Specificity determinants of a bifunctional signal transduction pathway in *Saccharomyces cerevisiae.*

by

Radclyffe L. Roberts

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Signature of Author_____
Department of Biology
September 4, 1997

Certified by_____
Gerald R. Fink
Professor of Biology
Thesis supervisor

Accepted by_____
Frank Solomon
Professor of Biology
Chairman, Committee for Graduate studies

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Abstract

Saccharomyces cerevisiae diploid strains starved for nitrogen enter a pseudohyphal growth mode, forming filaments of elongated cells that invade the growth substrate. After extended incubation on rich medium, haploid strains enter an invasive growth mode with many similarities to pseudohyphal development, including filament formation and agar penetration. Both of these developmental events require *STE20*, *STE11*, *STE7* and *STE12*, elements of a conserved mitogen-activated protein kinase (MAPK) cascade required for mating in haploid cells. Thus, a single MAPK cascade controls two distinct developmental programs in the same cell type: mating and filamentous growth. To monitor the outputs of the filamentous growth outputs of this bifunctional MAPK cascade, a transcriptional reporter was developed: *FG(TyA)-lacZ*. Studies of the regulation of *FG(TyA)-lacZ* and *FUS1-lacZ*, a mating specific reporter, demonstrate that mating pheromones induce *FUS1-lacZ* but not *FG(TyA)-lacZ* while conditions that stimulate filamentous growth induce *FG(TyA)-lacZ* but not *FUS1-lacZ*. A high degree of signal specificity is achieved.

Activated alleles of the small G proteins *RAS2* and *CDC42* induce both filamentous growth and *FG(TyA)lacZ*. This induction depends upon *STE20*, *STE11*, *STE7*, and *STE12*, indicating that *RAS2* and *CDC42* activate the bifunctional MAPK cascade during filamentous growth. *BMH1* and *BMH2*, redundant 14-3-3 protein homologs, are not essential for mating MAPK cascade signaling, though they are required for *FG(TyA)-lacZ* signaling and pseudohyphal development. Activated alleles of *RAS2* and *CDC42* induce pseudohyphal development and *FG(TyA)-lacZ* signaling in Bmh^+ strains but not in *ste20* or *bmh1 bmh2* mutant strains. Thus, *RAS2* and *CDC42* activate the bifunctional MAPK cascade during filamentous growth via the 14-3-3 proteins.

Thesis supervisor: Gerald R. Fink, American Cancer Society Professor of Genetics at M. I. T. and Director of the Whitehead Institute for Biomedical Research.

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Chapter one

Rising yeast: a paradigm for MAPK cascade specificity

Radclyffe L. Roberts and Gerald R. Fink

Internal energy stores languish at midnight and you hit the speed dial to order some essential nutrients. Within minutes a red hot pizza arrives at your door. Munching on your meal, you notice the neighbors house is on fire! Glucocorticoid levels surge as you reach for the phone and dial 911. Within minutes the police and firemen arrive at your door. What determines the specificity of these signaling events? The signal transduction pathway (telephone, encryption codes, processing center, and miles of cables) is virtually the same in both cases, and yet subtle differences in the primary signals elicited radically different responses. Specificity is vital. It would be disastrous to dial 911 and end up with a bubbling sausage special, and the firemen might not be keen on serving you dinner. Cells make similar decisions, rapidly and accurately initiating developmental programs to adapt to changes in their environment. The capacity of cells to utilize a single pathway to specify multiple developmental fates is an emerging theme in modern signal transduction. A new paradigm for signal transduction specificity is being revealed in the filamentous tendrils of *Saccharomyces cerevisiae*.

Setting the stage

Ordered filaments of elongated cells, or pseudohyphae, formed by strains of the brewers yeast *Saccharomyces cerevisiae* were first described in the late nineteenth century (Hansen, 1886). However, this developmental event was overlooked for many years as most laboratory strains are not dimorphic (Liu et al., 1996). In 1991 pseudohyphal development in *S. cerevisiae* was "re-discovered" (Gimeno et al., 1992). During the intervening century, *S. cerevisiae* had blossomed into the most powerful eukaryotic genetic model system in modern biology. With the tools of molecular genetics in hand, researchers quickly began to dissect the regulatory pathways controlling pseudohyphal development.

Pseudohyphal development requires elements of a MAPK cascade.

When starved for nitrogen, dimorphic diploid strains enter a pseudohyphal growth mode, forming filaments of elongated cells that invade the growth substrate (Gimeno et al., 1992)(Figure 1). Elements of the yeast mating pathway, a conserved mitogen-activated protein kinase (MAPK) cascade, are required for pseudohyphal development (Liu et al., 1993). MAPK cascades require the sequential activation and phosphorylation of a series of protein kinases (Crews et al., 1992; Kyriakis et al., 1992; Lange-Carter et al., 1993). These pathways are ubiquitous, controlling cell proliferation and differentiation in *C. elegans*, *D. melanogaster*, fungi, mammals and other eukaryotes (Blumer and Johnson, 1994; Brunner et al., 1994; Foster, 1993; Lackner et al., 1994; Pazin and Williams, 1992; Schlessinger and Ullrich, 1992; Wu and Han, 1994). In *S. cerevisiae*, several MAPK cascades have been described that control mating, osmotic sensitivity, cell wall integrity and sporulation (reviewed in Herskowitz, 1995). One of the best studied MAPK cascades is the mating pathway of *S. cerevisiae* (Sprague, 1991).

Figure 1.1

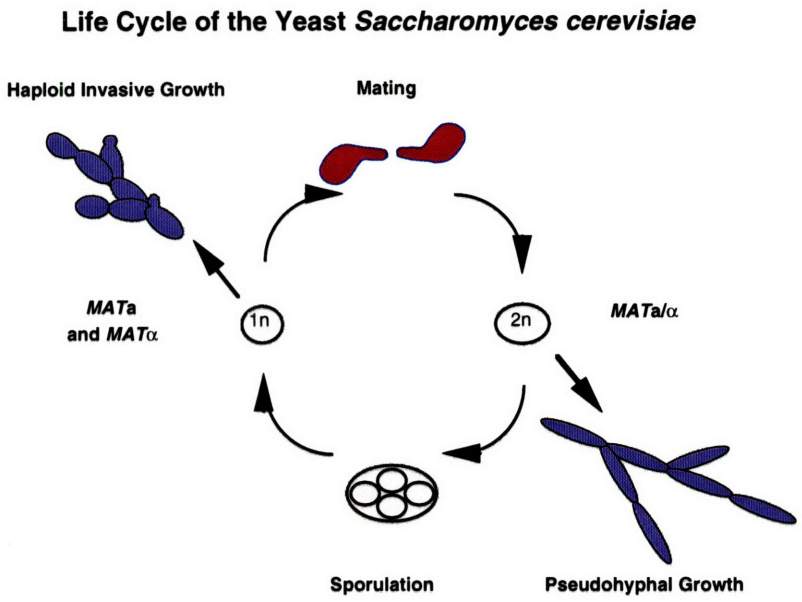

Figure 1.1

Life cycle of the yeast *Saccharomyces cerevisiae*.

S. cerevisiae can can exist in either a haploid (n) or diploid (2n) state. Haploid cells have two mating types: *MATa* and *MATα*. When cells of opposite mating type encounter one another they mate to form a diploid (2n) zygote. Diploid strains starved for nitrogen and carbon enter meiosis and begin sporulation, generating four haploid spores. In contrast, diploid strains are starved for nitrogen in the presence of a fermentable carbon source enter a pseudohyphal growth mode, forming filaments of elongated cells that penetrate the growth substrate. Haploid strains have a similar invasive filamentous growth mode that occurs after prolonged incubations on rich medium: haploid invasive growth.

The mating MAPK cascade of *S. cerevisiae*

In *S cerevisiae* haploid cells, mating is triggered by the binding of a peptide pheromone to its cognate receptor, leading to dissociation of a heterotrimeric G protein (Dietzel and Kurjan, 1987), and subsequent activation of a series of protein kinases in a conserved MAPK cascade (Reviewed by (Sprague and Thorner, 1992)). Ste20p, a $p65^{PAK}$ homolog, is proposed to link the G protein to the kinase cascade (Leberer, 1992; Ramer and Davis, 1993). The MEKK homolog Ste11p (Rhodes, 1990; Stevenson et al., 1992) and the MEK homolog Ste7p, are sequentially activated (Cairns et al., 1992), and the latter phosphorylates the MAPK homologs Fus3p and Kss1p (Gartner et al., 1992; Zhou et al., 1993), which are partially redundant for mating functions (Elion et al., 1991; Elion et al., 1991). Ste5p is thought to nucleate the assembly of the Ste11p, Ste7p and Fus3p protein kinases into a complex (Choi et al., 1994; Kranz et al., 1994; Marcus et al., 1994), which may phosphorylate the transcription factor Ste12p (Elion et al., 1993). In turn, Ste12p binds to DNA sequences known as pheromone responsive elements (PREs) and activates the transcription of mating-specific genes such as *FUS1* (Dolan and Fields, 1990; Trueheart et al., 1987). Activation of this MAPK cascade leads to the induction of the transcriptional reporter *FUS1-lacZ*, the development of mating projections, cell cycle arrest, and formation of a diploid zygote (Figures 1.1 and 1.2).

Mating MAPK cascade components control diploid pseudohyphal development.

Elements of the mating pheromone response MAPK cascade are essential for pseudohyphal development in diploid strains (Liu et al., 1993). Mutations in *STE20*, *STE11*, *STE7*, or *STE12* block the filament formation, cell elongation, and agar penetration of pseudohyphal cells. The finding that both mating and filamentous

growth utilize elements of the same MAPK cascade raised the question: how does a single signal transduction pathway specify two different developmental sequences? In one model, cell-type specific factors determine MAPK cascade specificity. According to this model, stimulation of the MAPK cascade in haploid *MAT***a** or *MAT*α cells results in the activation of mating specific events, whereas stimulation of this pathway in diploid *MAT***a**/α cells results in pseudohyphal development (Figure 1.1).

Defining a bifunctional MAPK cascade: haploid invasive growth.

The discovery of haploid invasive growth, a novel developmental event in the life cycle of *S. cerevisiae*, demonstrated that filamentous growth is not restricted to *MATa*/α diploid yeast strains (Figure 1.1). Haploid invasive growth shares many features with diploid pseudohyphal development, including filament formation and agar penetration (Roberts, 1994). Strikingly, the same MAPK cascade components necessary for diploid pseudohyphal development (*STE20*, *STE11*, *STE7*, and *STE12*) are also required for haploid invasive growth (Roberts, 1994). Moreover, mating specific components (*STE2*, *STE3*, *STE4*, *STE18* and *STE5*), which are expressed and functional in these cells, are not required for invasive growth. Importantly, the mating pathway reporter *FUS1-lacZ* is not induced during haploid invasive growth (Roberts, 1994). This result suggests that this bifunctional MAPK cascade regulates distinct sets of target genes for mating and filamentous growth. Thus, a single MAPK cascade controls two different developmental events in a single cell-type: mating and invasive growth (Figure 1.2). Moreover, cell-type specific factors are not the primary determinants of the differential outputs of this bifunctional MAPK cascade in haploid cells.

Figure 1.2

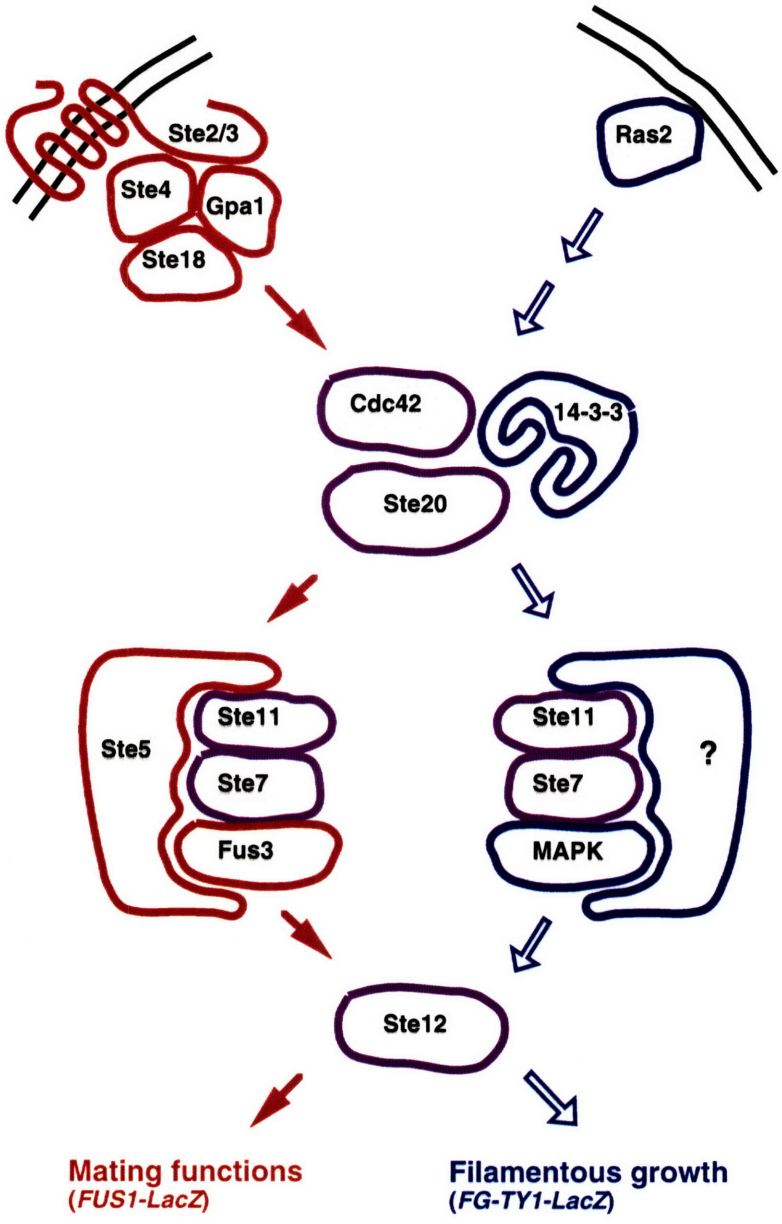

Figure 1.2
A Bifunctional MAPK Cascade.

Mating specific components are red. Filamentous growth specific components are blue. Shared components are purple.

Table 1.1

Plasmid	- pheromone	+ pheromone
vector	8.6	885
RAS2^{VAL19}	5.4	331

Table 1.1
RAS2^{VAL19} does not induce the mating reporter *FUS1-lacZ*.

MATa haploid yeast strain 10650-4d containing the *FUS1-lacZ* reporter pJB207 (B2146) and control vector pRS316 (B1820) or *RAS2^{VAL19}* (B2255) were incubated for three hours in the absence (-) or presence (+) of 5 μM alpha pheromone. Extracts were prepared and assayed for β-galactosidase activity normalized to total protein as described (Mosch et al., 1996).

Table 1.2

Strain	pheromone concentration	*FG(TyA)-lacZ* activity
STE+	5000 nM	49
STE+	500 nM	44
STE+	50 nM	44
STE+	5 nM	58
ste5	5000 nM	40

Table 1.2
Mating pheromone does not induce *FG(TyA)-lacZ* at low concentrations or in a *ste5* mutant.

MATa haploid yeast strains L5528 (*STE+*) and L5554 (*ste5)* containing the *FG(TyA)-lacZ* reporter plasmid (B3160) were incubated for three hours in the presence of mating pheromone at the concentrations indicated. Extracts were prepared and assayed for β-galactosidase activity normalized to total protein as described (Mosch et al., 1996).

A transcriptional reporter for filamentous growth

In order to monitor the outputs of the filamentous growth MAPK cascade, a transcriptional reporter was developed: *FG(TyA)-lacZ*. This reporter consists of a small fragment of the TY1 transposon that contains a *STE12* binding site (PRE) and a putative *TEC1* (a transcription factor) binding site (Company et al., 1988; Errede et al., 1980; Laloux et al., 1994; Sprague and Thorner, 1992). *FG(TyA)-lacZ* expression requires *STE20, STE11, STE7, TEC1,* and *STE12,* and it is strongly induced by either the dominant active *STE11-4* kinase, a truncation allele of *STE20,* or overexpression of *STE20* or *STE12* (Mosch et al., 1996; Roberts, 1997). This reporter is not regulated by the mating MAPK cascade. Its expression does not require the mating specific MAPK cascade components *STE2, STE3, STE4, STE18,* or *STE5,* and it is not induced by mating pheromones (Mosch et al., 1996). Thus, *FG(TyA)-lacZ* provides an accurate and specific assay for the activity of the filamentous growth MAPK cascade. Furthermore, these data support the idea that distinct transcriptional targets are induced during mating and filamentous growth. Specifically, mating pheromones induce *FUS1-lacZ* but not *FG(TyA)-lacZ,* whereas conditions that stimulate filamentous growth induce *FG(TyA)-lacZ* but not *FUS1-lacZ.* These two reporters are critical for monitoring the differential outputs of this bifunctional MAPK cascade.

Determining the specificity of a bifunctional MAPK cascade

Elements of a single MAPK cascade control two developmental programs in the same cell type: mating and filamentous growth. How do cells distinguish between the different stimuli that activate this bifunctional MAPK cascade and initiate the correct developmental program specific to each primary signal? This

question of signal specificity can be broken down into three distinct steps. First, what are the primary signaling components that activate the pseudohyphal development MAPK cascade and how do mating and pseudohyphal components differentially activate the pathway during each process? Second, how is the specificity of these primary signals maintained and faithfully transmitted through the shared MAPK cascade components? Finally, how are these signals translated into the correct transcriptional outputs specific to each process?

Activating the MAPK cascade during filamentous growth

The pheromone receptors and G protein involved in activating the mating MAPK cascade are not required for filamentous growth (Liu et al., 1993; Roberts, 1994). What are the upstream elements that activate this bifunctional MAPK cascade activation during filamentous growth? In mammals, flies, worms and fission yeast, Ras regulates cell proliferation and differentiation via conserved MAPK cascades (reviewed in Burgering and Bos, 1995; McCormick, 1994; van der Geer et al., 1994). *RAS2*^{Val19}, a dominant activated form of *RAS2*, stimulates both filamentous growth and expression of *FG(TyA)-lacZ* (Mosch et al., 1996). Full induction depends upon elements of the filamentous growth MAPK cascade: *STE20*, *STE11*, *STE7*, and *STE12* (Mosch et al., 1996). Importantly, *RAS2*^{Val19} does not induce the mating pathway reporter *FUS1-lacZ*. Thus, *RAS2* activates this bifunctional MAPK cascade during filamentous growth.

The Rho family G proteins Cdc42hs and Rac1 act downstream of Ras in mammalian cells (Khosravi-Far et al., 1995; Prendergast et al., 1995). Similarly, *CDC42* is a potent regulator of filamentous growth and *FG(TyA)-lacZ* expression in *S. cerevisiae* (Mosch et al., 1996). Stimulation of both filamentous growth and *FG(TyA)-lacZ* by activated alleles of *CDC42* (*CDC42*^{Val12} or *CDC42*^{Leu61}) depends upon

STE20. In addition, dominant negative *CDC42*^{Ala118} blocks *RAS2*^{Val19} activation, suggesting that *CDC42* acts downstream of *RAS2*. Unlike *RAS2*, *CDC42* has been implicated in mating MAPK cascade functions, but its precise role is uncertain (Leberer, 1997; Peter, 1996; Simon et al., 1995). Thus, filamentous growth in budding yeast is regulated by an evolutionarily conserved signaling pathway that controls cell morphology and transcription.

