Chromatin Modulation at the FLO11 Promoter of *Saccharomyces cerevisiae* by HDAC and Swi/Snf Complexes

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ABSTRACT Cell adhesion and biofilm formation are critical processes in the pathogenicity of fungi and are mediated through a family of adhesin proteins conserved throughout yeasts and fungi. In *Saccharomyces cerevisiae*, Flo11 is the main adhesin involved in cell adhesion and biofilm formation, making the study of its function and regulation in this nonpathogenic budding yeast highly relevant. The *S. cerevisiae FLO11* gene is driven by a TATA-box-containing promoter that is regulated through one of the longest regulatory upstream regions (3 kb) in yeast. We reported recently that two chromatin cofactor complexes, the Rpd3L deacetylase and the Swi/Snf chromatin-remodeling complexes, contribute significantly to the regulation of *FLO11*. Here, we analyze directly how these complexes impact on *FLO11* promoter chromatin structure and dissect further the interplay between histone deacetylases, chromatin remodeling, and the transcriptional repressor Sfl1. We show that the regulation of chromatin structure represents an important layer of control in the highly complex regulation of the *FLO11* promoter.

THE initiation of transcription is one of the main targets of gene expression regulation and is determined by regulator binding sequences both within the promoter region and in more distant regions. In yeasts, where the true promoter and associated regulatory sequences are usually close together, the combined regions are often referred to as "the promoter," and for simplicity we will follow this convention in this work. Even though promoter regulation depends mainly on the identity of bound regulators, either activators or repressors, and on the position and affinities of their binding sites, it has become clear that the accessibility of the promoter region is an equally important level of control (Li *et al.* 2007). In eukaryotes, DNA accessibility is governed through chromatin structure, whereby DNA is packaged into nucleosomes. Nucleosomes are assemblies of basic histone proteins on a stretch of 147 bp of DNA. They can adopt different positions along the DNA and may contain different histone variants or post-translationally modified histones (Kamakaka and Biggins 2005; Kouzarides 2007; Li *et al.* 2007). Histone modifications or changes in nucleosome positions are strategies to alter DNA accessibility and therefore gene expression. Accordingly, the analysis of chromatin structure and dynamics has become a key area in the study of promoter regulation. Indeed, many multi-protein complexes that affect chromatin have been described, and these are often recruited to promoters by transcriptional regulators or through DNA-binding subunits of their own.

Histone deacetylase (HDAC) complexes, for example, may either activate or repress gene transcription and are involved not only in transcription initiation but also in elongation (Kurdistani and Grunstein 2003a; Li *et al.* 2007). There are >10 HDAC enzymes known in *Saccharomyces cerevisiae*, grouped into three classes: I, II, and III. The class I member Rpd3 is the most studied and contributes to either repression or activation of many genes (Vidal and Gaber 1991; Kadosh and Struhl 1997, 1998; De Nadal *et al.* 2004; Puig *et al.* 2004). Rpd3 is the HDAC subunit of the Rpd3L and Rpd3S complexes, together with associated proteins such as Sap30, Pho23, Sin3, Cti6, Sds3, or Rxt2. Sds3 and Pho23 are exclusive subunits of Rpd3L, while Rco1 is an exclusive subunit of the Rpd3S complex (Carrozza *et al.* 2005).

Another example of promoter regulation through chromatin is the repositioning, remodeling, or disassembly of

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nucleosomes mediated by a family of ATP-dependent Snf2-type helicases that are often part of large multisubunit complexes (Clapier and Cairns 2009). The founding member of this family is the Swi/Snf complex, which contains the Swi2/Snf2 ATPase subunit and 11 more subunits. Several of these subunits are involved in the activation of different genes. Moreover, the Swi/Snf complex may interact with HDACs in gene regulation (Deckert and Struhl 2002; Sertil *et al.* 2007).

We are particularly interested in the regulation of *FLO11* expression. *FLO11* encodes the main cell-surface protein involved in cell–cell and cell-surface adhesion in *S. cerevisiae* (Lo and Dranginis 1998; Reynolds and Fink 2001). Adhesion is critical for infection in pathogenic fungi, such as *Candida albicans* (Verstrepen *et al.* 2004). In the nonpathogenic yeast *S. cerevisiae*, adhesion is also essential for various properties, including flocculation, biofilm formation, or the dimorphic switch, which are responses to environmental stress (Gimeno *et al.* 1992; Guo *et al.* 2000; Verstrepen and Klis 2006). As *S. cerevisiae* is a highly tractable organism with many genetic and molecular tools, it represents a very powerful model for studying adhesion in fungi and dissecting the regulation of *FLO11* gene expression (Reynolds and Fink 2001; Verstrepen *et al.* 2004; Verstrepen and Klis 2006). The *FLO11* promoter, ~3 kb in length, is the largest described in *S. cerevisiae*. Its regulation is very complex, with many different inputs and pathways affecting *FLO11* expression (Madhani and Fink 1997; Rupp *et al.* 1999; Pan and Heitman 2000, 2002; Kohler *et al.* 2002; Kuchin *et al.* 2002; Braus *et al.* 2003; Zeitlinger *et al.* 2003; Halme *et al.* 2004; van Dyk *et al.* 2005; Bumgarner *et al.* 2009; Octavio *et al.* 2009). We have previously described that a high level of *FLO11* expression is required for the formation of a liquid surface biofilm (Fidalgo *et al.* 2006). Moreover, we showed that only the promoter carried by the "flor" *Saccharomyces* yeast strain (used to produce sherry wine) is able to reach this level of expression. The promoter of this *FLO11* allele bears several point mutations and a deletion of 111 bp that confers a higher level of expression than common laboratory alleles (Fidalgo *et al.* 2006).

Nonetheless, it remains an open question through which mechanism these point mutations and the deletion lead to higher expression levels. To gain further mechanistic insights, we previously performed a genetic screen for factors activating *FLO11* expression. In this way, we identified subunits of the Swi/Snf nucleosome-remodeling complex and the HDAC complexes as important *FLO11* activators (Barrales *et al.* 2008), suggesting that the *FLO11* promoter may also be strongly regulated at the chromatin level.

Here, we extend our investigation into the role of these two chromatin-related complexes, the HDAC and Swi/Snf, in *FLO11* regulation. We analyzed if these complexes impact on the chromatin structure of the *FLO11* promoter and show that it is substantially altered by the removal of HDAC or Swi/Snf subunits. As no subunit with deacetylase activity was identified in our previous screen, we tested several candidate enzymes for their effect on *FLO11* expression, finding that the Hos2 and Rpd3 deacetylases activate *FLO11* in a partially redundant manner. Surprisingly, the role of these HDAC subunits was not mirrored by the loss of Pho23, a subunit of the Rpd3L deaceylation complex. Our data confirm an additional layer of *FLO11* regulation at the level of chromatin through an intricate interplay between histone modifications and nucleosome remodeling.

Materials and Methods

Strains, media, and genetic methods

The *S. cerevisiae* strains used in this study are listed in Table 1. PCR-mediated disruption (Lorenz *et al.* 1995) was used for gene deletions. Double deletions were carried out as described (Guldener *et al.* 1996). Plasmids and disruption cassettes were introduced using the LiAc/SSDNA/PEG procedure (Gietz *et al.* 1995). Standard yeast extract peptone dextrose (YEPD) contained 2 or 0.5% of glucose. Synthetic complete dextrose (SCD) medium without the appropriate amino acids was used for plasmid selection. When necessary, YEPD medium was supplemented with 200 mg/liter geneticin for selection of geneticin-resistant transformants.

Expression analysis

For *FLO11*, *ICR1*, and *SFL1* gene expression analysis, cells were incubated overnight in YEPD liquid medium at 30°, transferred to fresh YEPD medium, and incubated to an optical density at 600 nm (OD_{600}) of ~0.8. Cells were washed with ice-cold water and total RNA was isolated (QIAGEN RNeasy Mini Kit). For the Northern blot analysis, total RNA was separated by denaturing formaldehyde agarose gel electrophoresis and transferred overnight by capillary action to nylon membranes. The 400-bp regions at the 5′ end of the *FLO11* or *SCR1* genes were used as probes (labeled using the AMERSHAM Megaprime DNA Labeling System Kit), and radioactive bands were visualized using a Molecular Dynamics PhosphorImager. The complete images obtained were manipulated using Adobe Photoshop CS2 to convert them into grayscale format and adjust levels. For expression analysis by quantitative RT-PCR (qRT-PCR) cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche), and cDNA was quantitatively measured in triplicate with the ABI Prism 7000 sequence detection system. *SCR1* cDNA was used for normalization. Primers used are listed in the Supporting Information, Table S1.

Flow cytometry

To quantify GFP levels, cells were grown in SCD medium without uracil overnight at 30°, washed and resuspended in fresh medium, and incubated until they reached an OD_{600} of 0.8. Cells were pelleted, washed, and resuspended in 50 mM sodium citrate. The fluorescence of 20,000 cells was measured using a FACSCalibur flow cytometer (Becton Dickinson) with a 530/30 band-pass filter.

