# Large-scale Analysis of Filamentous Growth in *Saccharomyces cerevisiae* and the Related Human Fungal Pathogen, *Candida albicans*

**by**

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# Chapter 1

# Introduction

## 1.1. Yeast as a model organism:

*Saccharomyces cerevisiae* has long served as an exceptional model organism for studies of genetics and cell biology. It has many of the desired traits of a model organism; in particular, many yeast genes possess orthologs in higher eukaryotes, including mammals (Botstein *et al.* 1997). Bakers' yeast is readily available, and it is easy as well as economical to grow. In addition, it is easily maintained in the laboratory due to its small size, minimal nutritional requirements and non-pathogenic nature. It has a short life cycle of 90 minutes, speeding up experimental data collection. Of particular importance, it exists as both a stable haploid and diploid, facilitating genetic manipulation. The size of the haploid genome is $1.2 \times 10^7$ bp, consisting of sixteen chromosomes ranging in size from 230,000 bp to 2,352,000 bp (Suter *et al.*, 2006). The yeast genome was the first eukaryotic genome to be successfully sequenced in 1996 (Goffeau *et al.*, 1996), and its annotation, furthermore, developed a basic blueprint for gene-finding studies in related lower eukaryotes. The genome is relatively compact with approximately 6,400 genes comprising 70% of the total sequence. The sequence data and annotations are publicly available through the Saccharomyces Genome Database (SGD) at www.yeastgenome.org.

Since cell processes in yeast are typically conserved in higher eukaryotes (Melese and Hieter, 2002), numerous studies in yeast over the years have led to a wealth of information on basic cellular processes, such as cell cycle progression, DNA replication and repair, protein translation, and metabolism. Yeast is an increasingly popular model for pharmacological/drug discovery studies as well (Outeiro and Giorgini, 2006); since 40% of yeast proteins are similar in sequence to human proteins, orthologs of human disease related genes can be studied informatively in yeast. In addition, yeast is an excellent model for the study of pathogenic fungi (Truckses *et al.*, 2004). Most genes in fungi such as the human fungal pathogen, *Candida albicans*, and the corn smut fungus, *Ustilago maydis*, have orthologs in the budding yeast, and a lot of knowledge has been gained by studying the functions of these genes in *S. cerevisiae* (Lengeler *et al.*, 2000).

### 1.2. Yeast genomics:

The budding yeast has proven to be an equally strong model for the development of genomic and proteomic technologies. With its compact genome and simple genetics, *S. cerevisiae* has provided the test template for the development of many large-scale experimental approaches. Numerous genomic methodologies have been developed to identify functions for the roughly 6,000 genes in yeast (reviewed in Kumar and Snyder, 2001; Scherens and Goffeau, 2004) and the resulting data sets, while not quite accomplishing that goal, have contributed significantly to our knowledge base of eukaryotic biology — particularly since cell

processes in yeast are typically conserved in higher eukaryotes as mentioned earlier. Following are a few examples of various genomic approaches used by different groups:

### *1.2.1. Transposon-based gene disruption:*

In the mid-90's, Burns *et al.* (Burns *et al.*, 1994) generated a collection of yeast gene disruption mutants by transposon mutagenesis, and in several subsequent studies, this approach was expanded (Ross-Macdonald *et al.*, 1999; Kumar *et al.*, 2000), and modified (Kumar *et al.*, 2004) to ultimately generate a collection of transposon insertion mutants encompassing nearly 3,800 yeast genes. This collection provided the first genome-scale reagent resource suitable for systematic phenotypic screens of genes contributing to cellular stress.

The transposon mutagenesis approach is conceptually straightforward. Transposons are mobile genetic elements that randomly integrate themselves into the genome (Craig, 1997). Transposons are present in many organisms and have been used as tools in the laboratory for decades. Each transposon used in the studies referenced above was derived from a bacterial transposon, either Tn3 (Hoekstra *et al.*, 1991) or Tn7 (Biery *et al.,* 2000), and was modified to contain bacterial and yeast selectable markers, a lacZ reporter gene and a 3XHA (haemaglutinnin) epitope (Ross-Macdonald *et al.*, 1997). The lacZ reporter gene is terminated by a series of stop codons; so, insertion of the transposon into a coding sequence truncates the gene at the site of insertion, disrupting gene function in most cases. A random insertion in-frame with the surrounding gene coding sequence also results in the production of a β-galactosidase (β-gal) fusion

protein, a useful indicator of gene expression. The transposon also contains a lox site at both ends that leads to the reduction of the transposon to a 274-bp 3xHA element upon expression of the Cre recombinase in yeast (Cre-lox recombination). This results in the production of a full-length HA-epitope-tagged protein that can be used for protein localization studies and for studies of essential genes. Thus, the modified transposon was designed to be multifunctional, generating a gene disruption, reporter fusion, and epitope-tagged allele through a single insertion.

The transposon was used for large-scale mutagenesis in yeast as follows. Briefly, a yeast genomic DNA library was transposon-mutagenized *in vitro* (Kumar *et al.*, 2004) or *in vivo* (Ross-Macdonald *et al.,* 1999) in *Escherichia coli*; in either case, plasmids were isolated in 96-well format, digested to release a fragment of transposon-mutagenized yeast genomic DNA, and the DNA fragment was transformed into yeast. By homologous recombination, each fragment integrates at its corresponding genomic locus, replacing its genomic copy. Transformants were subsequently assayed for β-gal activity to identify insertions within protein-coding genes. Productive transposon insertions producing β-gal were selected into a separate collection, and corresponding plasmids were sequenced to identify the exact site of transposon insertion in each mutant. In total, this collection encompasses over 28,000 yeast mutants, containing insertions affecting approximately 3,800 genes. This collection of yeast transposon insertion mutants has subsequently been utilized for many phenotypic screens (Kumar *et al.*, 2004). For example, Jin *et al.* screened 3,627

transposon insertion alleles in a filamentous strain of yeast and identified 309 genes that played a role in pseudohyphal growth, a developmental process in yeast induced by certain environmental stress conditions (Jin *et al.*, 2008).

### *1.2.2. Genome-wide gene deletions:*

With the fully sequenced yeast genome available as a resource for gene-finding, an international consortium of laboratories undertook an approach in the late 1990s to systematically delete every annotated gene in the genome (Winzeler *et al.*, 1999). In *Saccharomyces cerevisiae*, precise start-codon-to-stop-codon gene deletions can be achieved using polymerase-chain reaction (PCR)-mediated gene disruption (Baudin *et al.,* 1993). In 1999, Winzeler *et al.* used a high-throughput-based modification of this approach to delete 2,026 yeast genes, creating heterozygous deletion mutants, as well as 1,620 homozygous and haploid deletion strains (Winzeler *et al.*, 1999). By this approach, a PCR-amplified selectable marker (KanMX, conferring resistance to geneticin (Wach *et al.*, 1994) flanked by two sequences corresponding to regions upstream and downstream of each gene was introduced into a yeast strain. Due to the high rate of homologous recombination in yeast, the PCR fragment replaced the targeted gene, creating a deletion mutant. To identify each deletion mutant, two 'molecular barcodes', 20-base unique sequences, were introduced into the PCR fragment flanking the selectable marker (Shoemaker *et al.*, 1996).

This preliminary collection of yeast gene deletion mutants was used to assay for mutant phenotypes as follows (Giaever *et al.,* 2002). A collection of deletion strains was pooled together under certain conditions, and the presence

of each strain in the pool was ascertained by PCR amplification of the barcodes and subsequent hybridization to a high-density array of the barcode complements. The absence of hybridized spots on the array for a particular gene deletion indicated that that gene is essential for growth under the particular condition in which the strains were grown. In this study, phenotypes of more than 500 deletions were assayed, and 40% of these mutants showed quantitative growth defects in either rich or minimal medium. This method can be used on a large scale to simultaneously examine the sensitivity of all deletion strains under any growth condition. In particular, this approach highlights the foresight of the consortium in including molecular barcodes within the individual deletion cassette.

### *1.2.3. Synthetic genetic interactions:*

In the quest to expand our knowledge of genetic/regulatory networks in yeast, a new method was developed by Tong et al. enabling the analysis of synthetic genetic interactions on a large scale (Tong *et al.,* 2001). As the name suggests, synthetic interactions involve a pair of mutations that result in a phenotype not observed in either single mutant; for example, the deletion of two non-essential genes may result in cell death. Such interactions can help uncover functions of redundant genes, as researchers may screen a specific mutant for second-site mutations that either suppress or enhance the original phenotype. Specifically, the synthetic genetic array (SGA) method involved the systematic construction of double-mutants by crossing a query mutation to an array of ~4700 haploid deletion mutants. Double-mutant progeny were subsequently scored for viability.

Any inviable progeny would indicate a synthetic lethal interaction between the two deleted genes, thus identifying functional relationships between genes. This method has been used successfully in a number of studies to identify genetic interactions and thus create genetic interaction networks in yeast (Davierwala *et al.*, 2005; Boone *et al.*, 2007).

### *1.2.4. Genome-wide gene overexpression constructs:*

In complement to gene deletion-based approaches, gene overexpression screens are a classic and informative methodology by which gene function may be investigated. In this method, a yeast strain is transformed with a DNA library constructed on a high copy vector or under an inducible promoter such as *GAL1*, and the resulting transformants are plated and screened for a particular phenotype. For example, Jin *et al.* screened 2,043 genes under the control of a galactose-inducible promoter in a filamentous yeast strain and identified 199 filamentous growth genes (Jin *et al.,* 2008). Overexpression screens have also been successfully used to identify yeast genes conferring cellular resistance to drug treatment as a method to find new drug targets (Launhardt *et al.,* 1998; Luesch *et al.,* 2005; Sopko *et al.*, 2006).

### *1.2.5. Microarray analysis:*

DNA microarray technology was reported by Schena and colleagues in 1995 as a novel approach by which gene expression may be characterized on a large scale (Schena *et al.,* 1995). By this methodology, cDNA products were amplified by PCR and were mechanically deposited on microscope slides; mRNA

extracted under desired growth conditions was fluorescently labeled and hybridized against the arrayed cDNA products. Hybridization of labeled mRNA to spotted cDNA was detected by laser scanning, such that fluorescent signal at any cDNA spot indicated expression of that gene. Without belaboring the obvious, variations of microarray approaches have been used in numerous studies since to measure gene expression in organisms with sequenced genomes.

In yeast, microarray approaches have been used extensively to measure gene expression upon exposure of cells to different conditions and stresses (DeRisi *et al.,* 1997; Lashkari *et al.,* 1997). For example, in a groundbreaking study, Gasch *et al.* (Gasch *et al.,* 2000) used microarray technology to profile gene expression in yeast under a wide variety of cell stresses and environmental growth conditions, identifying an extensive transcriptional program underlying cellular stress responses. Another more recent example is a study by Ma *et al.*, who performed a microarray analysis of differential gene expression during early pseudohyphal growth in a filamentous strain of budding yeast (Ma *et al.,* 2007). This study identified a wide variety of genes and pathways differentially expressed during this process and, more importantly, revealed a new link between filamentous growth and autophagy, another cellular stress response.

### *1.2.6. Genome-wide yeast two-hybrid studies:*

The yeast two-hybrid method was developed by Fields and Song in 1989 to identify protein-protein interactions in yeast (Fields and Song, 1989). The classic two-hybrid method is conceptually simple; interaction between two target proteins

results in the reconstitution of a functionally active transcription factor and corresponding reporter gene expression. Specifically, the two-hybrid approach utilizes the yeast GAL4 transcriptional activator, which consists of two separable domains, a DNA binding domain and a DNA activation domain. Each of these domains is fused to one of two test proteins; interaction between the test proteins brings the Gal4p DNA-binding and activation domains into close proximity, thereby restoring Gal4p transcriptional activity and Gal4p-target gene expression.

The two-hybrid approach has been used extensively and is very amenable to high throughput applications, as evidenced by two independent large-scale studies identifying protein-protein interactions across the S. cerevisiae proteome (Uetz *et al.*, 2000; Ito *et al.,* 2001). The first study by Uetz *et al.* identified 957 protein-protein interactions involving 1,004 proteins, whereas the second study by Ito *et al.* identified 4,549 two-hybrid interactions among 3,278 proteins generating a network linking most of the yeast proteins across the proteome.

### 1.3. Filamentous growth in yeast:

In response to certain nutrient limiting conditions in the external environment, some strains of budding yeast (e.g., the Σ1278b strain) undergo a significant change in growth form, transitioning from single, oval shaped cells to chains of elongated cells (Gimeno *et al.* 1992; Madhani and Fink 1998). This filamentous growth form occurs both in haploid and diploid cells of this strain; however, nitrogen limitation triggers this growth form in diploids (Gimeno *et al.*, 1992), whereas in haploid cells, carbon limitation is the trigger (Cullen and Sprague,

2000). Various developmental processes occur to effect this change, such as a delay in the G2-Mitotic transition phase of the cell cycle that leads to elongated cell morphology (Gimeno *et al.*, 1994; Kron *et al.,* 1994). In addition, the budding pattern changes from axial (in haploid cells) or bipolar (in diploid cells) to a unipolar pattern, where the daughter cell buds off the mother opposite the bud scar (Roberts and Fink, 1994; Cullen and Sprague, 2002). This leads to the formation of chains of cells, called pseudohyphae. Pseudohyphal filaments can be differentiated from the true hyphae of fungi because pseudohyphae exhibit distinct cell walls.

Pseudohyphal cells spread out on the surface of solid growth substrate; in addition, pseudohyphae invade the solid media on which they grow (Madhani 2000). This developmental process is believed to be a way for cells to forage for nutrients in times of scarcity. Other inducing signals that lead to pseudohyphal/filamentous growth are the presence of short chain alcohols, such as butanol or propanol; these alcohols mimic the metabolic by-products that would occur when amino acids are used as a nitrogen source during nitrogen deprivation (Lorenz *et al.*, 2000). Though filamentous growth occurs in both haploid and diploid yeast cells, in haploids, filaments don't extend on the surface as much as in diploids (Gancedo, 2001). Hence, filamentous growth in haploids is generally referred to as invasive growth, though for the purpose of this text, filamentous growth will be used throughout to describe both developmental programs.

Various aspects of filamentous growth in the budding yeast share similarity with the hyphal growth of fungi, including the important human fungal pathogens, *Candida albicans* and *Cyptococcus neoformans* (Gagiano *et al.,* 2002). The signaling pathways and their components leading to filamentous growth in *S. cerevisiae* are conserved in these fungi. Thus, an understanding of the signaling cascades and elements involved in filamentous growth in yeast will be useful in learning more about the mechanism of pathogenesis in these organisms.

## 1.4. Regulatory pathways leading to filamentous growth:

In yeast there are at least two known signaling pathways leading to filamentous growth – the Mitogen Activated Protein Kinase (MAPK) pathway and the cAMP-dependent Protein Kinase A (PKA) pathway (Kron, 1997; Madhani and Fink, 1998). Both these pathways are triggered by nitrogen or carbon limitation, and hence both signaling pathways lead to filamentous growth in haploids or diploids.

### *1.4.1. The Filamentous MAPK Pathway:*

The general mechanism of the evolutionarily conserved MAPK pathway involves an extracellular signal that sets off a cascade of signaling events culminating in the transcription of appropriate genes to effect the correct cellular response (Elion, 2000; Qi and Elion, 2005). This cascade consists of a mitogen-activated protein kinase kinase kinase (MAPKKK) that transfers its phosphate to a mitogen-activated protein kinase kinase (MAPKK), which in turn activates a

mitogen-activated protein kinase (MAPK). MAPKs are activated by dual phosphorylation of conserved threonine and tyrosine residues. Upon activation by the cascade, MAPKs often relocate to the nucleus where they activate proteins such as transcription factors by covalently attaching a phosphate to side chains of Serine or Threonine within a consensus motif on the target protein. This leads to the expression of pathway specific genes, resulting in an appropriate response to the extracellular signal.

In response to certain nutrient limiting conditions such as low nitrogen in the media, the GTP-binding protein Ras2p is activated which binds and activates the guanine nucleotide exchange factor Cdc42p. This in turn activates GTP-Cdc24p which interacts with the p21-activated kinase (PAK), Ste20p. Ste20p in turn activates the MAPK cascade consisting of the MAPKKK Ste11p, the MAPKK Ste7p and the filamentous growth-specific MAPK, Kss1p (Liu *et al.*, 1993). Kss1p in its unphosphorylated cellular form is bound to the filamentous growth-inhibitory proteins, Dig1p and Dig2p as well as the transcription factor, Ste12p (Madhani *et al.*, 1997). When Kss1p is activated by phosphorylation from the MAPK cascade, it phosphorylates Ste12p, leading to dissociation of Dig1p and Dig2p. Ste12p when released binds to another filamentous growth-specific transcription factor, Tec1p and these two proteins as a complex bind to target genes containing a Filamentous and invasive Response Element (FRE) in their promoter region.

As is common amongst various MAPK pathways in most eukaryotes, the yeast filamentous MAPK pathway also shares components with other yeast MAPK pathways, not only within the MAPK cascade, but also upstream and

downstream of the cascade (Liu *et al.*, 1993; O'Rourke and Herskowitz, 1998). The budding yeast has five other MAP Kinases involved in different pathways – Fus3p the mating MAPK, Hog1p for osmosensing, Slt2p and Mlp1p for cell integrity maintenance and Smk1p, the sporulation-specific MAPK. However erroneous crosstalk is limited in the cell by various mechanisms (Schwartz and Madhani, 2004). For example, the mating pathway not only shares three cascade components with the filamentous growth pathway, but also the downstream transcription factor, Ste12p is a common target. To prevent activation of filamentous growth during induction by mating pheromones, the mating-specific MAPK, Fus3p induces degradation of the Tec1p transcription factor (Bao *et al.*, 2004; Brückner *et al.,* 2004). Thus Tec1p is no longer available to bind with Ste12p at FREs and there is no activation of filamentous growth target genes.

### *1.4.2. cAMP-dependent /PKA pathway:*

The cAMP/PKA pathway in yeast regulates diverse cell processes such as cell growth, sporulation, bud site selection, aging, autophagy and filamentous growth (Broach 1991; Sun *et al.*, 1994; Madhani and Fink, 1998; Pan and Heitman, 1999; Budovskaya *et al.* 2004). Like the filamentous growth MAPK pathway, it is also induced by nutrient limitation and involves activation of protein kinase A (PKA) upon increase in cellular levels of cyclic-AMP (Palecek *et al.*, 2002). This occurs through two mechanisms – one involving the GTP-binding protein, Ras2p and the other involves a G-protein coupled receptor, Gpr1p (Jiang *et al.*, 1998). Gpr1p is bound to a heterotrimeric G-protein $\alpha$ subunit Gpa2p that acts as a nutrient sensor. Upon nutrient limitation, Gpa2p stimulates production of cyclic-

AMP by activating the adenylate cyclase gene, *CYR1*. Ras2p in its GTP-bound form also stimulates production of cAMP via Cyr1p. cAMP binds to PKA, a tetrameric protein consisting of two regulatory subunits encoded by *BCY1* and two catalytic subunits encoded by *TPK1*, *TPK2* and *TPK3*. Upon, cAMP-binding, PKA dissociates, releasing the catalytic subunits. The Tpk2p subunit binds to downstream target genes, such as Flo8p, a filamentous growth transcription factor, subsequently leading to activation of filamentous growth.

### 1.5. Different approaches to studying filamentous growth in yeast:

Filamentous growth in *Saccharomyces cerevisiae* has been studied for approximately 20 years. Different groups have undertaken various approaches to study this fascinating developmental process. Besides the studies of single genes or pathways that have helped identify a number of genes involved in filamentous growth, a slew of genomic and proteomic approaches have also been used. A few of the studies that aim to identify filamentous growth genes and their regulation are mentioned below.

To identify genes involved in filamentous growth, Jin and colleagues screened 4,528 gene disruption and overexpression mutants in the haploid Σ1278b yeast strain, thereby identifying 487 filamentous growth genes. Besides identifying the genes involved in filamentous growth, other approaches have been implemented to determine the regulatory mechanisms by which these genes are controlled. For example, a microarray analysis by Ma *et al.* was used to measure differential gene expression during filamentous growth, identifying a

wide variety of genes and pathways differentially expressed during early filamentous growth (Ma *et al.,* 2007). To measure protein abundance during filamentous growth on a large scale, a mass spectrometry-based approach has been undertaken (personal communication). The authors have found a number of genes in various cellular processes that show differential abundance during filamentous growth.

In Chapter 2 of this text, I describe a study of filamentous growth genes regulated by localization. Here, the entire set of 125 yeast protein kinases were tagged with the Venus variant of Yellow Fluorescent Protein (vYFP) at the C-terminus and localized under conditions of vegetative as well as filamentous growth. Six proteins changed localization under filamentous growth conditions; in addition, a network of regulated protein kinase localization during filamentous growth was also revealed.