What links *RAS2* and *CDC42* to the bifunctional MAPK cascade during filamentous growth? Recent experiments show that the *S. cerevisiae* 14-3-3 homologs *BMH1* and *BMH2* are critical for coupling *RAS2* and *CDC42* to the pseudohyphal development MAPK cascade. Specifically, activated alleles of *RAS2* and *CDC42* induce pseudohyphal development and *FG(TyA)-lacZ* signaling in Bmh$^+$ strains but not in *ste20* (p65^{PAK}) or *bmh1 bmh2* mutant strains (Roberts, 1997). Furthermore, Bmh1p and Bmh2p associate with Ste20p *in vivo*. Three alleles of *BMH1* encode proteins defective for *FG(TyA)-lacZ* signaling and association with Ste20p, yet these alleles complement other 14-3-3 functions (Roberts, 1997). Moreover, *BMH1* and *BMH2* are not essential for mating MAPK cascade functions (Roberts, 1997). Therefore, the 14-3-3 proteins are specifically required for RAS/MAPK cascade signaling during pseudohyphal development in *S. cerevisiae*.

How do *RAS2*, *CDC42*, and the 14-3-3 proteins activate Ste20p during filamentous growth? *In vitro*, Cdc42p binds Ste20p and stimulates its kinase activity (Zhao et al., 1995). Notably, the *CDC42* binding domain of *STE20* is necessary for filamentous growth but not mating (Leberer, 1997; Peter, 1996). The 14-3-3 proteins may also regulate the activity of Ste20p (Roberts, 1997). One possibility is that oligomerization of Ste20p is essential for its activation, as has been suggested for Raf1 (Farrar et al., 1996; Luo et al., 1996), and that the 14-3-3 proteins are vital for this event. Ste20p is capable of forming oligomers *in vivo*, but the functional significance of these complexes has not been ascertained (Roberts unpublished).

Together, these data suggest that Ras2p activates Cdc42p, which binds and activates Ste20p in concert with the 14-3-3 proteins.

Determining signal specificity of common MAPK cascade components

After the primary signaling components differentially activate the pathway, the signal must be propagated through the common MAPK cascade components without losing specificity. In one model, accessory proteins direct the association of common components of the pathway with their appropriate targets (Figure 1.2). For example, Ste5p assembles Ste11p, Ste7p, and Fus3p into a protein complex that may modify Ste12p, shifting it to a state that activates the transcription of mating specific genes (Choi et al., 1994; Kranz et al., 1994; Marcus et al., 1994). No sequence homolog or functional analog of *STE5* has yet been found for filamentous growth, or any other MAPK cascade. However, the absence of a sequence homolog does not preclude the existence of a scaffold protein that might drive the assembly of a filamentous growth specific kinase signaling complex. Do these scaffold components prevent promiscuous protein pairings that could lead to crossed signals? We find that deletion of *STE5* does not make *FG(TyA)-lacZ* expression responsive to mating pheromone (Table 1.1). Thus, *STE5* is not essential for maintaining signal specificity. Nevertheless, scaffold molecules like *STE5* may help ensure MAPK cascade specificity by sequestering different signaling complexes and directing them to the proper targets.

The MAPK cascade homologs Fus3p and Kss1p have redundant functions in mating and both proteins bind Ste7p (Elion et al., 1991; Elion et al., 1991) Kss1p is required for filamentous growth and *FG(TyA)-lacZ* expression in haploids, and it may also play a positive role in pseudohyphal development (Mosch et al., 1996; Roberts, 1994). In contrast, Fus3p plays a negative role in filamentous growth.

Specifically, haploid *fus3* mutant strains show increased invasive growth and *FG(TyA)-lacZ* expression that depends on Ste7p and Ste12p (Mosch et al., 1996; Roberts, 1994). Surprisingly, *fus3 kss1* haploid strains and diploid strains are filamentous (Liu et al., 1993; Roberts, 1994). These data suggest that Kss1p promotes filamentous growth while Fus3p antagonizes it. In summary, these two MAPKs may help determine the specificity of the shared MAPK cascade components Ste11p and Ste7p and differentially activate the downstream transcription factor Ste12p.

Determining the transcriptional outputs of the bifunctional MAPK cascade

The transcription factor *STE12* is required for both mating and filamentous growth, and yet distinct transcriptional targets are induced during each developmental event. Specifically, environmental or genetic ($RAS2^{VAL19}$) conditions that promote filamentous growth induce *FG(TyA)-lacZ* but not *FUS1-lacZ*, whereas mating pheromones induce *FUS1-lacZ* but not *FG(TyA)-lacZ*. How is this specificity achieved? The *FUS1* promoter contains four tandem copies of a Ste12p binding site (PRE), and this motif is both necessary and sufficient to confer mating pheromone inducible transcription (Hagen et al., 1991). In contrast, promoters with only a single PRE are not strongly pheromone inducible (Hagen et al., 1991). Thus, Ste12p-Ste12p cooperativity is critical for the induction of mating specific transcripts.

How does *STE12* regulate *FG(TyA)-lacZ*? The first clue is that *TEC1*, a TEA/ATTS transcription factor, is required for filamentous growth but not mating (Gavrias et al., 1996; Mosch and Fink, 1997). Moreover, *FG(TyA)-lacZ* expression depends on *TEC1*, and this reporter contains a putative Tec1p binding site adjacent to a PRE (Laloux et al., 1994). Indeed, Ste12p and Tec1p bind cooperatively to *FG(TyA)-lacZ*. Binding requires the presence of both proteins and it is abolished by

point mutations in the binding site of either protein (Madhani, 1997). These results imply that Ste12p-Tec1p complexes induce filamentous growth specific transcripts while Ste12p-Ste12p complexes induce mating specific transcripts. Thus, *TEC1* is important for determining the spectrum of genes induced by *STE12* during mating and filamentous growth.

The basal signaling hypothesis

The presence of elements of a conserved MAPK cascade evokes images of growth factors and pheromones that induce the pathway under the appropriate conditions. However, no such inducer has yet been discovered for filamentous growth. Moreover, although deletion of MAPK cascade components blocks filamentous growth and activated alleles stimulate it, there is no direct evidence for distinct activation states of this MAPK cascade. Until a physiologic inducer is found that specifically activates this pathway, we cannot rule out the possibility that signaling via the filamentous growth MAPK cascade is a consitutive process rather than a regulated one. In this light, we define the basal signaling hypothesis: A constant, low level of pathway activity, independent of the developmental state of the cell, is required for cellular differentiation. According to this model, the filamentous growth MAPK cascade may not ever be 'activated' during filamentous growth and constitutive 'basal' signaling may be necessary for this developmental event. The basal signaling hypothesis may describe signal transduction pathways in other systems as well.

Antagonism between different MAPK cascade functions

Why use a single MAPK cascade (instead of two pathways) to control two different developmental events? If each function of the pathway antagonizes the other one, this may help prevent the simultaneous initiation of different developmental outcomes (mating and filamentous growth). More generally, we define the functioanl antagonism hypothesis: Antagonism between components of a multifunctional pathway that are specific to different pathway outcomes ensures that these outcomes are mutually exclusive. The finding that *RAS2*^{Val19}, which strongly stimulates filamentous growth, reduces pheromone induction of the *FUS1-lacZ* supports this model (Mosch et al., 1996, Table 1.1). Similarly, *FUS1-lacZ* activity is lower in cells undergoing invasive growth on plates than in non-invasive cells in liquid culture (Roberts, 1994). Furthermore, genetic studies of *FUS3* and *KSS1* suggest a functional antagonism between these two MAPK homologs (Roberts, 1994). Antagonism between mating and filamentous growth components could arise from direct competition for binding to limiting components such as Ste11p or Ste7p. Alternatively, it might arise from driving one or more common MAPK components, such as *STE20* or *STE12*, into mutually exclusive functional states. For example, Ste12p may be driven into different protein complexes (ie: Ste12p-Ste12p versus Ste12p-Tec1p) with distinct DNA binding and transcriptional activation profiles. Regardless of the mechanism, antagonism between the mating and filamentous growth components of this bifunctional MAPK cascade may help ensure that the developmental outcomes are mutually exclusive.

Parallels in other systems

MAPK cascades are critical for cell proliferation and differentiation in a wide array of eukaryotes (Blumer and Johnson, 1994). The finding that a single MAPK cascade controls two distinct developmental sequences is emerging as a general

theme. In *D. melanogaster*, a dominant activated allele of the MAPK *rolled* disrupts the development of several tissues (Brunner et al., 1994). Perhaps the closest parallel to the bifunctional MAPK cascade in *S cerevisiae* is the behavior of PC12 cells in response to epidermal growth factor (EGF) and nerve growth factor (NGF). EGF induces transient MAPK activation and cell division in these cells while NGF induces prolonged activation and nuclear translocation of MAPK, causing the same cells to arrest growth and initiate neuronal differentiation (Traverse et al., 1992). However, in PC12 cells overexpressing the EGF receptor, EGF triggers prolonged MAPK activation and nuclear localization, and induces neuronal differentiation (Dikic et al., 1994; Traverse et al., 1994). Thus, the strength of the signal determines the developmental outputs of this MAPK cascade. To test if the same principle governs the *S. cerevisiae* mating/filamentous growth bifunctional MAPK cascade, we exposed cells carrying the *FG(TyA)-lacZ* reporter to varying concentrations of mating pheromone. If 5 μM pheromone strongly induces *FUS1-lacZ* but not *FG(TyA)-lacZ*, could much lower pheromone doses induce *FG(TyA)-lacZ*? We find that *FG(TyA)-lacZ* expression is not significantly induced by mating pheromone concentrations between 5 nM and 5μM (Table 1.2). This suggests that the specificity of this bifunctional MAPK cascade is not strictly a function of signal strength.

A paradigm for MAPK cascade specificity

The discovery that elements of a single MAPK cascade control mating and filamentous growth in *S. cerevisiae* raises many questions. Most importantly, how do cells distinguish between two different stimuli that activate the same MAPK cascade and initiate the developmental program specific to each primary signal? Unlike PC12 cells, yeast cells are not fooled by simply altering the strength of the signal. Nor are they confused by the elimination of the Ste5p scaffold protein.

These and other data suggest that the specificity of the primary signal is maintained at all steps of the pathway, thereby ensuring that the proper developmental program is executed in each case.

A model for the generation, maintenance, and execution of MAPK cascade specificity is emerging (Figure 1.2). During mating, a pheromone receptor (Ste2p or Ste3p) and heterotrimeric G protein (Ste4p, Ste18p and Gpa1p) are stimulated, while filamentous growth is triggered by Ras2p, Cdc42p, and the 14-3-3 proteins. These upstream signaling components differentially activate the MAPK cascade via Ste20p and Ste11p. Once the MAPK cascade has been activated, scaffold molecules like Ste5p help maintain signal specificity. In turn, MAPKs (Fus3p or Kss1p) determine pathway outputs by differentially activating the transcription factors Ste12p and Tec1p. Finally, distinct Ste12p-Ste12p and Ste12p-Tec1p complexes determine the spectrum of genes regulated by this bifunctional MAPK cascade. Functional antagonism between mating and filamentous growth components helps ensure that the outputs of this bifunctional MAPK cascade are mutually exclusive. Future studies should help elucidate the molecular mechanisms underlying each of these events. Because elements of the *S. cerevisiae* bifunctional MAPK cascade are highly conserved in evolution, this model system should provide a conceptual framework for understanding signal transduction in all eukaryotes.

In short, every time yeast order pizza, they get pizza. Every time they dial 911, the police and firemen come. For them, Ras2p and the 14-3-3 proteins specify the pizza delivery number while the mating pheromone receptors and heterotrimeric G protein specify the 911 number. Ste20p, Ste11p, and Ste7p constitute their telephone and other shared hardware and Fus3p and Kss1p are distinct output cables from their phone company signal processing center. Finally, Ste12p-Ste12p and Ste12p-Tec1p complexes are the phones in the firehouse and pizza parlor and *FUS1-lacZ* and *FG(TyA)-lacZ* are the firefighters and pizza respectively. The mechanisms

underlying signal specificity in yeast should provide a paradigm for many other eukaryotic signaling events. In the meantime, we can all indulge our late night pizza habits without fearing the wrath of volunteer firefighters.

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Chapter two

Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth

Radclyffe L. Roberts and Gerald R. Fink

Whitehead Institute for Biomedical Research and Department of Biology, M. I. T., Cambridge, Massachusetts 02142

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Summary

Diploid *Saccharomyces cerevisiae* strains starved for nitrogen undergo a developmental transition from a colonial form of growth to a filamentous pseudohyphal form. This dimorphism requires a polar budding pattern and elements of the MAPK cascade signal transduction pathway essential for mating pheromone response in haploids. We report here that haploid strains exhibit an invasive growth behavior with many similarities to pseudohyphal development, including filament formation and agar penetration. Haploid filament formation depends on a switch from an axial to a bipolar mode of bud site selection. Filament formation is distinct from agar penetration in both haploids and diploids. The same components of the MAPK cascade necessary for diploid pseudohyphal development (*STE20*, *STE11*, *STE7*, and *STE12*) are also required for haploid invasive growth. Thus, haploid yeast cells can enter either of two developmental pathways: mating or invasive growth, both of which depend on elements of a single MAPK cascade. These results provide a novel developmental model to study the dynamics of signal transduction, with implications for higher eukaryotes.

Introduction

Mitogen-activated protein kinase (MAPK) signal transduction pathways are critical for many developmental events in eukaryotes, and require the sequential activation and phosphorylation of a series of protein kinases (reviewed by (Blumer and Johnson, 1994). Activation of MAPK requires phosphorylation by activated MAPK/ERK kinase (MEK or MAPKK) (Crews et al., 1992). MEK activation is achieved by phosphorylation by RAF1 or MEK kinase (MEKK or MAPKKK) (Kyriakis et al., 1992; Lange-Carter et al., 1993). Homologs of each of these

components have been identified in a large number of eukaryotes. In the nematode *Caenorhabdtis elegans*, vulval induction requires signaling through the MAPK homolog *Sur1/Mpk1* (Lackner et al., 1994; Wu and Han, 1994). In *Drosophila melanogaster*, the MAPK homolog *rolled* is critical for photoreceptor development and dorso-ventral patterning during embryogenesis (Brunner et al., 1994). In mammalian cells, MAP kinases are important in a wide array of cytokine and growth factor responses (Foster, 1993; Pazin and Williams, 1992; Schlessinger and Ullrich, 1992).

In the yeast *Saccharomyces cerevisiae*, several independent MAPK cascades have been characterized. One pathway mediates the response to hyperosmotic conditions through the MAPK cascade homologs *PBS2* (MEK) and *HOG1* (MAPK), which stimulate glycerol accumulation (Brewster et al., 1993). A second pathway controls cell wall biosynthesis through *PKC1* (a protein kinase C homolog), and the protein kinases *BCK1* (MEKK), *MKK1* and *MKK2* (MEK), and *MPK1* (MAPK) (Blumer and Johnson, 1994). The best defined MAPK cascade controls the mating of haploid cells through *STE11* (MEKK), *STE7* (MEK), and *FUS3* and *KSS1* (MAPK).

In *S. cerevisiae* haploid cells, mating is triggered by pheromones, and leads to the fusion of two cells of opposite mating type to form a diploid. The primary signaling event in this pathway, the binding of the peptide pheromone to its cognate receptor (Ste2p in *MAT***a** cells and Ste3p in *MAT*α cells), results in GDP/GTP exchange on the alpha subunit (Gpa1p) of a heterotrimeric G protein (Dietzel and Kurjan, 1987). The β (Ste4p) and γ (Ste18p) subunits then stimulate the activation of a series of protein kinases (Whiteway et al., 1989). Ste20p is proposed to link the G protein to the kinase cascade (Leberer, 1992; Ramer and Davis, 1993). The MEKK homolog Ste11p (Rhodes, 1990; Stevenson et al., 1992), and the MEK homolog Ste7p, are sequentially activated (Cairns et al., 1992), and the latter phosphorylates the MAPK homologs Fus3p and Kss1p (Gartner et al., 1992; Zhou et al., 1993), which are

partially redundant for mating functions (Elion et al., 1991; Elion et al., 1991). Ste5p is thought to nucleate the assembly of the Ste11p, Ste7p and Fus3p protein kinases into a complex (Choi et al., 1994; Kranz et al., 1994; Marcus et al., 1994), which phosphorylates the transcription factor Ste12p (Elion et al., 1993). Fus3p also phosphorylates the cyclin-dependent kinase inhibitor Far1p (Elion et al., 1993), which mediates cell cycle arrest (Chang and Herskowitz, 1990). In turn, Ste12p activates the transcription of mating specific genes including *FUS1* (Dolan and Fields, 1990), and genes linked to the transposon Ty (Ciriacy et al., 1991; Van Arsdell et al., 1987). Thus, activation of the MAPK cascade in two haploid cells of opposite mating type arrests vegetative growth and leads to the fusion of the two cells to form a diploid.

Elements of the mating pheromone response MAPK cascade are essential for pseudohyphal development in diploid strains (Liu et al., 1993). When starved for nitrogen, diploid cells undergo a developmental transition from a single cell yeast form to a filamentous pseudohyphal form. Pseudohyphal filaments are composed of chains of long thin cells which radiate away from the colony, and penetrate the agar substrate on which they are grown (Gimeno et al., 1992). Mutations in *STE20*, *STE11*, *STE7*, or *STE12* block the filament formation, cell elongation, and agar penetration of pseudohyphal cells (Liu et al., 1993).

The finding that both mating and filamentous growth utilize elements of the same MAPK cascade raises the question of how the same signal transduction pathway can specify two different developmental sequences. Specifically, the Ste12p transcription factor must activate mating specific genes in the haploid, and pseudohyphal specific genes in the diploid. In one model, stimulation of the MAPK cascade in haploid *MAT***a** or *MAT*α cells results in the activation of mating specific events, whereas stimulation of this pathway in diploid *MAT***a**/α cells results in pseudohyphal development. According to this model, factors specific to each cell

type determine the ensemble of genes that are regulated by Ste12p. For example, **a**1 and α2 may repress the activation of mating specific genes, such as *FUS1*, in the *MAT***a**/α diploid cell, allowing the induction of genes specific to filamentous growth under conditions of nitrogen starvation.

