Analysis of Polygenic Mutants Suggests a Role for Mediator in Regulating Transcriptional Activation Distance in *Saccharomyces cerevisiae*

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ABSTRACT Studies of natural populations of many organisms have shown that traits are often complex, caused by contributions of mutations in multiple genes. In contrast, genetic studies in the laboratory primarily focus on studying the phenotypes caused by mutations in a single gene. However, the single mutation approach may be limited with respect to the breadth and degree of new phenotypes that can be found. We have taken the approach of isolating complex, or polygenic mutants in the lab to study the regulation of transcriptional activation distance in yeast. While most aspects of eukaryotic transcription are conserved from yeast to human, transcriptional activation distance is not. In *Saccharomyces cerevisiae*, the upstream activating sequence (UAS) is generally found within 450 base pairs of the transcription start site (TSS) and when the UAS is moved too far away, activation no longer occurs. In contrast, metazoan enhancers can activate from as far as several hundred kilobases from the TSS. Previously, we identified single mutations that allow transcription activation to occur at a greater-than-normal distance from the *GAL1* UAS. As the single mutant phenotypes were weak, we have now isolated polygenic mutants that possess strong long-distance phenotypes. By identification of the causative mutations we have accounted for most of the heritability of the phenotype in each strain and have provided evidence that the Mediator coactivator complex plays both positive and negative roles in the regulation of transcription activation distance.

KEYWORDS transcription; Mediator; polygenic; *Saccharomyces cerevisiae*

THE correct communication between a transcriptional activator and its target gene is of crucial importance to ensure properly regulated transcription. While most fundamental aspects of transcription initiation are conserved throughout eukaryotes, the distance over which transcriptional activation can occur varies greatly between yeast and metazoans. In the compact *Saccharomyces cerevisiae* genome, where intergenic distances are small and upstream activation sequences (UASs) are generally found within 450 base pairs (bp) of the transcription start site (Goffeau *et al.* 1996; Kristiansson *et al.* 2009), it is important that activation occurs over only a short distance to activate the correct target gene. In contrast, in the much larger metazoan genomes, enhancers that activate transcription are often located several kilobases away, with some enhancers as far as a megabase from a target gene (Bulger and Groudine 2011; Buecker and Wysocka 2012; Erokhin *et al.* 2015). While many studies have focused on understanding how enhancers function over a long distance to choose the correct target (Krivega and Dean 2012), there is less understanding of the regulation of transcriptional activation distance in yeast and how it differs from that in metazoans.

Early studies of yeast UAS elements suggested that transcriptional activation distance is limited (Guarente and Hoar 1984; Struhl 1984). More recent work systematically measured the dependence of transcriptional activation on the distance between the *GAL1* UAS and a core promoter (Dobi and Winston 2007) and demonstrated that transcriptional activation by Gal4 diminishes with increasing distance. This study also suggested that activation distance is repressed by particular factors and by chromatin structure, as loss-of-function

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mutations that allow long-distance activation of a reporter were identified in several genes, including *SIN4* and *SPT2*, which regulate transcription, *SPT10*, which regulates histone levels, and the *HTA1-HTB1* gene pair, which encodes histones H2A and H2B.

These results suggested that the control of activation distance in yeast involves the contributions of many factors, an idea supported by two additional results. First, although several mutants were identified that allow long-distance activation, their phenotypes were modest, with only a low level of expression of a reporter for long-distance activation over a distance of 800 bp. Second, attempts were made to isolate stronger mutants by selection for mutations that enhance the phenotype of a *sin4Δ* mutant. This selection successfully resulted in the isolation of a second mutation that strengthened the mutant phenotype in a *sin4Δ* background, but which conferred no detectable phenotype when present as a single mutant (J. Leeman, K. C. Dobi, and F. Winston, unpublished results). The isolation of such an enhancer mutation suggested the existence of other factors that regulate activation distance that might never be found by mutant selections when analysis is restricted to single mutants. Therefore, we have isolated polygenic mutants to study strains with stronger long-distance activation phenotypes.

Classical genetic studies in model organisms usually focus on single mutations to facilitate gene identification and to understand gene function. However, many traits found in nature are polygenic (or complex), caused by the combined effects of mutations in many genes, resulting in a range of phenotypes (Mackay *et al.* 2009; Mackay 2014). In humans, many diseases that have a genetic component are polygenic, including type 2 diabetes, schizophrenia, and hypertension. A major challenge in human genetics today is identifying the causative mutations that contribute to these diseases (Manolio *et al.* 2009; Womack *et al.* 2012).

S. cerevisiae has also been a focus for studies of natural genetic variation and polygenic traits (Liti and Louis 2012), as strains found in nature display a broad range of phenotypic variance (Ehrenreich *et al.* 2009; Liti and Louis 2012). Studies of yeast strain natural variance have identified the causative alleles for a number of polygenic traits including sporulation efficiency (Deutschbauer and Davis 2005; Ben-Ari *et al.* 2006; Gerke *et al.* 2006), high temperature growth (Steinmetz *et al.* 2002), translation efficiency (Torabi and Kruglyak 2011), and wine alcoholic fermentation (Salinas *et al.* 2012). A major advantage of yeast in studies of natural variation caused by multiple mutations is the ability to perform allele replacements, allowing direct tests for causative mutations.

In addition to studies of natural variation in yeast, the isolation and analysis of polygenic mutants has recently been initiated in laboratory studies to analyze specific processes, including resistance to high pH (Romano *et al.* 2010), the origin of multicellularity (Koschwanez *et al.* 2013), and the ability to grow under nutrient limitations (Gresham *et al.* 2008). In our current study, we have isolated polygenic mutants to study the regulation of transcriptional activation distance. Using a dual reporter system, we have isolated three polygenic mutants that allow strong long-distance activation. We have identified causative mutations in the genes *MOT3*, *GRR1*, *MIT1*, *MSN2*, and *PTR3* that contribute to this activation and we show that strong long-distance activation in these mutants is not merely a result of generally stronger transcriptional activation. Furthermore, we have fully recapitulated the mutant phenotype of one of the polygenic mutants from the identified causative mutations and have partially recapitulated the phenotype of the other two. Finally, our results have suggested a role for the Mediator coactivator complex in regulating long-distance activation.

Materials and Methods

S. cerevisiae strains

All *S. cerevisiae* strains used in this study (Supporting Information, Table S1) are isogenic with a *GAL2*$^+$ derivative of S288C (Winston *et al.* 1995) and were constructed by standard allele replacement methods or crosses. All PCR primers are listed in Table S2. The dual reporter strains used to select mutants that allow long-distance activation were derived from previously described strains (Dobi and Winston 2007). These strains contained two reporters constructed within large nonessential open reading frames (ORFs), *bph1*::*kanMX-UAS$_{GAL1}$799-HIS3* and *dug2*::*TRP1-UAS$_{GAL1}$806-URA3*, and *sin4Δ0*::*LEU2*. To construct the *URA3* reporter, the *HIS3* ORF was replaced with the *URA3* ORF. Both reporters contain the *HIS3* TATA element, transcription start sites, and transcription termination sequence. Allele replacements, either to correct a mutant allele to wild type or the reverse, were done by a two-step transformation method, using strains in which the *URA3* ORF was deleted from the *dug2* reporter. Allele replacements to correct mutant alleles to wild type were made in strains FY3056, FY3057, and FY3058. Mutant reconstructions to replace wild-type alleles with mutations were done in *sin4Δ0*::*LEU2* strains. The first step in each case was the integration of *URA3* at the relevant site. DNA containing *URA3* was made by PCR using pRS406 (Sikorski and Hieter 1989) as template DNA. For replacement of *URA3* by wild-type alleles, PCR fragments ~500 bp in length were synthesized using DNA from strain FY3046, the wild-type parental strain, as a template. PCR fragments were used to transform the *URA3* transformants, followed by selection for 5-FOA resistance. For replacement of *URA3* by mutant alleles, PCR fragments ~500 bp in length were amplified from genomic DNA prepared from strains 1.2 (FY3049; for mutations in *MOT3*, *GRR1*, and *SGM1*), 2.3a (FY3052; for mutations in *MIT1*, *PTR3*, and YOR019W), or 2.3b (FY3053; for a mutation in *MSN2*). Correct alleles were all verified by DNA sequencing. Complete deletions of the *MOT3*, *GRR1*, *MIT1*, *PTR3*, and *MSN2* ORFs were constructed by replacement with *URA3*, using pRS406 as template DNA to generate PCR fragments with *URA3*. Strains with the longer reporter distances of 1397 bp and 2027 bp contain the *HIS3* reporter in the same

location as in the 799-bp reporter, with the *GAL1* UAS located at the greater distances. For construction of strains that overexpressed *MED2*, *MED3*, and *CDK8*, plasmids were used from a previously described collection (Hu *et al.* 2007). All spot tests and most Northerns contain two control strains: a strain with a wild-type *HIS3* gene (strain FY76, labeled "*HIS3*") and a strain with the two reporters but no mutations that contribute to long-distance activation (strain FY3045, labeled "*SIN4*").

Media

Rich (YPD) and synthetic complete (SC) dropout media were prepared as previously described (Rose *et al.* 1990). SC Gal and SC −His Gal media contained 2% galactose as the carbon source. YP raffinose contained 2% raffinose as the carbon source and YP Gal contain 2% galactose. Specified concentrations of 3-aminotriazole (3AT) were added to SC −His Gal medium.

Selection and screen to isolate polygenic mutants that allow long-distance activation

Starting with strains FY3046 and FY3047, strains of opposite mating type that each contain *sin4Δ*, we selected for mutants that allow long-distance activation using the selection and screening procedure diagrammed in Figure 1A. For each round of selection, 10 independent cultures of each strain were grown in liquid YPD overnight at 30°. From each culture, 200 μL were plated onto each of two SC −His Gal 3AT plates, one of which was UV irradiated (Winston 2008). The first round of mutants was selected using 1 mM 3AT, the second round using 5 mM 3AT, and the third round using 10 mM 3AT. Plates were incubated at 30° and colonies that grew were purified on SC −His Gal 3AT. Mutants 1.1, 2.1, 2.2, and 2.3a were isolated spontaneously, while strains 1.2 and 2.3b were isolated after UV mutagenesis. Strains 2.3a and 2.3b were derived from the same original colony of FY3047, but were separated for the third and final round of selection. Mutant candidates were retested by dilution spot tests for 3AT resistance and for whether the His^+ phenotype was dependent on galactose. Mutants showing stronger 3AT resistance than the parent strains were then tested for *HIS3* and *URA3* messenger RNA (mRNA) levels by Northern analysis. Mutants that showed increased mRNA levels for both reporters relative to the parent strain were used for further selection.

Bulk segregant analysis and high-throughput sequencing of yeast segregant pools to identify candidate mutations

To identify candidate mutations, we performed bulk segregant analysis (Brauer *et al.* 2006; Wenger *et al.* 2010). First, pools of strains were generated for bulk segregant analysis by backcrossing polygenic mutant 1.2 to FY3046 and backcrossing the other two polygenic mutants, 2.3a and 2.3b, to FY3047. For each cross, approximately 300 tetrads were dissected. The long-distance activation phenotypes of the progeny were tested by replica plating to SC −His Gal media with 0 mM, 1 mM, 5 mM, and 10 mM 3AT. The mutant pools for each cross were composed of segregants with the same 10-mM 3AT-resistance phenotype as in the polygenic mutant parent. The wild-type pools for each cross were composed of segregants that had the phenotype of the *sin4Δ0* single mutant, which is sensitivity to 1 mM 3AT. Each pool contained at least 40 segregants, based on prior bulk segregant analysis studies (Brauer *et al.* 2006; Wenger *et al.* 2010), and the mutant and wild-type pools from each backcross contained the same number of segregants: 45 segregants (1.2), 48 segregants (2.3a), and 42 segregants (2.3b). Strain 1.2 segregants were all derived from complete tetrads while strain 2.3a and 2.3b segregants were from a mixture of complete and incomplete tetrads. To prepare genomic DNA for sequencing, a culture of each segregant was grown in YPD to saturation at 30°. For each pool, 1 ml of each culture was combined, the pooled culture was split into six fractions, and DNA was purified from each fraction as previously described (Rose *et al.* 1990).

