Genetic variation in *Saccharomyces cerevisiae*: Circuit diversification in a signal transduction network

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ABSTRACT

The connection between genotype and phenotype was assessed by determining the adhesion phenotype for the same mutation in two closely related yeast strains, S288c and Sigma, using two identical deletion libraries. Previous studies, all in Sigma, had shown that the adhesion phenotype was controlled by the filamentation MAPK (fMAPK) pathway, which activates a set of transcription factors required for the transcription of the structural gene *FLO11*. Unexpectedly, the fMAPK pathway is not required for *FLO11* transcription in S288c despite the fact that the fMAPK genes are present and active in other pathways. Using transformation and a sensitized reporter, it was possible to isolate *RPI1*, one of the modifiers that permits the bypass of the fMAPK pathway in S288c. *RPI1* encodes a transcription factor with allelic differences between the two strains: The *RPI1* allele from S288c but not the one from Sigma can confer fMAPK pathway independent transcription of *FLO11*. Biochemical analysis reveals differences in phosphorylation between the alleles. At the nucleotide level the two alleles differ in the number of tandem repeats in the ORF. A comparison of genomes between the two strains shows that many genes differ in size due to variation in repeat length.

INTRODUCTION

Recent advances in DNA sequencing have identified many nucleotide polymorphisms in the human genome, but it has been challenging to associate this genetic variation to specific phenotypic differences among individuals for complex traits (DICKSON et al. 2010; JAKOBSDOTTIR et al. 2009; MANOLIO et al. 2009). This difficulty has been variously attributed to both genetic and non-genetic factors (CARLBORG and HALEY 2004; DICKSON et al. 2010; HARTMAN et al. 2001; KORBEL et al. 2007). Among the genetic factors are: many genes
contributing a small effect to the final phenotype (QTLs) and complex (epistatic) gene interactions. The baker’s yeast *Saccharomyces cerevisiae*, with its compact and easily manipulated genome, offers the potential for identifying the relevant polymorphisms and, more importantly, identifying the molecular basis for the phenotypic differences.

Sequence studies comparing *S. cerevisiae* to other yeast species that diverged by 20 million years advanced our understanding of yeast evolution, but did not address how small genetic differences impact phenotypes (Kellis *et al.* 2003). Other studies have examined large numbers of both feral and laboratory *S. cerevisiae* strains, but have focused on population structure and evolutionary origins of the strains rather than the problem of connecting genotype to phenotype (Liti *et al.* 2009; Schacherer *et al.* 2009).

More recently, insights into the genotype to phenotype problem have been gained from linkage studies using modern genotyping techniques. Several examples can be seen in the cross of the wild vineyard strain RM11 to the standard laboratory strain S288c. A number of traits have been examined using this cross, including gene expression, cell morphology, resistance to DNA damaging agents, and telomere length (BREM *et al.* 2002; Demogines *et al.* 2008; Gatbonton *et al.* 2006; Nogami *et al.* 2007). The genetic complexity for most of these traits is high, with many of them influenced by more than three loci. By examining large pools of progeny, recent techniques have further increased the ability to map relevant loci, however it is still challenging to determine the exact alleles responsible and to understand how those alleles affect the phenotype (Connelly and Akey 2012; Ehrenreich *et al.* 2010).

Recent studies developed a model system that enables a comprehensive assessment of phenotypic differences for the same mutation in the two genetic backgrounds S288c and Σ1278b (Sigma) (Dowell *et al.* 2010). The two strains have very similar genomic sequences: their
divergence of ~0.3% is similar to that between unrelated humans. To assess functional differences between these two strains, ~5100 genes were deleted in Sigma for comparison with the same set of deletions in S288c (Dowell et al. 2010; Winzeler et al. 1999). The analysis identified strain specific essential genes. The basis for the strain specificity was likely a complex set of background modifiers.

Here we compare these deletion libraries for the genes that control the key morphogenetic trait of adhesion/filamentation. In Sigma, adhesion requires the filamentation mitogen activated kinase (fMAPK) pathway, but our library comparison showed that S288c can adhere in the absence of the fMAPK pathway. Although fMAPK independent adhesion is a complex genetic trait, we devised a transformation protocol that enabled the isolation of RPII, one of the modifiers responsible for the bypass of the fMAPK pathway. RPII is a transcription factor that is polymorphic between S288c and Sigma; the RPII allele from S288c (RPII$^{S288c}$) confers fMAPK pathway independence by activating FLO11 transcription, whereas the RPII allele from Sigma (RPII$^{Sigma}$) cannot. RPII$^{S288c}$ confers fMAPK pathway independence in either genetic background. Moreover, there is a biochemical difference between the alleles; RPII$^{S288c}$, but not the RPII$^{Sigma}$ is hyperphosphorylated in both S288c and Sigma. The two forms of RPII differ in the number of tandem repeats in the ORF. A comparison of the S288c and Sigma genomes shows that many other genes with intragenic tandem repeats are highly polymorphic with respect to repeat size, a polymorphism that has been associated with phenotypic changes (Verstrepen et al. 2005).
MATERIAL AND METHODS

