Cell, Vol. 116, 405–415, February 6, 2004, Copyright ©2004 by Cell Press

Genetic and Epigenetic Regulation of the *FLO* Gene Family Generates Cell-Surface Variation in Yeast

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Summary

The *FLO* gene family of *Saccharomyces cerevisiae* includes an expressed gene, *FLO11*, and a set of silent, telomere-adjacent *FLO* genes. This gene family encodes cell-wall glycoproteins that regulate cell-cell and cell-surface adhesion. Epigenetic silencing of *FLO11* regulates a key developmental switch: when *FLO11* is expressed, diploid cells form pseudohyphal filaments; when *FLO11* is silent, the cells grow in yeast form. The epigenetic state of *FLO11* is heritable for many generations and regulated by the histone deacetylase (HDAC) Hda1p. The silent *FLO10* gene is activated by high-frequency loss-of-function mutations at either *IRA1* or *IRA2*. *FLO10* is regulated by the same transcription factors that control *FLO11*: Sfl1p and Flo8p, but is silenced by a distinct set of HDACs: Hst1p and Hst2p. These sources of epigenetic and genetic variation explain the observed heterogeneity of cell-surface protein expression within a population of cells derived from a single clone.

Introduction

Pathogenic microorganisms have evolved diverse mechanisms that generate phenotypic variation at the cell surface in response to the host environment. The repertoire for this phenotypic variation is often a family of genes encoding cell-surface proteins, each of which has diverged in sequence and function. A common motif among different microorganisms is that one gene family member is expressed and the other family members serve as a silent reservoir of variation. By switching surface expression from one family member to another, a pathogen can evade detection by the immune system or alter interactions with host tissues (a classic example are the *VSG* genes in *Trypanosome bruceii* (see Pays et al., 1994 for a review).

Fungi express several gene families encoding cell-surface glycoproteins that confer different adherence and immunogenic properties to the fungal cell wall. In pathogens such as *Candida albicans* (*ALS* genes) and *Candida glabrata* (*EPA* genes), the proteins encoded by these gene families are responsible for adherence to mammalian tissues (Cormack et al., 1999; De Las Penas et al., 2003; Hoyer, 2001). In *Pneumocystis carinii* (*MSG* genes), these genes encode the primary cell-surface antigen recognized by the host immune system (Stringer and Keely, 2001). In *Saccharomyces cerevisiae* (*FLO* genes), proteins encoded by these genes confer adherence to agar, solid surfaces and other yeast cells (Guo et al., 2000; Reynolds and Fink, 2001). In these examples, the protein encoded by each family member is capable of producing distinct cell-surface properties and serves as a resource for cell-surface variation. In addition, many of these fungal gene families are found near telomeres, a location that may play an important role in their regulation and evolution.

S. cerevisiae has five known members of the *FLO* gene family: *FLO1*, *5*, *9*, *10*, and *11*. *FLO1*, *5*, *9*, and *10* are adjacent to their respective telomeres (~10 to 40 kb from the telomeres), whereas *FLO11* is neither adjacent to a telomere nor a centromere. In the Σ1278b genetic background, *FLO11* is the only expressed member of this family (Guo et al., 2000); the telomere-proximal *FLO* genes are silent. *FLO11* expression is required for several important developmental transitions in yeast, including adhesion to agar and plastic surfaces (Reynolds and Fink, 2001), sliding motility (Reynolds and Fink, 2001), and pseudohyphal filament formation (Gagiano et al., 1999; Lo and Dranginis, 1998). When the silent *FLO* genes are expressed by a heterologous promoter, they confer adhesive phenotypes distinct from those produced by *FLO11*: Flo1p does not promote adherence to agar or plastic, but enhances cell-to-cell adherence that causes flocculation. Flo10p generates phenotypes that overlap those of both Flo1p and Flo11p; Flo10p promotes adhesion and pseudohyphal filamentation, but also enhances cell-to-cell adherence (Guo et al., 2000). Although these silent *FLO* genes provide a reservoir of cell-surface variation, the regulatory mechanisms that permit access to this silent information have been unexplored.

This study demonstrates that expression of the *FLO* genes, which regulate the cell-surface properties of yeast, is under both genetic and epigenetic control. Diploid yeast strains filament in response to nitrogen starvation, but this response is heterogeneous: some cells initiate the filamentation program, whereas other adjacent cells remain in the yeast form. This variation is the consequence of the metastable silencing of the *FLO11* gene; *FLO11* is expressed to produce filamentous cells, but silent in yeast form cells. Haploid strains do not filament, but mutate at a high frequency to express another telomere-linked *FLO* gene, *FLO10*, resulting in hyperinvasion and flocculence. Like *FLO11*, the expression of *FLO10* is metastably silenced. This epigenetic silencing of *FLO10* and *FLO11* integrates both promoter and genomic positional information to produce variegated expression.

Results

Epigenetic Silencing of *FLO11* Regulates Pseudohyphal Development

Flo11 protein expression was visualized by immunofluorescence using strains containing a fully functional

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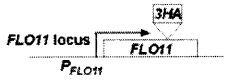
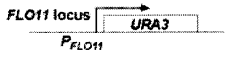
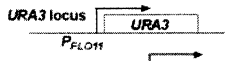
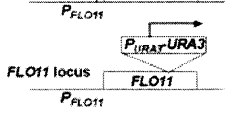
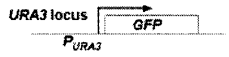
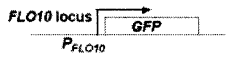
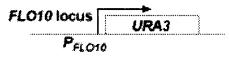
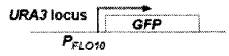
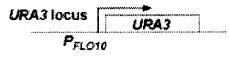
Table 1. Expression Constructs Used in This Study

Expression Construct		Silencing
FLO11::HA		+
P_{FLO11}-*URA3*		+
ura3::P_{FLO11}-*URA3*		-
flo11::URA3		-
P_{URA3}-*GFP*		-
P_{FLO10}-*GFP*		+
P_{FLO10}-*URA3*		+
flo10Δ::URA3		-
ura3::P_{FLO10}-*GFP*		-
ura3::P_{FLO10}-*URA3*		-

This table illustrates the expression constructs used to study epigenetic silencing at *FLO10*, *FLO11*, and *URA3* and summarizes the silencing effect observed for each construct.

Flo11p tagged with triple HA epitopes (Guo et al., 2000; Table 1). A clonal population of cells arising from a single haploid cell is not homogeneous: some cells express Flo11p, staining brightly with the HA-specific antibodies, whereas other cells from the same culture do not express Flo11p and fail to stain (Figure 1A).

To demonstrate whether heterogeneous Flo11p expression is due to transcriptional regulation of the *FLO11* gene, the chromosomal *FLO11* ORF was replaced with the *URA3* ORF to generate a P_{FLO11}-*URA3* allele (Table 1). With this allele, cells that have active transcription from the *FLO11* promoter will express the *URA3* gene and will be Ura$^+$ and 5-FOA sensitive, whereas cells that are either inactive at the *FLO11* promoter or have a silenced *FLO11* promoter will be Ura$^-$ and 5-FOA^R. A clonal population of cells bearing the chromosomal P_{FLO11}-*URA3* allele, when grown initially under nonselective conditions, contains some cells that are Ura$^+$ and others that are 5-FOA^R (Figure 1C). This heterogeneity agrees with the immunofluorescence analysis: a population derived from a single *FLO11* cell contains some cells that express the *FLO11* gene and others that do not.