In addition to elements of the mating MAPK cascade, filament formation also requires a series of polarized cell divisions determined by the site of bud emergence of each new cell in the growing chain. Diploid cells bud in the bipolar mode, where the first bud of a virgin mother emerges from the free end of the cell, and subsequent buds emerge from either the birth or free end of the cell (Freifelder, 1960). The birth end is defined as the region where each cell is attached to its mother; the opposite end is the free end (Freifelder, 1960). Pseudohyphal cells bud in a unipolar mode rather than the bipolar mode typical of diploid yeast form cells (Kron et al., 1994). In the unipolar mode, the first bud and all subsequent buds emerge from the free end of the cell. Successive rounds of unipolar budding produce a chain of first daughters oriented away from the cell that initiated the chain. In contrast, most haploid cells bud in an axial mode of bud site selection, and do not readily form filaments when starved for nitrogen (Gimeno et al., 1992). In the axial mode, each new bud emerges from the birth end of a cell (Freifelder, 1960). Successive rounds of axial budding produce a cluster of cells rather than the ordered chain of cells required to form a filament (Gimeno and Fink, 1992). The bud site selection mode is determined by a regulatory cascade of *BUD* genes (Chant et al., 1991; Chant and Herskowitz, 1991; Gimeno and Fink, 1992). A mutation in *BUD1* (*RSR1*) disrupts the bipolar budding pattern of diploids (Bender and Pringle, 1989) and drastically reduces their ability to form filaments (Gimeno et al., 1992).

In this chapter we describe a haploid invasive growth behavior with several similarities to diploid pseudohyphal development. Specifically, haploids switch their pattern of bud site selection from an axial to a bipolar pattern and consequently

form filaments. In addition, they penetrate the agar substrate on which they are grown. Haploid filament formation and agar penetration depend on the same pheromone response MAPK cascade components required for diploid pseudohyphal growth. Thus, in a single cell the same signal transduction pathway can mediate two distinct developmental sequences, showing that the differential outputs of the pathway do not require cell type specific factors.

Results

Haploid cells are capable of agar penetration and filament formation on rich medium

*MAT***a** and *MAT*α haploid strains of the Σ1278b background grown on rich medium plates (YPD) for several days begin to penetrate the agar beneath each colony (Figure 2.1, Table 2.1). A population of cells that have penetrated the agar can be observed by washing the top portion of the colony off the agar surface with water. The remaining cells are judged to have penetrated the agar surface because they are inaccessible to a microdissection needle unless the needle pierces the agar. After three days of growth, when the mass of cells on top of the agar has already slowed its growth, the number of cells that have penetrated the agar increases steadily, suggesting that agar penetration may be triggered by nutrient limitation. However, haploid agar penetration may not be related to ammonia deprivation because the addition of ammonium sulfate to YPD does not diminish agar penetration. Remarkably, isogenic *MAT***a**/α diploid cells do not efficiently penetrate agar under these conditions except after extremely long incubations, and the few cells that remain in the agar have the yeast form (Figure 2.1).

Figure 2.1

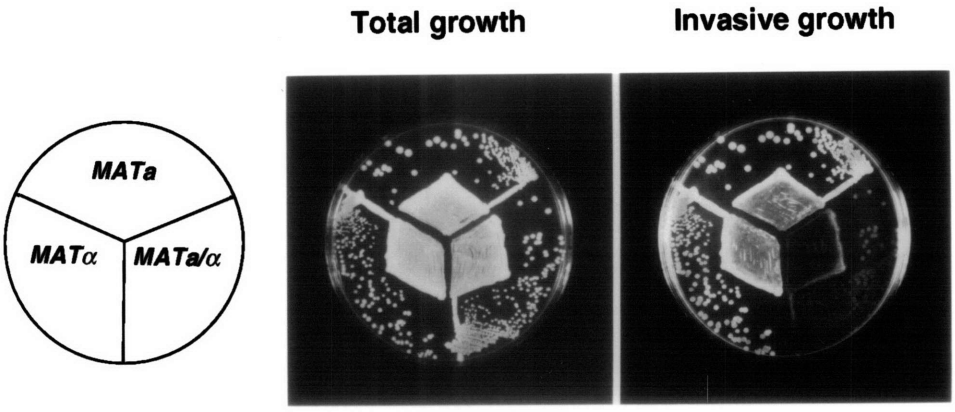

Figure 2.1

Invasive growth of haploid strains.

Haploid *MATα* (L5487) and *MATa* (L5528) strains were patched on YPD with an isogenic diploid MATa/α strain (L5721) and incubated as described in Materials and Methods. The plate was photographed before (total growth), and after (invasive growth) washing the cells off the agar surface.

Table 2.1

Strain	Genotype	Source
L5528	*MATa, ura3-52, his3::hisG*	This study
L5487	*MATα, ura3-52, leu2::hisG*	This study
L5466	*MATa, ste2::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5549	*MATa, ste4::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5579	*MATa, ste18::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5585	*MATa, ste20::TRP1, ura3-52, trp1::hisG*	Liu et al 1994
L5554	*MATa, ste5::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5565	*MATa, ste11::URA3::TRP1, ura3-52, trp1::hisG*	Liu et al 1994
L5559	*MATa, ste7::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5743	*MATa, fus3::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5491	*MATa, kss1::URA3::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5722	*MATa, fus3::LEU2, kss1::URA3::LEU2, ura3-52, leu2::hisG*	This study
L5723	*fus3::LEU2, ste7::LEU2, ura3-52, leu2::hisG*	This study
L5724	*fus3::LEU2, ste12::LEU2, ura3-52, leu2::hisG*	This study
L5573	*MATa, ste12::LEU2, ura3-52, leu2::hisG*	Liu et al 1994
L5742	*MATa, far1::URA3, ura3-52, his3::hisG*	This study
L5721	*MATa/α, ura3-52/ura3-52, his3::hisG/HIS3, leu2::hisG/LEU2*	This study
L5729	*MATa/α, bud1::URA3/bud1::URA3 ura3-52/ura3-52, his3::hisG/HIS3, leu2::hisG/LEU2*	This study
L5741	*MATa/α, ste12::LEU2/STE12, leu2::hisG/leu2::hisG*	This study
L5726	*MATa, bud1::URA3, ura3-52, his3::hisG*	This study
L5727	*MATa, bud2::LEU2, ura3-52, leu2::hisG*	This study
L5725	*MATa, bud3::URA3, ura3-52, his3::hisG*	This study
F1432	*MATa, bud4, ura3-52*	Gimeno and Fink 1994
L5740	*MATa, bud5::URA3, ura3-52, his3::hisG*	This study
L5728	*MATa, ura3-52, his3::hisG, ura3::FUS1-lacZ-URA3*	This study

Table 2.1

Strains used in this study.

All strains are congenic to the Σ1278b genetic background (Grenson et al., 1966; Liu et al., 1993).

Figure 2.2

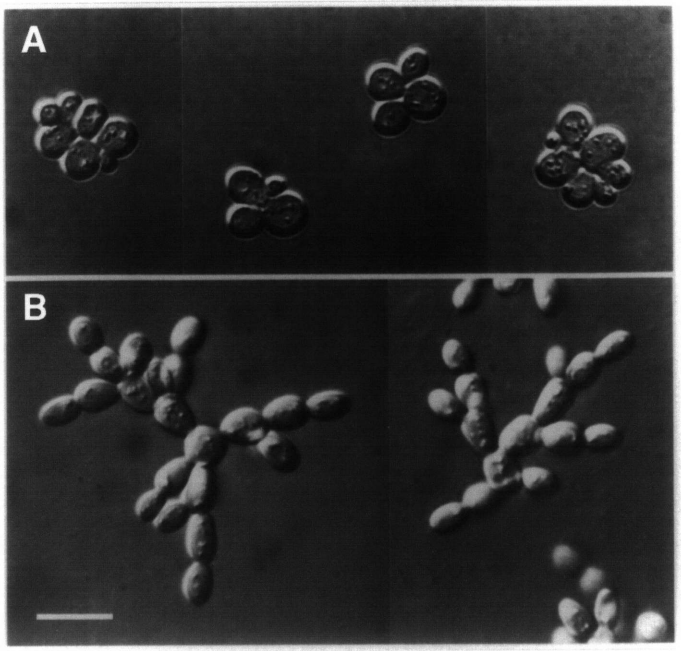

Figure 2.2

Filament formation during haploid invasive growth.

The haploid *MAT**a*** strain L5528 was photographed during exponential growth in YPD liquid (Panel A), and during invasive growth in a YPD plate (Panel B). The scale bar represents 10 microns.

The haploid cells remaining in the agar after washing the plate form networks of filaments underneath each colony (Figure 2.2). The cells of these haploid filaments are not as elongated as those observed in diploid filaments, though some may have a slightly greater length to width ratio than they do during exponential growth (1.43 +/- 0.02 as compared with 1.07 +/- 0.01) (mean +/- standard error of the mean). Even after prolonged incubations these haploid filaments do not extend beyond the perimeter of the colony, but are restricted to the agar immediately beneath it. Thus, we define the combination of filament formation and agar penetration that occurs underneath each colony on rich medium as haploid invasive growth.

Bud site switching during haploid invasive growth

Haploid filament formation is unexpected because haploid cells bud in the axial mode, a pattern inconsistent with filament formation. Even if the cells fail to separate after cytokinesis, reiteration of this budding pattern produces clumps of cells, not the sequential extensions required for filament formation (Figure 2.2). To determine the growth pattern of our strains, we compared the budding pattern of cells during exponential and invasive growth by staining their bud scars with calcofluor, a fluorescent compound that binds the chitin ring at the mother-daughter junction.

Cells were divided into three classes based on the distribution of their bud scars: axial, bipolar, and anomalous (see Materials and Methods). Haploid cells in exponential phase either on plates or in liquid bud in a very strict axial mode, where all the bud scars are clustered together at one end of the cell (Figure 2.3, Table 2.2). Even after extended growth periods in liquid medium, these cells continue to bud in an axial mode (Table 2.3). Remarkably, invasive haploid cells from washed plates

exhibit a bipolar pattern of bud scar distribution, with scars clustered at both ends of the cell. The frequency and appearance of these bipolar haploids is comparable to that of diploids in the yeast form (Figure 2.3, Table 2.2). Cells in the population that do not manifest the bipolar pattern could be either cells in the axial mode that have not switched, or cells that have switched but are in the unipolar pattern rather than the bipolar pattern (Kron et al., 1994). Because the distribution of bud scars is the same for cells dividing in the axial and unipolar patterns, these two modes could not be distinguished by this method.

The finding that haploid cells can switch from axial to bipolar budding on plates suggested that this switch is critical for filament formation. To determine directly whether the cells in haploid filaments have the diploid cell division motif, we recorded the growth of filaments in the plate by time lapse photography. In a high proportion of the cells in haploid filaments (52/70), the first bud of a virgin mother emerges from the free end of the cell. This pattern, typical of bipolar budding in diploid strains, permits the formation of filaments through a series of polarized cell divisions. The bipolar pattern contrasts with that of haploids in exponential growth, where all new buds emerge from the birth end of the cell (78/78). Thus, haploids grown on plates can switch their budding pattern from an axial to a bipolar mode and form filaments. This switch demonstrates that bud site selection is not a fixed parameter determined by cell type, but rather a dynamic process sensitive to environmental influences.

Figure 2.3

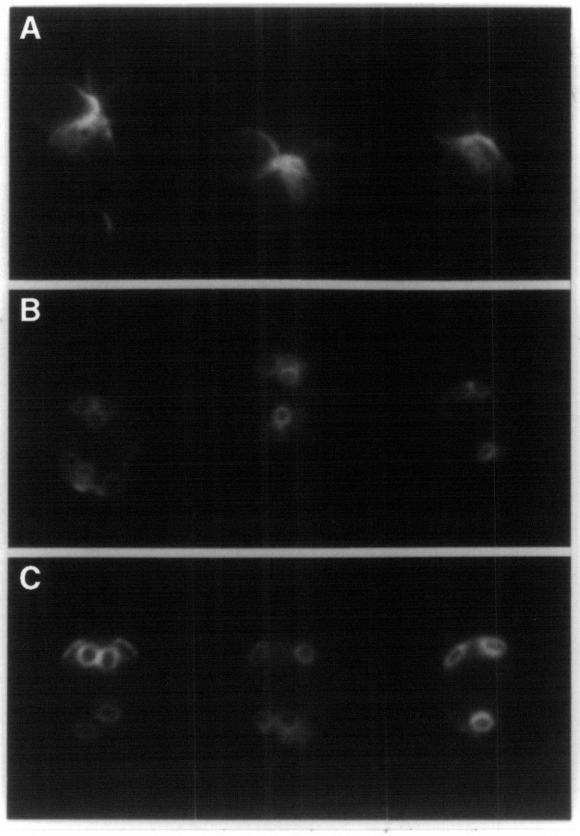

Figure 2.3

Bud scar distribution.

Bud scars were stained and visualized for haploid *MAT**a*** strain L5528 during exponential growth (Panel A), and invasive growth (Panel B), and for the isogenic diploid *MAT**a**/α* strain L5721 during exponential growth (Panel C).

Table 2.2

	Bud scar distribution			
Sample	**axial**	**bipolar**	**anomalous**	**n**
***MATa* exponential**	**98**	**1**	**1**	**500**
***MATa* below agar**	**30**	**62**	**8**	**800**
***MATa/α* exponential**	**28**	**66**	**6**	**200**

Table 2.2

Haploid bipolar budding.

The distribution of bud scars was scored for the haploid strain L5528 during exponential and invasive growth on plates, and for the diploid strain L5721 during exponential growth in liquid. Numbers represent the percent of cells in each class (see Materials and Methods) and the sample size (n).

Table 2.3

Bud scar distribution

Sample	axial	bipolar	anomalous	n
MATa exponential	98	1.0	1.0	400
MATa 2 days	80	15	4.3	391
MATa 20 days	85	8.0	7.0	100

Table 2.3

Haploid cells do not switch budding patterns in liquid.

The distribution of bud scars was scored for the haploid strain L5528 after growth in YPD liquid for various amounts of time. Numbers represent the percent of cells in each class (see Materials and Methods) and the sample size (n).

Genes that regulate haploid bud site switching.

The bud site selection pattern of invasive haploid cells is remarkably similar to that of diploid MATa/α cells. The bipolar cells are not diploids because, when excised from the agar, they give rise to cells capable of mating with cells of the appropriate mating type. Because the mating type loci control bud site selection patterns, we wished to determine whether the bipolar budding of haploid cells during invasive growth could result from de-repression of the silent mating type cassettes at HMLα and HMRa. To test this we introduced a disruption of the HML locus (*hml::LEU2*) and examined the budding pattern and invasive behavior of *MAT*a and *MAT*α *hmlα::LEU2* strains. These mutant haploid strains were indestinguishable from their wild-type counterparts in their ability to invade agar, form filaments, and switch their pattern of bud site selection (Table 2.4). These data suggest that haploid bipolar budding is not caused by modulations of the mating type loci.

The products of five *BUD* genes affect the position of bud site selection. *BUD3* and *BUD4* are required for the axial pattern of bud site selection in haploids; both *bud3* and *bud4* haploid strains exhibit bipolar budding (Table 2.4). Our analysis of the cells beneath the colony shows that both *bud3* and *bud4* haploids form filaments similar to those formed by Bud$^+$ strains (data not shown).

Three gene products, *BUD1*, *BUD2*, and *BUD5*, are required for both the axial and bipolar budding patterns. Haploid *bud1*, *bud2*, and *bud5* mutant strains exhibit a random distribution of bud emergence sites on the cell surface. Unlike isogenic Bud$^+$ strains, these mutants do not switch to the bipolar budding pattern (Table 2.4), or form filaments in our plate assay (data not shown).

Table 2.4

Bud scar distribution

Sample	Exponential growth			Invasive growth		
	axial	bipolar	anomalous	axial	bipolar	anomalous
Bud+	98	1.5	0.0	26	66	8.0
bud1	14	14	72	9.0	20	72
bud2	12	13	75	2.0	21	77
bud3	21	64	15	26	59	15
bud4	23	65	12	20	65	15
hmlα MATa	-	-	-	24	67	9.0
hmlα MATα	-	-	-	30	65	5.0

Table 2.4

The budding pattern switch requires BUD genes and not HMLα.

The distribution of bud scars was scored for haploid strains (*Bud+* L5528), (*hmlα MATα* RRY990), (*hmla MATa* RRY991), (*bud1* L5726), (*bud2* L5727), (*bud3* L5725), and (*bud4* F1432) during exponential growth in liquid and invasive growth on plates. Numbers represent the percent of cells in each class (see Materials and Methods) for a sample size of n = 200.

Figure 2.4

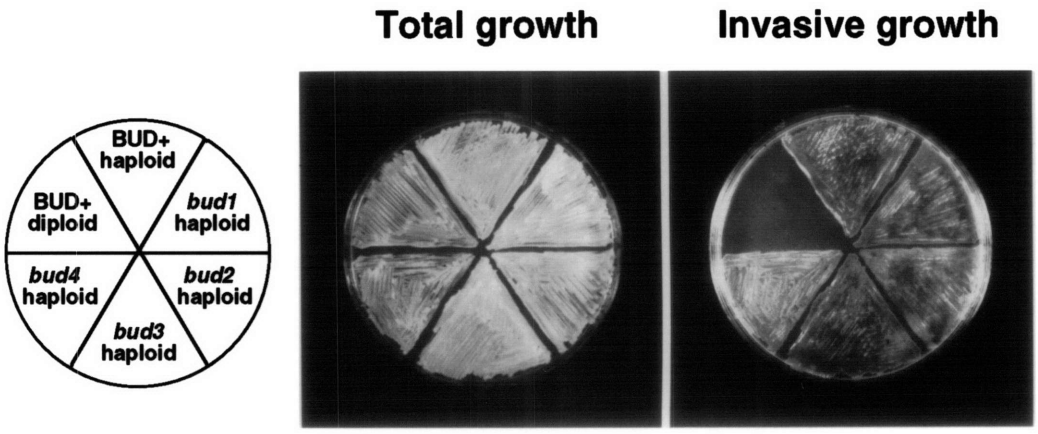

Figure 2.4

Haploid invasive growth and bud site selection.

Haploid strains (*Bud+* L5528), (*bud1* L5726), (*bud2* L5727), (*bud3* L5725), (*bud4* F1432), and the diploid (*Bud+/Bud+* L5721) were patched on a YPD plate and incubated as described in Materials and Methods. The plate was photographed before (total growth) and after (invasive growth) washing the cells off the agar surface.

Agar penetration does not require BUD gene functions

Haploid *bud1*, *bud2*, and *bud5* mutants fail to form filaments on rich medium, but they penetrate agar as well as Bud$^+$ strains (Figure 2.4). Therefore, a polar mode of bud site selection is not necessary for agar penetration, although it is necessary for filament formation. To test whether *BUD* genes are also required for diploid filamentous growth, we constructed diploid strains homozygous for a deletion of *BUD1*. These strains bud randomly and fail to form filaments on plates (SLAD) that induce pseudohyphal growth, but they form normal proportions of long cells, which penetrate the agar below each colony (data not shown). On rich medium used to observe haploid invasive growth (YPD), diploid Bud$^+$ strains do not efficiently penetrate agar (Figure 2.1, Figure 2.4), even though they exhibit the bipolar budding pattern necessary for filamentous growth. Taken together these data suggest that filament formation and agar penetration are distinct functions in both haploids and diploids. Thus, a polar mode of bud site selection is neither necessary nor sufficient for agar penetration; other factors must regulate this behavior.