### 1.6. *Candida albicans*, the human fungal pathogen:

*Candida albicans* is the primary fungal pathogen of humans. It lives as a harmless commensal in the gastrointestinal and genitourinary tract as well as on the skin and mucus membranes. However, in favorable conditions, such as when the immune system is compromised due to immunosuppressive treatment in patients who have undergone organ or bone marrow transplants, or patients undergoing cancer chemotherapy, it can proliferate and disseminate in the body (Berman and Sudbery, 2002; Noble and Johnson, 2007). Once it reaches the bloodstream it can invade tissues, causing life-threatening candidial infections of

the bloodstream, liver, kidneys, spleen or central nervous system. In otherwise healthy individuals, extended antibiotic treatment that eliminates competing bacteria can cause local mucosal infections, such as oral thrush or vaginitis. In patients with HIV/AIDS severe oropharyngeal or oesophageal candidiasis are more common than invasive candidiasis. Due to various complications in individuals, invasive candidial infections lead to death in approximately 40% of patients (Wisplinghoff *et al.,* 2004). *Candida* has become an increasingly important pathogen, being one of the main factors of nosocomial infections.

Treatment of candidial infections typically involves use of an azole antifungal such as fluconazole or itraconazole but as the disease tends to recur, azole-resisistant *Candida albicans* tend to develop. Development of new anti-fungal drugs is hence extremely important in order to treat candidiasis and prevent its recurrence in patients.

### 1.7. Challenges to genetic studies in *Candida albicans*:

There have been reports in the literature of isolating *Candida albicans* as early as 1839; however, it is only since the 1990s that laboratories began studying this important human pathogen in earnest (Noble and Johnson, 2007). There are various aspects to *Candida* that hindered progress in understanding its biology. Besides the fact that *Candida albicans* is a human pathogen, and hence care must be taken when working with this organism, a host of other issues have made it challenging to conduct experiments. For one, as *Candida* does not exist as a stable haploid, genetics is all the more cumbersome. Though there has

been some evidence of a sexual cycle in some engineered laboratory strains, this is an incomplete cycle lacking meiosis, hence leading to a tetraploid organism after mating of the diploid cells (Noble and Johnson, 2007). To study recessive mutations, it is therefore necessary to mutate both copies of a gene to observe any effect.

Exogenously supplied DNA tends to integrate in *Candida* by homologous recombination, as it also does in *S.cerevisiae*; however, there are often cases of genes integrating via non-homologous recombination as well, so caution must be exercised in interpreting results from transformation-based approaches (Pla *et al.*, 1996). Transformation efficiencies are extremely low in *Candida*, and large quantities of transforming DNA are required to achieve reasonable results. There are no naturally occurring replicating plasmids such as the 2μ plasmid in budding yeast. Certain plasmids that have been engineered to contain autonomous replicating sequences (ARS) invariably integrate into the *Candida* genome (Berman and Sudbery, 2002). *C.albicans* also translates the CUG codon as Leucine instead of Serine (Santos *et al.*, 1997); so, the use of heterologous markers and tags was previously restricted to those that lack this codon in their sequence. Finally, since *Candida* is naturally resistant to antibiotics like G418/kanamycin (Fonzi and Irwin, 1993), many drug-resistance genes cannot be used as selection markers.

However, numerous studies over the past ten years have served to facilitate experimental analysis in *Candida albicans*. For one, transformation efficiencies have been improved by trial and error of different experimental

conditions (Walther and Wendland, 2003). New heterologous markers have been designed, such as *NAT1* (Shen *et al.,* 2005; Schaub *et al.*, 2006) and new reference strains like *leu2Δ/leu2Δ* (Noble and Johnson, 2004) have been developed. GFP and its variants have been redesigned with a modified CUG codon (Morschhauser *et al.,* 1998). Thus, numerous advances by multiple laboratories across the world have finally made it possible to perform more sophisticated experiments in *C.albicans*.

Genome sequencing of the heterozygous diploid *Candida albicans* began in 1996 by the Stanford Genome Technology Center (Jones *et al.*, 2004). It was completed and annotated in 2004, with revisions made as recently as 2006 (Nantel, 2006; van het Hoog *et al.,* 2007). *Candida* has a haploid genome size of 16Mb with approximately 6,500 ORFs. The sequence data and annotations are freely available through the Candida Genome Database (CGD) at www.candidagenome.org. Since the sequencing of the genome was completed, there have been numerous genomic studies as well as individual gene/pathway studies on different aspects of *Candida albicans* biology.

### 1.8. Morphogenesis of *Candida albicans*:

*Candida* is known to exist in multiple growth forms – yeast, pseudohyphae, hyphae and chlamydospores (Sudbery *et al.,* 2004; Whiteway and Oberholzer, 2004). It adopts these various forms in response to its environment. Unlike most other fungal pathogens, *Candida* is not found in nature but is isolated from clinical samples from individuals. The yeast and hyphal forms are both found in

clinical isolates. Yeast form cells are oval cells that divide by budding like in *S.cerevisiae*. They are typically found at acidic pH of approximately 4, at high cell densities of greater than $10^6$ cells, and at low growth temperatures of 30°C. Yeast cells form pseudohyphae at a slightly higher pH of 6 and 35°C temperature, in addition to other triggers such as low nitrogen and high phosphate.

Pseudohyphae are similar in form to those in *S.cerevisiae* – they consist of chains of elongated cells with distinct cell walls, in contrast to hyphal cells that have no constriction at the mother-bud junction and have a parallel cell wall along their length. The width of pseudohyphal cells is also greater, with a minimum width of about 2.8 μm, whereas hyphal cells have a width of approximately 2μm (Sudbery *et al.,* 2004). Hyphae are formed from yeast cells in response to many different conditions such as serum, N-acetylglucosamine, physiological temperature of 37°C and neutral pH, and microaerophillic conditions. In addition, macrophage engulfment and iron deprivation also trigger the yeast-hyphal transition. Chlamydospores are large cells with thick cell walls that are formed mainly from hyphae under nutrient-poor micoraerophillic conditions at low temperatures (Whiteway and Oberholzer, 2004).

### 1.9. Regulatory pathways leading to hyphal development in *Candida*:

The ability to switch between the yeast, pseudohyphal and hyphal forms is believed to be important for virulence. This was inferred from a study of *Candida* mutants that were unable to undergo a switch to the hyphal form – these mutants

were avirulent in a mouse model of pathogenesis (Lo *et al.,* 1997). Also, mutants that can't undergo hyphal growth have decreased virulence in various *in vivo* and *ex vivo* models of candidial infections (Zheng *et al.,* 2004; Thewes *et al.,* 2007). It has been suggested that yeast form cells play a role in dissemination while the hyphal form is important for invasion of host tissue (Gow, 2002). Thus the yeast-to-hyphal switch is an important factor to consider in understanding the pathogenesis of this organism.

Studies of the yeast-to-hyphal transition have revealed the existence of multiple regulatory pathways that respond to various environmental triggers – some of them being the MAPK pathway (Kohler and Fink, 1996; Román *et al.,* 2007), the cAMP-dependent PKA pathway (Biswas *et al.,* 2003) and the RAM pathway (Song *et al.,* 2008). The MAPK and PKA pathways are amongst the better-characterized ones and are analogous to those found in the yeast-to-pseudohyphal switch in *Saccharomyces cerevisiae* (Dhillon *et al.*, 2003). The RAM network regulates many cell processes in eukaryotes, including hyphal development in *C.albicans* (Nelson *et al.*, 2003; Song *et al.,* 2008).

### *1.9.1. MAPK pathway:*

The MAPK pathway that regulates the yeast-hyphal transition in *C.albicans* is triggered by various environmental conditions acting through the GTP-binding single Ras protein homolog, Ras1p (Biswas *et al.,* 2007). When Ras1p is activated, it binds and activates the guanine nucleotide exchange factor Cdc42p, in turn activating Cst20p, analogous to the PAK kinase, Ste20p in yeast. Additional proteins of the cascade include the MAPKK, Hst7p and the MAPK,

Cek1p, homologous to Kss1p. The transcription factors downstream of this cascade are Cph1p, the Ste12p yeast homolog, and Tec1p, homologous to *S.cerevisiae* Tec1p.

### *1.9.2. cAMP-dependent PKA pathway:*

Like in the budding yeast, the cAMP/PKA pathway in *C. albicans* involves activation of protein kinase A (PKA) upon increase in cellular levels of cyclic-AMP (Biswas *et al.,* 2003). Activation of this pathway occurs via the amino acid-sensing G protein-coupled receptor system Gpr1p-Gpa2p. Ras1p in its GTP-bound form also stimulates production of cAMP by activation of the adenylate cyclase gene, *CDC35*. PKA in *C.albicans* consists of a regulatory subunit, Bcy1p and two catalytic subunits, Tpk1p and Tpk2p. When cAMP binds to PKA, the subunits dissociate, and the catalytic subunits phosphorylate and activate the downstream transcription factor, Efg1p, leading to cAMP-dependent morphogenesis.

### *1.9.3. RAM network:*

The RAM (Regulation of Ace2 and Morphogenesis) network consists of six genes present in both *C. albicans* and *S. cerevisiae* – namely, *CBK1*, *MOB2*, *KIC1*, *HYM1*, *TAO3* and *SOG2* (Song *et al.*, 2008). The RAM pathway regulates diverse cell processes in both eukaryotes, such as cell separation, daughter cell-specific localization of the transcription factor Ace2p, cell wall integrity, and polarized growth (Jansen *et al.,* 2006, Nelson *et al.*, 2003). In addition, the RAM network is involved in hyphal development in *C.albicans*. In *S.cerevisiae*, Mob2p

binds Cbkp1 and is responsible for its activation and correct localization. The Kic1p kinase functions upstream of the Mob2p-Cbk1p complex, and Kic1p likely activates these proteins. Hym1p interacts with both Cbk1p and Kic1p and is responsible for localization of this complex. Cbk1p is a serine/threonine protein kinase in the Ndr/LATS family; it phosphorylates Ace2p, which regulates numerous targets contributing to wild-type cell polarity (Mazanka *et al.,* 2008). The role of the RAM network proteins in hyphal development in *Candida* has been shown by a number of independent studies on Cbk1p (McNemar and Fonzi, 2002; Uhl *et al.,* 2003) Mob2p (Song *et al.* 2008) and the Ace2p transcription factor (Kelly *et al.,* 2004).

## 1.10. Genomic studies of hyphal growth in *Candida albicans*:

Various approaches have been undertaken over the years to understand the signaling pathways leading to hyphal development in *Candida albicans*. These studies have included both single gene / pathway studies as well as recent large-scale genomic studies (Bruno and Mitchell, 2004).

### *1.10.1. Transposon mutagenesis:*

There have been two major large-scale transposon mutagenesis studies that have been used to investigate related aspects of hyphal development. The first study used transposon mutagenesis to identify genes involved in the yeast-to-hyphal transition. Using *in vitro* transposition, Uhl and colleagues created and

screened 18,000 heterozygous mutant strains that led to the identification of 146 genes involved in this process (Uhl *et al.*, 2003).

Another study by Davis *et al.* employed transposons to identify essential genes and regulators of alkaline pH-induced hyphal growth (Davis *et al.,* 2002). Out of 253 transposon insertions, they identified three genes required for this process; the authors also identified 36 essential genes, twenty of which were homologs of essential genes in *S.cerevisiae*.

### *1.10.2. Repressible gene collection:*

In an attempt to identify essential genes in *C.albicans*, Roemer *et al.* created a collection of strains that contained 1152 genes whose expression was regulated by the antibiotic, tetracycline (Roemer *et al.*, 2003). In this study, one allele was deleted from each strain, and the other was placed under control of a tetracycline-repressible promoter. Using this method, the authors identified 567 essential *C.albicans* genes; importantly, only 61% of these genes had essential orthologs in *S.cerevisiae*.

### *1.10.3. Microarray analysis:*

The sequencing of the genome in the late 1990's subsequently led to the production of microarrays contaning the ORFs of the entire genome of *Candida albicans*. This has resulted in numerous studies of the transcriptional response of genes under various conditions. For example, a microarray analysis of 6,333 predicted ORFs in cells undergoing hyphal growth was performed which,

amongst other findings, led to the identification of several genes involved in virulence (Nantel *et al.*, 2002).

Another study by Mulhern and colleagues (Mulhern *et al.,* 2006) used transcriptional profiling to identify genes regulated by the Ace2p transcription factor of the RAM network during hyphal development. They showed that *ACE2* regulates genes involved in cell separation and glycolysis, and is required for hyphal growth under hypoxic conditions.

Thus, the development of new tools and techniques for genetic and genomic studies in *C.albicans* has resulted in a host of new information on the regulatory pathways leading to hyphal development, besides identifying various elements involved in the pathogenesis of this important human pathogen.

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# Chapter 2

## Analysis of the Yeast Kinome Reveals a Network of Regulated Protein Localization During Filamentous Growth

### 2.1. Introduction

In eukaryotes, protein function is regulated through mechanisms controlling transcription, translation, post-translational modification, protein degradation, and subcellular localization. In recent years, global and/or systematic studies have been employed to consider the majority of these regulatory mechanisms across a wide set of genes and proteins. DNA microarray technologies (DeRisi, 1997; Gasch *et al.*, 2001) and mass spectrometry-based approaches (Gygi *et al.*, 1999; Tang *et al.*, 2005; Roth *et al.*, 2006) have cataloged genome-wide changes in transcriptional levels, protein abundance, and post-translational modifications; however, our understanding of regulated protein localization remains cursory, constructed piecemeal from individual reports of a given protein whose function is regulated by its localization.

Protein localization has been investigated most intensely in *S. cerevisiae* (Kumar *et al.*, 2002; Huh *et al.*, 2003), and reports of regulated protein localization have surfaced frequently in yeast-based studies. For example, a

number of yeast proteins, such as the $G_1$ cyclins Cln2p and Cln3p, are regulated by differential compartmentalization during cell cycle progression (Edgington and Futcher, 2001). The transcription factor Pho4p, involved in phosphate metabolism, is predominantly cytoplasmic under conditions of phosphate sufficiency, but localizes to the nucleus during phosphate starvation (O'Neill *et al.*, 1996). Components of the yeast Slt2p mitogen-activated protein kinase (MAPK) cell wall integrity pathway localize at sites of polarized growth in response to mating factor (Mazzoni *et al.*, 1993; Buehrer and Errede, 1997). Collectively, from these and other studies (Shimada *et al.*, 2000), we infer that a sizable protein complement may be regulated by differential localization in yeast, with protein kinases constituting one group particularly subject to such regulation.

Protein kinases play a prominent role in many developmental processes, and filamentous differentiation in yeast provides a strong example. In certain strains of yeast (e.g., Σ1278b), MAPK and cAMP-dependent protein kinase (PKA) pathways mediate a stress-induced transition to a multicellular, filamentous growth form (Gimeno and Fink, 1994; Kron, 1997; Cullen and Sprague, 2000; Erdman and Snyder, 2001). Specifically, nitrogen stress or growth in the presence of short chain alcohols initiates a developmental program characterized by the formation of filamentous chains of cells, called pseudohyphae (Lorenz *et al.*, 2000). During filamentous growth, yeast cells delay in $G_2$/M, exhibit an elongated morphology, display an altered budding pattern, remain physically attached after cytokinesis, and invade their growth substrate (Gimeno *et al.*, 1992; Kron, 1997; Madhani and Fink, 1998). These

morphological and genetic changes are brought about through signaling pathways encompassing the Kss1p MAPK cascade (Ste11p, Ste7p, and Kss1p) and PKA (Liu *et al.*, 1993; Pan *et al.*, 2000). In yeast, PKA consists of the regulatory subunit Bcy1p and the catalytic subunit isoforms Tpk1p, Tpk2p, and Tpk3p (Robertson and Fink, 1998). As yeast cells undergo filamentous growth, these kinase-based signaling modules function with additional genes and pathways governing cell polarity, bud site selection, and cell cycle progression (Chandarlapaty and Errede, 1998; Miled *et al.*, 2001; Madhani *et al.*, 1999). Extensive regulatory mechanisms are in place to coordinate signaling pathways during filamentous growth (Gimeno and Fink, 1994; Borneman *et al.*, 2006), and the subcellular distribution of yeast kinases is likely controlled as part of this regulation.

To consider the degree to which protein localization is regulated during eukaryotic cell growth, and, specifically, the extent to which this contributes to the filamentous growth response, we screened all protein kinases in the budding yeast for differential localization during filamentous growth. This analysis revealed six kinases localized evenly across the cell during vegetative growth but localized predominantly in the nucleus under conditions of filamentous growth. Through localization-based epistasis studies, we found that the kinases form part of an interdependent network of regulated protein localization — the first such network identified in any eukaryote. Our results indicate a "regulatory/subordinate" relationship among kinases within this subnetwork; using deletion mutants and kinase-dead alleles, we show that kinase

translocation, in many cases, requires the presence/activity of another kinase. In addition, this study implicates the functionally uncharacterized Ser/Thr kinase Ksp1p in filamentous growth, while highlighting, in broader terms, the need to consider similar regulatory mechanisms in other eukaryotes.

## 2.2. Materials and Methods

### *2.2.1. Yeast strains and growth conditions:*

The filamentous yeast strains Y825 and Y825/6 are derived from Σ1278b (Gimeno *et al.*, 1992). The genotype of haploid Y825 is *MAT***a** *ura3-52 leu2*Δ0; the genotype of diploid Y825/6 is *ura3-52/ura3-52 leu2*Δ*0/leu2*Δ*0*. The non-filamentous strain Y2269 is of the S288c genetic background and is a derivative of strain BY4743 (Giaever *et al.*, 1999). Deletion mutants were constructed in strain Y825 using a one-step PCR-based gene disruption strategy (Baudin *et al.*, 1993) with the G418 resistance cassette from plasmid pFA6a-KanMX6 (Longtine *et al.*, 1998). The *sks1-K39R* kinase-dead allele was generated by site-directed mutagenesis using oligonucleotides described in Yang and Bisson (1996). Kinase-dead alleles were obtained or generated on low-copy plasmids, except the *ksp1-K47D* allele, which is carried on pYEPs29K47D, described in Fleischmann *et al.* (1996).

Standard growth medium for microscopy was prepared using 0.17% Yeast Nitrogen Base (YNB) without amino acids and ammonia, 2% glucose and 5mM ammonium sulfate (Guthrie and Fink, 1991). Haploid filamentous growth was

induced in this standard medium supplemented with 1% (vol/vol) butanol (Lorenz *et al.*, 2000) or on SLAD plates (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, 50μM ammonium sulfate, with essential amino acids) plus 1% (v/v) butanol (Lorenz *et al.*, 2000; Gimeno *et al.*, 1992). Diploid filamentous growth was induced in liquid low-nitrogen medium (0.17% YNB without amino acids and ammonia, 2% glucose and 50μM ammonium sulfate) (Gimeno *et al.*, 1992). Invasive growth was assayed on YPD medium or synthetic complete (SC) –Ura as indicated (Guthrie and Fink, 1991; Lorenz *et al.*, 2000).

### *2.2.2. Construction of yeast kinase-fluorescent protein fusions:*

In this study, we constructed a Gateway-compatible (Invitrogen Corporation, CA) yeast vector for the recombination-based cloning of promoter-gene cassettes as carboxy-terminal fusions to fluorescent protein (Walhout *et al.*, 2000). This Gateway vector was constructed from the centromeric yeast shuttle vector YCp50 (Rose *et al.*, 1987). To construct pDEST-vYFP, the Venus variant of yellow fluorescent protein (vYFP) (Nagai *et al.*, 2002) was amplified by PCR from pBS7 (Yeast Resource Center, University of Washington) for introduction into the *Sph*I-*Sal*I site of YCp50. Subsequently, the YCp50 vector carrying vYFP was digested with *Sph*I and made blunt with $T_4$ DNA Polymerase (New England Biolabs, MA). Gateway cassette A (Invitrogen Corporation, CA) was ligated with the blunt-ended vector, and *Eco*RI was used to identify the orientation of the cassette.

In total, we cloned 119 kinase genes into pDEST-vYFP. Briefly, we

amplified by PCR each open reading frame along with 1 kb of upstream genomic DNA for introduction into pDEST-vYFP; PCR primers contained phage lambda *att* sites allowing for recombination with the *att*-containing destination vector according to protocols described in Gelperin *et al.* (2005). Upon cloning, the PCR product creates a translational fusion between the 3'-end of the gene and vYFP, with a 10-codon linker encoding NPAFLYKVVI. The six remaining yeast kinase genes (*MEC1, HRK1, RIM15, TEL1, TOR1,* and *TOR2*) proved difficult to clone and were instead chromosomally tagged at their 3-ends with vYFP in the filamentous strain Y825/6; the genes *HRK1*, *RIM15*, *TEL1*, and *TOR1* were also chromosomally tagged in Y825 as well. Integrated alleles were generated by standard protocols using the vYFP-KanMX6 cassette from pBS7 (Yeast Resource Center, University of Washington).