Genomic libraries were prepared for DNA sequencing using the Illumina TruSeq DNA PCR-Free Sample Preparation kits. Using PCR-free sample preparation prevents additional mutations from being introduced during library amplification. The resulting libraries were sequenced on a single lane of an Illumina HighSequation 2000, producing 108-nucleotide single end reads and greater than 80-fold coverage for each library. The sequence reads, compiled in a FASTQ file, were mapped to the *S. cerevisiae* S288C genome using BWA (Li and Durbin 2009) producing a SAM file. Using SAMTools (Li *et al.* 2009), this SAM file was converted to a BAM file, which was searched for single-nucleotide polymorphisms (SNPs) using Freebayes (Garrison and Marth 2012). Freebayes generated a VCF file containing the SNPs. We then calculated, using a PERL script (File S1), the frequency of all SNPs in two matched pools: (1) segregants with the wild-type phenotype and (2) segregants with the mutant phenotype. SNPs with a higher frequency in the mutant pool were further considered as candidate SNPs that might be causative. There was no strict threshold cutoff; however, SNPs were considered candidates if they were present at a frequency >50% in pool 2. The SNPs were subsequently computationally verified by observing their frequency and location in the BAM file containing all of the aligned reads, using Integrative Genomics Viewer (IGV) (Robinson *et al.* 2011). Once the SNPs were confirmed by these methods, they were further confirmed to be present in the mutant parent and absent in the wild-type parent by Sanger DNA sequencing.

Dilution spot tests

Cultures were grown in liquid YPD at 30° to saturation. The next day, the cells were pelleted and resuspended in sterile water to adjust them to the same concentration based on OD_{600}. Then 10-fold serial dilutions were made in sterile water into a microtiter dish. The dilutions were spotted on different media, and the plates were incubated at 30° for 4 days unless otherwise noted.

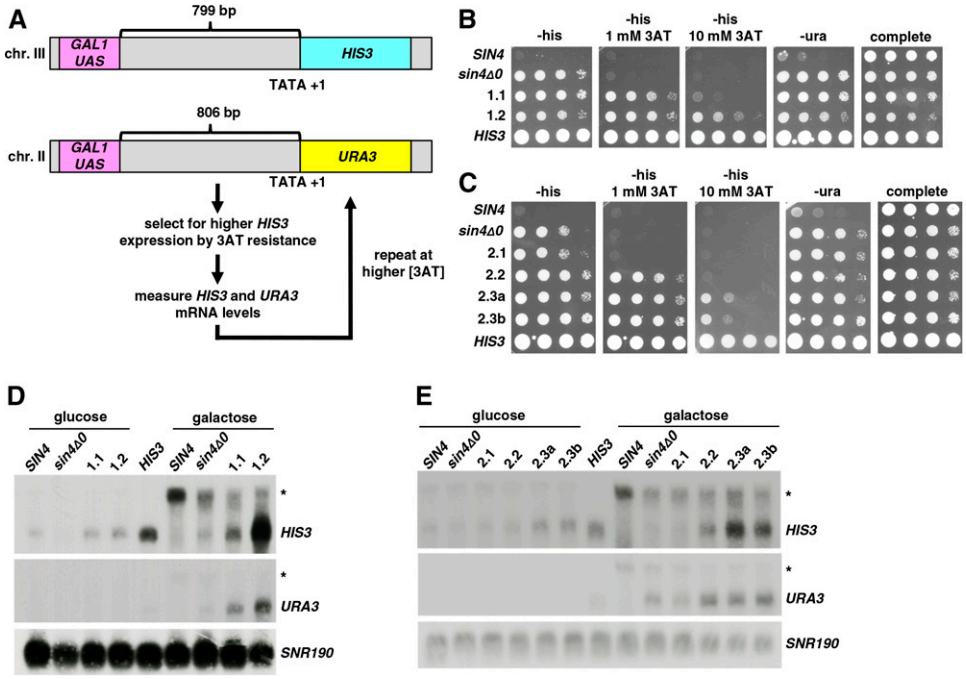

Figure 1 Isolation of polygenic mutants that allow long-distance transcriptional activation. (A) The diagram illustrates the steps for selection and screening for mutants that allow transcription of reporters with the *GAL1* UAS at a distance that is normally nonpermissive for activation. (B and C) Dilution spot tests to assay expression of the long-distance reporters after each round of mutant isolation. All plates contain galactose as the carbon source and the indicated levels of 3AT. (D and E) Northern analysis was done to measure the levels of *HIS3* and *URA3* RNAs after each round of mutant isolation. *SNR190* served as the loading control. The Northern presented in E was chosen for best overall quality, where the other two replicates showed greater increases in *URA3* levels in 2.3a and 2.3b as compared to 2.2 (data not shown). The asterisks note the longer transcripts that likely initiate near the *GAL1* UAS (see text and Dobi and Winston 2007). The two control strains in this and later figures are a strain with a wild-type *HIS3* gene (strain FY76, labeled "*HIS3*") and a strain with the two reporters but no mutations that contribute to long-distance activation (strain FY3045, labeled "*SIN4*").

Northern analysis

RNA isolation and Northern hybridization experiments were performed as previously described (Ausubel *et al.* 1991). Strains were grown to ~1 × 10^7 cells/ml in YP raffinose and then divided with one part shifted to YPD and the other shifted to YP Gal for 1 hr each. Northern hybridization analysis was conducted with probes to the coding regions of *HIS3* (+400 to +651, where +1 is the ATG), *URA3* (+206 to +727), and *SNR190* (+1 to +190), which served as a loading control. Each Northern experiment was done at least three times with independent cultures.

Western analysis

Western blots were performed on a carboxy-terminally tagged Med3-FLAG fusion that was constructed by standard procedures, using a FLAG tag with a glycine linker (Funakoshi and Hochstrasser 2009). For Western blot analysis, protein extracts were prepared as follows. Briefly, 10-ml cultures grown to ~1 × 10^7 cells/ml in YP raffinose, were shifted to YP Gal for 1 hr, and then the cells were pelleted and frozen at −70°. Cells were resuspended in 1 ml cold dH_2O, 150 μl of YEX buffer (7.4 g NaOH, 7.5 ml β-mercaptoethanol, 92.5 ml dH_2O) was added, and samples were incubated on ice for 10 min. A total of 150 μl of cold 50% TCA/50% dH_2O was added and samples were incubated on ice for 10 min. Cells were pelleted at 12,500 rpm for 5 min at 4° and the supernatant was carefully removed. Samples were resuspended in 200 μl of 2× Laemmli buffer (2.5% SDS, 60 mM Tris base, 6.25 mM EDTA, pH 8.0, protease inhibitors), boiled for 5 min, and quantitated by Bradford assay to assure equal loading. To each sample, 5× Laemmli buffer B was added [50% glycerol 100 mM Tris base 0.1–0.05% (w/v), Bromophenol blue, 400 mM dithiothretol 10% 2-mercaptoethanol], and samples were heated to 95° for 5 min before loading on an 8% SDS-polyacrylamide gel. Primary antibodies for Western blot analysis were anti-FLAG antibody (Sigma M2) or anti-Pgk1 (Life Technologies). Secondary antibodies were LI-COR goat antimouse. Imaging was performed using the AERIUS Automated Imaging System and were quantified using ImageJ.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (Helmlinger *et al.* 2008). ChIP of Med3 was performed using a Med3-FLAG fusion. The strains used for ChIP were deleted for the endogenous *GAL1* UAS to allow detection of Med3 association with the *GAL1* UAS at the long-distance reporter. Briefly, 50-ml cultures grown to ~1 × 10^7 cells/ml in YP raffinose, were shifted to YP Gal for 1 hr and were then cross-linked (1% formaldehyde, 30 min). Next, the cells were broken open by bead beating to achieve 100% cell lysis. Wild-type cells were lysed after six rounds of bead beating and the *mot3-1 grr1-1 sin4Δ0* mutant required nine rounds of bead beating to achieve the same amount of lysis. The chromatin fraction was isolated and sheared to 100- to 300-bp fragments using a Bioruptor sonicator (Diagenode). Immunoprecipitations (IPs) were performed overnight at 4° with 1 μg of anti-FLAG antisera (Sigma M2). IPs were coupled to 50 μl of protein G-Sepharose beads (GE Healthcare Life Sciences) at 4° for 4 hr. The beads

were washed and eluted, and the eluate was reverse cross-linked overnight at 65° and incubated with proteinase K and glycogen for 2 hr at 37°. DNA was purified by phenol-chloroform extraction and precipitated in ethanol overnight at 80°. ChIP DNA was analyzed by qPCR on the Stratagene MX3000P cycler. Each sample was analyzed in triplicate and quantified by comparison to a standard curve (10-fold serial dilutions of input DNA). Relative percent IP (%IP) values were calculated by dividing the %IP (calculated as IP/input) at the regions of interest by the %IP at an intergenic background locus.

Microarray experiments

RNA was purified, labeled, and hybridized to Agilent expression microarrays (8× 15k custom printed yeast arrays) (AMADID 017566) as previously described (McIsaac *et al.* 2011). Reference RNA from strain FY3054 was labeled with Cy3 and experimental RNA was labeled with Cy5. RNA from FY3054 grown in YP raffinose was used as the reference for all arrays. After washing, arrays were scanned using Agilent Feature Extractor software version 9.5. Genes that had flagged features marked as unreliable were excluded from data analysis; this resulted in the analysis of 5610 genes. The raw signal intensity values were floored to a value of 350 (values of <350 were set to 350). The log2 ratio was calculated using the floored data values. Each microarray was performed twice for wild type (FY3054), 1.2 (FY3056), 2.3a (FY3057), 2.3b (FY3058), *sin4Δ mot3-1 grr1-1* (FY3061), and *sin4Δ mot3-1 mit1-1* (FY3067). Analysis of the log2 ratios revealed that one of the 1.2 replicates and one of the 2.3a replicates were unusable, so these were discarded from analysis. For the remaining replicates, the log2 ratios were averaged and these averages were used for subsequent analysis. We used a two-fold cutoff for calling RNA levels as increased or decreased (log2 value of 1 or −1). Hierarchical clustering was performed using Cluster 3.0 with average linkage using the Pearson correlation distance as the metric of similarity between genes (de Hoon *et al.* 2004). K-means clustering was performed with MultiExperiment Viewer using a setting of 10 clusters and using Euclidean distance as the metric of similarity between genes (Saeed *et al.* 2003, 2006).

Data availability

File S1 describes the code used to analyze the sequencing results. Yeast strains are listed in Table S1 and are available upon request. Table S2 lists all oligonucleotide sequences used. Table S3 lists crosses used for bulk segregant analysis. Table S4 lists sequencing coverage. Table S5 lists causative mutant candidates. Table S6 lists microarray results. Table S7 describes enrichment for transcriptional effects on adjacent gene pairs. Figure S1 shows evidence for disomy in two mutants. Figure S2 presents Western analysis of Med3 levels. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) (Edgar *et al.* 2002) and are accessible through GEO series accession no. GSE72093.

Results

Selection of polygenic mutants that allow increased transcription activation distance

To study the regulation of transcriptional activation distance, we decided to isolate polygenic mutants, based on two sets of previous results. First, the single mutants that we previously identified that allow long-distance activation (Dobi and Winston 2007) have modest phenotypes, with weak expression of the *HIS3* reporter. Second, additional pilot mutant selections demonstrated that we could obtain double mutants with stronger phenotypes, but when the mutations were separated genetically, one of the mutations often caused no detectable phenotype. Therefore, we rationalized that the isolation of polygenic mutants would allow the isolation of mutants with stronger phenotypes as well as the identification of additional genes involved in the regulation of activation distance.

We selected for polygenic mutants that allow long-distance activation using the dual reporter strains described in *Materials and Methods* (Figure 1A). Each strain also contained a *sin4Δ0* mutation, as *sin4* mutants were the strongest and most frequent class of single mutants previously isolated (Dobi and Winston 2007). Two reporters were used, as selection using only a single reporter often identified *cis*-acting mutations and chromosomal rearrangements in addition to the desired class of *trans*-acting mutations (C. T. Reavey and F. Winston, unpublished results).

Beginning with single colonies, one from strain FY3046 and the other from strain FY3047, multiple rounds of selection and screening for increased long-distance activation were performed (*Materials and Methods*), resulting in the isolation of three strains that allowed strong long-distance activation, named 1.2 (FY3049), 2.3a (FY3052), and 2.3b (FY3053). The latter two strains derived from the same original colony of FY3046, but were separated for the third and final round of selection. Additional rounds of selection were attempted for each, but did not yield any mutants with increased long-distance activation. We will refer to these strains as 1.2, 2.3a, and 2.3b to denote their lineage relationship.

