SIGNALING SPECIFICITY IN THE FILAMENTOUS GROWTH PATHWAY OF *SACCHAROMYCES CEREVISIAE*

by

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Cells convey information through signaling pathways. Distinct signaling pathways often rely on similar mechanisms and may even use the same molecules. With a variety of signals conveyed by pathways that share components, how does the cell maintain the integrity of each pathway?

Budding yeast provides an example of multiple signaling pathways utilizing the same components to transduce different signals. The mating pathway, the high osmolarity glycerol (HOG) pathway and the filamentous growth (FG) pathway each respond to different environmental conditions and generate unique cellular responses. Despite the individuality of the pathways, they each contain a core group of the same signaling proteins. How does the cell generate a variety or responses utilizing the same group of proteins? Both the mating and HOG pathways utilize scaffolding factors that concentrate pathway components to the location of activation and in the case of the mating pathway alter the kinetics of the interaction. In addition, negative regulatory mechanisms operate in both the mating and HOG pathways. These negative regulatory mechanisms are understood in detail for the mating pathway but not for the HOG pathway. Mechanisms for providing specificity for the FG pathway are as yet unknown.

The purpose of this work is to elucidate the mechanisms that provide specificity to the FG pathway. The search for specificity factors was done through both a random mutagenesis screen and a synthetic genetic array screen, looking for mutants in which activation of the FG pathway led to inappropriate activation of the HOG pathway. The

random mutagenesis screen resulted in a large number of mutants that I organized into five complementation groups. The identity of the gene mutated in the largest complementation group was sought using a variety of methods including complementation with the yeast deletion collection and whole genome sequencing. A synthetic genetic array was screened as an alternative method to identify genes necessary for FG pathway specificity. These experiments have resulted in a list of candidate genes, but thus far have not yet led to any discernable mechanism for maintenance of FG pathway specificity.

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CHAPTER I

AN INTRODUCTION TO SIGNALING PATHWAYS AND SPECIFICITY MECHANISMS IN YEAST

Mitogen Activated Protein Kinase (MAPK) pathways are found in a wide variety of eukaryotic organisms where they control responses to numerous physiological signals and developmental cues. The yeast Saccharomyces cerevisiae has five MAPK pathways that individually control the mating response, the response to hyperosmolarity (HOG), aspects of the response to nutrient deprivation (FG), cell wall integrity (CWI) and spore wall assembly. Each of these pathways responds to unique environmental stimuli and generates a unique response. In wild type cells the signal in each pathway is insulated from other pathways; activation of one pathway does not lead to cross-activation of other pathways.

Introduction to Yeast MAPK Signaling Pathways

The mating pathway, the most thoroughly studied of all yeast MAPK pathways, controls mating by haploid cells. Haploid yeast cells can reproduce vegetatively, replicating their chromosomes and budding off new daughter cells (hence budding yeast) when nutrients are abundant. However, they can also undergo cell fusion to generate a diploid cell. This mating process involves two distinct haploid cell types, called **a** and alpha (α), each of which produces a secreted peptide pheromone and expresses a cell surface receptor for the cognate pheromone. Binding of pheromone to receptor creates a transmembrane signal that activates a trimeric G protein. Activation of the trimeric G

protein requires exchange of GDP for GTP in the α subunit and dissociation of the βγ subunits from the complex [1]. This dissociation initiates the signaling cascade comprised of a series of kinases and a number of other pathway components. The kinases in the mating pathway are Ste20, Ste11, Ste7 and Fus3 [2]. Cofactors that play a role in the pathway are Cdc42, Ste50, Ste5 and Dig1/2 [2]. These kinases and other proteins function together to activate transcription factors and other pathway targets thereby changing the transcriptional profile, the localization of proteins and the status of the cell cycle. Activation of the mating MAPK pathway through its extracellular receptor ensures that the mating responsive genes are only activated under appropriate conditions for mating.

The HOG pathway functions to maintain the turgor pressure in the cell under conditions of hyperosmotic stress. The yeast cell needs to maintain solute concentrations internally to match the extracellular environment. To do so, the HOG pathway senses the external environment and initiates the production of glycerol to balance the internal solute concentration with the external solute concentration. This pathway is activated by a branched MAPK system. There are two different activating receptors and kinase cascades that coalesce at Pbs2, the MAPKK of the HOG pathway, the Sln/Ssk branch and the Sho1/Msb2 branch. The Sln/Ssk branch of the pathway is not pertinent to this work and will not be discussed further. The Sho1/Msb2 branch of the HOG pathway is integral to this work and will be touched upon throughout this dissertation. The Sho1/Msb2 branch of the HOG pathway contains a number of components similar to the mating pathway and the filamentous growth pathway. As with the mating pathway there is a central core kinase cascade consisting of Ste20, Ste11, Pbs2 and Hog1 [2]. The receptors

that activate this core cascade are Sho1, Msb2 and Hkr1 [3]. The mechanism by which the HOG pathway is activated by these receptors is not known at present. Activation of the kinase cascade results in the activation of a number of transcription factors, particularly Hot1, which activate genes involved in glycerol production and cell cycle regulation [4]. Regulation of internal glycerol concentration by the HOG pathway allows the cell to adjust its internal osmotic content in relation to the external osmotic environment.

The filamentous growth (FG) pathway enables yeast to respond to nutrient limitation (glucose and nitrogen deprivation) and alter their growth from vegetative to a filamentous style [5] [6]. Activation of the filamentous growth pathway changes the budding pattern and cell shape of yeast. Under nutrient limiting conditions yeast cells elongate (change from round to more rod shaped) and switch from axial to unipolar budding [5]. Activation of the filamentous response produces more flocculins, which cause the daughter cells to stick to their mother cell [7]. Flocculation together with unipolar budding produces chains of daughter cells growing away from the mother cell; this growth pattern may enable daughter cells to encounter a new environment with a greater nutrient supply. It happens that byproducts of normal yeast metabolism (certain alcohols) also promote filamentous growth as part of a quorum sensing phenomenon [8]. Hence, as population density increases, cells deplete the environment of the preferred nutrient source, simultaneously generating a secondary energy source and quorum sensing molecule, the alcohol. This promotes filamentous growth and causes the cells to bud away from the initial environment towards a possibly more nutrient rich environment.

There are two branches to the filamentous growth pathway, a cAMP pathway that turns on the transcription factor Flo8 and the MAPK pathway that activates the transcription factors Ste12 and Tec1 [9]. Both of these pathways converge at the *FLO11* locus [9]. The *FLO11* gene itself is a member of the flocculin family and is one of the major players in helping daughter cells stick to their mother cells [7]. The cAMP pathway, though part of the FG system is not pertinent to this work and will not be discussed further. The FG MAPK kinase pathway (referred to from this point as the FG pathway) displays similarities with both the mating and HOG MAPK pathways. The known receptors for the pathway are Sho1 and Msb2. Msb2 negatively regulates the FG pathway [10]. Msb2 repression of FG is relieved by cleavage of its mucin domain by a yapsin, which is upregulated during nutrient limiting conditions [11]. Cleavage of Msb2, along with activation of Sho1 by an unknown mechanism, activates the MAPK cascade that includes Ste20, Ste11, Ste7 and Kss1 [2]. Kss1, when phosphorylated, relieves the inhibition of the Kss1/Dig1/Dig2/Ste12 complex [12]. Unphosphorylated Dig1/Dig2 inhibits Ste12 from positively interacting with filamentous response elements (FRE) in the promoter region of filamentous responsive genes [13]. When active, Ste12/Tec1 heterodimers regulate the activity of filamentous responsive genes such as *FLO11* [14]. Regulation of filamentous responsive genes through the FG pathway allows the cell to sense the nutrient availability in the environment and respond in the most effective manner for the propagation of its progeny.