### *2.2.3. Live cell microscopy:*

Cells were grown overnight, diluted to an $OD_{600}$ of roughly 0.1 and grown in standard medium or inducing conditions as required. Filamentous growth in haploid strains was induced by inoculating diluted cultures into standard growth medium supplemented with 1% butanol for 4 hours at 30°C. Diploid filamentous strains were induced as follows: overnight cultures were centrifuged, washed and inoculated at an $OD_{600}$ of 0.1 into low-nitrogen medium at 30°C for 4 hours before observation. Osmotic stress and heat stress were induced by growing diluted overnight cultures in standard growth medium to mid-log phase, followed by either 20 minutes growth in medium containing 0.4M NaCl (Ferrigno *et al.*, 1998) or by 20 minutes growth at 37°C (Ferrigno *et al.*, 1998). 4,6-diamidino-2-

phenylindole (DAPI) was added at a final concentration of 2μg/ml for 30 minutes to stain DNA, marking the nucleus and mitochondria.

#### *2.2.4. Phenotypic assays for filamentous growth defects:*

Colony morphology of deletion mutants was observed by streaking mid-log cultures grown in YPD onto SLAD plates supplemented with 1% (v/v) butanol (Lorenz *et al.*, 2000) and incubating at 30°C for 10 days. Invasive growth was determined by the standard plate-washing assay of Gimeno *et al.* (1992). For this assay, mid-log phase cultures were spotted onto YPD plates and incubated for 5 days at 30°C; surface cells were then washed off under a gentle stream of water.

## 2.3. Results

#### *2.3.1. Localization of yeast kinases during filamentous growth:*

The yeast proteome encompasses 125 protein kinases, defined from data sets deposited in the *Saccharomyces* Genome Database as of August 2006 (www.yeastgenome.org). For purposes of this analysis, we include the regulatory subunit of protein kinase A, Bcy1p. The yeast kinase complement, or kinome, is listed in Supplementary Figure SF2-1 (appendix), along with relevant functions and protein localization data describing the subcellular distribution of each protein in non-filamentous lab strains (e.g., S288c). To investigate the subcellular dynamics of these kinases during filamentous growth, we constructed a unique plasmid-based collection of kinase-fluorescent protein fusions. This

plasmid-based approach is well suited for localization studies in non-standard genetic backgrounds, such as in the filamentous strain Σ1278b; the Σ1278b genetic background is preferred for studies of filamentous growth (Gimeno *et al.*, 1992), and existing reagents for protein localization studies are not available in this strain. As part of this study, we designed a centromeric yeast shuttle vector, pDEST-vYFP, for the expression of cloned genes as chimeric proteins in which the Venus variant of yellow fluorescent protein (vYFP) is fused to the carboxy terminus of the target protein (Nagai *et al.*, 2002). The pDEST-vYFP vector carries phage lambda *att* sites for recombination-based cloning, accommodating gene coding sequences along with 1 kb of upstream promoter sequence. By virtue of this vector, genes are expressed under control of their native promoters at nearly endogenous levels.

In total, we cloned 119 kinase genes into pDEST-vYFP and subsequently introduced the kinase-vYFP fusion into the filamentous strain Σ1278b by the approach outlined in Figure 2-1. For six kinases (*MEC1, HRK1, RIM15, TEL1, TOR1,* and *TOR2*), C-terminal fusions to vYFP were generated as integrated alleles in Σ1278b (described in Materials and Methods). The kinase-vYFP chimeras were screened by fluorescence microscopy for differential localization under vegetative and filamentous growth conditions in both haploid and diploid strains of Σ1278b, since the filamentous growth response differs according to ploidy (Gancedo, 2001). A full listing of protein kinase localizations under these conditions is presented in Supplementary Table ST2-1 (appendix).

From this analysis, we found six kinases with altered subcellular

distribution under filamentous growth conditions (Figure 2-2). The proteins Bcy1p, Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p distributed evenly over the cell under conditions of vegetative growth but localized predominantly to the nucleus during filamentous growth. In each case, this nuclear shift was striking, with approximately 80-95% of observed fluorescence concentrated within the nucleus. The differential localization of these kinases was evident in both haploid cells (shown in Figure 2-2) and diploid cells for Bcy1p, Fus3p, Kss1p, Sks1p, and Tpk2p; however, Ksp1p did not localize strongly to the nucleus during filamentous growth in diploid yeast.

As mentioned previously, Bcy1p, Tpk2p, and Kss1p are known components of filamentous growth PKA and MAPK cascades, respectively. Fus3p is the MAPK mediating the yeast mating response (Elion *et al.*, 1993; Choi *et al.*, 1999), and Sks1p is a serine/threonine kinase involved in the cellular response to glucose limitation (Vagnoli and Bisson, 1998). The cellular function of Ksp1p is unknown, although its overexpression is known to suppress mutations in *SRM1*, a nucleotide exchange factor required for nucleocytoplasmic trafficking of macromolecules (Fleischmann *et al.*, 1996).

To determine if the observed localization shifts are specific to the yeast filamentous growth response, we further screened the six yeast kinases identified above for differential localization during osmotic stress (in 0.4 M NaCl) and heat stress in Σ1278b. Bcy1p is known to localize differentially during heat stress (Griffioen *et al.*, 2001); however, the other kinase-vYFP fusions were distributed evenly over the cytoplasm and nucleus under the conditions tested. Thus, the

observed nuclear shifts do not represent general stress responses and are likely specific to conditions inducing filamentous growth.

### *2.3.2. Phenotypic analysis of nuclear-localized kinases:*

To clarify the potential roles of these six kinases in filamentous growth, we deleted each corresponding kinase gene in a haploid derivative of Σ1278b for phenotypic analysis of filamentation (Figure 2-3). Deletion mutants were screened for colony and cell morphologies, and invasive growth was assayed by the standard plate-washing assay of Gimeno *et al.* (1992). Filamentous growth phenotypes were consistent across all assays. Hyperfilamentous growth was evidenced by extended peripheral filamentation, elongated cell morphology, and increased invasive growth relative to wild type; the converse phenotypes were present in mutants defective in filamentous growth. In our assays, deletion of *KSP1*, *KSS1*, and *TPK2* resulted in decreased filamentous growth, while the *fus3*Δ mutant was hyperfilamentous. Deletion of *BCY1* and *SKS1* did not affect filamentous growth. These phenotypic results are consistent with our understanding of filamentous growth functions associated with Bcy1p, Fus3p, Kss1p, and Tpk2p; however, Ksp1p had not been implicated previously in filamentous growth, and, in this context, the strong effect of Ksp1p on filamentous growth is particularly noteworthy.

The kinase shifts reported here were identified using proteins tagged at their carboxy termini with vYFP; however, carboxy-terminal protein modifications can, in some cases, disrupt function. To consider the functionality of these kinase-vYFP chimeras, we assessed the ability of Fus3p-vYFP, Ksp1p-vYFP,

Kss1p-vYFP, and Tpk2p-vYFP to complement corresponding deletion mutants for filamentous growth phenotypes. In each case, introduction of the kinase-vYFP fusion restored wild-type filamentous growth. The Bcy1p-vYFP chimera complemented the heat-sensitive phenotype of a *bcy1*Δ mutant, as assayed by the method described in Toda *et al.* (1987). The functionality of Sks1p-vYFP was not considered, because its null phenotype is not easily assayed.

### *2.3.3. Interdependent kinase translocation to the nucleus during filamentous growth:*

The regulated localization of Bcy1p, Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p during filamentous growth and the known interplay between kinases in signaling pathways led us to consider the possibility that the observed nuclear translocation of these kinases may be interdependent: the presence of one kinase may be required for the localization shift of another. To address this explicitly, we systematically examined the subcellular localization of these six kinases in mutant backgrounds individually deleted for one of the five other kinases. For example, the subcellular distribution of Bcy1p was examined under conditions of vegetative and filamentous growth in five haploid mutant backgrounds: *fus3*Δ, *ksp1*Δ, *kss1*Δ, *sks1*Δ and *tpk2*Δ. The remaining five kinases were also examined accordingly, and the results from this study are presented in Figure 2-4.

Interestingly, the nuclear shift observed for Bcy1p, Fus3p, Ksp1p, and Sks1p is dependent in each case upon the presence of at least one other kinase (Figure 2-4A and B). Fus3p is distributed evenly across the cytoplasm and

nucleus under conditions inducing filamentous growth in a haploid strain deleted for *KSP1*. The nuclear shift of Bcy1p requires the presence of Ksp1p, Kss1p, and Sks1p. The predominantly nuclear localization of Ksp1p during filamentous growth is lost in strains deleted for *BCY1*, *FUS3*, *SKS1*, or *TPK2*. The deletion of any kinase in this subset disrupts the nuclear shift of Sks1p under filamentous growth conditions. In contrast, the localization of Kss1p and Tpk2p during filamentous growth is unaffected by the deletion of these kinases.

Many kinases in this localized subgrouping exhibit reciprocal relationships (Figure 2-4C). The deletion of *BCY1* affects the butanol-induced nuclear localization of Sks1p, and, reciprocally, deletion of *SKS1* disrupts the nuclear shift of Bcy1p. We observed similar effects for the kinase pairs Bcy1p-Ksp1p, Fus3p-Ksp1p, and Ksp1p-Sks1p. Other kinase pairs, however, exhibit a unidirectional "regulatory/subordinate" relationship; for example, the presence of Fus3p is required for the nuclear shift of Sks1p, but *SKS1* is not required for wild-type localization of Fus3p under conditions of filamentous growth. Similarly, Tpk2p contributes to the regulated localization of Ksp1p and Sks1p, but the reciprocal relationships are not evident. Kss1p also contributes to the butanol-induced nuclear shift of Bcy1p and Sks1p, although neither gene product affects the localization of Kss1p during filamentous growth.

Taken collectively, the six kinases identified here form part of an interdependent network of regulated protein localization during filamentous growth (Figure 2-4D). It is likely that additional proteins contribute to the regulated localization of these kinases; thus, the relationships indicated in Figure

2- 4 represent a portion of a potentially larger network. In particular, our results highlight the key regulatory roles of Kss1p and Tpk2p; both proteins are required for the wild-type localization of members of this subnetwork (i.e., Bcy1p, Ksp1p, and Sks1p), but neither is affected by the presence of any other kinase tested here. Ksp1p plays a central role in this network, affecting the localization of Bcy1p, Fus3p, and Sks1p, while itself requiring the presence of Bcy1p, Fus3p, Sks1p, and Tpk2p for wild-type localization under conditions of filamentous growth. This is particularly interesting since Ksp1p had not been implicated previously in filamentous growth. Downstream or subordinate roles are also evident within this subnetwork; in particular, all five other kinases are required for the nuclear localization of Sks1p, while its presence is only required for the wild-type localization of Bcy1p and Ksp1p.

This localization-based interdependence suggests regulatory relationships that may be investigated through traditional epistasis studies with double-deletion mutants. For example, as outlined above, the localization of Ksp1p is affected upon deletion of *BCY1*, *FUS3*, *SKS1*, or *TPK2*; thus, we deleted each of these genes individually in a strain deleted for *KSP1* to assess the impact of each mutation on the *ksp1*Δ filamentous growth phenotype. The results from this analysis are shown in Supplementary Figure SF2-4 (appendix). The *ksp1*Δ*fus3*Δ mutant exhibited exaggerated filamentous growth, as assessed by examination of colony morphology, cell morphology, and invasive growth; this mimics the phenotype of *fus3*Δ. As expected, the *ksp1*Δ*tpk2*Δ double mutant shows no filamentous growth; however, deletion of *BCY1* and *SKS1*, respectively, restores

yeast filamentous growth in a *ksp1*Δ genetic background. At minimum, this indicates a filamentous growth effect associated with *sks1*Δ. It is interesting that the *ksp1*Δ phenotype is masked by the phenotype associated with the other gene deletion in each double mutant. From this, Ksp1p may serve an upstream role in filamentous growth pathways; however, it is very difficult to interpret these results, since we have no clear evidence that Ksp1p actually functions in a linear pathway with the PKA and/or MAPK modules.

### *2.3.4. Kinase activity is required for the nuclear translocation of dependent proteins:*

Kinases serve both structural and catalytic functions in signaling pathways, phosphorylating target proteins but also, in many cases, acting as scaffolds facilitating protein-protein interactions (Madhani and Fink, 1997; Choi *et al.*, 1994). To specifically determine the role of kinase phosphorylation in controlling subordinate protein translocation to the nucleus, we examined the subcellular distribution of Bcy1p, Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p in kinase-dead mutants of Fus3p (*fus3-K42R*), Ksp1p (*ksp1-K47D*), Kss1p (*kss1-K42R*), Sks1p (*sks1-K39R*), and Tpk2p (*tpk2-K99R*) (Madhani *et al.*, 1997; Zeitlinger *et al.*, 2003; Fleischmann *et al.*, 1996; Yang and Bisson, 1996; Demlow and Fox, 2003); Bcy1p was omitted from this study, because it does not possess a kinase domain. As indicated in Supplementary Figure SF2-5 (appendix), each kinase-dead mutant exhibits filamentous growth phenotypes mirroring those observed upon gene deletion.

In this analysis, we individually introduced each kinase-dead allele into a

haploid Σ1278b strain deleted for the corresponding kinase gene and analyzed the subcellular localization of kinases dependent upon this protein for nuclear translocation during filamentous growth (Figure 2-5). For example, the localization of Ksp1p and Sks1p was observed in a kinase-dead *fus3-K42R* mutant; the localization of Bcy1p, Fus3p, and Sks1p was assessed in the *ksp1-K47D* mutant, and so on. In each case, kinase activity was required for the observed localization shift. As shown in Figure 2-5, Ksp1p-vYFP and Sks1p-vYFP were evenly distributed across the nucleus and cytoplasm in a strain bearing the *fus3-K42R* allele under conditions of butanol-induced filamentous growth. Similar results were observed over the full panel of mutants tested. Thus, the kinase activity of the identified proteins is required for the nuclear translocation of dependent kinases. It should be noted that the required phosphorylation events may be indirect. Also, observed protein localization was not affected in any kinase-dead mutant under conditions of vegetative growth (Supplementary Figure SF2-6) (appendix).

### *2.3.5. The nuclear translocation of Ksp1p is required for wild-type filamentous growth:*

As evidenced by its null phenotype, Ksp1p is required for butanol-induced filamentous growth in haploid yeast; however, the importance of its observed localization shift during filamentous growth cannot be explicitly determined from this finding. To consider this point more directly, we generated a modified form of Ksp1p unable to translocate into the nucleus. Specifically, we fused a nuclear export sequence (NES) to the 3′ end of *KSP1*-vYFP, as illustrated in Figure 2-

6A. The Ksp1p-vYFP-NES chimera was expressed from the native *KSP1* promoter on the low-copy destination vector described in Figure 2-1. For this analysis, the NES was derived from the Rev protein of HIV-1 (Fritz *et al.*, 1995), which has been used previously to direct nucleo-cytoplasmic trafficking in yeast (Murphy and Wente, 1996). Attachment of this NES prevented the nuclear shift of Ksp1p under conditions of butanol-induced filamentous growth (Figure 2-6A). Phenotypic analysis of this NES-tagged mutant revealed significantly reduced filamentous growth as compared to a haploid *ksp1*Δ strain expressing the Ksp1p-vYFP chimera: surface-spread filamentation and invasive growth was lost in the NES-tagged strain, and its cell morphology was rounded rather than elongated under conditions inducing filamentous growth (Figure 2-6B). The *ksp1-K47D* kinase-dead mutant is also defective in filamentous growth (Supplementary Figure SF2-5) (appendix); therefore, the nuclear translocation of Ksp1p and its kinase activity are both required for wild-type filamentation, suggesting that Ksp1p likely phosphorylates one or more nuclear proteins during the filamentous growth response.

## 2.4. Discussion

From numerous individual examples, it is clear that the function of a protein can be controlled through mechanisms regulating its subcellular distribution; however, the extent to which this form of regulation occurs has not been investigated on a large scale. Accordingly, we undertook the first systematic analysis of differential protein localization for any protein set in any eukaryote,

and using the yeast kinome as the subject, we identified an interdependent subnetwork of proteins whose intracellular localization is tightly regulated during filamentous growth. This study, therefore, describes an underappreciated type of regulatory network, yields insight into the degree to which differential protein localization serves as a widespread regulatory mechanism, and also identifies the kinase Ksp1p as a new filamentous growth gene.

The plasmid collection described here constitutes a unique and versatile resource for the yeast scientific community, in complement to a very useful set of chromosomally integrated GFP-fusions presented in Huh *et al.* (2003). Obviously, however, our plasmid collection is not without its limitations; carboxy-terminal fusions may perturb the functions of some proteins, and this is an important consideration in using these plasmids. For example, carboxy-terminal tagging is typically problematic in analyzing isoprenylated gene products and geranylgeranylated proteins (Bhattacharya *et al.*, 1995), as well as proteins modified with palmitoyl and farnesyl groups (Roth *et al.*, 2006; Sun *et al.*, 2004). While some proteins of the cell wall, endoplasmic reticulum, and peroxisomes may be adversely affected by carboxy-terminal modification, we do expect the majority of yeast proteins to function normally as C-terminal FP fusions (Pelham *et al.*, 1988). Plus, the alternative approach of amino-terminal tagging poses greater potential for protein mislocalization.

By live-cell imaging of kinase-YFP chimeras, we found Bcy1p, Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p differentially localized to the nucleus under filamentous growth conditions. These kinases have been characterized to

varying degrees, and the pathway context of each is presented in Figure 2-7, along with a summary of our localization data. In many cases, the observed localization shifts can be reconciled easily with corresponding protein functions. Specifically, PKA phosphorylates nuclear proteins, such as the filamentous growth transcription factor Flo8p (Rupp *et al.*, 1999), under filamentous growth conditions; the nuclear shift of Bcy1p and Tpk2p likely enables PKA to selectively phosphorylate nuclear-localized targets during filamentation. The filamentous growth MAPK Kss1p phosphorylates an incompletely defined set of nuclear proteins, including the transcriptional activator Ste12p. Kss1p does not shift its localization in response to mating factor (Ma *et al.*, 1995), and this filamentous growth-induced shift may constitute one mechanism ensuring Kss1p signaling specificity.

In regards to this study, it should be noted that filamentous growth can be induced by conditions of nitrogen deprivation as well as by growth in the presence of short-chain alcohols, such as butanol. Classically, pseudohyphal growth refers to a form of filamentous growth induced in diploid yeast by conditions of nitrogen deprivation on solid medium, wherein the yeast strain exhibits both surface-spread filamentation and invasive growth (Gimeno *et al.*, 1992; Gancedo, 2001). Haploid strains of yeast undergo invasive growth on rich medium, but do not exhibit extensive surface-spread filamentation (Roberts and Fink, 1994). Growth in butanol can be used to induce filamentation in haploid and diploid yeast, yielding morphological properties resembling pseudohyphal growth, even in liquid medium (Lorenz *et al.*, 2000). As compared to nitrogen

deprivation, butanol induction involves several underlying genetic differences; for example, Lorenz *et al.* (2000) report that numerous upstream nutrient-sensing genes required for classic pseudohyphal growth are not required for butanol-induced filamentous growth. We, however, identified at least two of these genes (*GPA2* and *GPR1*) in a disruption screen for genes essential in butanol-induced haploid filamentous growth (Jin *et al.*, 2008); thus, further analysis will be required to understand the genetic basis of these induction mechanisms. To consider both induction schemes, in this study, we assayed kinase localizations in diploid yeast under conditions of nitrogen stress and in haploid yeast by growth in butanol. Furthermore, we have endeavored to make clear the growth conditions used in each study throughout this text.

As indicated from our data, Ksp1p represents an important new filamentous growth gene, since its deletion inhibits all characteristic filamentous growth landmarks in haploid yeast: cell elongation, surface-spread filamentation, and invasive growth. Furthermore, the localization shift of Ksp1p is required for filamentous growth, as is its kinase activity. This strongly suggests that Ksp1 phosphorylates one or more nuclear proteins as an essential step in the yeast filamentous growth response. At present, Ksp1p has no confirmed targets. In vitro phosphorylation studies using protein microarrays identify 187 putative substrates for Ksp1p (Ptacek *et al.*, 2005); however, none of these putative substrates belong to known filamentous growth pathways. The nuclear shift of Ksp1p requires the presence of *BCY1*, *TPK2*, *FUS3*, and *SKS1*, and, at minimum, the kinase activity of Fus3p. Ksp1p is not an established target of

these kinases, and the effect of Fus3p phosphorylation may be indirect. Interestingly, Ksp1p is a target for Hsf1p, Pho85p, and Pcl1p — hence its predicted involvement in the cell cycle and in the yeast general stress response (Hashikawa *et al.*, 2006; Dephoure *et al.*, 2005). Since filamentous growth is coordinated with the cell cycle and general stress response machinery, Ksp1p may play an important role in the signaling link between these processes.