Table 1 Yeast strains used in this study

Strains	Genotype	Source
133d	*MAT***a** *ura3-52*	Fidalgo *et al.* (2006)
133d *pho23Δ*	*MAT***a** *ura3-52 pho23Δ::KanMX4*	Barrales *et al.* (2008)
133d *hos1Δ*	*MAT***a** *ura3-52 hos1Δ::KanMX6*	This study
133d *hos2Δ*	*MAT***a** *ura3-52 hos2Δ::KanMX6*	This study
133d *hos3Δ*	*MAT***a** *ura3-52 hos3Δ::KanMX6*	This study
133d *rpd3Δ*	*MAT***a** *ura3-52 rpd3Δ::KanMX6*	This study
133d *hda1Δ*	*MAT***a** *ura3-52 hda1Δ::KanMX6*	This study
133d *hos2Δ rpd3Δ*	*MAT***a** *ura3-52 rpd3Δ hos2Δ::KanMX6*	This study
133d *sfl1Δ*	*MAT***a** *ura3-52 sfl1Δ::KanMX6*	This study
133d *flo8Δ*	*MAT***a** *ura3-52 flo8Δ::KanMX4*	Barrales *et al.* (2008)
133d *flo8Δ sfl1Δ*	*MAT***a** *ura3-52 sfl1Δ flo8Δ::KanMX4*	This study
133d *snf5Δ*	*MAT***a** *ura3-52 snf5Δ::KanMX4*	Barrales *et al.* (2008)
133d *snf5Δ sfl1Δ*	*MAT***a** *ura3-52 sfl1Δ snf5Δ::KanMX6*	This study
133d *pho23Δ sfl1Δ*	*MAT***a** *ura3-52 sfl1Δ pho23Δ::KanMX6*	This study
L5684	*MAT***a** *ura3-52 leu2Δ*	G. R. Fink
L5684 *pho23Δ*	*MAT***a** *ura3-52 leu2Δ pho23Δ::KanMX6*	Barrales *et al.* (2008)
L5684 *hos1Δ*	*MAT***a** *ura3-52 leu2Δ hos1Δ::KanMX6*	This study
L5684 *hos2Δ*	*MAT***a** *ura3-52 leu2Δ hos2Δ::KanMX6*	This study
L5684 *hos3Δ*	*MAT***a** *ura3-52 leu2Δ hos3Δ::KanMX6*	This study
L5684 *rpd3Δ*	*MAT***a** *ura3-52 leu2Δ rpd3Δ::KanMX6*	This study
L5684 *hda1Δ*	*MAT***a** *ura3-52 leu2Δ hda1Δ::KanMX6*	This study
L5684 *hos2Δ rpd3Δ*	*MAT***a** *ura3-52 leu2Δ rpd3Δ hos2Δ::KanMX6*	This study
L5684 *sfl1Δ*	*MAT***a** *ura3-52 leu2Δ sfl1Δ::KanMX6*	This study
L5684 *flo8Δ*	*MAT***a** *ura3-52 leu2Δ flo8Δ::KanMX4*	Barrales *et al.* (2008)
L5684 *flo8Δ sfl1Δ*	*MAT***a** *ura3-52 leu2Δ sfl1Δ flo8Δ::KanMX4*	This study
L5684 *snf5Δ*	*MAT***a** *ura3-52 leu2Δ snf5Δ::KanMX6*	Barrales *et al.* (2008)
L5684 *snf5Δ sfl1Δ*	*MAT***a** *ura3-52 leu2Δ sfl1Δ snf5Δ::KanMX6*	This study
L5684 *pho23Δ sfl1Δ*	*MAT***a** *ura3-52 leu2Δ sfl1Δ pho23Δ::KanMX6*	This study
L5684 *flo8Δ hda1Δ*	*MAT***a** *ura3-52 leu2Δ hda1Δ::KanMX6 flo8Δ::hphMX4*	This study
L5684 *snf5Δ hda1Δ*	*MAT***a** *ura3-52 leu2Δ hda1Δ::KanMX6 snf5Δ::hphMX4*	This study
L5684 *pho23Δ hda1Δ*	*MAT***a** *ura3-52 leu2Δ hda1Δ::KanMX6 pho23Δ::hphMX4*	This study
L5684 pho23:Myc	*MAT***a** *ura3-52 leu2Δ PHO23-13Myc::KanMX4*	This study
L5684 rpd3:Myc	*MAT***a** *ura3-52 leu2Δ RPD3-13Myc::KanMX4*	This study

β-*Galactosidase Assay*

The β-galactosidase assays were performed essentially as described previously (Rose and Botstein 1983). Cells were grown in SCD medium without uracil overnight at 30°, washed and resuspended in fresh medium, and incubated until they reached an OD_{600} of 0.8. Specific β-galactosidase activity was then measured and normalized to the total protein content in each extract.

Chromatin immunoprecipitation analysis

The cells were incubated in YEPD liquid medium overnight at 30°, transferred to fresh YEPD medium, and incubated until they reached an OD_{600} of ~1. Cells for L5684 *pho23:Myc* and L5684 *rpd3:Myc* strains were incubated 4 hr in 0.5% glucose medium . Cells were cross-linked with 1% formaldehyde for 20 min at room temperature. In the case of Rpd3 and Pho23 chromatin immunoprecipitation (ChIP), cells were pretreated with 10 mM dimethyl adipimidate dihydrochloride for 45 min as described previously (Kurdistani and Grunstein 2003b). Cross-linking was quenched by adding glycine to a final concentration of 125 mM. The cells were washed twice with ice-cold 0.9% NaCl, resuspended in HEG150 buffer [150 mM NaCl, 50 mM HEPES (pH 7.6), 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride] and lysed with a FastPrep FP120 (two times for 15 sec at power 4.5 with a 60-sec pause on ice). Chromatin was sheared to an average size of 500-bp fragments by sonication using a Bioruptor (Diagenode, three times for 30 sec with a 60-sec pause, position on high, ice-water bath). Chromatin immunoprecipitation was performed as described (Strahl-Bolsinger *et al.* 1997). The antibodies used were anti-histone H3 antibody from Abcam (ab1791), anti-acetyl-histone H4 from Millipore (06-866), and anti-cMyc from Santa Cruz Biotechnology (9E10). Immunoprecipitated and input DNA was quantitatively measured in triplicates with the ABI Prism 7000 sequence detection system. Primers used are listed in Table S1.

Chromatin analysis

The preparation of yeast nuclei (Almer *et al.* 1986) and chromatin analysis of nuclei by restriction nucleases and MNase digestion with indirect end-labeling was performed as described (Svaren and Horz 1995; Gregory and Horz 1999). Secondary cleavage was with *Xcm*I (+584), to monitor the whole promoter, or with *Xba*I (−461 in strain 133d, −460 in L5684), to analyze in more detail the promoter 5′ region. The probes are PCR products corresponding to bases

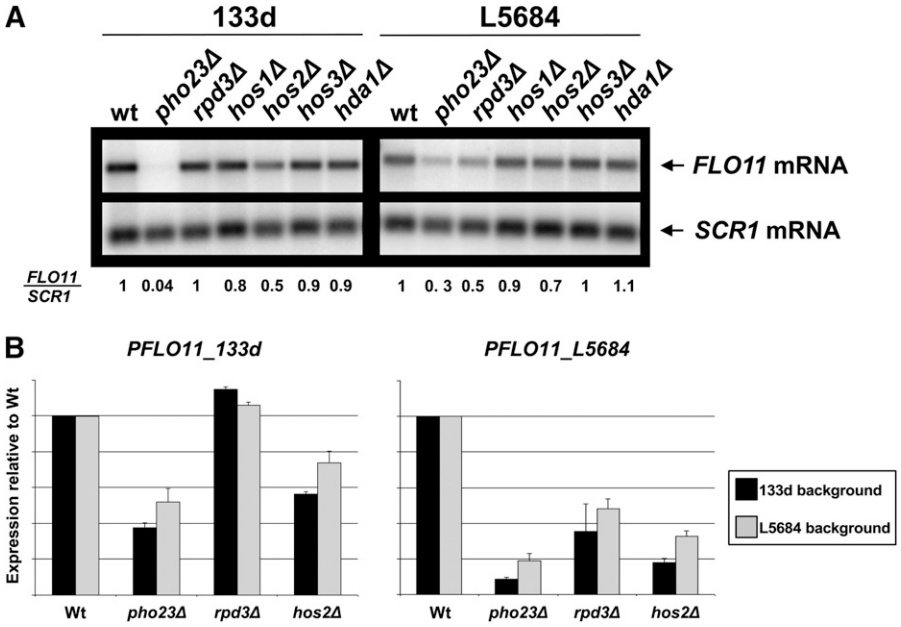

Figure 1 Strain-dependent activation of *FLO11* transcription by the Hos2 or Rpd3 deacetylases. (A) Northern blot analysis of *FLO11* mRNA levels in the indicated mutants of 133d (flor) or the L5684 (laboratory) yeast strains. *SCR1* mRNA was probed as loading control. Numbers below the blot indicate *FLO11* expression levels normalized to *SCR1* and the respective quotient of the wild type. (B) The differential roles of deacetylases are specific to the promoter alleles. Expression levels were measured by flow cytometry or β-galactosidase assays for strains transformed with a plasmid containing the GFP ORF under the control of the 133d *FLO11* promoter (*PFLO11_133d*) or the lacZ ORF under the control of the laboratory one (*PFLO11_L5684*), respectively, and normalized to wild type. Error bars represent the standard deviation of three biologically independent measurements.