Elements of the pheromone response pathway are required for haploid invasive growth

Since mutations in the mating signal transduction pathway block both the filament formation and agar penetration of diploid strains, we reasoned that this pathway might control these behaviors in haploids as well. Haploid strains carrying disruptions of various pheromone response pathway genes were tested for their ability to penetrate agar. Strikingly, the same components of the MAPK cascade necessary for diploid pseudohyphal growth are required for haploid invasive

growth. As in diploids, the pheromone receptor (*STE2*), and the G protein (*STE4*, *STE18*) are not required for haploid agar penetration. Similarly, the scaffold protein (*STE5*) that nucleates assembly of the signaling kinases is not required, nor is the cyclin-dependent kinase inhibitor (*FAR1*) (Figure 2.5 and data not shown). In contrast, other components of the MAPK cascade, including *STE20*, *STE11* (MEKK), *STE7* (MEK), and the transcription factor *STE12*, are essential for agar penetration (Figure 2.5). As in diploids (Liu et al., 1993), the *ste20* mutant shows the strongest phenotype of all the mutants in this pathway. In each case the defect in invasive growth of these strains is completely linked to the *ste* deletion (Figure 2.6), and agar penetration can be restored by introduction of the appropriate *STE* gene. It is difficult to assess whether the non-invasive *ste* mutants are completely defective in filament formation because our assay requires examining the cells remaining in the agar. Nevertheless, the few cells that do remain after washing the plate do not appear to form filaments. Therefore, we conclude that these mutant strains are defective in agar penetration and probably also filament formation.

A MAPK is required for haploid invasive growth

Haploid strains containing deletions of the Fus3p and Kss1p MAPK homologs were tested for their ability to penetrate agar and form filaments. These genes are partially redundant for an essential mating function (Elion et al., 1991; Elion et al., 1991; Liu et al., 1993), but are not required for filament formation in diploids (Liu et al., 1993), Haploid *kss1* strains show greatly reduced agar penetration (Figure 2.7), demonstrating that Kss1p promotes haploid invasive growth. In contrast, *fus3* strains penetrate agar more vigorously than *FUS3* strains, suggesting that Fus3p inhibits haploid invasive growth (Figure 2.7). Surprisingly, the double mutant *fus3 kss1* penetrates agar as well as the wild-type *FUS3 KSS1* strain. Since the *fus3 kss1*

double mutant penetrates agar better than the *kss1* single mutant, we hypothesized that there might be a novel MAPK, partially redundant with Kss1p, which is inhibited by the presence of a functional *FUS3* gene. To test whether the enhanced invasiveness of the *fus3* mutant strain was dependent upon a functional signal transduction pathway, we analyzed *fus3 ste7* and *fus3 ste12* mutants. These double mutant strains have the non-invasive phenotype of the *ste7* and *ste12* single mutant strains (Figure 2.7). We tested deletions of the MAPK homologs *HOG1* and *MPK1* and found that these kinases do not play a role in haploid invasive growth (data not shown). Taken together, these data support a positive role for Kss1p in haploid invasive growth, and raise the possibility of a heretofore unidentified MAPK.

STE pathway components are not required for bud site switching

To determine whether the pheromone response MAPK cascade controls the bud site switching essential for haploid filament formation, we examined the pattern of bud site selection in *ste* strains. Since certain *ste* mutants penetrate agar poorly, we analyzed the budding pattern of cells collected from colonies on the agar surface. All the *ste* mutants in the pheromone response pathway still switch their budding pattern from an axial to a bipolar mode when grown for several days on YPD plates (Table 2.5). Although the proportion of cells that switch from axial to bipolar budding is not as great for the cells on the agar surface as for those below the surface (compare Table 2.2 and Table 2.5), it is clear that the *ste* mutants retain the capacity to switch to the bipolar pattern.

Figure 2.5

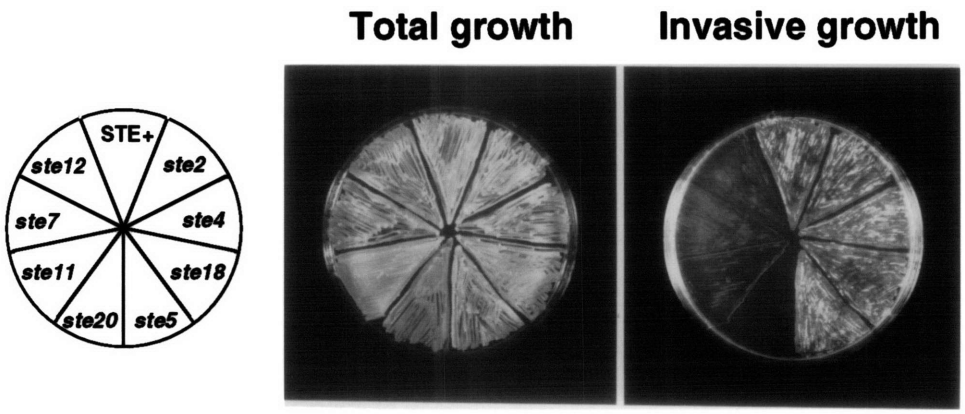

Figure 2.5

Mating MAPK cascade components are required for agar penetration.

Haploid *MAT**a*** strains (*STE*+ L5528), (*ste2* L5466) (*ste4* L5549), (*ste18* L5579), (*ste5* L5554), (*ste20* L5585), (*ste11* L5565), (*ste7* L5559), (*ste12* L5573), were patched on a YPD plate and incubated as described in Materials and Methods. The plate was photographed before (total growth) and after (invasive growth) washing the cells off the agar surface.

Figure 2.6

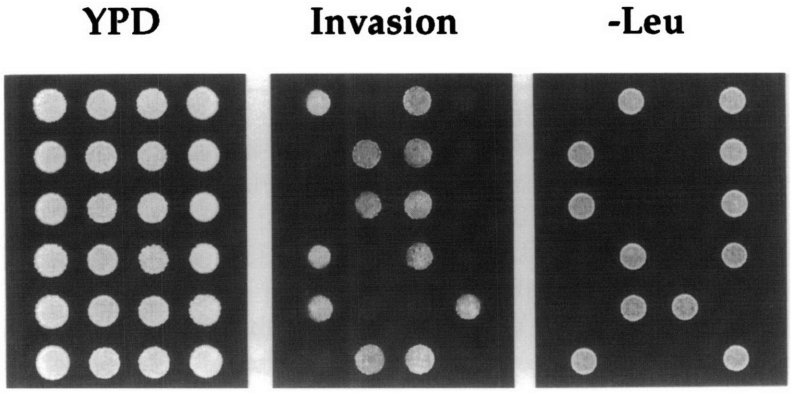

Figure 2.6

Invasive growth is linked to *STE12*.

The diploid strain *(ste12::LEU2/STE12, leu2/leu2* L5741) was sporulated and subjected to tetrad analysis. Spores were assayed for total growth (YPD), agar penetration (Invasion), and the ability to grow on medium lacking leucine (-Leu).

Figure 2.7

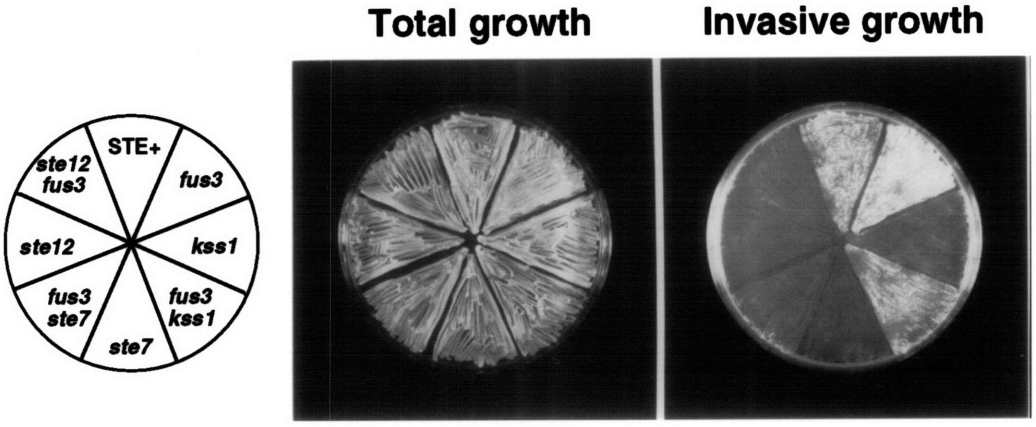

Figure 2.7

The role of MAPKs in haploid agar invasion

Haploid strains (*STE+* L5528), (*fus3* L5479), (*kss1* L5491), (*fus3, kss1* L5722), (*ste7* L5559), (*fus3, ste7* L5723), (*ste12* L5573), and (*fus3, ste12* L5724) were patched on a YPD plate and incubated as described in Materials and Methods. The plate was photographed before (total growth) and after (invasive growth) washing the cells off the agar surface.

Table 2.5

Bud scar distribution

Sample	axial	bipolar	anomalous
STE+	53	42	5
ste2	45	46	9
ste4	43	50	7
ste18	45	47	8
ste20	46	48	6
ste5	43	53	4
ste11	59	37	4
ste7	51	43	6
fus3	46	50	4
kss1	50	42	8
fus3, kss1	46	48	6
ste12	50	42	8

Table 2.5

The budding pattern switch does not require STE genes.

The distribution of bud scars was scored for haploid *MATa* strains (*Ste+* L5528), (*ste2* L5466) (*ste4* L5549), (*ste18* L5579), (*ste20* L5585), (*ste5* L5554), (*ste11* L5565), (*ste7* L5559), (*fus3* L5479), (*kss1* L5491), (*fus3, kss1* L5722), and (*ste12* L5573) after five days of growth on a YPD plate. Numbers represent the percent of cells in each class (see Materials and Methods) for a sample size of n = 200.

Table 2.6

Mating transcription induction

Sample	β-galactosidase activity
*MAT*a exponential growth + αF	250
*MAT*a exponential growth	2.2
*MAT*a on agar surface	0.40
*MAT*a beneath agar surface	0.39

Table 2.6

FUS1-lacZ is not induced during haploid invasive growth.

β-galactosidase activity was assayed in cells of the haploid *MAT**a*** strain L5728 during exponential growth in YPD liquid in the presence and absence of mating pheromone, and after five days of growth on a YPD plate. Numbers represent β-galactosidase activity normalized to total protein (see Materials and Methods).

Mating-specific transcription is not induced during haploid invasive growth

Since haploid cells express all of the genes required for induction of pheromone responsive genes, it is possible that mating specific genes such as *FUS1* are activated in the cells under the colony during the induction of haploid invasive growth. To test this possibility, we compared the expression levels of a *FUS1-lacZ* construct in haploid cells that had penetrated the agar or were cultured in the presence of mating pheromone. This reporter provides a sensitive assessment of the transcriptional induction of mating specific genes. Transcription of *FUS1-lacZ* is induced a hundred fold during the pheromone response, and this induction is mediated by the transcription factor Ste12p (Dolan and Fields, 1990; Hagen et al., 1991). These assays show that there is no induction of *FUS1* in cells above or below the agar in the absence of mating pheromone (Table 2.6). Thus, the signaling cascade and the transcription factor Ste12p presumably induce a set of genes specific to haploid invasive growth that is distinct from pheromone-inducible genes.

DISCUSSION

A comparison of haploid invasive growth and diploid pseudohyphal growth

Haploid invasive growth is a developmental program with many similarities to diploid pseudohyphal growth. First, in both haploids and diploids, filament formation and agar penetration appear to be induced by nutrient limitation. Second, both cell types form filaments through a series of polarized cell divisions that depend on a polar pattern of bud site selection. Furthermore, filament formation is distinct from agar penetration in both cell types, as both haploid and diploid *bud* strains are unable to form filaments, but still penetrate agar. Perhaps

the most striking similarity is that elements of the pheromone response MAPK signal transduction pathway, including *STE20, STE11, STE7* and *STE12*, are required for triggering these developmental events in both haploids and diploids.

However, there are differences between haploid invasive growth and diploid pseudohyphal growth. Diploids form filaments readily on low ammonia medium, but poorly on rich medium. In contrast, haploids do not appear to form filaments on low ammonia medium, but do form them on rich medium. Moreover, diploid filaments extend beyond the perimeter of the colony, whereas haploid filaments are only observed beneath the colony. In addition, cells in diploid pseudohyphal filaments are more elongated than those in haploid filaments. Despite these differences, haploid invasive growth and diploid pseudohyphal growth share several common motifs and require many of the same genes, suggesting that they are different manifestations of a common developmental pathway.

Haploid filamentous growth requires bud site switching

Bud site selection is not a fixed parameter determined by cell type, but rather a dynamic process sensitive to environmental influences. Haploid cells switch from an axial to a bipolar mode of bud site selection when grown on rich medium for an extended period. In the bipolar mode, the first bud emerges on the free end of the cell, but subsequent buds can emerge from either end. Diploid cells switch from a bipolar to a unipolar budding pattern when starved for nitrogen (Kron et al., 1994). In the unipolar budding pattern, the first bud and all subsequent buds emerge from the free end of the cell. Thus, upon nutrient deprivation, both diploids and haploids switch their pattern of bud site selection and form filaments.

The polar budding pattern provides the architectural motif for the growth and extention of a filament. In polar budding the first bud of each virgin mother

emerges from the free end of the cell, away from her mother. The switch in diploids from bipolar to unipolar budding ensures that all new growth projects in filaments away from the colony. The switch in haploids from axial to bipolar budding is also consistent with filament formation. Both the bipolar chain and the unipolar chain are composed of a lineage of buds born from the free end of each new cell in the chain. However, in the bipolar pattern the emergence of subsequent buds on the birth end of cells can lead to secondary fronds projecting back towards the cell that originated the chain. This difference may explain why the haploid filaments we observe are not as extensive or ordered as pseudohyphal filaments. Mutations in *BUD1*, *BUD2*, and *BUD5* disrupt the polar budding pattern and consequently block filament formation. Thus, reiteration of the polar budding pattern is a critical element of filament formation. Although the pathway controlling haploid bud site switching is not well understood, our data suggest it does not require modulation of the mating type loci.

Filament formation is distinct from agar penetration

Filament formation can be genetically dissected from agar penetration and cell elongation. Haploid *bud1*, *bud2*, and *bud5* mutants penetrate agar as efficiently as Bud$^+$ strains, even though these mutants do not form ordered filaments. Similarly, diploid *bud1/bud1* mutants penetrate agar and form clusters of long cells on medium which induces pseudohyphal growth, though they too fail to form filaments. Thus, the pattern of bud site selection determines the ability of a strain to form ordered filaments, but does not affect agar penetration or cell elongation. Mutations in the *STE20*, *STE11*, *STE7*, and *STE12* genes block agar penetration and filamentous growth in both haploids and diploids, but do not affect bud site switching in haploids. Filament formation and agar penetration depend on

signaling through elements of this MAPK cascade, but each process is ultimately controlled by distinct downstream morphological pathways.

MAPK cascade components control haploid invasive growth

We report here that three protein kinases of the mating pheromone response MAPK cascade, Ste20p, Ste11p (MEKK), and Ste7p (MEK), and the transcription factor Ste12p, are required for haploid invasive growth. These same components are required for pseudohyphal growth in diploids (Liu et al., 1993). The mating pheromone receptors and the G protein, which transmit the primary signal in the pheromone response, are not required for either haploid invasive growth or diploid pseudohyphal growth. It is significant that the pheromone receptors and G protein are not required for haploid invasive growth, because the genes encoding these proteins are transcribed and the proteins are functional in haploids, whereas they are not in diploids (Liu et al., 1993; Whiteway et al., 1989). The mating pheromone response MAPK signal transduction pathway may be key to filamentous growth in all fungi. In the human pathogen *Candida albicans*, the *STE12* homolog *CPH1* plays a role in hyphal formation (Liu et al., 1993). In *Ustilago maydis*, the *STE7* homolog *Fuz7* is required for mating functions, filamentous growth, and pathogenesis (Banuett and Herskowitz, 1994). Unlike *Saccharomyces*, in *Ustilago* the pheromones and their receptors, encoded by the *a* locus, also play a role in filamentous growth (Bolker et al., 1992).

The role of MAPKs in haploid invasive growth

The *FUS3* and *KSS1* MAPKs are partially redundant for an essential function in the haploid pheromone response (Elion et al., 1991; Elion et al., 1991). The single

mutants are fertile, but the *fus3 kss1* double mutant is completely sterile. The *FUS3 kss1* single mutant is defective in agar penetration and filament formation, whereas the *fus3 KSS1* mutant is enhanced. The role of the *FUS3* and *KSS1* genes in haploid invasive growth is unexpected because these genes are not required for diploid pseudohyphal growth. Formally, Kss1p plays a positive role in haploid invasive growth and Fus3p plays a negative role. Epistasis analysis shows that the enhanced agar penetration of the *fus3* mutant depends upon the function of two other components of this pathway, *STE7* and *STE12*.

These data raise the possibility that Kss1p may be partially redundant with a novel MAPK in the invasive growth signaling pathway; either can activate Ste12p so that it promotes invasive growth. The presence of Fus3p might interfere with the function of the postulated MAPK, thereby reducing the invasive growth signal. This model explains the strength of the haploid invasion phenotypes that we observe: strongest in *fus3 KSS1*, intermediate in *FUS3 KSS1* and *fus3 kss1*, and weakest in *FUS3 kss1*. The postulated MAPK is unlikely to be either *MPK1* or *HOG1*, two other MAPKs present in yeast, because deletions of these genes do not block haploid invasive growth. Alternatively, there may not be a third MAPK involved in haploid invasive growth. Instead, Kss1p could promote, and Fus3p antagonize, the Ste7p dependent Ste12p activation required for haploid invasive growth.

Elements of a single MAPK cascade control two developmental events in the same cell type

Our finding that components of the pheromone response signal transduction pathway are required for both mating, and haploid invasive growth demonstrates that a single MAPK cascade can activate two different developmental outcomes in a

haploid cell. The target of this cascade, the transcription factor Ste12p, is required for both processes, presumably to regulate the expression of genes specific to each developmental program. Since mating specific genes are not detectably induced during haploid invasive growth, Ste12p must have at least two functional states: one that controls the transcription of mating specific genes, and another that controls the transcription of genes specific to haploid invasive growth. Each Ste12p state might have a distinct phosphorylation pattern which determines DNA binding specificity, association with other transcription factors, or both.

How do haploid cells distinguish between two different stimuli that activate the same signal transduction pathway and initiate the developmental program specific to each primary signal? In one model, accessory proteins are activated that direct the association of common components of the pathway with their appropriate targets (Figure 2.8). For example, in the response to mating pheromone, Ste5p assembles Ste11p, Ste7p, and Fus3p into a protein complex which modifies Ste12p, shifting it to a state that activates the transcription of mating specific genes (Choi et al., 1994; Kranz et al., 1994; Marcus et al., 1994). According to this model, upon induction of the haploid invasive growth pathway, a scaffolding protein analogous to Ste5p mediates the assembly of Stellp and Ste7p with a MAPK distinct from Fus3p. The protein complex modifies Ste12p, shifting it to a state that activates the transcription of genes specific to haploid invasive growth (Figure 2.8). This explains why the pheromone receptors, G protein, *FUS3* and *STE5* are not necessary for haploid invasive growth, whereas *STE20*, *STE7*, *STE11* and *STE12* are essential. Other models that do not require a MAPK are consistent with these data.