To determine whether the state of *FLO11* expression is reversible, cells were isolated from colonies grown on 5-FOA, grown on YPD media overnight, and re-plated onto SC, SC-Ura, and SC+5-FOA media. If the Ura$^-$ colonies that grew up were silenced irreversibly (e.g., by mutation), then there should be very few Ura$^+$ colonies emerging from this population under nonselective growth. In contrast, Ura$^-$ cells selected on 5-FOA again generate both Ura$^+$ and Ura$^-$ cells at the same frequencies as cells not grown on 5-FOA (Figure 1C). The ability of Ura$^-$ cells selected on 5-FOA to generate both Ura$^-$ and Ura$^+$ cells shows that the transition between *FLO11* silenced (Ura$^-$) and *FLO11* desilenced (Ura$^+$) is reversible and switches back and forth frequently.

The metastable silencing of *FLO11* is similar to position-effect silencing described for subtelomeric transgenes (Gottschling et al., 1990). To determine whether *FLO11* silencing is dependent upon its genomic location, the P_{FLO11}-*URA3* allele was moved to the *URA3* locus, which is located ~12 kb from the centromere on the left arm of chromosome V (see Table 1). The *ura3::*P_{FLO11}-*URA3* strains fail to grow on media containing 5-FOA (Figure 1C), suggesting that the *FLO11* promoter is not silenced when positioned at the *URA3* locus. Therefore, silencing of *FLO11* is position dependent.

However, in contrast to telomere silencing, silencing of *FLO11* is promoter specific. When the *URA3* gene with its own promoter is placed at the *FLO11* locus, it is not silenced (*flo11::URA3*, Figure 1C), suggesting that factors that specifically recognize the *FLO11* promoter regulate silencing at this locus. A candidate for this promoter-specific factor is Sfl1p, which inhibits expression of *FLO11* (Pan and Heitman, 2002). Indeed, Sfl1p is required for silencing at the *FLO11* promoter (Figures 1B and 1C), suggesting that Sfl1p recognition may be a necessary step in the silencing of the *FLO11* gene.

Genome-wide studies have demonstrated that Hda1p participates in the deacetylation of large continuous subtelomeric regions of the yeast genome (Robyr et al., 2002). These regions extend much further away from the telomeres than Sir2p-mediated silencing effects and encompass a region that includes the *FLO11* gene. The *hda1*$^-$ P_{FLO11}-*URA3* strain fails to grow on media containing 5-FOA (Figure 1C), demonstrating that Hda1p is necessary for silencing *FLO11*. Since Hda1p deacetylation appears to be restricted to specific regions of the genome, the requirement for Hda1p might explain the position dependence of *FLO11* silencing; although the factors that localize Hda1p activity are still unknown. In addition, Hda1p is recruited to specific promoters by Tup1p (Wu et al., 2001), which in turn has been shown to be recruited to the *FLO11* promoter by Sfl1p (Conlan and Tzamarias, 2001). This suggests a pathway by which Sfl1p recruits Hda1p to silence *FLO11*.

Mutations that disrupt the function of the yeast Ku proteins, the Sir complex, or the Sir2p homologs, Hst1-4p, show no effect on *FLO11* silencing as measured by the activity of the P_{FLO11}-*URA3* allele (data for *sir2*$^-$ shown; Figure 1C). The failure of these genes involved in telomere silencing to affect *FLO11* expression is not entirely surprising because *FLO11* is quite far from its telomere (>40 kb).

The phenotypic consequences of *FLO11* switching are striking in diploid cells, where *FLO11* expression is required for the transition from yeast form cells to pseudohyphae. To visualize Flo11p expression in diploids during pseudohyphal development, diploid strains homozygous for the *FLO11::HA* allele were grown on media that induce pseudohyphal growth (see Experimental Procedures). The expression of Flo11p on the surface of nitrogen-starved diploid cells is variegated; some cells express Flo11p and some do not, confirming

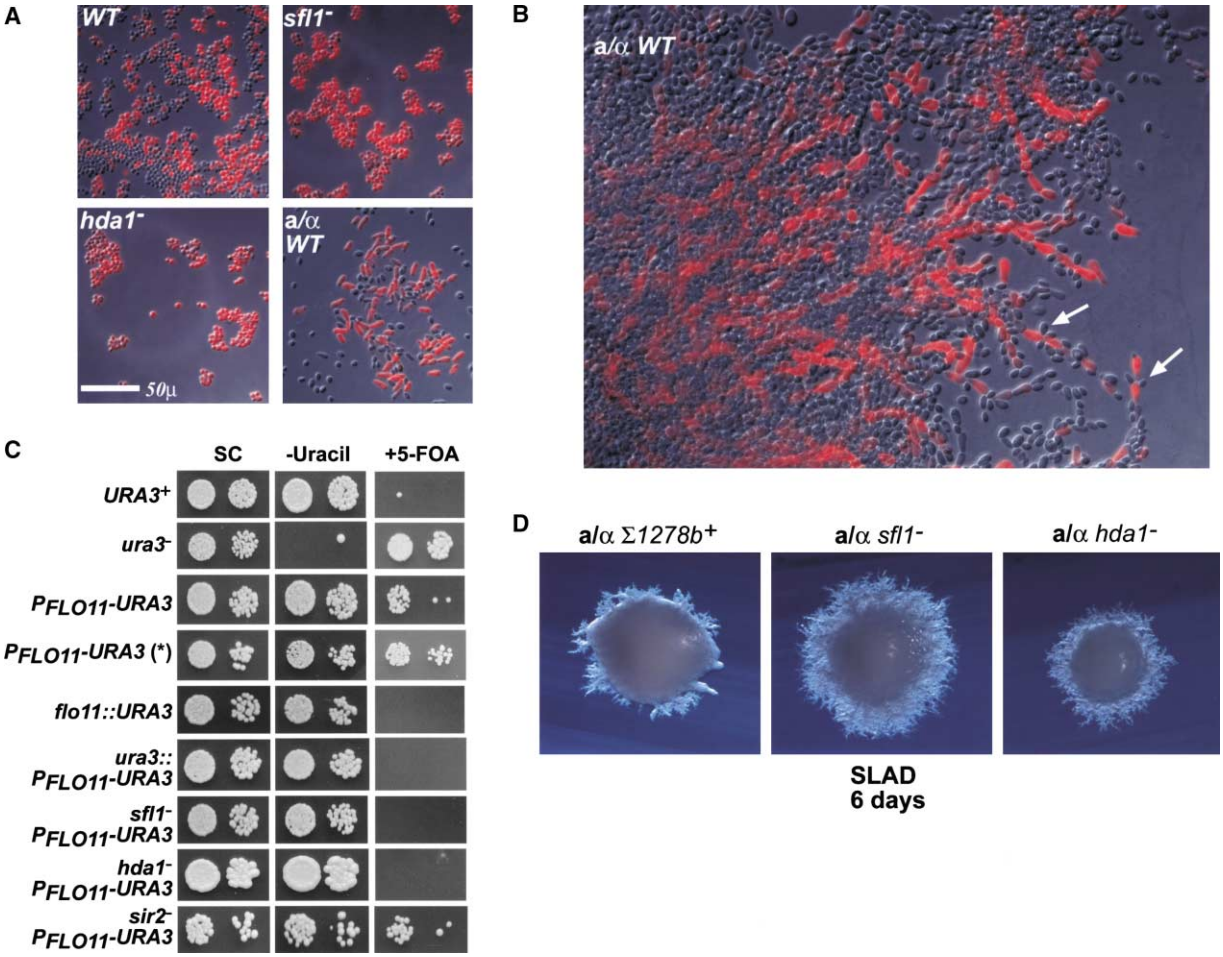


Figure 1. Epigenetic Control of *FLO11* Expression Regulates Pseudohyphal Differentiation

(A) Immunofluorescence analysis reveals variegated expression of Flo11p at the cell surface. All strains carry the *FLO11::HA* allele to visualize Flo11p expression on a cell-by-cell basis. Images show an overlay of HA-targeted immunofluorescence over a Nomarski image of the same field. Variegated expression is observed in a wild-type haploid strain, but is lost in strains that lack Sfl1p or Hda1p. Diploids (a/α) were grown on solid nitrogen starvation (SLAHD) media to induce pseudohyphal differentiation. Flo11p-HA expression in diploids is strongly correlated with the filamentous growth form ($p = 6.1x10^{-64}$).

