104	pRS314	Not1/Xho1/**Ava1/ Kpn1**	Tup1 TS 245/246 AA, 567A under Tup1 promoter creates Ava1, Kpn1 sites SEQUENCED	TRP1
pSG105	pRS314	Not1/Xho1/**BsrG1 /Kpn1**	Tup1 T318, S567A under Tup1 promoter creates BsrG1, Kpn1 sites made from ligation of BstE11/Xho1 fragment of pSG103 into backbone of pSG101	TRP1
pSG106	pRS314	Not1/Xho1/**EcoRV**	Tup1 S490 under Tup1 promoter creates EcoRV site SEQUENCED	TRP1
pSG107	pRS314	Not1/Xho1/**Ava1/ BsrG1/Kpn1**	Tup1 T/S245/6 T318A, S490A, S567A under Tup1 promoter creates Ava1, EcoRV, BsrG1, Kpn1 sites SEQUENCED	TRP1
pSG108	pRS314	Not1/Xho1/**Bsm1**	Tup1 S393A under Tup1 promoter creates Bsm1 sites SEQUENCED	TRP1

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pSG109	pRS314	Not1/Xho1/**Ava1/ EcoRV/BsrG1/Kp n1/Bsm1**	Tup1 T/S245/6, T318A, S393A, S490A, S567A under Tup1 promoter creates Ava1, EcoRV, BsrG1, Kpn1, Bsm1 sites SEQUENCED	TRP1
pSG110	pRS314	Not1/Xho1/**BssH1**	Tup1 S/T520A under Tup1 promoter creates BssH1 sites SEQUENCED	TRP1
pSG111	pRS314	Not1/Xho1 **Ava1/EcoRV/Bsr G1/Kpn1/Bsm1/ BssH1**	Tup1 T/S245/6, T318A, S490A, S/T520, S567A under Tup1 promoter creates Ava1, EcoRV, BsrG1, Kpn1, BssH1 sites SEQUENCED	TRP1

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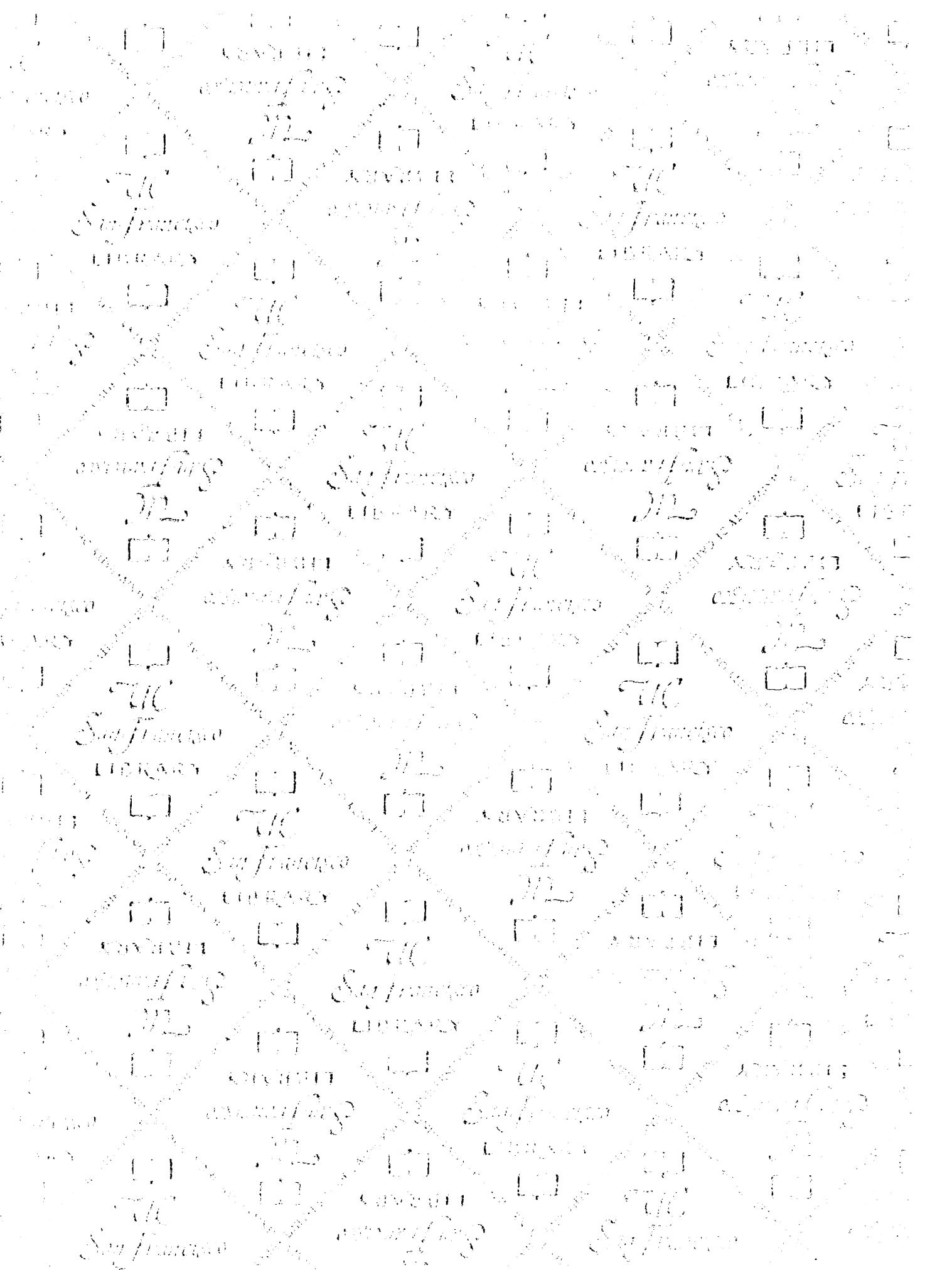
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