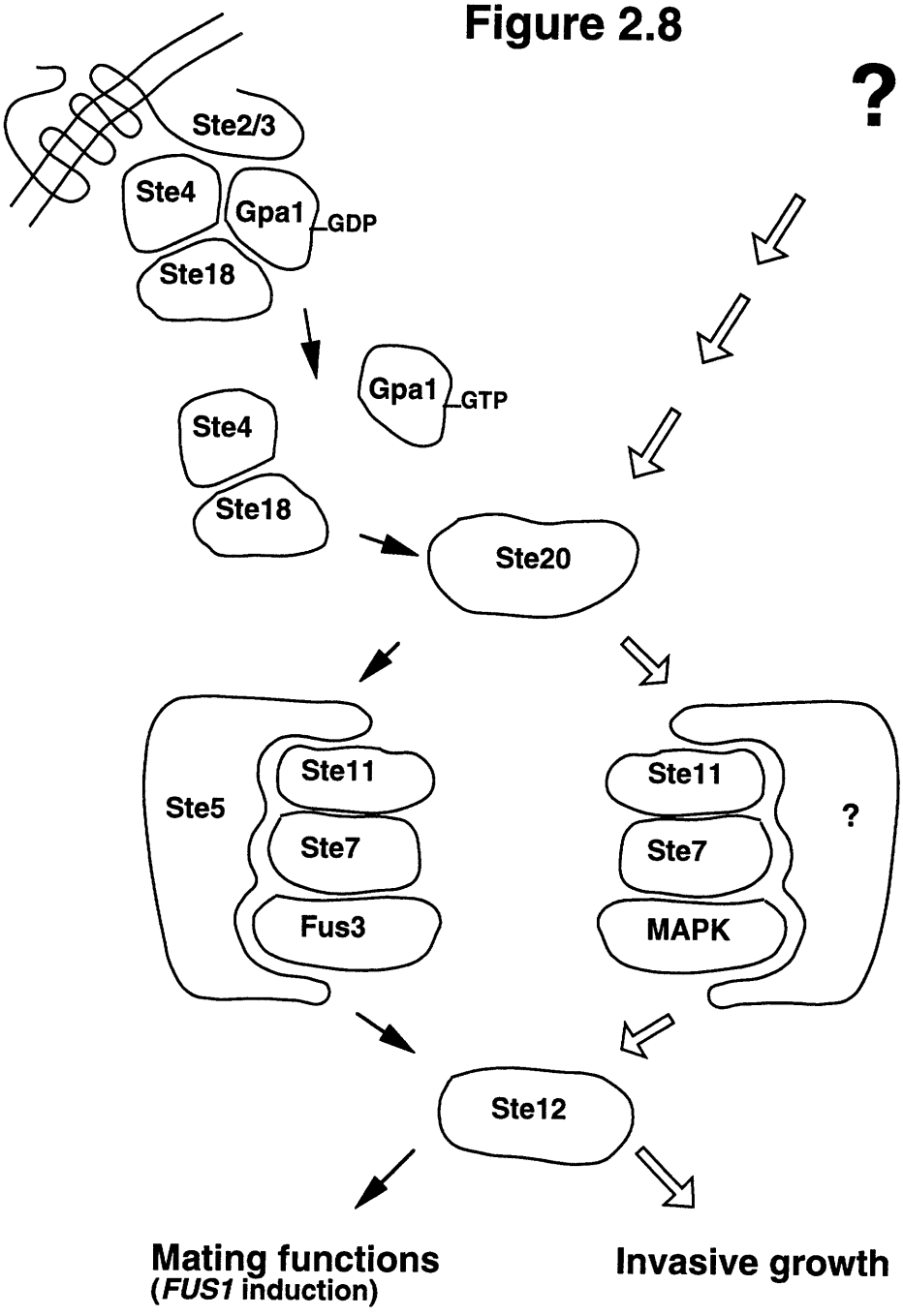

Figure 2.8 A bifunctional MAPK cascade.

A paradigm for generating diversity from a signal transduction pathway

Our observation that elements of a single MAPK cascade control two distinct developmental sequences could emerge as a general theme. MAPK cascades have been implicated in development and differentiation in many different organisms. In mammalian cells, a wide array of growth factors and cytokines can activate a MAP/ERK kinase cascade which stimulates proliferation or differentiation (Blumer and Johnson, 1994). In *Drosophila melanogaster*, a dominant activated allele of a single MAPK gene disrupts the development of several tissues (Brunner et al., 1994), though in this case each of these processes occurs in distinct cell types at different times during development.

There are precedents for two distinct signals acting through a single MAPK cascade in the same cell type. For example, in cardiac myocytes both endothelin (ET-1) and acidic fibroblast growth factor (aFGF) activate a MAPK, and induce hypertrophy in these cells (Bogoyevitch et al., 1994). ET-1 acts through G protein-coupled serpentine receptors and phopholipase Cβ, while aFGF activation is mediated by a family of tyrosine kinase receptors. These two agonists act through disparate primary signaling events, but both pathways converge on the MAPK to mediate the same physiologic response. Perhaps the closest parallel to the dual functionality of the MAPK cascade in *S cerevisiae* is the behavior of PC12 cells in response to epidermal growth factor (EGF) and nerve growth factor (NGF) (Traverse et al., 1992). EGF induces transient ERK/MAPK activation and cell division in these cells. In contrast, NGF induces prolonged activation and nuclear translocation of ERK/MAPK, causing the same cells to arrest growth and initiate neuronal differentiation. Identification of the primary signals and the putative novel MAPK required for yeast filamentous growth should help to elucidate the mechanism for achieving diversity from a single cascade.

Materials and Methods

Strains and growth conditions

All yeast strains used in this study are described in Table 2.1, and are congenic to the Σ1278b genetic background (Grenson et al., 1966; Liu et al., 1993). Standard yeast culture medium was prepared essentially as described (Sherman et al., 1986). YPD plates (2% yeast extract (DIFCO), 4% Bacto peptone (DIFCO), 2% glucose, 2% Bacto-agar (DIFCO), and 0.03% L-tryptophan) were allowed to cool, polymerize, and dry three days at room temperature before use. Low ammonia medium (SLAD) for scoring pseudohyphal growth was prepared as described (Gimeno et al., 1992). Strains scored for pseudohyphal filament formation were streaked on SLAD plates and observed after 24, 48 and 72 hours of growth at 30°C. Long cells which had penetrated the agar were examined after washing the cells off the agar surface with deionized water.

Cells in exponential growth phase in liquid were prepared by incubating cells in YPD medium on a roller drum at 30°C until the culture reached OD_{600} 0.6. Cells in exponential growth phase on plates were prepared by plating 3 mls of cells at OD_{600} 0.6 (see above) on a YPD plate, and incubating them an additional eight to ten hours at 30°C.

Invasive growth assay

Strains were patched on YPD plates with a toothpick, being careful to avoid scratching the agar surface. Patches were allowed to grow at 30°C for three days, and incubated at room temperature for an additional two days. For tetrad analysis,

haploid cells were transferred from liquid cultures in a microtiter dish to a YPD plate. Since these samples started at a higher cell density than the patches previously described, invasive growth was scored after two days of growth at 30°C. Samples were photographed with a 35mm camera using Technical Pan (Kodak) film, and a gentle stream of deionized water was then used to rinse all the cells from the agar surface. The agar was never directly rubbed or otherwise disrupted. Microscopic analysis revealed that the cells remaining after washing were below the focal plane of the agar surface and inaccessible to a microdissection needle unless the needle pierced the agar. Plates were allowed to dry briefly, and then photographed again as described above. Cells that had penetrated the agar were excised with a toothpick for subsequent manipulations.

Filament formation was scored after two days of invasive growth at 30°C because the number of cells in the agar after five days was too high to distinguish individual filaments. Cells remaining in the agar after washing the plate were overlayed with a coverslip and scored by direct microscopic examination for the presence of filaments. For the purpose of this study, a filament is defined as a chain of cells attached to one another which project in an ordered pattern away from the first cell of the chain.

Photomicroscopy

Preparations of cells in exponential growth were prepared as described above, placed on a slide with a coverslip, and compressed gently to ensure that all the cells in each clump were in the same focal plane. Cells undergoing invasive growth were cultured as described above and prepared for observation by excising the washed agar from the plate and placing it between two coverslips. Samples were viewed with Nomarski optics on a Zeiss Axioscop, and photographed with a 35mm

camera using Technical Pan (Kodak) film. To determine the axial ratio of cells, we prepared samples and photographed them as described above. Images were printed onto photographic paper and directly measured. The length was measured along the longest axis of each cell, and the width was measured at the midpoint of the longest axis (Gimeno et al., 1992). The mean length/width ratios are presented for each sample along with the standard error of the mean.

Bud scar staining

Invasive cells were prepared as described above and excised from washed plates with a toothpick. Cells in exponential growth phase were prepared as described above. Samples were forced through a 24 guage needle ten times to separate cells as much as possible, and rinsed in water once before fixation. Cell suspensions were fixed at room temperature for two hours in 3.7% formaldehyde. Samples were rinsed twice in water, and resuspended in 200 µl of a fresh stock of 1 mg/ml calcofluor white (Fluorescent Brightener #28 F6259 Sigma) in water. Samples were stained at room temperature in the dark for ten minutes, and washed three times in water before observation.

Bud scars were visualized by fluorescence microscopy on a Zeiss Axioscop, and photographed with a 35mm camera using TMAX 400 (Kodak). Cells with two to ten clear bud scars were divided into three bud scar distribution classes: Axial, cells with all bud scars at one end of the cell immediately adjacent to one another; bipolar, cells with two or more bud scars with at least one scar at each end of the cell (the birth end and the free end); anomalous, cells with bud scar distributions other than axial or bipolar. Numbers in the tables represent the percent of cells in each class for a sample of n cells.

Time-lapse photomicroscopy

A plate of invading cells was prepared as described above, and a coverslip was placed over the cells remaining in the agar. Each plate was taped down on a Zeiss WL light microscope stage, maintained at 30°C, and photographed at ninety minute intervals over a fourteen hour period with a 35mm camera using Technical Pan film (Kodak). Budding was observed and classified according to the site where the first bud of a virgin mother emerged. None of the cells present on the plate at time zero was scored, as their birth end could not always be determined. Instead, buds which emerged after the first time point and subsequently initiated buds of their own were defined as virgin mothers. The birth end of a virgin mother was defined as the region adjacent to the site where she was attached to her mother, and the free end was defined as the end of the cell opposite the birth end (Freifelder, 1960). The position of bud site emergence of these virgin mothers was determined by direct microscopic observation.

Exponential growth samples were prepared as described above and subjected to time lapse analysis. In these preparations, we find that mother and daughter cells remain attached to one another even after being subjected to high shear forces. This site of attachment defines the birth end of each cell (Freifelder, 1960). However, because the majority of these cells are in large clumps, we could not always identify the first bud of a virgin mother, and simply scored new buds as emerging from the free end or the birth end of the cell.

β-galactosidase assays

β-galactosidase assays were performed essentially as described (Trueheart et al., 1987). Exponential growth phase samples were prepared as described above, and

split into two samples. These samples were spun, resuspended in 5 mls YPD pH4 with or without 5 μM alpha factor, and incubated at 30°C for an additional two hours before being harvested. Cells undergoing invasive growth were prepared as described above. Before washing the plate, cells on the agar surface were gently scraped away and harvested. Separate plates grown under the same conditions were washed as described, and the cells excised from the agar were harvested. All cells were washed with water once before being lysed to remove contaminating protein. β-galactosidase activity was normalized to the total protein in each extract according to the equation: (OD_{420} x 1.7)/(0.0045 x mgs/ml protein x extract volume x time).

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Chapter Three

FG(TyA)-lacZ discriminates between activation of the bifunctional MAPK cascade during mating and filamentous growth

Radclyffe L. Roberts, Hans-Ulrich Mösch, and Gerald R. Fink

Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142.

All of the experiments described in this chapter were designed and executed by RLR. Based on his results with *TEC1*, HUM conceived of using pIL30 as a reporter and he repeated several of the experiments described here. Part of this work has been published: PNAS *93*, 5352-5356 (1996).

Summary

Because both mating and filamentous growth require the same transcription factor *STE12*, we sought a *STE12*-dependent transcriptional reporter that discriminates between the signals that induce mating and filamentous growth. We describe here the transcriptional reporter *FG(TyA)-lacZ*, whose expression correlates extremely well with activity of the filamentous growth MAPK cascade. Full expression of *FG(TyA)-lacZ* requires *STE20*, *STE11*, *STE7*, *TEC1*, and *STE12*. Overexpression of *STE20* or *STE12* or activated alleles of *STE11* (*STE11-4*) or *STE20* (*STE20ΔN*) induce *FG(TyA)-lacZ* expression. Moreover, this reporter is specific to filamentous growth. *FG(TyA)-lacZ* expression does not require the mating specific MAPK cascade components *STE2*, *STE3*, *STE4*, and *STE5*, and its expression is not induced by mating pheromone. Nitrogen starvation, which stimulates filamentous growth, also induces *FG(TyA)-lacZ*. Thus, *FG(TyA)-lacZ* provides an accurate and specific assay for the activity of the filamentous growth MAPK cascade.

Introduction

When starved for nitrogen diploid yeast strains enter a pseudohyphal growth mode, forming filaments of elongated cells that penetrate the agar substrate on which they are grown (Gimeno et al., 1992). Haploid strains grown on rich medium exhibit an invasive growth mode with many similarities to pseudohyphal development, including filament formation and agar penetration (Roberts, 1994). Both haploid invasive growth and diploid pseudohyphal development require elements of a conserved MAPK cascade, including *STE20*, *STE11*, *STE7* and the transcription factor *STE12* (Liu et al., 1993; Roberts, 1994). In addition, activated alleles of *STE11* (*STE11-4*) and *STE20* (*STE20ΔN*) induce filamentous growth. The

effects of *STE20ΔN* are blocked by a mutation that changes the conserved catalytic lysine to an arginine (*STE20ΔNK649R*). Overexpression of *STE20* or *STE12* also induces filamentous growth. Together, these results indicate that this MAPK cascade is important for filamentous growth.

MAPK cascades typically modulate the transcription of one or more target genes. These target genes provide useful reporters to monitor MAPK cascade activity in response to various environmental and genetic manipulations. The ideal reporter has several properties: 1 - It is strongly transcriptionally induced (or repressed) during the process of interest. 2 - Its regulation depends on elements of the pathway of interest. 3 - It is a physiologic target important for the process during which it is induced. *FUS1-lacZ*, the yeast mating MAPK cascade reporter, is the gold standard in this respect. Its expression is induced approximately 100 fold in response to mating pheromones and this induction depends elements of the mating MAPK cascade (Hagen et al., 1991). Moreover, *FUS1* encodes a gene important for cell fusion, one of the final steps leading toward diploid formation (Trueheart et al., 1987). Thus, *FUS1-lacZ* provides an accurate and sensitive reporter for the mating MAPK cascade. *FUS1-lacZ* is not induced by *STE12* during filamentous growth. Therefore we sought a transcriptional reporter for the filamentous growth MAPK cascade.

Elements of the yeast transposon TY1 have been shown to regulate the transcription of closely linked genes (Errede et al., 1980). A small fragment within the TY1 transposon confers transcriptional regulation by the mating type locus (Company et al., 1988). This fragment contains a *STE12* binding site (PRE) and a putative Tec1p binding site (Company et al., 1988; Laloux et al., 1994). Strikingly, transcription of *FG(TyA)-lacZ*, a construct containing this element, depends on *STE11*, *STE7*, *STE12* and *TEC1* (Company et al., 1988; Laloux et al., 1994). Because these genes are required for filamentous growth, we wished to determine whether

this fragment could serve as a reporter for the transcriptional outputs of the filamentous growth MAPK cascade.

Results

FG(TyA)-lacZ expression requires the filamentous growth MAPK cascade.

Expression of the transcriptional reporter *FG(TyA)-lacZ* (previously described as pIL30) correlates extremely well with activity of the filamentous growth MAPK cascade. Previous work had shown that *FG(TyA)-lacZ* responds to *STE11*, *STE7* and *STE12* (Laloux et al., 1994). We find that both haploid and diploid strains containing mutations in *STE20*, *STE11*, *STE7*, and *STE12* show reduced *FG(TyA)-lacZ* expression (Table 3.1). Furthermore, *FG(TyA)-lacZ* is strongly induced by the dominant active *STE11-4* kinase, a truncation allele of *STE20*, and overexpression of *STE20* or *STE12* (Table 3.2). Thus, *FG(TyA)-lacZ* is a sensitive *STE12*-dependent transcriptional reporter that reflects the activity of the filamentous growth kinase cascade.

FG(TyA)-lacZ expression does not depend on the mating MAPK cascade.

Does *FG(TyA)-lacZ* specifically reflect the outputs of the filamentous growth MAPK cascade or is it also responsive to the mating MAPK cascade? To test this, we exposed *MAT**a*** cells carrying *FG(TyA)-lacZ* to mating pheromone under conditions that induce the mating-specific reporter approximately 100 fold. There was essentially no difference in β-galactosidase activity between cells exposed to mating pheromone and untreated control cells (Table 3.1). Moreover, strains carrying disruptions of mating specific components, including *STE2*, *STE4*, *STE18*, *STE5* show levels of *FG(TyA)-lacZ* expression comparable to wild-type strains (Table 3.1).

Furthermore, *FG(TyA)-lacZ* activity in MAPK mutants mirrors the extent of their invasive growth phenotypes, *fus3* > *fus3*, *kss1* > *kss1*, rather than their mating phenotypes (Table 3.1). Thus, *FG(TyA)-lacZ* expression is not responsive to the mating MAPK cascade.

Nutrient availability regulates *FG(TyA)-lacZ* expression.

Do environmental conditions that stimulate filamentous growth induce *FG(TyA)-lacZ* expression? To test this we exposed strains carrying the *FG(TyA)-lacZ* reporter to a variety of different media conditions. Low ammonium medium that promotes pseudohyphal development (SLAD) induces *FG(TyA)-lacZ* (Table 3.1 and Table 3.3). However, prolonged incubation in YPD liquid medium, which does not promote filamentous growth, also induces *FG(TyA)-lacZ* (Table 3.3). Most other media manipulations do not significantly alter *FG(TyA)-lacZ* expression (Table 3.3). Notably, the effects of *STE11-4* and SLAD medium on *FG(TyA)-lacZ* are approximately additive (Table 3.2). Together, these data suggest that the *FG(TyA)-lacZ* reporter is induced by media conditions that promote filamentous growth, but that the filamentous growth MAPK cascade is not the primary conduit for this nutritional regulation.

Table 3.1

β-galactosidase activity

Haploid genotype			Diploid genotype		
STE		101	*STE*		10
STE	+ α-factor	97	*STE*	(Low N)	89
STE	(Low N)	330	*ste20*		2.2
ste2		55	*ste11*		0.75
ste4		88	*ste7*		1.4
ste18		91	*fus3 kss1*		14
ste20		33	*ste12*		0.66
ste5		100			
ste11		10			
ste7		2.5			
fus3		130			
kss1		29			
fus3 kss1		66			
ste12		1.7			

Table 3.1

Expression of *FG(TyA)-lacZ* in haploid and diploid strains.

FG(TyA)-lacZ expression was measured in exponentially growing cells in nitrogen-rich medium (synthetic complete medium), or in cells grown for three days on nitrogen-poor medium (SLAD)(low N). Where indicated (+ α-factor), exponentially growing cells were exposed to 5 μM alpha factor for two hours. β-galactosidase assays were performed with cellular extracts as described. Activities are expressed in nanomoles O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min x mg protein.