Strains, Media, Microbiological Techniques, and Growth Conditions: Yeast strains used in this study are derived from S288c and Σ1278b. Standard yeast media were prepared and genetic manipulation techniques were carried out as described (GUTHRIE and FINK 2002). Adhesion assays were carried out by densely patching strains onto YPD or SC plates. These were grown overnight at 30°C and then replica plated onto YPD or SC plates. The replica plates were grown at 30°C for three days and then washed. The S288c strain expresses FLO1 which leads to flocculation that can influence agar adhesion phenotypes. To compare agar adhesion between S288c and Sigma, which does not express FLO1, the washes were performed by partially filling the petri dishes with 10mM EDTA (which disrupts FLO1 dependent aggregates) and gentle shaking at approximately 75rpm on an orbital shaker. To visualize the difference between the strains, the media used for both the adhesion and transcription assays was optimized for intrinsic growth differences between S288c and Sigma (e.g. flocculation and mother-daughter cell separation). However, the controls intrinsic to each experiment always permitted a comparison between strains grown under the same media conditions. To induce pseudohyphal growth, single cells were microdissected and grown on SLAD media (GIMENO et al. 1992).

The S288c library was constructed using previously published methods (VOYNOV et al. 2006). Each of the 4705 deletion strains in the standard S288c flo8 library was transformed with a CEN/ARS plasmid carrying the Sigma FLO8 gene under the control of its own promoter. The 4633 FLO8 deletion strains successfully recovered from these transformations formed the S288c deletion library. Screening the S288c library and the comparable Sigma deletion library for adhesion uncovered 599 deletions with decreased adhesion (Ahs⁻) (Tables S1-S3). Only 46 deletions affected adhesion the same way in both strains (Table S3).
For qPCR and ChIP, cells were grown overnight in liquid media as noted, diluted to OD600=0.25, and grown to OD600=4-4.5. For protein preparations, cells were grown as for qPCR in synthetic complete media.

Yeast strains carrying gene deletions were constructed by PCR amplification of kanamycin-resistance gene cassettes from the yeast deletion library (WINZELER et al. 2000) with approximately 200 bases of flanking sequence. Correct integrants were identified by PCR, with the exception of tec1Δ, which was additionally checked by Southern blot using standard techniques (BROWN 2001). FLO11 promoter swaps were carried out by first deleting the FLO11 promoter with the URA3 cassette. The reciprocal swap was carried out by PCR amplifying the sequences from each strain and using the PCR products to transform the opposite strain from which the sequence was amplified. The same procedure was performed for the RPII swaps but with only the ORF sequences. 3xFLAG tagged constructs were created by amplifying the URA3 cassette from PRS306 using a primer (BCP534) that contained the 3x FLAG epitope. This construct was then subject to another round of PCR to add 50bp of flanking homology to the RPII c-terminus. The resulting PCR product was used for transformation. The haploid MATa deletion collection was transformed with plasmid pHL1 using previously published protocols (LIU et al. 1996; VOYNOV et al. 2006).

**GFP measurements:** Cultures for GFP measurements were grown overnight in liquid YPD in 96 well plates and then pelleted and resuspended in water. Samples were transferred to Corning 96 well black clear-bottom plates and OD600 and GFP fluorescence was measured in a Tecan Safire2 plate reader. For backcrosses, high fluorescing progeny were backcrossed to the low fluorescing Sigma tec1Δ for three generations.
**tec1Δ bypass screen:** The *CLN2* PEST sequence was added to the end of the *HIS3* gene to target the protein product to the proteasome. Without this modification, a Sigma *FLO11pr-HIS3*, *tec1Δ* strain produces enough His3p protein from the *FLO11* promoter to be His⁺, even in relatively high concentrations of the His3p competitive inhibitor 3-aminotriazole. The *HIS3-PEST* construct was created by Infusion PCR cloning (Clontech) the PEST sequence from *CLN2* immediately upstream of the *HIS3* stop codon in PRS315. The *CLN2* PEST sequence was amplified using primers BCP316 and BCP317 and PRS315 was linearized by PCR using primers BCP320 and BCP321. To create the *FLO11pr-HIS3-PEST* strain, the *HIS3-PEST* construct was PCR amplified with primers BCP249 and BCP324. These primers have homology to replace the endogenous *FLO11* ORF with the *HIS3-PEST* ORF, and the PCR product was transformed into *yBC172*. Transformants were selected on -HIS media and then correct transformants were screened for by PCR. *TEC1* was deleted in *FLO11pr-HIS3-PEST* transformants by PCR transformation.

The *FLO11pr-HIS3-PEST, tec1Δ* strain was transformed with an S288c CEN/ARS genomic library (Rose *et al.* 1987). Transformants were first selected for 24 hours on -URA plates, and then replica plated onto -URA, -HIS plates plus 5mM 3-AT.

We obtained approximately 300 His⁺ transformants out of over 15,000 total transformants, we examined if the His⁺ phenotype was dependent upon the plasmid by selecting for strains that had lost the plasmid on 5-FOA. After 5-FOA selection, these strains were examined, by dilution series, on -HIS plates.