The nuclear shifts of Fus3p and Sks1p are surprising. Fus3p is phosphorylated by Ste7p and translocates to the nucleus in response to mating pheromone, where it phosphorylates Ste12p and the filamentous growth transcription factor Tec1p. In the latter case, phosphorylation of Tec1p targets it for degradation, thereby inhibiting the filamentous growth pathway during mating (Bao *et al.*, 2004; Elion *et al.*, 1993; Choi *et al.*, 1999). During filamentous growth, the function of Fus3p in the nucleus is unclear, since it presumably cannot phosphorylate Tec1p under these conditions. Fus3p may still be phosphorylated by Ste7p and may still phosphorylate Ste12p during filamentous growth. Fus3p kinase activity is required for the nuclear translocation of Ksp1p and Sks1p during filamentation, suggesting that it possesses previously unappreciated roles in the yeast filamentous growth response. The role of Sks1p in filamentous growth is also unclear, as its known functions are apparently distinct from those mediating filamentous growth, and its deletion does not affect filamentation. In this context, kinase deletion phenotypes must be interpreted with caution, since kinases are known to engage in significant cross talk and compensatory activity (Madhani and Fink, 1997; McClean *et al.*, 2007). Thus,

the phenotype of a deleted kinase may be masked by activity from another kinase not normally functioning in a given process.

The observed localization shifts raise an interesting question regarding the mechanism by which these kinases translocate into the nucleus. Little has been reported describing nuclear localization signals (NLSs) in these proteins, and sequence analysis by the program PSORTII (Nakai and Horton, 1999) suggests the presence of a putative NLS in only Ksp1p. Thus, the remaining kinases are likely ferried into the nucleus through interaction with another protein. For example, under conditions of temperature stress in S288c, Bcy1p translocates from the nucleus to the cytoplasm through an interaction with the protein Zds1p (Griffioen *et al.*, 2001). Similar interactions with other proteins may allow for the filamentous growth-induced translocation of the kinase subset reported here, although no obvious candidates are evident from interaction data sets.

It is tempting to extrapolate our findings over the yeast proteome as a whole; however, we expect that the kinome is particularly subject to this form of regulatory control and that the overall percentage of yeast proteins regulated by differential localization will be less than the 5% rate (6 of 125) observed here. We do expect the transcription factor complement in yeast to be similarly regulated by subcellular localization to a high degree, and, in complement to this study, it would be interesting to screen the full set of yeast transcription factors for differential localization during filamentous growth.

Collectively, this study presents the first large-scale analysis of differential protein localization in any eukaryote, and the kinase network identified here

defines a previously overlooked mechanism of regulatory control during yeast cell growth and development. Conceptually, regulated protein localization provides a level of specificity to otherwise promiscuous kinase activities, and the network-based structure of this regulated localization confers greater specificity still. The implications of these findings extend beyond our understanding of yeast cell biology. Similar mechanisms are assuredly at play in higher eukaryotes as well, and subsequent investigations in higher organisms will clarify the degree to which these localization-based regulatory networks are evolutionarily conserved.

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**Figure 2-1 Schematic overview of the construction and subsequent screening of carboxy-terminal YFP fusions**. Primers for PCR amplification were designed with 5'-att sites such that resulting PCR products could be cloned into the pDEST-vYFP vector by phage lambda-based recombination. The resulting gene fusion carries a 10 codon linker between the target open reading frame and YFP. Subsequent screening steps are as indicated, with a diagrammatic representation of a protein differentially localized to the nucleus under conditions of filamentous growth.

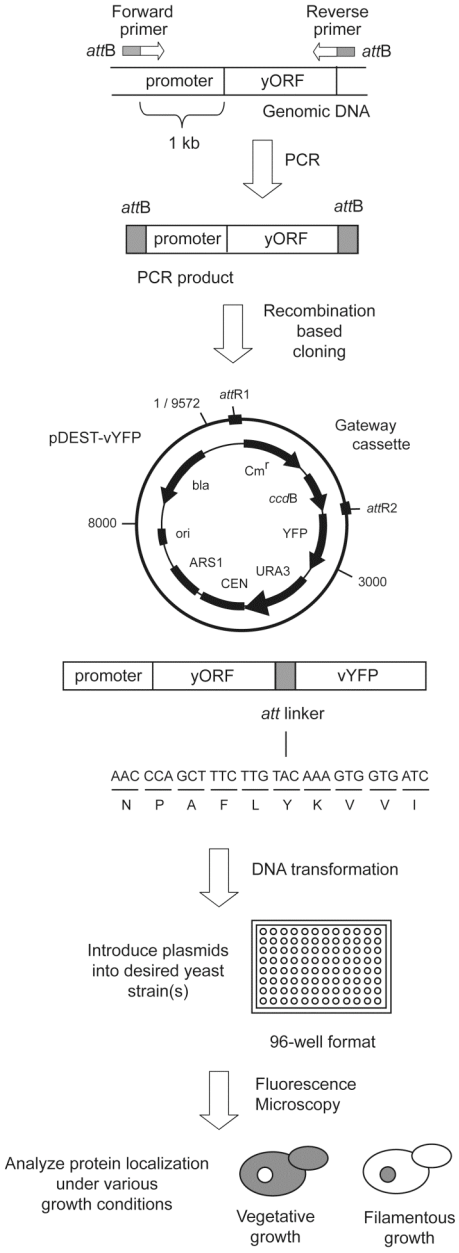


**Figure 2-2 Differential localization of the protein kinases Bcy1p, Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p.** Kinase-vYFP fusions were visualized by fluorescence microscopy (left image) under vegetative growth conditions and during filamentous growth. Yeast cells were stained with the DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI; center image) to visualize the nucleus and mitochondria. The yeast cell shape and vacuoles were imaged by differential interference contrast (DIC) microscopy (right image).

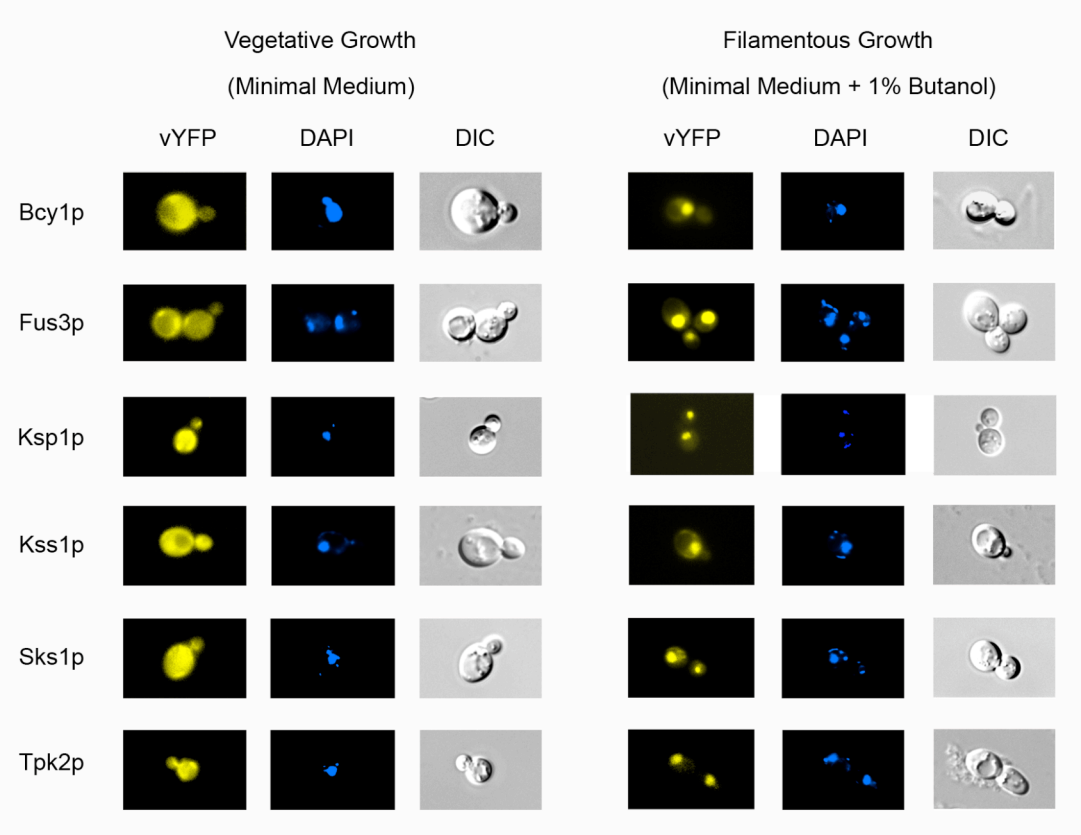


**Figure 2-3 Phenotypic analysis of kinase deletion mutants in the filamentous Σ1278b genetic background.** Each haploid deletion mutant was assayed for: (A) surface-spread filamentation; (B) cell morphology; and (C) invasive growth. Surface-spread filamentation was assayed on SLAD medium (Materials and Methods) supplemented with 1% butanol. Cells from these colonies were inoculated into a small volume of water for differential interference contrast (DIC) microscopy. Invasive growth was assayed on YPD medium as described in Materials and Methods. The Σ1278b background strain Y825 served as a wild-type control for these filamentous growth assays.

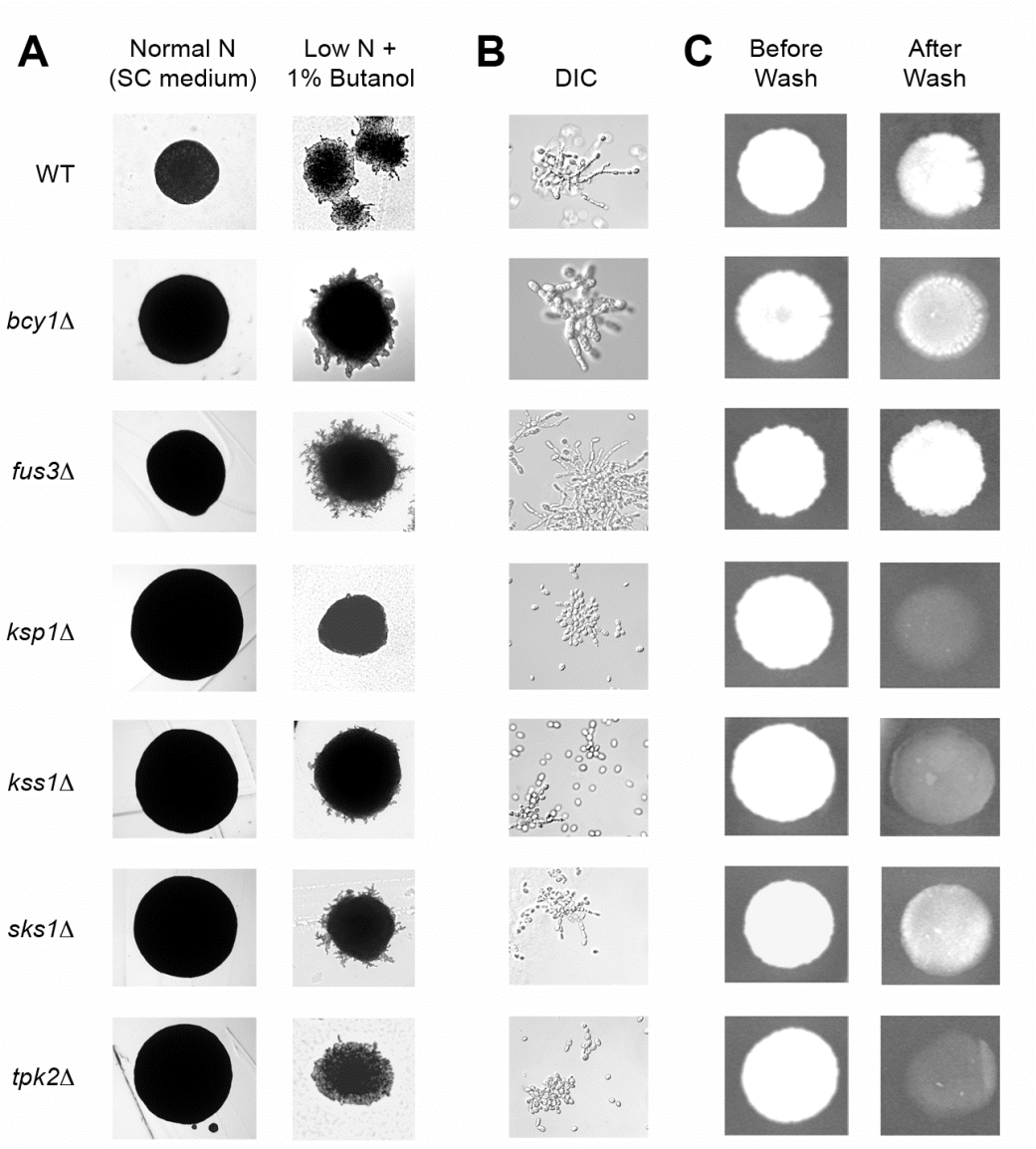


**Figure 2-4 Interdependent localization of yeast kinases during filamentous growth.** (*A*) Each kinase-vYFP fusion was visualized by fluorescence microscopy under filamentous growth conditions in a haploid strain of Σ1278b deleted for the indicated kinase. DAPI-stained and DIC images are provided. Images of the kinase-vYFP fusions under vegetative growth conditions are presented in Supplementary Figure SF2-2 (appendix). The mixed distribution of these kinases over the cytoplasm and nucleus signifies a loss of nuclear localization under filamentous growth conditions upon deletion of the indicated kinase gene. Only kinases exhibiting a loss of nuclear localization are shown here. (*B*) Matrix of kinase localizations in gene deletion backgrounds under conditions of filamentous growth. The results corresponding to the images shown in part A are boxed in red. Images of nuclear-localized kinase-vYFP fusions unaffected by the indicated gene deletions are presented in Supplementary Figure SF2-3 (appendix). (*C*) Localization-based regulatory relationships among yeast kinases. Each forward pointed arrow indicates that the given kinase is required for the wild-type localization of the subordinate kinase; for example, Fus3p is required for the wild-type nuclear localization of Sks1p during filamentous growth. Reciprocal relationships between kinases are indicated by the circular arrows in the left column. (*D*) The network of regulated protein localization between Bcy1p, Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p is illustrated here; arrows are drawn as described for part C. Double-sided arrows indicate that the localization of the given kinase pair is reciprocally affected under filamentous growth conditions.

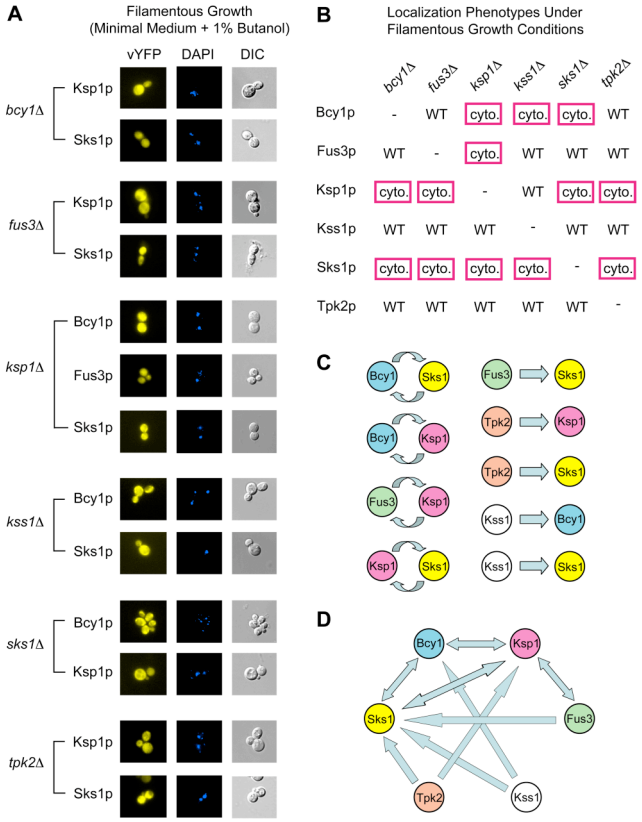


**Figure 2-5 The observed nuclear translocations are dependent upon the kinase activity of Fus3p, Ksp1p, Kss1p, Sks1p, and Tpk2p, respectively.** Each indicated kinase-vYFP fusion was imaged in a strain carrying the kinase-dead allele listed to the left. For example, the subcellular distribution of Ksp1p and Sks1p is shown in a haploid fus3D strain carrying the fus3-K42R kinase-dead allele on a low-copy plasmid. DAPI-stained and DIC images are as described previously. Note the mixed distribution of each kinase over the cytoplasm and nucleus upon loss of the indicated kinase activity. Images of these kinases in the kinase-dead background strains under vegetative growth conditions are presented in Supplementary Figure SF2-6 (appendix). Scale bar, 3 mm.

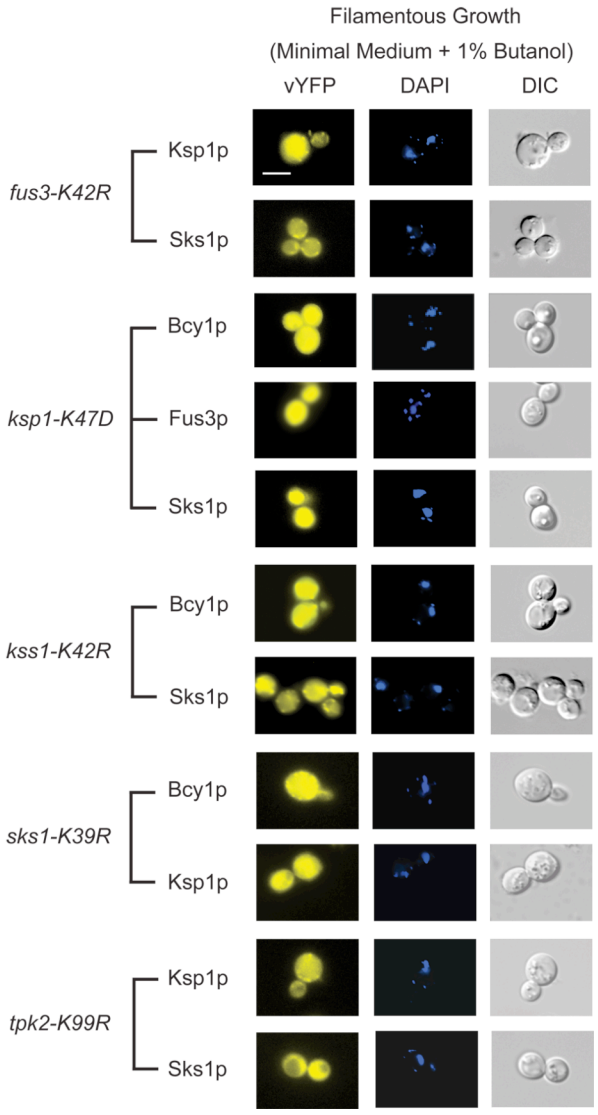


**Figure 2-6 Tagging Ksp1p with a nuclear export sequence (NES) abolishes filamentous growth.** (A) Schematic diagram of the NES-tagged form of Ksp1p, where the NES (encoding LQLPPLERLTLD) is fused at the carboxy terminus of the Ksp1p-vYFP chimera. As visualized by fluorescence microscopy, addition of the NES prevents the butanol-induced nuclear translocation of Ksp1p. DIC and vYFP images are shown. Scale bar, 3 mm. (B) Phenotypic analysis of NES-tagged Ksp1p. For comparison, we have included a haploid ksp1D strain carrying a centromeric plasmid with the KSP1-vYFP fusion under transcriptional control of the native KSP1 promoter; this strain exhibits wild type filamentous growth properties. Addition of the NES results in decreased filamentous growth, evidenced in a loss of surface-spread filamentation, rounded cell morphology, and decreased invasive growth. Scale bar, 3 mm.