(B) Cells isolated from filamentous diploid colonies allow the in situ visualization of filamentous and yeast form cells. The budding of yeast form cells off of a phalanx of filamentous cells (arrows) can be seen.

(C) The P_{FLO11}-*URA3* expression construct reveals the silenced (5-FOA^R) and desilenced (Ura$^+$) states of the *FLO11* promoter. Two equivalent 10-fold dilution spots are shown for each plate. P_{FLO11}-*URA3* (*) indicates that these cells were isolated from 5-FOA media, grown on YPD, then re-plated onto SC, -Uracil, and 5-FOA plates. See Table 1 for more detailed descriptions of the *URA3* expression constructs.

(D) Silencing of *FLO11* regulates the transition from yeast to filamentous growth. In Σ1278b strains, the initiation of filamentation at the colony periphery is heterogeneous, whereas in *FLO11*-desilenced *sfl1*$^-$ or *hda1*$^-$ mutants, the cells at the periphery of the colony are uniformly filamentous.

that *FLO11* is also metastably silenced under the conditions that stimulate pseudohyphal development (Figure 1A). The elongated, pseudohyphal cell types generally express Flo11p on their surface: 90.2% of filamentous cells (n = 174) versus 2.1% of yeast form cells (n = 188) express Flo11p, demonstrating that filamentation and Flo11p surface expression are tightly associated ($p = 6.1 \times 10^{-64}$).

The expression of Flo11p can be visualized in a phalanx of pseudohyphal cells if the colonies are treated gently prior to staining. As shown in Figure 1B, the cells that make up the intact filament express Flo11p, whereas the yeast form cells that surround the filament do not. Moreover, Flo11p-expressing cells can be seen to be dividing to produce yeast form cells that do not express Flo11p on their surface (Figure 1B, arrows). The proximity of these two forms, filamentous (*FLO11* on) and yeast (*FLO11* off), suggests that their differentiation is not the result of differing environmental inputs. Rather, it demonstrates that the epigenetic control of *FLO11* expression determines the differentiation of yeast and filamentous forms.

The strong correlation between the expression of Flo11p and pseudohyphal filamentation suggests that changes in silencing at *FLO11* should affect the pattern of pseudohyphal development. During diploid pseudohyphal filamentation, the developmental transition from yeast to pseudohyphal form does not occur in

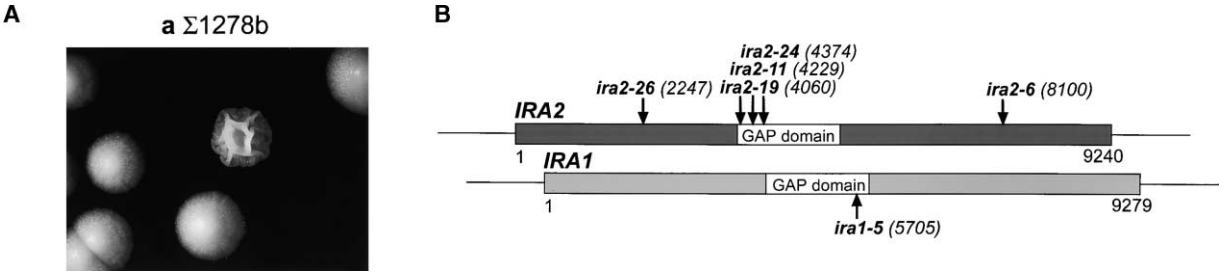


Figure 2. Spontaneous, High-Frequency Mutations at *IRA1* and *IRA2* Produce Wrinkled Colony Morphology Variants

(A) A switch in colony morphology is the result of high-frequency mutations at *IRA1* or *IRA2*. Wrinkled colonies were isolated from haploid strains at a frequency of about 1×10^{-3} (see Experimental Procedures for details of frequency analysis). Genetic analysis of these wrinkled colonies revealed that the phenotypic switch from smooth to wrinkled colony morphology is due to mutations in the *IRA1* and *IRA2* genes.

(B) Several *IRA1* and *IRA2* mutations were cloned and sequenced to identify the loss-of-function mutations in these alleles. The location of each of the polymorphisms in both the *IRA1* and *IRA2* genes are shown. The consensus GTPase-activating domain (Tanaka et al., 1990) is shown for each gene. 4/6 (*ira1-5*, *ira2-19*, *ira2-24*, *ira2-26*) of the mutations sequenced are frameshift mutations, whereas *ira2-6* and *ira2-11* are transversions.

concert among all the cells at the periphery of the colony. Some cells initiate pseudohyphal filaments, while adjacent cells continue to divide in a yeast form pattern (Figure 1D). In contrast, desilenced homozygous *sfl1*$^-$ or *hda1*$^-$ diploid colonies both produce an increased level of pseudohyphal filamentation (Figure 1D). This analysis of cell-by-cell distribution of Flo11p expression helps clarify previous reports of the enhancement of pseudohyphal filamentation in *sfl1*$^-$ mutants (Robertson and Fink, 1998). Loss of silencing produces an altered distribution of *FLO11* expression: from a heterogeneous expression in wild-type colonies to a constitutive expression in homozygous *sfl1*$^-$ or *hda1*$^-$ colonies. Desilenced cells can then participate in pseudohyphal filamentation leading to the hyperfilamentous phenotype observed in *sfl1*$^-$ or *hda1*$^-$ homozygotes. Therefore, metastable silencing at the *FLO11* locus regulates the transition to pseudohyphal development in response to nitrogen starvation.

Phenotypic Switching in *S. cerevisiae* Results from Mutations in *IRA1* or *IRA2*

In addition to the epigenetic variegation of *FLO11* gene expression, *S. cerevisiae* is capable of altering its cell-surface properties via mutations that activate the expression of other *FLO* gene family members. These mutants appear as wrinkled colony morphology variants that have increased adhesion to the agar. Variants that have stably switched to a wrinkled colony morphology (Figure 2A) appear at a frequency of 1.1×10^{-3} (see Experimental Procedures for details). The wrinkled colony morphology phenotype results from a single mutation in one of two complementation groups.

Complementation analysis of independently arising wrinkled isolates ($n = 96$) demonstrated that these wrinkled variants result from loss-of-function mutations in either *IRA1* or *IRA2*, the yeast Ras GTPase-activating proteins. The wrinkled *ira1*$^-$ and *ira2*$^-$ mutants are phenotypically indistinguishable and occur at similar frequencies. One *ira1*$^-$ and five *ira2*$^-$ alleles from wrinkled variants were cloned (see Supplemental Data at http://www.cell.com/cgi/content/full116/3/405/DC1) and sequenced (Figure 2B). Each of the cloned mutations results from a different nucleotide change within the *IRA1* or *IRA2* ORFs. The *ira1-5* allele contains an insertion of a T-A base pair at position 5705 bp of the *IRA1* ORF, among a stretch of ten A-T and T-A base pairs. Several frameshift mutations were also found among the *ira2*$^-$ alleles. The *ira2-19*, *ira2-24*, and *ira2-26* alleles are all frameshift mutations resulting from a deletion or insertion of an A-T or T-A base pair within stretches of 8–14 successive A-T and T-A base pairs. The other two *ira2*$^-$ mutations we sequenced, *ira2-6* and *ira2-11*, are transversions (C to G and C to A respectively). Therefore, the colony morphology variation observed in *S. cerevisiae* is a high-frequency genetic event, which can be traced to sequence polymorphisms in either the *IRA1* or *IRA2* genes.