Specificity Mechanisms for MAPK Pathways in Yeast

Each of the pathways discussed above recognize an extracellular stimulus and translates that signal into a unique cellular response. While each of these pathways (mating, HOG and FG) plays a unique role in the cell, the proteins in each pathway are not unique. There is significant overlap between the proteins in each of these pathways, particularly the core MAPK proteins. See figure 1. How is it that the same proteins can generate a variety of cellular responses? The answer lies in a variety of mechanisms that provide specificity to each pathway.

There are a number of recurring themes that are known to provide specificity for various pathways. These themes are scaffolding, negative regulation, localization, and pathway specific components. Scaffolds, though initially found in yeast, have since been found in other eukaryotes such as humans. Scaffolds can be simple docking sites for various pathway components, bringing them in closer proximity to one another to enhance the processivity of signaling. Some scaffolds play more nuanced roles in their pathways. Negative regulation of one pathway by another is a different mechanism for maintaining specificity. By turning off similar pathways farther downstream instead of at the head of each pathway, the system has more flexibility in the final decision of which pathway to fully activate. Once the decision to activate one pathway over another has been made, the cell still has the

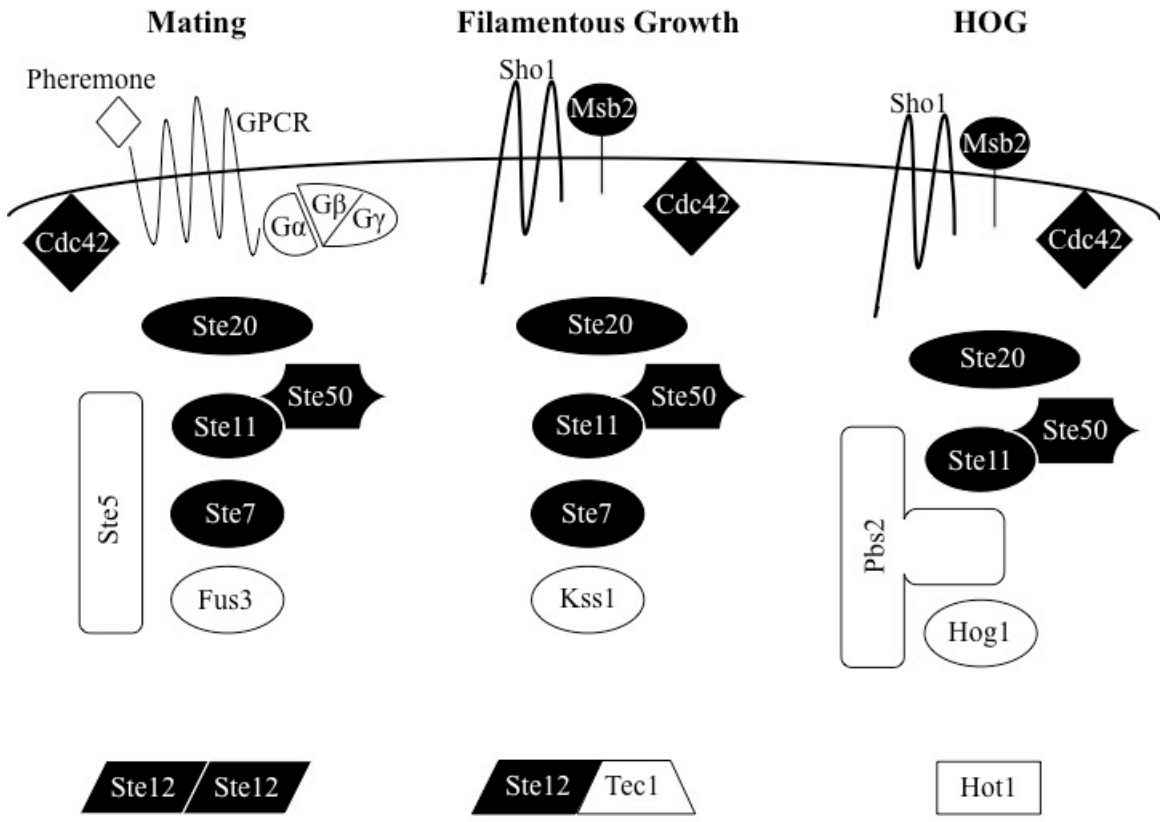


Figure 1

Proteins in solid black are found in common between multiple pathways. Proteins specific to individual pathways are shown in white.

ability to stop the response of the unwanted pathway. Pathway components can be isolated from members of other pathways by cellular compartmentalization or localization to different areas of the cell. Pathways can also take advantage of components that are specific to one particular pathway. Cells can utilize one or a number of these mechanisms in the same signaling pathway to ensure that only the proper pathway responds to a particular stimulus.

The mating pathway utilizes a combination of mechanisms to maintain signaling specificity. The receptors that recognize pheromone are unique to the mating pathway, as is the MAPK Fus3. The kinase cascade, though similar to other MAPK pathways, utilizes an additional factor not present in other pathways, Ste5. Ste5 was identified as the first scaffolding factor [15]. As a scaffold, it interacts with all three members of the core kinase cascade and Gβ at the plasma membrane [16]. Initially it was thought of as a passive factor whose only role was as a docking site for other pathway components to interact. We have come to learn that Ste5 is more of an active participant in the mating pathway. Interaction of Fus3 with Ste5 makes Fus3 a better substrate for Ste7 [17]. The increased k_{cat} for the Ste7-Fus3 phosphorylation reaction when Ste5 is present, along with the close proximity of the proteins, allows Fus3 to outcompete Kss1 for phosphorylation by Ste7 during mating conditions [17]. Ste5 has also been implicated in the degradation of Ste11, thereby negatively regulating the pathway [18].

Besides the scaffolding factor Ste5, the mating pathway also utilizes negative regulation of the FG pathway to maintain specificity. The MAPK Fus3, once active, initiates the ubiquitin dependent degradation of Tec1 [19] [20]. Tec1 is part of the Ste12/Tec1 heterodimeric transcription factor that activates filamentous responsive

genes. Degradation of Tec1 prevents activation of any FG responsive genes during the mating response. This combination of specificity mechanisms functions to ensure that presence of pheromone in the environment activates only the mating pathway and mating responsive genes.

Specificity in the HOG pathway is maintained by a number of known mechanisms, but the full extent of the mechanisms that provide specificity to the HOG pathway has yet to be elucidated. First, the MAPKK Pbs2 is not only a kinase, but also a scaffold [21]. As with the mating pathway, the scaffold is thought to bring pathway components together and localize them at the membrane for optimal interaction between the activating receptors and the MAPK cascade. Second, there are unique components in the HOG pathway including the receptor Hkr1, the scaffold/kinase Pbs2 and the MAPK Hog1 that only function in the HOG pathway [3] [21]. Hog1 has been shown to be necessary for maintaining specificity of the HOG pathway [22]. In particular, deletion of Hog1 or Pbs2 causes activation of the mating pathway in the presence of osmolytes [22]. It has been inferred that there may be a negative regulatory mechanism that turns off other pathways during HOG activation, but such a mechanism has yet to be fully proven [23]. The HOG pathway utilizes many of the same mechanisms for specificity as the mating pathway, but the complete picture has yet to be established for the HOG pathway; how does Hog1 provide specificity and how does the HOG pathway negatively regulate the other MAPK pathways?

