Genome-wide Analysis of the Functions of a Conserved Surface on the Corepressor Tup1[□]

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The general transcriptional repressor Tup1 is responsible for the regulation of a large, diverse set of genes in *Saccharomyces cerevisiae*, and functional homologues 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 article, 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 by 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 >300 genes under standard growth conditions (DeRisi *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 *et al.*, 1997; Fisher and Caudy, 1998; Levanon *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 *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 *et al.*, 2000; Bone and Roth, 2001; Wu *et al.*, 2001; Zaman *et al.*, 2001; Robyr *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 *et al.*, 1994; Tzamarias and Struhl, 1994; Edmondson *et al.*, 1996; Varanasi *et al.*, 1996; Zhang *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 *et al.*, 1994; Garcia-Higuera *et al.*, 1996; Smith *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 *et al.*, 1994). Sprague *et al.* (2000) 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 article, 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 by 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 immunoprecipitation (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.

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A

B

Figure 1. Point mutants of Tup1. (A) Ribbon diagrams of the WD domain of Tup1 (282–713 aa) depicting the five amino acids selected for mutation to alanine. (B) Western blots showing expression levels of mutant Tup1 proteins.

MATERIALS AND METHODS

Plasmids

Plasmid pKK602 was described previously (Komachi and Johnson, 1997). Plasmid pAJ201 contains two LexA operators at the *Sma*I site in pLGΔ312S (Guarente and Mason, 1983). The α -specific gene reporter pKK78 contains three *Mata*2 operator sites inserted into the *Sma*I 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 800 base pairs (bp) of sequence upstream of the *TUP1* open reading frame (ORF) into the *Not*I/*Xho*I site of pRS424 (Sikorski and Hieter, 1989). They are pSG82 (wild-type 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 800-bp *TUP1* upstream sequence fused directly to the LexA protein sequence.

Yeast Strains

The *S. cerevisiae* strains used in this study were all generated from a parental strain of genotype MAT α *ura*3-52, *lys*2-801^{amb}, *ade*2-101^{och}, *leu*2- Δ 1, *his*3- Δ 200, *trp*1- Δ 1, which was descended from the original S288c strain. SGY84 (*tup1* Δ) 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 ~200 bp of ORF homology on either side. Growth on 5-fluoroorotic 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 MAT α strain matching the parental S288c strain (SGY69) and sporulated to generate MAT α 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*- Δ 1 deletion restored. SGY146 and SGY200 both have doubling times of ~100 min in YPD at 30°C, and we do not believe the differences at the *TRP1* locus are significantly influencing our results under the growth conditions used in these experiments (our unpublished data). The wild-type strain used in the microarrays was either SGY70 (the parental MAT α strain described above) or SGY92 in which the *trp1*- Δ 1 deletion was restored.

SGY212, SGY220, and SGY226 are MAT α strains with pKK78 integrated at the *ADE2* locus and wild-type *TUP1*, *tup1*^{R447A}, and *tup1* Δ at the genomic *TUP1* locus, respectively. SGY213, SGY221, and SGY227 are the matching MAT α strains. SGY215 is SGY70 with pKK602 integrated at the *ADE2* locus.

SGY219 was made by selecting for the replacement of a *tup1* Δ 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 MAT α 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 Significance Analysis of Microarrays (SAM) Analysis

Microarrays of cDNA ORFs (~6100 spots) were performed as described previously (<http://derisilab.ucsf.edu/microarray/protocols.html>; Green and Johnson, 2004). Each of the microarrays was done six times (from independently grown cultures), except for the *tup1* Δ and *tup1*^{R447A} microarrays, which were done seven times (data available as Supplemental 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 data sets, except *tup1* Δ and *tup1*^{R447A}, upon which 5000 permutations were performed. The delta values for all data sets were selected as the value that resulted in the lowest false discovery rate (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 Ausubel (1988). 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 Biotechnology (Lake Placid, NY) (catalog no. 06-719). Antibodies against Tup1 were generated using a bacterially expressed full-length Tup1-glutathione *S*-transferase (GST) fusion. 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 ~1 was used for each immunoprecipitation. Extracts were sonicated seven times for 12 s by using a Branson sonifier 450 at 50% output power. ChIPs were

analyzed by QPCR in a DNA Engine Opticon machine (MJ Research, Watertown, MA). PCR products were between 200 and 400 bp.

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 immunoprecipitation (IP). The amount of immunoprecipitated DNA in each IP was normalized for input and the measurements from α and α strains for each mutant were averaged. This average was then divided by the average of the measurements for *tup1 Δ* α and α strains to produce a relative level of enrichment for each mutant. QPCR reactions were done one to three 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 α -specific reporter, the ratios of QPCR measurements for the α 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 with 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.

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*, *Caenorhabditis elegans*, and mice) were added to this alignment much of the conservation at this surface was maintained (our unpublished data). 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 over-represented 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 *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 (our unpublished data).

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 by using SAM methodology (Tusher *et al.*, 2001). In brief, SAM ranks each gene based on the magni-

tude of the change in expression and the reproducibility of that measurement in duplicate experiments. SAM also estimates the number of false positives (FDR) in a set of genes corresponding to a selected threshold of significance. We chose a threshold of significance for each of our data sets that yielded the lowest FDR for that data set, 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 data sets. 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 up-regulated, implying a loss of negative regulation. The few genes whose expression levels decrease in the point mutants generally show small magnitudes of changes in expression, and we believe this likely reflects indirect effects of Tup1-mediated repression.