54 strains required the library plasmid to be His⁺, and the plasmid from these strains was
isolated and the ends of the insert were sequenced. Potential bypass strains were identified by examining the overlapping regions among the inserts.

**qPCR:** Total RNA was obtained by standard acid phenol extraction from 2 ml of culture. The Qiagen QuantiTect Reverse Transcription Kit was used to remove residual genomic DNA and reverse transcribe the RNA templates to generate cDNAs. Aliquots of cDNA were used in real-time PCR analyses with reagent from Applied Biosystems and the ABI7500 real-time PCR system.

**Chromatin IP:** Protocols have been described (LEE et al. 2006). Briefly, IPs were performed with Dynal Protein G magnetic beads pre-incubated with antibodies against FLAG-epitope (Sigma M2). To examine enrichment, SYBR Green qPCR (Applied Biosystems) was performed on IP and WCE using gene specific primers.

**Protein manipulations:** Total protein was extracted using standard TCA precipitation with slight modifications (GRAHAM 2001). Namely, after TCA precipitation the acetone wash was omitted and instead the cells were washed once with 1M Tris-pH8. For phosphatase assays, 5µl of total protein was treated with 2 µl lambda phosphatase (NEB) for 2 hours at 30°C and the reaction was stopped by adding 6x Laemmli loading buffer to 1x concentration and boiling for 10 minutes. Samples were run out on a 10% TGX gel (BioRad 456-1036S). The phosphorylation of RPII causes it to run as a diffuse smear and the amount of signal is distributed across this entire range. To visualize phosphorylated RPII alongside phosphatase
treated $RPI1$, up to five times the amount of phosphorylated $RPI1$ was loaded. Blotting against FLAG was performed using HRP-conjugated anti-FLAG M2 antibody (Sigma A8592).

**Bioinformatics:** Gene ontology term enrichment was performed using the AMIGO term enrichment tool version 1.8 (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment).

To find intragenic repeats, the EMBOSS program ETANDEM (RICE et al. 2000) was used to screen the sequences of all $S. cerevisiae$ (S288c version 2010 downloaded from SGD in April 2011) and the $\Sigma_{1278b}$ strain (Sigma downloaded from http://mcdb.colorado.edu/labs1/dowelllab/pubs/DowellRyan/ in October 2010) for repeat units of length 3 to 500 bp. For each ORF, we compared the length in the two strains. We screened 6685 ORFs in S288c and 6450 ORFs in Sigma. A total of 6439 ORFs were common to both strains. Of these 6439 ORFs, 5928 were identical in length. Of the remaining 511 ORFs, 127 ORFs differed in total length by at least 6 bp and showed a length difference in the repeat region of at least 6 bp. We eliminated an additional 11 ORFs because of large truncations in either the 5' or 3' region of the ORF accounting for the length differences between strains. All but 9 of the length differences in the 116 ORFs were a multiple of 3. These discrepancies could be due to sequencing errors. The length of the ORF was longer in Sigma for 60 ORFs (43 ORFs with bp difference of 6 to 33, 17 ORFs with bp differences of 36 or greater). A total of 56 ORFs were longer in S288c (43 ORFS with bp difference of 6 to 33, 13 ORFs with bp differences of 36 or greater).

**Repeat length PCRs:** Primers flanking the repeat region were designed using PRIMER3 (ROZEN and SKALETSKY 2000). PCR products were visualized on 10% polyacrylamide gels.
RESULTS

Creation of an S288c FLO8 deletion library: Systematic deletion library comparison of S288c and Sigma for the adhesion phenotype required the creation of a new S288c FLO8 library because the progenitor to the standard S288c deletion library carries a flo8 mutation that prevents adhesion to agar. When S288c flo8 is transformed to Flo8^+, it adheres in a FLO11 dependent fashion (LIU et al. 1996). We next assayed the entire library for the adhesion phenotype (Adh^+ or Adh^-) and identified deletions in the S288c library with the Adh^- phenotype.

The fMAPK pathway is required for adhesion and FLO11 transcription in Sigma but not in S288c: Comparison of the loss of adhesion mutants in the Sigma and S288c deletion libraries revealed that many genes have strain specific roles in adhesion (Tables S1-S3). The strain specificity of the Ahs^- phenotypes is not attributable to an integrated FLO8 in the Sigma library, but a plasmid borne FLO8 in the S288c library. The Ahs^- phenotype was the same in 28/30 deletions tested from the S288c deletion library whether FLO8 was plasmid-borne or integrated at the resident FLO8 locus (replacing the flo8 allele). All strains pursued further had the FLO8 gene integrated at its native locus in S288c.

The comparison of S288c and Sigma adhesion mutants revealed that the fMAPK pathway is required for adhesion in Sigma but it is not required for adhesion in S288c (Figure 1A). Strains carrying deletions in kinase genes - STE7, STE11, KSS1 - and the transcription factor genes - STE12, and TEC1 - have a clear adhesion defect in Sigma but adhere well in S288c (Figure 1A). qPCR measurements revealed that wild-type S288c and S288c tec1Δ both show strong expression of FLO11, whereas Sigma tec1Δ has a 50-fold decrease in FLO11 RNA levels.
relative to the wild-type control (Figure 1C). The distinct requirement for the fMAPK pathway in Sigma but not in S288c suggests that adhesion is controlled differently in the two strains.

