Promoter-dependent Roles for the Srb10 Cyclin-dependent Kinase and the Hda1 Deacetylase in Tup1-mediated Repression in *Saccharomyces cerevisiae*[□]

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The Tup1–Ssn6 complex has been well characterized as a *Saccharomyces cerevisiae* general transcriptional repressor with functionally conserved homologues in metazoans. These homologues 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-dependent kinase 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 ~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, more than 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.

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. Homologues of Tup1 have been identified in several other organisms (for example, *unc-37* in *Caenorhabditis 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 *et al.*, 1997; Fisher and Caudy, 1998; Levanon *et al.*, 1998; Grbavec *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 *et al.*, 2001). Hyperacetylation of Tup1-repressed genes also is seen when Tup1 is deleted (Bone and Roth, 2001; Davie *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 *et al.*, 2002). However, a clear link between loss of Hda1-mediated 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 using a naked DNA template (Herschbach *et al.*, 1994; Redd *et al.*, 1997). In addition, several components of the PolIII transcriptional machinery (Rgr1, Sin4, Rox3, Hrs1, and Srbs8-11) have been identified in genetic screens for loss of Tup1-mediated repression (Sakai *et al.*, 1990; Kuchin *et al.*, 1995; Wahi and Johnson, 1995; Song *et al.*, 1996; Carlson, 1997). A few also have been shown to physically interact with the Ssn6–Tup1 complex (Gromoller and Lehming, 2000; Papamichos-Chronakis *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 *et al.*, 2001; Borggreffe *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 *et al.*, 1998; Kuchin and Carlson, 1998; Song and Carlson, 1998; Lee *et al.*, 2000). Furthermore, expression microarray experiments demonstrated some overlap between genes repressed by Srb10 and by Rox1, a

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DNA-binding protein that recruits the Tup1-Ssn6 complex and is responsible for repressing hypoxia-induced genes (Holstege *et al.*, 1998; Becerra *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, multilayer system for establishing Tup1-mediated gene repression.

MATERIALS AND METHODS

Yeast Strains

The *S. cerevisiae* strains used in this study were all generated from a parental strain of genotype MAT α *ura3-52*, *lys2-801^{amb}*, *ade2-101^{och}*, *leu2- Δ 1*, *his3- Δ 200*, *trp1- Δ 1*, which was a descendent of the original S288c strain. SGY201 (*srb10^{D304}*) was made by transforming a full-length open reading frame (ORF) fragment containing the mutation into a strain in which the *SRB10* locus has been replaced with *URA3*, leaving $\approx$ 200 bp of ORF homology on either side. Growth on 5-fluoroorotic acid selected for a strain in which the mutated *SRB10* ORF had been integrated at the *SRB10* genomic locus. SGY160 (*hda1 Δ*), SGY84 (*tup1 Δ*), and SGY203 (*srb10^{D304}hda1 Δ*) were constructed by transforming the parental strains with polymerase chain reaction (PCR) products of the *TRP1* gene flanked by homologous sequences of the appropriate target gene. SGY167 (*tup1 Δ hda1 Δ*) was made by crossing SGY160 and SGY83 (MAT α *tup1 Δ*) and SGY205 (*srb10^{D304}tup1 Δ hda1 Δ*) was made by repeating the protocol for mutating *SRB10* described above but using SGY167 as the starting strain. The gene knockouts we made resulted in *TRP1* strains, so we replaced the *trp1- Δ 1* 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 ($\sim$ 6100 spots) were performed as described previously (<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) by 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 *tup1 Δ* 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 by 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 *tup1 Δ* upon which 5000 permutations were performed. The delta values for all datasets except *hda1 Δ* were selected as the value that resulted in the lowest FDR calculated for the 90th percentile *d*-scores. The FDRs for the *hda1 Δ* data were higher in general than those of the other mutants, 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 *hda1 Δ* data suggested an alternative delta value selection. *HDA1* was the only gene substantially down-regulated in the SAM Plot of the *hda1 Δ* data, so we used its sole inclusion in the set of significantly down-regulated 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 down-regulated genes. This added 33 genes to the set of significant genes for the *hda1 Δ* strain (and only increased the FDR by four percentage points) compared with the set of genes resulting from the delta

value corresponding to the lowest FDR. The degree of overlap between the significant genes for the *tup1 Δ* strain and the sets of significant genes for the *hda1 Δ* strain by using these two delta values was essentially unchanged, so we elected to allow for a slightly higher FDR and to use the larger *hda1 Δ* significant gene set.