Despite the variety of mechanisms known to provide specificity to MAPK pathways, it is not known what mechanism(s) are utilized by the FG pathway to prevent pathways like the mating and HOG pathways from becoming activate during glucose or

nitrogen limiting conditions. A large number of FG pathway components are utilized in either the mating pathway or HOG pathway. There are very few known FG specific pathway components such as Kss1 that could prevent activation of other pathways. It is reasonable to predict that mechanisms to prevent the activation of the mating and HOG pathways by the FG pathway during nutrient limiting conditions do exist. It is possible that the FG pathway utilizes a known mechanism for specificity through factors as yet unidentified. It is also within reason to speculate that the FG pathway may utilize a novel mechanism for specificity. This work has set out to identify factors that are providing specificity to the FG pathway.

CHAPTER II

RANDOM MUTAGENESIS SCREEN FOR SPECIFICITY FACTORS

As an initial step toward discovering the mechanisms that govern signaling specificity in the filamentous growth pathway I sought mutants in which activation of that pathway led to inappropriate readout in the HOG pathway. To this end I created a reporter in which the *HIS3* gene was driven by the HOG pathway dependent *STL1* promoter. In an otherwise WT strain, the *STL1-HIS3* reporter is the only copy of *HIS3* in the cell, thus making the strain his^- unless the HOG pathway is activated. The HOG pathway is typically activated by addition of osmolyte. I reasoned that the HOG pathway can also be activated by abnormal crosstalk from another MAPK pathway, such as the FG pathway, in mutants defective for specificity mechanisms. I used reporter-bearing strains to select for mutants that are histidine prototrophs when cells are exposed to conditions that activate the FG pathway. I used two different strategies to search for such mutants. In the first strategy I used a chemical mutagen (EMS) to mutagenize the genome randomly. In the second strategy I used synthetic genetic analysis (SGA) to introduce the *STL1-HIS3* reporter into the yeast deletion collection. The first strategy allows not only for isolation of loss of function mutants, but also partial loss of function mutants and dominant mutants in essential and non-essential genes. The second strategy only identifies loss of function mutants in non-essential genes, but has the virtue that the affected gene is identified immediately. I first describe the results obtained with the random mutagenesis strategy (Chapter II) and then with the SGA strategy (Chapter III).

Random Mutagenesis Screen

Taking a forward genetic approach to the issue of specificity factor identification resulted in a screen for mutants that have lost specificity for the filamentous growth MAPK pathway. One result of the loss of specificity in the filamentous growth pathway is the cross activation of other MAPK pathways such as the mating and HOG pathways. Other labs have used such cross activation based screens to identify factors in pathways such as the HOG pathway [24]. The logic behind the screen is that specificity mutants can be identified from a pool of random mutants by the activation of a reporter in another pathway such as the HOG pathway. The reporter system I choose to utilize is a growth-based reporter based on the promoter of the *STL1* gene. *STL1* is a HOG sensitive gene. Posas et al showed that under salt stress, transcription of the *STL1* gene increased by approximately 90 fold [25]. The basal level of gene expression was also very low [25]. The combination of low basal activity and strong HOG activation makes *STL1* an ideal promoter for use in the screen. The *STL1* promoter was fused to the coding region of the *HIS3* gene, generating a growth based reporter (strains SY4316 and SY4318). See figure 2. Mutagenized strains plated on FG pathway activating media lacking histidine will only grow when the reporter is activated by a mutation that loses specificity to the FG pathway and produces the *HIS3* gene product. Randomly generated mutants were created in both the **a** and α mating types. Three independent cultures of the strain SY4316 and four independent cultures of the strain SY4318 were treated with EMS, plated directly onto media lacking fermentable carbon and histidine to identify potential mutants (see materials and methods).

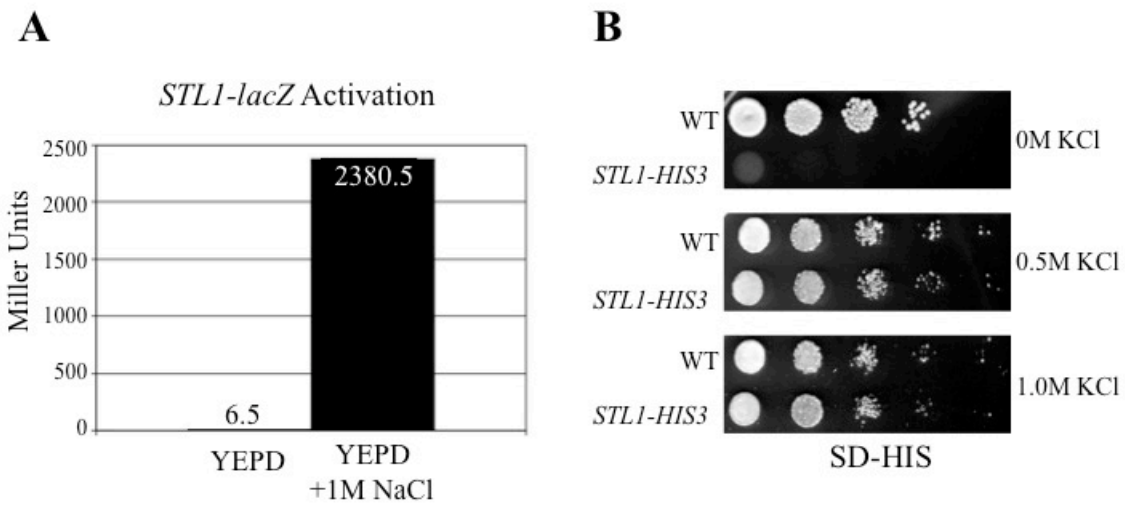


Figure 2

A-β galactosidase assay of the *STL1*-lacZ reporter. The addition of osmolyte to the media activates the reporter resulting in an increase in β-galactosidase activity. **B**-*STL1-HIS3* is a growth based reporter. Addition of osmolyte to the medium activates the reporter and generates histidine, allowing for growth on medium lacking histidine.

Of approximately 600 mutants isolated by random mutagenesis and subjected to phenotypic and genetic assessment, the most robust of the 112 recessive mutants that grew on 20 mM 3-aminotriazole were chosen for further analysis.

With respect to phenotype, I sought to determine the magnitude of the potential signal in the HOG pathway by measuring the degree of resistance to 3-aminotriazole. I also asked whether any elements of filamentous growth were retained by the mutants by assessing bud pattern, cell elongation and agar invasion. With respect to genetic analysis, I first determined whether the mutations indeed affected signaling specificity; that is, actually required signaling in the FG pathway rather than causing constitutive activation on the STL1 promoter by some other means. Subsequently, I determined if the mutations were dominant or recessive and grouped the recessive mutants into complementation groups.

Phenotypic Assessment

It is possible that different mutants activate the *STL1-HIS3* reporter to varying degrees. In order to class mutants together with others of similar activity, I assayed growth on various levels of 3-aminotriazole. 3-aminotriazole is a competitive inhibitor of the His3 enzyme. If cells are to grow on media containing 3-aminotriazole, they must produce enough *HIS3* gene product, the enzyme imidazoleglycerol-phosphate dehydratase that catalyzes the sixth step of histidine biosynthesis, to out compete the inhibition of 3-aminotriazole [26], [27]. Therefore, the higher the concentration of 3-aminotriazole that cells can grow on, the more *HIS3* gene product they are producing. The relative activity of the reporter can then be compared among mutants based on the

highest level of 3-aminotriazole each mutant can grow on. I screened the mutants on 5, 10, 15, 20 and 40 mM 3-aminotriazole. This allowed for the grouping of the mutants based on their reporter activities. Most of the mutants fell in the middle of the 3-aminotriazole range. 51% of α mutants and 44% of **a** mutants grew on 20 mM 3-aminotriazole. Very few mutants had either enough reporter activity to grow on 40mM 3-aminotriazole or so little activity that they only grew on 5mM 3-aminotriazole. See figure 3B. In order to determine the growth phenotype in later genetic experiments, it became necessary to limit the mutants I worked with to one level of reporter activity. The group of mutants that were able to grow on 20 mM aminotriazole were chosen for their robustness of growth and the adequate number of mutants in that group. For the genetic studies discussed later in this work, all of those experiments were done with the group of mutants that grew on 20 mM 3-aminotriazole.