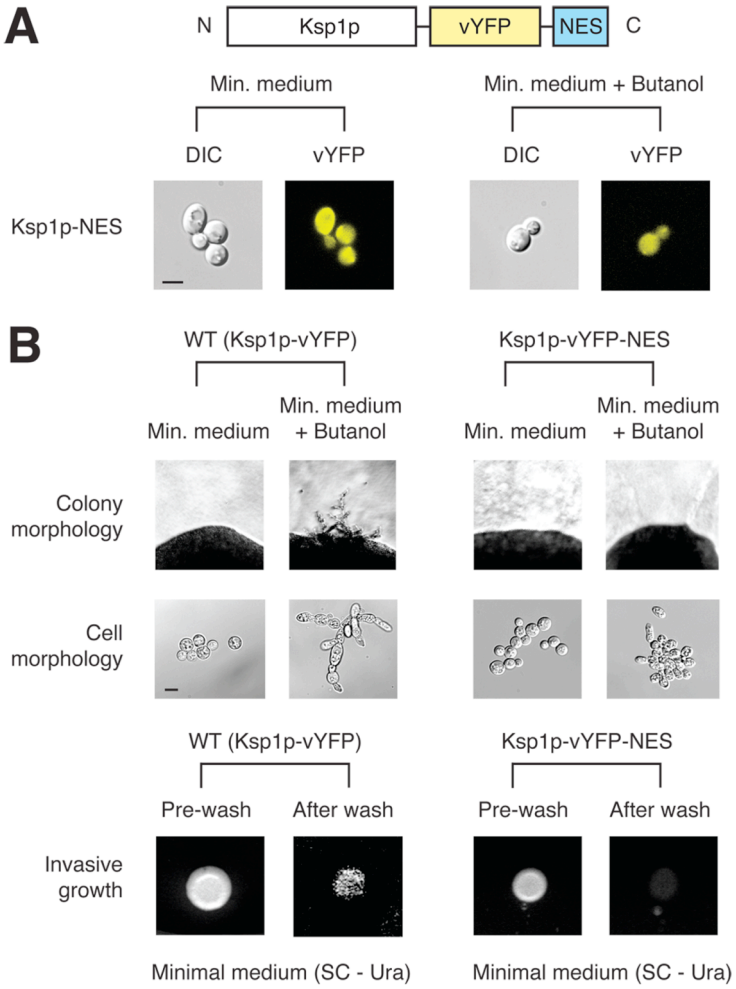
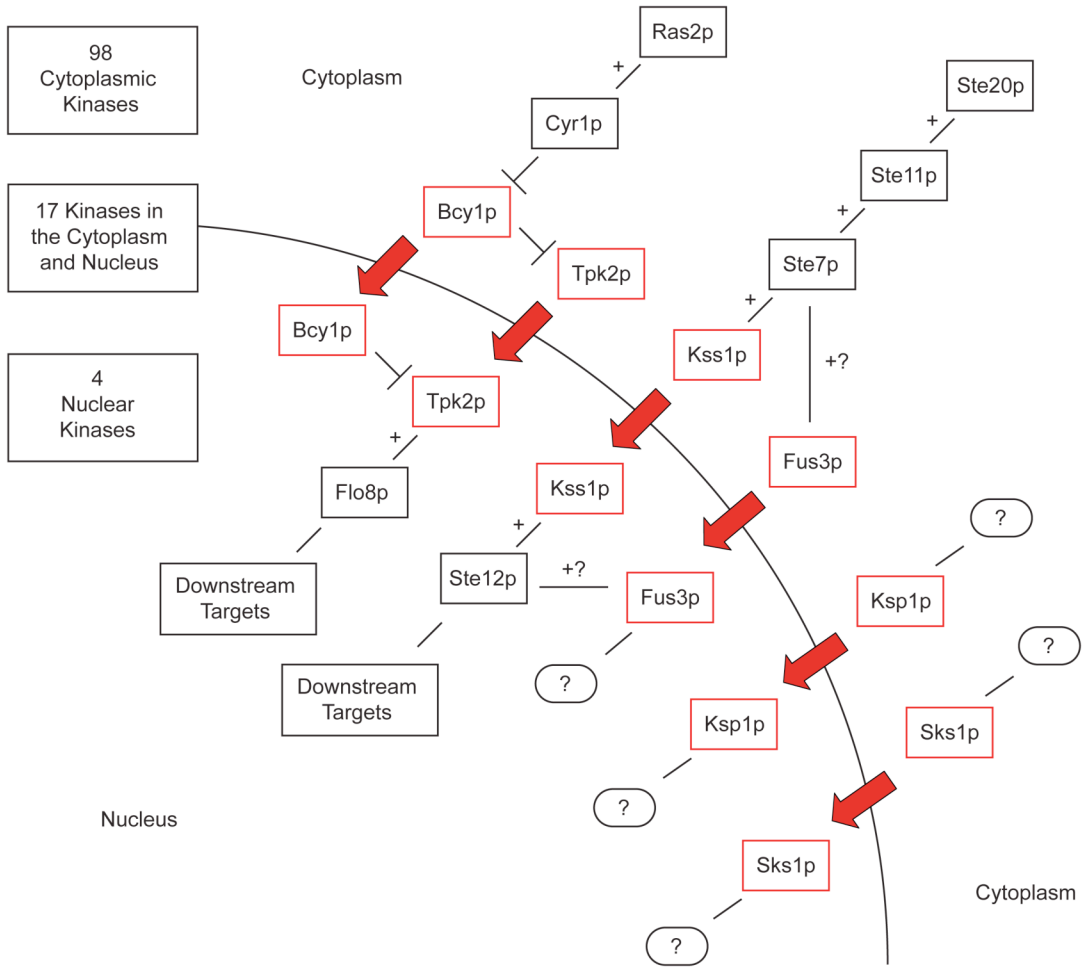


**Figure 2-7 Subcellular localization of the yeast kinome during filamentous growth.** This diagram summarizes the localization of 125 protein kinases, constituting the yeast kinome, under conditions of filamentous growth. In total, 119 kinases do not shift localization during the transition to filamentous growth, and the localization of these kinases is tallied in the upper left. For simplicity, Yck3p, which localizes to the vacuole, is included in the cytoplasmic compartment. The six kinases that shift localization between vegetative and filamentous growth conditions are boxed in red, and the pathway context of each kinase is shown. Flo8p and Ste12p are included as representative targets of Tpk2p and Kss1p, respectively. Speculative and unknown interactions are indicated with question marks.



## 2.5 Appendix

### Supplementary Figures and Tables

**Figure SF2-1 The yeast kinase complement, or kinome, is indicated, along with Gene Ontology terms describing the subcellular compartment to which each kinase has been localized in non-filamentous strains of yeast**. Functions related to filamentous growth, mating, and cell cycle progression are color-coded alongside each kinase.

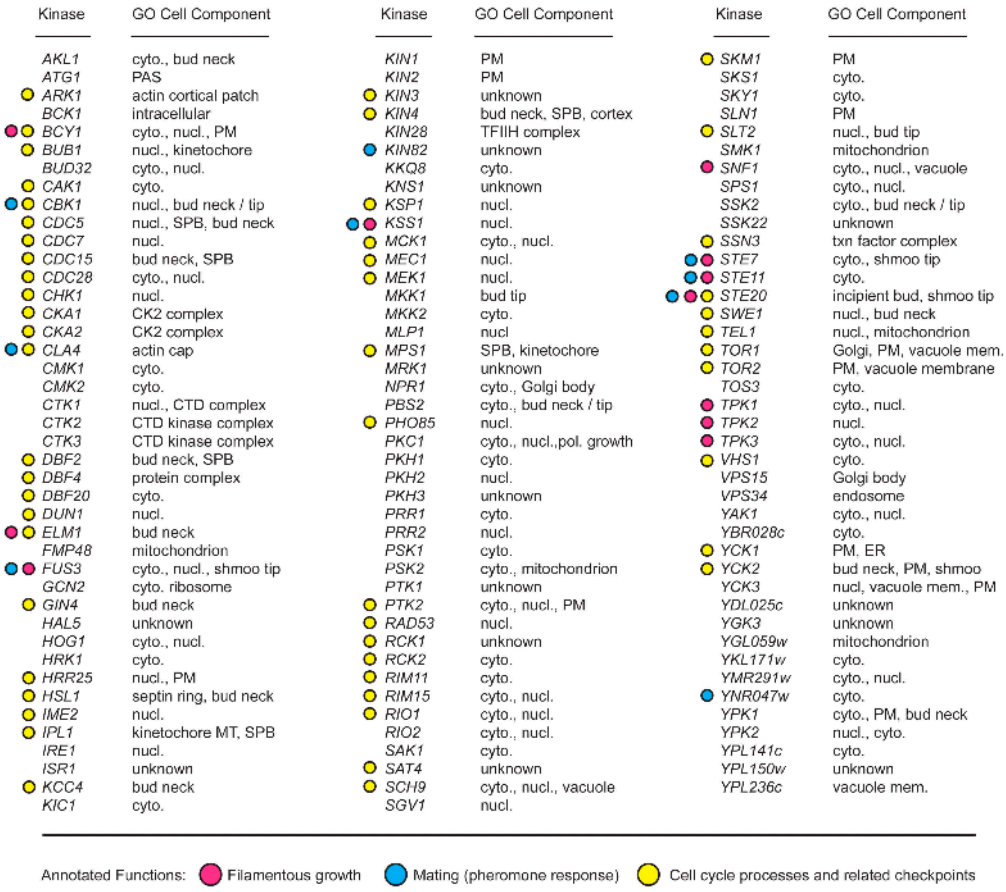


**Figure SF-2-2 Subcellular distribution of kinase-vYFP chimeras in the indicated kinase deletion mutants under vegetative growth conditions**. Yellow fluorescence and DIC images are presented. Scale bar, 3μm.

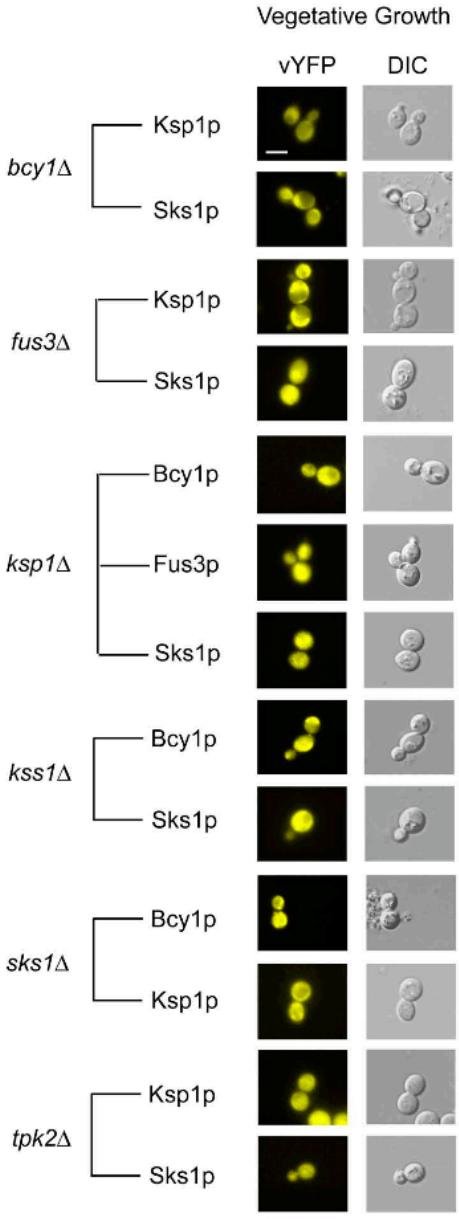


**Figure SF-2-3 Nuclear-localized kinase-vYFP chimeras in the indicated deletion mutants.** The images presented here identify nuclear shifts unaffected by the respective gene deletions. Yellow fluorescence and DIC images are presented. Scale bar, 3μm.

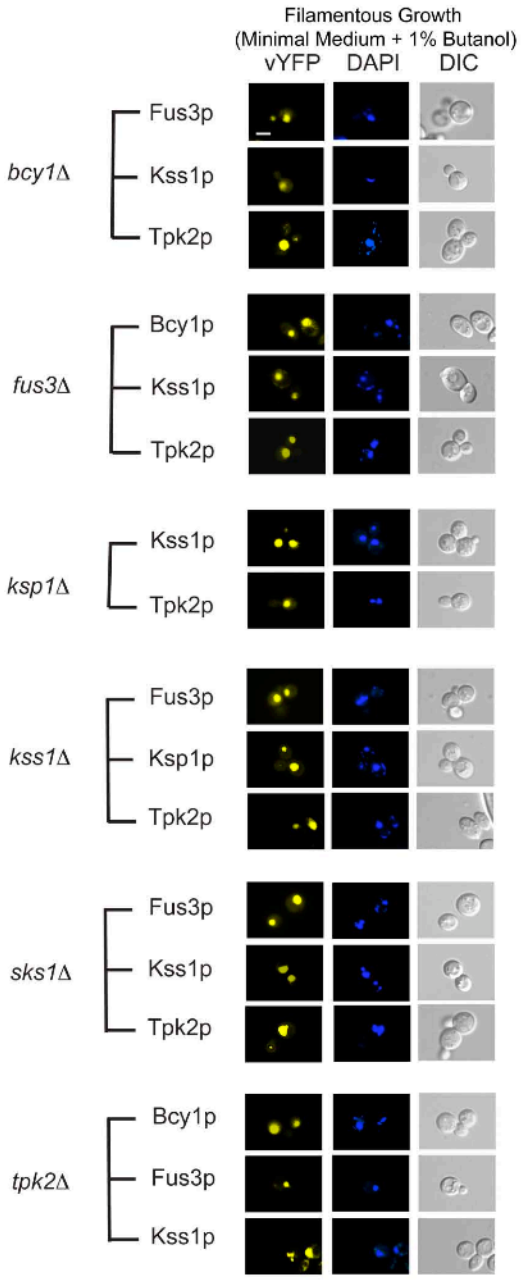


**Figure SF-2-4 Filamentous growth phenotypes of the indicated double deletion mutants**. Colony morphology was assessed on synthetic complete (SC) medium and on SLAD plates supplemented with 1% butanol. Cells from these colonies were scraped into water for differential interference contrast microscopy. Invasive growth was assayed on YPD medium; plates were washed after 5 days growth.

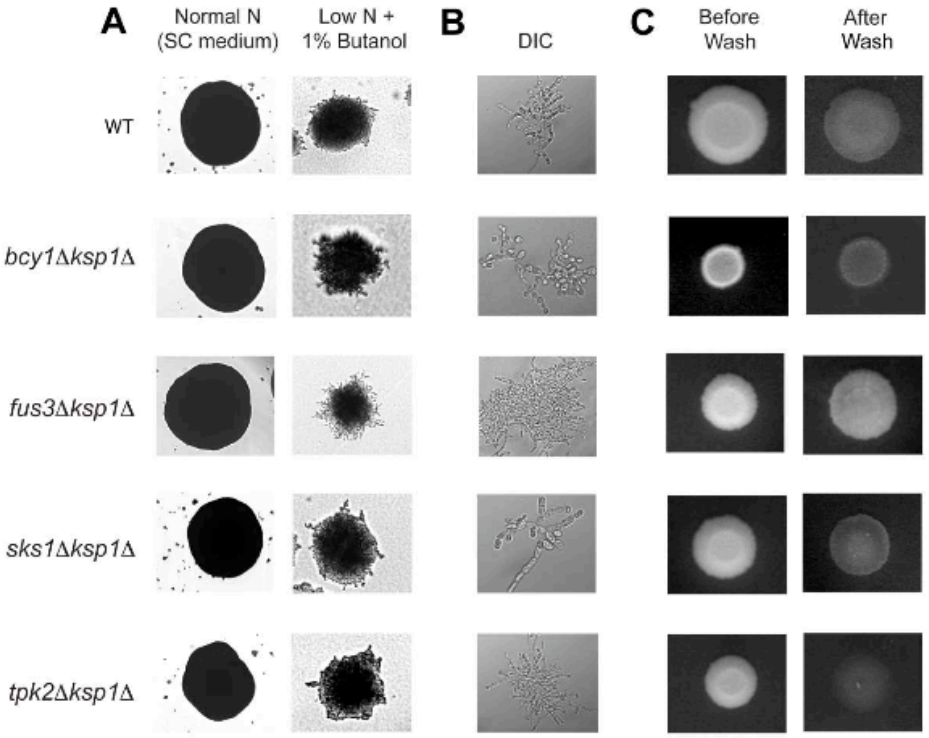


**Figure SF-2-5 Filamentous growth phenotypes of kinase-dead mutants**. Colony morphology was determined on SC medium (normal nitrogen levels) and on SLAD medium supplemented with 1% butanol. Cells were scraped from colonies for DIC microscopy. Invasive growth was assayed on SC-Ura medium; plates were washed after growth for 5 days.

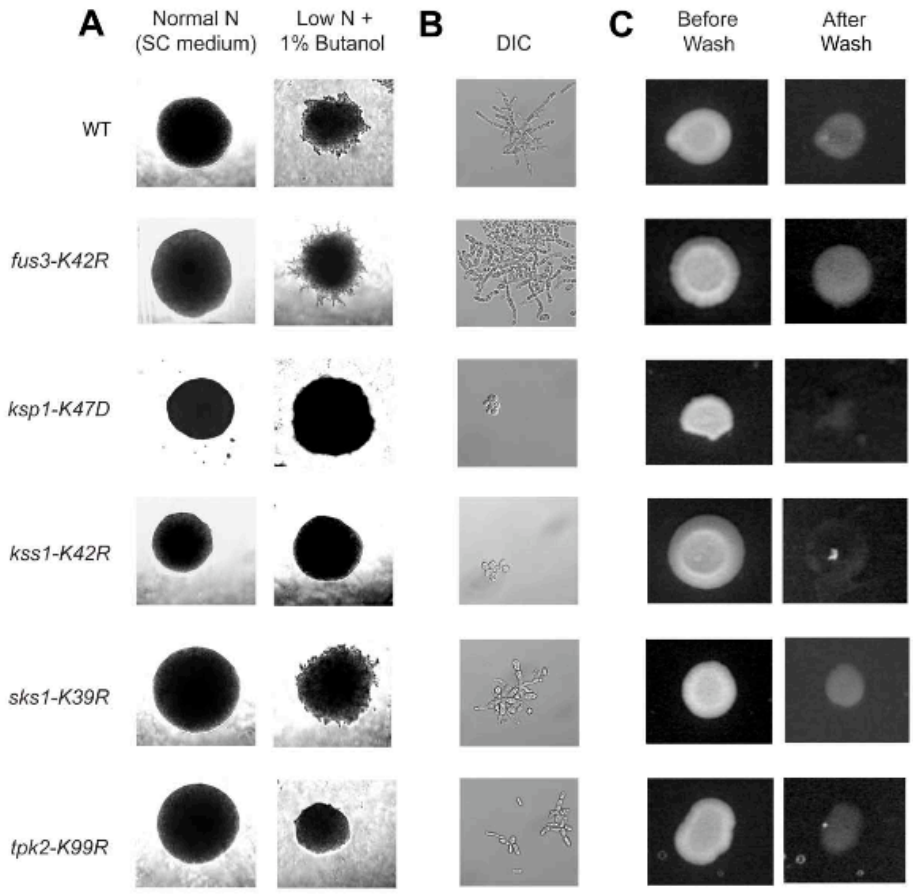


**Figure SF-2-6 Subcellular distribution of kinase-vYFP chimeras in haploid yeast strains carrying the indicated kinase-dead alleles under vegetative growth conditions.** Yellow fluorescence and DIC images are presented. Scale bar, 3μm.