Chromatin Immunoprecipitation (ChIP) and Quantitative PCR (Q-PCR)

Antibodies against acetylated lysine 18 of histone H3 were purchased from Upstate Biotechnology (Lake Placid, NY) (catalog no. 07-354). Cultures were cross-linked with formaldehyde for 5 min, and ChIPs were performed with slight modifications as described previously (Strahl-Bolsinger *et al.*, 1997). Extract from 50 to 100 ml of culture at OD₆₀₀ $\sim$ 1 was used for each immunoprecipitation (IP). Extracts were sonicated 10 times for 12 s by using a Branson sonifier 450 at 50% output power. ChIPs were quantitated by Q-PCR in a DNA Engine Opticon machine (MJ Research, Watertown, MA). PCR products were between 200 and 400 bp. 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 from a particular ChIP experiment. Enrichment levels for each analyzed gene are averages of data from two to four independent ChIPs. Enrichment for α -specific genes was calculated separately for the two cell types, but for all other non-cell type controlled genes data for both α - and α -cells were averaged.

RESULTS

Deletion of TUP1 Derepresses a Large Group of Diverse Genes

One of the first articles describing the expression microarray technique included a set of data for a *tup1 Δ* mutant (DeRisi *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 seeming to be slightly down-regulated in a *tup1 Δ* strain (Hughes *et al.*, 2000b). 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 *tup1 Δ* 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 *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 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 *tup1 Δ* strain.

Three hundred and thirty-four genes passed this standard and are considered significantly derepressed in the *tup1 Δ* mutant. Many of these genes also were reported in the initial published *tup1 Δ* dataset (using a $\geq$ 2-fold increase in expression cut-off), but more than 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 *tup1 Δ* microarray experiments allowed us to de-

Table 1. GO classifications for Tup1-regulated gene

Category	Not found in Derisi <i>et al.</i> set		Included in Derisi <i>et al.</i> set	
	No.	%	No.	%
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	

termine 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 *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 that 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* or *HDA1* or containing a mutant of Srb10 that lacks kinase activity (*srb10^{D304}*) (Liao *et al.*, 1995; Ansari *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 in order to identify differentially regulated genes. We analyzed datasets representing at least four duplicates for each strain by 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 *Materials and 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 example, a fairly standard cutoff of ≥ 2 -fold change in expression worked well for identifying significant genes in the *tup1Δ* dataset, but it 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 is up-regulated (derepressed)

compared with 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 the *tup1Δ* strain to those of each of the other three mutant strains. We focused only on derepressed genes because the down-regulation 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 one-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 down-regulate the activity of several transcriptional activators, which could account for many of the genes derepressed in the *srb10^{D304}* microarrays (Chi *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 Tup1-controlled genes, but rarely was the level of derepression in the mutant strains equal to the full derepression measured in a *tup1Δ* 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 *hda1Δtup1Δ*, *srb10^{D304}tup1Δ*, and *srb10^{D304}hda1Δtup1Δ*. The expression profiles of Tup1-repressed genes in these strains closely resemble that of the *tup1Δ* strain (unpublished data). 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 (unpublished data) 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-mediated Repression

The microarray experiments presented above allow us to divide the larger set of Tup1-controlled genes into five sub-

Table 2. Some previously unassigned Tup1-regulated genes

ORF	Gene Name	Molecular Function	
YOR348C	PUT4	amino acid permease activity / neutral amino acid transporter activity	Transport
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	
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	
YIL015W	BAR1	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	
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	Cell Wall
YMR135C	GID8	molecular_function unknown	
YFL058W	THI5	molecular_function unknown	
YDL244w	THI13	molecular_function unknown	
YMR096W	SNZ1	molecular_function unknown / protein binding	
YNL274C		oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as	
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	Stress Response
YGR032W	GSC2	1,3-beta-glucan synthase activity	
YDL049C	KNH1	molecular_function unknown	
YKR076W	ECM4	molecular_function unknown	
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	
YMR175W	SIP18	phospholipid binding	Stress Response
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

	No. of significant genes			FDR ^a (%)
	Total	Up-regulated	Down-regulated	
<i>tup1Δ</i>	354	334	20	0.12
<i>hda1Δ</i>	133	132	1	11.4
<i>srb10^{D304}</i>	217	166	51	0.24
<i>srb10^{D304}hda1Δ</i>	327	277	50	0.13

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

classes based on the influence of the other mutations on gene expression. These subclasses are described as follows: 1) genes derepressed in the *tup1Δ* and *srb10^{D304}* strains; 2) genes derepressed in the *tup1Δ* and *srb10^{D304}hda1Δ* strains but not the single *hda1Δ* and *srb10^{D304}* mutant strains; 3) genes derepressed in the *tup1Δ*, *hda1Δ*, *srb10^{D304}*, and *srb10^{D304}hda1Δ* strains; (4) genes derepressed in the *tup1Δ* and *hda1Δ* strains; and 5) genes derepressed in the *tup1Δ* 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 *et al.*, 1990; Zitomer and Lowry, 1992; Balasubramanian *et al.*, 1993; Amillet *et al.*, 1995; Treitel and Carlson, 1995; Ozcan and Johnston, 1996; Deckert *et al.*, 1998; Lutfiyya *et al.*, 1998; Johnston, 1999; Lee *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 used at a particular promoter.