Table 3.2

Plasmid	Medium	β-galactosidase activity
None	high N	11
None	low N	89
STE11-4	high N	41
STE11-4	low N	360
STE20 2μ	low N	285
STE20ΔN	low N	302
STE20ΔN K649R	low N	65
Gal-STE12	low N	420

Table 3.2

Induction of *FG(TyA)-lacZ* in *STE+* diploid strains.

FG(TyA)-lacZ expression was measured in exponentially growing diploid cells in nitrogen-rich medium (synthetic complete medium)(high N), or in cells grown for three days on nitrogen-poor medium (SLAD)(low N). Strains containing no insert, *STE11-4* (B2616), *STE20* 2μ (B3422), *Gal-GST-STE20ΔN*, *Gal-GST-STE20ΔN K649R*(B3486), or *Gal-STE12* (B2065) are indicated. β-galactosidase assays were performed with cellular extracts as described. Activities are expressed in nanomoles O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min x mg protein.

Table 3.3

Medium	Liquid/plate	Relative *lacZ* activity STE+ Haploids	STE+ Diploids
-6aa OD_{600} = 0.6	liquid	1.0	1.0
-6aa dex	liquid	1.3	1.2
-6aa gal	liquid	1.2	1.6
-6aa no sugar	liquid	-	1.0
YNB	liquid	1.4	0.8
YPD	liquid	1.6	2.2
SLAD	liquid	2.0	1.5
-6aa dex	plate	2.1	1.4
-6aa dex + aF	plate	2.0	1.3
-6aa gal	plate	2.2	1.8
GNA	plate	2.3	1.9
NGS	plate	0.9	1.0
NGS + NH_4SO_2	plate	1.1	1.2
SLAD	plate	3.1	3.2
48 hrs -6aa dex	liquid	4.1	4.2

Table 3.3

Nutrient regulation of *FG(TyA)-lacZ* in *STE+* strains.

FG(TyA)-lacZ expression was measured in strains RRY1045 (L5978) (*MATa/α*) and RRY3 (L5528)(*MATa*) transformed with *FG(TyA)-lacZ* HIS3 and pRS316 exposed to different media conditions (See materials and methods). β-galactosidase assays were performed as described. A relative lacZ activity of 1.0 corresponds to 100 (10 for diploids) nanomoles O-nitrophenyl-β-D-galacto-pyranoside hydrolyzed/min x mg protein for haploids.

Discussion

FG(TyA)-lacZ expression correlates extremely well with the activity of the filamentous growth MAPK cascade. Specifically, *FG(TyA)-lacZ* levels depend on MAPK cascade components and this reporter is induced by activated alleles or overexpression of MAPK cascade components. Furthermore, *FG(TyA)-lacZ* is not responsive to mating pheromones. Thus, *FG(TyA)-lacZ* provides an accurate and specific reporter for the activity of the filamentous growth MAPK cascade.

Although *FG(TyA)-lacZ* expression reflects the activity of the filamentous growth MAPK cascade, it is important to realize that this is not the only output of the pathway. As yet, no physiologic target of the MAPK cascade has been identified that is critical for filamentous growth. Moreover, the pathway for filamentous growth may not be a single unbranched sequence. Although mutations in *STE11*, *STE7*, and *STE12* completely block *FG(TyA)-lacZ* induction by $RAS2^{VAL19}$, they do not completely block induction of cell elongation, suggesting that *RAS2* has outputs independent of the MAPK cascade and *FG(TyA)-lacZ* important for cell elongation (See Mosch et al., 1996 Figure 1). By comparison, even though *FUS1-lacZ* provides a useful and accurate reporter for the mating MAPK cascade, it is not the only output of the mating pathway and its expression can be uncoupled from some mating functions. For example, mutations in *FAR1*, a cyclin-dependent kinase inhibitor, block the cell-cycle arrest but not *FUS-lacZ* transcription induced by mating pheromone (Chang and Herskowitz, 1990).

The transcription factor *STE12* specifically induces *FUS1-lacZ* during mating and *FG(TyA)-lacZ* during filamentous growth. How is this specificity achieved? The *FUS1* promoter contains four tandem copies of a Ste12p binding site (PRE), and this motif is both necessary and sufficient to confer mating pheromone inducible transcription (Hagen et al., 1991). In contrast, promoters with only a single PRE are

not strongly pheromone inducible (Hagen et al., 1991). Thus, Ste12p-Ste12p cooperativity is critical for the induction of mating specific transcripts.

How does *STE12* regulate *FG(TyA)-lacZ*? The first clue is that *TEC1*, a TEA/ATTS transcription factor, is required for filamentous growth but not mating (Gavrias et al., 1996; Mosch and Fink, 1997). Moreover, *FG(TyA)-lacZ* expression depends on *TEC1*, and this reporter contains a putative Tec1p binding site adjacent to a PRE (Laloux et al., 1994). Recent studies show that Ste12p and Tec1p bind cooperatively to *FG(TyA)-lacZ* (Madhani, 1997). This implies that Ste12p-Tec1p complexes induce filamentous growth specific transcripts (ie: *FG(TyA)-lacZ*) while Ste12p-Ste12p complexes induce mating specific transcripts (ie: *FUS1-lacZ*). Thus, *TEC1* may determine the spectrum of genes induced by *STE12* during mating and filamentous growth.

FG(TyA)-lacZ may not adequately measure all of the signals that regulate filamentous growth. Its expression correlates extremely well with the activity of the the bifunctional MAPK cascade during filamentous growth and not during mating. However, there is not an exact correlation between the expression of this reporter and the phenotypic manifestations of filamentous growth. For example, *ste7*, *ste11* and *ste12* mutants show very low *FG(TyA)-lacZ* activity, yet these strains are slightly invasive and make some filaments. These sterile mutants completely block $RAS2^{VAL19}$ induction of *FG(TyA)-lacZ*, but only partially block its induction of cell elongation (See chapter four). Thus, there is not a perfect correlation between *FG(TyA)-lacZ* activity and filamentous growth. Furthermore, nutrient regulation of *FG(TyA)-lacZ* does not completely correlate with the visual assays for filamentous growth or with the activity of the MAPK cascade. For example, starvation for nitrogen induces *FG(TyA)-lacZ* and filamentous growth, but *FG(TyA)-lacZ* is also induced after prolonged incubation in YPD medium, whereas filamentous growth is not. Additionally, $RAS2^{VAL19}$ and *STE11-4*, which induce the reporter in the

presence of high ammonia, still require nitrogen starvation to induce pseudohyphal development. Moreover, the effects of *STE11-4* and SLAD medium on *FG(TyA)-lacZ* are approximately additive. Together, these data suggest that nutrient regulation of *FG(TyA)-lacZ* activity may be independent of the filamentous growth MAPK cascade.

One explanation for these discrepancies is that the *FG(TyA)-lacZ* construct is derived from the TY transposon rather than the promoter of a gene important for filamentous growth. Thus, there could be elements present in the *FG(TyA)-lacZ* construct that are not present in authentic targets of the MAPK cascade. Similarly, *FG(TyA)-lacZ* may be missing regulatory elements present in bona fide MAPK cascade target promoters. The *TEC1* promoter contains a filamentous response element (FRE) similar to the one in *FG(TyA)-lacZ*, with the Ste12p and Tec1p binding sites in a different configuration. This element may be important for filamentous growth (Madhani and Fink 1997). Additional genes with promoters containing FREs important for filamentous growth may be identified. Nevertheless, *FG(TyA)-lacZ* reporter activity correlates extremely well with the activity of the filamentous growth MAPK cascade and this reporter provides a critical tool for dissecting the outputs of this pathway.

Materials and Methods

β-Galactosidase assays

β-Galactosidase assays were performed with extracts from exponentially growing strains (OD_{600} of 0.5 - 1.0) (Rose and Botstein, 1983). For pheromone induction, cells in exponential growth phase were washed into 5 ml of fresh medium with or without 5 μM α-factor, and incubated at 30°C for 2 hr before being harvested. For assays under nitrogen starvation conditions, cells in exponential

growth phase were washed with 2% glucose or 3% galactose, spread on SLAD or SLAG plates, incubated at 30°C for 3 days (SLAD) or 22 h (SLAG), and harvested by resuspension from the plates. Cells were always washed once with water prior to lysis to remove external enzyme. Broken cell extracts were assayed for β-galactosidase activity at 37°C (Rose and Botstein, 1983). The activities were normalized to the total protein in each extract using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). β-Galactosidase specific activity = (OD_{420} x 1.7)/(0.0045 x protein concentration x extract volume x time).

Strains RRY1045 (L5978)(*MATa/α*) and RRY3 (L5528)(*MATa*) transformed with *FG(TyA)-lacZ HIS3* and pRS316 were grown overnight in synthetic complete medium (-6aa). From this stock, 200µl was diluted into each of six tubes containing 10 mls of synthetic medium (-6aa) and grown at 30°C until the cultures reached OD_{600} = 0.6. Samples were spun at 2500 rpm, washed with sterile deionized water, spun again, and resuspended in 1.0 ml of water. At this point 400µl of each strain was added to tubes containing 10 mls of media (see table for details), or spread onto different types of plates (see table). Liquid cultures were incubated for three hours at 30°C, spun, rinsed in water, transferred to an eppendorf tube, spun, resuspended in 250µl of breaking buffer, and frozen. Samples on plates were incubated sixteen hours at 30°C, scraped off the plates, rinsed in 1.0 ml of water, spun, resuspended in 250µl of breaking buffer, and frozen. The original overnight cultures were incubated for forty eight hours and 500µl was rinsed in water and frozen in breaking buffer as described above. Samples were assayed for β-galactosidase activity and protein concentration as described.

Acknowledgments

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Chapter four

Ras2 signals via the Cdc42/Ste20/MAPK module to induce filamentous growth in *Saccharomyces cerevisiae*

Hans-Ulrich Mösch, Radclyffe L. Roberts and Gerald R. Fink

Whitehead Institute for Biomedical Research and Department of Biology, M.I.T., Cambridge, MA 02142

RLR performed the bulk of the experiments with the *FG(TyA)-lacZ* reporter. HUM performed the bulk of the phenotypic analysis of yeast strains. Many experiments were repeated by both HUM and RLR to confirm results. This work has been published: PNAS *93*, 5352-5356 (1996).

Summary

RAS2^{Val19}, a dominant activated form of *S. cerevisiae RAS2*, stimulates both filamentous growth and expression of the transcriptional reporter *FG(TyA)-lacZ* but does not induce the mating pathway reporter *FUS1-lacZ*. This induction depends upon elements of the conserved mitogen-activated protein kinase (MAPK) cascade that is required for both filamentous growth and mating, two distinct morphogenetic events. Full induction requires the *STE20*, *STE11*, and *STE7* protein kinases and the transcription factor *STE12*. Moreover, the Rho family protein *CDC42*, a conserved morphogenetic G protein, is also a potent regulator of filamentous growth and *FG(TyA)-lacZ* expression in *S. cerevisiae*. Stimulation of both filamentous growth and *FG(TyA)-lacZ* by *CDC42* depends upon *STE20*. In addition, dominant negative *CDC42*^{Ala118} blocks *RAS2*^{Val19} activation, placing *CDC42* downstream of *RAS2*. These results suggest that filamentous growth in budding yeast is regulated by an evolutionarily conserved signaling pathway that controls cell morphology.

Introduction

The small G protein Ras plays a key role in signaling processes that regulate proliferation and differentiation in eukaryotes. In mammals, flies, worms and fission yeast, Ras regulates conserved mitogen-activated protein kinase (MAPK) pathways, that convey signals from the plasma membrane to the nucleus (reviewed in (Burgering and Bos, 1995; McCormick, 1994; van der Geer et al., 1994)). In metazoans, Ras mediates two different pathways, one via Raf, a serine/threonine kinase, and another distinct from the Raf/MAPK pathway that involves Rho family proteins such as Cdc42hs (Daum et al., 1994; Khosravi-Far et al., 1995; Prendergast et al., 1995). The situation in fission yeast is similar to that in metazoans: the Ras homolog ras1 triggers two distinct signaling pathways (Chang et al., 1994), a mating pathway involving a Raf-like serine/threonine kinase (byr2) and a morphogenetic pathway involving a Rho family protein (Cdc42sp) and a $p65^{PAK}$ kinase homolog, Shk1 (Marcus et al., 1995).

In the budding yeast *S. cerevisiae*, by contrast, neither *RAS1* nor *RAS2* (the two redundant *RAS* genes) has been linked to any of the MAPK signaling cascades that control mating, osmotic sensitivity, the protein kinase C pathway or sporulation in this organism (reviewed in (Herskowitz, 1995)). The sole characterized target of Ras in *S. cerevisiae* is adenylate cyclase (for review see (Broach and Deschenes, 1990)). Activation of Ras in *S. cerevisiae* results in elevated intracellular cAMP levels that in turn activate the A kinase, which is composed of an inhibitory subunit Bcy1 and a catalytic subunit encoded by three redundant genes *TPK1*, *TPK2*, and *TPK3*. Activation of the A kinase either by a dominant $RAS2^{Val19}$ mutation or *bcy1* null mutation confers sensitivity to heat shock and nutrient starvation, depletes carbohydrate reserves, and blocks sporulation.

Recent work on *S. cerevisiae* has revealed that activated *RAS2*^{Val19} stimulates filamentous growth (Gimeno et al., 1992), a morphogenetic pathway that requires the p65^{PAK} kinase homolog *STE20*, the *STE11* (MEKK) and *STE7* (MEK) protein kinases and the transcription factor *STE12* --- genes that constitute part of the MAPK module required for mating (Liu et al., 1993). However, the upstream effectors of mating, the pheromone receptors (*STE2, STE3*), the heterotrimeric G protein (*GPA1, STE4,* and *STE18*), and two MAPKs (*FUS3,* and *KSS1*) are not required for filamentous growth. In addition, recent evidence suggests that the Rho family protein Cdc42 may regulate the *STE20*/MAPK module for pheromone signaling (Simon et al., 1995; Zhao et al., 1995). Here, we show that (i) *RAS2* stimulates filamentous growth and the *FG(TyA)-lacZ* reporter depending on *STE20, STE11, STE7* and *STE12*; (ii) *RAS2* regulation via the *STE20*/MAPK module is independent of A kinase; (iii) *CDC42* is a potent regulator of filamentous growth, and is likely to act downstream of *RAS2*.

Results

RAS2 stimulation of filamentous growth depends upon *STE20, 11, 7* and *12*

To test whether enhancement of filament formation by a constitutively activated *RAS2* protein requires the filamentous *STE20*/MAPK cascade, we expressed the dominant *RAS2*^{Val19} allele in strains carrying disruptions of *STE20, STE11, STE7,* and *STE12* and assayed both the *FG(TyA)-lacZ* reporter and filamentous growth under nitrogen starvation conditions. The *ste20* mutation completely blocks the *RAS2*^{Val19} enhancement of filamentous growth and the *ste11, ste7,* and *ste12* mutations partially block this enhancement (Figure 4.1). These morphological observations were corroborated by measurement of *FG(TyA)-lacZ*

expression in these strains. *RAS2^{Val19}* induces *FG(TyA)-lacZ* (about 8 fold in diploids and 3 fold in haploids) under conditions that induce filamentous growth and this enhancement is completely dependent upon *STE20*, *STE7* and *STE12* (Figure 4.2). In contrast, *FUS1-lacZ* expression is not induced by *RAS2^{Val19}* either in the presence or absence of alpha factor (Table 4.1). Taken together, these results suggest that *RAS2* regulates filamentous growth by activating the filamentous growth MAPK cascade.

RAS2 activation of _FG(TyA)-lacZ_ does not occur via the A kinase

We tested whether activation of the A kinase was involved in the stimulation of *FG(TyA)-lacZ* by *RAS2*. Neither deletion of *BCY1* nor overexpression of *TPK1* significantly enhances *FG(TyA)-lacZ* expression (Figure 4.3) or filamentous growth. The results with the reporter are critical here because the *bcy1* mutation has morphological abnormalities that could confuse the analysis. *RAS2^{Val19}* depletes storage carbohydrates and makes cells sensitive to heat shock (Broach and Deschenes, 1990). We find that double mutants between *RAS2^{Val19}* or *bcy1* and *ste20*, *ste11* or *ste7* are still sensitive to heat shock (Figure 4.4). Thus, *RAS2* regulation of filamentous growth via the *STE20*/MAPK cascade is independent of A kinase and A kinase phenotypes appear to be independent of the *STE20*/MAPK cascade.

Figure 4.1

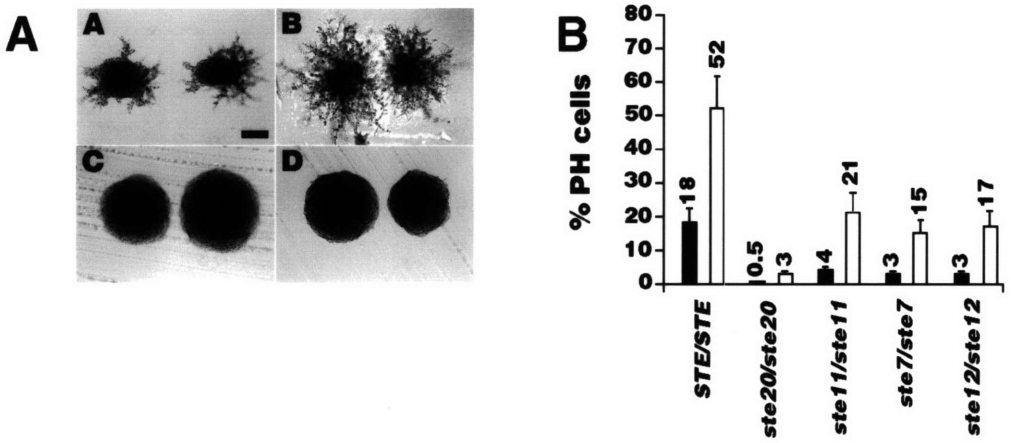


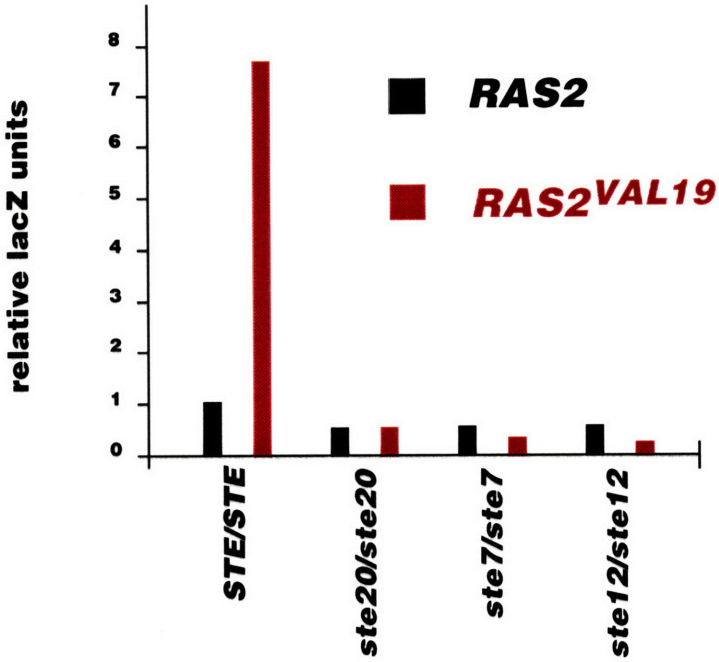
Figure 4.1

Filamentous growth stimulation by *RAS2*^{Val19} depends on *STE20*, *STE11*, *STE7*, and *STE12*.