I infer from the His^+ phenotype of the mutants that signaling has bled from the FG pathway to the HOG pathway (see below for discussion of this inference). However, even if this inference is correct, some signaling in the FG pathway may be retained. Moreover, the architecture of the FG pathway is not known. There may be mutations that affect one or a few aspects of the filamentous growth phenotype, but not other aspects. I therefore assayed the mutants for various filamentous phenotypes.

The mutants were assayed for their ability to invade agar using the plate washing assay (see materials and methods). 23% of **a** mutants lost the ability to invade agar under filamentous growth conditions. 28% of α mutants lost their ability to invade agar under filamentous conditions. For an example of mutants that fail to invade see the boxed strains in figure 3A. There were a small number of mutants that appeared to have

constitutive agar invasion under conditions not conducive for filamentous growth. The ability of some mutants to maintain their invasive properties while others do not suggests a fundamental difference between those categories of mutants, either mutations in different genes, or alleles that affect different aspects of protein function. For mutants that have lost their ability to invade agar, it is possible that the affected gene(s) is necessary for proper transduction of the FG signal as well as for insulating that signal from the HOG pathway. Those strains that show proper agar invasion are likely to have mutations in a gene(s) that is not essential for proper FG signal transduction, but does play a role in maintaining FG pathway specificity. Mutations that constitutively activate agar invasion may be in a gene(s) that negatively regulates FG activity under non-invasive conditions as well as insulating the signal to only the FG pathway. While genes in each of these categories are presumed to play some role in limiting signaling to the FG pathway, they may be expected to do so in different ways.

Cells growing filamentously exhibit cell elongation and unipolar budding. These two phenotypes are both dependent on the filamentous pathway, but are independent of each other. Mutants display both, one or none of these phenotypes. Mutants were looked at under the microscope to determine if they displayed either, both or none of these two phenotypes. Mutants were found that lacked the elongation phenotype, or lacked the elongation and unipolar budding phenotype. The majority of mutants appeared to maintain the unipolar budding phenotype and the elongation phenotype, particularly when the mutants maintain their ability to invade agar. See figure 3B for examples

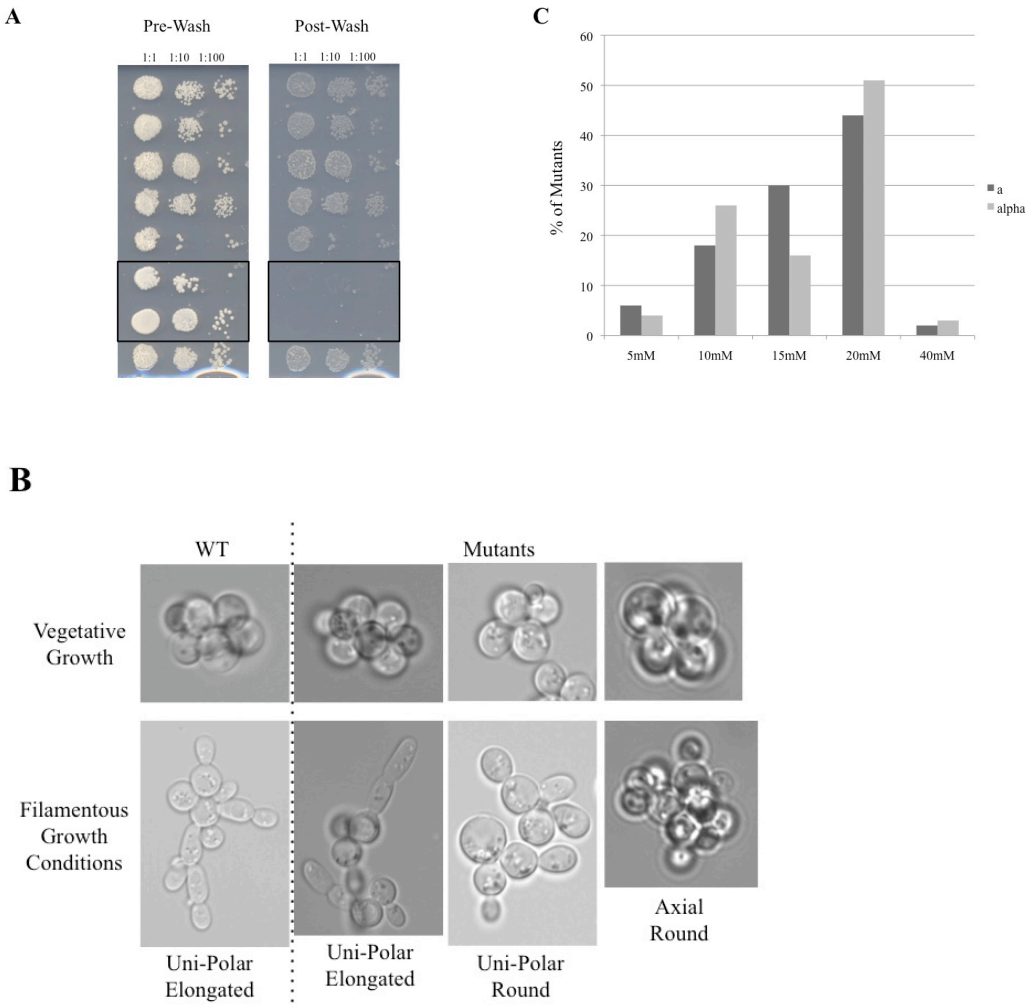


Figure 3

A-Invasive growth assay of randomly generated mutants. Mutants that have lost the ability to invade agar fail to leave a scar on the plate as seen in the boxed section. **B**-Percent of mutants that activate the *STL1-HIS3* reporter enough to grow on various levels of 3-aminotroazole. The majority of mutants are able to grow on 20mM 3-aminotriazole. **C**-Morphology of various mutants. There are three categories of mutants, those with both phenotypes, elongation and unipolar budding, those that only elongate and finally those with neither.

of the variety of phenotypes found among the mutants. No mutants were found that maintained the elongation phenotype, but lost the unipolar budding phenotype.

Genetic Assessment

The mutants I am interested in are those that are dependent on activation of the filamentous growth pathway for reporter activity. It is possible to generate mutants that activate the HOG pathway independently of FG pathway activity, meaning they turn on the HOG pathway without a signal from the FG pathway. Such mutants could be generated by constitutive mutations in HOG pathway components. For example, a mutation that constitutively activates Pbs2 or Hog1 will activate the reporter without a signal from the FG pathway. To identify the mutants that are not dependent on the FG pathway, I plated the mutants on medium that contains fermentable carbon, but lacks histidine. Those mutants that grow on this type of medium are activating the reporter without input from the FG pathway and are not specificity mutants. See figure 4. 30% of **a** and 13% of α mutants were found to be independent of the filamentous growth pathway. These mutants were not studied further. The FG pathway dependent mutants were further characterized.