We chose to restrict further analysis of the genes that were significantly derepressed in the point mutants to those that had been previously identified as targets of Tup1-mediated repression (Green and Johnson, 2004). Using these gene sets, we first determined the degree of overlap between the data sets of the point mutants. *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 data sets with the set of genes derepressed by *tup1^{R447A}* (Figure 2B). Moreover, there was considerable overlap between the data sets from any two of the other mutants (our unpublished data). 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 α -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 mu-

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 up-regulated in each mutant data set are available in Supplemental Table 1. (B) Venn diagrams depicting the overlap between sets of significantly derepressed Tup1-regulated genes in the point mutants. Percentages displayed are the percent of the individual mutant data sets that overlaps with the *tup1^{R447A}* data set. As explained in *Results*, only those genes previously shown to be targets of Tup1-mediated repression are included in this analysis. (C) Cluster diagram of six independent microarrays for each strain; displayed are all significantly up-regulated 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 with a wild-type strain. Microarray data for each strain is available in Supplemental Table 2.

tant 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}* data sets. *HSP12* and *SPI1* are newly identified Tup1-controlled genes that were identified

Figure 3. Recruitment of Tup1 point mutants to regulated promoters. (A) Table of expression microarray data for Tup1 point mutants compared with a wild-type strain. (B) ChIP analysis for Tup1 protein; bars represent the average of one to three QPCRs each on material from three independent ChIP experiments; data were collected for both cell (α and α) types for each strain. Error bars represent the 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) Log₁₀-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.

as derepressed by all five of the point mutants in our SAM analysis.

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 (nonspecific) 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 pro-

motors (*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

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. The average of the units for the LexA (not fused to Tup1) strain is designated as 1 $\times$ repression. The fold repression for each of the other strains is then determined by dividing the average units for the LexA strain by the average units of the respective strains. Strains used in this experiment are SGY286, SGY287, SGY288, SGY289, SGY290, SGY291, and SGY292. The significance of the differences between the β -galactosidase measurements for the wild-type and mutant Tup1-LexA fusions was determined using the Student's *t* test. By this method, we conclude that all of the mutants are significantly defective for repression compared with wild-type Tup1 ($p < .05$) except *tup1*^{Y489A}. The measurements for the *tup1*^{Y489A} mutant had a much larger scatter and, although the average value suggests a decrease in repression function, the significance of this value is questionable. (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 are 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.

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* seems to be greater than that of wild-type Tup1, but we do not believe this is biologically significant. Regardless, 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-regulated genes in a *tup1* Δ 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 *tup1* Δ strain (median increases in expression over a wild-type strain of 2.4- and 2.9, 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 because 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 Defects 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 (with the exception of *tup1*^{Y489A}) 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 by 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 by using antibodies against Tup1 itself and obtained the same result (our unpublished data). 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

A Repression of α 2op-LacZ Reporter Construct

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

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 (435 $\times$), and the percentages of repression in *tup1 Δ* and *tup1^{R447A}* strains 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 are 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.

tup1^{E463A} strains. Unlike the other subsets of Tup1-repressed genes, this group was typically fully derepressed compared with 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 Mata2, the sequence-specific DNA-binding protein regulating the *a*-specific genes. The *a*-specific genes are normally on in *a* cells, in which Mata2 is not present, and off in α cells, which express Mata2. By comparing the levels of expression in α and *a* cells (repressed vs. derepressed conditions) of a gene controlled by Mata2, we can determine the level of repression of that gene (Figure 5A, see 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 (*tup1 Δ*). ChIP experiments showed that at this *a*-specific reporter, the *tup1^{R447A}* mutant was not properly recruited to the promoter in α cells expressing Mata2 (Figure 5B). In contrast, Mata2, the sequence-specific DNA-binding protein responsible for recruiting Tup1, was properly bound to the *a*-specific reporter (our unpublished ChIP data), so we conclude that the absence of *tup1^{R447A}* from the reporter results from the inability of the mutant protein to interact with Mata2.

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 *tup1 Δ* , 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 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 seem 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 Mata2. Finally, a third subset of Tup1-regulated genes is derepressed by only *tup1^{R447A}*. These genes are only slightly derepressed compared with the magnitudes of changes in expression seen in a *tup1 Δ* strain but are considered significant by SAM analysis. The genes in this subset do not seem 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

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 Mata2 shown in blue. (B) Model depicts the two situations created by the mutants described in this article. In a wild-type strain, Tup1 is recruited to regulated promoters through an interaction with a DNA-binding protein (pink oval) and then represses transcription via mechanisms involving chromatin-modifying factors and the general transcriptional machinery. For one set of Tup1-regulated genes (the α -specific genes), three of the point mutations disrupted an interaction with a DNA-binding protein (Mata2). 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.

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

For a small group of Tup1-repressed genes, the α -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 α -specific operator even though Mata2, 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 Mata2 (Komachi and Johnson, 1997). This earlier result, combined with our ChIP data and the observation that the α -specific genes are fully derepressed in the three mutants compared with levels seen in a *tup1* Δ strain, support the conclusion that these three residues of Tup1 are critical for the interaction between Tup1 and the sequence-specific DNA-binding protein Mata2. 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 α -specific genes, confirming

that the mutations cause defects in repression beyond their inability to bind Mata2. These data indicate that two functions of Tup1—recruitment to α -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 seem 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 *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 *et al.*, 1996; Redd *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.

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