(A) Filamentous growth of STE/STE (A and B) and *ste20/ste20* mutant (C and D) diploid strains containing control plasmid YCp50 (A and C) or plasmid YCp50-RAS2Val19 (B and D) after 4 days of growth on synthetic low ammonia dextrose (SLAD) medium. The scale bar in panel A applies to panels A, B, C, and D and represents 100 μM.

(B) Quantitative measurement of pseudohyphal cell development upon nitrogen starvation. *STE/STE* and mutant *ste/ste* diploids containing YCp50 (black bars) or YCp50-RAS2Val19 (white bars) were grown for 4 days on SLAD medium. The percentage of cells that had undergone pseudohyphal development (% PH cells) was then determined (for details see materials and methods). Cells were considered as pseudohyphal, when their length/width ratio was greater than two.

Figure 4.2



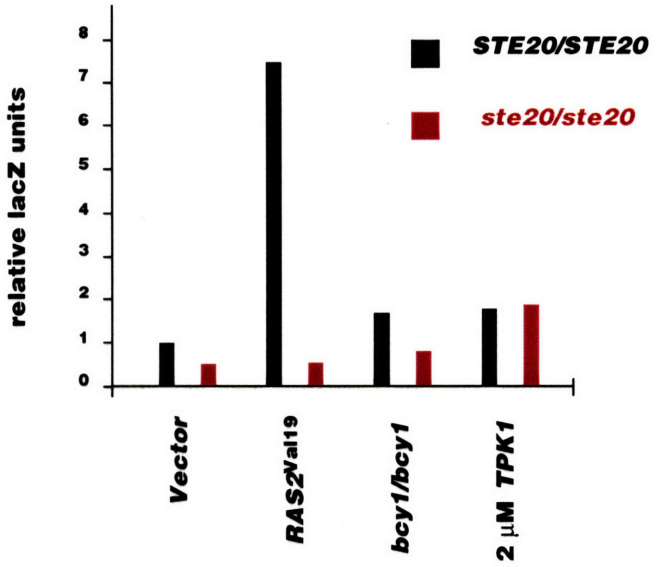
Strain genotype	Plasmid genotype	Relative *FG(TyA)-lacZ* activity
STE+/STE+	control vector	1.0
STE+/STE+	*RAS2^{Val19}*	0.5
ste20/ste20	control vector	7.6
ste20/ste20	*RAS2^{Val19}*	0.5
ste11/ste11	control vector	0.5
ste11/ste11	*RAS2^{Val19}*	0.5
ste7/ste7	control vector	0.6
ste7/ste7	*RAS2^{Val19}*	0.3
ste12/ste12	control vector	0.6
ste12/ste12	*RAS2^{Val19}*	0.2

Figure 4.2

RAS2^{Val19} induces *FG(TyA)*-lacZ in a *STE20-*, *STE7-* and *STE12*-dependent manner

Strains were grown on synthetic low ammonia dextrose medium (SLAD) for 3 days, and *FG(TyA)-lacZ* expression was measured as a function of β-galactosidase activity in cellular extracts from these strains. Numbers represent β-galactosidase activities normalized to the activity measured in wild-type diploid strains under nitrogen starvation conditions, with a value of 1 corresponding to 89 nmoles O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min x mg protein (See Table 3.1). The relevant genotype of each strain and plasmid is indicated.

Figure 4.3



Strain genotype	Plasmid genotype	Relative *FG(TyA)-lacZ* activity
STE+/STE+	control vector	1.0
ste20/ste20	control vector	0.4
STE+/STE+	*RAS2^{Val19}*	7.6
ste20/ste20	*RAS2^{Val19}*	0.5
STE+/STE+, bcy1/bcy1	control vector	1.7
ste20/ste20, bcy1/bcy1	control vector	0.6
STE+/STE+	2μ *TPK1*	1.8
ste20/ste20	2μ *TPK1*	1.9

Figure 4.3

High A kinase levels do not induce *FG(TyA)-lacZ* expression comparable to *RAS2^{Val19}*.

Strains were grown on synthetic low ammonia dextrose medium (SLAD) for 3 days, and *FG(TyA)-lacZ* expression was measured as a function of β-galactosidase activity in cellular extracts from these strains. Numbers represent β-galactosidase activities normalized to the activity measured in wild-type diploid strains under nitrogen starvation conditions, with a value of 1 corresponding to 89 nmoles O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min x mg protein (See Table 3.1). The relevant genotype of each plasmid is indicated.

Figure 4.4

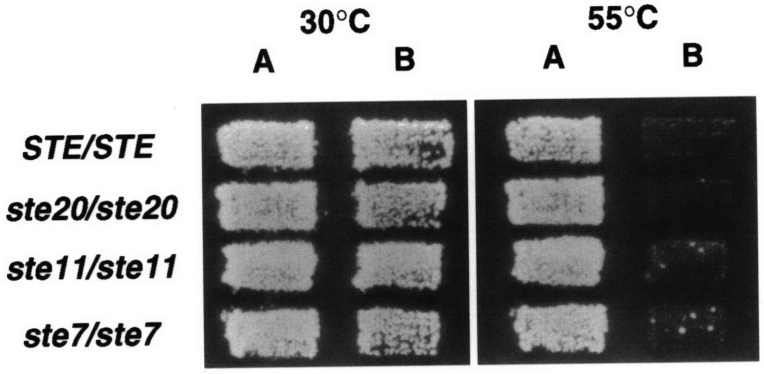


Figure 4.4

Mutations in the filamentous growth MAPK cascade do not block the heat shock sensitivity induced by *RAS2*^{VAL19}.

Diploid yeast strains carrying a control vector (A) or *RAS2*^{VAL19} (B) were exposed to 30°C or 55°C for fifteen minutes and then replica plated and incubated at 30°C. Samples were photographed after twenty four hours at 30°C. The genotype of each strain is indicated at left.

RAS2* stimulates filamentous growth via *CDC42

To determine whether stimulation of filamentous growth by *RAS2* depends upon *CDC42*, a Rho-type GTP binding protein, we analyzed the effects of various dominant alleles of *CDC42* (Ziman et al., 1991). Expression of the *CDC42* alleles was controlled by growing strains with *pGAL-CDC42* constructs on raffinose to avoid the inhibition of growth caused by high levels of these mutant proteins. Under these conditions expression of the dominant negative allele $CDC42^{Ala118}$ completely suppressed filamentous growth (Figure 4.5). In contrast, the dominant active *CDC42* alleles $CDC42^{Val12}$ and $CDC42^{Leu61}$ induce both filamentous growth and the *FG(TyA)-lacZ* reporter (Figure 4.5 and Figure 4.6). This induction is completely dependent upon the presence of a functional *STE20* gene, showing that *CDC42* activates filamentous growth via *STE20*.

Double mutants containing the activated *RAS2* allele and the dominant negative *CDC42* allele ($RAS2^{Val19}$, $CDC42^{Ala118}$) fail to form filaments (Figure 4.7) and have lower expression of *FG(TyA)-lacZ* than does the $RAS2^{Val19}$ single mutant (Figure 4.8). In contrast, the $CDC42^{Ala118}$ allele does not block the *STE11-4* enhancement of *FG(TyA)-lacZ* expression (Figure 4.8). Taken together these data suggest that *CDC42* is required for *RAS2* activation of filamentous growth and acts downstream of *RAS2*.

Figure 4.5

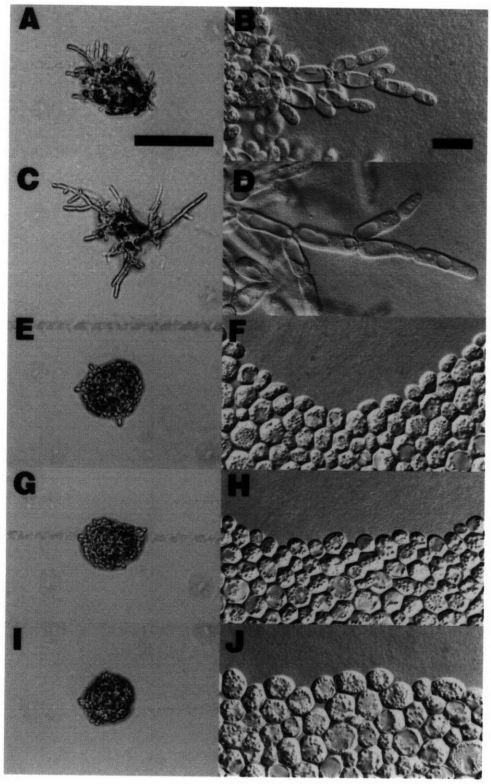
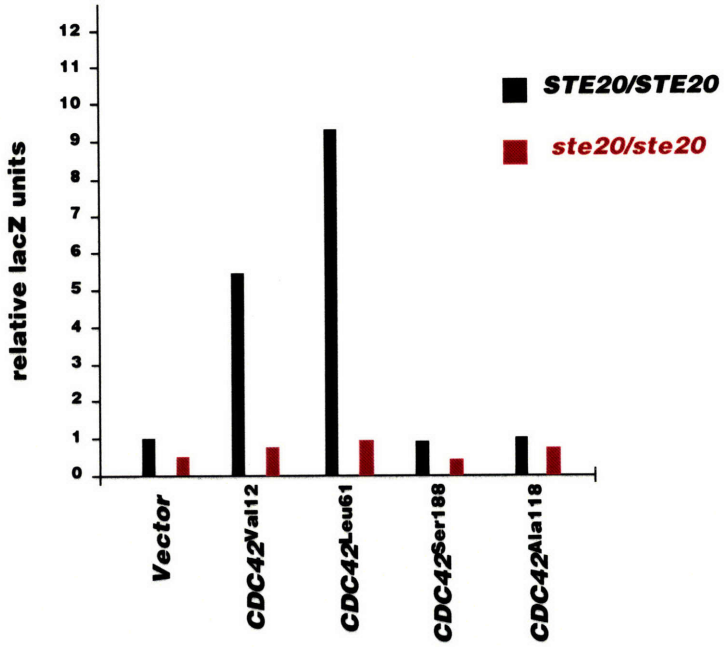


Figure 4.5

Stimulation of filamentous growth by *CDC42* depends upon *STE20.*

STE/STE (A, B, C, D, I, and J) and *ste20/ste20* mutant (E, F, G, and H) diploids containing control plasmid pRS315 (A, B, E, F), plasmid pGAL-CDC42Leu-61 (C, D, G, and H) or plasmid pGAL-CDC42Ala-118 (I, and J) were streaked on synthetic low ammonia raffinose (SLAR) medium (A through H) or SLAR medium containing 0.1% of galactose (I and J), and incubated at 30°C. Pictures of representative microcolonies were taken after 17 hours of growth using a light microscope (A, C, E, G, and I). After 4 days, pseudohyphal development of cells at the edges of the colonies was visualized under the microscope using Nomarski optics (B, D, F, H, and J). The scale bar in panel A applies to panels A, C, E, G and I and represents 50 μM; the scale bar in panel B applies to panels B, D, F, H, and J and represents 10 μM.

Figure 4.6



Strain genotype	Plasmid genotype	Relative *FG(TyA)-lacZ* activity
STE+/STE+	control vector	1.0
ste20/ste20	control vector	0.4
STE+/STE+	*CDC42^{Val12}*	5.2
ste20/ste20	*CDC42^{Val12}*	0.8
STE+/STE+	*CDC42^{Leu61}*	9.2
ste20/ste20	*CDC42^{Leu61}*	1.1
STE+/STE+	*CDC42^{Ser188}*	0.9
ste20/ste20	*CDC42^{Ser188}*	0.4
STE+/STE+	*CDC42^{Ala118}*	1.1
ste20/ste20	*CDC42^{Ala118}*	0.7

Figure 4.6

CDC42 alleles stimulate *FG(TyA)-lacZ* activity via *STE20*.

FG(TyA)-lacZ expression was measured in strains grown for 20 hours on synthetic low ammonia galactose medium (SLAG), and β-galactosidase assays were performed with cellular extracts. Numbers represent β-galactosidase activities normalized to the activity measured in wild-type diploid strains under nitrogen starvation conditions, with a value of 1 corresponding to 89 nmoles O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min x mg protein (See Table 3.1). Strain and plasmid genotypes are indicated.

Figure 4.7

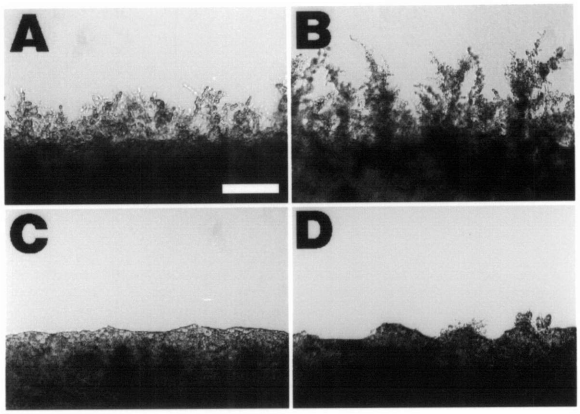
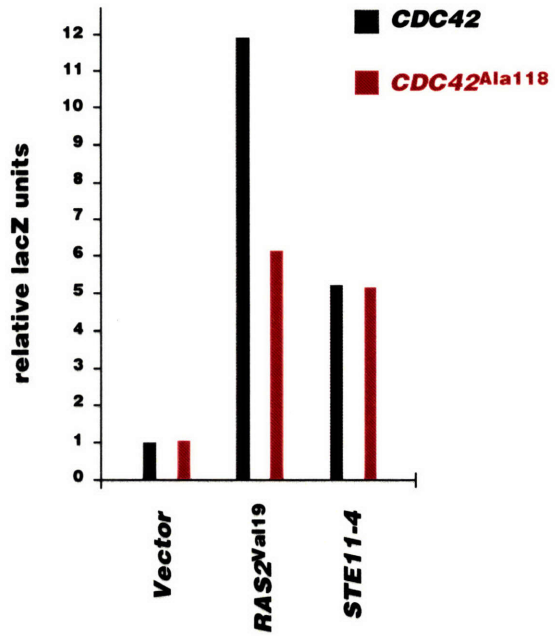


Figure 4.7

Genetic interactions between *RAS2* and *CDC42* during filamentous growth.

Strain CGX179 (*ura3/ura3, leu2/leu2*) was transformed with plasmids YCp50 and pRS315 (A), YCp50-RAS2Val19 and pRS315 (B), YCp50 and pGAL-CDC42Ala-118 (C), or YCp50-RAS2Val19 and pGAL-CDC42Ala-118 (D) and grown on SLAR medium containing 0.1% of galactose. After 4 days of growth, pseudohyphal development of strains was visualized under a microscope and photographed. The scale bar in panel A represents 100 μM.

Figure 4.8



Plasmid genotype	Plasmid genotype	Relative *FG(TyA)-lacZ* activity
PGAL control	vector control	1.0
PGAL::CDC42^{Ala118}	vector control	0.9
PGAL control	*RAS2^{Val19}*	11.8
PGAL::CDC42^{Ala118}	*RAS2^{Val19}*	6.1
PGAL control	*STE11-4*	5.4
PGAL::CDC42^{Ala118}	*STE11-4*	5.3

Figure 4.8

CDC42^{Ala118} partially blocks *FG(TyA)-lacZ* induction by *RAS2^{Val19}* but not *STE11-4*.

Wild-type strains were grown for 20 hours on synthetic low ammonia galactose medium (SLAG), and *FG(TyA)-lacZ* expression was measured as a function of β-galactosidase activity in cellular extracts from these strains. Numbers represent β-galactosidase activities normalized to the activity measured in wild-type diploid strains under nitrogen starvation conditions (See Table 3.1), with a value of 1 corresponding to 89 nmoles O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min x mg protein. The relevant genotype of each plasmid is indicated.

Table 4.1

Plasmid	- pheromone	+ pheromone
vector	8.6	885
STE11-4	39	1023
RAS2^{Val19}	5.4	331

Table 4.1

RAS2^{VAL19} does not induce the mating reporter *FUS1-LacZ*.

MATa haploid yeast strain 10650-4d containing the *FUS1-LacZ* reporter pJB207 (B2146) and control vector pRS316 (B1820), *STE11-4* (B2616), or *RAS2^{VAL19}* (B2255) was incubated for three hours in the absence (-) or presence (+) of 5 μM alpha pheromone. Extracts were prepared and assayed for β-galactosidase activity normalized to total protein as described.

Discussion

These experiments resolve some of the disparity between the function of the Saccharomyces *RAS* genes and the *RAS* genes from other organisms. In fission yeast, flies, worms and humans, Ras protein activity signals through an evolutionarily conserved MAPK cascade to activate gene expression. In fission yeast, for example, extracellular mating factors activate Ras, which signals through a MAPK cascade to stimulate mating (Chang et al., 1994). However, in *S. cerevisiae* no role for Ras in mating has been discovered despite the involvement of a conserved MAPK cascade consisting of *STE20*, *STE11*, *STE7*, and two MAPK homologs (*FUS3* and *KSS1*). Activation of this MAPK cascade by mating pheromone is readily measured by the transcriptional reporter *FUS1-lacZ*. However, $RAS2^{Val19}$does not induce *FUS1-lacZ*; in fact it reduces *FUS1-lacZ* induction by pheromone. Previous work had shown that some elements of the mating MAPK cascade also function in vegetative cells to promote filamentous growth (Liu et al., 1993). Although $RAS2^{Val19}$ was known to stimulate filamentous growth (12), it was not clear whether this stimulation occurred via the mating MAPK cascade.

Our study shows that in *S. cerevisiae RAS2* signals through the MAPK cascade for filamentous growth. Mutations in the MAPK cascade (*ste20*, *ste11*, *ste7*, *ste12*) block the stimulation of filamentation by $RAS2^{Val19}$. Moreover, the *FG(TyA)-lacZ* reporter, which responds to the activity of the *STE20*, *STE11*, *STE7* kinase cascade in vegetative cells but not to mating pheromones is induced by $RAS2^{Val19}$. Our double mutant data place *CDC42* in the pathway downstream from *RAS2*.