+87 to +553 from the ATG of the *FLO11* ORF of strain 133d for the *Xcm*I secondary cleavage and to bases −975 to −589 for the *Xba*I secondary cleavage. Probes generated from strain 133d genomic DNA worked equally as well as probes generated from strain L5684 DNA (data not shown). The complete images obtained were manipulated using Adobe Photoshop CS2 to convert them into grayscale format and adjust levels. Sometimes parts of the image were reorganized to avoid space between lines.

Results

FLO11 transcriptional activation exhibits an allele-specific requirement for Rpd3 and Hos2 deacetylases

We showed previously that many subunits of the Rpd3L complex (Pho23, Sds3, Rxt2, Sap30, and Ash1) are involved in the activation of *FLO11* expression; this occurred even when using glucose-containing media as the sole carbon source, where *FLO11* expression should be repressed. These subunits activate the expression of both the "flor" and the laboratory alleles of *FLO11* (Barrales *et al.* 2008). The Rpd3L complex is recruited to specific promoters through the DNA-binding proteins Ash1 and Ume6 (Carrozza *et al.* 2005). Once recruited, the HDAC complex subunit Rpd3 is responsible for repressing or activating target gene transcription via its deacetylase activity (Kadosh and Struhl 1997; Kurdistani *et al.* 2002; De Nadal *et al.* 2004). However, in our screen we did not identify any catalytic HDAC subunits as a *FLO11* activator. This could be because (i) the screen did not reach saturation, (ii) the activation by the Rpd3L complex does not require HDAC activity, or (iii) other histone deacetylases present in *S. cerevisiae* act redundantly with the components of the Rpd3L complex in *FLO11* activation.

To directly address these possibilities, we first examined the effect of deleting *RPD3* on *FLO11* expression in both flor (133d) (Fidalgo *et al.* 2006) and laboratory (L5684) strains. Given possibility (iii), we also examined the effect of deleting other candidate deacetylases (*HOS1*, *HOS2*, *HOS3*, and *HDA1*). In the laboratory L5684 strain, *FLO11* expression was reduced mainly in the *rpd3Δ* mutant (Figure 1A), which supports a role for Rpd3-mediated deacetylase activity in *FLO11* regulation. Interestingly, a smaller but significant reduction in *FLO11* expression was also observed in the *hos2Δ* mutant (Figure 1A). However, in the flor 133d strain, the deletion of *rpd3Δ* did not affect *FLO11* expression, but was reduced only by the loss of *HOS2* (Figure 1A). The Rpd3L complex subunit Pho23, previously identified as a *FLO11* activator (Barrales *et al.* 2008), was included as a positive control. Interestingly, the decrease in *FLO11* expression in both the 133d strain/*hos2Δ* and the L5684 strain/*rpd3Δ* mutants was smaller than the corresponding decrease in either *pho23Δ* mutant (Figure 1A).

The strain-specific requirements for different HDACs in *FLO11* activation could be due directly to the differences in the *FLO11* promoter or indirectly to other differences in their genetic background. To distinguish between these possibilities, we transformed the L5684 and 133d strains with a plasmid harboring the GFP gene under the control of the *FLO11* promoter from the 133d strain ($pFLT_{133d}GFP$) or the lacZ ORF controlled by the *FLO11* promoter from the L5684 strain (B3782) (Rupp *et al.* 1999). This allowed us to use flow cytometry and β-galactosidase assays to measure the effect of *PHO23*, *HOS2*, and *RPD3* deletions on the activity of both *FLO11* promoters in the two genetic backgrounds. As expected, *FLO11* expression directed by the flor *FLO11* promoter was reduced in the flor strain background by the deletion of *PHO23* and *HOS2*, but not of *RPD3* (Figure 1B). On the other hand, a reduction in expression was observed in

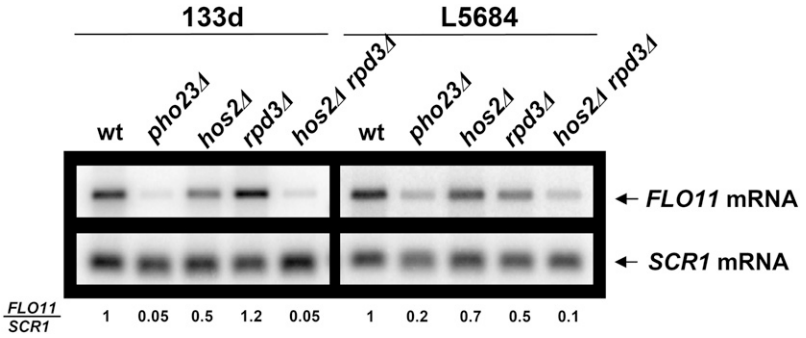

Figure 2 Hos2 and Rpd3 synergistically activate *FLO11* expression. Northern blot analysis as in Figure 1A, but including the *hos2Δ rpd3Δ* double mutant in 133d and L5684 strains.

the three mutants when the laboratory *FLO11* promoter was analyzed in the laboratory background (Figure 1B). The reduction in expression observed in the *hos2Δ* mutant using this assay was stronger than the one observed by Northern blot. However, a difference in the medium used in these two assays could explain this difference. These data suggest that our assay faithfully recapitulates *FLO11* promoter regulation. As before, the effect of the *PHO23* deletion was somewhat stronger than that of the *RPD3* or *HOS2* deletions (Figure 1B). Significantly, the expression levels observed for the flor and laboratory promoters were unaffected by the genetic background of the strain used (Figure 1B). This confirms the results shown in Figure 1A and argues that the different deacetylase requirements are due to differences in the promoter sequence between the two *FLO11* alleles.

Rpd3 and Hos2 deacetylases redundantly activate FLO11 expression

As Rpd3 and Hos2 were differentially involved in activating different *FLO11* promoter alleles, we considered the possibility that these two deacetylases might be acting partially redundantly. To test this, we generated *hos2Δ rpd3Δ* double mutants in both the 133d and L5684 strain backgrounds and found that the double-mutant combination resulted in a significantly stronger reduction in *FLO11* expression in both cases (Figure 2). Thus the activity of Hos2 and Rpd3 on *FLO11* expression appears to be partially redundant in both strains, with each deacetylase probably having additional allele-specific functions. As the levels of *FLO11* expression in the double mutants were approximately the same as in the corresponding *pho23Δ* mutants (Figure 2), it seems likely that the redundant activities of both deacetylases are mediated through the Rpd3L complex.

Pho23, but not HDACs Rpd3 and Hos2, has a clear role in generating a nucleosome-depleted FLO11 promoter region

Rpd3 mainly deacetylates histones H4 and H3 (Kurdistani and Grunstein 2003a), acting redundantly with Hos2 in the case of histone H4 (Sharma *et al.* 2007). If Hos2 and Rpd3 are the catalytic subunits of the complexes involved in *FLO11* activation, we would expect to see changes in histone acetylation at the *FLO11* promoter in single or double mutants for these catalytic subunits, as well as in *pho23Δ* mutants. To check this prediction, we used ChIP to measure levels of acetylated H4 at specific points along the *FLO11* promoter in the 133d and L5684 strains and compared the effect of single *pho23Δ*, *hos2Δ*, and *rpd3Δ* mutants as well the double *hos2Δ rpd3Δ* mutant on H4 deacetylase activity (Figure 3, A–E). Surprisingly, the levels of H4 acetylation at the promoter were somewhat decreased by the loss of *PHO23* and *HOS2* in both backgrounds (Figure 3, A–C). H4 acetylation levels were almost unchanged in *hos2Δ rpd3Δ* double mutants and increased only in the 133d strain/*rpd3Δ* mutant, even though we had not observed an effect on *FLO11* expression in this strain (Figure 3, A, D, and E; also see Figures 1 and 2). Thus, we did not observe a clear-cut relationship between the effects on expression levels and histone H4 acetylation.

In this experiment, the amount of histone H3 was used as a control to determine total nucleosome occupancy. However, when we directly compared levels of histone H3 between wild-type and mutant strains, we obtained some very interesting results. In wild-type L5684 and 133d strains, the *FLO11* promoter showed strikingly low levels of nucleosome occupancy, especially over the central region (Figure 3F). Given the differential *FLO11* expression in these two strains, it suggests that this promoter nucleosome depletion is not a consequence of high transcription levels. In contrast, nucleosome occupancy in the *FLO11*-coding region was low only in the 133d background, presumably reflecting the high expression levels of this strain (Figure 3F).