Altered Cell-Surface Properties Result from *FLO10* Activation

Ira$^-$ mutants display phenotypes consistent with the activation of additional *FLO* gene family members. Ira$^+$ strains require *FLO11* for haploid-invasive growth, whereas Ira$^-$ strains do not. As shown in Figure 3A, a *FLO11*$^+$ strain remains attached to the surface of the agar, whereas a *flo11*$^-$ strain washes off easily. However, the isolated Ira$^-$ mutants no longer require *FLO11* for adhesion. An *ira2*$^-$ *flo11*$^-$ strain is significantly more adherent than an *IRA2*$^+$ *flo11*$^-$ strain (similar results are observed with *ira1*$^-$ mutants). This Flo11p-independent haploid adhesion of Ira$^-$ strains depends upon the normally inactive *FLO10* gene, as *ira2*$^-$ *flo11*$^-$ *flo10*$^-$ mutants do not adhere to agar (Figure 3A). In a higher stringency assay for adhesion (Figure 3A, scrubbed), the *ira2*$^-$ *flo11*$^-$ strains are even more adherent than the *FLO11*$^+$ strain.

The wrinkled Ira$^-$ mutants also have a significant flocculation phenotype (cells adhere and aggregate into large clumps that sediment much more quickly than dispersed cells; Figure 3B). This flocculation in liquid media is also dependent upon *FLO10* gene activity as loss of *FLO10* function leads to a loss of flocculation in the Ira$^-$ mutants (Figure 3B).

Additionally, the strikingly wrinkled surface of these Ira$^-$ colonies is dependent upon the expression of the Flo11p cell-surface adhesin (Figure 3C). This wrinkled surface morphology is likely due to increased *FLO11*

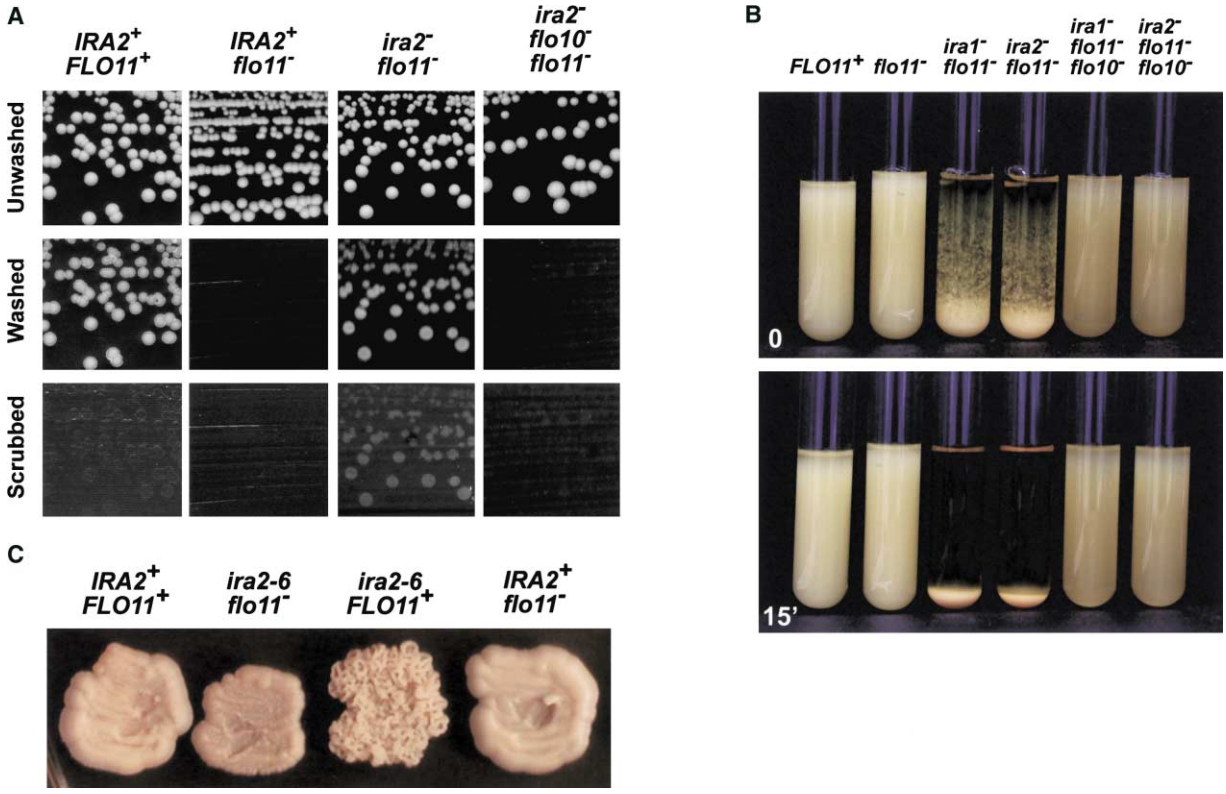


Figure 3. Ira⁻ Phenotypes Require *FLO* Gene Activity

(A) Flo11p-independent agar adhesion in Ira⁻ strains requires the activity of the *FLO10* gene.

(B) Flocculation of Ira⁻ mutants requires *FLO10* gene activity. The top panel shows liquid cultures immediately after resuspension by vortexing. The bottom panel shows the same cultures after 15 min.

(C) The wrinkled colony morphology phenotype of Ira⁻ mutants is dependent upon an intact *FLO11* gene.

gene expression as mutations that upregulate *FLO11* (*sfl1*⁻ mutants show a similar wrinkled morphology), or *GAL1* promoter-driven expression of the *FLO11* gene (data not shown), also manifest the wrinkled colony morphology phenotype.

These results suggest that altered expression of *FLO10* and *FLO11* plays a role in several of the phenotypes produced in wrinkled Ira⁻ mutants. To determine whether the expression of either of these genes is altered in variant strains, total RNA was isolated from a smooth Ira⁺ strain and two rough Ira⁻ variants (*ira1-12* and *ira2-6*). Northern analysis of these RNA samples (Figure 4) demonstrates that Ira⁻ mutants have higher levels of *FLO11* (2-fold) and *FLO10* (12-fold) message. Activation of *FLO10* and *FLO11* expression in Ira⁻ strains is dependent upon Ras2p and the filamentation MAP kinase and PKA pathways (Rupp et al., 1999 for *FLO11* and Supplemental Figure S1 on *Cell* website for *FLO10*). Messages for the other known adhesins, *FLO1*, *5*, and *9*, could not be detected by Northern analysis in either Ira⁺ or Ira⁻ strains. Therefore, mutations at the *IRA* loci result in a phenotypic switch in colony morphology and altered cell-surface adhesion. Several of these new traits are dependent on transcriptional activation of *FLO10*.

Epigenetic Regulation of *FLO10*

To determine whether the epigenetic silencing effects observed at *FLO11* extend to other *FLO* gene family members, a *TRP1*-tagged *Aequorea victoria GFP* gene (Heim et al., 1994) was inserted into the *FLO10* locus, such that *GFP* gene expression would be regulated by the *FLO10* promoter (see Table 1). As a control, the fluorescence generated by GFP expression was analyzed when transcription of the *GFP* gene is controlled by the endogenous *URA3* promoter on chromosome V (P_{URA3}-*GFP*). The expression of GFP in the *URA3* promoter-regulated construct is homogeneous (Figure 5A).

In contrast, *GFP* regulated by the endogenous *FLO10* promoter (P_{FLO10}-*GFP*) has a very different pattern of expression. In Ira⁺ strains, the *FLO10* promoter is inactive and no *GFP* expression is observed. In *ira1*⁻ strains (or *ira2*⁻ strains; data not shown), *GFP* expression driven by the *FLO10* promoter is variegated. Some cells have a high level of *GFP* expression, whereas other cells have no evident GFP fluorescence (Figure 5A).

