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Identification of Novel Activation Mechanisms for *FLO11* Regulation in *Saccharomyces cerevisiae*

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ABSTRACT

Adhesins play a central role in the cellular response of eukaryotic microorganisms to their host environment. In pathogens such as *Candida* spp. and other fungi, adhesins are responsible for adherence to mammalian tissues, and in *Saccharomyces* spp. yeasts also confer adherence to solid surfaces and to other yeast cells. The analysis of *FLO11*, the main adhesin identified in *Saccharomyces cerevisiae*, has revealed complex mechanisms, involving both genetic and epigenetic regulation, governing the expression of this critical gene. We designed a genomewide screen to identify new regulators of this pivotal adhesin in budding yeasts. We took advantage of a specific *FLO11* allele that confers very high levels of *FLO11* expression to wild "flor" strains of *S. cerevisiae*. We screened for mutants that abrogated the increased *FLO11* expression of this allele using the loss of the characteristic fluffy-colony phenotype and a reporter plasmid containing GFP controlled by the same *FLO11* promoter. Using this approach, we isolated several genes whose function was essential to maintain the expression of *FLO11*. In addition to previously characterized activators, we identified a number of novel *FLO11* activators, which reveal the pH response pathway and chromatin-remodeling complexes as central elements involved in *FLO11* activation.

UNICELLULAR eukaryotes are generally able to adhere to different surfaces or cells in response to environmental conditions. This capability is essential for developmental processes such as the dimorphic switch, flocculation, and biofilm formation (Gimeno *et al.* 1992; Guo *et al.* 2000; for review see Verstrepen and Klis 2006). Pathogenic yeast such as *Candida albicans* and *Candida glabrata* require this adhesive property for infectivity (reviewed in Verstrepen *et al.* 2004). Proteins involved in this adhesion phenotype are grouped into families called adhesins, represented, for example, by the *FLO* family in *Saccharomyces cerevisiae* (Guo *et al.* 2000) and by the *ALS* (reviewed in Hoyer 2001) and *EPA* (De Las Penas *et al.* 2003) proteins in *C. albicans*.

Expression of adhesins is regulated by environmental stress such as nitrogen or carbon source depletion, growth on alcohol as the sole carbon source, or pH changes. Moreover, upregulation of adhesins is critical prior to entry into the pathogenic program of some microorganisms (for review see Hoyer 2001; Verstrepen and Klis 2006).

In *S. cerevisiae*, Flo11p/Muc1p (Lambrechts *et al.* 1996) is the main cell-surface protein involved in adhesion-related phenotypes (Lo and Dranginis 1998; Reynolds and Fink 2001). The ability to study cell adhesion in a genetically tractable system such as *S. cerevisiae* and the analysis of Flo11p as a model system for adhesins has significantly contributed to our understanding of mechanisms leading to cell adhesion and the regulatory pathways governing adhesin expression.

Control of *FLO11* expression is relatively complex. Its promoter covers a region of ~3 kb, one of the largest promoters to be found in the whole *S. cerevisiae* genome, containing at least four activation sequences and nine repression domains. Most of these regulatory regions are targets for the MAPK pathway, the cAMP cascade, and the Gnc4p-controlled signaling pathway (Pan and Heitman 1999; Rupp *et al.* 1999; Braus *et al.* 2003). The MAPK pathway converges on Ste12p activation (Madhani and Fink 1997). Ste12p then activates Tec1p, which can bind specific *FLO11* promoter sequences independently or in combination with Ste12p to induce *FLO11* expression (Madhani and Fink 1997; Rupp *et al.* 1999; Kohler *et al.* 2002; Zeitlinger *et al.* 2003). Activation of the cAMP pathway induces phosphorylation of Flo8p and Sfl1p transcription factors, promoting the release of Sfl1p-P and the binding of Flo8p-P to the *FLO11* promoter, which in turn leads to the transcriptional activation of the *FLO11* gene (Pan and Heitman 2002). *FLO11* expression is also activated via Phd1p and Ash1p, two transcription factors that function independently of the MAPK and cAMP pathways (Pan and Heitman 2000). On the other hand, *FLO11* expression is repressed through two other transcription factors, Nrg1p and Nrg2p, both negatively regulated by Snf1p (Kuchin *et al.* 2002).

Mss11p has been described as the pivotal element underlying all of these regulatory networks controlling

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FLO11 expression. It is essential for *FLO11* activation via the MAPK cascade, the cAMP pathways, and Phd1p/Ash1p, as well as for repression through the Nrg1p and Nrg2p proteins (van Dyk *et al.* 2005). In addition to the Mss11p-related networks, *FLO11* is also regulated by amino acid starvation via the Gcn4p-controlled signaling pathway, which is required for *FLO11* activation (Braus *et al.* 2003).

Apart from these well-known signaling pathways, *FLO11* expression is also subjected to epigenetic silencing, in both a positional and a promoter-specific way, probably through the Sfl1p transcription factor (Halme *et al.* 2004). Hda1p is the histone deacetylase responsible for this silencing effect. In addition to this silencing mechanism, a role for Rme1p and Msn1p in *FLO11* activation has been suggested, hypothetically acting through a chromatin-dependent mechanism (Sidorova and Breeden 1999; van Dyk *et al.* 2005).

In budding yeasts, Flo11p is involved in a wide repertoire of phenotypic variations involved in adapting to adverse environmental conditions including filamentation, invasive growth, flocculation, and adherence to solid surfaces. The central role that this adhesin plays in response to environmental changes probably explains the complexity of its regulation. We have recently described a *FLO11* allele (named *FLO11F*), found in certain wild "flor" strains of *S. cerevisiae*, which is highly expressed and confers a number of additional properties to these yeasts, such as the formation of compact fluffy colonies and the ability to form a buoyant biofilm in liquid media required during sherry wine production (Fidalgo *et al.* 2006).

To better analyze the characteristics of this particular *FLO11F* gene, we generated a haploid flor-laboratory hybrid strain (133d) containing *FLO11F* instead of the laboratory *FLO11* allele (*FLO11L*). The 133d strain behaves as a conventional laboratory strain, but manifests all the *FLO11F*-associated phenotypes found in wild flor yeasts (Fidalgo *et al.* 2006). These phenotypes include a very high level of *FLO11F* expression, even in media containing a high glucose concentration, where the *FLO11L* allele is in a repressed state, and fluffy colonies, an easily distinguishable phenotype associated with *FLO11F* expression (Fidalgo *et al.* 2006). On the basis of these distinctive properties, we have developed a genomewide screen, utilizing insertional mutagenesis to isolate positive regulators required for *FLO11* expression. Using this powerful approach, we have identified several novel activators of *FLO11*. Further investigation allowed us to establish that the pH response pathway is a new pathway controlling *FLO11* expression and that chromatin-remodeling complexes are central elements involved in *FLO11* activation.