Interestingly, neither the promoter in either strain, nor the coding region in the 133d strain, was nucleosome depleted in the *pho23Δ* mutant (Figure 3, F and G). This is consistent with the greatly *FLO11*-reduced expression of this mutant (Figures 1 and 2) and suggests that Pho23 is somehow involved in maintaining an open chromatin structure at the *FLO11* promoter. A similar but somewhat less pronounced increase in nucleosome occupancy was observed for the *hos2Δ* mutant in the 133d background (Figure 3, F and H), which also correlates with the lower expression levels in this strain (Figures 1 and 2). Conversely, the *rpd3Δ* single mutant showed increased nucleosome occupancy in the *FLO11* promoter region mainly in the upstream region in the L5684 but not in the 133d strain (Figure 3, F and I, amplicon 2). Moreover, there was a general tendency toward higher nucleosome occupancy levels

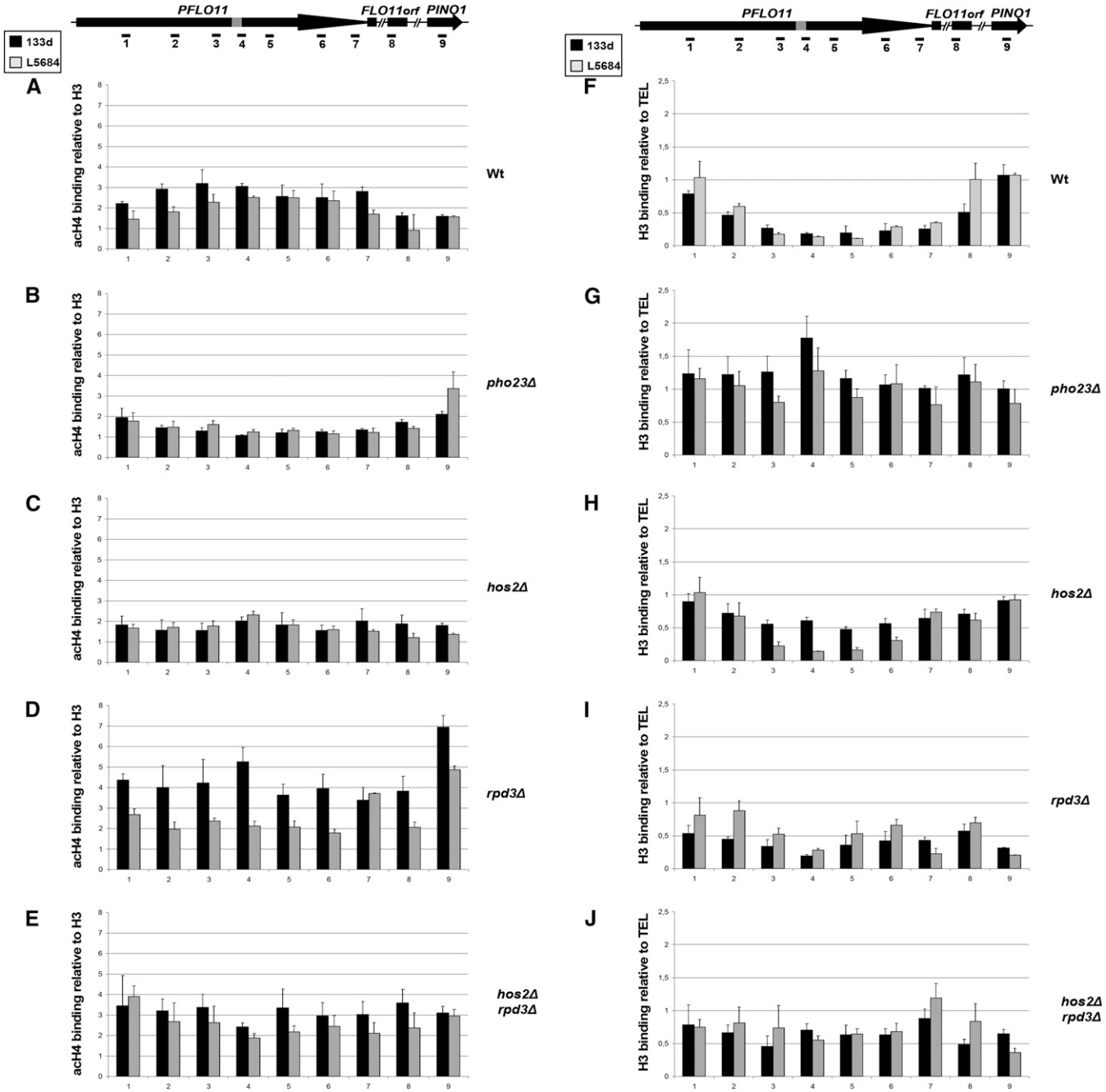

Figure 3 Mutant phenotypes of histone depletion and acetylation at the *FLO11* promoter. The level of acetylated histone H4 (A–E) and total histone H3 (F–J) occupancy over the *FLO11* promoter and coding region as well as over the *INO1* promoter was monitored by ChIP assay in the indicated wild type and mutants for both the 133d and the L5684 strain. Acetylated histone H4 levels are shown relative to histone H3 levels, and histone H3 levels were normalized to an amplicon at the telomere. Error bars represent the standard deviation of three biologically independent measurements. Diagrams above the panels show amplicon positions. The gray box in the *FLO11* promoter corresponds to the 111-bp region that is deleted in the flor promoter allele. As a positive control, we used an amplicon at the *INO1* promoter to test H3 levels and observed a strong decrease in nucleosome occupancy in the *rpd3Δ* and *hos2Δ rpd3Δ* mutants (F, I, and J) in agreement with increased *INO1* transcription in these mutants (Rundlett *et al.* 1998).

at the *FLO11* promoter in the *hos2Δ rpd3Δ* double mutant. Thus, there was a correlation between an increase in promoter nucleosome occupancy and a decrease of *FLO11* expression. Nonetheless, it remains to be determined if the increase in nucleosome occupancy is a cause or a consequence of decreased expression. Interestingly, the difference in nucleosome occupancy between *pho23Δ* and *rpd3Δ hos2Δ* mutants (Figure 3, G and J), even with the same level of *FLO11* expression, suggests that Pho23 is actively involved in maintaining a nucleosome-depleted *FLO11* promoter and that this effect is at least partially independent of Rpd3 and Hos2.

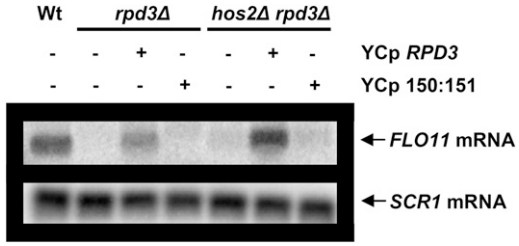

Figure 4 The enzymatic activity of Rpd3 is required for *FLO11* activation in the laboratory strain. Northern blot analysis (as in Figure 1A) of *FLO11* mRNA from wild type (L5684) and *rpd3Δ* or *hos2Δ rpd3Δ* mutant transformed (+) or not (−) with the plasmid containing either the *RPD3* wild-type gene (YCp *RPD3*) or the *RPD3* allele defective for deacetylase activity (YCp 150:151).

Deacetylase activity of Rpd3 is required for FLO11 expression

To study if the reduction in *FLO11* expression obtained in the laboratory strain was due to the loss of Rpd3 deacetylase activity, we transformed L5684 strain yeasts harboring *RPD3* and *HOS2 RPD3* deletions with a plasmid containing either the wild-type *RPD3* allele or a point mutant allele where His150 and His151 were replaced with alanines resulting in defective deacetylase activity (De Nadal *et al.* 2004). The level of *FLO11* expression was restored only if transformed with the wild-type but not with the mutant allele (Figure 4), showing that the enzymatic activity of Rpd3 is essential for complete *FLO11* activation in the laboratory L5684 background.

Rpd3-dependent activation of the laboratory FLO11 allele may be partly due to reduced Sfl1 repression

Rpd3 has been described to activate the expression of some genes by counteracting the effect of gene repressors (Sertil *et al.* 2003, 2007). Sfl1 and the Rpd3L complex have recently been described to mediate antagonistic regulatory effects on the *FLO11* promoter regulation (Bumgarner *et al.* 2009). Sfl1 is one of the main repressors of *FLO11* expression, and a region for Sfl1 binding has been identified in the *FLO11* promoter (Pan and Heitman 2002). This region includes the 111-bp sequence that is absent in the 133d strain. Thus, an explanation of why Rpd3 deacetylase has an effect in the laboratory allele but not in the flor one could be that Sfl1 is counteracted by Rpd3 in yeast carrying the laboratory *FLO11* allele but not the flor one. To check this hypothesis, we deleted *SFL1* from both laboratory (L5684) and flor (133d) backgrounds. Northern blot analysis showed that, as predicted, Sfl1 had a repressor effect on the L5684 strain but not on the 133d one (Figure 5A). To check a possible relation between this repressor and Rpd3, we generated a *rpd3Δ sfl1Δ* double mutant. As controls, we also generated double mutants with *PHO23*, as a member of the complex, and with *FLO8* and *SNF5*, as other *FLO11* activators, to discard a general effect. In the L5684 strains, *FLO11* expression was completely restored by the deletion of *SFL1* in all the activator mutants except *flo8Δ*, where we observed only a partial restoration (Figure 5A). As expected, the reduced expression of *FLO11* in the 133d strain produced by deletion of the described activators was not restored in *sfl1Δ* double mutants (Figure 5A). These data suggest that Rpd3 deacetylase may be required to counteract *FLO11* repression by Sfl1 and explain why in the 133d strain—where this repression does not exist—we do not observe an activator effect of this deacetylase. The question of why the activation of *FLO11* by the Swi/Snf and Rpd3L complexes is Sfl1-independent and much stronger in the 133d background than in the laboratory one is an open question that remains to be studied.