To dissect the *FLO10* silenced and desilenced states, we replaced the endogenous *FLO10* ORF with the *URA3* ORF (Table 1). The phenotype of *ira1*⁻ P_{FLO10}-*URA3* strains is both Ura⁺ and 5-FOA^R (Figure 5B). This result is consistent with the previous observation that *GFP* expression is variegated when driven by the *FLO10* promoter.

To determine whether the silenced state of *FLO10* is reversible, cells bearing the P_{FLO10}-*URA3* allele were isolated from 5-FOA grown colonies, grown on YPD media overnight, and re-plated onto SC, SC-Ura, and SC+5-FOA media. The frequency of Ura⁺ colonies is

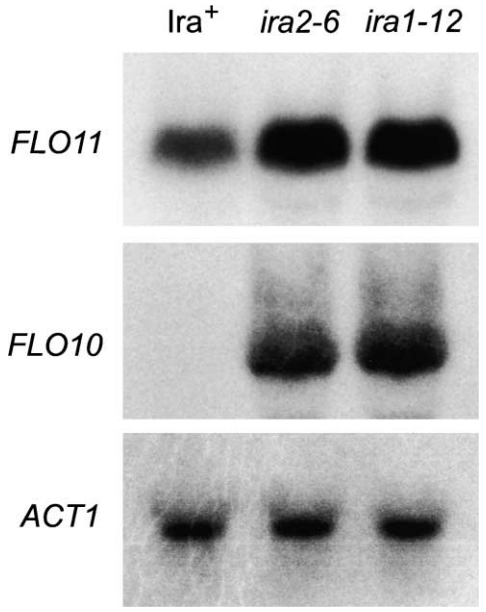


Figure 4. Northern Analysis of Ira⁻ Mutants Reveals Increased Levels of *FLO11* and *FLO10* Transcripts

Total RNA was prepared from harvested cells by using a hot acid phenol protocol. Fifteen micrograms total RNA/sample was separated on a denaturing formaldehyde gel. Northern blotting was performed as described (Sambrook et al., 1989). Ira⁻ mutants display a 2- to 5-fold increase in *FLO11* transcript and a 10- to 20-fold increase in *FLO10* transcript. Oligonucleotides used to generate *ACT1*, *FLO10*, and *FLO11* probes are described in Supplemental Table S2 on the *Cell* website.

roughly equivalent to that observed in strains that have not been grown on 5-FOA (Figure 5B), suggesting that silencing at *FLO10*, like at *FLO11*, is metastable and switches back and forth frequently.

To establish whether the silencing at *FLO10* requires sequences specific to the *FLO10* promoter, a *flo10*::*URA3* allele was constructed. This allele is similar to the P_{FLO10}-*URA3* allele used earlier, except that in this second strain, *URA3* is regulated by its own promoter (Table 1) and located at the *FLO10* locus on chromosome XI. An *ira1*⁻ *flo10*::*URA3* strain does not silence the *URA3* gene. It grows on SC-Ura media but fails to grow on 5-FOA media (Figure 5B). Therefore, the silencing observed at the *FLO10* locus is likely to require factors that specifically recognize and associate with the *FLO10* promoter.

Sfl1p is a likely candidate for directing silencing to the *FLO10* promoter as Sfl1p represses transcription at both *FLO11* (Pan and Heitman, 2002; Robertson and Fink, 1998) and *FLO10* (Supplemental Figure S1 on *Cell* website) and is also required for silencing at *FLO11* (Figure 1C). An *sfl1*⁻ mutant fails to silence the P_{FLO10}-*URA3* allele (Figure 5B), suggesting that Sfl1p recognition of the *FLO10* promoter may be important for the promoter specificity of silencing at *FLO10*.

To determine whether the epigenetic silencing of the *FLO10* gene is position dependent, we constructed strains in which either the P_{FLO10}-*GFP* or the P_{FLO10}-*URA3* allele was moved to the *URA3* locus (Table 1). Neither of these alleles is silenced at the *URA3* locus (Figures 5A and 5B). Therefore, silencing of the *FLO10* promoter is dependent on its genomic positioning. Since *FLO10* silencing is dependent on its subtelomeric location (*FLO10* is only ~17 kb from a telomere, much closer than *FLO11*), strains containing deletions of several genes required for telomere silencing were constructed and tested for silencing of the *FLO10* promoter. Sir3p and Sir4p associate with Sir2p at the telomeres and silent mating loci and is required for silencing at these sites (Haber and George, 1979; Moazed et al., 1997; Moretti et al., 1994; Rine and Herskowitz, 1987; Rine et al., 1979). Deletion of the *SIR3* gene also disrupts this complex and also disrupts silencing at the *FLO10* locus in Ira⁻ cells (Figure 5B). A similar result is observed in Ira⁻ cells carrying deletions of the genes *YKU70* or *YKU80* (data not shown; similar to *sir3*⁻ mutants). These genes encode homologs of the mammalian Ku proteins and are required for telomere silencing, but not silencing at *HML* or *HMR* (Mishra and Shore, 1999). Since the Ku and Sir proteins are involved in regulating regional silencing effects, the position dependence of the silencing at the *FLO10* promoter is likely to be mediated by these proteins.

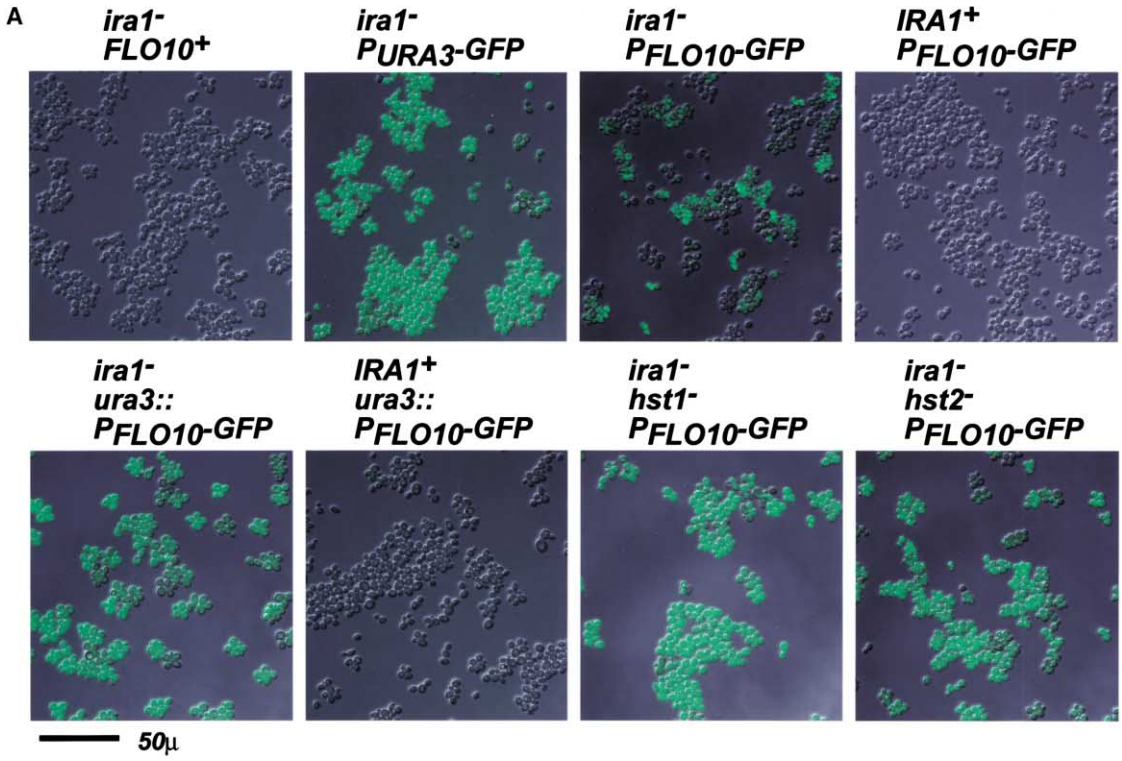
Silencing of *FLO10* Requires Hst1p and Hst2p, which Associate with the *FLO10* Promoter