MATERIALS AND METHODS

Strains, plasmids, media, and genetic methods: The yeast strains used in this study are listed in Table 1. Most of directed deletions were carried out by amplifying the alleles containing the target gene replaced with KanMX4 in the strain BY4741. These alleles were amplified by PCR with oligonucleotide primers flanking the target open reading frame. The PCR products were then used to transform the 133d strain by using the lithium acetate/single-strand DNA/PEG procedure (Gietz *et al.* 1995). PCR-mediated disruption (Lorenz *et al.* 1995) was used for other gene deletions. Double deletions using the same marker were performed as described in Guldener *et al.* (1996). Standard YPED and synthetic complete dextrose medium (SCD) lacking the appropriate amino acids for plasmid or transposon selection were used. The YPED medium was supplemented with 200 mg/liter geneticin for selection of geneticin-resistant transformants. Solid media contained 2% agar.

To obtain the plasmid pFLT$_{133d}$GFP, the 133d *FLO11* promoter was amplified by PCR and cloned into the *Eco*RI site of pRS316. The GFP was cloned downstream from the *FLO11F* promoter into the *Sma*I–*Ksp*I sites, and the *ADH1* terminator was cloned downstream from the GFP into the *Sac*II site. Bacterial transformations and plasmid isolation were performed as described (Sambrook and Russell 2001).

Yeast mutagenesis: The 133d strain was mutagenized by transformation with *Not*I-cleaved DNA carrying random *Tn3::lacZ::LEU2* insertions (Burns *et al.* 1994). Yeast cells carrying the transposon as a recombinational replacement of the genomic copy with the transposon-mutagenized version were selected on SCD with auxotrophic supplements lacking leucine.

The site of the insertion in selected mutants was determined by plasmid rescue and DNA sequence analysis as described (Burns *et al.* 1994). Briefly, mutant yeast cells were transformed with linearized pRSQ1 plasmid. Transformants were selected on SCD plates lacking both leucine and uracil. Yeast genomic DNA from each mutant was recovered and digested with *Eco*RI or *Eco*RV. The fragments were circularized and recovered in bacteria. Plasmids were sequenced using a primer complementary to the 5′-end of the transposon. DNA homology searches were performed using the *Saccharomyces* Genome Database.

Northern blot analysis: To analyze *FLO11* gene expression, cells were incubated in YPED liquid medium overnight at 30° and then transferred to fresh YPED medium and incubated to an optical density at 600 nm (OD_{600}) of ~0.8. If the analysis was in glucose-rich medium, then cells were collected and RNA extraction was performed as described below. If the analysis was in low-glucose medium, then cells were washed and transferred to YPED with 0.2% of glucose for 2 hr. Cells were washed with cool water, and total RNA was isolated with the QIAGEN (Valencia, CA) RNeasy mini kit, separated by formaldehyde denaturing agarose gel electrophoresis, and transferred overnight by capillary action to nylon membranes. The 400-bp regions at the 5′-end of *FLO11* and *ACT1* genes were then used to probe the membranes. The radioactive bands were visualized and quantified using a Molecular Dynamics PhosphoImager.

Flow cytometry: To quantify the GFP levels in the obtained mutants, cells were grown in YPED overnight at 30° and then replaced to a fresh YPED medium and incubated to an OD_{600} of 0.8. Just prior to analysis, cells were pelleted, washed, and resuspended in 50 mm sodium citrate. The fluorescence of 10,000 cells was measured using a FACSCalibur flow cytometer (Becton Dickinson) with a 530/30 band-pass filter.

Light and fluorescence microscopy: To study colony morphology, single-colony photographs were taken directly from petri plates using a Leica DMRE microscope with a ×10 objective. To analyze colony fluorescence, a Leica MZFL*III* stereomicroscope was used.

Invasive growth assay: The plate washing assay was performed as described (Roberts and Fink 1994) with several

TABLE 1

Yeast strains used in this study

Strains	Genotype	Source/reference
133d	*MAT***a** *ura3-52*	Fidalgo *et al.* (2006)
133dL[a]	*MAT***a** *ura3-52 leu2*Δ	This study
133d *flo8*Δ	*MAT***a** *ura3-52 flo8*Δ::*KanMX4*	This study
133d *msn1*Δ	*MAT***a** *ura3-52 msn1*Δ::*KanMX4*	This study
133d *mss11*Δ	*MAT***a** *ura3-52 mss11*Δ::*KanMX4*	This study
133d *ash1*Δ	*MAT***a** *ura3-52 ash1*Δ::*KanMX4*	This study
133d *gal11*Δ	*MAT***a** *ura3-52 gal11*Δ::*KanMX4*	This study
133d *tup1*Δ	*MAT***a** *ura3-52 tup1*Δ::*KanMX4*	This study
133d *sap30*Δ	*MAT***a** *ura3-52 sap30*Δ::*KanMX4*	This study
133d *pho23*Δ	*MAT***a** *ura3-52 pho23*Δ::*KanMX4*	This study
133d *rxt2*Δ	*MAT***a** *ura3-52 rxt2*Δ::*KanMX4*	This study
133d *sds3*Δ	*MAT***a** *ura3-52 sds3*Δ::*KanMX4*	This study
133d *snf5*Δ	*MAT***a** *ura3-52 snf5*Δ::*KanMX4*	This study
133d *snf2*Δ	*MAT***a** *ura3-52 snf2*Δ::*KanMX4*	This study
133d *yta7*Δ	*MAT***a** *ura3-52 yta7*Δ::*KanMX4*	This study
133d *rim20*Δ	*MAT***a** *ura3-52 rim20*Δ::*KanMX4*	This study
133d *rga2*Δ	*MAT***a** *ura3-52 rga2*Δ::*KanMX4*	This study
133d *rdr1*Δ	*MAT***a** *ura3-52 rdr1*Δ::*KanMX4*	This study
133d *rri2*Δ	*MAT***a** *ura3-52 rri2*Δ::*KanMX4*	This study
133d *bud4*Δ	*MAT***a** *ura3-52 bud4*Δ::*KanMX4*	This study
133d *ena1*Δ	*MAT***a** *ura3-52 ena1*Δ::*KanMX4*	This study
133d *atp10*Δ	*MAT***a** *ura3-52 atp10*Δ::*KanMX4*	This study
133d *gph1*Δ	*MAT***a** *ura3-52 gph1*Δ::*KanMX4*	This study
133d *kre11*Δ	*MAT***a** *ura3-52 kre11*Δ::*KanMX4*	This study
133d *yhr177w*Δ	*MAT***a** *ura3-52 yhr177w*Δ::*KanMX4*	This study
133d *snf6*Δ	*MAT***a** *ura3-52 snf6*Δ::*KanMX4*	This study
133d *swi3*Δ	*MAT***a** *ura3-52 swi3*Δ::*KanMX4*	This study
133d *rim101*Δ	*MAT***a** *ura3-52 rim101*Δ::*KanMX6*	This study
133d *rim20*Δ*rim101*Δ	*MAT***a** *ura3-52 rim101*Δ *rim20*Δ::*KanMX4*	This study
L5684	*MAT***a** *ura3-52 leu2*Δ	G. R. Fink
L5684 *mss11*Δ	*MAT***a** *ura3-52 leu2*Δ *mss11*Δ::*KanMX6*	This study
L5684 *snf5*Δ	*MAT***a** *ura3-52 leu2*Δ *snf5*Δ::*KanMX6*	This study
L5684 *pho23*Δ	*MAT***a** *ura3-52 leu2*Δ *pho23*Δ::*KanMX6*	This study
L5684 *tup1*Δ	*MAT***a** *ura3-52 leu2*Δ *tup1*Δ::*KanMX6*	This study
L5684 *rim101*Δ	*MAT***a** *ura3-52 leu2*Δ *rim101*Δ::*KanMX6*	This study
BY4741[b]	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *met15*Δ	Euroscarf