To analyze the strength of long-distance activation at each round of mutant isolation, we assayed both 3AT resistance (a phenotypic readout of *HIS3* expression levels) and the levels of the *HIS3* and *URA3* reporter mRNAs by Northern analysis. Our results show that the mutants generally show an increased level of 3AT resistance after each round of selection (Figure 1, B and C), as well as increased *HIS3* and *URA3* mRNA levels (Figure 1, D and E). In addition, all the mutants require galactose for their phenotypes, showing that, as expected, the activation of the reporter is galactose dependent. In addition to the *HIS3* and *URA3* transcripts, we also see a longer transcript for each reporter. Previous 5′-RACE experiments showed that the *HIS3* long transcript initiates proximal to the UAS (Dobi and Winston 2007). Although the level of this long transcript is variable and we do not understand its regulation, we note that transcripts initiating near enhancers have been observed in metazoans (Ashe *et al.*

1997; Kong *et al.* 1997; Carninci *et al.* 2005; Manak *et al.* 2006). Overall, our results demonstrate the successful isolation of mutants with strong long-distance activation phenotypes.

Identification of candidate causative mutations by bulk segregant analysis and whole-genome sequencing

To identify candidate causative mutations, we used bulk segregant analysis followed by whole-genome sequencing (*Materials and Methods*). Briefly, the three polygenic mutant strains, 1.2, 2.3a, and 2.3b, were crossed to *sin4Δ0*, the resulting diploids were sporulated, and ~300 tetrads were dissected and analyzed for each cross (Table S3). Consistent with multiple mutations causing the strong 3AT-resistance phenotype, the progeny displayed a continuum of 3AT-resistance phenotypes ranging from sensitivity to 1 mM 3AT (the phenotype of the *sin4Δ0* single mutant parent) to resistance to 10 mM 3AT (the phenotype of the polygenic mutant parent).

To identify candidate mutations based on DNA sequence, progeny with each parental phenotype were pooled, each pool was sequenced (Table S4), and candidate mutations were identified as those present in the mutant (3AT-resistant) pool at a level >50%, while present in the 3AT-sensitive pool at ~50%. We set the threshold below 100% for the mutant pool as we considered the possibility that different combinations of causative mutations might be sufficient to confer the strong mutant phenotype. By this reasoning, every mutation might not be required in every segregant to produce the strong mutant phenotype. To qualify as a candidate, we also required that the putative mutant allele be present in the original polygenic mutant parent and the wild-type allele to be present in the *sin4Δ* single mutant parent. Using these requirements, we established a list of genes to be tested for causality (Table S5).

Identification of causative mutations in lineage 1

To test the candidate mutations present in strain 1.2 for causality, we replaced them with the wild-type allele and then tested the mutant phenotypes. In these experiments, strains contained only the *HIS3* reporter as the allele replacement method required that *URA3* not be expressed. Our results showed that replacement of either *grr1-1* or *mot3-1* causes a reduction in 3AT resistance (Figure 2A). In contrast, replacement of another mutation, *sgm1-1*, with the wild-type allele does not alter 3AT resistance. The *sgm1-1* mutation was likely present at a high frequency in the mutant pool because *SGM1* is linked to *GRR1*. Sanger sequencing of *GRR1* and *MOT3* showed that the *grr1-1* arose in the first round of selection while *mot3-1* arose in the second round (Figure 3). These results demonstrate that *grr1-1* and *mot3-1* are causative.

As a complementary approach, we tested whether we could recapitulate the original mutant phenotypes of strain 1.2 by integrating the causative mutations into a wild-type background. To do this, we constructed a *sin4Δ0 mot3-1 grr1-1* triple mutant, as well as all possible combinations of double mutants, and analyzed the strains for their phenotypes by 3AT resistance and Northern analysis. Significantly, the

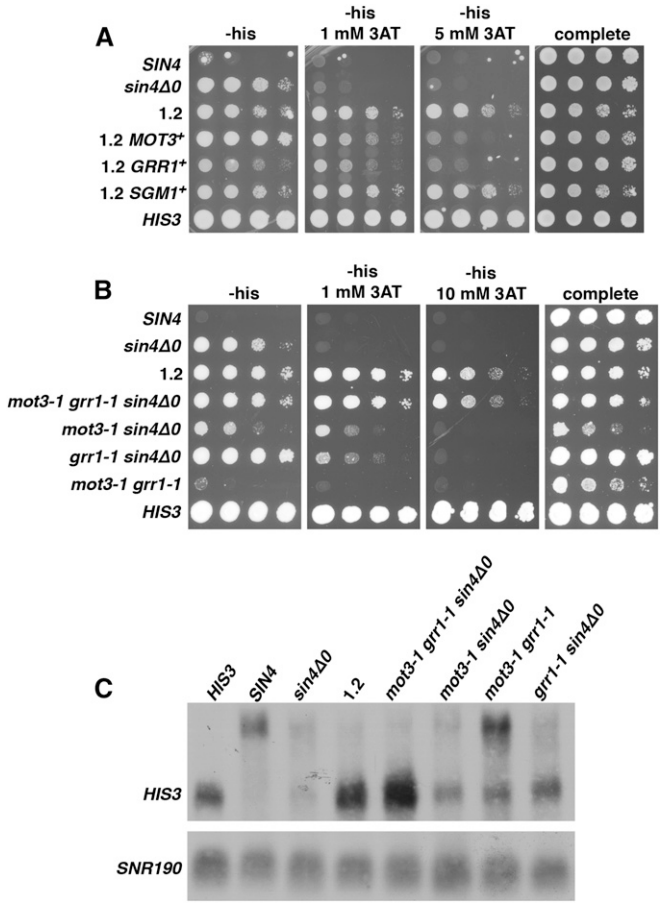

Figure 2 Tests to identify causative mutations in lineage 1. (A) Allele replacements were done to test each candidate mutation. Starting with polygenic mutant 1.2, three mutations *mot3-1*, *grr1-1*, and *sgm1-1* were each replaced with the wild-type allele and were then tested for long-distance activation by expression of the *HIS3* reporter on the plates shown. All plates contain galactose as the carbon source. Plates were incubated at 30° for 4 days. (B) To test the sufficiency of the identified causative mutations, beginning with the *sin4Δ* strain, FY3055, strains were constructed that contained each mutation singly (data not shown), as different combinations of double mutants, and as the *mot3-1 grr1-1 sin4Δ0* triple mutant. All plates contain galactose as the carbon source. Plates were incubated at 30° for 4 days. (C) Northern analysis was done to measure *HIS3* RNA levels in the reconstructed mutants and to compare the levels to that in the original polygenic mutant 1.2.

sin4Δ0 mot3-1 grr1-1 triple mutant is resistant to 10 mM 3AT and shows a similar level of *HIS3* mRNA expression as strain 1.2 (Figure 2, B and C), strongly suggesting that these three mutations account for the strain 1.2 mutant phenotypes. In contrast, all of the double mutant combinations have much weaker phenotypes, especially the *mot3-1 grr1-1* mutant. From these results, we conclude that the *sin4Δ0*, *mot3-1*, *and grr1-1* mutations together can account for the strain 1.2 mutant phenotype.

Identification of causative mutations in lineage 2

Similar steps were taken with strains 2.3a and 2.3b to determine the causative mutations. By replacement with wild-type alleles, our results showed that *ptr3-1* is causative in both

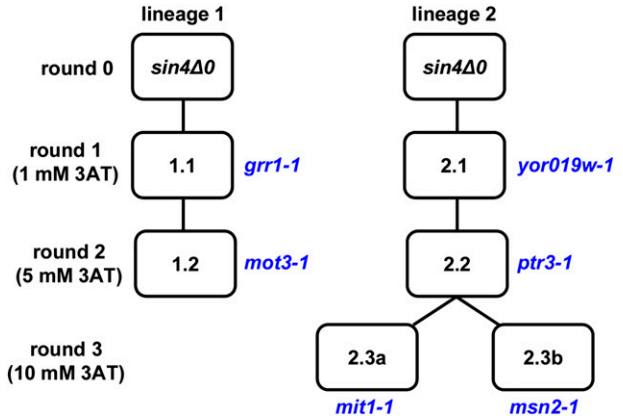

Figure 3 Lineages of the polygenic mutants. Diagrammed are the steps in the isolation of the polygenic mutants 1.2, 2.3a, and 2.3b. Shown in blue are the causative mutations isolated after each round. The strains 2.3a and 2.3b were derived independently from 2.2 as described in the text.

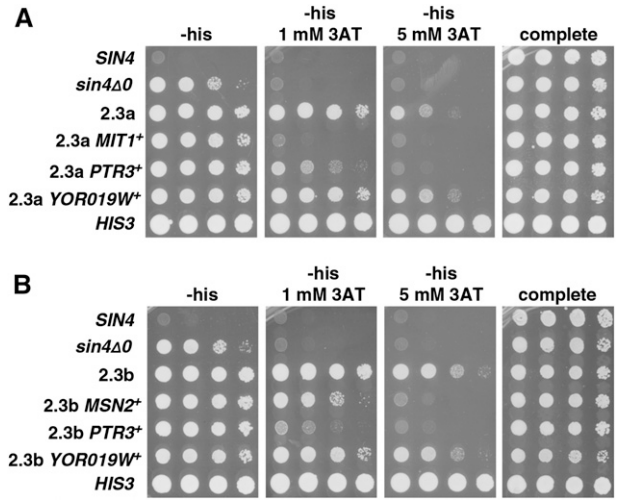

Figure 4 Tests to identify causative mutations in lineage 2. (A and B) Allele replacements were done to test each candidate mutation. Starting with polygenic mutants 2.3a and 2.3b, the mutations *mit1-1*, *ptr3-1*, *yor019w1-1*, and *msn2-1* were each replaced with the wild-type allele and were then tested for long-distance activation by expression of the *HIS3* reporter on the plates shown. All plates contain galactose as the carbon source.

strains 2.3a and 2.3b, while *mit1-1* is causative in 2.3a and *msn2* in 2.3b (Figure 4). Our results indicate that *yor019w-1* contributes very weakly to the mutant phenotype, as replacement with the wild-type allele causes a very modest reduction in growth on 3AT media in the 2.3b strain background. Sanger sequencing of each relevant gene in strains 2.1, 2.2, 2.3a, and 2.3b showed that *yor019w-1* arose in the first round of selection, *ptr3-1* in the second round, and *mit1-1* and *msn2-1* independently in the third round (Figure 3).

As we did for lineage 1, we tested whether combining the identified causative mutations can recapitulate the phenotypes of strains 2.3a and 2.3b. Our results show that, while each reconstructed strain can activate the long-distance reporter, the phenotypes are more modest than those of the original strains 2.3a and 2.3b (Figure 5). Therefore, we are able to partially recapitulate the phenotype of 2.3a and 2.3b from the known causative mutations, although there is some missing heritability.

Some of the missing heritability can be accounted for by aneuploidy. Analysis of the DNA sequencing results for strains 2.3a and 2.3b suggests that they are both disomic for chromosome III, the chromosome that contains the *HIS3* reporter, and that only 2.3a is also disomic for chromosome XIV (Figure S1). Additional genetic analysis showed that the chromosome III disomy contributes to the strength of the phenotype in these strains, based on increased 3AT resistance, although it does not completely account for the missing heritability (data not shown).

The mit1-1 and mot3-1 mutations do not cause loss of function

To test whether the causative mutations confer their phenotypes due to loss of function, we deleted each causative gene in the polygenic mutants, replacing the original mutant allele, and tested the mutant phenotypes. From these results, the deletion alleles *grr1Δ0*, *ptr3Δ0*, and *msn2Δ0* each confer the same level of 3AT resistance as the original alleles (Figure 6), suggesting that the original alleles cause a loss of function. As *MSN2* has a paralogue, *MSN4*, we also tested the effect of *msn4Δ0* and of *msn2Δ0 msn4Δ0* in a *sin4Δ0*, *ptr3-1*, *and yor019w-1* background. We found that *msn4Δ0* confers a level of 3AT resistance similar to *msn2Δ0*, while the *msn2Δ0 msn4Δ0* combination allows greater 3AT resistance (data now shown), consistent with distinct roles for the two proteins (Berry and Gasch 2008). Therefore, loss of the factors Grr1, Ptr3, Msn2, and Msn4 contributes to long-distance activation.