The fMAPk pathway in Sigma activates FLO11 transcription for haploid adhesion and diploid filamentation (Liu et al. 1993; Lo and Dranginis 1998; Roberts and Fink 1994). To determine whether the fMAPK pathway is dispensable for diploid filamentation in S288c, we constructed diploid S288c strains. Filamentation in the S288c tec1Δ/tec1Δ strain is indistinguishable from wild-type, whereas the Sigma tec1Δ/tec1Δ strain has a filamentation defect (Figure 1B). A hybrid S288c/Sigma tec1Δ/tec1Δ strain is able to filament, showing that the ability of S288c to bypass an fMAPK defect for filamentation is dominant. Homozygous diploid S288c flo11Δ/flo11Δ and Sigma flo11Δ/flo11Δ strains failed to form filaments. Thus, FLO11 function is required for adherence and filamentation in both S288c and Sigma even though the requirement for the fMAPK pathway is restricted to Sigma.

**Differences in the FLO11 promoter sequence do not account for S288c fMAPK independent FLO11 expression**: Reciprocal promoter swap strains were used to determine whether the sequence differences between the S288c and Sigma FLO11 promoters (FLO11prS288c and FLO11prSigma respectively), could account for the fMAPK independence of S288c. S288c FLO11prSigma adhered like a wild-type S288c as did S288c FLO11prSigma tec1Δ showing that FLO11prS288c is not necessary for fMAPK independent adhesion of S288c cells (Figure 2). FLO11 RNA levels in the S288c FLO11prSigma strain were consistent with the adhesion phenotypes; specifically, in S288c there was no significant difference in FLO11 RNA levels, regardless of the promoter, or the presence of a tec1Δ (Figure S1A).
The $FLO11pr^{S288c}$ does not promote $FLO11$ transcription as efficiently in Sigma as it does in S288c. This difference is reflected both in the adhesion assay and the qPCR measurement of $FLO11$ RNA levels (Figure 2 and S1B). Nevertheless, the $FLO11pr^{S288c}$ in Sigma is $TEC1$ dependent for both adhesion and $FLO11$ transcription, whereas it is $TEC1$ independent in S288c. These results imply that the sequence differences in the promoters are not responsible for the fMAPK independence of S288c.

**The strain difference in $FLO11$ regulation is genetically complex:** Crosses between the adherent S288c $tec1\Delta$ strain to the non-adherent Sigma $tec1\Delta$ did not yield a simple segregation pattern for adherence:non-adherence. Analysis of 24 complete meiotic tetrads produced novel phenotypes (24/96 progeny were clearly adherent, 56/96 were non-adherent, and 16/96 displayed various partially adherent phenotypes) (Figure S2). Backcrosses of the F1 adherent progeny to the Sigma $tec1\Delta$ strain continued to yield non-Mendelian segregations and novel adherent phenotypes.

We considered the possibility that the failure to isolate modifiers by backcrosses was due to the lack of robustness of the adhesion assay. Moreover, agar adhesion can be affected by both transcriptional and post-transcriptional regulation of $FLO11$ (VOYNOV et al. 2006; WOLF et al. 2010). In addition, $FLO11$ manifests epigenetic switching between on and off states (BUMGARNER et al. 2009; HALME et al. 2004). To quantitatively assess the $FLO11$ phenotype we used a $FLO11pr::GFP$ construct to monitor the segregation of $FLO11$ transcription in S288c $tec1\Delta$ x Sigma $tec1\Delta$ crosses. These crosses directly examined the variation affecting $FLO11$ transcription, yet the segregation of GFP fluorescence was still complex in both the F1 generation and subsequent backcrosses (Figure S3).
Tetrad analysis of crosses between the adherent wild-type S288c and Sigma strains provided further insight into the cause of the anomalous segregation patterns. Since both wild type strains were adherent, we expected the F1 progeny would all be adherent. However, many of the F1 progeny were non-adherent (Figure S4). This data suggest that polymorphisms between wild-type Sigma and S288c combine in the progeny to suppress FLO11 expression. This situation considerably complicates using either conventional tetrad genetic analysis or bulk segregation analysis to find alleles that bypass the fMAPK pathway. Isolation and analysis of any of the many polymorphisms contributing to fMAPK independence required another approach.

**Transformation permits the isolation of a modifier from S288c conferring fMAPK independent expression of FLO11:** To overcome the challenges of mapping polymorphisms for fMAPK independent adhesion, we developed a transformation protocol to select for plasmids carrying S288c genes that bypass the fMAPK pathway. The selection required replacement of the FLO11 ORF with a HIS3-PEST construct in the Sigma tec1Δ strain. This PEST modification enabled the visualization of slight differences in FLO11 expression when selecting for His⁺ transformants. The Sigma FLO11pr-HIS3-PEST, tec1Δ strain is His⁻ whereas the S288c FLO11pr-HIS3-PEST, tec1Δ strain is His⁺. Modifiers from S288c that could bypass the requirement for the fMAPK pathway in Sigma were obtained by transforming the Sigma FLO11pr-HIS3-PEST, tec1Δ strain (His⁻) with a S288c CEN/ARS genomic library (ROSE et al. 1987) and selecting for His⁺ transformants.