[a] Strain used for the mutagenesis.
[b] For deletions of genes using the *KanMX4* marker, mutants for target genes in this background were used.

modifications. Cells were grown in YPED overnight at 30° and then replaced to a fresh YPED medium and incubated to an OD_{600} of ~0.8 and then cells were spotted onto YPED, incubated for 4 days at 28°, and photographed. Plates were then washed under a stream of water by rubbing with Digralsky spreader and then photographed again.

Assay for adherence to plastic and hydrophobicity: Assays for adherence to the wells of a polystyrene 96-well microtiter plate and hydrophobicity were carried out as described (Reynolds and Fink 2001) with minor modification. For adherence to plastic assays, cells were grown in YPD to an OD_{600} of ~0.8, collected, washed, and resuspended in YPD to an OD_{600} of 1. Cells (0.1 ml) were transferred to the wells of a microtiter plate and incubated for 1 hr at 28°. The cells were then stained with 1% crystal violet, and the wells washed repeatedly with water and photographed. For quantification, the crystal violet was solubilized by adding 100 μl of SDS 10%, plates were incubated for 15 min, and then wells were mixed with 100 μl of water and the absorbance at 530 nm (A530) was measured using a microplate reader. For hydrophobicity assay, cells were grown in SCD to an OD_{600} of ~0.8 and then 1.2 ml of the culture was overlaid with 600 μl of octane and vortexed for 3 min. The OD_{600} of the aqueous layer was taken and the relative difference with the initial OD_{600} was used to determine the percentage of hydrophobicity.

RESULTS

High levels of *FLO11F* expression are necessary to confer the fluffy-colony morphology shown by wild flor yeast: Naturally occurring *S. cerevisiae* flor yeast show a fluffy-colony morphology in contrast to the smooth morphology of *S. cerevisiae* laboratory strains. 133d is a haploid flor-laboratory hybrid strain harboring the *FLO11F* allele from wild flor yeasts (Fidalgo *et al.* 2006) and produces fluffy colonies (Figure 1A). This phenotype is *FLO11F* dependent, since a *FLO11F* loss-of-function

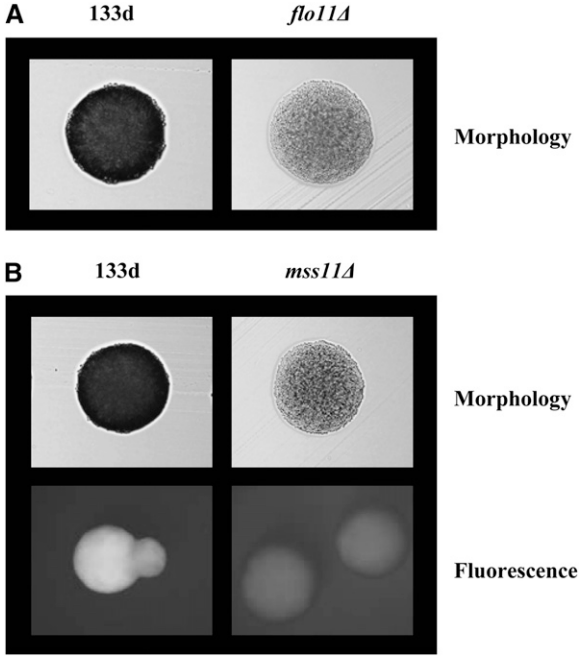

Figure 1.—Colony morphology and fluorescence level. (A) Deletion of *FLO11F* in 133d generates smooth colonies. (B) Deletion of *MSS11* in 133d pFLT$_{133d}$GFP generates smooth nonfluorescent colonies in contrast to the fluffy fluorescent colonies for 133d pFLT$_{133d}$GFP. The smooth colonies are semitransparent in contrast to the opaque, dark, fluffy colonies (133d).

mutant forms smooth colonies, which are easily distinguishable from fluffy colonies by visual inspection (Figure 1A). As the main difference between the laboratory *FLO11L* and flor *FLO11F* alleles is the higher level of expression conferred by the *FLO11F* promoter (Fidalgo *et al.* 2006), it strongly suggests that the fluffy-colony phenotype is directly related to elevated *FLO11* expression. To confirm this relationship, *MSS11*, one of the main *FLO11* activators (van Dyk *et al.* 2005), was deleted in the 133d strain. Deletion of *MSS11* yielded smooth colonies (Figure 1B) similar to those observed for *FLO11F*-deleted cells. This relationship between colony morphology and *FLO11* expression allowed us to develop a screening strategy to detect novel *FLO11* activators. To ensure that changes in colony morphology are a direct result of decreased *FLO11* expression, we generated a plasmid containing a GFP reporter under the control of the *FLO11F* promoter from the 133d strain (pFLT$_{133d}$GFP). Deletion of *MSS11* in 133d pFLT$_{133d}$GFP yielded smooth nonfluorescent colonies, in contrast to the fluffy fluorescent colonies of the 133d pFLT$_{133d}$GFP strain (Figure 1B). Therefore, we can use 133d pFLT$_{133d}$GFP as a novel method for rapidly identifying new positive regulators involved in *FLO11* expression on the basis of colony morphology and fluorescence changes.