In contrast, *mot3Δ0* and *mit1Δ0* do not confer the same mutant phenotype as the *mot3-1* and *mit1-1* alleles, respectively, as each deletion mutation weakens the original mutant phenotype (Figure 6). In the case of the *mot3Δ0* mutation, this is a partial effect, as we see three strengths of mutant phenotypes (*mot3-1* > *mot3Δ0* > *MOT3*$^+$), while in the case of the *mit1* alleles, the difference is clearer as *mit1Δ0* confers the same phenotype as *MIT1*$^+$. Both the *mot3-1* and *mit1-1* mutations encode changes in the DNA binding domains of these transcription factors (Madison *et al.* 1998; Cain *et al.* 2012), as *mot3-1* causes an N388H change in a position that directly contacts DNA (Grishin *et al.* 1998), while *mit1-1* causes an H187R change at a position that likely causes a conformational change of the DNA binding domain (Lohse *et al.* 2014) (M. B. Lohse and A. D. Johnson, personal communication). These results demonstrate that *mit1-1* and *mot3-1* are not loss-of-function alleles.

Combinatorial analysis of transcription factor mutations

We were interested in testing for genetic interactions between the mutations isolated in the different lineages. To do this, we constructed a new series of mutant strains, testing combinations of the *mot3*, *mit1*, and *msn2* mutations. Our

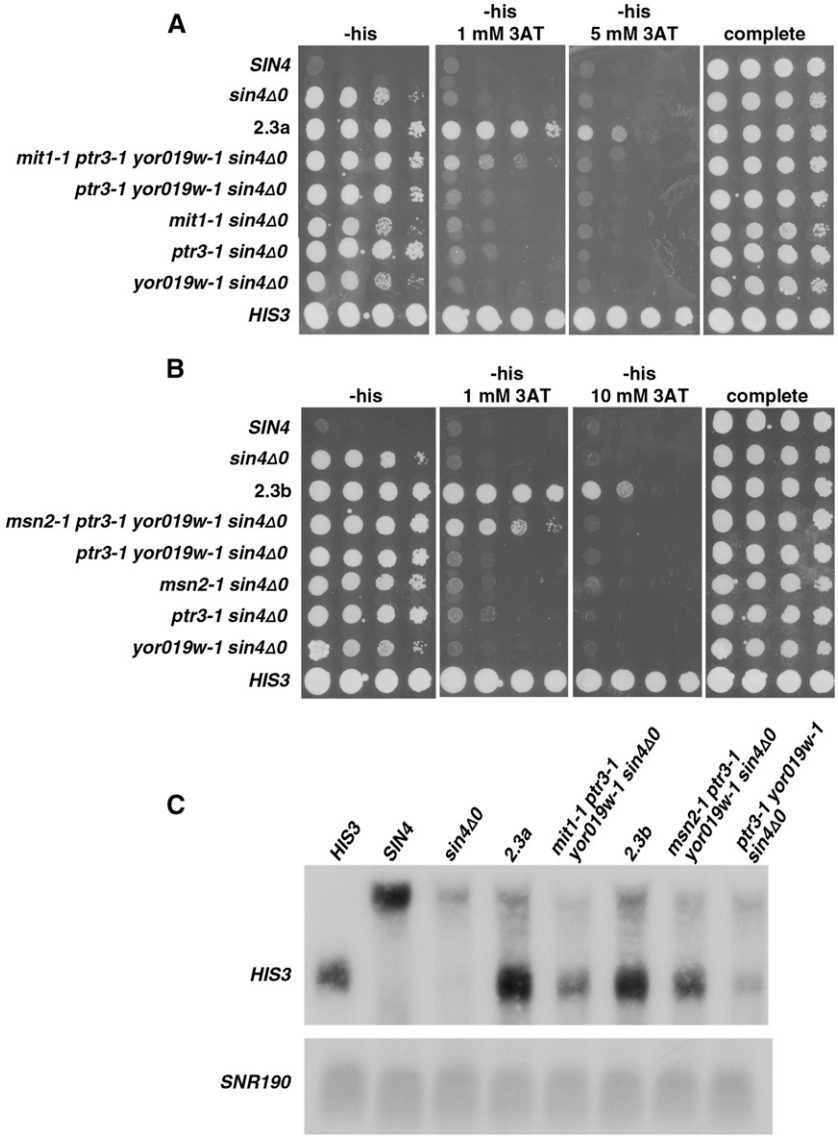

Figure 5 Attempts to reconstruct the phenotypes of strains 2.3a and 2.3b from causative mutations. (A and B) To test the sufficiency of the identified causative mutations for strains 2.3a and 2.3b, beginning with the *sin4Δ* strain, FY3055, strains were constructed that contained each mutation singly (data not shown), as different combinations of double and triple mutants, and as the quadruple mutants. All plates contain galactose as the carbon source. Pictured is the highest 3AT concentration with strong 2.3a or 2.3b growth on day 4. (C) Northern analysis was done to measure *HIS3* RNA levels in the reconstructed mutants and to compare the levels to that in the original polygenic mutants 2.3a and 2.3b.

results (Figure 7) show that combining the mutations in a *sin4Δ* background both increases 3AT resistance and causes corresponding increases in *HIS3* mRNA levels. These results show that interactions between the mutations are not confined to the lineages in which they arose.

Analysis of shorter and longer activation distances

Previous studies have suggested a correlation between activation strength and activation distance (Carey *et al.* 1990), suggesting that our mutants might allow long-distance activation by a general increase in Gal4 activation strength. By this model, we expect to observe increased activation in our mutant backgrounds even over a short distance. However, when we tested this idea by analyzing expression of a *HIS3* reporter where activation occurs over a distance of only 280 bp, we found that the highest level of activation occurs in the wild-type strain, with a lower level in the polygenic mutant and an even lower level in a *sin4Δ* mutant (Figure 8A). These results show that the mutants do not act through an overall increase in activation strength and may specifically have acquired the ability to activate over long distances.

To test whether the mutant strains are able to activate transcription at distances greater than the 799 bp in our reporter, we constructed and tested two longer reporters of 1397 bp and 2027 bp. All three reconstructed polygenic mutants strains show the ability to activate, albeit modestly, at these longer distances (Figure 8B). Surprisingly, there appears to be stronger activation with the 2027-bp reporter strain than with the 1397-bp reporter strain. Attempts to detect transcription at these long distances by Northern analysis were not successful, presumably due to the low level of the transcripts. We additionally tested the polygenic mutants at each distance for growth using glucose as the carbon source and did not see any growth on SC −His selective medium (data not shown), suggesting that there are not other activator sites within the reporter contributing to the

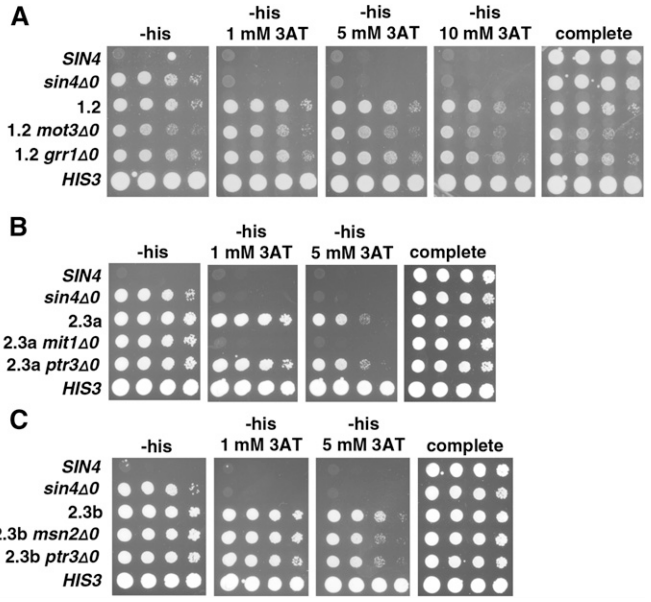

Figure 6 Tests of of loss-of-function mutations. To test whether the mutations isolated in polygenic mutants 1.2, 2.3a, and 2.3b cause a loss of function, each causative allele was replaced by a complete deletion of the gene and the strains were compared to the original polygenic mutants. All plates contain galactose as the carbon source.

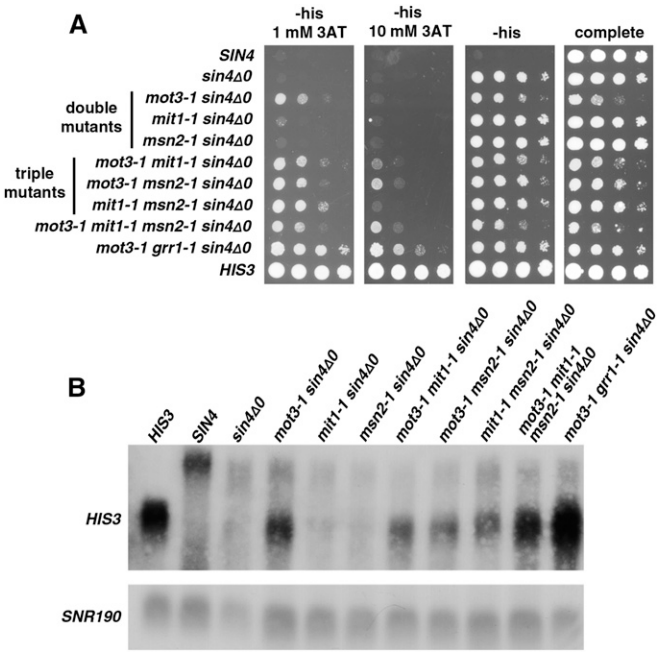

Figure 7 Combinations of mutations from different lineages allow long-distance activation. (A) To test whether mutations isolated in different lineages can function together to allow long-distance activation, we constructed the strains shown in the figures and tested them for 3AT resistance by spot tests. All plates contain galactose as the carbon source. (B) The same strains were also assayed for *HIS3* RNA levels by Northern analysis. Strains are compared to the original *sin4Δ0* parent and the reconstructed lineage 1.2 strain, *mot3-1 grr1-1 sin4Δ0*.

phenotype in the 1397- and 2027-bp reporter strains. Our results strongly suggest that the polygenic mutants are able to activate transcription at distances up to 2 kb.

A role for the Mediator coactivator complex in regulation of activation distance

Several lines of evidence suggest a role for the Mediator coactivator complex in the regulation of transcription activation distance. Our initial studies of long-distance activation showed that Sin4 and Rgr1 of Mediator repress long-distance activation (Dobi and Winston 2007; De Santa *et al.* 2010; Kim *et al.* 2010; K. C. Dobi, C. T. Reavey, and F. Winston, unpublished results). While our current study did not isolate additional mutations in genes encoding Mediator components, a recent study (Gonzalez *et al.* 2014) has connected Grr1, a ubiquitin ligase, to Mediator function, as it suggested a role for Grr1 in regulating the stability of Med3 of Mediator. That study demonstrated that Grr1 normally destabilizes Med3, dependent upon Med3 phosphorylation by Cdk8, another Mediator component. In addition, previous studies have provided biochemical and genetic evidence that Mediator is required for activation by Gal4 (Park *et al.* 2000; Jeong *et al.* 2001; Bryant and Ptashne 2003; Kuras *et al.* 2003; Larschan and Winston 2005; Reeves and Hahn 2005).

Based on these previous studies, we tested for roles for Med3, Cdk8, and other Mediator components in long-distance activation by double mutant analysis with *sin4Δ*. Both the *sin4Δ0 med2Δ0* and *sin4Δ0 med3Δ0* double mutants show complete loss of long-distance activation (Figure 9A). In contrast, the *sin4Δ0 cdk8Δ0* double mutant shows a modest increase in long-distance activation relative to the *sin4Δ0* single mutant. These results suggest that Med2 and Med3 are required for long-distance activation and Cdk8 plays a role in repressing it.

Given the proposed role of Grr1 in controlling Med3 levels, we tested whether the levels of Mediator proteins may play a role in long-distance activation. To do this, we overexpressed *MED2*, *MED3*, and *CDK8*. Our results show that overexpression of *MED3* in some of our polygenic mutants led to an increase in long-distance activation, based on the level of 3AT resistance (Figure 9B), while overexpression of *MED2* and *CDK8* had no detectable effect (data not shown). The overexpression of *MED3* in wild-type and *sin4Δ0* strains had no effect, suggesting that overexpression of *MED3* enhances long-distance activation only in a *grr1-1* mutant background.