Sequence analysis of the plasmids capable of conferring the His⁺ phenotype to the Sigma FLO11pr-HIS3-PEST, tec1Δ strain identified several genes (including TEC1 itself). A gene with
a relevant S288c polymorphism should have a sequence difference from its Sigma allele and the
ability to confer the His\(^+\) phenotype (bypass the \textit{tec1}\(\Delta\) defect) when integrated in the
chromosome in single copy. \textit{RPI1}\(^{S288c}\) was the only gene obtained that fulfilled these criteria.
When \textit{RPI1}\(^{S288c}\) replaced \textit{RPI1}\(^{Sigma}\) in the chromosome, the Sigma \textit{FLO11pr-HIS3-PEST, tec1}\(\Delta\)
strain was His\(^+\). Moreover \textit{RPI1}\(^{S288c}\) and \textit{RPI1}\(^{Sigma}\) differ in numerous SNPs and stretches of
intragenic repeats that differ in length (Figure 3, S5 and S6).

\textbf{\textit{RPI1}\(^{S288c}\) but not the \textit{RPI1}\(^{Sigma}\) is a bypass suppressor of the fMAPK pathway:} Consistent
with the hypothesis that the \textit{RPI1}\(^{S288c}\) has an allele specific role in \textit{FLO11} expression, deletion of
\textit{RPI1}\(^{S288c}\) in S288c results in a strong adhesion defect and decreased \textit{FLO11} RNA, whereas
deletion of \textit{RPI1}\(^{Sigma}\) in Sigma does not (Figure 4A-C). To further characterize the allele
specificity of \textit{RPI1}, we swapped \textit{RPI1} alleles between the strains. S288c \textit{RPI1}\(^{Sigma}\) displayed an
adherence phenotype and \textit{FLO11} RNA levels that were not significantly different than an \textit{rpi1}\(\Delta\),
suggesting that \textit{RPI1}\(^{Sigma}\) is not functional in \textit{FLO11} regulation (Figure 4A and 4B). Deletion of
\textit{TEC1} in S288c \textit{RPI1}\(^{Sigma}\) does not further decrease adhesion or \textit{FLO11} levels. Reciprocally, the
Sigma \textit{RPI1}\(^{S288c}\) strain had \textit{FLO11} mRNA levels that were comparable to wild-type, and when
\textit{TEC1} is deleted, Sigma \textit{RPI1}\(^{S288c}\) \textit{tec1}\(\Delta\) had more \textit{FLO11} RNA than the Sigma \textit{RPI1}\(^{Sigma}\) \textit{tec1}\(\Delta\),
but less than wild-type (Figure 4C). These results show that the \textit{RPI1}\(^{S288c}\) allele promotes \textit{FLO11}
expression and can partially bypass the \textit{tec1}\(\Delta\); however, the \textit{RPI1}\(^{Sigma}\) allele is unable to bypass
\textit{tec1}\(\Delta\).
**Rpi1p interaction with the FLO11 promoter is Rpi1p allele specific:** To determine whether the difference in fMAPK independent FLO11 expression is a consequence of differences in the ability of Rpi1p$^{\text{Sigma}}$ and Rpi1p$^{\text{S288c}}$ to interact with the FLO11 promoter, we performed chromatin immunoprecipitation (ChIP), and tested for enrichment of the FLO11 promoter. Rpi1p$^{\text{S288c}}$ interacts with the FLO11 promoter with a peak around -1300bp (Figure 5A), the site bound by other positive activators of FLO11 such as Tec1p, and Flo8p (BORNEMAN et al. 2006; ZEITLINGER et al. 2003). Immunoprecipitation of the Rpi1p$^{\text{S288c}}$ allele enriches for the FLO11 promoter regardless of the strain background. In contrast to Rpi1p$^{\text{S288c}}$, immunoprecipitation of the Rpi1p$^{\text{Sigma}}$ results in strain-background-specific enrichment for this same region of the FLO11 promoter. When the Rpi1p$^{\text{Sigma}}$ is immunoprecipitated from a Sigma strain, it enriches for the FLO11 promoter; when it is immunoprecipitated from an S288c strain it does not.