Isolation of *FLO11F* low-expression-level mutants: To generate low *FLO11F* expression mutants, the 133d pFLT$_{133d}$GFP strain was mutagenized by integrative transformation with a yeast genomic library carrying random *Tn3::LEU2::lacZ* gene insertions (Burns *et al.* 1994). First, we screened for reduced colony fluorescence in 182,000 Leu$^+$ transformants. Colonies exhibiting reduced fluorescence were selected and streaked onto selective media to analyze colony morphology, and only smooth colonies were chosen. Following these two rounds of selection, we were left with 63 candidate mutant strains.

To identify the position of transposon insertion in each of the 63 mutants, the mutants were subjected to plasmid rescue and DNA sequencing (Burns *et al.* 1994). We found that they represented 45 independent insertions affecting 25 different genes (Table 2). To ensure that single insertions in the identified genes were responsible for the *FLO11F* low-expression phenotype, we performed directed deletion of each of the 25 identified genes in the 133d pFLT$_{133d}$GFP strain and determined colony morphology and fluorescence of the deleted mutants. Sixteen of these genes showed a smooth morphology and reduced fluorescence when deleted (Table 2). As expected, a number of the identified genes correspond to known *FLO11* regulators (Table 2). Of these, *MSS11*, *FLO8*, *MSN1*, and *ASH1* have been previously described to encode *FLO11* activators (Gagiano *et al.* 1999b; Pan and Heitman 1999; Rupp *et al.* 1999; Pan and Heitman 2000). *TUP1* encodes a protein that has been previously described as a component of the general corepressor Tup1-Cyc8, a complex involved in the repression of *FLO11* expression (Conlan and Tzamarias 2001). Here, in contrast, we have identified Tup1p as a *FLO11F* activator, which, when deleted, reduces *FLO11* expression as judged by colony morphology and reporter expression. Thus, our findings suggest that Tup1p can act as an activating factor, at least in the 133d pFLT$_{133d}$GFP genetic background.

We also identified Gal11p as an activator of *FLO11F*. *GAL11* encodes an element of the mediator complex, which acts by transferring information from enhancers and other regulatory elements to the RNApolII. This mediator complex is required for the transcriptional control of all RNApolII-dependent genes (Bjorklund and Gustafsson 2005).

Interestingly, our screen allowed us to identify a number of novel *FLO11F* transcriptional activators. These include Rim20p, which is involved in the alkaline pH response pathway (Xu and Mitchell 2001; Boysen and Mitchell 2006), and a large number of genes involved in chromatin remodeling, such as components of the histone deacetylase Rpd3L complex (*SAP30*, *PHO23*, *RXT2*, and *SDS3*) (Carrozza *et al.* 2005) and the SWI/SNF complex (*SNF2*, *SNF5*) (Cairns *et al.* 1994; Peterson *et al.* 1994, 1998).

Deacetylases remove the acetate groups from the acetylated lysine residues in the histones amino-terminal domains (Rundlett *et al.* 1996; Kadosh and Struhl

TABLE 2

Genes found in the screen

Gene	No. of independent insertions in the gene	Colony morphology in directed deletions	Short description
FLO8	1	Smooth	Transcription factor
MSN1	6	Smooth	Transcriptional activator
MSS11	2	Smooth	Transcription factor
ASH1	2	Smooth	Transcription factor
GAL11	3	Smooth	Component of the Mediator complex
TUP1	1	Smooth	General repressor of transcription
SAP30	4	Smooth	Subunit of a histone deacetylase complex
PHO23	4	Smooth	Component of the Rpd3 histone deacetylase complex
RXT2	3	Smooth	Subunit of the histone deacetylase Rpd3L complex
SDS3	1	Smooth	Component of the Rpd3p/Sin3p deacetylase complex
SWI1	1	Lethal	Subunit of the SWI/SNF chromatin-remodeling complex
ARP7	1	Lethal	Component of both the SWI/SNF and RSC chromatin-remodeling complexes
SNF5	3	Smooth	Subunit of the SWI/SNF chromatin-remodeling complex
SNF2	1	Smooth	Catalytic subunit of the SWI/SNF chromatin-remodeling complex
YTA7	2	Smooth	Protein of unknown function
RIM20	1	Smooth	Protein involved in proteolytic activation of Rim101p in response to alkaline pH
RGA2	1	Fluffy	GTPase-activating protein
RDR1	1	Fluffy	Transcriptional repressor
RRI2	1	Fluffy	Subunit of the COP9 signalosome (CSN) complex
BUD4	1	Fluffy	Protein involved in bud-site selection and required for axial budding pattern
ENA1	1	Fluffy	P-type ATPase sodium pump
ATP10	1	Fluffy	Mitochondrial inner membrane protein
GPH1	1	Fluffy	Nonessential glycogen phosphorylase
KRE11	1	Fluffy	Protein involved in biosynthesis of cell-wall β-glucans
YHR177W	1	Fluffy	Putative protein of unknown function

The number of independent transposon insertions in each gene is shown in the second column. The fourth column is a short description, obtained from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/), of the proteins encoded by the identified genes.

1998). Usually, Rpd3p acts by repressing gene transcription, although in some genes an activation activity has been described. (Vidal and Gaber 1991; Kadosh and Struhl 1997, 1998; De Nadal *et al.* 2004; Puig *et al.* 2004).

The SWI/SNF ATP-dependent remodeling complex regulates gene expression by remodeling chromatin structure and altering histone acetylation patterns (Peterson and Workman 2000). This multi-subunit complex is able to delocalize histone octamers to generate nucleosome-free regions available for transcription factors (Whitehouse *et al.* 1999). In addition to *SNF2* and *SNF5*, we also isolated *ARP7* and *SWI1* as *FLO11* activators. Directed deletions against the latter genes showed lethality, and no further analysis was possible. However, because these two genes also belong to the SWI/SNF complex (Cairns *et al.* 1994; Peterson *et al.* 1994), their isolation further supports a role for the SWI/SNF complex in *FLO11* activation.

Another candidate *FLO11* activator, related to chromatin remodeling, is *YTA7*, encoding an ATPase with a bromo-like domain (Jambunathan *et al.* 2005). Yta7p is involved in binding to acetylated histones and other chromatin-associated proteins (Zeng and Zhou 2002; Yang 2004; De La Cruz *et al.* 2005), similar to those present in components of the SWI/SNF complex. *YTA7* has also been identified in the DPB4 chromatin-remodeling complex, which binds to DNA regions near the *FLO11* gene (Tackett *et al.* 2005). Thus, the discovery of several genes encoding components of pH response and chromatin-remodeling complexes in our screen suggests that these pathways may play an important role in controlling *FLO11* activation.

The role of the remaining nine genes identified in *FLO11* expression was not confirmed by directed deletion. In these cases, the phenotype observed in the original mutant could have been caused by additional insertions or by a dominant-negative effect. Further experiments are required to discern if these genes play any role in regulating *FLO11* expression.