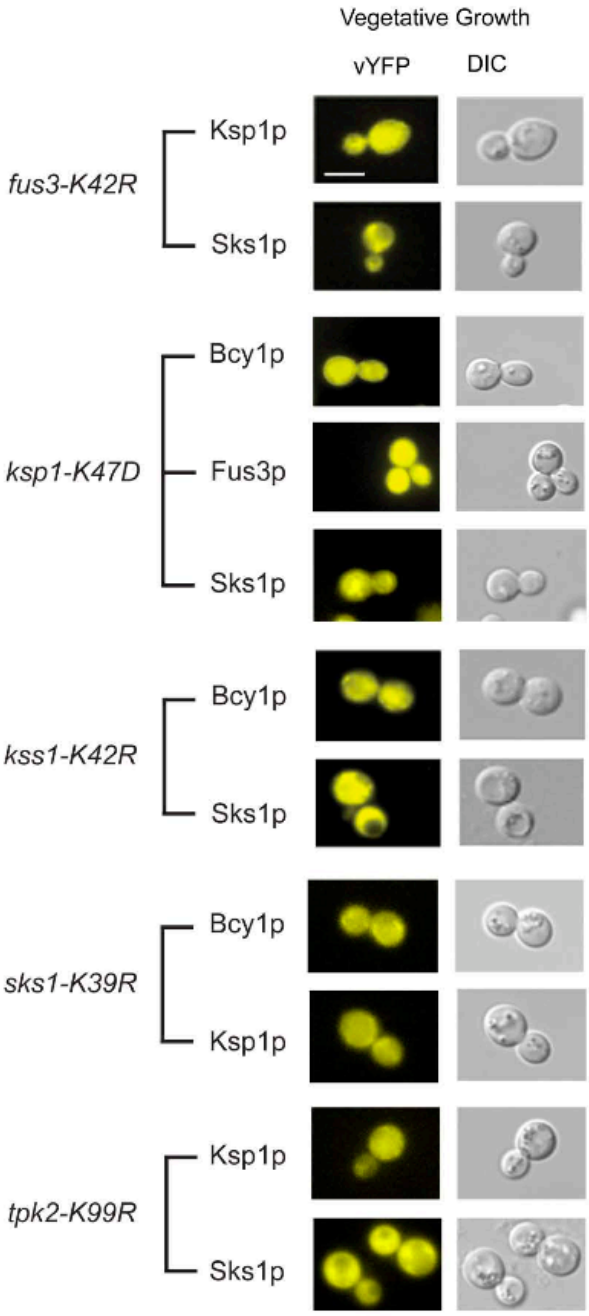


Table ST-2-1 Kinase localization in haploid filamentous yeast (Y825).

| Kinase | Vegetative Growth | Filamentous Growth (1% Butanol) |
|---|---|---|
| *AKL1* | Cytoplasm | Cytoplasm |
| *ATG1* | Cytoplasm | Cytoplasm |
| *ARK1* | Punctate | Punctate |
| *BCK1* | Cytoplasm | Cytoplasm |
| *BCY1* | Cytoplasm | Nucleus |
| *BUB1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *BUD32* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *CAK1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *CBK1* | Cytoplasm | Cytoplasm |
| *CDC15* | Cytoplasm | Cytoplasm |
| *CDC28* | Cytoplasm | Cytoplasm |
| *CDC5* | Cytoplasm (faint) | Cytoplasm (faint) |
| *CDC7* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *CHK1* | Cytoplasm | Cytoplasm |
| *CKA1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *CKA2* | Cytoplasm | Cytoplasm |
| *CLA4* | Cytoplasm | Cytoplasm |
| *CMK1* | Cytoplasm | Cytoplasm |
| *CMK2* | Cytoplasm | Cytoplasm |
| *CTK1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *CTK2* | Nucleus | Nucleus |
| *CTK3* | Nucleus | Nucleus |

| Kinase | Vegetative Growth | Filamentous Growth (1% Butanol) |
|---|---|---|
| *DBF2* | Cytoplasm | Cytoplasm |
| *DBF20* | Cytoplasm | Cytoplasm |
| *DBF4* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *DUN1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *ELM1* | Cytoplasm (faint), Bud neck | Cytoplasm (faint), Bud neck |
| *FMP48* | Mitochondrion, Cytoplasm (faint) | Mitochondrion, Cytoplasm (faint) |
| *FUS3* | Cytoplasm | Nucleus |
| *GCN2* | Cytoplasm | Cytoplasm |
| *GIN4* | Cytoplasm | Cytoplasm |
| *HAL5* | Cytoplasm (faint) | Cytoplasm (faint) |
| *HOG1* | Cytoplasm | Cytoplasm |
| *HRK1* | Cytoplasm | Cytoplasm |
| *HRR25* | Cytoplasm | Cytoplasm |
| *HSL1* | Bud neck | Bud neck |
| *IME2* | Cytoplasm (faint) | Cytoplasm (faint) |
| *IPL1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *IRE1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *ISR1* | Cytoplasm | Cytoplasm |
| *KCC4* | Bud, Cytoplasm | Bud, Cytoplasm |
| *KIC1* | Cytoplasm | Cytoplasm |
| *KIN1* | Cytoplasm | Cytoplasm |
| *KIN2* | Cytoplasm (faint) | Cytoplasm (faint) |
| *KIN28* | Nucleus | Nucleus |
| *KIN3* | Cytoplasm | Cytoplasm |

| Kinase | Vegetative Growth | Filamentous Growth (1% Butanol) |
|---|---|---|
| *KIN4* | Cytoplasm | Cytoplasm |
| *KIN82* | Cytoplasm | Cytoplasm |
| *KKQ8* | Cytoplasm | Cytoplasm |
| *KNS1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *KSP1* | Cytoplasm | Cytoplasm |
| *KSS1* | Cytoplasm | Cytoplasm |
| *MCK1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *MEC1* | Cytoplasm | Cytoplasm |
| *MEK1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *MKK1* | Cytoplasm | Cytoplasm |
| *MKK2* | Cytoplasm | Cytoplasm |
| *MLP1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *MPS1* | Cytoplasm | Cytoplasm |
| *MRK1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *NPR1* | Cytoplasm | Cytoplasm |
| *PAK1* | Cytoplasm | Cytoplasm |
| *PBS2* | Cytoplasm | Cytoplasm |
| *PHO85* | Cytoplasm | Cytoplasm |
| *PKC1* | Cytoplasm | Cytoplasm |
| *PKH1* | Cytoplasm | Cytoplasm |
| *PKH2* | Cytoplasm | Cytoplasm |
| *PKH3* | Cytoplasm (faint) | Cytoplasm (faint) |
| *PRR1* | Cytoplasm | Cytoplasm |
| *PRR2* | Cytoplasm | Cytoplasm |

| Kinase | Vegetative Growth | Filamentous Growth (1% Butanol) |
|---|---|---|
| *PSK1* | Cytoplasm | Cytoplasm |
| *PSK2* | Cytoplasm | Cytoplasm |
| *PTK1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *PTK2* | Cytoplasm | Cytoplasm |
| *RAD53* | Nucleus | Nucleus |
| *RCK1* | Cytoplasm | Cytoplasm |
| *RCK2* | Cytoplasm | Cytoplasm |
| *RIM11* | Cytoplasm | Cytoplasm |
| *RIM15* | Cytoplasm | Cytoplasm |
| *RIO1* | Cytoplasm | Cytoplasm |
| *RIO2* | Cytoplasm | Cytoplasm |
| *SAT4* | Cytoplasm | Cytoplasm |
| *SCH9* | Cytoplasm | Cytoplasm |
| *SGV1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *SKM1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *SKS1* | Cytoplasm | Nucleus |
| *SKY1* | Cytoplasm | Cytoplasm |
| *SLN1* | Cytoplasm | Cytoplasm |
| *SLT2* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *SMK1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *SNF1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *SPS1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *SSK2* | Cytoplasm | Cytoplasm |
| *SSK22* | Cytoplasm (faint) | Cytoplasm (faint) |

| Kinase | Vegetative Growth | Filamentous Growth (1% Butanol) |
|---|---|---|
| *SSN3* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *STE11* | Cytoplasm | Cytoplasm |
| *STE20* | Cytoplasm | Cytoplasm |
| *STE7* | Cytoplasm | Cytoplasm |
| *SWE1* | Cytoplasm | Cytoplasm |
| *TEL1* | Mitochondrion, Cytoplasm | Mitochondrion, Cytoplasm |
| *TOR1* | Cytoplasm (faint) | Cytoplasm (faint) |
| *TOR2* | Cytoplasm (faint) | Cytoplasm (faint) |
| *TOS3* | Cytoplasm | Cytoplasm |
| *TPK1* | Cytoplasm | Cytoplasm |
| *TPK2* | Cytoplasm | Cytoplasm |
| *TPK3* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *VHS1* | Cytoplasm | Cytoplasm |
| *VPS15* | Cytoplasm (faint) | Cytoplasm (faint) |
| *VPS34* | Cytoplasm (faint) | Cytoplasm (faint) |
| *YAK1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *YBR028C* | Cytoplasm | Cytoplasm |
| *YCK1* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *YCK2* | Cytoplasm | Cytoplasm |
| *YCK3* | Vacuole | Vacuole |
| *YDL025C* | Cytoplasm (faint) | Cytoplasm (faint) |
| *YGK3* | Cytoplasm (faint) | Cytoplasm (faint) |
| *YGL059W* | Cytoplasm (faint) | Cytoplasm (faint) |
| *YKL171W* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |

| Kinase | Vegetative Growth | Filamentous Growth (1% Butanol) |
|---|---|---|
| *YMR291W* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *YNR047W* | Cytoplasm | Cytoplasm |
| *YPK1* | Cytoplasm | Cytoplasm |
| *YPK2* | Cytoplasm, Nucleus | Cytoplasm, Nucleus |
| *YPL141C* | Cytoplasm | Cytoplasm |
| *YPL150W* | Cytoplasm (faint) | Cytoplasm (faint) |
| *YPL236C* | Cytoplasm (faint) | Cytoplasm (faint) |

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# Chapter 3

## Synthetic Genetic Analysis of the RAM network During Hyphal Development in *Candida albicans*

### 3.1. Introduction

*Candida albicans* is the most prevalent fungal pathogen of humans. It lives as a commensal in the gastrointestinal and genitourinary tract of humans and is normally harmless. However, in certain favorable conditions, such as upon a decrease in host immunity or extended antibiotic treatment that eliminates competing bacteria, it proliferates and causes local mucosal infections as well as serious systemic infections (Mitchell, 1998; Pappas *et al.*, 2004). Systemic infections normally occur in immunocompromised patients that have undergone bone marrow or organ transplants or in patients undergoing chemotherapy. HIV/AIDS patients also almost always develop both local candidial infections of the esophagus or the oropharynx as well as invasive candidial infections, though the latter are less common (de Repentigny *et al.* 2004). Invasive candidial infections lead to death in approximately 40% of cases, making *Candida albicans* an extremely important pathogen (Wisplinghoff *et al.*, 2004). An additional complication is that extended anti-fungal treatment due to the recurring nature of the disease has led to resistant strains of *Candida* (Enoch *et al.*, 2006). This in

turn has led to the use of stronger anti-fungals that invariably have undesirable side effects. Thus there is an urgent need for newer drugs with more specificity to treat these infections.

Candida exists in multiple morphological forms, the most important being yeast, pseudohyphal and hyphal. It is believed that the ability to switch forms is tightly linked to its virulence (Mitchell, 1998). This is seen in both *in vivo* as well as *ex vivo* models of candidiasis where mutant *C.albicans* strains that could not produce hyphae had decreased virulence (Loh *et al.*, 1997; Jayatilake *et al.,* 2006). It is believed that the yeast form is important for dissemination in the bloodstream whereas the pseudohyphal and hyphal forms help in invading tissues and destroying macrophages after ingestion, thus evading the host immune system (Gow, 2002). An understanding of hyphal development is important in the design of drugs to inhibit this process and thus halt disease progression. In this context there have been a number of studies to delineate the intracellular signaling machinery that leads to hyphal development (reviewed in Biswas *et al.*, 2007; Cottier and Mühlschlegel, 2009; Hall *et al.,* 2009).

A number of signaling pathways that lead to hyphal growth have been identified in *Candida albicans* (Song *et al.*, 2008; Dhillon *et al.,* 2003). One of these, the RAM (Regulation of Ace2p and Morphogenesis) network is an evolutionary conserved pathway that regulates various processes in eukaryotes such as cell separation, daughter cell-specific transcription and polarized growth (Nelson *et al.* 2003; Voth *et al.,* 2005; Walton *et al.,* 2006). In *C.albicans* this pathway is also involved in hyphal development (Song *et al.*, 2008). The RAM

network is comprised of six genes (Figure 3-1), present in both *Saccharomyces cerevisiae* and *C.albicans*. One of these genes, *CBK1* has been studied in *S.cerevisiae* and is shown to phosphorylate the transcription factor, Ace2p which leads to Ace2p localization to the daughter cells (Mazanka *et al.,* 2008; Sbia *et al.,* 2008). Other elements of this network are however not identified, nor is it known how the RAM network interacts with the other morphogenetic signaling pathways. In *S.cerevisiae*, a connection between the RAM network and the Protein Kinase A (PKA) pathway has been suggested (Schneper *et al.,* 2004). The cyclicAMP-dependent PKA pathway is a conserved eukaryotic signaling pathway that regulates various processes in the cell, such as cell growth, bud site selection and filamentous/hyphal growth (Broach 1991; Pan and Heitman 1999; Giacometti *et al.*, 2009). Schneper *et al.* showed that an overexpression of one of the catalytic subunits of PKA, namely *TPK1*, suppressed budding defects of a *cbk1* null mutant, thus suggesting that these pathways may function in parallel. In *C.albicans*, *CBK1* has been shown by at least two groups to be required for hyphal development (McNemar *et al.,* 2002; Uhl *et al.,* 2003). The Ace2p transcription factor of the RAM network has been shown to play a role in hyphal development and regulates a number of metabolic genes under hypoxic conditions (Mulhern *et al.* 2006).

In this study we examine how the RAM network regulates hyphal developmental using large-scale synthetic genetic analysis. Synthetic genetic analysis has been used successfully in *S.cerevisiae* in a number of studies (Davierwala *et al.*, 2005; Boone *et al.*, 2007). Synthetic interactions involve a pair

of mutations that result in a phenotype not observed in either single mutant; for example, the deletion of two non-essential genes may result in cell death. Such interactions can help uncover functions of redundant genes, as researchers may screen a specific mutant for second-site mutations that either suppress or enhance the original phenotype. Tong *et al.* (Tong *et al.*, 2001) developed a method enabling synthetic genetic analysis on a large scale in *S.cerevisiae*, called the synthetic genetic array (SGA) method. This involved the systematic construction of double-mutants by crossing a query mutation to an array of ~4700 haploid deletion mutants. Double-mutant progeny were subsequently scored for viability. Any inviable progeny would indicate a synthetic lethal interaction between the two deleted genes, thus identifying functional relationships between genes. The fact that *S.cerevisiae* exists as a stable haploid facilitated this kind of analysis.

Synthetic genetic analysis on a large scale has so far not been attempted in *C.albicans.* One of the reasons is the difficulty in generating strains mutant at two different loci due to the fact that it lacks a viable haploid stage. To circumvent this issue, we have made use of the property of gene haploinsufficiency in this large-scale synthetic genetic analysis. Haploinsufficiency describes the situation when mutation in one allele of a given gene results in a measurable phenotype. A large-scale transposon mediated gene disruption study in *C.albicans* previously made use of haploinsufficiency to identify genes involved in the yeast-hyphal transition (Uhl *et al.,* 2003). One of the genes identified was *CBK1* (Cell wall Biosynthesis Kinase), a Ser/Thr kinase of the RAM network. In *S.cerevisiae*,

Haarer *et al.* studied synthetic genetic interactions of haploinsufficient genes and successfully identified over 200 actin-interacting genes that showed defects in the actin cytoskeleton (Haarer *et al.*, 2007). This is known as complex haploinsufficiency (CHI) where a strain with two heterozygous mutations displays a more severe phenotype than either single mutant. This method can be successfully used to identify genes with similar function. In this study, we have used CHI in a *C.albicans* strain heterozygous for the RAM network kinase gene, *CBK1*. We identified 151 genes showing decreased hyphal growth relative to the *CBK1* heterozygote, including 44 genes that genetically interact with *CBK1*. Our results have also established a possible connection between the RAM and PKA pathways in hyphal development, besides identifying 17 putative targets of the RAM network transcription factor, Ace2p.

## 3.2. Materials and Methods

### *3.2.1. Strains and growth conditions:*

CAMM-292 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434/*cbk1*-Δ1::*his*G/*CBK1)* (McNemar and Fonzi, 2002) was used as the parental strain for the transposon mutagenesis. CAI4 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434) and CAMM-29 (*ura3*Δ::*imm*434/*ura3*Δ::*imm*434/*cbk1*-Δ1::*his*G-*URA3-hisG*/*CBK1)* were used for comparison of hyphal growth phenotypes (McNemar and Fonzi, 2002).

Standard yeast media was used for growth of strains with slight modifications. More specifically, strains for transformation were grown in liquid

YPD medium containing 80mg/l uridine at 30°C(Bensen et al., 2005). URA+ transformants were grown on standard SC-URA plates at 30°C. Screening of insertion mutants was carried out on solid Spider medium at 37°C for 5 days (Uhl *et al.,* 2003).

### *3.2.2. Transposon Mutagenesis:*

C.*albicans* strain WO-1 pEMBLY23 genomic DNA library (NIH AIDS Research & Reference Reagent Program; Slutsky *et al.*, 1987; Baldari *et al.*, 2005) was used as the starting material for the transposon mutagenesis. Mutagenesis was performed *in vitro* using the GPS3-Mutagenesis system from NEB, with 80ng of DNA library and 20ng of donor plasmid. The donor plasmid was first modified to contain the Candida-specific *URA3-dpl200* cassette (Wilson *et al.,* 2000) at the *Spe*I restriction site to enable selection of *C.albicans* transformants. A total of nine independent mutagenesis reactions were performed. The transposon-inserted library plasmids were transformed into DH5$\alpha$ cells and selected on LB+Amp+Kan plates. Plasmids were recovered by Maxiprep (Qiagen), digested with *Pvu*II to release the genomic DNA fragments and transformed into *C.albicans* strain CAMM-292 using a standard lithium acetate transformation protocol (Geitz *et al.*, 1992). Slight modifications of this protocol were used, namely heat shock temperature of 44°C for 20 minutes (Walther and Wendland, 2003) before plating. Each fragment integrates into the genome via homologous recombination. The resultant double heterozygotes were selected on SC-URA plates.

### *3.2.3. Screening for hyphal growth phenotypes:*

URA+ transformants were grown overnight in liquid SC-URA medium in 96-well plates and replica-plated using a hand-pinning tool onto Spider media in duplicate. Plates were incubated at 37°C for 5 days. Colonies with altered hyphal growth relative to the starting strain CAMM-292 were scored as positive. These were retested on Spider plates and incubated as before to confirm the phenotype.

### *3.2.4. Identification of transposon insertions:*

Transposon insertion sites were identified by 3'RACE (rapid amplification of cDNA ends) as follows. Total cellular RNA was extracted (Ambion Ribopure) from the transposon-inserted strains, reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and a 3' RACE adapter. A fragment of the *URA3* marker from each strain was amplified by PCR using primers complementary to the 3'RACE adapter and the 3'end of *URA3* gene. The PCR product contains the transposon insertion site in the genome. PCR products were cloned into a TA vector followed by sequencing and BLASTN analysis. Gene sequences were compared and identified using the Candida Genome Database (www.candidagenome.org).

### *3.2.5. Confirmation of synthetic interactions:*

*CBK1* was re-integrated into each double heterozygote using plasmid pMM4 (McNemar and Fonzi 2002) which carries the entire *CBK1* ORF and its promoter region. Since the selectable marker in pMM4 is *URA3*, similar to the transposon

marker used, we had to cure the strain of this marker before reintroducing *CBK1*. To do this, we used the standard 5'FOA (5' fluoroorotic acid) selection method (Boeke *et al.,* 1987). 5'FOA is a compound that is converted to fluorodeoxyuridine, a toxic intermediate, by yeast cells containing a wild type *URA3* gene. Thus only cells that do not possess a wild type *URA3* gene are able to survive on media containing 5'FOA with uracil added exogenously. The double heterozygotes were first grown in liquid YPD with 80mg/l uridine for 48 hours to encourage loss of the *URA3* marker (Monteoliva et al., 1996). A dilution was then plated on Synthetic Complete media containing 80mg/l uridine and 1mg/ml 5'FOA. Colonies were picked at random and replica plated onto YPD + uridine plates and SC-Ura plates. Colonies on YPD + uridine plates that did not grow on SC-Ura were scored as positive i.e. they had lost the *URA3* marker. These were then picked as candidates for transformation of *CBK1*.

For the *CBK1* transformation, pMM4 was linearized with *BsrG*I and transformed into each transposon-inserted Ura- strain. The resultant *CBK1*-integrated transformants were selected on SC-URA. These were then screened on Spider medium as earlier and an increase in hyphal growth compared to that of the double heterozygote was scored as positive. Integration of *CBK1* was confirmed by standard PCR amplification of a fragment of *CBK1*.

## 3.3. Results

### *3.3.1. Construction of double heterozygotes:*

We performed transposon mutagenesis across the *C.albicans* genome in a *CBK1* heterozygous mutant strain in order to screen for double mutants with a hyphal growth defect more severe than the *CBK1* heterozygote. Transposon mutagenesis is a useful method for large-scale mutagenesis of various organisms. It has been used often in *S.cerevisiae* to perform genome-wide gene disruptions in a relatively quick and straightforward method (Burns *et al.,* 1994; Ross-McDonald *et al.* 1999; Kumar *et al.* 2000; Kumar *et al.,* 2004). In *C.albicans*, two groups have made use of this method independently to generate homozygous (Davis *et al.,* 2002; Richard *et al.*, 2005) and heterozygous (Uhl *et al.*, 2003) mutants to study various aspects of hyphal development.

As a resource for large-scale screening, transposon insertion mutants offer both advantages and disadvantages. The advantage of using transposon-mediated gene disruption is that the mutagenic event is not biased towards annotated genes (Ross-McDonald *et al.* 1999), so uncharacterized open reading frames (ORFs) are also disrupted. Transposon insertion mutants can be used to generate a greater variety of mutant alleles than can be obtained using a single treatment with chemical mutagens or ultraviolet radiation (Kumar and Snyder, 2001). Furthermore, both coding and non-coding regions may be disrupted depending upon the site of insertion; so, the phenotypic consequences of disrupted regulatory regions, like promoters and terminators, can also be studied.