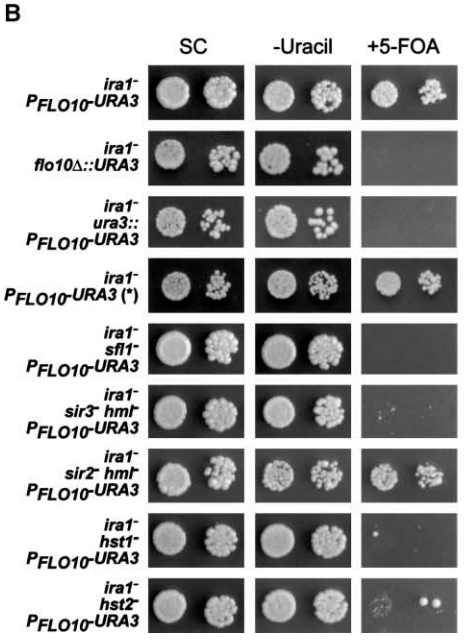
The pattern of variegated expression observed for *FLO10* is similar to the patterns of expression for ectopic genes regulated by telomere silencing (Gottschling et al., 1990). Data from localization experiments (Kennedy et al., 1997; Martin et al., 1999), expression of ectopic promoters (Gottschling et al., 1990), and genome-wide expression analysis (Wyrick et al., 1999) suggest that telomere silencing requires the activity of the NAD^+-dependent histone deacetylase protein, Sir2p. The P_{FLO10}-*URA3* allele is still effectively silenced Ira⁻ *sir2*⁻ strains (Figure 5B), demonstrating that Sir2p does not play a role in silencing *FLO10*.

To determine whether any of the other Sir2p homologs, Hst1p-Hst4p (Brachmann et al., 1995), play a role in *FLO10* silencing, Ira⁻ mutants lacking each of the *HST* genes were assayed for silencing of the P_{FLO10}-*URA3* allele. Both *HST1* and *HST2* are necessary for silencing at the *FLO10* promoter (Figures 5A and 5B), whereas deletions of either *HST3* or *HST4* have no effect on silencing at *FLO10*.

Chromatin immunoprecipitations of *MYC* epitope-tagged alleles of *HST1* and *HST2* were analyzed by PCR probes to determine whether Hst1/2p silencing of *FLO10* is through interaction of these HDAC proteins with the *FLO10* promoter. A region of the *FLO10* promoter (−900 to −1175) is enriched in the immunoprecipitate fraction of Myc-tagged Hst1p and Hst2p strains over untagged strains (Figure 5C). Different regions of the *FLO10* promoter (−2050 to −1800 and −550 to −300) as well as a probe to the *URA3* promoter show little enrichment in the immunoprecipitate of tagged fractions over untagged fractions (Figure 5C).

To test whether Sfl1p is necessary for recruitment of the Hst1/2p HDACs to the *FLO10* promoter, *sfl1*⁻ strains were analyzed for association of both Hst1p and Hst2p with the *FLO10* promoter. Loss of Sfl1p function leads to a disassociation of both Hst1p and Hst2p with the Hst1/2p-associated region described above. Immuno-





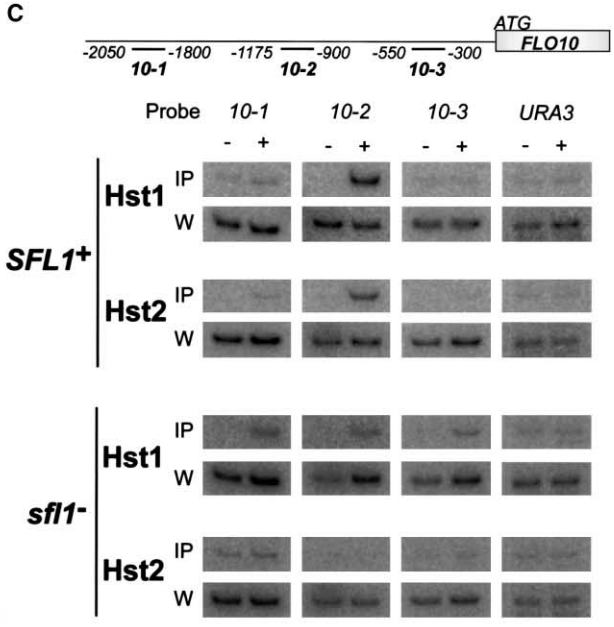


Figure 5. Telomere Position Effects at *FLO10* Are Mediated by Hst1p and Hst2p Association

(A) *FLO10* gene expression shows position-effect variegation. The *FLO10* ORF was replaced with a *TRP1*-tagged *GFP* gene such that *GFP* expression was regulated by the genomic *FLO10* promoter (P_{FLO10}-*GFP*, see Table 1 for expression constructs). As a control, a similar construct was generated at the *URA3* locus, such that the *URA3* promoter was responsible for *GFP* expression (P_{URA3}-*GFP*).

(B) The P_{FLO10}-*URA3* expression construct reveals the silenced (5-FOA^R) and desilenced (Ura$^+$) states of the *FLO10* promoter. Two equivalent 10-fold dilution spots are shown for each plate. The *ira1*$^-$ P_{FLO10}-*URA3* (*) cells were initially selected on 5-FOA and grown on YPD before plating on SC, -Uracil, and 5-FOA media. As is observed at *FLO11*, silencing of the *FLO10* promoter produces both Ura$^+$ and 5-FOA^R populations of cells.

(C) Chromatin immunoprecipitation demonstrates Sfl1p-dependent association of Myc-tagged Hst1p and Hst2p with the *FLO10* promoter. *SFL1*$^+$ or *sfl1*$^-$ strains carrying either MYC-tagged *HST1* or *HST2* alleles (*HST1* and *HST2*; + lanes) or untagged alleles (− lanes) were analyzed by chromatin immunoprecipitation. Quantitative PCR reactions from immunoprecipitate (IP) fractions were compared to those from whole-cell extract (W) to determine the levels of enrichment. Precipitation of fixed whole-cell extract samples with anti-Myc antibodies produced an enrichment of a fragment of the *FLO10* promoter (bases −900 to −1175) in both Myc-tagged Hst1p (43.19-fold) and Hst2p (6.93-fold) strains. In *sfl1*$^-$ strains, IP enrichment of this fragment is substantially decreased for both Myc-tagged Hst1p (15.2-fold less enrichment in an *sfl1*$^-$ strain) and Hst2p (5.2-fold less enrichment in an *sfl1*$^-$ strain), demonstrating that Sfl1p is important for the association of these HDAC proteins with the *FLO10* promoter.

precipitates of 9Myc-tagged Hst2p show no enrichment for the Hst1/2-associated region over untagged immunoprecipitates in *sfl1*$^-$ strains (Figure 5C). This suggests that Sfl1p regulates two distinct pathways that converge upon the *FLO10* promoter: (1) Sfl1p represses *FLO10* gene expression through inhibition of the Flo8p transcriptional activator (Pan and Heitman, 2002 and Supplemental Figure S1 on *Cell* website) and (2) Sfl1p recruits the HDAC proteins Hst1p and Hst2p to the *FLO10* promoter to silence *FLO10* gene expression in a subpopulation of Ira$^-$ cells (Figure 5C).