***FLO11F* expression is reduced in all selected mutants:** To analyze and quantify *FLO11F* expression in the 14 selected deletion mutants, Northern blot analysis (Figure 2) and flow-cytometry-mediated GFP quantification on mutant yeasts transformed with $pFLT_{133d}GFP$ (data not shown) were performed. Consistent with our

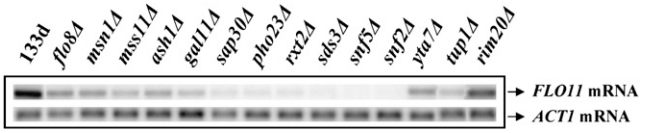

FIGURE 2.—*FLO11F* expression levels in the deleted mutants. Northern blot analysis was performed to determine *FLO11F* expression level. *ACT1* was used as probe for loading control.

previous observation, all 14 mutants expressed greatly reduced levels of *FLO11F*, as determined by both Northern blot and flow cytometry. The deletion of *TUP1* resulted in low levels of *FLO11F* expression, confirming the role of this protein as an *FLO11F* activator. As expected for *RIM20*, given its mild effect on colony morphology, the effect of the deletion in *FLO11F* expression was the lowest observed. When genes encoding components of the two chromatin-remodeling complexes, Rpd3L and SWI/SNF, were deleted, *FLO11F* expression was almost completely abolished. In contrast, *YTA7* deletion did not result in such a dramatic drop in *FLO11F* expression, suggesting that *FLO11F* regulation via *YTA7* is distinct from that achieved through the other chromatin-remodeling complexes identified in this screen. Surprisingly, deletion of previously described *FLO11L* activators (*MSN1*, *MSS11*, *FLO8*, and *ASH1*) also resulted in a more moderate decrease in *FLO11F* expression. Thus, *FLO11F* activation is more dependent on chromatin-remodeling complexes than previously described activators.

***FLO11*-related phenotypes are altered in the isolated mutants:** To study the role of the identified genes on a number of *FLO11*-dependent phenotypes, we analyzed the effect of the deletion for the selected genes on cellular hydrophobicity, invasive growth, and solid surface biofilm (LO and DRANGINIS 1998; GAGIANO *et al.* 1999b; REYNOLDS and FINK 2001).

In the majority of cases, effects on *FLO11F* expression and cellular hydrophobicity were closely related (Figure 3A). The largest effect on hydrophobicity was shown by mutants for genes involved in chromatin remodeling, where the decrease in hydrophobicity was similar to that shown by *flo11F*Δ. The only exception for this class of genes was *yta7*, which exhibited a weaker reduction in hydrophobicity, although this correlated with the smaller reduction of *FLO11F* expression. The only example in which hydrophobicity did not correlate with *FLO11F* expression was observed for *TUP1*. Remarkably, the hydrophobicity level for the *tup1*Δ mutant was similar to that observed for the wild-type strain, despite the fact that the *FLO11F* expression level was dramatically reduced to levels similar to *mss11*Δ or *gal11*Δ mutants.

Next we examined the ability of each of the selected mutants to form solid surface biofilms. Quantification of biofilm formation determined that the quality of the

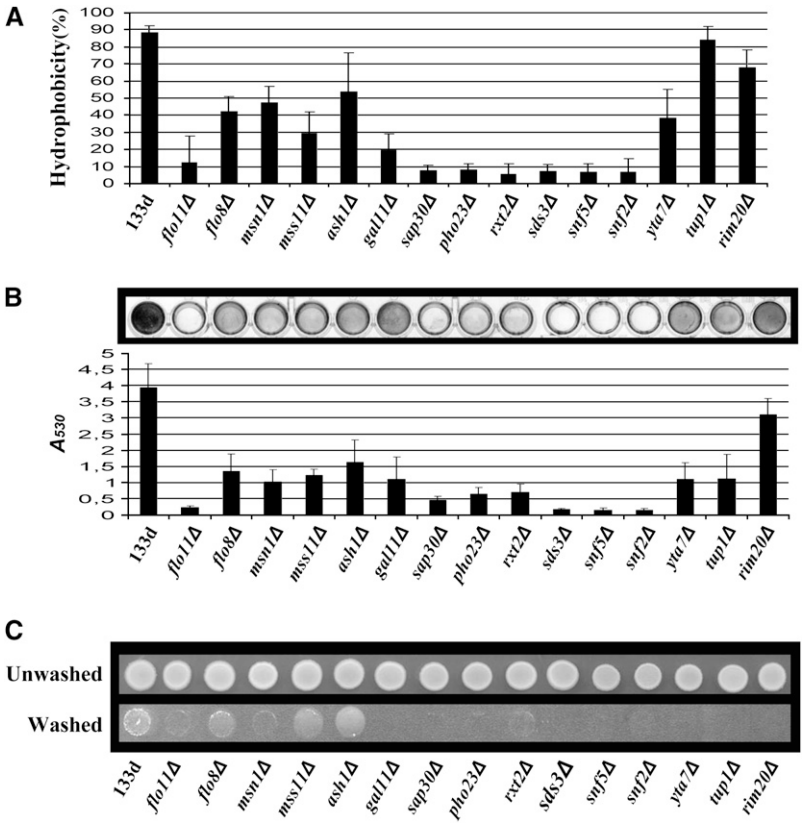

FIGURE 3.—*FLO11F*-related phenotypes in the deleted mutants. (A) Hydrophobicity. Cells cultures were overlaid with octane and mixed. The OD_{600} of the aqueous layer was taken and the relative difference with the initial OD_{600} was used to determine the percentage of hydrophobicity. (B) Biofilm on solid surface. Exponentially growing cells were placed in microtiter plate wells and incubated for 1 hr at 28°. The cells were then stained with 1% crystal violet, and the wells were washed repeatedly with water and photographed. For biofilm quantification, the crystal violet was solubilized using SDS (10%), and the absorbance at 530 nm (*A530*) was measured. Data presented represent averages of three independent assays. Error bars correspond to standard deviation. (C) Invasive growth. Dots of exponentially growing cells were spotted on YPED solid medium and photographed before (unwashed) and after (washed) washing under a stream of water.