If Med3 levels affect long-distance activation, we might also expect to see an increase in either Med3 levels or Med3 recruitment to the long-distance reporter in our mutants that are permissive for long-distance activation. To test these possibilities, we measured Med3 levels and performed ChIP of FLAG-Med3. By Western analysis, we did not detect any change in Med3 levels in a mutant that allows strong long-distance activation (Figure 9C, Figure S2). However, by ChIP, we measured a modest 1.5- to 2-fold increase in the recruitment of Med3 in the *mot3-1 grr1-1 sin4Δ0* mutant (Figure 9D). This increase is not seen in the *sin4Δ* single mutant, suggesting that all three mutations are required for elevated Med3 recruitment. Additionally, we see a 1.5-fold increase of Med3

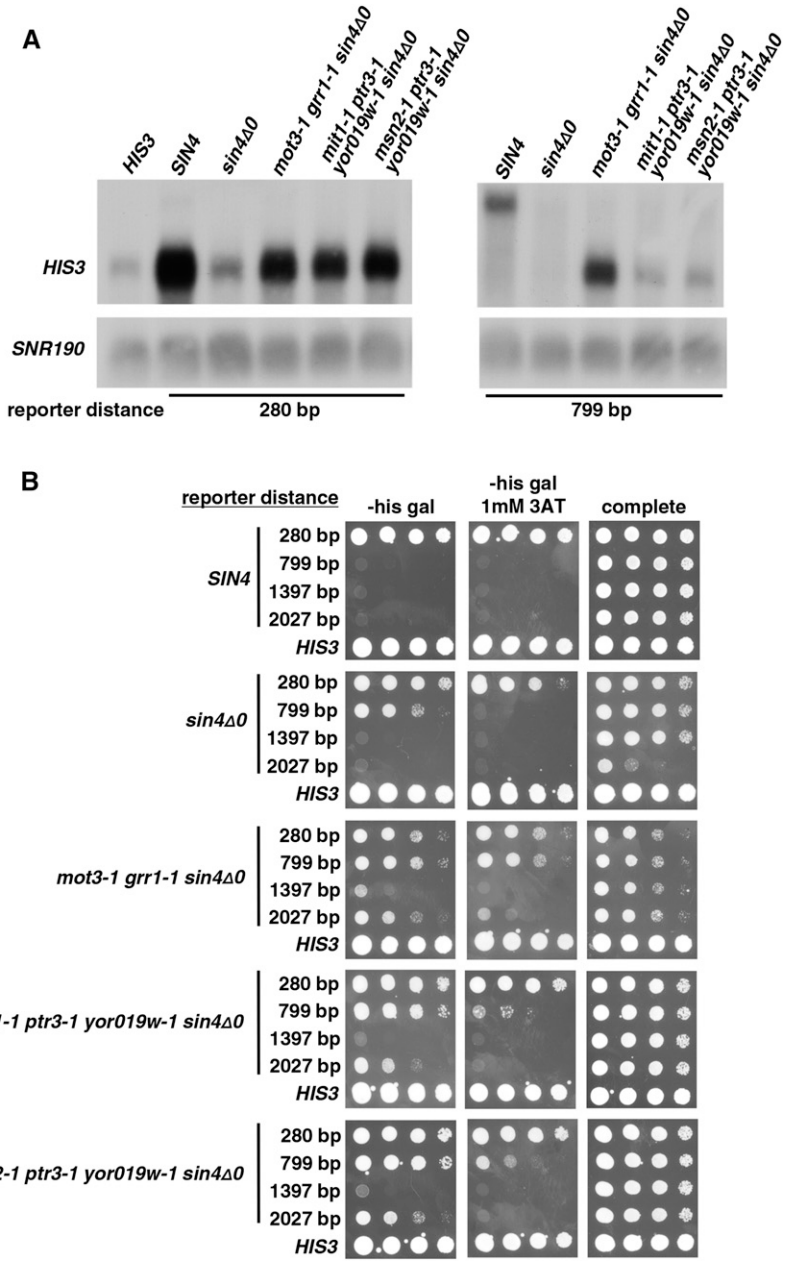

Figure 8 Tests of activation at shorter and longer distances. (A) Northern analysis was performed to test the polygenic mutants for activation of a reporter with a shorter distance of 280 bp and to compare the relative levels of activation to the long-distance reporter with a distance of 799 bp. (B) Spot tests were done to measure the level of 3AT resistance in strains that carry reporters with four different distances between the *GAL1* UAS and the *HIS3* TATA: 280 bp, 799 bp, 1397 bp, and 2027 bp. For reasons we do not understand, we reproducibly observed poor growth of the *sin4Δ* mutant with the 2027-bp reporter on complete medium. All plates contain galactose as the carbon source. Plates were incubated at 30° for 3 days.

at the *CCW12* UAS, a known Med3 binding site, suggesting Mediator recruitment may also be increased at other sites in the genome. These results suggest that the combination of mutations in the polygenic mutant results in an increased level of Mediator recruited to the UAS, which may be important in long-distance activation.

Discussion

In this study, we have isolated and analyzed yeast mutants that are capable of strong transcriptional activation over distances much greater than can occur in wild type. To identify these strains, we used a dual reporter system that allowed multiple rounds of selection and screening for an increased level of long-distance activation, resulting in the isolation of polygenic mutants. Importantly, these mutants do not generally increase all transcriptional activation; over the short distance that is typical of most yeast promoters, the mutants actually activate more poorly than wild type, suggesting they have become specifically permissive for long-distance activation. Although the different polygenic mutants acquired distinct sets of mutations, combining mutations from different lineages also allows strong long-distance activation, showing that specific mutant interactions are not necessary for this phenotype. Given the approximately additive contribution of each mutation, long-distance activation can be considered to be a quantitative trait. Our results suggest that many factors contribute to regulate long-distance activation in yeast where it is normally repressed, and possibly, in larger eukaryotes where long-distance activation normally occurs.

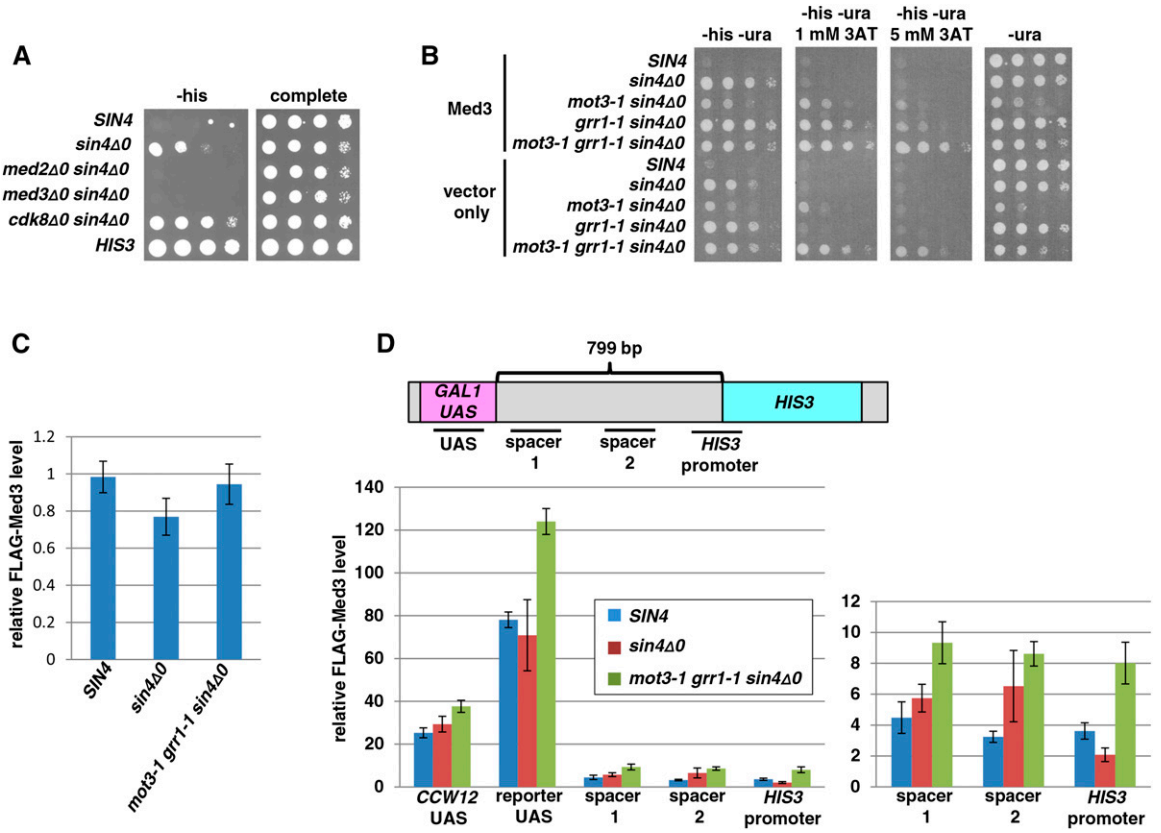

Figure 9 Tests for the requirements of Mediator components. (A) Spot tests to determine the requirement for Mediator components Med2, Med3, and Cdk8. All plates contain galactose as the carbon source. Plates were incubated at 30° for 3 days. (B) Spot tests to determine the phenotype of overexpression of *MED3* from a high-copy-number plasmid. All plates contain galactose as the carbon source. (C) To determine the level of Med3 protein in our strains, Western analysis was performed. Shown is a bar graph showing the mean and standard error of three experiments normalized to the levels of Pgk1. *T*-tests indicated no significant difference between Med3 levels in WT, *sin4Δ0*, and *mot3-1 grr1-1 sin4Δ0* for $P < 0.05$. (D) At the top is a diagram of the *HIS3* reporter showing the regions assayed for Med3 association by ChIP. At the bottom left is a bar graph showing the ChIP results. The *CCW12* promoter is a previously characterized site where Mediator is known to associate. Shown are the mean and standard error for six experiments (WT and *mot3-1 grr1-1 sin4Δ0*) or five experiments (*sin4Δ0*). *T*-tests indicated a significant difference between WT and *mot3-1 grr1-1 sin4Δ0* at all primer sets for $P < 0.05$. Shown at the bottom right are the same data for three of the regions, plotted on a different scale to make the results easier to view.

Our results have implicated the Mediator coactivator complex in the regulation of transcriptional activation distance and suggest that some of the mutations that we have isolated strengthen Mediator with respect to long-distance activation. Interestingly, human Mediator has been shown to be required for long-distance activation by enhancers in human embryonic stem cells (Kagey *et al.* 2010). Our current results, as well as our previous studies (Dobi and Winston 2007; K. C. Dobi, C. T. Reavey, F. Winston, unpublished results) show that the ability of Mediator to activate transcription over a long distance is normally repressed by the Mediator components Sin4, Rgr1, and Cdk8 and is dependent upon the Mediator components Med2 and Med3. Interestingly, previous results demonstrated a positive role for Cdk8 at the *GAL1* UAS (Larschan and Winston 2005), suggesting that the role of Cdk8 may depend upon the distance between the *GAL1* UAS and the target gene. Our results are also consistent with earlier results that showed positive roles for Med2, Med3, and Gal11/Med15 in activation by Gal4 (Piruat *et al.* 1997; Lee *et al.* 1999; Myers *et al.* 1999; Bryant and Ptashne 2003; Kuras *et al.* 2003; Reeves and Hahn 2005; Ansari and Morse 2012, 2013). Finally, previous results showed that the tail domain of Mediator (Med2, Med3, and Med15) can be recruited independently of the rest of Mediator in a *sin4Δ* mutant (Zhang *et al.* 2004; He *et al.* 2008; Ansari *et al.* 2009, 2012). Our results, showing a modestly increased level of Med3 recruitment have not distinguished between recruitment of the tail domain or the entire complex. Tests of additional Mediator components will shed light on the role of the rest of the complex in the regulation of activation distance.

Our isolation of a *grr1* mutation also implicates Mediator based on recent evidence that Grr1 destabilizes Med3 in a Cdk8-dependent fashion (Gonzalez *et al.* 2014). However, in contrast to that study, we did not detect elevated levels of Med3 in a *grr1Δ* background. This difference may be because we measured Med3 levels with the *MED3* gene in single copy, while Gonzalez *et al.* (2014) made their observations when *MED3* was overexpressed from a plasmid. Since we failed to

detect a change in Med3 levels, Grr1 may normally repress long-distance activation by controlling the level of other Mediator components or by a mechanism independent of Mediator.