There is no observable *FLO10* expression in Ira$^+$ silencing mutants (*sir3*, *yku70*, *yku80*, *hst1*, and *hst2*; Supplemental Figure S2), supporting a model in which the silencing of the *FLO10* promoter by Hst1/2p is a separate pathway from the PKA-dependent activation of *FLO10* in Ira$^-$ cells (Supplemental Figure S1). Since coimmunoprecipitation of Hst1p and Hst2p with the *FLO10* promoter is observed in Ira$^+$ cells (Figure 5C), this silencing potential may be present at the *FLO10* locus even when it is inactive.

Discussion

The experiments described here demonstrate that rapid variation in *S. cerevisiae* cell-wall glycoproteins results from both epigenetic and genetic regulation of the *FLO* gene family. Although there are five members of this family in *S. cerevisiae*, *FLO11* is the only member that is expressed in the Σ1278b background (Guo et al., 2000). *FLO11* gene expression is required for key developmental transitions in yeast, including adhesion to agar and plastic surfaces, sliding motility, and pseudohyphal filament formation (Gagiano et al., 1999; Lo and Dranginis, 1998; Reynolds and Fink, 2001).

Genetic and immunofluorescent analyses (Figure 1) demonstrate that epigenetic silencing determines whether cells express the cell-surface protein Flo11p and transition to a filamentous developmental form in the presence of the appropriate environmental signal. There are several remarkable features of silencing at *FLO11*. First, silencing of *FLO11* is a binary switch, where *FLO11* expression (silenced = OFF, desilenced = ON) determines the developmental outcome for individual cells. The state of this switch is inherited for several generations; the phalanges of filamentous cells seen in Figure 1B record lineages of more than ten generations in which *FLO11* remains in the expressed state. Second, silencing at *FLO11* is both promoter specific and position dependent. These considerations suggest that both global (gene nonspecific) and promoter-specific factors contribute to the establishment of *FLO11* silencing (Figure 6). The dual nature of silencing at *FLO11* contrasts with most previously described positional silencing effects, which are defined by their promoter-independent ability to silence transcriptional activity. Finally, silencing at the *FLO11* gene (~46 kb from the end of chromosome IX) reveals the presence of telomere-independent positional silencing effects within the yeast genome. Telomere-independent silencing has also been described for the rDNA locus (Smith and Boeke, 1997) and for the *HIS4* locus (Jiang and Stillman, 1996), suggesting that metastable silencing of genes may be more widespread than had been previously imagined.

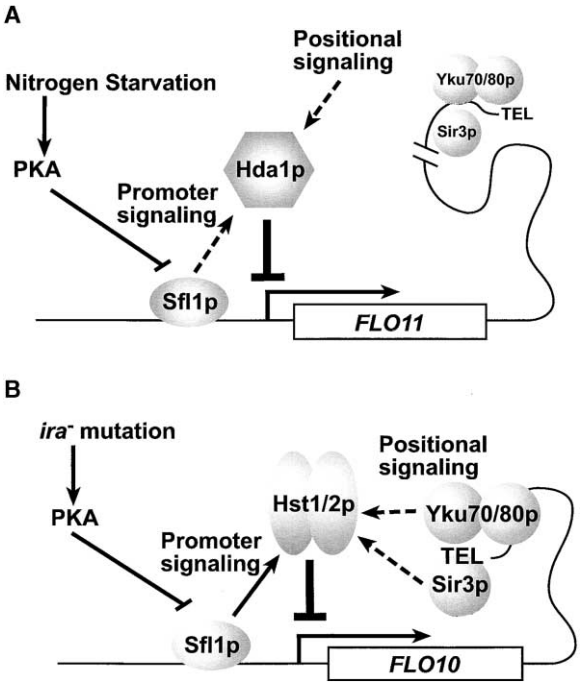


Figure 6. Silencing at *FLO10* and *FLO11* Integrates both Promoter-Specific and Genomic Positional Information

(A) Hda1p regulates telomere-independent positional silencing of *FLO11*. Sfl1p, which binds the *FLO11* promoter, is required for epigenetic silencing of the *FLO11* gene (Figure 1) and likely provides the promoter specificity to *FLO11* silencing. Although it is clear that genomic position regulates *FLO11* silencing (Figure 1C), the nature of the positional determinants remains unclear. Silencing of *FLO11* also requires the Hda1p histone deacetylase (Figure 1), which is likely to integrate both positional and promoter-specific (Sfl1p) information.

(B) Silencing of *FLO10* is dependent upon the Hst1p and Hst2p HDACs. These HDAC proteins integrate promoter and positional information through interactions with Sfl1p and the telomere-associated Ku and Sir proteins.

The epigenetic silencing of *FLO11* explains the variation in filamentation within a genetically homogeneous colony of yeast. When *S. cerevisiae* colonies are starved for nitrogen (Figures 1B and 1D), they produce pseudohyphal filaments (Gimeno et al., 1992). However, only a subset of cells participate in forming filaments. *FLO11* is expressed in the filamentous cells and silent in the nonfilamentous cells. This switch is controlled by Hda1p and Sfl1p; diploid strains lacking either Sfl1p or Hda1p show constitutive filamentation along the edge of the colonies (Figure 1D). Therefore, silencing regulates the differentiation of cell types: Cells expressing Flo11p at their surface develop as pseudohyphae, whereas Hda1p-silenced cells do not express Flo11p and remain in the yeast form.

The ability of genetically identical cells to switch between these two morphologically distinct forms could be important for survival. In the human fungal pathogen *C. albicans*, any mutation that locks the cells in either a yeast form or a hyphal form (Braun and Johnson, 1997; Lo et al., 1997), or prevents the transition from yeast to filamentous forms during infection (Saville et al., 2003), severely reduces the pathogenicity of this fungus. The

requirement for both yeast and hyphal forms during *C. albicans* infection suggests that there is a division of labor; the filamentous forms may represent the foraging form of the fungus, allowing it to find more favorable environments, whereas the yeast form is the colonizing form, growing more effectively under conditions where nutrients are available. Metastable regulation of yeast-pseudohyphal differentiation by silencing allows a clonal population of *S. cerevisiae* to test both of these different phenotypes without committing all the cells in a colony to one developmental form or another.

Saccharomyces can produce additional cell-surface variation when other members of the *FLO* gene family are expressed. The other Flo proteins (Flo1p, Flo5p, Flo9p, and Flo10p) are capable of producing adhesive phenotypes distinct from those of Flo11p, yet only Flo11p is normally expressed. This situation in yeast is analogous to what has been observed in several other microorganisms (De Las Penas et al., 2003; Howell-Adams and Seifert, 2000; Mehr and Seifert, 1998; Stringer and Keely, 2001; Weiden et al., 1991), where each member of a gene family produces distinct cell-surface properties, yet only a limited number are expressed.

Numerous mechanisms, including gene conversion (Bernards et al., 1981; Pays et al., 1983a, 1983b), recombination (Howell-Adams and Seifert, 2000; Mehr and Seifert, 1998), *trans*-splicing (Stringer and Keely, 2001), and epigenetic regulation (De Las Penas et al., 2003; Michels et al., 1984; Rudenko et al., 1995) are utilized by these microorganisms to produce a switch in surface protein expression. In *S. cerevisiae*, a mutational mechanism governs access to the reservoir of cell-surface variation provided by the *FLO* genes (Figure 2). Surface expression of additional *FLO* genes is accomplished through mutation of the *IRA1* or *IRA2* genes (Figure 4). These loci are genetically unstable, producing loss of function *ira*$^-$ mutations at a high ($\sim 10^{-3}$) frequency. The source of this high mutation frequency is unclear, although it is evident that this genetic instability is not shared across the rest of the genome. Mutations in the *CAN1* gene appear at a much lower frequency ($\sim 10^{-6}$; see Experimental Procedures). The *ira1*$^-$ and *ira2*$^-$ mutants have novel adhesive phenotypes, many of which result from the transcriptional activation of the *FLO10* gene (Figures 3 and 4). This transcriptional activation is dependent upon both the MAP kinase and cAMP-regulated PKA pathways (Supplemental Figure S1 on *Cell* website) and is likely due to increased Ras activity in these strains (Russell et al., 1993).