To know if the activation of *FLO11* mediated by both complexes in the laboratory background is a consequence of *SFL1* expression regulation, we performed qRT-PCR to measure the level of *SFL1* expression in the *snf5Δ* and *pho23Δ* mutants. As can be observed in Figure 5B, *SFL1* expression was increased in the *snf5Δ* mutant but not in the *pho23Δ* one, indicating that at least part of the activation effect observed for the Swi/Snf complex could be mediated through *SFL1* repression. The fact that *SFL1* transcription is unaffected in the *pho23Δ* mutant, and that in the 133d strain *FLO11* activation by the Swi/Snf and Rpd3L complexes was Sfl1-independent, suggests a direct effect of these complexes on the *FLO11* promoter.

Sfl1 has been proposed to recruit the Hda1 repressor to the *FLO11* promoter, contributing to an epigenetic regulation (Halme *et al.* 2004; Octavio *et al.* 2009). To check if deletion of *HDA1* restores *FLO11* expression in a similar way to *sfl1Δ* mutants, we deleted *HDA1* in *flo8Δ*, *snf5Δ*, and *pho23Δ* single mutants. However, as is shown in Figure 6A, the drop in *FLO11* expression is not restored in any of the double mutants.

On the other hand, Sfl1 has been described to repress *FLO11* expression by increasing the amount of *ICR1* noncoding RNA (ncRNA), which is transcribed in the *FLO11* promoter region. Sfl1 activates *ICR1* ncRNA transcription by repressing the transcription of *PWR1* ncRNA that partially interferes with the *ICR1* one. In this interesting regulatory mechanism, the Rpd3L complex has been described to have the opposite role (Bumgarner *et al.* 2009, 2012). We carried out qRT-PCR to study the level of *ICR1* ncRNA in mutants from both backgrounds (Figure 6B) and to study the effect of histone deacetylases and Sfl1 over this regulation. As it has been previously described, *ICR1* ncRNA amounts increased significantly in the *pho23Δ* and *rpd3Δ* mutants in the L5684 laboratory background (Figure 6B). Interestingly, this increase was not observed in the 133d flor background (Figure 6B). Moreover, the increase in the *ICR1* ncRNA in an *rpd3Δ* mutant was abolished in a double mutant with *SFL1* (Figure 6B). These observations support the idea that the Rpd3L complex counteracts the repression of *FLO11* expression exerted by Sfl1 through the regulation of *ICR1*. Deletion of *HOS2* had no significant effect over *ICR1* ncRNA expression (Figure 6B), indicating that the redundant role of Rpd3 and Hos2 in controlling *FLO11* expression is not mediated

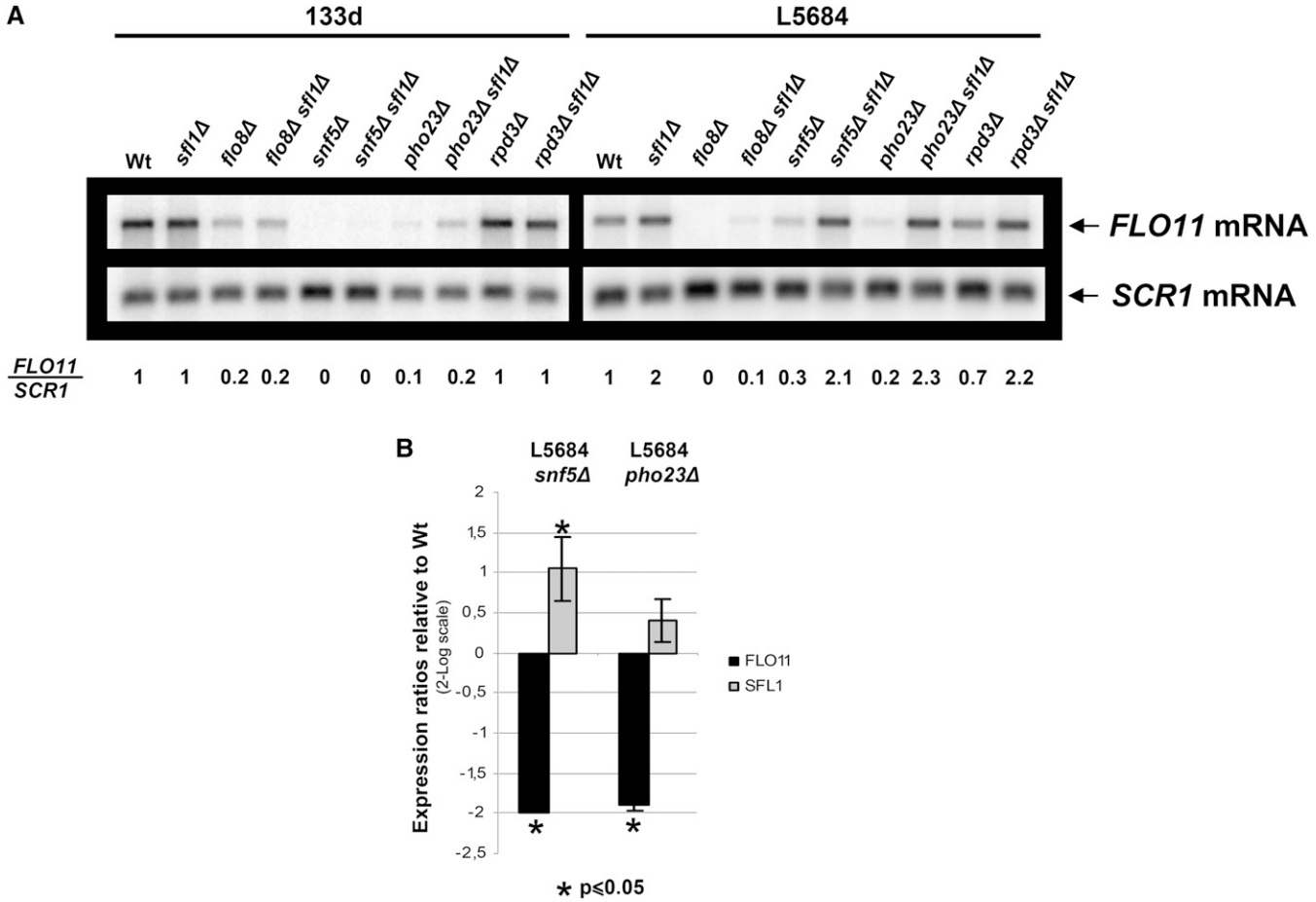

Figure 5 Sfl1-mediated repression is important for *FLO11* regulation but is absent in the 133d strain. (A) Northern blot analysis of *FLO11* mRNA, as described in Figure 1A, for the indicated wild-type and mutant strains. (B) qRT-PCR analysis of *FLO11* and *SFL1* expression in the laboratory strain. Expression was normalized to *SCR1* expression and to wild type. Error bars represent the standard deviation of three biologically independent measurements.

by regulating this ncRNA. This result and the fact that the ncRNA system seems to be unregulated in the 133d strain support the idea that the opposing actions of Rpd3 and Sfl1 on *FLO11* expression via ncRNA is the additional mechanism by which Rpd3 affects laboratory *FLO11* allele expression independently of its redundant activity with Hos2.