Random mutagenesis can generate both dominant and recessive mutations. For a number of genetic experiments described later in this work it is important to know if the mutation is dominant or recessive. To this end, all mutants were mated to the wild-type strain of the opposite mating type. The resulting diploids were then screened for *STL1-HIS3* reporter activity. Those mutants that activated the reporter with a single copy of the mutant gene are dominant. Those mutants that failed to activate the reporter with only a

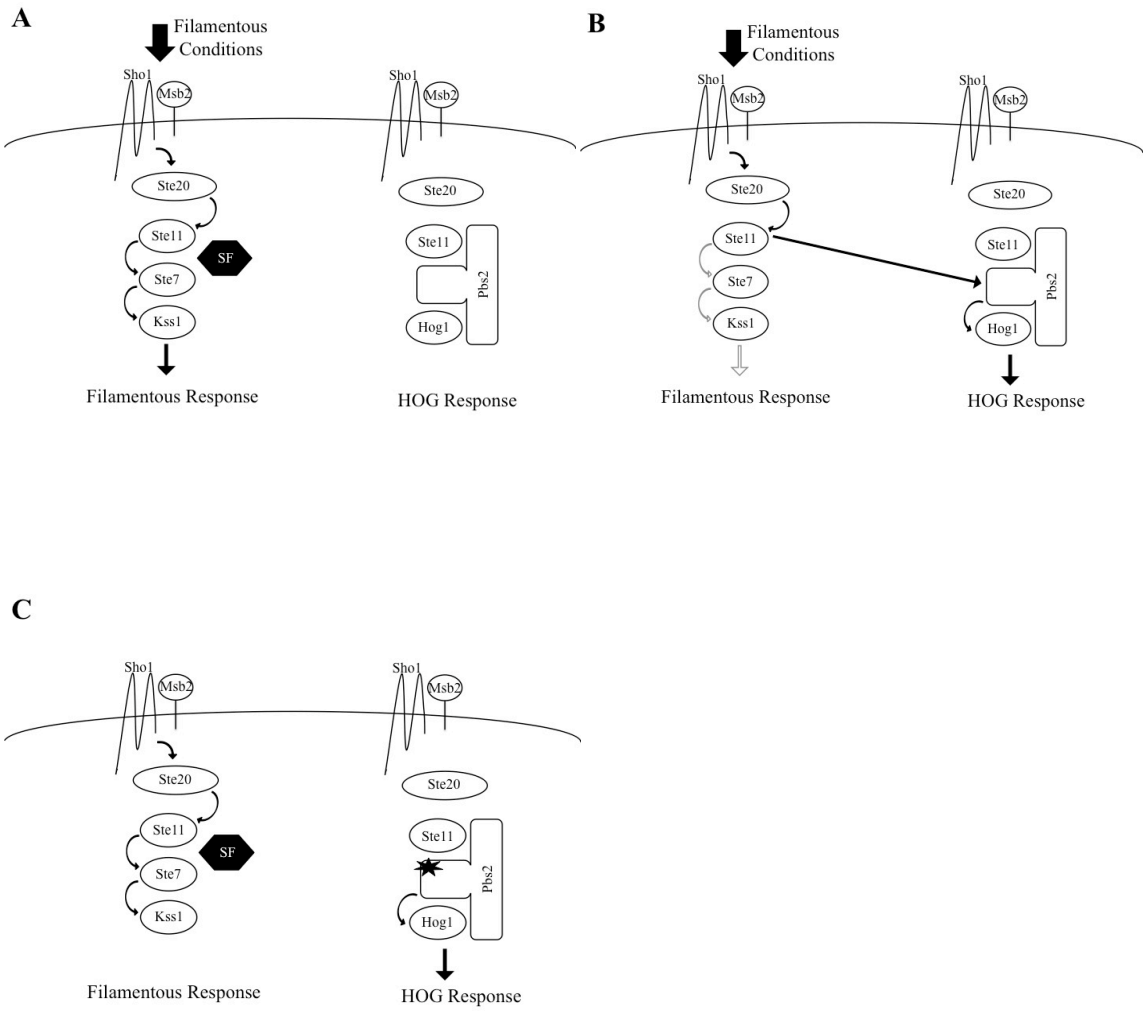


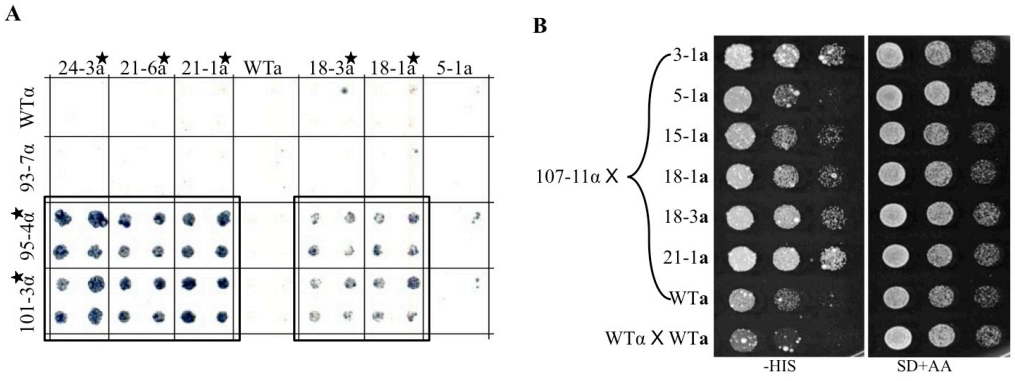
Figure 4

A-The presence of a specificity factor prevents the signal from leaking to the HOG pathway. **B**-Mutation of the specificity factor prevents it from functioning and allows the signal to leak over to the HOG pathway, activating the HOG response. **C**-Constitutive mutants in the HOG pathway activate the HOG response without input from the FG pathway.

single mutant gene are recessive. Only the recessive mutants were used for further genetic tests. Of the mutants screened, 51 **a** mutants and 34 α mutants were found to have dominant mutations. 68 **a** mutants and 171 α mutants were found to have recessive mutations.

Complementation Tests

Due to the fact that the mutants were generated randomly, the identity and number of the mutated genes is not known. I performed complementation tests to determine the number of genes identified by the mutagenesis screen. Mutants in mating type **a** were mated to mutants in mating type α and the His phenotype of the resulting diploids assayed. Diploids that fail to activate the *STL1-HIS3* reporter have mutations that complement each other and are located in distinct genes. Mutants from the 20mM 3-aminotriazole group were tested in this manner and most of the mutants fell into one large complementation group, classified as group 1. There were a number of individual mutants that fell into their own complementation groups, groups 2 through 5. This test was done on the RoTor robot in four fold coverage for each mating. See figure 5A. Diploids of mutants that fall in the same complementation group are seen to grow, while diploids of mutants not in the same complementation group fail to grow. Results were repeated with spot testing. See figure 5B. Mutant 107-11α was mated to a variety of **a** mutants. All diploids grew except that created by mating with strain 5-1**a**, which does not belong to complementation group 1. The list of mutants assigned to each complementation group can be found in figure 5C.



C

Group 1	
3-1a	23-2α
15-1a	33-1α
18-1a	59-6α
18-3a	88-2α
21-1a	95-4α
21-5a	101-3α
21-6a	105-4α
24-3a	105-9α
27-6a	107-11α
33-10a	

Group 2	Group 3
5-1a	93-7α

Group 4	Group 5
27-1α	103-4α

Figure 5

A-Colonies of matings between mutants using the ROTOR robot. The boxes show growing colonies. Mutant strains marked with stars all belong to the same complementation group, group 1. **B-**Similar results shown by spot test. **C-**List of mutants belonging to each complementation group.