The requirement for Mediator for long-distance activation in yeast suggests a possible mechanism for activation, as Mediator is required for the enhancer to loop to the core promoter in human embryonic stem cells (Kagey *et al.* 2010). To date, we have little evidence either for or against looping as a mechanism in yeast long-distance activation. Our previous results show that looping does occur at our long-distance reporter; however, it was detected in both wild-type and mutant strains (Dobi and Winston 2007). Possibly, looping is necessary but not sufficient for long-distance activation. Tracking, another proposed mechanism for long-distance activation by enhancers, is unlikely, as we previously showed that long-distance activation still occurs in the presence of a terminator between the UAS and core promoter (Dobi and Winston 2007). Clearly, additional experiments are required to elucidate the mechanism for the long-distance activation that occurs in our mutants, including tests for a role of cohesin, which interacts with Mediator to generate looping between enhancers and core promoters in human cells (Kagey *et al.* 2010).

The roles of the other factors identified by our mutations remain to be determined. Three of the factors, Mot3, Mit1, and Msn2, are site-specific DNA-binding transcriptional regulators. While the *msn2* mutation clearly causes loss of Msn2 function, the *mot3* and *mit1* mutations are not loss-of-function mutations. Both the *mot3-1* and the *mit1-1* alleles are predicted to cause changes in their DNA binding domains, which could result in a novel function for either transcription factor, causing direct or indirect effects on transcriptional activation at the long-distance reporters. The isolation of the *ptr3-1* mutation was surprising, as Ptr3 is known to be important for nutrient sensing and transport (Forsberg *et al.* 2001; Forsberg and Ljungdahl 2001). Our results suggest the possibility of additional functions for Ptr3. Conceivably, the *ptr3-1* mutation or other mutations we identified could affect RNA stability instead of synthesis.

An important question is whether our polygenic mutants allow long-distance activation from endogenous UAS elements in the yeast genome. To begin to address this question, we measured mRNA levels in several of our mutants using microarrays after growth in three carbon sources: glucose, galactose, and raffinose (data not shown). Our results showed that all of the mutants have altered mRNA levels for a large number of genes, including both increased and decreased levels, and that the set of affected genes significantly overlaps between the three lineages of polygenic mutants (Reavey 2013) (Table S6). As our reporter uses the Gal4 activator, we specifically looked at the genes adjacent to the *GAL* genes for elevated levels in galactose-grown cells, but did not observe any increase. However, our results show that the number of adjacent gene pairs with increased mRNA levels is significantly greater than would be predicted if the effects were random (Table S7), suggesting possible cases of long-distance activation.

Our isolation and analysis of polygenic mutants that allow long-distance activation, as well as other studies that have taken a similar polygenic approach (for example, Gresham *et al.* 2008; Romano *et al.* 2010; Koschwanez *et al.* 2013), shows that this is a feasible genetic approach to study topics in yeast biology that might not otherwise be accessible. In our case, we were able to find a phenotype that is apparently not possible in single mutants. Furthermore, we identified genes relevant to the regulation of transcription activation distance that would not have been identified by limiting our studies to single mutants. Given the straightforward methods available to identify causative mutations in yeast, we believe that this type of approach will be useful for studying other aspects of gene regulation and yeast biology.

Acknowledgments

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Supplementary information

Analysis of polygenic mutants suggests a role for Mediator in regulating transcriptional activation distance in *Saccharomyces cerevisiae*

Caitlin T. Reavey*, Mark J. Hickman‡, Krista C. Dobi*[1], David Botstein†[2], and Fred Winston*

File S1

PERL script to calculate the frequency of all SNPs in two matched pools

```
# Usage: cerevisiae_VCF_diff <vcf-file> <min-counts> <min-fraction>
# Input file must be a VCF file created from two samples (e.g., WT and mutant).
# When calling the function, the user must specify the minimum reads needed in
each sample
# (this study used 10) and the minimum fraction of an alternative allele
observed in either
# of the two samples (this study used 0.5).
# This program will ask which column is the WT and which column is the mutant.
# Output file is a tab-delimited file with same name as input file, plus the
date.
# Output file contains the allele descriptions and their counts.
# "FRACTION_DIFF_REF" shows the fraction difference of the reference allele: WT
minus mutant.
# The closer this value is to 1 (or -1), the greater the difference between WT
and mutant.

#!/usr/bin/perl
use warnings;
use strict;
use List::Util qw( min max );

my $input = shift;
my $date = ((localtime)[5]+1900)."_".((localtime)[4]+1)."_".((localtime)[3]);
my $output = $input.$date.".txt";

open IN, $input or die $!;
open OUT, ">$output" or die $!;
select OUT;

my $min_num = shift;
my $min_fraction = shift;

my
%chr=(chrI=>1,chrII=>2,chrIII=>3,chrIV=>4,chrV=>5,chrVI=>6,chrVII=>7,chrVIII=>8,

      chrIX=>9,chrX=>10,chrXI=>11,chrXII=>12,chrXIII=>13,chrXIV=>14,chrXV=>15,ch
rXVI=>16,chrMito=>17,"2-micron"=>18);
my %column;

$/="\n";

my $labels = join "\t", qw/CHROM POS REF_ALLELE ALT_ALLELE1 ALT_ALLELE2
ALT_ALLELE3 NUM_REF_WT NUM_ALT1_WT NUM_ALT2_WT NUM_ALT3_WT NUM_REF_MUT
NUM_ALT1_MUT NUM_ALT2_MUT NUM_ALT3_MUT FRACTION_DIFF_REF/;
print $labels,"\n";

my $WT_column = "-1";
my $mutant_column = "-1";

while (<IN>) {
      my @row = split /\t/, $_;
```

```
my @new_row;

#  Meta-information lines
if ($row[0] =~ /^##/) {next;}

# header line:  determine order of columns
elsif ($row[0] =~ /^#[A-Za-z0-9]/) {
      my $count=0;

      for (@row) {
            print STDOUT "$count: $_\n";
            $column{$_} = $count;
            $count++;
      }
      print STDOUT "Which column is the WT sample? ";
      chomp ($WT_column = <STDIN>);
      print STDOUT "Which column is the mutant sample? ";
      chomp ($mutant_column = <STDIN>);
      next;
}

# data line (i.e., with mutation)
else {
      $new_row[0] = $chr{$row[$column{"#CHROM"}]};
      $new_row[1] = $row[$column{POS}];
      $new_row[2] = $row[$column{REF}];

      my @MUTATIONS = split /,/, $row[$column{ALT}];
      if (!defined$MUTATIONS[0] || ($MUTATIONS[0] eq "."))
{$new_row[3] = "";}
      else {$new_row[3]=$MUTATIONS[0];}
      if (!defined$MUTATIONS[1] || ($MUTATIONS[1] eq "."))
{$new_row[4] = "";}
      else {$new_row[4]=$MUTATIONS[1];}
      if (!defined$MUTATIONS[2] || ($MUTATIONS[2] eq "."))
{$new_row[5] = "";}
      else {$new_row[5]=$MUTATIONS[2];}

      my %format_column;
      my @format = split /:/, $row[$column{FORMAT}];
      my $count=0;
      my $RA="RA"; my $AA="AA";
      for (@format) {
            $format_column{$_} = $count;
            if ($_ eq "RO")        { $RA = "RO"; }
            if ($_ eq "AO")        { $AA = "AO"; }
            $count++;
      }

      if ($row[$WT_column] =~ /:/) {
            my @WT = split /:/, $row[$WT_column];
            chomp (@WT);
            $new_row[6] = $WT[$format_column{$RA}];
            my @AA1 = split /,/, $WT[$format_column{$AA}];
```

```
                  if (!defined$AA1[0] || ($AA1[0] eq "."))      {$new_row[7] =
0;}
                  else {$new_row[7]=$AA1[0];}
                  if (!defined$AA1[1] || ($AA1[1] eq "."))      {$new_row[8] =
0;}
                  else {$new_row[8]=$AA1[1];}
                  if (!defined$AA1[2] || ($AA1[2] eq "."))      {$new_row[9] =
0;}
                  else {$new_row[9]=$AA1[2];}
            }
            else {
                  @new_row[6..9] = ("?","?","?","?");
            }

            if ($row[$mutant_column] =~ /:/) {
                  my @MUT = split /:/, $row[$mutant_column];
                  chomp (@MUT);
                  $new_row[10] = $MUT[$format_column{$RA}];
                  my @AA2 = split /,/, $MUT[$format_column{$AA}];
                  if (!defined$AA2[0] || ($AA2[0] eq "."))      {$new_row[11] =
0;}
                  else {$new_row[11]=$AA2[0];}
                  if (!defined$AA2[1] || ($AA2[1] eq "."))      {$new_row[12] =
0;}
                  else {$new_row[12]=$AA2[1];}
                  if (!defined$AA2[2] || ($AA2[2] eq "."))      {$new_row[13] =
0;}
                  else {$new_row[13]=$AA2[2];}
            }
            else {
                  @new_row[10..13] = ("?","?","?","?");
            }

      }

      my $WT_sum = 0;
      my $mut_sum = 0;

      for (@new_row[6..9]) {
            if ($_ ne "?") {$WT_sum=$WT_sum+$_;}
      }
      for (@new_row[10..13]) {
            if ($_ ne "?") {$mut_sum=$mut_sum+$_;}
      }

      if ( $WT_sum >= $min_num && $mut_sum >= $min_num ) {
            if ( ( ( max(@new_row[7..9]) / $WT_sum) >= $min_fraction) ||
            ( ( max(@new_row[11..13]) / $mut_sum) >= $min_fraction) ) {
                  $new_row[14] = ($new_row[6]/$WT_sum)-($new_row[10]/$mut_sum);
                  my $line = join "\t", @new_row;
                  print $line, "\n";
            }
      }

}
```

Table S1. *S. cerevisiae* strains

Strain	Genotype
FY76	*MAT***a** *lys2-128δ*
FY3045	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX*
FY3046 (2.0)	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX*
FY3047 (1.0)	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX*
FY3048 (1.1)	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX grr1-1*
FY3049 (1.2)	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX mot3-1 grr1-1 sgm1-1 rim8-1 tma108-1 sgf73-1*
FY3050 (2.1)	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX yor019w-1*
FY3051 (2.2)	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX ptr3-1 yor019w-1*
FY3052 (2.3a)	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX mit1-1 ptr3-1 yor019w-1*
FY3053 (2.3b)	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3-natMX msn2-1 ptr3-1*

	yor019w-1
FY3054	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806*
FY3055	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3*
FY3056	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 grr1-1 sgm1-1 rim8-1 tma108-1 sgf73-1*
FY3057	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mit1-1 ptr3-1 yor019w-1*
FY3058	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 msn2-1 ptr3-1 yor019w-1*
FY3059	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3- 1*
FY3060	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 grr1-531*
FY3061	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 grr1-1*
FY3062	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UASGAL1806 mot3-1 grr1-1*
FY3063	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UASGAL1 799-HIS3 dug2Δ::TRP1-UASGAL1806 mot3-1 msn2-1*
FY3064	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UASGAL1 799-HIS3 dug2Δ::TRP1-UASGAL1806 msn2-1 sin4Δ0*

FY3065	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UASGAL1 799-HIS3 dug2Δ::TRP1-UASGAL1806 mit1-1 msn2-1*
FY3066	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UASGAL1 799-HIS3 dug2Δ::TRP1-UASGAL1806 mit1-1 sin4Δ0*
FY3067	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UASGAL1 799-HIS3 dug2Δ::TRP1-UASGAL1806 mot3-1 mit1-1*
FY3068	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UASGAL1 799-HIS3 dug2Δ::TRP1-UASGAL1806 mot3-1 mit1-1 msn2-1*
FY3069	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2Δ::TRP1-UAS_{GAL1}806 MIT1 ptr3-1 yor019w-1*
FY3070	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2Δ::TRP1-UAS_{GAL1}806 mit1-1 PTR3 yor019w-1*
FY3071	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2Δ::TRP1-UAS_{GAL1}806 mit1-1 ptr3-1 YOR019W*
FY3072	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2 Δ::TRP1-UAS_{GAL1}806 MSN2 ptr3-1 yor019w-1*
FY3073	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2 Δ::TRP1-UAS_{GAL1}806 msn2-1 PTR3 yor019w-1*
FY3074	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2 Δ::TRP1-UAS_{GAL1}806 msn2-1 ptr3-1 YOR019W*
FY3075	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2Δ::TRP1-UAS_{GAL1}806 mot3Δ0::URA3 grr1-1*
FY3076	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS_{GAL1} 799-HIS3 dug2Δ::TRP1-UAS_{GAL1}806 mot3-1 grr1Δ0::URA3*