This difference between Rpi1p$^{\text{S288c}}$ and Rpi1p$^{\text{Sigma}}$ promoter binding is also observed at the promoter of MIT1, previously identified as a target of Rpi1p and a “master regulator” of FLO11 transcription (CAIN et al. 2011; WANG et al. 2011; ZEITLINGER et al. 2003). However, Wang et al. and Cain et al. provided only strain specific analyses of MIT1 and RPI1 function: The Mit1p$^{\text{Sigma}}$ protein was shown to bind to the FLO11 promoter in Sigma, and Rpi1p$^{\text{S288c}}$ has been reported to localize to the promoter of MIT1$^{\text{S288c}}$ in S288c. Our ChIP data show that Rpi1p$^{\text{S288c}}$ localizes to the MIT1 promoter, regardless of strain background, but Rpi1p$^{\text{Sigma}}$ localizes to the MIT1 promoter only in the Sigma background (Figure 5B). Furthermore, Rpi1p$^{\text{S288c}}$ requires a functional MIT1 to suppress a defect in the fMAPK pathway in both S288c and Sigma. Rpi1p$^{\text{Sigma}}$ can interact with both the FLO11 and MIT1 promoters in Sigma, but not in S288c. Thus, Rpi1p$^{\text{Sigma}}$ must be structurally different from Rpi1p$^{\text{S288c}}$ and require additional factors to function.
The Rpi1p protein is differentially phosphorylated in the two strains: Analysis of the Rpi1p protein showed that Rpi1p$^{S288c}$ is structurally different from Rpi1p$^{\Sigma}$. Figure 6 shows that 3x-FLAG-tagged Rpi1p$^{S288c}$ extracted from S288c and visualized on Western blots runs as a diffuse species different from the Rpi1p$^{\Sigma}$ band from Sigma. When the Rpi1p$^{S288c}$ is expressed in Sigma, it again runs as a diffuse higher molecular weight species, but when Rpi1p$^{\Sigma}$ is expressed in S288c, it runs as a single band (Figure 6).

To determine whether the difference between the isoforms of Rpi1p is due to phosphorylation, protein extracts were treated with lambda phosphatase. The broad Rpi1p$^{S288c}$ band collapsed to a single band. This change in migration pattern occurs regardless of the strain background that expresses Rpi1p$^{S288c}$. Treatment of Rpi1p$^{\Sigma}$ with phosphatase changed its migration only if the protein was obtained from a Sigma strain. These experiments show that Rpi1p$^{\Sigma}$ has strain specific phosphorylation and likely has a different phosphorylation pattern than Rpi1p$^{S288c}$. This altered phosphorylation pattern of Rpi1p$^{\Sigma}$ may account for its inability to activate $FLO11$ transcription in either strain.

The RPI1 polymorphism is not restricted to laboratory strains: The striking difference in the control of $FLO11$ transcription between these two strains could be attributed to their long term culture in the laboratory. Indeed, all S288c strains have a nonsense mutation in $FLO8$ and many have a mutation in the $KSS1$ gene as well, both affecting $FLO11$ expression (Elion et al. 1991; Liu et al. 1996). However, an assessment RPI1 sequences shows that the S288c-like polymorphisms are widespread and found in both feral and laboratory strains (Figure S6). Thus,
the expansion and contraction of \textit{RPII} appears to be a common avenue for diversity both in the laboratory and in the wild.

\textbf{Intragenic tandem repeats are highly polymorphic within a species:} The difference in repeat length between the \textit{RPII} alleles of S288c and Sigma led us to ask how many other genes differ in this way. Previous studies focused on cell surface proteins and have found profound phenotypic consequences for changes in the size of an internal repeat region (Fidalgo \textit{et al.} 2008; Levansky \textit{et al.} 2007; MacDonald \textit{et al.} 1993; Sheets and St Geme 2011; Tan \textit{et al.} 2010; Verstrepen \textit{et al.} 2005), but it is difficult to perform genome-wide examinations of repeat length changes because few organisms have multiple genomes of sufficiently high quality to compare repeat regions. With the release of the Sigma genome, this comparison can be done because both the S288c and Sigma genomes are of a high enough quality to ask, like \textit{RPII}, how many genes differ in size due to repeat length changes? By computationally comparing the size of every ORF between S288c and Sigma, we identified 107 genes that differ in length due to in-frame expansions or contractions of intragenic repeat sequences (Table S4). The set of genes with intragenic repeat length differences includes genes involved in diverse biological processes, including transcription, chromatin modification, and signal transduction. To ensure that these differences are not due to sequencing errors, 24 of these length differences were verified by PCR (Fig. 7 and S7). 22 of 24 genes show the predicted size difference, confirming the size differences predicted from the genome sequences reflected length differences in the repeats.
DISCUSSION

Individuals within a species may signal gene expression through different pathways: Our analysis of comparable deletion libraries in two inter-fertile strains of *Saccharomyces cerevisiae* (Sigma and S288c) with nearly identical genomes (Dowell et al. 2010), allowed us to ask the question: Do the same signal transduction pathways control development in both strains? Previous mutational analyses identified the fMAPK pathway as required for adhesion and FLO11 transcription in Sigma (Cook et al. 1996; Lorenz and Heitman 1998; Roberts and Fink 1994). A recent comprehensive genome-wide analysis of the Sigma deletion library for adhesion, filamentation and biofilm formation again uncovered the fMAPK genes (Ryan et al. 2012). Therefore, the finding that S288c does not require the fMAPK pathway was unanticipated. This functional difference is not a consequence of gene duplication but rather involves distinct genes encoding two separate pathways, each capable of eliciting the same phenotype. The two strains differ by polymorphisms in the transcription factor RPI1; the $RPI1^{S288c}$ allele is active and suppresses the loss of function of the fMAPK pathway; the $RPI1^{Sigma}$ allele is inactive and incapable of suppressing of a defect in the fMAPK pathway.