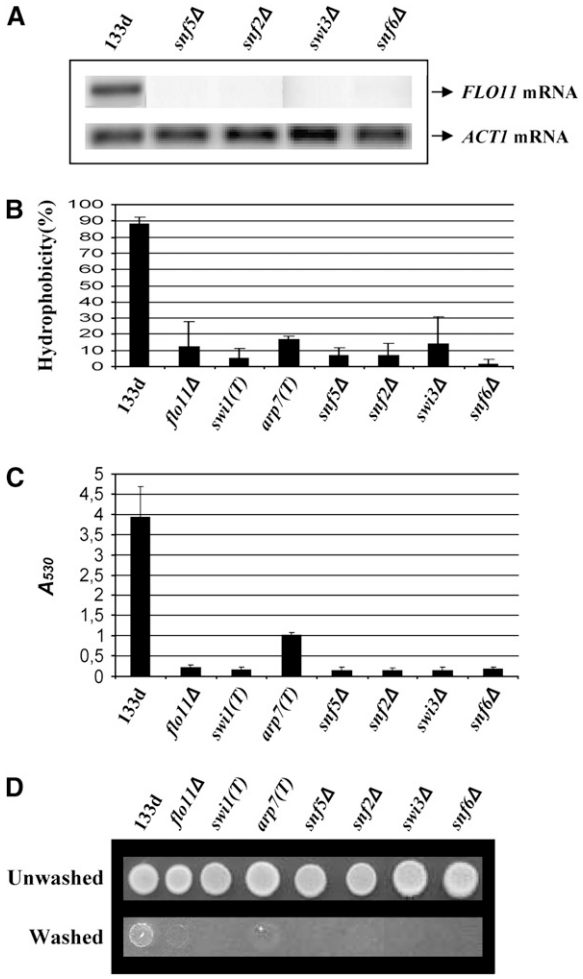

FIGURE 4.—*FLO11F* expression and related phenotypes in mutants for *SWI/SNF* complex members. (A) *FLO11F* expression. Northern blot analysis using *FLO11F* as a probe. *ACT1* was used as a loading control. B–D represent hydrophobicity, biofilm on solid surface, and invasive growth, respectively, performed as mentioned in Figure 3. For hydrophobicity and biofilm on solid surface, data presented represent averages of three independent assays. Error bars correspond to standard deviation. *swi1(T)* and *arp7(T)* are the original transposon insertion mutants isolated during the screen, because full deletion of these genes caused lethality.

biofilm formed by each mutant was closely related to the level of *FLO11F* expression (Figure 3B). It is significant that, in contrast to hydrophobicity, biofilm formation in *tup1*Δ mutants corresponded to its reduced expression of *FLO11F*.

Similar to their effect on hydrophobicity and solid biofilm formation, the very low levels of *FLO11F* expression in mutants for genes involved in chromatin remodeling did not allow invasive growth (Figure 3C). However, the decrease in *FLO11F* expression observed for the remaining mutants did allow invasive growth to occur in *flo8*Δ, *mss11*Δ, and *ash1*Δ but not in *msn1*Δ, *gal11*Δ mutants, nor in *rim20*Δ, which showed the highest level of *FLO11F* expression. These observations

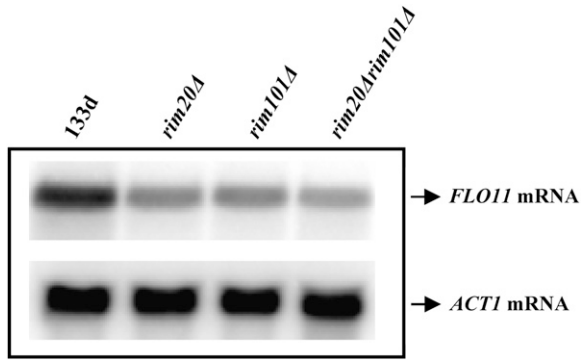

FIGURE 5.—*FLO11F* expression in *rim20*Δ, *rim101*Δ, and *rim20*Δ*rim101*Δ mutants. *FLO11F* expression was measured by Northern blot using *ACT1* as probe for loading control.

suggest that the latter three genes affect invasive growth in a *FLO11*-independent manner.

Other SWI/SNF complex members are involved in *FLO11F* activation: In this screen, we have identified four members of the SWI/SNF complex. Deletions of *SNF2* and *SNF5* in the 133d strain generate the highest decrease in *FLO11F* expression, while deletions of SWI1 and ARP7 were found to be lethal. The SWI/SNF complex is composed of 11 subunits (PETERSON *et al.* 1998). *SWI3* and *SNF6* encode proteins that have been shown to copurify with Swi1p, Snf2p, and Snf5p (CAIRNS *et al.* 1994; PETERSON *et al.* 1994). To determine whether other members of the SWI/SNF complex are also involved or essential for *FLO11F* regulation, we deleted *SWI3* and *SNF6* from the 133d strain. *FLO11F* expression level decreased in *swi3*Δ and *snf6*Δ mutants to the same level as in *snf2*Δ and *snf5*Δ mutants (Figure 4A). As with other members of this complex identified in our screen, including the isolated *swi1* and *arp7* mutants [*swi1(T)* and *arp7(T)*], loss of *SWI3* and *SNF6* resulted in the loss of all the characteristic *FLO11F*-dependent phenotypes (Figure 4, B–D). Thus our results clearly show that the SWI/SWF complex is essential for *FLO11* activation.

Rim20p activates *FLO11F* via Rim101p: Rim20p is necessary for the proteolytic activation of Rim101p, the budding yeast homolog of the *Aspergillus nidulans* PacC protein (XU and MITCHELL 2001). Rim101p is a transcription factor involved in the response to alkaline pH (LAMB *et al.* 2001). Loss of *RIM101* results in phenotypes similar to those observed in the *rim20*Δ mutant, including weaker invasive growth (XU and MITCHELL 2001). To establish a role for Rim101p in *FLO11F* activation, we deleted the *RIM101* gene from the 133d strain and compared its *FLO11F* expression to that of the *rim20*Δ mutant by Northern blot analysis. We found that the loss of *RIM101* resulted in a reduction in the level of *FLO11F* expression similar to that observed for the *rim20*Δ mutant (Figure 5). Moreover, we found that all of the *FLO11F*-related phenotypes were also affected in a similar manner (data not shown). To confirm that *FLO11F* activation by

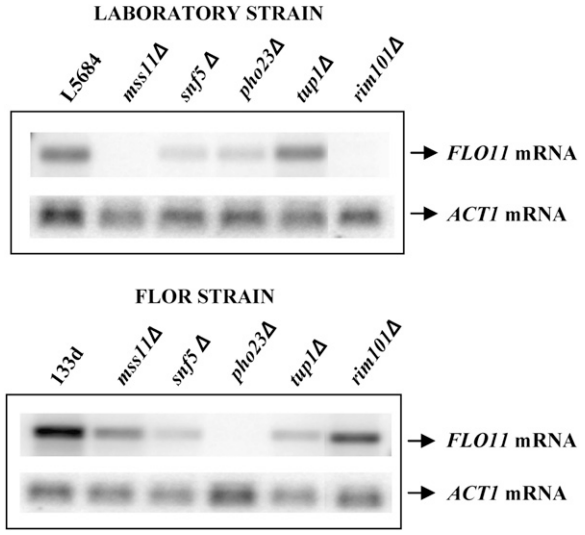

Figure 6.—Expression of *FLO11* in L5684 (Σ1278b background) and 133d derivative strains. A gene representing any of the main group of genes identified in the screening was analyzed. Cells were grown on YEPD with a low amount of glucose to mimic the natural derepressed state of the *FLO11* promoter in the 133d strain. Expression was measured by Northern blot using *ACT1* as a probe for loading control.