FY3077	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mit1Δ0::URA3 ptr3-1 yor019w-1*
FY3078	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mit1-1 ptr3Δ0::URA3 yor019w-1*
FY3079	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 msn2Δ0::URA3 ptr3-1 yor019w-1*
FY3080	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 msn2-1 ptr3-Δ0::URA3 yor019w-1*
FY3081	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$280-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806*
FY3082	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$1397-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806*
FY3083	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$2027-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806*
FY3084	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$280-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3*
FY3085	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$1397-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3*
FY3086	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$2027-HIS3 dug2 Δ::TRP1-UAS$_{GAL1}$806-URA3*
FY3087	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$280-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 grr1-1*
FY3088	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$1397-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 grr1-1*

FY3089	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$2027-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 grr1-1*
FY3090	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$280-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mit1-1 ptr3-1 yor019w-1*
FY3091	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$1397-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mit1-1 ptr3-1 yor019w-1*
FY3092	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$2027-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mit1-1 ptr3-1 yor019w-1*
FY3093	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$280-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 msn2-1 ptr3-1 yor019w-1*
FY3094	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$1397-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 msn2-1 ptr3-1 yor019w-1*
FY3095	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$2027-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 msn2-1 ptr3-1 yor019w-1*
FY3096	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 UAS$_{GAL1}$-10Δ::hphMX*
FY3097	*MATα his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 UAS$_{GAL1}$-10Δ::hphMX MED3-FLAG::natMX*
FY3098	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 UAS$_{GAL1}$-10Δ::hphMX sin4Δ0::LEU2*
FY3099	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 UAS$_{GAL1}$-10Δ::hphMX sin4Δ0::LEU2 MED3-FLAG::NatMX*
FY3100	*MAT**a** his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3*

	dug2Δ::TRP1-UAS$_{GAL1}$806 UAS$_{GAL1}$-10Δ::hphMX sin4Δ0::LEU2 mot3-1 grr1-1
FY3101	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 lys2-128δ bph1Δ::kanMX-UAS$_{GAL1}$799 dug2 Δ::TRP1-UAS$_{GAL1}$806 UAS$_{GAL1}$-10Δ::hphMX sin4Δ0::LEU2 mot3-1 grr1-1 MED3-FLAG::NatMX*
FY3102	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 MOT3 grr1-1 sgm1-1 rim8-1 tma108-1 sgf73-1*
FY3103	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 GRR1 sgm1-1 rim8-1 tma108-1 sgf73-1*
FY3104	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806 mot3-1 grr1-1 SGM1 rim8-1 tma108-1 sgf73-1*
FY3105	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$799 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3 ptr3-1*
FY3106	*MAT***a** *his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3 yor019w-1-1*
FY3107	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$799 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3 ptr3-1 yor019w-1*
FY3108	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3 mit1-1 ptr3-1 yor019w-1*
FY3109	*MATα his3Δ200 ura3Δ0 leu2Δ0 trp1Δ63 lys2-128δ sin4Δ0::LEU2 bph1Δ::kanMX-UAS$_{GAL1}$ 799-HIS3 dug2Δ::TRP1-UAS$_{GAL1}$806-URA3 msn2-1 ptr3-1 yor019w-1*
FY2650	*MAT***a** *his3Δ200 lys2-128d leu2Δ0 ura3Δ0 trp1Δ63 sin4Δ0::LEU2 med3Δ0::LEU2*

	bph1Δ::kanMX-UAS$_{GAL1}$799-HIS3
FY2651	*MAT***a** *ade8-140 his3Δ200 lys2-128d leu2Δ0 ura3Δ0 trp1Δ63 sin4Δ0::LEU2 med2Δ0::URA3 bph1Δ::kanMX-UAS$_{GAL1}$799-HIS3*
FY2657	*MATα ade8-140 his3Δ200 lys2-128d leu2Δ0 ura3Δ0 trp1Δ63 sin4Δ0::LEU2 cdk8Δ0::URA3 bph1Δ::kanMX-UAS$_{GAL1}$799-HIS3*

Strains isolated during the mutant isolation are indicated by additional numbers in parentheses which indicate the stage when they were isolated. Lineage 1 has strains 1.0, 1.1, and 1.2, and lineage 2 has strains 2.0, 2.1, 2.2, 2.3a, and 2.3b.

Table S2. Oligonucleotides

Oligo	Purpose	Sequence
FO8064	replace *HIS3* ORF with *URA3* ORF in *dug2* reporter	TATACTAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGATGTCGAAAGCTACATATAA
FO8065	replace *HIS3* ORF with *URA3* ORF at *dug2* reporter	TATATATATCGTATGCTGCAGCTTTAAATAATCGGTGTCATTAGTTTTGCTGGCCGCATC
FO6834	Northern probe of *HIS3*	GATCTTTCGAACAGGCCGTA
FO4995	Northern probe of *HIS3*	ACCTTTGGTGGAGGGAACAT
FO481	Northern probe of *URA3*	CCATGGAGGGCACAGTTAAGCCGC
F0483	Northern probe of *URA3*	CCCTTCCCTTTGCAAATAGTCCTC
FO1324	Northern probe of *SNR190*	GGCCCTGATGATAATG
FO1325	Northern probe of *SNR190*	GGCTCAGATCTGCATG
FO3662	delete *URA3* from *dug2* reporter	GTTGGTTGGGTGACCCAACAAATCAT
FO3663	delete *URA3* from	CTATTGAATACTTTAGACAAAATCTCA

	dug2 reporter	
FO10433	integrate *URA3* at site of *mot3* mutation	TCGCAACAAAGACATTTTCTATGCCCTTGGTGCTTAAGCAAGATTGTACTGAGAGTGCAC
FO10434	integrate *URA3* at site of *mot3* mutation	AATTGTTGTAGTTAAAGATGATGTTGTTTTTCTTGAGTTCCTGTGCGGTATTTCACACCG
FO10277	replace *URA3* with *mot3* allele	ACGACAGCACCTAACCATCC
FO10278	replace *URA3* with *mot3* allele	TCTTCATTTTCGGGAGCTGT
FO10271	integrate *URA3* at site of *grr1* mutation	AAAAAAATCCAAGAGTTTCTGGTTGTTATAGAGAAACGTAAGATTGTACTGAGAGTGCAC
FO10272	integrate *URA3* at site of *grr1* mutation	TTTAAGGTTGTCTAGCTCAATTTCGTTCAGTATTTTTTTTCTGTGCGGTATTTCACACCG
FO10273	replace *URA3* with WT *GRR1* allele	CGCTGAACGGGATTGACATA
FO10274	replace *URA3* with WT *GRR1* allele	GCTTGGAATGGCAGTATGCA
FO10435	integrate *URA3* at site of *sgm1* mutation	AAAGAAAAAAATGATATTGAAGAAAAATATCAAACTGCCTAGATTGTACTGAGAGTGCAC

FO10436	integrate *URA3* at site of *sgm1* mutation	GTCTCTCCAAGTCTTTCACTTCCAAAACTGCCTTCGAATGCTGTGCG GTATTTCACACCG
FO10281	replace *URA3* with *sgm1* allele	TCAAAGGCCAACTGGGATAG
FO10282	replace URA3 with *sgm1* allele	TCGCCAAGTTTTGACATTGA
FO10263	integrate *URA3* at site of *mit1* mutation	AAGCTGAAGAGACCTCCATTCAACTCGATTGAAAATTTACAGATTGTA CTGAGAGTGCAC
FO10264	integrate at site *of mit1* mutation	ATTTTGTTTTATATCTTTTACAGAATAATAAGAGACAATACTGTGCGG TATTTCACACCG
FO10265	replace *URA3* with *mit1* allele	GTACTGATTCCGCCGTCATT
FO10266	replace *URA3* with *mit1* allele	TCAGGGGAGTGGAAGAGTTG
FO10431	integrate *URA3* at site of *ptr3* mutation	AACTTCAACCAACTAACAGAGCAATCGTCATCTTCACTCTAGATTGTA CTGAGAGTGCAC
FO10432	integrate *URA3* at site of *ptr3* mutation	TATCAAGAAATCATTGGAAAGTTTGCAAAAACGTTGGCTCCTGTGCG GTATTTCACACCG
FO10269	replace *URA3* with *ptr3* allele	GCACATGATCTGGACGAAGA

FO10270	replace *URA3* with *ptr3* allele	ATGGGGAATCTCGACACGTA
FO10397	integrate *URA3* at site of *yor019w* mutation	CCAGCTTTAAGAATGCTTTGATAGGCAATGGGTCGAAAAAAGATTGT ACTGAGAGTGCAC
FO10398	integrate *URA3* at site of *yor019w* mutation	CTGAGGAAGAATATGGTATTAAAGATTTTCTAAACTTTGTCTGTGCGG TATTTCACACCG
FO10363	replace *URA3* with *yor019w* allele	CCCAGCATTCAAGAAGGAAG
FO10364	replace *URA3* with *yor019w* allele	GCACCGGCACTTTTAACTTT
FO10453	integrate *URA3* at site of *msn2* mutation	AAGTGTCGTAATAGAATCAACAAAGGAACTCGAGGAGAAAAGATTGT ACTGAGAGTGCAC
FO10454	integrate *URA3* at site of *msn2* mutation	GGTCGTTCGTTAGAGTGAACAGATCTCACATGCCTTTTCACTGTGCG GTATTTCACACCG
FO10451	replace *URA3* with *msn2* allele	TATCACCATTTCCCACAGCA
FO10452	replace *URA3* with *msn2* allele	TGACAAGCAAATGGTCGTTC
FO10323	delete *MOT3* with *URA3*	TAGGCAAATAGTAAAGGGACATATCATATTTGAGCAATGAAGATTGT ACTGAGAGTGCAC

FO10324	delete *MOT3* with *URA3*	ATGAGTGGGAAGGGATATTTTGTGTGTCTATAAAGTCTATCTGTGCG GTATTTCACACCG
FO10317	delete *GRR1* with *URA3*	AAACAGTTTTGCGGTTTCCTTTATACTAAGAAGGTCTATAAGATTGTA CTGAGAGTGCAC
FO10318	delete *GRR1* with *URA3*	AAAGGTGTAGTAGGACAGTAAGTATTCAATGAAATACAACTGTGCGG TATTTCACACCG
FO10519	delete *MIT1* with *URA3*	CAACATTTCAACTAGACACAAGGACAACGTAAATTTCTAAAGATTGTA CTGAGAGTGCAC
FO10520	delete *MIT1* with *URA3*	AAGAAAAGAAAATCGGAGTACTTTTTTAAAATATATTTACCTGTGCGG TATTTCACACCG
FO10521	delete *PTR3* with *URA3*	ACACATACATAGGTACGAAATACACAACTGATAGGCGTTCAGATTGT ACTGAGAGTGCAC
FO10522	delete *PTR3* with *URA3*	GTATACCAGAACCTTAAACATACGTATATATTTAGATGCACTGTGCG GTATTTCACACCG
FO7014	delete *MSN2* with *URA3*	TTTTTCAACTTTTATTGCTCATAGAAGAACTAGATCTAAAAGATTGTAC TGAGAGTGCAC
FO7015	delete *MSN2* with *URA3*	TTATGAAGAAAGATCTATCGAATTAAAAAAATGGGGTCTACTGTGCG GTATTTCACACCG
FO7018	delete *MSN4* with *URA3*	TTCGGCTTTTTTTTCTTTTCTTCTTATTAAAAACAATATAAGATTGTAC TGAGAGTGCAC
FO7019	delete *MSN4* with *URA3*	TAGCTTGTCTTGCTTTTATTTGCTTTTGACCTTATTTTTTCTGTGCGGT ATTTCACACCG
FO10461	replace *kanMX-GAL1* with *URA3*	ATTCACAACTTTGGTCAAACGCCTTTACAAATATTTCAGGAGATTGTA CTGAGAGTGCAC