The discovery of $RPI1^{S288c}$ as a bypass suppressor of the fMAPK pathway provides insight into the mechanism by which allelic polymorphisms can buffer the effect of mutations and rewire a signaling pathway. Although previous studies have identified many QTL’s in intraspecies crosses of *S. cerevisiae*, many of these polymorphisms have not in been connected to differences in function. As with the adhesion phenotype, each of the polymorphisms may have only a modest effect on the phenotype, making it difficult to isolate and assess the mechanism of action. We were able to tune the conditions so that we could use transformation to select for modifiers such as $RPI1$ that only partially restore FLO11 expression.
The presence of $RPI1^{S288c}$ in S288c means that loss of function of any member of the fMAPK pathway will fail to manifest an adhesion phenotype because $FLO11$ can now be activated by $RPI1^{S288c}$. Even $MSB2$, the protein believed to be the sensor for the fMAPK pathway, is not needed for S288c adhesion (Table S2). The activation of $FLO11$ by $RPI1^{S288c}$ raises the question: What is upstream of $RPI1$ in S288c? Our genome-wide screen of the S288c library for strains with adhesion defects identified a number of potential candidates that do not have adhesion/filamentation defects in Sigma. In the future a systematic analysis of these is likely to identify those genes required for $RPI1$ activation.

**The evolution of circuit diversification begins within a species:** Comparing species that evolved from a common ancestor before and after the whole genome duplication (WGD) (Kellis et al. 2004; Wapinski et al. 2007) has elucidated the gradual rewiring of transcription circuits in the fungal lineage. For example, yeast species post WGD have two proteins controlling the ribosomal protein stress response, a positive ($IFH1$) and a negative ($CRF1$) regulator, whereas organisms that did not undergo the WHG have a single ancestral protein with both positive and negative activities (Wapinski et al. 2010). Post-WHG, the duplicate genes specialized with one losing a positive function and the other a negative, while both retaining “stress response control”.

The plasticity of these regulatory networks is most dramatically seen in the comparison of the regulatory circuit that regulates mating type in the human fungal pathogen *Candida albicans* with that of *Saccharomyces cerevisiae*. The ensemble of genes controlling mating is largely conserved in the two organisms; however, the a-specific genes in Candida are under
positive control by the a2 protein and in *S. cerevisiae* they are under negative control by the alpha2 protein. This transition from positive to negative regulation of the a-specific genes involved slight changes over evolutionary time in both the cis-acting elements in the promoters of the a-specific genes and the trans-acting regulatory proteins a2 and alpha2 (Tsong et al. 2006).

These variations in regulatory control observed in different species, which evolved over evolutionary time, must have arisen from variations that occurred within a single species and subsequently became fixed as sexual isolation took place. As we have shown, such variation in the circuitry of key signaling pathways exists among contemporary members of the same species. This apparent redundancy in *FLO11* activation raises the question: why are the two pathways retained? Despite the overlapping functions of the fMAPK pathway and *RPI1*, the organization of these genes into complex networks likely imposes constraints on the loss of one or the other of these activation pathways. The elements of the fMAPK pathway that have been conserved in both S288c and Sigma (Ste20p, Ste11p, Ste7p, Ste12p) are under strong positive selection because they have cross-pathway functions in additional signal transduction pathways (mating, osmotic-sensing). Since *RPI1* regulates the cell wall under different conditions, it is also likely to function in conjunction with many pathways (Puria et al. 2009; Sobering et al. 2002; Wang et al. 2011). The finding that *RPI1* not only localizes to the *FLO11* promoter but also the *MIT1* promoter (Wang et al. 2011), itself a transcriptional activator of *FLO11* and many other genes (Cain et al. 2011), is consistent with the idea that *RPI1* is also constrained by its participation in many regulatory networks.
**RPII**<sup>S288c</sup> and **RPIISigma** differ by intragenic tandem repeat expansions: Although the two **RPII** alleles differ by several nucleotide changes, the most striking difference is the alteration in the size of a repeat region present in the coding sequence of the gene. These repeat polymorphisms in **RPII** are present in wild isolates of yeast as well as in many laboratory strains (Figure S6). Some wild isolates have the **RPII**<sup>S288c</sup> length repeat and others have the **RPII**<sup>Sigma</sup> length.

Repeats within a coding sequence create enormous flexibility for the evolution of diversity within a species. Because repeats can expand and contract at high frequencies, they permit a species to adapt to changing environments without becoming irreversibly committed to a phenotype (RANDO and VERSTREPEN 2007). Although SNPs remain the major type of variation between S288c and Sigma, over 100 genes differ in size due to repeat length differences. These data suggest that in a cross between S288c and Sigma these size polymorphisms could generate as many as $2^{100}$ genotypes in a cross. Phenotypic effects from even a tiny fraction of this variation would provide ample grist for evolution’s mill.