A limitation of this approach is that the transposon insertion collection is not comprehensive; transposition is a random process, making it difficult to obtain an insertion affecting each yeast gene. In addition, the transposon might insert into a gene in such a way that it does not disrupt gene function. Finally, the specific collection of mutants that is obtained is dependent on the specific genomic library that is used as the receptor plasmids. In spite of this, transposon-mediated gene disruption remains an extremely useful way to generate large collections of mutants and is one of the few widely available methods applicable for large-scale mutagenesis in *C. albicans.*

In our study, we made use of a transposon derived from the Tn7 bacterial transposon (Biery *et al.,* 2000), and modified it to contain a *C.albicans*-specific *URA3* selectable marker. We used this modified transposon in an *in vitro* mutagenesis reaction with a *C.albicans* genomic DNA library derived from the WO-1 strain (NIH AIDS Research & Reference Reagent Program; Slutsky *et al.*, 1987; Baldari *et al.,* 2005). After amplification of the transposon-inserted plasmid library in Escherichia coli, the plasmids were transformed into the *CBK1* heterozygote to generate a total of 6,528 transposon-inserted strains that were heterozygous at *CBK1* and a second locus (Figure 3-2).

### *3.3.2. Identification of genes displaying complex haploinsufficiency with CBK1:*

To identify genes that synthetically interact with *CBK1* to produce a hyphal growth defect, we performed a phenotypic screen. We first considered the media to use in the complex haploinsufficiency screen to ensure it was sensitive enough

to detect small changes in hyphal growth that would be brought about by disrupting a single allele. Two previous studies had independently identified *CBK1* as a gene which when disrupted, showed a hyphal growth defect on certain media, namely, solid Spider medium (McNemar and Fonzi, 2002; Uhl *et al.,* 2003). The Fonzi group isolated the *CBK1* gene and subsequently tested both heterozygous and homozygous mutants for hyphal growth defects on various media, including Spider. Uhl and colleagues tested various media and concluded that certain media such as Spider, was sensitive enough to detect slight differences in hyphal growth phenotypes such as might occur with haploinsufficiency. We tested a couple of different conditions, namely Media 199 and Spider media, both in liquid and on plates and at two different temperatures, 30°C and 37°C as reports in the literature cite both as valid hyphal growth temperatures (Antley and Hazen, 1988; McNemar and Fonzi, 2002).

We initially compared hyphal growth of the wild type CAI-4 strain, the *CBK1* heterozygote and the *CBK1* homozygote and decided that the condition that produced the best results, i.e. allowed us to distinguish phenotypes better was solid Spider medium at 37°C (Figure 3-3). We tested our entire collection of 6,528 insertion mutants for alterations in hyphal growth by spotting cultures on 96-well format plates in duplicate and incubating them for 5 days. Hyphal growth phenotypes were compared to the original *CBK1* heterozygous strain. A decrease in hyphal growth, namely reduction or absence of peripheral hyphae or wrinkling at the center was scored as positive. A representative plate obtained from the screen is shown in Figure 3-4. After retesting these strains in duplicate

as earlier, 441 out of 1,810 showed consistent hyphal growth defects and were considered as our final set of putative *CBK1* interactors.

The genes disrupted by the transposon insertions were identified using the 3'RACE (Rapid Amplification of cDNA Ends) as described in the methods section. Identification of 151 insertion mutants revealed 53 unique genes that potentially interact with *CBK1*/RAM network during hyphal growth. The full list of genes is shown in Table 3.1. Of these, 16 genes were found to be regulated by the RAM network and 9 genes were regulated by the PKA pathway. We also identified 22 new genes involved in hyphal development, besides confirming 24 previously characterized hyphal growth genes.

An important issue in the study of hyphal growth phenotypes in *C.albicans* concerns expression of the *URA3* gene. *URA3* is a conserved gene and encodes for orotidine-5'-phosphate decarboxylase, a pyrimidine biosynthesis enzyme. Though it is a popular selection marker, being one of the first few markers available in *C.albicans*, some studies have shown that differences in expression of this gene can lead to hyphal growth defects (Lay *et al.,* 1998; Bain *et al.*, 2001). This defect can be reversed by addition of uridine to the growth medium (Sundstrom *et al.,* 2002; Uhl *et al.*, 2003). To confirm that the hyphal growth phenotypes observed were due to the transposon insertions and not *URA3*-position related effects, we retested the identified insertion mutants on Spider medium containing uridine. All the mutants tested showed hyphal growth defects, just as on the original Spider plates (data not shown), confirming that *URA3* expression was not responsible for the phenotypes.

### *3.3.3. Confirmation of true synthetic interactions with CBK1:*

A critical issue we had to contend with in our complex haploinsufficiency screen was to distinguish hyphal growth defects occurring due to genetic interactions with *CBK1* as opposed to defects occurring due to the second-site mutations alone. Since our aim was to identify genes that genetically interact with *CBK1* during hyphal growth, it was important to distinguish phenotypes resulting from such genetic interactions versus hyphal growth defects occurring due to the transposon-mediated disruption of genes.

To confirm that the genes we identified were true synthetic interactors of *CBK1*/RAM network, we performed a complementation test. *CBK1* on a plasmid was re-introduced by transformation into each double heterozygous strains as described in Methods. Transformants were screened on Spider medium for 5 days at 37°C as in the previous screen. Hyphal growth phenotypes were compared to the original transposon-inserted double heterozygotes and those that showed less severe hyphal growth defects were considered as true synthetic interactors of *CBK1*. Out of the 53 genes, only seven (*ALS4, CCT7, CYS3, HGT6, RPS12, SSD1* and *19.334*) showed a severe hyphal growth defect after *CBK1*-reintroduction indicating that the transposon insertion itself was responsible for the phenotype. The majority showed wild type phenotype with peripheral hyphae and some wrinkling at the center of growth (Figure 3-5).

### *3.3.4. RAM network genes and putative Ace2p targets identified:*

Three classes of interacting genes are likely to be identified using a complex haploinsufficiency screen such as this. One class of genes is those genes that function in the same pathway as *CBK1* (the RAM pathway) if, for example, partial loss of activity in two components of the pathway leads to an additive effect. This would lead to a more severe phenotype compared to loss of just one component, namely, *CBK1*. Another class of genes is genes that belong to a parallel pathway leading to the same outcome (i.e. hyphal growth). A third class of genes would be genes that are defective in the general fitness of the strain and so show hyphal growth defects – these would likely be identified as slow growing strains.

We identified sixteen genes from our screen that had been previously characterized as RAM network-regulated genes by the fact that they have altered expression in an *ace2*Δ mutant (Mulhern *et al.*, 2006). Our CHI screen thus successfully identified genes with a direct mechanistic relationship to the query mutant, the *CBK1* heterozygote. In the same previous study of the comparative transcriptional profiles of a *Candida albicans ace2*Δ mutant and a wild type strain, Mulhern *et al.* defined a candidate consensus binding site for Ace2p, namely AACCAGC. We analyzed the promoter regions of our list of 53 identified genes and found this binding site in 17 of the genes; the sequences differed by no more than one base at positions other than the core sequence, xxCCAxC (Table 2). Out of these, 12 had not been previously identified as potential Ace2p targets. Further work will need to be done to confirm that these are true Ace2p targets.

### *3.3.5. The RAM network and PKA pathway function in parallel during hyphal growth:*

We closely examined the set of 53 identified genes, using the Candida Genome Database as well as the existing literature and identified nine genes (*ADE4, ALS4, HGT6, HGT7, MAF1, MP65, UCF1, YKE2* and *19.3644*) that were regulated by the cAMP-dependent PKA pathway (Bahn *et al.,* 2007). No genes regulated by other hyphal growth pathways, such as *RIM101*, *HOG1* or the *CPH1* MAPK pathway, were found amongst our list. The RAM and PKA pathways both regulate genes involved in metabolism, cell wall and ergosterol biosynthesis, new bud formation, polarized growth and hyphal development. With this in mind, we compared two recent studies done in *C.albicans* of the transcriptomes of mutants of both pathways; an *ace2*Δ mutant (Mulhern *et al.*, 2006) and a *cap1*Δ (adenylate cyclase-related protein) mutant (Bahn *et al.*, 2007). This comparison showed that these pathways regulate a set of 11 genes common to both pathways, besides regulating other pathway-specific genes.

The presence of a substantial number of PKA-regulated genes in our synthetic genetic screen as well as the transcriptional data from the literature suggests a possible link between the PKA pathway and *CBK1*/RAM network in *C.albicans*. In *S.cerevisiae*, as mentioned earlier, it has been suggested that the PKA and RAM pathway function in parallel in bud site selection (Schneper *et al.,* 2004). The same study identified the small GTPase *RHO1* as a possible common target of both pathways. *CBK1* seems to negatively regulate *RHO1* since null mutations in GTPase activating proteins (GAPs) in a *cbk1Δ*

background cause a synthetic slow growth phenotype. In our study, we identified an uncharacterized GAP (*RGD3*) that is homologous to a RhoGAP (*RGD2*) in *S. cerevisiae*. This evidence, along with the fact that about 17 % of our set of identified genes consists of PKA pathway regulated genes, suggests that the PKA pathway and the RAM network function in parallel in *C. albicans* to regulate hyphal development.

### 3.4. Discussion

*Candida albicans* is undoubtedly an important human pathogen and the center of a host of studies that aim to understand its pathogenesis. It causes both local mucosal infections in healthy individuals as well as life-threatening systemic candidial infections in immunocompromised patients. These infections are typically treated with azole antifungals such as fluconozale or itraconazole, however, as this disease tends to recur, azole-resistant non-albicans *Candida* species emerge (Rex *et al.*, 1995). Further treatment involves the use of pharmacological agents that have more side effects or that need to be administered intravenously (Enoch *et al.,* 2006). Thus there is a dire need to design new drugs with more specificity and fewer undesirable side effects. As its ability to cause disease is closely linked to its ability to transition between morphological yeast and hyphal forms, morphogenesis in *C.albicans* has been the focus of many studies. By understanding components involved in this process, new drug targets can be identified and subsequently used in pharmacological studies. The fact that *Candida* exists as a diploid without a

viable haploid stage has somewhat hampered studies of gene function, resulting in less than optimal studies in this organism. Though single gene / pathway studies are extremely important, large-scale genomic studies allow faster data collection and should speed up the process of drug discovery.

With the sequencing of the *C.albicans* genome in 2004, many groups have made use of this data for large-scale studies such as genome-wide transcriptional analysis (Nantel *et al.*, 2002; Mulhern *et al.*, 2006; Bahn *et al.,* 2007), transposon-mediated gene disruptions (Davis *et al.,* 2002; Uhl *et al.*, 2003; Nobile and Mitchell, 2006), and essential gene identification using gene deletion and promoter-replacement (Roemer *et al.*, 2003). In this study we performed the first ever large-scale synthetic genetic screen in *Candida albicans*. This study has not been attempted so far in *Candida* though there are numerous examples of it being used successfully in *S.cerevisiae* to identify genes and networks involved in cellular processes (Davierwala *et al.*, 2005; Boone *et al.*, 2007). We used transposons to disrupt genes across the genome in a strain heterozygous for a RAM network kinase, *CBK1*. Using complex haploinsufficiency (CHI), we examined the mechanism by which the RAM network regulates hyphal development and showed that this method can be successfully used to identify genes and networks involved in hyphal growth in *C.albicans*.

We uncovered a potential link between the RAM network and the cAMP-dependent PKA pathway and believe that these pathways function in parallel to regulate hyphal development in this species. In addition, we identified several

potential targets of the Ace2p transcription factor of the RAM network, and upon confirmation, will show that a CHI screen can identify genes with a mechanistic relationship to the query mutation. We also identified a considerable number of new genes involved in hyphal development, including several uncharacterized genes. Our study has confirmed previously characterized hyphal growth genes as well. We have shown how CHI is useful not only to identify genes in the same pathway as the starting mutant, but also identify genes in other networks that interact with this pathway and to uncover genes with redundant functions. Using the property of haploinsufficiency eliminates the need to perform the laborious process of disrupting both alleles; this facilitates studies in an asexual diploid such as *Candida*. The transposon-based gene disruption method is useful for large-scale mutagenesis in *Candida,* as it is relatively quick and not as cumbersome as PCR-mediated gene deletions.

Transposon-mediated gene disruption methods are not without limitations. As mentioned before, transposons insert randomly into the genome but there is a certain level of bias since they insert more frequently into some areas of the genome compared to others. Also, it is difficult to determine the completeness of the starting genomic DNA library. In spite of these limitations, our screen revealed far more relevant results than what typically can be achieved from a large-scale screen. Many of the identified genes are regulated by the RAM network which includes cell wall genes like *RBT5*, metabolic genes, *ADH1, LEU42* and *MET2* and genes involved in glucose response – *HGT6, PGK1, ENO1*. Seventeen potential Ace2p targets were identified, including twelve new

target genes. In addition, a substantial number were genes regulated by the PKA pathway, another pathway involved in hyphal developmental in *Candida*. This, along with other evidence mentioned earlier, supports the idea that the RAM and PKA pathways function in parallel to regulate hyphal growth. The PKA pathway-regulated genes include glucose transporters *HGT6* and *HGT7*, and a number of known hyphal-growth related genes such as the agglutinin family gene *ALS4*, cytoskeleton modulator *YKE2*, cell surface protein *MP65* and PKA pathway-specific genes, *UCF1* and the uncharacterized *19.3644*. Besides these, 22 new hyphal-growth genes involved in various cell processes have been identified such as stress responsive genes *HSP60* and *HSP30*; cytoskeletal genes *ACT1* and *CCT7*, transport genes *CCC1*, ribosomal genes *RPS1*, *RPS12* and *RPL15A* and a number of uncharacterized genes.

Morphogenesis is a complex process as evidenced by the fact that there are at least six signaling pathways identified in *Candida* (Brown and Gow, 1999). Though these are triggered by a host of inducing agents, such as low nitrogen, carbon source, high phosphate and physiological conditions such as serum and high temperatures, there are certain inducing agents specific to certain pathways. For example, the *RIM101* pathway is activated in response to alkaline conditions (Davis *et al.*, 2000), the *HOG1* pathway responds to oxidative stress (Román *et al.*, 2005). Studies of mutants of the *CPH1* MAPK pathway have shown that disruption of certain MAPK cascade genes leads to hyphal growth defects when the inducer is Spider medium (that uses mannitol as a carbon source) but continue to form hyphae when serum is used as the inducing agent (Köhler and

Fink, 1996). In this context, it would be interesting to perform similar CHI screens using different inducing conditions and compare the resulting gene sets. This would help delineate the specific triggers and possibly help uncover the sensors that lead to activation of these pathways. Another interesting possibility is to repeat the CHI screen with different query mutants in different pathways. Though this would involve a substantial amount of work, it would help uncover the connections between networks in *Candida* morphogenesis and ultimately lead to construction of a genome-wide network map of this process. Information gained from this would undoubtedly aid in uncovering important pharmacological targets, the ultimate goal of all these studies.

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**Figure 3-1 Simplified schematic of the Regulation of Ace2p and Morphogenesis (RAM) pathway.** Interaction of the network proteins leads to activation of the kinase Cbk1p which phosphorylates the transcription factor Ace2p. This leads to activation of target genes involved in diverse cellular processes like cell separation, polarized growth, metabolism and hyphal growth.

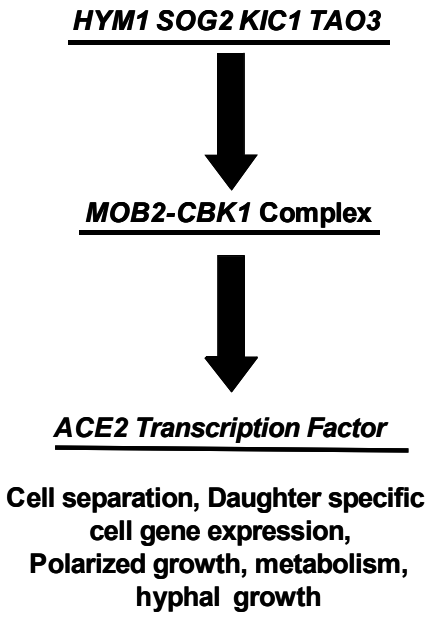


**Figure 3-2 Schematic overview of the construction of C. albicans Tn7 transposon library and insertional mutagenesis of cbk1Δ/CBK1.** The Tn7-transposon donor plasmid is modified to contain the URA3dpl200 cassette by PCR amplification of the cassette using primers with SpeI sites in the flanking sequences and subsequent ligation to SpeI-digested donor plasmid. The C. albicans genomic DNA library is subjected to in vitro mutagenesis. Tn7-mutagenized inserts contained in the resulting library are released from the plasmid by digestion with PvuII, which cuts at two sites flanking the position where the C. albicans genomic DNA inserts were cloned into the original donor plasmid. The resulting linear DNA was transformed into the ura-, cbk1Δ/CBK1 strain. The transposon cassettes are directed by the flanking sequences of C. albicans DNA and insert into the chromosomes by homologous recombination. Transformants are subsequently screened on Spider media for hyphal growth defects.

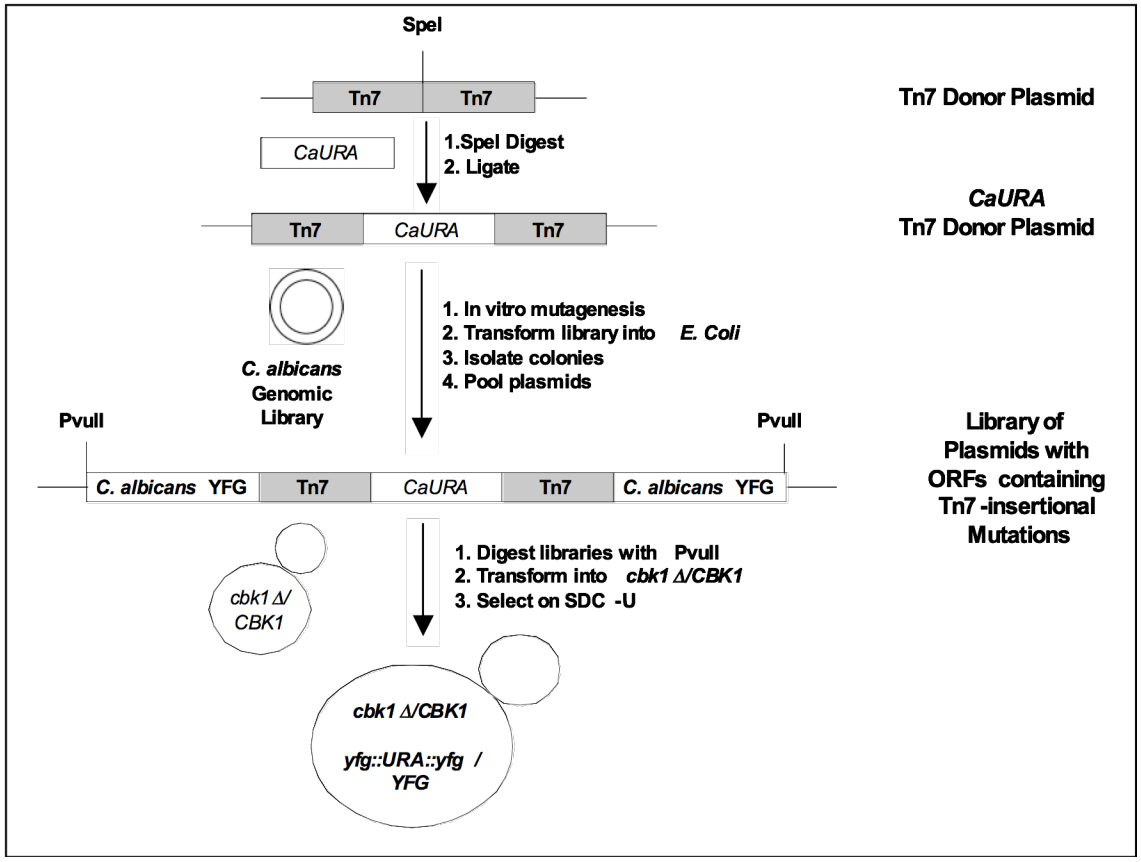


**Figure 3-3 Comparison of colony and cellular morphology of wild type CAI4, heterozygous mutant cbk1Δ/CBK1 and null mutant cbk1Δ/ cbk1Δ strains.** (A) Strains were spotted on Spider + uridine medium and incubated at 37°C for 5 days to induce hyphal growth. (B) Strains were grown overnight in YPD+ uridine at 30°C and then inoculated into liquid Spider + uridine at 37°C for 6 hours to induce hyphal growth. Strains were (C) spotted on YPD + uridine and incubated at 30°C or (D) grown in liquid YPD + uridine at 30°C as vegetative growth controls.