FO10462	replace *kanMX-GAL1* with *URA3*	AAGATTGTCTTCTCAAATATTGGCTTCATTGGAACCTTACCTGTGCG GTATTTCACACCG
FO6828	replace *URA3* with endogenous *BPH1* sequence	TTACCCAGGCGCTGTAAATC
FO6829	replace *URA3* with endogenous *BPH1* sequence	GGTTACCTGAAACCGAATGC
FO2143	integrate *kanMX-GAL1* at 280 bp	TCTGGGACATGATTAATTTCGAACTGAATTCGAGCTCGTTTAAAC
FO2054	integrate *kanMX-GAL1* at 280 bp	AAATATCGTAAAGATAAGCATTCGGGAATTCGAGCTCGTTTAAAC
FO3085	integrate *kanMX-GAL1* at 1397 bp	TATGAAGCTTTGGCTTCCCTGGAAAATGCTGAATTCGAGCTCGTTTA AAC
FO3086	integrate *kanMX-GAL1* at 1397 bp	AGATATGAAGATACTATCATAGCTGAGGAATCATCGCTTCGCTGATT AATTACCC
FO6666	integrate *kanMX-GAL1* at 2027 bp	CGCAAGAATCACGGGGATATGACGGTTAGCTGAATTCGAGCTCGTT TAAAC
FO6667	integrate *kanMX-GAL1* at 2027 bp	AGTTTCCAAACAAAGACTTCGTGCTTTAGG TCATCGCTTCGCTGATTAATTACCC
FO10659	Flag-tag MED3	GAACAATCTGGAATTAGGTGGTCTGAACATGGATTTCTTGCGGATCC CCGGGTTAATTAA
FO10660	Flag-tag MED3	TATACAGATAATTACTATCTTGGATACATAGATGCACCAGGAATTCGA GCTCGTTTAAAC

FO10241	delete endogenous $UAS_{GAL1-10}$ with hphMX	TATACATATCCATATCTAATCTTACTTATATGTTGTGGAAGGGAACAA AAGCTGG
FO10242	delete endogenous $UAS_{GAL1-10}$ with hphMX	GCAGCTTTTCCATTTATATATCTGTTAATAGATCAAAAATTATAGGGC GAATTGG
FO10663	ChIP qPCR *CCW12* UAS	GGAAATCAGACGCCAATAGC
FO10664	ChIP qPCR *CCW12* UAS	GCCACCCTCACCTCACTAAC
F02947	ChIP qPCR reporter UAS	AGGCACATCTGCGTTTCAG
FO10665	ChIP qPCR reporter UAS	ACGCTTAACTGCTCATTGCT
FO10679	ChIP qPCR *BPH1* spacer 1	GCGAAGCGATGAGTAAGGTT
FO10680	ChIP qPCR *BPH1* spacer 1	GCGAAGCCCCTCCAGTATAA
FO2295	ChIP qPCR *BPH1* spacer 2	ATCGTAAAGATAAGCATTCGGT
FO4216	ChIP qPCR *BPH1* spacer 2	TCGTGATAACAGCGCATCTC

FO4993	ChIP qPCR *HIS3* promoter	GGGCTTTCTGCTCTGTCATC
FO5062	ChIP qPCR *HIS3* promoter	CGCAAAAGGTCGCAATATCT
FO10671	ChIP qPCR Chr V untranscribed control	GCTAGGTGAGAGAAAGCAAAGG
FO10672	ChIP qPCR Chr V untranscribed control	TACGATCTTAGTTCCAATGGTGA

Table S3. Crosses for bulk segregant analysis

Cross	Total tetrads	Segregants with strong phenotype	Complete tetrads
FY3049 (1.2) x FY3046	282	66 (6.8%)	172 (61%)
FY3052 (2.3a) x FY3047	297	59 (6.4%)	134 (45%)
FY3053 (2.3b) x FY3047	340	42 (4.1%)	129 (38%)

Table S4. Sequencing coverage of segregant pools

Cross	Segregants	Pool size (segregants)	Number of mapped reads	Fold coverage
FY3049 (1.2) x FY3046	Wild type	45	12,115,678	89
	Mutant	45	12,364,877	85
FY3052 (2.3a) x FY3047	Wild type	48	13,097,764	101
	Mutant	48	14,174,495	97
FY3053 (2.3b) x FY3047	Wild type	42	11,391,104	80
	Mutant	42	38,280,078	260

In each cross, wild-type segregants were those that were sensitive to 1 mM 3AT and mutant segregants were those resistant to 10 mM 3AT.

Table S5. Causal mutant candidates

Mutant strain	Candidate gene	Amino acid change	Causative?	Mutant reads/total read
FY3049 (1.2)	*MOT3*	N388H	Yes	73/74
	GRR1	L181stop	Yes	67/79
	SGM1	L407S	No	79/95
	SGF73	P84L	No	46/69
	TMA108	A458V	No	42/67
	RIM8	P52L	No	46/68
FY3052 (2.3a)	*PTR3*	S363stop	Yes	68/68
	YOR019W	N553K	Weakly	48/97
	MIT1	H187R	Yes	81/81
FY3053 (2.3b)	*PTR3*	N553K	Yes	231/235
	YOR019W	N553K	Weakly	62/259
	MSN2	C652stop	Yes	252/252

Causality was determined by allele replacement for most of the listed mutations as described in Materials and Methods. For three mutations, in *SGF73*, *TMA108*, and *RIM8*, allele replacement was not done. Instead, these mutations were tested during reconstruction of the polygenic mutant and were shown to not confer any mutant phenotype.

Table S6. Number of genes with a two-fold or greater change in RNA levels in the polygenic mutants

		Strain			
	Genes		*sin4Δ0 mot3-1*		
Carbon source	affected	1.2	*grr1-1*	2.3a	2.3b
Glucose	Up	416	182	145	167
	Down	158	79	62	82
Galactose	Up	474	133	146	184
	Down	426	139	78	133
Raffinose	Up	313	152	180	172
	Down	135	84	123	141

Shown for each strain is the number of genes whose RNA levels change by two-fold or more when the strains are grown in YP medium with the indicated carbon sources. The actual strains used are as follows: 1.2, FY3056; *sin4Δ0 mot3-1 grr1-1*, FY3061; 2.3a, FY3057; 2.3b, FY3058.

Table S7. Adjacent gene pairs enrichment

		Promoter Orientation								
		Convergent			Divergent			Tandem		
		E	O	p-value	E	O	p-value	E	O	p-value
Raffinose	1.2	2.75	8	2.10E-03	1.87	15	1.90E-10	6.07	22	1.20E-07
	sin4Δ0 mot3-1 grr1-1	0.613	4	4.30E-04	0.344	4	3.00E-05	0.943	5	4.40E-04
	2.3a	1.08	2	9.50E-02	0.636	5	5.40E-05	1.85	11	6.30E-07
	2.3b	0.873	4	2.10E-03	0.514	8	4.30E-09	1.91	9	3.20E-05
Glucose	1.2	5.77	19	3.00E-06	3.63	18	1.10E-08	4.87	23	4.80E-10
	sin4Δ0 mot3-1 grr1-1	0.760	5	1.40E-04	0.488	3	1.60E-03	0.340	4	2.90E-05
	2.3a	0.726	5	1.10E-04	0.323	3	3.50E-04	0.468	8	2.00E-09
	2.3b	1.02	7	1.20E-05	0.424	5	5.70E-06	0.726	8	8.00E-08
Galactose	1.2	6.07	22	1.20E-07	11.89	24	6.00E-04	14.99	35	2.90E-06
	sin4Δ0 mot3-1 grr1-1	0.943	5	4.40E-04	2.238	9	1.20E-04	0.754	4	1.10E-03
	2.3a	1.85	11	6.30E-07	1.40	8	1.60E-05	1.40	11	3.20E-08
	2.3b	1.91	9	3.20E-05	1.77	10	2.70E-06	2.08	16	1.00E-10

Shown for each strains are the expected number (E) of adjacent gene pairs upregulated given the overall number of upregulated genes, the observed (O) number of upregulated gene pairs, and the p-value for the significance in difference between observed and expected. Analysis was performed for convergent, divergent, and tandem promoter orientation when the strains are grown in YP medium with the indicated carbon sources. The actual strains used are as follows: 1.2, FY3056; *sin4Δ0 mot3-1 grr1-1*, FY3061; 2.3a, FY3057; 2.3b, FY3058.

Supplemental Figure 1

A

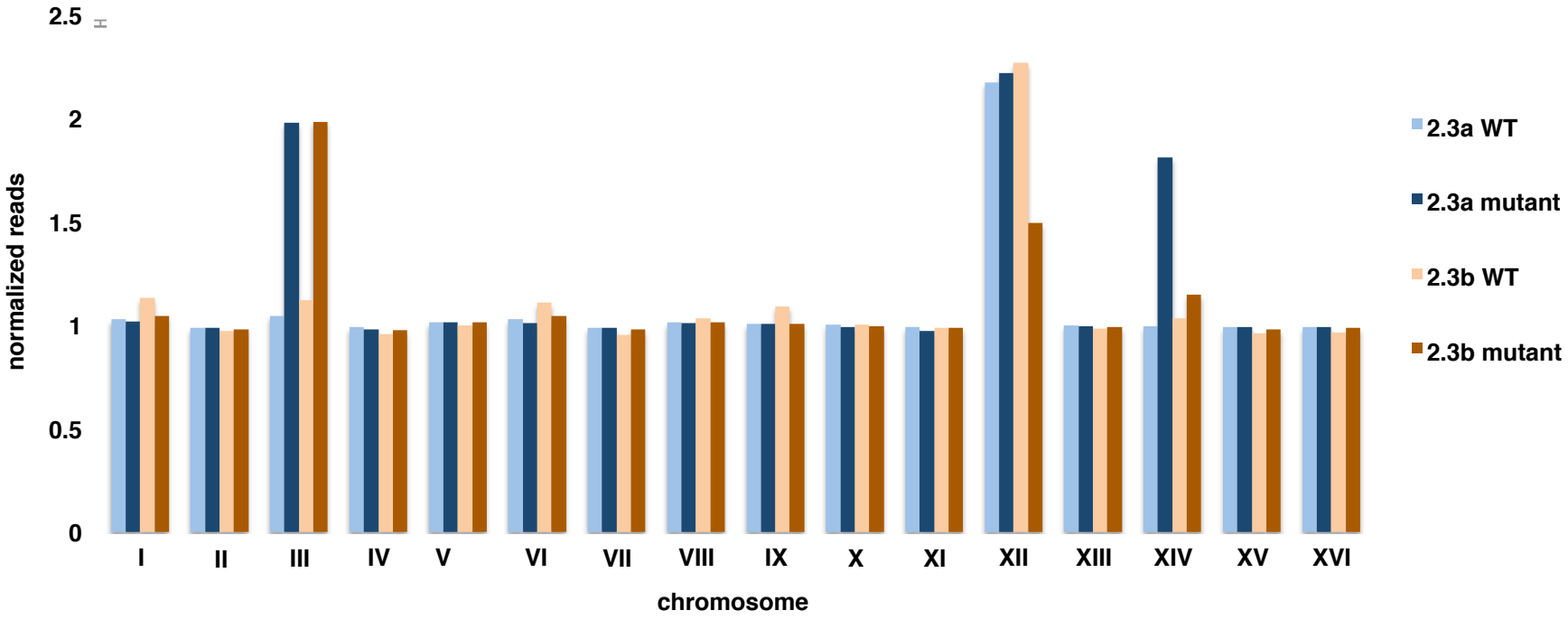

Figure S1. Evidence for disomy in two of the polygenic mutants. (A) Shown are the normalized levels of sequencing reads across each *S. cerevisiae* chromosome. The elevated reads for chromosomes III and XIV are likely caused by disomy for those chromosomes. The elevated reads for chromosome XII are likely an artifact of the repeated rDNA.

Supplemental Figure 2

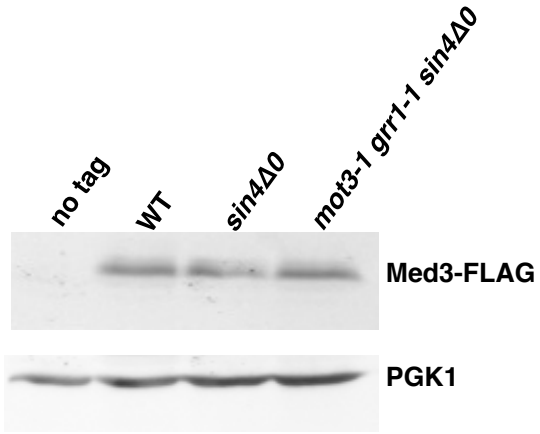

Figure S2. Western analysis of Med3. Shown is a representative western looking at levels of Med3-FLAG. Quantitation of three experiments is shown in Figure 9C.