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Figure 1 - The fMAPK pathway is not required for FLO11 expression in S288c. (A) Adhesion assays performed on S288c strains (right half of the plate), or Sigma strains (left half of the plate). The same plate is shown before (top) and after (bottom) washing. (B) Pseudohyphal growth on SLAD media for diploid Sigma, S288c, or Sigma/S288c hybrids. (C) qPCR assay of FLO11 transcript levels was performed on Sigma and S288c strains that were WT or tec1Δ. Mean FLO11 levels normalized to ACT1 levels are presented ± SD. (*) P < 0.01 compared to WT.

Figure 2 - S288c with {FLO11}^{prSigma}::FLO11 is fMAPK independent. Agar adhesion assays performed on S288c strains (right half of the plate), or Sigma strains (left half of the plate) in the FLO11 promoter swap experiment (see text). The same plate is shown before (left) and after (right) washing. Strains with their endogenous FLO11 promoter are labeled with their relevant genotype. Strains carrying a swapped FLO11 promoter are labeled numerically: (1) S288c FLO11pr^{Sigma}::FLO11; (2) S288c FLO11pr^{Sigma}::FLO11, tec1Δ; (3) Sigma FLO11pr^{S288c}::FLO11, tec1Δ; (4) Sigma FLO11pr^{S288c}::FLO11.

Figure 3 - RPI1 alleles vary in the number of intragenic repeats. The S288c and Sigma alleles of RPI1 have intragenic repeats, but the repeat lengths differ between the two strains. The schematic illustrates the alignment of the two alleles. The boxes represent individual repeat elements and arrow heads represent locations of SNPs. Empty areas represent the shortened repeat length in that allele.

Figure 4 - RPI1^{S288c} can partially bypass the fMAPK pathway for agar adhesion and FLO11 expression. (A) Agar adhesion of S288c and Sigma strains carrying reciprocal allele swaps of RPI1. The top row shows adhesion assays performed on S288c strains grown on YPD and the bottom row shows adhesion assays performed on Sigma strains grown on synthetic media (see METHODS). The same plates are shown before and after washing. qPCR assay of FLO11 transcript levels performed on (B) S288c strains grown in synthetic media and (C) Sigma strains grown on YPD. Mean FLO11 levels normalized to ACT1 levels are presented ± SD. ** P < 0.01. Strains with their endogenous RPI1 allele are labeled with their relevant genotype. Strains carrying a swapped RPI1 allele are labeled numerically: (1) S288c RPI1^{Sigma}; (2) S288c RPI1^{Sigma}, tec1Δ; (3) Sigma RPI1^{S288c}; (4) Sigma RPI1^{S288c}, tec1Δ.
Figure 5 - *RPII*<sup>S288c</sup> shows strain independent localization to the *MIT1* and *FLO11* promoters. Localization of Rpi1p using FLAG tagged alleles in Sigma and S288c assayed by ChIP followed by qPCR for enrichment at (A) -1.3kb in the *FLO11* promoter and (B) -1kb in the *MIT1* promoter. Data were normalized to *ACT1* and are expressed as the mean fold enrichment ± SD. * P < 0.01 compared to untagged.

Figure 6 - The Rpi1p<sup>S288c</sup> protein is hyperphosphorylated. Western blot analysis of Rpi1p phosphorylation state in strains expressing either 3x flag tagged *RPI1*<sup>S288c</sup> or *RPI1*<sup>Sigma</sup>. Samples were treated with either buffer or lambda phosphatase.

Figure 7 - Many S288c genes differ from Sigma genes due to changes in intragenic tandem repeats. 24 of the 107 genes predicted to differ between S288c and Sigma in the length of internal repeats were examined by PCR. 22 of these genes had the predicted size difference. Five genes are shown and the results for the other genes are shown in Figure S7. *PGD1* and *SPT8* have two repeat regions that both change in size. For each pair the left sample is the S288c product and the right is the Sigma product.
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Figure 3

$RPI1$

S288c

Sigma
Figure 5

A

ChIP FLO11pr

|          | S288c^FL08 | Sigma |
|----------|------------|-------|
| untagged |            |       |
| RPI1     |            |       |
| RPI1 Sigma |          |       |
| untagged |            |       |
| RPI1 Sigma |          |       |
| RPI1 S288c |          |       |

B

ChIP MIT1pr

|          | S288c^FL08 | Sigma |
|----------|------------|-------|
| untagged |            |       |
| RPI1     |            |       |
| RPI1 Sigma |          |       |
| untagged |            |       |
| RPI1 Sigma |          |       |
| RPI1 S288c |          |       |
Figure 6

| Strain          | S288c<sup>FLO8</sup> | Sigma |
|-----------------|-----------------------|-------|
| RPI1 allele     | S288c                | Sigma | Sigma | S288c |
| λ phosphatase   | -                    | +     | -     | +     | -     | +     |

[Image of gel electrophoresis pattern]
Figure 7