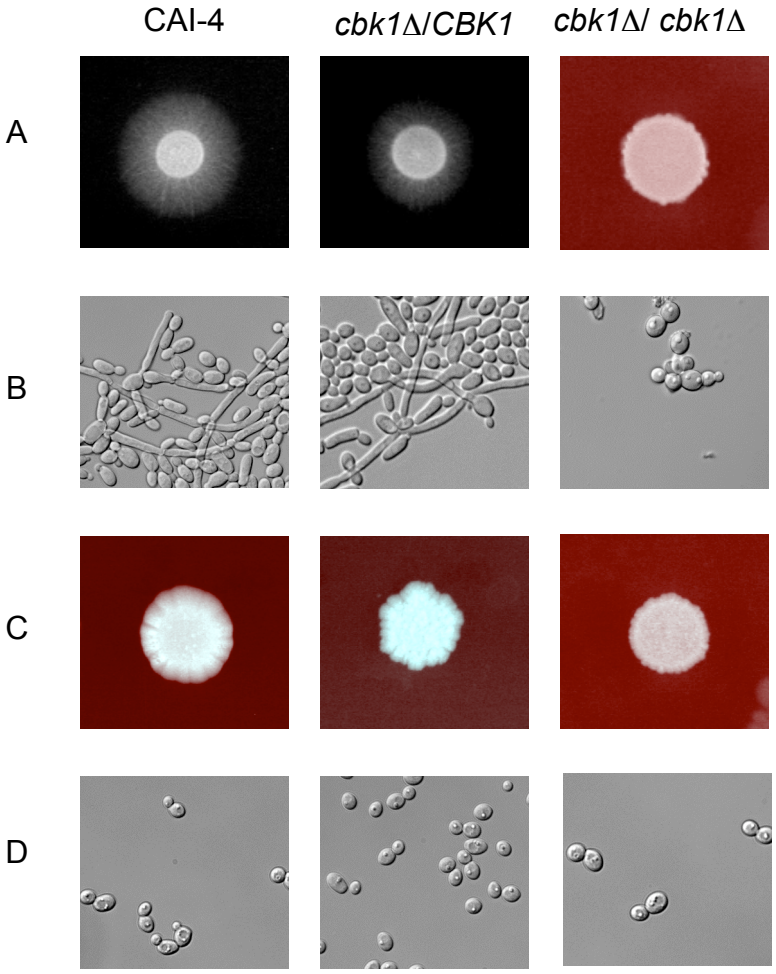


**Figure 3-4 Screening of Tn7 transposon-inserted transformants on Spider plates for hyphal growth phenotypes.** Transformants were grown overnight in SC-Ura and then spotted in 96-well format using a hand pinning tool on Spider medium. Plates were incubated at 37°C for 5 days. Colonies showing reduced or null hyphae as compared to cbk1Δ/CBK1 were scored as positive (example of such colonies shown by the pointed arrow). These were retested on Spider medium as earlier to confirm hyphal growth phenotypes.

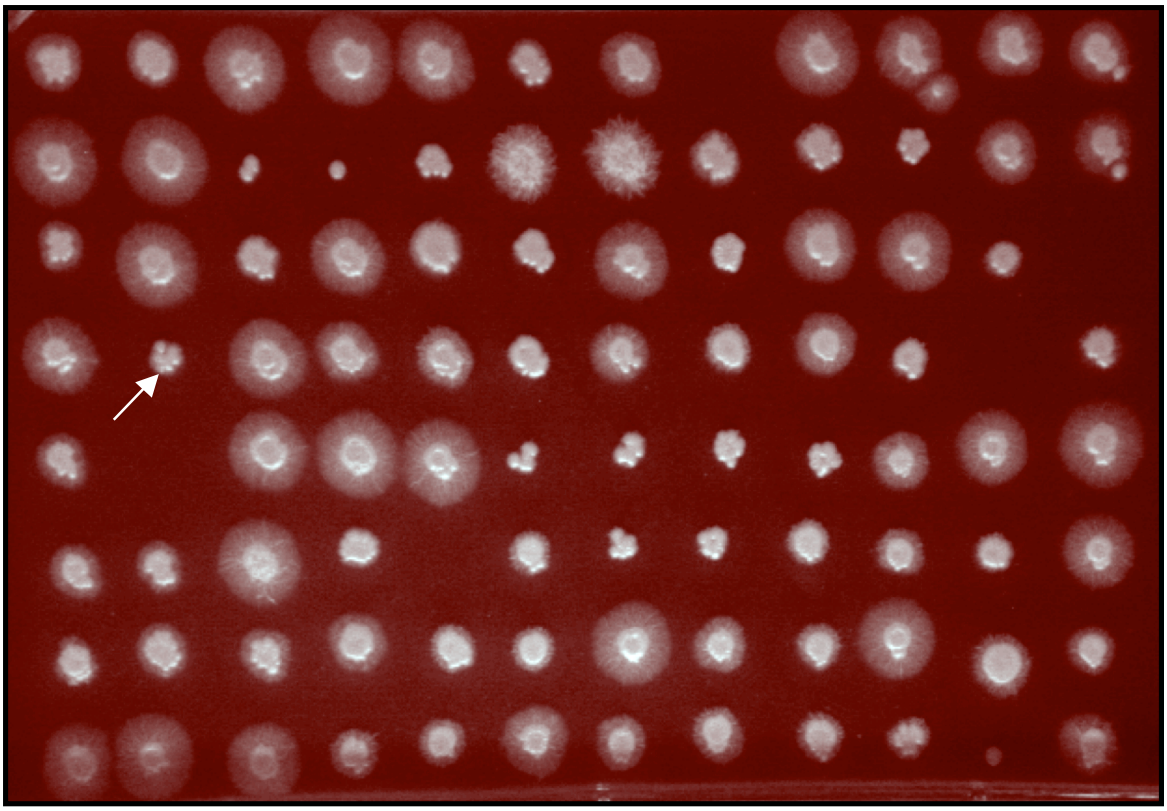
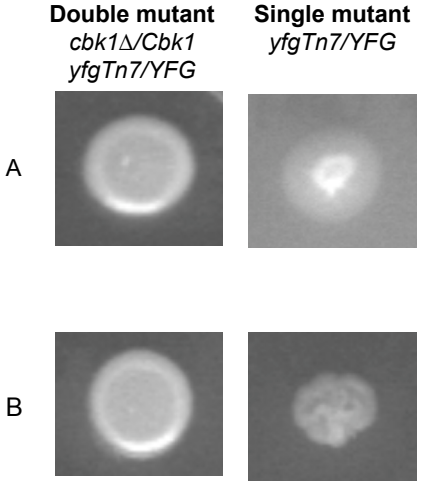


**Figure 3-5 Secondary phenotypic screen to identify double mutants displaying complex haploinsufficiency.** CBK1 on integrative plasmid pMM4 was re-transformed into the double heterozygous mutants to generate single heterozygous strains wild type for CBK1. These transformants were grown overnight in SC-Ura and then spotted on Spider medium. Plates were incubated at 37°C for 5 days. Hyphal growth phenotypes of the single heterozygous Tn7-insertion mutants were compared to the original double heterozygous mutants. (A) Group I: Single heterozygous strains showing reduced hyphal growth defects as compared to the double heterozygous mutant. Phenotype of the UCF1 mutant is shown and is a representative image of all strains within this group. (B) Group II: Single heterozygous strains with no reduction in hyphal growth defects as compared to the double heterozygous mutant. Phenotype of the HGT6 mutant is shown and is a representative image of all strains within this group.




| | | | | | | |
|---|---|---|---|---|---|---|
| ACT1 | ENO1 | MAF1 | PPA1 | SFL1 | VPS13 | 19.6596 |
| ADE4 | FAA4 | MET2 | RBT5 | SMI1 | YKE2 | 19.263.1 |
| ADH1 | GPD2 | MP65 | RGD3 | SNZ1 | 19.1152 | |
| AIM38 | HGT7 | NSP1 | RNR22 | SSS1 | 19.133 | |
| ATP3 | HSP60 | OCH1 | RPB5 | TRM1 | 19.2048 | |
| CCC1 | KER1 | PGK1 | RPL15A | TRX1 | 19.3644 | |
| COX8 | LEU42 | POL5 | RPS1 | UCF1 | 19.384 | |

| | | |
|---|---|---|
| ALS4 | HGT6 | 19.334 |
| CCT7 | RPS12 | |
| CYS3 | SSD1 | |

**Table 3-1 List of identified genes from the screen.** A brief description of the genes derived from the Candida Genome Database (candidagenome.org) is presented here. The last column classifies the genes into the following groups: (1) RAM pathway-regulated; (2) PKA pathway-regulated; (3) Genes previously linked to hyphal growth/biofilm formation and (4) Genes not previously linked to hyphal growth/biofilm formation.

| Gene | Description | Classification |
|---|---|---|
| *ACT1* | Actin; Regulated by growth phase and starvation | 4 |
| *ADE4* | Purine biosynthesis pathway? | 1, 2 |
| *ADH1* | Alchol dehtdrogenase | 1, 3 |
| *AIM38* | Predicted membrane protein. Mitochondrial? | 3 |
| *ALS4* | Adhesion and WT germ tube induction | 2, 3 |
| *ATP3* | Macrophage/pseudohypal induced. ATP synthesis? | 3 |
| *CCC1* | Putative Fe2+/Mn2+ transporter | 4 |
| *CCT7* | Cytoskeleton organization and biogenesis;protein folding | 4 |
| *COX8* | Cytochrome oxidase activity? | 4 |
| *CYS3* | Sulfur amino acid biosynthesis? Biofilm-upregulated | 1 |
| *ENO1* | Enzyme of glycolysis and gluconeogenesis; Biofilm regulated | 1, 3 |
| *FAA4* | Acyl-CoA synthase? | 1 |
| *GPD2* | Similar to glycerol 3-P dehydrogenase; pseudohyphal growth | 1, 3 |
| *HGT6* | Putative glucose transporter | 1, 2 |

| Gene | Description | Classification |
|---|---|---|
| *HGT7* | Putative glucose transporter | 2, 3 |
| *HSP30* | Similar to heat shock protein; fluconazole-downregulated | 4 |
| *HSP60* | Mitochondrial heat shock protein | 4 |
| *KER1* | Alkaline-induced PM protein; affects cell aggregation | 1, 3 |
| *KRE1* | 1,6-beta-D-glucan biosynthesis | 4 |
| *LEU42* | Leucine synthesis; upregulatd by human whole blood | 1, 3 |
| *MAF1* | Nucleocytoplasmic transport; synthesis of RNA Pol II | 2, 3 |
| *MET2* | Methionine synthesis; macrophage/pseudohyphal repressed | 1, 3 |
| *MP65* | Cell surface mannoprotein; induced upon germ tube formation | 2, 3 |
| *NSP1* | Essential component of nuclear pore complex? | 4 |
| *OCH1* | Alpha-1,6-mannosyltransferase; required for virulence | 3 |
| *PGK1* | Phosphoglycerate kinase; Biofilm-induced | 1, 3 |
| *POL5* | Induced upon biofilm formation | 3 |
| *PPA1* | Vacuolar ATPase? | 4 |
| *RBT5* | Cell wall protein involved in hemoglobin utilization | 1, 3 |
| *RGD3* | Rho GTPase activating protein? | 1 |

| Gene | Description | Classification |
|---|---|---|
| *RNR22* | Ribonucleoside diphosphate reductase | 1 |
| *RPL15A* | Protein component of large ribosomal subunit | 4 |
| *RPS1* | Ribosomal protein? Transcription induced during active growth | 4 |
| *RPS12* | Acidic ribosomal protein S12 | 4 |
| *SFL1* | Negative regulation of flocculence and filamentous growth | 3 |
| *SMI1* | Decreased mRNA abundance in cyr1 null hyphal cells | 1, 3 |
| *SNZ1* | Stationary-phase protein; induced on yeast-hyphal switch | 1, 3 |
| *SSD1* | Role in resistance to host antimicrobial peptides; virulence role | 3 |
| *SSS1* | Subunit of Sec61p translocation complex? | 4 |
| *TRM1* | tRNA methyltransferase; induced upon adherence to polysterene | 3 |
| *TRX1* | Similar to thioredoxin; Biofilm induced | 3 |
| *UCF1* | Upregulated by cAMP in filamentous growth | 2, 3 |
| *VPS13* | Vacuolar protein sorting? | 4 |
| *YKE2* | Cytoskeleton modulator? Induced upon yeast-hyphal switch | 2, 3 |
| *19.1152* | Induced in core stress response | 4 |
| *19.133* | Induced by nitric oxide | 4 |

| Gene | Description | Classification |
|---|---|---|
| *19.2048* | Transcription is positively regulated by Sfu1p (iron) | 4 |
| *19.263.1* | Uncharacterized | 4 |
| *19.3644* | Greater mRNA abundance in cyr1 homozygous null mutant | 2, 3 |
| *19.384* | Uncharacterized | 4 |
| *19.6596* | Transcriptionally regulated by Tac1p(drug), Mnl1p(stress) | 4 |

**Table 1-1 List of genes with consensus Ace2p binding sites in promoter regions.** Genes listed in bold font have decreased expression in ace2Δmutants by microarray analysis. Small case letter indicates position where identified sequence differs from the consensus sequence. All listed sequences differ by no more than one base at positions other than the core xxCCAxC. Position denotes the position of putative Ace2p binding site relative to the ATG start codon for the indicated ORF.

| *Gene* | Ace2 site (aaCCAgC) | Position |
|---|---|---|
| ***ADH1*** | AgCCAGC | -481 |
| ***ENO1*** | AACCAcC | -195 |
| *COX8* | AACCAaC | -448 |
| ***HGT6*** | AACCAaC | -146 |
| *HGT7* | AACCAcC | -141 |
| *HSP60* | AACCAaC | -318 |
| *MAF1* | AACCAaC | -279 |
| *NSP1* | AtCCAGC | -200 |
| *POL5* | AACCAaC | -57 |
| ***RGD3*** | AgCCAGC | -175 |
| *RPB5* | AACCAaC | -225 |
| *SFL1* | AACCAtC | -885 |
| ***SNZ1*** | AACCAaC | -431, -169 |
| *TRM1* | AACCAcC | -741 |
| *TRX1* | AACCAaC | -211 |
| *UCF1* | AACCAGC | -926 |
| *19.384* | AACCAaC | -89 |

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# Chapter 4

## Conclusions and Future Directions

The budding yeast *Saccharomyces cerevisiae* is well recognized as a preferred eukaryote for the development of genomic technologies and approaches. Yeast has proven to be useful in gaining knowledge about various cell processes in eukaryotes, such as the cell cycle, DNA replication and repair, protein translation and post-translational modifications (Melese and Hieter, 2002). A sizeable complement of genomic resources has been developed in yeast, and this genomic foundation is now informing a wide variety of disciplines. In my thesis, I make use of genomics to study a particular growth process, namely filamentous growth in budding yeast. Chapter 1 introduces baker's yeast as a model for eukaryotic cell biology and genetics and illustrates genomic studies that have been carried out by various groups. In this chapter, I also present the human fungal pathogen, *Candida albicans* and discuss work on filamentous (hyphal) growth that has been done by the *Candida* community.

Filamentous growth, as mentioned earlier is a developmental process that certain strains of yeast undergo. In response to certain stress conditions such as nitrogen limitation or the presence of short chain alcohols like butanol and

ethanol, the yeast cell changes form from the oval, budded form to the filamentous form, which consists of chains of elongated cells (Gimeno *et al.* 1992; Madhani and Fink 1998; Lorenz *et al.,* 2000). There have been numerous studies of this important process over the years, since it appears strikingly similar to the hyphal/filamentous growth of fungal pathogens. Fungal pathogens such as *Candida albicans* switch form from budded to filamentous (hyphal) like *S.cerevisiae* (Sudbery *et al.,* 2004). It has been suggested that there is a link between the ability of fungal pathogens to undergo hyphal growth and to cause disease. The switch between growth forms appears to be essential for virulence. This was first discovered in a *Candida* mutant that was defective in hyphal formation and was subsequently avirulent in a mouse model (Lo *et al.,* 1997). The signaling pathways leading to filamentous/hyphal growth share several components; in particular there is a strong degree of gene conservation between *C. albicans* and *S. cerevisiae*.

Studies of filamentous growth in budding yeast have been done in an attempt to delineate the signaling pathways, their sensors, the downstream targets, and the mechanisms by which these pathways interconnect with other related cellular processes (e.g., cell cycle progression, bud site selection, etc.). There have been studies of single genes/pathways as well as genomic studies making use of tools and techniques such as transposon mutagenesis, PCR-mediated gene deletions, microarrays, and mass spectrometry. Subcellular localization of proteins is one method of gene regulation that has not been studied in filamentous yeast on a large scale previously. In Chapter 2, I describe

a method of localizing the 125 protein kinases during filamentous growth. We identified six kinases that change localization under filamentous growth-inducing conditions. Further, we show how these kinases form part of an interdependent, localization-based regulatory network. We also identified a new filamentous growth gene, *KSP1*, whose nuclear translocation is required for filamentous growth. Thus, the localization of Ksp1p and the other kinases identified here is tightly controlled during filamentous growth, representing an overlooked regulatory component of this stress response.

As a follow up to this study, it would be informative to carry out a localization study of all the yeast transcription factors to see how many show differential localization during filamentous growth. The data from this study would no doubt help in forming a better picture of the network of regulated protein localization during this process. The identification of *KSP1* as a new filamentous growth gene was an interesting outcome from this study - further experiments to investigate the role of this gene in filamentous growth are expected to be highly informative.

It would be interesting to study further the mechanism by which these kinases translocate into the nucleus; only *KSP1* contains a putative nuclear localization signal (NLS) (as deduced from the program PSORTII). Previous studies have shown that Bcy1p translocates from the cytoplasm to the nucleus with an interacting protein, Zds1p during temperature stress in the S288C strain of budding yeast (Griffioen *et al.*, 2001). Accordingly, a study can be done to see if these six kinases are transported into the nucleus with interacting proteins

during filamentous growth. In yeast, cargo molecules are escorted to and from the nucleus by carrier proteins called karyopherins, of which 14 have been identified (Lusk *et al.*, 2002). A protein-protein interaction study using yeast two-hybrid or co-immunoprecipitation can be done to see if any of these carrier proteins interact with and hence, might be responsible for transport of these kinases. Alternatively, individually deleting each karyopherin and looking for a disruption in kinase nuclear translocation during filamentous growth would suggest that particular karyopherin is likely responsible for nuclear transport.

Another possible study could be to understand the timing and specificity of kinase phosphorylation during filamentous growth. Do the kinases translocate to the nucleus post-phosphorylation, and which kinases are responsible for phosphorylation? Finally, to help with identifying components and understanding the process of filamentous growth better, it would be useful to try and identify the targets of these kinases. These findings could then be applied to hyphal growth processes in fungal pathogens, taking us a step closer to understanding corresponding cellular process in these organisms.

Chapter 3 describes a genome-wide synthetic genetic screen in the human fungal pathogen *Candida albicans* in an attempt to identify elements involved in hyphal growth. A complex haploinsufficiency screen was carried out in a strain heterozygous for the RAM network kinase, *CBK1* to identify genes that genetically interact with the *CBK1*/RAM network. Genes were disrupted throughout the genome by making use of transposons that randomly insert into the genome. This generated double heterozygous strains that were disrupted at

the *CBK1* locus as well as a second, random locus. Screening these strains for defects in hyphal growth led to the identification of 151 strains with decreased hyphal growth as compared to the original heterozygote, *cbk1*Δ*/CBK1.* This set consists of genes disrupted at 53 unique loci, out of which many are regulated by the RAM network, bringing out the high relevance of this study.

We also identified 17 putative targets of the Ace2p transcription factor of the RAM network. These targets were identified on the basis of an identified consensus sequence (aaCCAgC) in the promoter region of these genes (Mulhern *et al.,* 2006). Of these 17 genes, 12 have not been identified before. To determine if these putative target genes are regulated by Ace2p, expression of these genes can be compared in the wild type strain, *ace2*Δ/*ace2*Δ and *cbk1*Δ/*cbk1*Δ mutants using quantitative RT-PCR. If a gene showed decreased expression in the mutants compared to the wild type strain, it would likely mean it is Ace2p- or Cbk1p-regulated. A further study could be done to check if Ace2p binds to the promoter regions of these genes. Biochemical tests such as a gel mobility shift assay or chromatin immunoprecipitation could be carried out to confirm Ace2p binding to the consensus binding site.

In this study, nine genes regulated by the PKA pathway were also identified, and along with other evidence mentioned earlier, led to the hypothesis that the PKA and RAM pathway function in parallel during hyphal growth. One way in which this hypothesis could be tested is to use quantitative RT-PCR to determine if the expression of these nine genes is increased in *cbk1*Δ or *ace2*Δ mutants as a compensatory effect. Further, a known inhibitor of the PKA pathway

(Cloutier *et al.,* 2003) could be added to the cells to see if this increased expression is now blocked. Another study could be done to determine whether defects in hyphal growth in *cbk1*Δ or *ace2*Δ mutants are exacerbated by PKA pathway-inhibitors or suppressed by PKA pathway-activating chemicals (Wolyniak and Sundstrom, 2007).

Besides confirming 24 genes previously known to play a role in hyphal development, this study also identified 22 new hyphal growth genes, including several uncharacterized genes. Further experiments could be done to characterize the role of these genes in hyphal growth that would add to the current information on hyphal development and, in turn, pathogenesis of this important human fungal pathogen.

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