In Ira$^-$ cells, the expression of *FLO10* is variegated, suggesting that *FLO10*, like *FLO11*, is regulated by metastable epigenetic silencing (Figure 6B). Although it is unclear if the telomere-associated Sir3p and Ku proteins act through Hst1p and/or Hst2p or through some independent mechanism at the *FLO10* locus, the genetic analysis demonstrates that both promoter-specific (Sfl1p) and position-dependent (Sir3p and Ku proteins) information is integrated in the silencing of *FLO10*.

As each *FLO* gene is capable of producing distinct adhesive phenotypes, access to the silent *FLO* genes permits yeast to display different cell-surface properties. This situation is analogous to VSG expression in trypanosomes (Pays et al., 1994) or the MSG switching in *P. carinii* (Stringer and Keely, 2001), where the transcriptional regulation of gene family members is a mechanism to generate diversity. In these examples, silent genes located near the telomeres provide a repository of information that, when accessed, can produce phenotypic variation. Interestingly, many of the genes found near the telomeres in yeast are members of multigene families (e.g., the *SUC*, *MAL*, *MEL*, *PAU*, and *HXT* gene families). It has been proposed (Otto and Yong, 2002) that novel gene functions can arise through the process of gene duplication and divergence. With this in mind, it is interesting that many of the genes found at metastably silenced loci, such as the yeast telomeres, are members of larger gene families. Perhaps these regions are favored for the evolution of new gene activities.

Experimental Procedures

Strains, Media, Microbiological Techniques, and Growth Conditions

Yeast strains are listed in Supplemental Table S1. For yeast strain construction, see Supplemental Data online. All strains are derived from Σ1278b (also known as MB1000; Brandriss and Magasanik, 1979; Grenson et al., 1966) and MB758-5B (Siddiqui and Brandriss, 1988). Standard yeast media, yeast transformations, and genetic manipulations were performed as described in Rose et al. (1990). To induce pseudohyphal differentiation, strains were grown on nitrogen-poor SLAHD media, which was prepared as described in Gimeno et al. (1992).

Indirect Immunofluorescence

Indirect immunofluorescent staining of HA-tagged Flo11p was performed as described in Guo et al. (2000). Briefly, cells were carefully isolated from colonies on plates. For diploid strains isolated off of SLAD plates, an effort was made to dig the majority of the colony out of the agar with a needle. Cells were fixed, washed, and stained with anti-HA antibodies (HA11; BAbCo) and Cy3-conjugated secondary antibodies (Goat Anti-Mouse IgG; Jackson ImmunoResearch), taking care to disturb the cells minimally at each step (cells were centrifuged at low speeds and resuspended gently with P1000 pipetting). Despite our efforts, many of the cells became dispersed (see Figure 1A: a/α *WT*), but in several instances, large clusters of cells were preserved (see Figure 1B), allowing the in situ observation of variegated Flo11p expression.

Isolation and Analysis of Spontaneous Ira$^-$ Mutants

Colonies were grown on YPD plates for 14 days. Cells were harvested from colonies, diluted in sterile water, and plated onto YPD plates at a density of between 300–500 colonies/plate. Wrinkled colony morphology variants (Figure 2A) could be observed at a frequency of roughly 1/1000. A cross of these wrinkled variants to a smooth strain demonstrated that the wrinkled phenotype (as well as the other phenotypes described in Figure 3) were linked to a single locus, segregating in a 2:2 pattern among tetrad ascospores. Complementation analysis among wrinkled mutants demonstrated that these spontaneous mutations segregated into two complementation groups. In a transposon library screen (Kumar et al., 2002), a transposon insertion in the *IRA2* gene was isolated, which recapitulated the phenotypes observed in the wrinkled variants. The isolated wrinkled variants fail to complement loss-of-function *ira1*$^-$ or *ira2*$^-$ mutations, suggesting that each wrinkled variant resulted from a mutation in either *ira1*$^-$ or *ira2*$^-$. Ira$^-$ mutants can also be easily identified by an iodine vapor staining assay. For iodine staining, about 30 grams of iodine crystals (Sigma) were placed evenly over the bottom of a glass dish. Plates with colonies to be tested were inverted over the iodine crystals for 2–5 min. Ira$^+$ strains stain bright red from exposure to iodine vapors, whereas *ira1*$^-$ and *ira2*$^-$ mutants stain yellow.

Iodine staining was used to identify Ira$^-$ mutants for frequency analysis. Eight independent colonies derived from single cells were grown on YPD plates for 14 days and harvested as described earlier

for identifying wrinkled variants. 10,000–20,000 viable cells were plated from each colony at a density of 300–500 colonies/plate, grown for three days, and assayed with iodine vapors to identify *ira*$^-$ mutants. The median frequency of mutations producing an Ira$^-$ phenotype was 1.08×10^{-3}, with a 95% confidence interval between 7.3×10^{-4} and 1.4×10^{-3}. The same colonies were assayed for mutations in the *CAN1* gene, which could be identified as canavanine resistant colonies. 1.0–2.0 $\times 10^7$ cells were assayed for canavanine resistance from each colony. The median frequency of *can1*$^-$ mutation was 1.54×10^{-6}, with a 95% confidence interval between 3.1×10^{-7} and 3.2×10^{-6}. Therefore the frequency of mutations at the *IRA* loci is roughly 1000-fold higher than the frequency seen at the *CAN1* locus.

Complementation Analysis

To determine if putative *ira*$^-$ mutants carried mutations in the *IRA1* or *IRA2* gene, complementation tests were performed using strains carrying *kan*^{r}-tagged deletion alleles of *IRA1* and *IRA2*. Loss-of-function mutations in these genes are recessive for all the phenotypes we have identified. Unknown *ira*$^-$ mutants were crossed to *ira1*$^-$ and *ira2*$^-$ strains and assayed for complementation of the *ira*$^-$ iodine staining phenotype. Complementation analysis of 96 putative *ira*$^-$ mutants demonstrated that all 96 were indeed *ira*$^-$ mutants, with mutations occurring in roughly similar frequencies at the two loci (31 *ira1*$^-$, 65 *ira2*$^-$).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitations of 9Myc-tagged Hst1p and Hst2p were performed as described in Knop et al. (1999). Briefly, cells were grown to between $O.D._{600}$ 0.8–1.2 units, harvested, and fixed with formaldehyde. Cells were lysed in the presence of protease inhibitors, sonicated to shear the chromatin, and the lysates were immunoprecipitated with anti-Myc antibodies (9E11) bound to Dynabeads M-450, pre-coated with pan-anti-mouse IgG (Dynal). The beads with bound protein/DNA complexes were washed and the protein/DNA complexes were eluted from the beads. Reversal of the crosslinks with TE/SDS was followed by ethanol precipitation of the DNA in the IP fraction. This DNA was analyzed by PCR for the presence of specific sequences in the *FLO10* promoter.

Acknowledgments

We thank D.G. Halme, R. Prusty, and R. Wheeler for their assistance and comments on the manuscript; R. Young for generously donating reagents to generate Myc-tagged Hst1p and Hst2p as well as anti-Myc antibodies. This work was supported by NIH grants GM35010 and GM40266 and the Abraham Siegel Fellowship to A.H.; G.R.F. is an American Cancer Society Professor.

Received: October 24, 2003
Revised: December 19, 2003
Accepted: December 30, 2003
Published: February 5, 2004

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