These complementation tests suggest the minimum number of genes that were mutated in the random mutagenesis screen is 5. It is likely that there are a number of alleles present in the mutant collection for the gene represented in complementation group 1. This experiment does not determine the actual identity of the gene mutated in group 1.

An attempt was made to determine the identity of the gene in complementation group 1 by complementation with a wildtype genomic plasmid library (see materials and methods). The plasmids isolated in this screen did not generate consistent phenotypes. The plasmids isolated were those that failed to complement the crosstalk phenotype (growth on medium lacking fermentable carbon and histidine). Propagation of those strains, transformation of the plasmids from those strains, and single gene complementation from those plasmids did not generate a reproducible phenotype (failure to complement). Due to the lack of reproducibility in the results the screen was abandoned for other methods of gene identification.

All members of complementation group 1 are predicted to have mutations in the same gene. It follows that alignment of the genomes from complementation group 1 would show random mutations throughout each genome, but only one gene would have mutations in all group members. Therefore, sequencing and subsequent alignment of group 1 genomes should provide an identity for the gene mutated in complementation group1. Sequencing of seven members of group 1 and background strains of both mating types was done after re-verification of complementation grouping. Alignment of the sequencing reads was done against the Sigma (Σ) reference genome sequence provided by the Boone Lab. A consensus sequence was compiled for each genome. Each mutant

genome was compared to the respective background strain sequence and single nucleotide polymorphisms (SNPs) were identified where the two sequences differed. A list of all genes containing SNPs was compiled for each member of complementation group 1 and the lists were cross-referenced between the mutants. Two genes that contained SNPs in all members of complementation group 1 were identified using this method, YBL105c and YBR150c.

YBL105c and YBR150c were further analyzed using the Integrated Genome Browser software to verify the presence and location of the SNPs in the α mutant strains. No conclusive sequencing of SNPs were found in either of the α mutant strains. Analysis showed some variation in the sequencing between the wildtype and mutants strains, but the quality of the sequence was not high enough to verify a SNP. It is likely that many of the SNPs designated in the original list are incorrect due to the inability of the SNP calling software to distinguish between high quality sequencing data and low quality sequencing data. I have been unable at this time to further characterize the other genes on the SNP list due to time constraints. It is possible that further analysis of the actual traces for each of the genes on the SNP list may lead to a candidate. For a full list of genes candidates see Table 1. This effort would be further aided by additional sequence data. The coverage of each genome varied widely and in some cases was much lower than expected. Additional coverage of each genome would increase the confidence in the consensus sequence and in turn SNPs identified from such sequence.

The yeast deletion collection is a collection of approximately 5,000 yeast strains, each with a different non-essential gene knocked out. I used the collection to attempt to identify the gene mutated in complementation group 1. A candidate from

complementation group one (107-11α) was crossed to the entire collection and the resulting diploids were screened for their ability to activate the *STL1-HIS3* reporter. Growth of a diploid on FG medium lacking histidine suggests that the particular deletion and the query mutation affect the same gene. This screen was done with deletion collections in both the non-filamentous (S288c) and filamentous (Σ) genetic backgrounds for a total of three screens, resulting in a list of 20 candidate genes. To determine whether those candidates were real, each gene was then independently knocked out in the wildtype *STL1-HIS3* reporter strain SY4316 and assayed for crosstalk. See figure 6A for a list of candidates. Of those 20 genes, a number of them were not correctly identified in their respective collections. That is, the locus that was ostensibly deleted in these strains did not contain the knockout cassette when tested by PCR. I infer that these candidates contain a deletion or other mutation that fails to complement the mutant, but the identity of that gene(s) is unknown. 11 candidates were successfully knocked out in the *STL1-HIS3* reporter strain. Of those 11 candidates, only the *nrg1Δ* strain showed a significant level of crosstalk.

The crosstalk generated in the *nrg1Δ* strain is fairly weak when compared to the original mutant crosstalk as shown in figure 6B. There are a number of reasons that the level of crosstalk may be different. One possibility is that the alleles of the gene isolated by chemical mutagenesis may behave differently than the full knockout. The *NRG1* locus was sequenced in the mutant strain used for the complementation test (107-11α) to identify any mutations that could be causing crosstalk. See figure 6C. The *NRG1* locus from mutant 107-11α from 500bp before the start codon, to 500bp past the stop codon, did not contain any mutations, insertions or deletions when compared to the Σ

A

ORF	Name	Locus Incorrect	Crosstalk
YDR049W			None
YDR043C	NRG1		+Weak
YFL033C	RIM15		None
YIR026C	YVH1		None
YOR005C	DNL4		None
YOL114C		✔	
YPR098C		✔	
YBL103C	RTG3		None
YOL109W	ZEO1	✔	
YOL156W	HXT11	✔	
YBR083W	TEC1		None
YIL151C	ESL1	✔	
YGR271C-A	EFG1	✔	
YJR096W		✔	
YMR115W	FMP24	✔	
YBR121C	GRS	✔	
YPR058W	YMC1		None
YNL109W			None
YNL108C			None
YFR036W	CDC26		None

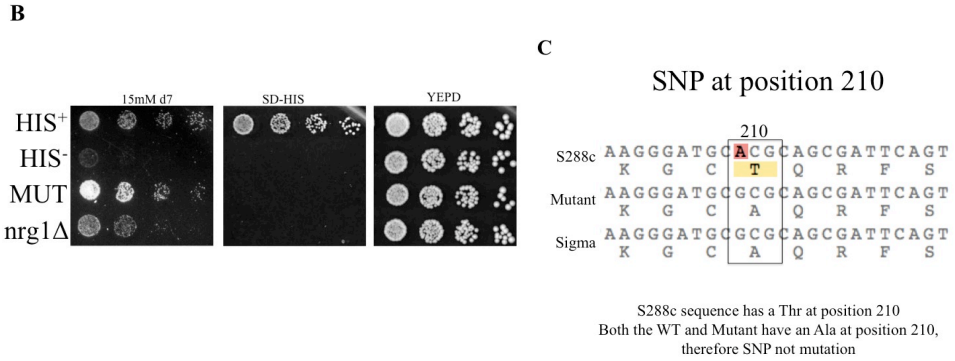


Figure 6

A-List of candidate genes from complementation with the deletion collection. **B**-Spot test of *nrg1*Δ strains on SLAG-HIS+15mM 3AT, SD-HIS and permissive medium (YEPD). **C**-Alignment of *NRG1* sequence at position 210 showing no change in the mutant in relation to the Sigma reference strain (wildtype).

background strain that it was derived from (SY4318). We know that the *nrg1*Δ strain failed to complement group 1 mutations and that the knockout generates reporter activity. However, with no mutation in the *NRG1* locus in the original mutants, it is unclear at present exactly how *NRG1* is involved in FG signaling to generate that phenotype. From the current knowledge of *NRG1* there are a number of plausible hypothesis for how *NRG1* can be tied to filamentous growth.

Nrg1 functions as a co-repressor in the presence of glucose to down regulate a variety of genes that are usually active only in low glucose conditions. Nrg1 has been implicated in the regulation of *FLO11* by its physical interaction with Snf1 [28] and by binding sites for Nrg1 in the *FLO11* promoter [29]. Deletion of *nrg1* and its homolog *nrg2* result in hyper-invasive cells [29]. Nrg1 is phosphorylated in response to stresses including NaCl, Sorbitol, alkaline pH and low glucose [30]. The levels of Nrg1 in the cell also increase during low glucose conditions, particularly in the presence of ethanol and glycerol [28]. The most plausible hypothesis is that Nrg1 functions as a negative regulator of FG pathway activity at the transcriptional level in the presence of glucose. In the absence of glucose Nrg1 might be necessary in a modified (phosphrylated) form to alter gene regulation, possibly by releasing co-repressor Cyc8/Tup1 or recruiting alternative regulatory factors.