Pho23 is important for the maintenance of proper nucleosome organization at the FLO11 promoter

Our observations described above and the strong effect on overall promoter nucleosome occupancy in the *pho23Δ* mutant (Figure 3, F and G) led us to look for direct effects of the Rpd3L complex on *FLO11* promoter chromatin. To this end, we assessed if there were changes in chromatin structure at the level of nucleosome positioning. First, we characterized *FLO11* promoter chromatin structure in both the 133d and L5684 wild-type strains by MNase indirect end-labeling analysis. We used YEPD medium, where the deletion of *PHO23* leads to an obvious drop in *FLO11* expression. We observed a characteristic pattern for each strain, which differed from that of free DNA (Figure 7, A and B). A small deletion in the ORF of the 133d flor *FLO11* allele caused a shift of the banding patterns relative to the L5684 laboratory strain if *Xcm*I was used for secondary cleavage (Figure 7A). Taking this shift into account, both patterns were quite similar to each other. However, closer examination suggested subtle differences in the region close to the 111-bp flor-specific promoter deletion. Downstream of the deletion, two distinct hypersensitive bands flanking a protected region were observed in the 133d strain (Figure 7A). In contrast, the laboratory strain showed more uniformly smeared accessibility in the same region. Using *Xba*I for secondary cleavage, which cuts closer to this region and allows us to zoom in on it (Figure 7B), we identified a protected area in the L5684 strain just at the position where the 111 bp are deleted in the flor strain; the flor strain showed a more pronounced hypersensitive band just downstream of this region. Even though subtle, these differences may be highly relevant because the 133d specific 111-bp deletion is the main sequence difference between the two promoter alleles. Moreover, targeted deletion of this region in the laboratory allele leads to increased *FLO11* expression (Fidalgo *et al.* 2006).

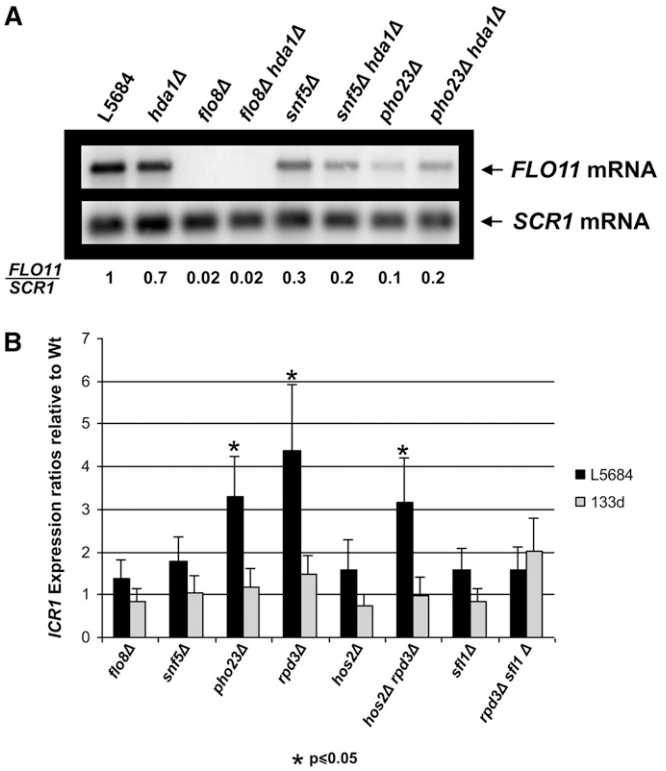

Figure 6 Rpd3 counteracts the action of Sfl1 on the laboratory *FLO11* allele by regulating *ICR1* ncRNA. (A) Northern blot analysis of *FLO11* mRNA, as described in Figure 1A, for the indicated wild-type and mutant strains. (B) qRT-PCR analysis of *ICR1* ncRNA expression in both laboratory and 133d strains. Expression was normalized to *SCR1* expression and to wild type. Error bars represent the standard deviation of three biologically independent measurements.

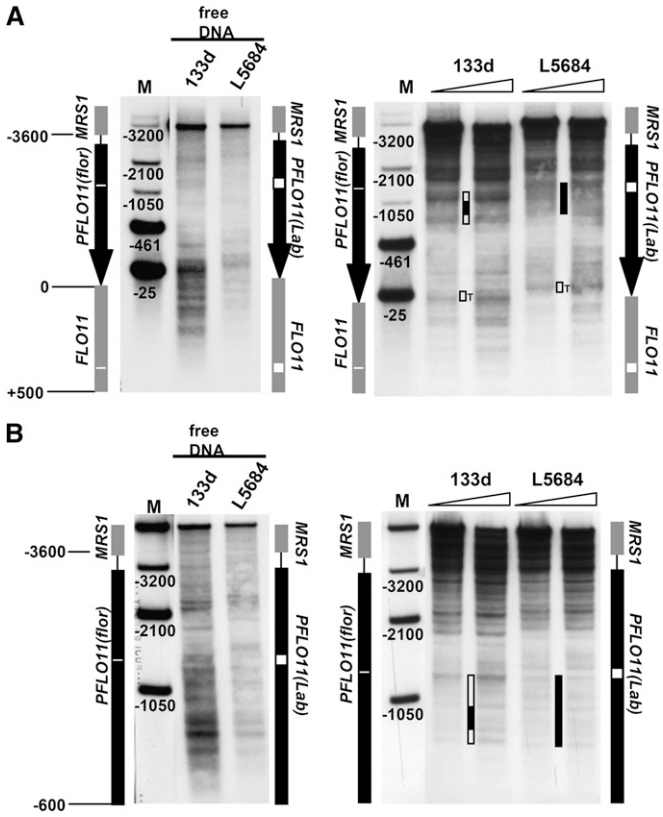

Figure 7 Chromatin structure at the *FLO11* promoter is generally very similar between flor and laboratory alleles but is distinct in the vicinity of the 111-bp deletion. MNase indirect end-labeling analysis of chromatin structure at the *FLO11* promoter in 133d and L5684 strains. Ramps above the lanes denote increasing MNase concentrations. MNase patterns of free DNA are shown for comparison. Secondary cleavage with *Xcm*I is shown in A and with *Xba*I in B. The five marker bands of lanes M in A were generated by double digestion with *Xcm*I and either *Stu*I, *Xba*I, *Hpa*I, XmnI, or *Afl*I (from bottom to top, corresponding to positions −25, −461, −1050, −2100, and −3200 bp from *FLO11* ATG). The three marker bands in lanes M of B were generated by double digestion with *Xba*I and *Hpa*I, *Xmn*I, or *Afl*I (from bottom to top). Diagrams outlining the *FLO11* promoter (black arrow in A and black line in B) and flanking ORFs (gray lines) are shown on either side of the blots. The regions where the flor allele has deletions in the promoter and ORF relative to the laboratory allele are marked by a white box for the laboratory and by a white line for the flor allele. Hypersensitive regions of interest are highlighted by open bars in between lanes, and more protected regions by solid bars. Note the protected region flanked by two bands next to the 111-bp deletion region (white box) in the L5684 allele in B. "T" denotes the TATA box region.

Next we asked whether or not Pho23 was involved in generating this chromatin structure at the *FLO11* promoter. Indeed, MNase mapping of the *FLO11* promoter chromatin structure in *pho23Δ* mutants revealed differences from the wild-type pattern in both flor and laboratory backgrounds (Figure 8). We observed changes in the banding pattern of the region around the 111-bp deletion and the appearance of additional very strong hypersensitive sites downstream of the TATA box, as well as in a somewhat more protected TATA box region (Figure 8A). A closer look at the upstream *FLO11* promoter region using secondary *Xba*I cleavage showed that the *pho23Δ* mutant patterns in the vicinity of the −1050 position were similar to the wild-type pattern in terms of the position of bands, but the relative band intensities were affected, especially in the laboratory background (Figure 8B). Importantly, these differences in intensities were not due to differences in the degree of digestion as the band intensities in the vicinity of the −2100 position were all very similar. These observations, combined with the histone H3 ChIP analysis (Figure 3B), suggest not only that the average occupancy levels of the *FLO11* promoter nucleosomes are increased in the *pho23Δ* mutant, but also that the nucleosomes are more strongly associated with the DNA.

The additional hypersensitive bands downstream of the TATA box were not present in the free DNA pattern (Figure 7A) and flank a protected region that may correspond to a strongly positioned nucleosome. Furthermore, the wild-type pattern showed a weak but substantial band at the −25 position, which is close to the TATA box itself. This band was also present in the free DNA pattern (Figure 7A), but became protected in the *pho23Δ* mutant patterns (Figure 8A). This may argue for a less accessible TATA box and a strongly positioned nucleosome downstream of the TATA box in this mutant. In summary, our findings suggest

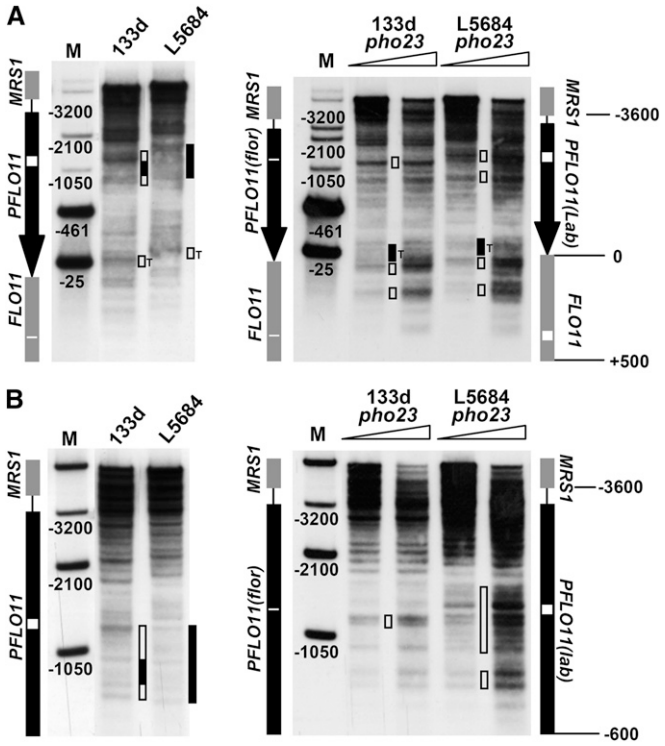

Figure 8 Pho23 is essential for the correct chromatin structure at the *FLO11* promoter. MNase indirect end-labeling analysis of the *FLO11* promoter, as in Figure 7 but including the *pho23Δ* mutants in the 133d and L5684 backgrounds. Secondary cleavage with *Xcm*I is shown in A and with *Xba*I in B. Wild-type patterns taken from Figure 7 are shown on the left for comparison.