Chromosomal Position Bias of Tup1-controlled Genes

We wanted to know whether 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 25 kb of a chromosome end (subtelomeric regions) (Robyr *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, 5 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 seem 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-mediated 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 *et al.*, 2001). ChIP microarrays also have demonstrated that the pattern of genes derepressed upon *TUP1* deletion significantly overlaps with acetylation patterns resulting from an *HDA1* deletion (Robyr *et al.*, 2002). These data, in addition to evidence of a physical interaction between Tup1 and Hda1, suggest that Tup1 recruits Hda1 to promoters to repress transcription (Wu *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 (unpublished data), 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, whereas *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 with a wild-type strain (Figure 4, A and C). We next examined the effect of an *HDA1* deletion on H3-K18 acetylation at Tup1-controlled genes. In the experiments shown in Figure 4, all of the Tup1-controlled promoters we tested are hyperacetylated at H3-K18 in an *hda1Δ* strain compared with a wild-type strain. However,

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) *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 with a wild-type strain. Lists of the significantly derepressed genes in each mutant strain are available as Supplementary Table 3.

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 seems that Hda1 is functioning at most (if not all) promoters directly repressed by Tup1 as part of a multicomponent 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 (unpub-

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 up-regulated genes in the *tup1Δ* microarray that had data for all 16 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) *tup1Δ* and *srb10^{D304}*, 2) *tup1Δ* and *srb10^{D304} hda1Δ*, 3) *tup1Δ*, *hda1Δ*, *srb10^{D304}*, and *srb10^{D304} hda1Δ*, 4) *tup1Δ* and *hda1Δ*, and 5) *tup1Δ* 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 with a wild-type strain.

Figure 3. Overlap of repressed subtelomeric genes. (A) Table shows the total number of significantly derepressed genes both within and outside of subtelomeric regions (≤ 25 kb 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.

lished data; Erdman *et al.*, 1998). The *FIG1* promoter is not hyperacetylated at H3-K18 in either the *tup1Δ* or *hda1Δ* strains compared with 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 *S. 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. Whereas 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 example, 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 reflect its role in mediating the cell's adaptive response to the external environment.

In this article, 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 the genes whose expression is significantly altered in a *tup1Δ* strain are up-regulated. Similarly, Hda1 seems to act primarily as a transcriptional repressor. In fact, no genes (save *HDA1* itself) are significantly down-regulated 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 one-third of Tup1-repressed genes. These data suggest that Hda1's main role in transcriptional regulation is to function with Tup1, whereas 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,

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 increase in acetylation in a mutant strain compared with 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 SE 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.

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 *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 also are repressed by Tup1, whereas only a minority of the genes deacetylated by Hda1 also are regulated by Tup1. This difference suggests that although 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. Although 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 previously and seems to fit well with the regulatory requirements of a repressor of diverse gene sets (Carlson, 1997; Lee *et al.*, 2000; Papamichos-Chronakis *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

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.

all subclasses of Tup1-repressed genes, and therefore do not seem to be subject to a particular repression mechanism simply because of 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 seem 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-controlled 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.* (2004) and Huisinga and Pugh (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 article exhibit this same propensity for SAGA-dominated transcriptional regulation, so although inclusion in this group does seem to be a characteristic of Tup1-repressed genes, it does not seem to dictate the influence of a particular repression mechanism.

Another model, proposed by Edmondson *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 *et al.*, 2002). In addition, our own initial microarray analysis of a strain containing this internally deleted Tup1 allele ($\Delta 129-282$ aa) revealed no affect on Tup1-mediated repression (unpublished data). Similarly, microarrays of a strain with the histone H3 tail deleted ($\Delta 1-28$) 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 *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 *et al.*, 2003). However, derepression in an Itc1 deletion is not complete, and microarrays of an *isw2* Δ strain do not demonstrate this loss of repression for the larger set of Tup1-controlled genes (Hughes *et al.*, 2000a). 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 ~450 kDa, could itself be sufficient to generate a significant degree of repression simply by interfering with transcriptional initiation conditions (Varanasi *et al.*, 1996; Redd *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 known from these studies whether the

complex remains intact (Papamichos-Chronakis *et al.*, 2002; Proft and Struhl, 2002; Mennella *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 not 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 seems 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 one-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.

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