CHAPTER III

STL1-HIS3 SYNTHETIC GENETIC ARRAY

The technology developed for the yeast synthetic genetic array is a useful tool for genetic manipulation and screening of the entire yeast genome. Using the yeast deletion collection and the SGA technology, one can incorporate additional genetic elements into each of the deletion strains. This technology is usually used for the pairing of additional gene knockouts with the entire deletion collection. This is done by mating a query strain containing the gene knockout of interest to the entire collection. Through a series of sporulation and haploid selection steps, a collection of haploid strains that now contain the knockout of interest and the original gene knockout are generated (see materials and methods). I have utilized this technology to insert the *STL1-HIS3* reporter into the filamentous (Σ) yeast deletion collection. This allows for the screening of any non-essential gene that is necessary for maintaining specificity in the FG pathway. The collection was mated to two independently isolated query strains (once to one and twice to the other). Each mated collection was then screened on three concentrations of 3-aminotriazole (5, 10 and 15 mM) and medium containing fermentable carbon and lacking histidine (SD-HIS) to eliminate candidates that were generating crosstalk independently of the FG pathway. Candidates had to grow from two of the three independently mated collections and fail to grow on the SD-HIS plates to qualify as candidates. 14 strains met those conditions. Of the 14 strains, all grew on 5 and 10 mM 3-aminotriazole. 5 of the 14 strains also grew on 15 mM 3-aminotriazole. The *STL1-HIS3* SGA screen resulted in 14 candidates that generate crosstalk from the FG pathway to the HOG pathway.

The location of each strain in the array is annotated for identification purposes. Cross-referencing the plate number with the row and column location should give an identity to each candidate. The plate numbers and locations along with the gene identities given for each location are described in Table 2 along with the screens that the candidates came from. To verify the phenotype of each candidate, the knockout cassette and surrounding sequence was to be transferred from the deletion collection strain to the SY4316 *STL1-HIS3* reporter strain. Unique primers were designed for each locus approximately 600 base pairs upstream and 600 base pairs down stream of the start and stop sites. The knockout cassette with flanking regions should result in a PCR product of approximately 2.5kb. The resulting PCR products did not correspond to the knockout cassette, but instead corresponded with the correct size for each wild-type locus. Each ORF was checked with PCR for the presence of the knockout cassette. For ORFs whose wild-type band size was similar to the knockout cassette size (between 2.5 and 3kb) digests were done to verify the absence of the kan^r cassette. No strains showed the correct size PCR fragment to indicate the presence of the knockout cassette. Those done by digest also failed to indicate the presence of the knockout cassette.

It is obvious that there is a discrepancy between the stated identity of each candidate and the actual gene knockout present in each strain. It was presumed that incorrect arraying of the collection could have altered the location of each strain. A number of auxotrophic strains and vma^- strains were checked for their presence in the array at both the presumed locations and any location within the respective quadrants. No strain was found to be correct in any of those locations. The plates were also tested

for altered orientation (flipped vertically or horizontally), but no such change in orientation was found.

It is not possible within the present time restriction to determine the identity of the 14 candidate genes. If one wanted to identify the genes in the 14 candidate strains, those genes could be identified by PCR. Isolation of genomic DNA from each strain and PCR using an internal primer for the deletion cassette and a set of randomized primers to prime from the genome outside the knockout cassette would generate a DNA fragment containing the genomic DNA on one side of the knockout cassette. Sequencing of the resulting PCR products would identify the location of one flank of the knockout cassette in the genome.

Without the identity of said candidate genes, the mechanism by which each candidate provides specificity to the FG pathway cannot be elucidated. Given that there are 14 candidates from the screen, it can be stated that there are up to 14 non-essential genes that are required for maintaining the specificity of the FG pathway. The number of different mechanisms represented by those 14 candidates is not known, but it is possible that some of those candidates represent the mechanism by which Nrg1 might provide specificity to the FG pathway.

APPENDIX

MATERIALS AND METHODS

Strains-Strains were propagated and transformations were done as in Burke et al 2000 [31].

Gene knockouts-PCR products were generated from respective strains from the yeast deletion collection. Each PCR product contained the knockout cassette and ~500 base pairs of locus specific homology on either side of the knockout cassette. Strains were transformed with PCR products and transformants were selected by expression of the knockout cassette marker (kan^r).

Reporter strain construction-*STL1-HIS3* reporter selection was done as in Horecka and Sprague 2000 [32]. The *CYC1* promoter in pSL1470 was swapped for 825 base pairs of the *STL1* promoter. The resulting plasmid was cut with NheI and transformed into SY3089 and SY3090. Ura^+ transformants were selected by growth on SD–Ura. Candidates were then streaked on 5FOA plates to select for Ura^- candidates. Ura^- candidates were tested for growth on SD-His and by PCR. These strains were named SY4316 and SY4318. Strain SY4318 had the *TRP1* locus knocked out with a kan^r cassette as in Longtine et al 1998 [33].

Filamentous media-Two types of filamentous inducing plates were used in various experiments. The first type, SLAG-His, were made as in Tong and Boone 2001 (SD protocol), substituting 2% glucose for 2% glycerol and using all amino acids excepting histidine [34]. The second type, SG-His plates, were made as in Burke et al

2000 with 2% glycerol and all amino acids except histidine. 0 to 40mM 3-aminotriazole was added to both types of media depending on the experiment.

SGA Query strain construction and assay-The *STL1-HIS3* query strain was generated by the PCR method. The *STL1-HIS3* cassette was created by homologous recombination between a pRS316 plasmid containing the *STL1* promoter and a PCR fragment from the Stevens lab plasmid 3454 containing *HIS3* fused to an HA tag and the natMX cassette. The resulting plasmid contained 825 base pairs of the *STL1* promoter fused to the *HIS3* coding region tagged with HA and nat^r. A PCR product was generated from the plasmid with 40 base pairs of homology to the *HIS3* locus on either side of the reporter cassette. The PCR product was transformed into the Sigma WT Y3295 strain provided by the Boone lab and nat resistant strains were selected. Candidates were tested for lack of growth on SD-HIS and by PCR. SGA was performed as in Tong and Boone 2001 with the media changed to select for leucine prototrophy [34]. Final analysis of the *STL1-HIS3 geneXΔ* collection was done on SLAG-HIS+5/10/15mM 3-aminotriazole plates incubated at 30 degrees C for 7 days. The collection was also plated on SD-HIS to remove any candidates that displayed FG independent crosstalk.

Dilution spot tests- 3AT and plate washing assay dilution spot tests were done with each strain equilibrated to .7 ODs with a final volume of 200 microliters and diluted 1:10 in 96 well plates. Cells were plated with a 48 prong device.

Random Mutagenesis- Reporter strains SY4316 and SY4318 were mutagenized with EMS according to Lawrence [35]. The mutagenized cells were plated on SG-HIS and selected for colonies that grew. Candidates were streaked and then patched on SG-

HIS plates. Mutants were selected from only those candidates that grew on all three plates (original, streak and patch).