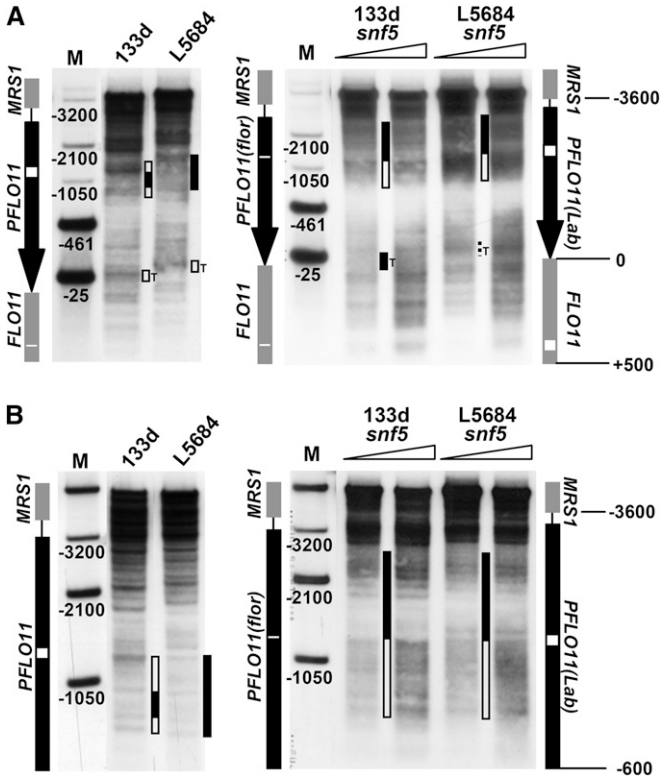

Figure 9 Snf5 is involved in nucleosome positioning at the *FLO11* promoter. MNase indirect end-labeling analysis as in Figure 7, but including the snf5*Δ* mutants in flor and laboratory backgrounds. Secondary cleavage with *Xcm*I is shown in A and with *Xba*I in B. Wild-type patterns taken from Figure 7 are shown on the left for comparison.

that Pho23 is important for correct *FLO11* promoter chromatin structure especially in the region of the 111-bp flor-specific deletion and around the TATA box.

Swi/Snf complex is involved in organizing FLO11 promoter chromatin structure

Contrasting types of interactions between Rpd3 and Swi/Snf complexes have been described at different target promoters. So, Rpd3 inhibits the recruitment of the Swi/Snf complex at several promoters that are repressed by the Rpd3 complex (Deckert and Struhl 2002). Conversely, it has been proposed that Rpd3 complex-mediated deacetylation helps the Swi/Snf complex to bind promoters where Rpd3 activates gene expression (Sertil *et al.* 2007). Because we have demonstrated that subunits of both complexes are strong activators of *FLO11* (Barrales *et al.* 2008) and because we saw substantial effects on *FLO11* promoter chromatin structure in the *pho23Δ* mutant (Figure 8), we repeated the *FLO11* promoter MNase indirect end-labeling analysis for the *snf5Δ* mutants in both backgrounds (Figure 9, A and B).

As before, we found substantial differences in the chromatin structure between the mutant and the wild-type in both backgrounds. Downstream of the 111-bp deletion there was more pronounced hypersensitivity, while upstream, close to the −2100 position, the region was somewhat more protected (Figure 9A). *Xba*I secondary cleavage revealed that the deletion is located just at the intersection between the more protected and more hypersensitive regions (Figure 9B). Moreover, the TATA box region (−25 position) in the 133d *snf5Δ* mutant appeared more protected than in the wild type, similar to that seen for the *pho23Δ* mutant (Figure 8A), but this difference was not as clear in the laboratory background. Thus, the Swi/Snf complex also has a role in shaping the *FLO11* promoter chromatin structure. Even though the *snf5Δ* mutant patterns were different from the *pho23Δ* mutant patterns, the same promoter subregions in the vicinity of the 111-bp deletion and the TATA box were affected.

Discussion

FLO11 promoter regulation at the chromatin level

Regulation of *FLO11* is exceedingly complex because of an unusually long promoter region and the numerous regulators that act on it. Recently, we added new elements to this complex system by showing that the Rpd3L and Swi/Snf complexes are major activators for *FLO11* expression (Barrales *et al.* 2008). These two complexes were previously described to control gene expression by alterations in chromatin structure through histone modification or chromatin remodeling,

respectively. Here we show that both complexes clearly affect chromatin structure at the *FLO11* promoter. Deletion of *PHO23* led to increased nucleosome occupancy over the whole *FLO11* promoter (Figure 3, F and G), to the protection of a hypersensitive site in the TATA box area, and, mostly for the laboratory strain, to the appearance of a more strongly positioned nucleosome in the region where the 111-bp flor-specific deletion is located (Figure 8, A and B). Similarly, deletion of *SNF5* resulted in protection of the TATA box region as well as in accessibility changes in the vicinity of the deletion, which is different from that observed in the *pho23Δ* mutant (Figure 9, A and B).

Our repeated observations of altered chromatin structure close to the 111-bp region led us to postulate a key role for this region in the regulation of *FLO11* expression. This may explain why a deletion of this region is sufficient to give rise to a new capability for *S. cerevisiae*, *i.e.*, overexpression of *FLO11* and the consequent formation of a biofilm on top of a liquid surface. Alteration of the sequence and nucleosome structure in this region could affect the accessibility for previously described regulators, such as the activator Flo8 and the wide-ranging repressor Sfl1. The Swi/Snf complex is thought to be required as a general activator for genes under the control of global repressors (Sertil *et al.* 2007). We demonstrated that the activation effect exerted by Swi/Snf in the laboratory strain may work in part by counteracting the repression by Sfl1. Swi/Snf is also described to enhance binding of Flo8 to the *STA1* promoter (Kim *et al.* 2004), a promoter very similar to the *FLO11* promoter. Interestingly, Flo8 binds to the *FLO11* promoter closely upstream of the 111-bp deletion region where the Swi/Snf complex maintains an accessible chromatin structure (Figure 9). Moreover, downstream of this region, Swi/Snf generates a protected area that includes a binding site for the Sfl1 repressor. Thus, the Swi/Snf complex could maintain a chromatin structure in this area that prevents Sfl1 binding and enhances binding of Flo8, resulting in *FLO11* activation. Interestingly, the repression exerted by Sfl1 is absent from the 133d flor strain, perhaps as a result of the changes to the *FLO11* genomic region in this strain. Nevertheless, a role for changes in the genetic background cannot be ruled out, and more studies must be done to better understand the important role of this repression and the basis for the differences between the two strains.

Regarding the Rpd3L complex, the effect of the *pho23Δ* mutation on chromatin structure in the 111-bp deletion region of the 133d strain was smaller than for the *snf5Δ* mutation, although a clear effect on total nucleosome promoter occupancy was observed. These results support the idea that this complex has a general function in *FLO11* activation, such as in maintaining the whole promoter in an open state.

All these data strongly support our hypothesis that these complexes directly affect chromatin structure at the *FLO11* promoter. This is further supported by the direct demonstration of Swi3 binding, a subunit of the Swi/Snf complex, to the *FLO11* promoter and coding region (Venters and Pugh 2009) and Rpd3 binding to the *FLO11* promoter (Bumgarner *et al.* 2009). We also observed a slightly increased occupancy of Pho23 and Rpd3 in the central region of the *FLO11* promoter (Figure S1). In addition to this direct effect, our evidence also suggests that the Swi/Snf complex could be indirectly activating *FLO11* by repressing *SFL1* expression.