Rim20p occurs via Rim101p, we generated a *rim20*Δ *rim101*Δ double mutant. The double mutant showed an expression level similar to that of the single mutants (Figure 5), suggesting that the two genes act in the same pathway to activate *FLO11F* expression. Thus, we can conclude that the alkaline pH response pathway is a novel regulatory mechanism for *FLO11* activation.

The identified genes also regulate *FLO11* expression in a Σ1278b background: The fact that the 133d strain has a higher level of *FLO11* expression than the laboratory L5684 strain (Σ1278b genetic background) provided a powerful tool that permitted us to undertake this screen for *FLO11* activators. However, given the differences between the flor *FLO11F* and laboratory *FLO11L* promoters and, potentially, the differences in the genetic background between 133d and L5684 strains, we extended our analysis of putative *FLO11F* activators to *FLO11L* expression in the L5684 laboratory strain. We tested the requirement for *FLO11L* expression by growing L5684 mutant strains on media in which the *FLO11L* promoter is derepressed (low glucose) (Kuchin *et al.* 2002) to mimic the high expression level of *FLO11F* observed in the 133d strain. The putative *FLO11* regulators that we chose to examine were *MSS11* as a member of the previously described *FLO11F* activators, *SNF5* as a member of the SWI/SNF complex, *PHO23* as a member of the Rpd3L complex, *RIM101* as a component of the pH response pathway, and *TUP1* because it had showed a role in *FLO11* regulation in the 133d strain different from the one described in laboratory strains. As expected, the deletion of *MSS11*, *RIM101*, *SNF5*, and *PHO23* yielded a decrease in *FLO11L* expression, confirming them as genes encoding general *FLO11* activators, capable of regulating *FLO11L* in the laboratory strain (Figure 6). However, the effects of these deletions on *FLO11* expression differed between L5684 and 133d yeast cells (Figure 6). This suggests that differences in the *FLO11* promoter and/or the genetic background do influence the degree to which these genes are required for *FLO11* activation. Deletion of *TUP1* has a low but detectable role in repressing *FLO11* expression in the L5684 strain, as previously described for laboratory strains (Conlan and Tzamarias 2001), while the 133d strain has an activator role (Figure 6). Further experiments to determine other factors involved in the role exchange for *TUP1* are in progress.

DISCUSSION

The discovery of a specific *FLO11* allele (*FLO11F*) in flor yeast has allowed us to perform an in-depth study of the activation mechanisms controlling *FLO11* expression. The promoter that drives expression in the *FLO11F* allele confers the highest level of expression known for this gene (Fidalgo *et al.* 2006). This property has been crucial for the development of a powerful strategy for characterizing the mechanisms involved in *FLO11* activation in *S. cerevisiae*. Using this method, we identified a number of known *FLO11* activators multiple times, validating the methodology. Nevertheless, other known *FLO11* activators were isolated only once, indicating that the screen may not have reached saturation (see Table 2). Among the known genes identified, it is worth highlighting the genes *MSN1* and *MSS11*, which have been previously postulated as the major *FLO11* activators (Lorenz and Heitman 1998; van Dyk *et al.* 2005). The fact that several activation pathways converge on Msn1p and Mss11p, combined with a requirement in the screen for a significant decrease in *FLO11F* expression, may explain why other proteins, previously described as *FLO11* activators, have not been identified. In addition to known components, our novel approach has allowed us to identify two new mechanisms for *FLO11* activation: the pH response pathway and chromatin remodeling.

Unicellular eukaryotes use complex systems to sense and adapt to extracellular environmental conditions. Flo11p is an essential protein involved in changes in cellular behavior in response to environmental alterations, such as carbon source depletion, nitrogen starvation, pheromone presence, etc. (Gagiano *et al.* 1999b; Pan and Heitman 1999; Lorenz *et al.* 2000; Gancedo 2001). It has been previously demonstrated that *FLO11*-dependent flocculation occurs only at acidic pH (Bayly *et al.* 2005). This observation, together with our results regarding the role of the Rim101p in *FLO11* activation, allow us to propose pH as a new input sensed by yeast to respond to changes in external acidity by modifying

FLO11 expression. Rim101p is the main transcription factor involved in the pH response of *S. cerevisiae*, which acts indirectly by repressing the expression of genes encoding transcriptional repressors such as *NRG1* and *SNP1* (Lamb and Mitchell 2003). Curiously, *FLO11* expression is repressed by *NRG1*, so Rim101p could act on *FLO11* activation via *NRG1* repression. However, the expression level for *FLO11F* in a double *rim101*Δ *nrg1*Δ mutant is not as high as for a *nrg1*Δ single mutant (data not shown), indicating that Rim101p can activate *FLO11* expression in an *NRG1*-independent way. Rim101p is proteolytically activated by Rim20p, and because the double *rim101*Δ*rim20*Δ mutant has the same effect in *FLO11* expression as the two single mutants (Figure 5), it suggests that the effect of Rim101p on *FLO11* regulation is dependent on its activation by Rim20p.

Most of the other genes identified either can be assigned to complexes involved in chromatin remodeling, (i) the Rpd3L and (ii) SWI/SNF complexes, or (iii) are in some way related to chromatin remodeling.

Rpd3L complex: The regulation of *FLO11* expression by histone deacetylase has been described previously (Halme *et al.* 2004). Hda1p was identified as an essential element of *FLO11* silencing. Here we have found an activation effect directed by the Rpd3L complex. In our screen, we have isolated *PHO23*, *SAP30*, *RXT2*, and *SDS3*, all of which encode members of the Rpd3L histone deacetylase complex (Carrozza *et al.* 2005). A single deletion of any one of these genes almost completely abolishes *FLO11F* expression (Figure 2). The Rpd3L complex is also composed of Rpd3p, Sin3p, Dep1, Ash1p, Ume1p, Cti6p, Rxt3p, and Ume6p (Carrozza *et al.* 2005). Of these components, only Ash1p has been previously described as being involved in the control of *FLO11* expression (Chandarlapaty and Errede 1998; Pan and Heitman 2000). Curiously, although *ASH1* was also isolated in the screen, the *FLO11F* expression level in the *ash1*Δ deletion mutant was not as strongly diminished as in the *pho23*Δ, *sap30*Δ, *rtx2*Δ, and *sds3*Δ mutants (Figure 2). Ash1p and Ume6p are the only known sequence-specific DNA-binding protein in the Rpd3L complex (Carrozza *et al.* 2005), suggesting that the role for Ash1p in *FLO11* activation might be the recruitment of the Rpd3L complex to the *FLO11* promoter. The reason that the effect of *ASH1* deletion is not as dramatic as for *pho23*Δ, *sap30*Δ, *rtx2*Δ, and *sds3*Δ could be that this recruitment activity is shared with other proteins. Rpd3p is the only protein with deacetylase activity in the Rpd3L complex, but was not identified in this screen. This might be because Rpd3p is not required for the *FLO11* activation by the Rpd3L complex or because the screen did not achieve saturation. Further studies to determine the requirement of Rpd3p deacetylase activity for *FLO11* activation are currently in progress.