Invasive Growth Assay-Strains were transferred from 96 well plates to YEPD plates with a 48 prong device. Plates were grown at 30 degrees Celsius for 3 days. The plates were then run under a stream of water and rubbed gently with a gloved hand to remove any cells that had not invaded into the plate.

Microscopy-Strains were grown in YEPD for 2 days. 8 ul of each culture were pipetted to a slide and viewed by DIC at 20X and 100X.

B-gal Assay- Two 10ml mid-log (.7 OD_{600}) cultures were grown of each strain at 30 degrees C. One culture from each strain was incubated for 10 to 60 minutes with 1M NaCl at 30 degrees C. Cells were then pelleted and lysed by vortexing each sample in .4ml buffer (50mM Tris pH8, 20mM NEM, 100mM NaCl, 0.5% Triton X, 1mM PMSF, Complete Protease Inhibitor) and ~150ul glass beads for 5 minutes. Samples were centrifuged for 2 minutes at 4 degrees C to remove precipitate. Between 1 and 10ul of cell prep was added to 190-199ul of Z buffer with CPRG. Each sample and a blank were assayed at OD_{578} every 30 seconds for 60 minutes. β-galactosidase units were caluculated by the following formula- 1000 x OD_{578} /(elaspsed time x (.1x concentration factor) x OD_{600}).

Plasmid Library Complementation- was done as in Rose et al 1987. Colonies were selected that grew on permissive (SD-URA) plates, but failed to grow on restrictive plates (SG-HIS).

Whole Genome Sequencing- Genomic preps were done as in the Yeast Genomic Prep for high quality DNA from the Hoskins/Hahn Lab 1997. Library construction was done by Jason Carrier of the Johnson Lab. The actual sequencing was done on an Illumina sequencer with 80 base single read sequencing by the University of Oregon sequencing facility. Assembly of the sequencing reads was done by Nicolas Stiffler using the sigma genome as reference. The Samtools program was used to generate the consensus sequence for each genome assembled. The genomes of each mutant were then compared to the WT genome. SNPs were marked when there was at least 10X coverage of the base and the consensus differed between the WT and mutant. Traces were analyzed on the Integrated Genome Viewer software.

Table 1-List of Candidate Genes from Genome Sequencing and Alignment

All α strains		All a Strains	a and α Strains
BAL001C	YBR044C	YAL026C	YBR105c
BAL002C	YBR118W	YBL105C	YBR150c
YAL051W	YBR150C	YBR128C	
YAL024C	YBR198C	YBR132C	
YAR035W	BBR010W	YBR150C	
YAR050W	YBR293W	YBR155W	
YBL107C	BBR016W	YBR200W	
YBL105C	BCL018C		
YBL103C	YCR038C		
YBL102W	YDR204W		
YBL082C	YDR247W		
YBL064C	YGR183C		
YBL023C	YLR450W		

Table 2-List of Candidates from *STL1-HIS3* SGA

ORF	GENE	PLATE	ROW	COLUMN	SCREEN 1	SCREEN 2	SCREEN 3
YJL048C	UBX6	14	1A	11	✔	✔	✔
YJL101C	GSH1	14	2B	2	✔	✔	✔
YJL207C		14	2B	8	✔	✔	✔
YPR067W	ISA2	13	5	2	✔	✔	
YJL088W	ARG3	13	6	24		✔	✔
YJL175W		13	8	8	✔	✔	✔
YKL189W	HYM1	13	12	7	✔		✔
YJR017C	ESS1	13	16	9	✔	✔	✔
YOL104C	NDJ1	12	9	23	✔	✔	✔
YLL012W	YEH1	9	16	24	✔	✔	
YBR037C	SCO1	5	7	4	✔	✔	
YCL011C	GBP2	5	16	15	✔	✔	
YPR098C		4	12	12	✔	✔	
YBL056W	PTC3	1	7	21	✔	✔	✔

Table 3-Yeast Strains

Strain	Genotype	Source
SY3089	Sigma MAT**a** *ura3-52*	Fink Lab
SY3090	Sigma MATα *ura3-52*	Fink Lab
SY4316	3089 *STL1pr-HIS3*	This Study
SY4318	3090 *trpΔ*::kan^r *STL1pr-HIS3*	This Study
SY4475	4316 mutant 3.1	This Study
SY4476	4316 mutant 5.1	This Study
SY4477	4316 mutant 15.1	This Study
SY4478	4316 mutant 18.1	This Study
SY4479	4316 mutant 18.3	This Study
SY4480	4316 mutant 21.1	This Study
SY4481	4316 mutant 21.5	This Study
SY4482	4316 mutant 21.6	This Study
SY4483	4316 mutant 24.3	This Study
SY4484	4316 mutant 27.7	This Study
SY4485	4316 mutant 33.10	This Study
SY4486	4318 mutant 23.2	This Study
SY4487	4318 mutant 27.1	This Study
SY4488	4318 mutant 59.6	This Study
SY4489	4318 mutant 88.2	This Study
SY4490	4318 mutant 93.7	This Study
SY4491	4318 mutant 95.4	This Study
SY4492	4318 mutant 101.3	This Study
SY4493	4318 mutant 103.4	This Study
SY4494	4318 mutant 105.4	This Study

Stain	**Genotype**	**Source**
SY4495	4318 mutant 105.9	This Study
SY4496	4318 mutant 107.11	This Study
Y3295	Sigma MATα *his3*::hisG *leu2Δ ura3Δ ho-*	Boone Lab
Σ collection	Sigma MAT**a** *abc1*Δ::kan^r *can1*Δ::*Ste2*pr-sp*HIS5 lyp1*Δ::*Ste3*pr-*LEU2 his3::hisG leu2Δ0 ura3Δ0*	Boone Lab
SY4512	4316 *ydr049wΔ*::kan^r	This Study
SY4513	4316 *ydr043cΔ*::kan^r	This Study
SY4514	4316 *yfl033cΔ*::kan^r	This Study
SY4515	4316 *yir026cΔ*::kan^r	This Study
SY4516	4316 *yor005cΔ*::kan^r	This Study
SY4517	4316 *yol114cΔ*::kan^r	This Study
SY4518	4316 *ypr098cΔ*::kan^r	This Study
SY4519	4316 *ybl103cΔ*::kan^r	This Study
SY4520	4316 *yol109wΔ*::kan^r	This Study
SY4521	4316 *yol156wΔ*::kan^r	This Study
SY4522	4316 *ybr083wΔ*::kan^r	This Study
SY4523	4316 *yil151cΔ*::kan^r	This Study
SY4524	4316 *ygr271c-AΔ*::kan^r	This Study
SY4525	4316 *yjr096wΔ*::kan^r	This Study
SY4526	4316 *ymr115wΔ*::kan^r	This Study
SY4527	4316 *ybr121cΔ*::kan^r	This Study
SY4528	4316 *ypr058wΔ*::kan^r	This Study
SY4529	4316 *ynl109wΔ*::kan^r	This Study
SY4530	4316 *ynl108cΔ*::kan^r	This Study
SY4531	4316 *yfr036wΔ*::kan^r	This Study

Table 4-Plasmids

Plasmid	Description	Source
CY751	pΔSS *CYC1*-lacZ	Johnson and Herskowitz 1985
CY4016	pΔSS *STL1*-lacZ	This Study
CY1845	*FUS1-HIS3*	Horecka and Sprague 2000
CY4040	*STL1-HIS3*	This Study
CY3964	pAG25 *natMX4*	Goldstein et al 1999
S3454	*HIS3-HA*-nat^r	Stevens Lab
CY4041	*STL1-HIS3-HA*-nat^r	This Study
CY1464	pRS316	ATCC

[36] [32] [37]

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