Activation of FLO11 by both Rpd3 and Hos2

Rpd3 and Hos2 have been described to have generally overlapping specificities in gene expression regulation, but with different functions—Rpd3 as a repressor and Hos2 as an activator (Wang *et al.* 2002). However, both deacetylases have been shown to act redundantly to positively regulate the expression of DNA damage-inducible genes (Sharma *et al.* 2007). Here we have observed a strong reduction of *FLO11* expression in the 133d strain when both *RPD3* and *HOS2* were deleted. However, there was no change in *FLO11* expression when only *RPD3* was deleted, suggesting that Rpd3 and Hos2 also act redundantly in activating this promoter. Rpd3 and Hos2 differentially contribute to *FLO11* activation, depending on the allele. Both Rpd3 and Hos2 activate the laboratory *FLO11* allele. While Rpd3 exerts a stronger effect on the laboratory allele, only Hos2 has a clear effect on the 133d flor allele. So we postulate a model in which a basal level of *FLO11* expression in both alleles is redundantly governed by Rpd3 and Hos2, for example, by promoting polymerase initiation complex assembly as described for *DAN1* regulation (Sharma *et al.* 2007). The Pho23-dependent accessibility at the TATA box region could correspond to this effect. Nonetheless, it could also be a consequence of reduced *FLO11* transcription levels. Recently, an expanded Rpd3 complex (Rpd3LE) has been described (Shevchenko *et al.* 2008). The Rpd3LE complex contains five additional members from the Set3 complex, including Hos2. Therefore, it is possible that both deacetylases are recruited to the *FLO11* promoter through this complex and take part in *FLO11* activation. Additionally, Rpd3 and Hos2 might have an extra role in this process. However, the extra effect of Rpd3 would not matter for regulation of the flor promoter allele.

In this work, we have shown that Sfl1 repression is a major regulator of *FLO11* expression in the laboratory strain but has no effect on its expression in the flor strain. Interestingly, we have found that reduced *FLO11* expression in the laboratory strain caused by a *rpd3Δ* single mutant is restored when *SFL1* is deleted, suggesting that at least part of the activation effect of Rpd3 could be due to its counteracting Sfl1 repression. Accordingly, the lack of such a repression mechanism in the flor allele could be the reason why the deletion of *RPD3* did not affect *FLO11* expression in this case.

Rpd3 and Sfl1 have been described to have opposing roles in controlling *FLO11* expression via the regulation of the transcription of the *ICR1* ncRNA. Rpd3 activates *FLO11* expression by repression of *ICR1* ncRNA and Sfl1 has the

opposite role (Bumgarner *et al.* 2009, 2012). We have observed that this effect of Rpd3 is not shared by Hos2, suggesting that this effect may be the additional mechanism independent of Hos2 observed for Rpd3. Moreover, we show that the *ICR1* regulation seems to be lost in the 133d strain, further supporting this hypothesis.

To analyze the mechanism by which the histone deacetylase complex regulates *FLO11* transcription, we studied the levels of nucleosome occupancy as well as the degree of histone H4 acetylation along the promoter. Surprisingly, we did not observe a clear correlation between *FLO11* expression and nucleosome occupancy or H4 acetylation for the different mutants studied. This would suggest that the interplay of factors regulating *FLO11* expression leads to a more complex readout of promoter chromatin states than expected. Nonetheless, at this stage it is already very interesting that the deletion of *PHO23* had a stronger effect on nucleosome occupancy than was observed for the *rpd3Δ hos2Δ* double mutant. This suggests a novel function for the Rpd3L complex, where it can act to maintain a generally open state of *FLO11* promoter chromatin at least partly independently of Rpd3 and Hos2 histone deacetylases.

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Chromatin Modulation at the FLO11 Promoter of *Saccharomyces cerevisiae* by HDAC and Swi/Snf Complexes

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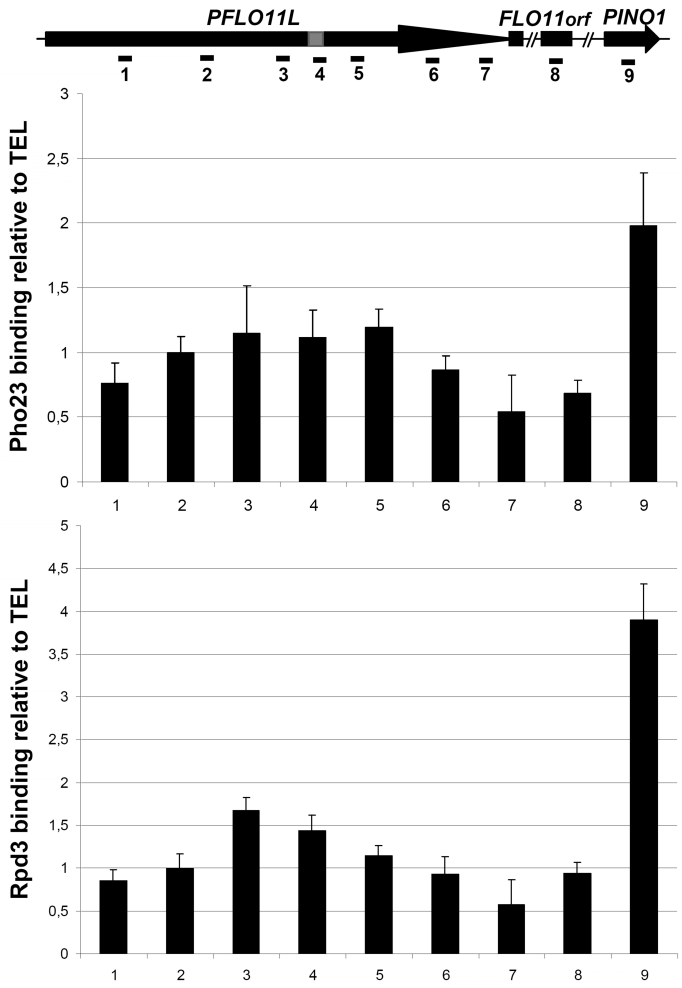

Figure S1 Pho23 and Rpd3 weakly bind the *FLO11* promoter. Pho23-13Myc or Rpd3-13Myc binding at *FLO11* promoter was measured by ChIP analysis and quantificated by qRT-PCR using amplicons over the promoter and open reading frame. Data are showed relative to a telomeric internal control (TEL). An amplicon of a region of the *INO1* promoter has been used as a positive control. Error bars represent the standard deviation of three biologically independent measurements. Diagrams at the top of the panels show amplicon positions. The gray box in the *FLO11* promoter corresponds to the 111 bp region that is deleted in the flor promoter allele.

Table S1 Primers used for qRT-PCR.

Primers	Sequence	Used for amplicon (Figure 3)
P*FLO11*ChIP5	ATGCCTTATAGCAACCAAGAAGCT	1
P*FLO11*ChIP6	TGGCGCTGTTCATTCCAAT	1
P*FLO11*ChIP15	TCCCCTAATGTATCCCTCATTTCA	2
P*FLO11*ChIP16	GCTCGGCTCTCGATGAGAAT	2
P*FLO11*ChIP1	TCCACCACATGAAACCTGCTACT	3
P*FLO11*ChIP2	AAATCTCACCCGTGGATCCTT	3
133dP*FLO11*u1	GATCTTTTCCTGGCTCCAATAGG	4
133dP*FLO11*u2	GCCCCAGAGAAGGACTAAAGACA	4
LP*FLO11*sfl1u1	AGTTTCTCGGAATGTGGCATTAC	4
LP*FLO11*sfl1u2	GGAAAATAACGGCCGAAACTCT	4
P*FLO11*FChIP17	AAAATTAGGCTTCACTGGTACGAGTT	5
P*FLO11*FChIP18	CTAAACGCTCGGACTGATTGC	5
P*FLO11*LChIP17	GAGTTTCGGCCGTTATTTTCC	5
P*FLO11*LChIP18	AGTCCATTCTTAGCCCCAAAGAA	5
P*FLO11*ChIP3	TTGCCTATCGGTGGTGTGATT	6
P*FLO11*ChIP4	GGCACTTTTAGGGTTGGGCAAT	6
P*FLO11*FChIP19	ATCTGAGGAATGTCCGTGTTCA	7
P*FLO11*FChIP20	AACCCTTCAACAACTTGTACTGGTAA	7
P*FLO11*LChIP19	TGAGGAATGTCCGTGTTCGA	7
P*FLO11*LChIP20	TGGGAACCCTTCAACAATTTGT	7
P*FLO11*orf1	CACCAGTAACTCCTGCCACTAATG	8
P*FLO11*orf2	TGCGCTTGCAGAACTTACGA	8
PIno1RT1	GAAATATGCGGAGGCCAAGTAT	9
PIno1RT2	TGCGCTTTCTCTGCTCCAT	9
TEL1aRT	GCGTAACAAAGCCATAATGCCTCC	for normalization
TEL1bRT	CTCGTTAGGATCACGTTCGAATCC	for normalization
FLO11RT1	CACCAGTAACTCCTGCCACTAATG	
FLO11RT2	TGCGCTTGCAGAACTTACGA	
SFL1RT1	CCGGCACATGTACCAAACTTT	
SFL1RT2	CCCGGTTTGTTGTTATTTTTCG	
ICR1RT1	TTGCCTATCGGTGGTGTGATT	
ICR1RT2	GGCACTTTTAGGGTTGGGCAAT	
SCR1RT1	AGCAAAGGTGACCCGTGATG	
SCR1RT2	CTGATGGCACCGCCAAAT	