SWI/SNF complex: Another major class of genes identified in our screen is composed of elements of the SWI/SNF ATP-dependent remodeling complex. Deletion for several members of this complex from the 133d strain resulted in an extremely low level for *FLO11F* expression (Figure 2) and in the abolishment of all phenotypes in which *FLO11* is involved (Figure 3).

Another promoter, almost identical to *FLO11*, exists in the *S. cerevisiae* (var. *diastaticus*) genome. This promoter governs the expression of *STA1*, *STA2*, and *STA3*, a gene family encoding for glucoamylase proteins. Most of the previously described pathways involved in *FLO11* regulation are also involved in *STA* regulation (Gagiano *et al.* 1999a,b, 2003; van Dyk *et al.* 2003). This promoter is directly controlled by the SWI/SNF complex. (Kim *et al.* 2004) According to our results and considering the regulatory similarities between *FLO11* and *STA* genes, we may assume that *FLO11* activation is also directly controlled by the SWI/SNF complex. In particular, as it has been previously demonstrated for *STA1* (Kim *et al.* 2004), we predict that certain transcription factors such as *FLO8* and *MSS11* recruit the SWI/SNF complex to the *FLO11* promoter. The fact that deletion for *FLO8* or *MSS11* in the 133d strain does not produce as dramatic an effect on *FLO11F* expression levels as the deletion for members of the SWI/SNF complex suggests that the recruitment of this complex to the *FLO11* promoter might also be mediated by other transcription factors. In this context, it is significant that a role for Ste12p and Tec1p transcription factors in recruitment of the SWI/SNF complex to the *STA1* promoter has been described. However, although these transcription factors are involved in *FLO11* activation in the laboratory strains (Madhani and Fink 1997; Rupp *et al.* 1999; Kohler *et al.* 2002; Zeitlinger *et al.* 2003), they are not required for *FLO11* activation in the 133d strain (data not shown).

YTA7 is another gene encoding a protein involved in chromatin remodeling that we found in our screen for *FLO11* activators. It encodes a protein with a bromo-like domain and has been found in the DPB4 complex, which is located in regions flanking transcriptionally silent areas (Tackett *et al.* 2005). It has been proposed that this complex is necessary to preserve the transcriptionally active state of regions adjacent to these silenced zones (Tackett *et al.* 2005). Significantly, the DPB4 complex has been found to bind sequences close to *FLO11* as well as other *FLO* genes (Tackett *et al.* 2005). Thus, this protein might play a role in countering the silencing effect that normally occurs close to the centromere where *FLO11* is located. The deletion of *YTA7* did not have as drastic an effect on *FLO11* expression as the loss of members of the SWI/SNF or Rpd3L complexes. This difference might make it more difficult to find other DPB4-complex-related genes using this screening methodology.

Genes related to chromatin remodeling: Another gene identified in our screen as a positive regulator of *FLO11F* in the 133d strain is *TUP1*, which is related to chromatin remodeling. Tup1p is a component of the

Tup1p-Cyc8p corepressor, which binds to underacetylated histone tails (Edmondson *et al.* 1996; Huang *et al.* 1997) and requires deacetylase activity for its repression function (Watson *et al.* 2000; Davie *et al.* 2002, 2003). Tup1p-Cyc8p has been described as an important repressor of a wide variety of genes (Keleher *et al.* 1992), although it has also been described as an activator in some cases (Conlan *et al.* 1999; Proft and Struhl 2002). In relation to *FLO11* regulation, Tup1p-Cyc8p has been described as a transcriptional repressor (Conlan and Tzamarias 2001). In contrast, here we have found that Tup1p can act as an activator of *FLO11* in the 133d strain (Figure 2). This suggests that differences in the *FLO11* promoter region or the genetic background of the 133d strain affect Tup1 function and result in conversion from transcription repressor to transcription activator for *FLO11*. However, a laboratory strain transformed with GFP under the control of the *FLO11* promoter from the 133d strain did not show an increase in GFP expression when *TUP1* was deleted (data not shown). These results suggest that changes in the promoter region may be responsible for modifying the activity of Tup1p-Cyc8p. The differential distribution of Tup1p within the *SUC2* promoter has been linked to changes in its activation or repression state (Boukaba *et al.* 2004). Moreover, the phosphorylation of additional elements that bind to Tup1p-Cyc8 may help maintain an activating state by promoting the recruitment of SAGA and SWI/SNF complexes as has been previously described (Proft and Struhl 2002). It remains to be determined if the changes in the *FLO11F* promoter result directly in an altered distribution of Tup1p-Cyc8p or indirectly, for example, via changes in the binding of proteins involved in the modification of Tup1p-Cyc8p. Another interesting finding regarding Tup1p activity is the observation that when *FLO11F* expression levels are decreased as a consequence of *TUP1* deletion in the 133d strain, all phenotypes related to *FLO11* activity are lost, except for cellular hydrophobicity, which is maintained at wild-type levels (Figure 3). This suggests that, in 133d, Tup1p might regulate the expression of other proteins involved in cellular hydrophobicity, which, when altered in a *tup1*Δ strain, can maintain cellular hydrophobicity in a *FLO11*-independent manner. One of these proteins could be Flo1p, a protein involved in flocculation. The gene encoding this protein is located in a region repressed by Tup1p (Fleming and Pennings 2001). Thus, Tup1p appears to act via at least two mechanisms to promote the floating capability of 133d yeast, maintaining the increased expression of *FLO11* while repressing other genes, such as those involved in flocculation.

Adhesin expression is essential for fungal pathogenesis. Our screen has revealed novel activators required for *FLO11* adhesin expression, which may provide new targets and strategies to overcome fungal infections. Of these novel regulators, our data suggest that chromatin remodeling plays a fundamental role in controlling *FLO11* expression. Further studies of *FLO11* regulation by these chromatin activators may help reveal underlying principles of gene regulation by chromatin modification.

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