Figure 4.9

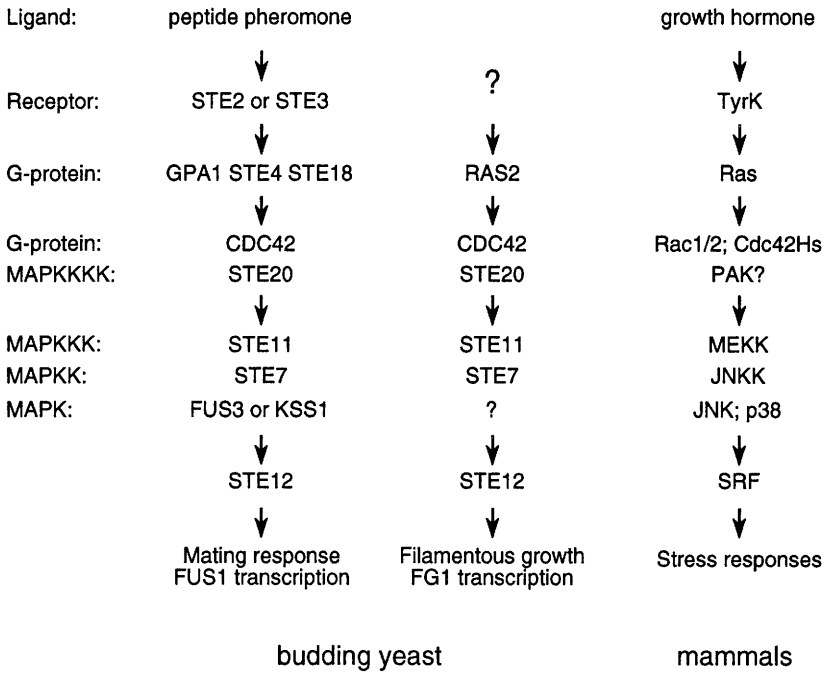


Figure 4.9

Common motifs in MAPK cascade signaling in yeast and mammals.

In yeast mating (left), binding of a peptide pheromone to a transmembrane receptor triggers dissociation of a heterotrimeric G protein that activates a series of protein kinases in a conserved MAPK cascade. These kinases ultimately activate a transcription factor that induces mating specific genes. In the mammalian stress response (right), the ligand binds a tyrosine kinase receptor which activates the small G proteins Ha-Ras, Rac1/2, and Cdc42hs, which in turn activate the MAPK cascade and transcription factor. The yeast filamentous growth pathway (center) is more similar to the mammalian stress response pathway as *RAS2* and *CDC42* stimulate the MAPK cascade.

This arrangement of the filamentous growth signaling pathway in Saccharomyces is similar to that described for mammalian cells and fission yeast (Figure 4.9). In mammalian cells Ha-Ras (Derijard et al., 1994)as well as Rac1 and Cdc42Hs have recently been shown to activate selectively the JNK/SAPK (MAP kinases), with Rac1 proposed as an intermediate between Ha-Ras and JNK/SAPK. In addition, in mammals Ras is thought to act via the Rho family proteins Rac1 and Cdc42Hs to control reorganization of the actin cytoskeleton (Khosravi-Far et al., 1995). A similar pathway has been described in *S. pombe*, where the ras1 protein is thought to regulate cell morphogenesis via cdc42sp (Chang et al., 1994). In addition, Shk1, a homolog of the *S. cerevisiae STE20* and mammalian $p65^{PAK}$ protein kinases, is thought to be a component of the Ras1/Cdc42sp signaling module (Marcus et al., 1994).

Previous work suggested the involvement of the A kinase in filamentous growth because overexpression of the cAMP phosphodiesterase (*PDE2*) reduced the stimulation of filamentous growth by $RAS2^{Val19}$ (Ward et al., 1995). However, our results indicate that *RAS2* regulation of filamentous growth via the *CDC42/STE20*/MAPK pathway is independent of A kinase activity. These findings could be explained if *RAS2* has a dual role in activation of filamentous growth; activation of a *CDC42/STE20*/MAPK signaling module (cAMP-independent) and stimulation of A kinase activity (cAMP-dependent). Our finding that mutations in *STE11*, *STE7*, and *STE12* do not completely block $RAS2^{Val19}$ induction of cell elongation and filament formation is consistent with this model.

Mating and filamentous growth require different G proteins for activation, a heterotrimeric G protein and Ras2p, respectively. How does *RAS2* regulate *CDC42* to activate *STE20* for filamentous growth? A number of recent studies indicate the existence of a protein complex including Cdc42p and Ste20p. This complex is thought to function in bud emergence, cell polarity, and pheromone signaling

(Zhao et al., 1995). The Ras-like protein Rsr1p is thought to recruit a protein complex containing Cdc42p to the site of the emerging bud thereby controlling cell polarity (Zheng et al., 1995). By analogy with Rsr1p, Ras2p could recruit a Cdc42p/Ste20p complex to the membrane thereby regulating cell morphology during filamentous growth. Indeed, recent work has suggested that *RSR1* function overlaps with that of *RAS1* and *RAS2* in cell cycle control (Morishita et al., 1995). Moreover, in mammalian cells a critical function of RAS seems to be to recruit the Raf kinase to the membrane where it is activated. The factors that control the orderly interchange of G proteins within Ste20p complexes should help to explain how a single MAPK cascade can activate two different developmental pathways.

Materials and methods

Media, growth conditions, and yeast strains

Standard yeast culture medium was prepared essentially as described (Sherman et al., 1986). Low ammonia medium for scoring pseudohyphal growth or β-galactosidase assays was prepared as described (Gimeno et al., 1992) with carbon sources at the following concentrations: SLAD (2% glucose), SLAR (3% raffinose), SLAR + 0.1% GAL (3% raffinose and 0.1% galactose), or SLAG (3% galactose).

All strains used in this study are congenic to the Σ1278b genetic background (Grenson et al., 1966; Liu et al., 1993). The derivation of *ste2*, *ste4*, *ste18*, *ste20*, *ste5*, *ste11*, *ste7*, *fus3*, *kss1*, and *ste12* strains has been described (Liu et al., 1993; Roberts, 1994). The *bcy1* mutation was introduced using the disruption plasmid p*bcy1-LEU2* (Toda et al., 1987). The *FG(TyA)-lacZ* transcriptional reporter was transformed into strains using centromere-based plasmids p*FG(TyA)-lacZ-URA3*, p*FG(TyA)-lacZ-LEU2* or p*FG(TyA)-lacZ-HIS3*, , and integrated *FUS1-lacZ* has been described (Liu et

al., 1993; Roberts, 1994). The *STE11-4* mutation (Stevenson et al., 1992) was expressed using plasmid pSL1509. For high copy expression of *STE20* or Tpk1, plasmids pRS426-*STE20* or YEp-*TPK1* (Toda, 1987), respectively, were used. The dominant $RAS2^{Val19}$ mutation allele was expressed using plasmids YCp50-$RAS2^{Val19}$or YCp*LEU2*-$RAS2^{Val19}$. Dominant *CDC42* alleles were expressed from a galactose-inducible promoter (*GAL1-10*) using plasmids p*GAL-CDC42*$^{Val-12}$, p*GAL-CDC42*$^{Leu-61}$, p*GAL-CDC42*$^{Ala-118}$, or p*GAL-CDC42*$^{Ser-188}$ (Ziman et al., 1991). Plasmids pRS315, pRS316, YCp50 or pRS426 were used as vector controls. Standard procedures were used for yeast transformation and genetics (Gietz et al., 1992; Ito et al., 1983; Sherman et al., 1986).

Plasmids

Plasmid p*FG(TyA)-lacZ-URA3* is plasmid pIL30 (Laloux et al., 1994)and contains a 97 base-pair fragment derived from transposon Ty1 harboring a Ste12p binding site in a *TDH3-lacZ* reporter gene expression system. Plasmids p*FG(TyA)-lacZ-LEU2* and p*FG(TyA)-lacZ-HIS3* were constructed by homologous recombination in yeast using p*FG(TyA)-lacZ-URA3* and a linear *ura3::LEU2* or *ura3::HIS3 URA3*-disruption cassette (Ma et al., 1987). Plasmid YCp*LEU2*-$RAS2^{Val19}$ was obtained by homologous re(Leberer, 1992)combination in yeast using plasmid YCp50-$RAS2^{Val19}$ and a linear *ura3::LEU2* disruption fragment. Plasmid pRS426-*STE20* was constructed by cloning a 4.9 kb *Kpn*I-*Not*I fragment from plasmid pSTE20-5 into high copy plasmid pRS426 .

β-Galactosidase assays

β-Galactosidase assays were performed with extracts from exponentially growing strains (OD_{600} of 0.5 - 1.0) (Rose and Botstein, 1983). For pheromone induction, cells in exponential growth phase were washed into 5 ml of fresh medium with or without 5 μM α-factor, and incubated at 30°C for 2 hr before being harvested. For assays under nitrogen starvation conditions, cells in exponential growth phase were washed with 2% glucose or 3% galactose, spread on SLAD or SLAG plates, incubated at 30°C for 3 days (SLAD) or 22 hours (SLAG), and harvested by resuspension from the plates. Cells were always washed once with water prior to lysis to remove external enzyme. Broken cell extracts were assayed for β-galactosidase activity at 37°C (Rose and Botstein, 1983). The activities were normalized to the total protein in each extract using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). β-Galactosidase specific activity = (OD_{420} x 1.7)/(0.0045 x protein concentration x extract volume x time).

Qualitative and quantitative assays of filamentous growth

The qualitative growth assay for filament formation, and light microscopy of microcolonies and invasive cells were performed as previously described (Gimeno and Fink, 1994; Gimeno et al., 1992). Quantitative pseudohyphal cell development was performed based on the method for characterization of cell shape in *Candida albicans* (Merson-Davies and Odds, 1989). Strains were grown on SLAD medium for 3 days at 30°C, and non-invasive cells were removed by washing the plates with water. Invasive cells were scraped off the plate, suspended in 50 μl of water, and analyzed for cell shape by light microscopy. Cell shape was quantified by determining the length to width (l/w) ratio (typically 200 cells) and dividing them

into the classes: Yeast form (YF) cells with a l/w ratio of smaller than 2; pseudohyphal (PH) cells with a l/w ratio of greater than 2. The data were verified by taking photographic pictures of a smaller number of cells and directly measuring cell dimensions.

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Chapter five

14-3-3 proteins are essential for RAS/MAPK cascade signaling during pseudohyphal development in *S. cerevisiae*

Radclyffe L. Roberts, Hans-Ulrich Mösch, and Gerald R. Fink

1-Whitehead Institute for Biomedical Research and Department of Biology, M. I. T., Cambridge, Massachusetts 02142. 2-Institute for Microbiology, Georg-August-University Göttingen, D-37077 Göttingen, Germany. 3-Corresponding author.

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Summary

14-3-3 proteins are highly conserved, ubiquitous proteins whose explicit functions have remained elusive. Here we show that the *S. cerevisiae* 14-3-3 homologs *BMH1* and *BMH2* are not essential for viability or mating MAPK cascade signaling, but they are essential for pseudohyphal development MAPK cascade signaling and other processes. Activated alleles of *RAS2* and *CDC42* induce pseudohyphal development and *FG(TyA)-lacZ* signaling in Bmh$^+$ strains but not in *ste20* (p65^{PAK}) or *bmh1 bmh2* mutant strains. Moreover, Bmh1p and Bmh2p associate with Ste20p *in vivo*. Three alleles of *BMH1* encode proteins defective for *FG(TyA)-lacZ* signaling and association with Ste20p, yet these alleles complement other 14-3-3 functions. Therefore, the 14-3-3 proteins are specifically required for RAS/MAPK cascade signaling during pseudohyphal development in *S. cerevisiae*.

Introduction

14-3-3 proteins, originally named for their migration pattern in two-dimensional gel electrophoresis, are abundant acidic proteins that form dimers (Aitken et al., 1992; Jones et al., 1995). Each polypeptide contains a cleft that could bind an alpha helix of a substrate protein, potentially dimerizing substrates or bringing different substrates into close proximity (Aitken et al., 1992; Jones et al., 1995; Liu et al., 1995; Xiao et al., 1995). 14-3-3 protein homologs are highly conserved throughout the eukaryotic kingdom. Mutants of *S. pombe* and some *S. cerevisiae* strains that lack 14-3-3 function are inviable (Ford et al., 1994; Gelperin et al., 1995; van Heusden et al., 1996).

14-3-3 proteins have been implicated in diverse processes. The two *S. cerevisiae* 14-3-3 homologs, *BMH1* and *BMH2*, may play a role in RAS signaling via

protein kinase A (PKA) (Gelperin et al., 1995). In *S. pombe*, two 14-3-3 homologs regulate a DNA damage checkpoint (Ford et al., 1994). In mammalian cell extracts, 14-3-3 proteins bind and modulate tryptophan hydroxylase and protein kinase C (PKC) activity, and regulate Ca^{2+}-dependent exocytosis (Acs et al., 1995; Chamberlain et al., 1995; Ichimura et al., 1995; Toker et al., 1992). *In vitro* biochemical studies suggest a role for 14-3-3 proteins in Ras/Raf/mitogen-activated protein kinase (MAPK) cascade signaling in vertebrate cell proliferation and differentiation (Fantl et al., 1994; Li et al., 1995). 14-3-3 isoforms bind directly to phosphoserine residues in the Raf kinase but the functional significance of this binding is uncertain (Freed et al., 1994; Fu et al., 1994; Irie et al., 1994; Michaud et al., 1995; Muslin et al., 1996; Suen et al., 1995).

In *S. cerevisiae*, several independent MAPK cascades have been characterized (Herskowitz, 1995). One MAPK cascade controls the pheromone-induced mating of haploid cells via *STE20* ($p65^{PAK}$), *STE11* (MEKK), *STE7* (MEK), *FUS3* and *KSS1* (MAPK), and the transcription factor *STE12*(Sprague and Thorner, 1992). The outputs of this MAPK cascade can be divided into several measurable events: induction of the transcriptional reporter *FUS1-lacZ*, the development of mating projections, cell cycle arrest, and formation of a diploid zygote. Mutations in *STE20*, *STE11*, *STE7*, and *STE12* block all of these events.

When starved for nitrogen, diploid yeast strains enter a pseudohyphal growth mode that depends on elements of the mating MAPK cascade (Gimeno et al., 1992; Liu et al., 1993). Pseudohyphal development is a composite of several distinct processes: cell elongation, filament formation, and agar penetration. All of these processes require the kinases *STE20*, *STE11*, and *STE7*, and the transcription factors *STE12* and *TEC1* (Gavrias et al., 1996; Liu et al., 1993). These transcription factors bind cooperatively to a filamentous response element (FRE) within the transcriptional reporter *FG(TyA)-lacZ* (Madhani, 1997). Full expression of *FG(TyA)-*

lacZ requires *STE20, STE11, STE7, TEC1,* and *STE12,* and this reporter is strongly induced by the dominant active *STE11-4* kinase (Laloux et al., 1994; Mosch et al., 1996). Thus, *FG(TyA)-lacZ* provides an accurate reflection of MAPK cascade signaling during pseudohyphal development.

The mating and pseudohyphal development MAPK cascades are activated by different sets of proteins. The pheromone receptors and heterotrimeric G protein involved in activating the mating MAPK cascade are not required for pseudohyphal development (Liu et al., 1993). The two yeast RAS homologs, *RAS1* and *RAS2,* regulate accumulation of the storage carbohydrate glycogen via cAMP and protein kinase A (*BCY1* and *TPK1, 2, 3*) (Cameron, 1988; Toda et al., 1987). A dominant activated form of *RAS2*, $RAS2^{VAL19}$, induces pseudohyphal development and *FG(TyA)-lacZ* transcription but does not induce the mating pathway reporter *FUS1-lacZ* (Mosch et al., 1996). Full induction requires *STE20, STE11, STE7, STE12.* Activated alleles of *CDC42* ($CDC42^{VAL12}$, $CDC42^{LEU61}$) also induce pseudohyphal development and *FG(TyA)-lacZ* in a *STE20*-dependent manner. Furthermore, dominant negative $CDC42^{ALA118}$ blocks $RAS2^{VAL19}$ activation, placing *CDC42* downstream of *RAS2.* Thus, the conserved G proteins *RAS2* and *CDC42* control activation of the pseudohyphal development MAPK cascade (Mosch et al., 1996).

The pathway for pseudohyphal development may not be a single unbranched sequence from *RAS2* to *STE12.* Although mutations in *STE11, STE7,* and *STE12* completely block *FG(TyA)-lacZ* induction by $RAS2^{VAL19}$, they do not completely block induction of cell elongation, suggesting that *RAS2* has outputs independent of the MAPK cascade important for cell elongation (See (Mosch et al., 1996) Figure 1). In support of this model, *ste20/ste20* mutant diploids show more pronounced defects in pseudohyphal cell elongation and invasion than *ste11/ste11* or *ste12/ste12* mutants, and these defects are only partially bypassed by *STE11-4* (Mosch et al., 1996); R. Roberts unpublished observation). In addition, when the Cdc42p binding site of

Ste20p is deleted, pseudohyphal development is blocked but mating MAPK cascade signaling via Ste11p, Ste7p and Ste12p is unaffected (Leberer, 1997; Peter, 1996). Thus, there may be a signaling bifurcation at the level of Ste20p that separates MAPK cascade signaling from aspects of cellular morphogenesis.

In this study we show that the yeast 14-3-3 proteins, Bmh1p and Bmh2p, are not strictly required for viability or mating MAPK cascade functions in *S. cerevisiae* strain Σ1278b, although they are essential for RAS/MAPK cascade signaling during pseudohyphal development. Furthermore, these proteins associate with Ste20p *in vivo*. We isolated three alleles of *BMH1* that encode proteins defective for *FG(TyA)-lacZ* signaling and association with Ste20p. These mutant proteins rescue the other phenotypes of *bmh1 bmh2* strains. Because elements of the *S. cerevisiae* pseudohyphal development RAS/MAPK cascade are highly conserved in evolution, the molecular mechanisms underlying their activation should provide a paradigm for signaling in all eukaryotes.

Results

14-3-3 proteins are not essential in all *S. cerevisiae* strains

Because 14-3-3 proteins have been implicated in RAS/MAPK cascade signaling in vertebrates, we wished to determine whether the 14-3-3 homologs of *S. cerevisiae*, *BMH1* and *BMH2*, are required for RAS/MAPK cascade signaling during pseudohyphal development. To this end, we deleted both of these genes and replaced their open reading frames with the *HIS3* gene in Σ1278b-derived strains (Table 1). The *bmh1 bmh2* double mutant strains resulting from a cross of *bmh1* x *bmh2* grow slightly more slowly than *BMH1 BMH2* strains on plates and have a relative plating efficiency of 63% when compared to isogenic *BMH1 BMH2* strains.

Neverthless, Σ1278b-derived yeast strains lacking both *BMH1* and *BMH2* are viable, in contrast to other *S. cerevisiae bmh1 bmh2* mutant strains(Gelperin et al., 1995; van Heusden et al., 1996).

Although *BMH1* and *BMH2* are the only members of the 14-3-3 family in the *S. cerevisiae* S288C genome (Cherry, 1997), we tested whether the viability of our double deletion could be ascribed to the presence of an additional 14-3-3 gene(s) present in our genetic background (Σ1278b). Low stringency Southern analysis of genomic DNA from *BMH1 BMH2* , *bmh1*, *bmh2*, and *bmh1 bmh2* strains using fragments of the *BMH1* and *BMH2* open reading frames as probes does not reveal any additional 14-3-3 homologs in our strains (data not shown). In addition, an anti-Bmh2p polyclonal antibody that readily detects Bmh1p and Bmh2p fails to bind any epitopes in *bmh1 bmh2* extracts (Figure 5.1). These data strongly suggest that *BMH1* and *BMH2* are the only 14-3-3 homologs in our Σ1278b-derived yeast strains. These strains enable us to study *in vivo* signaling in the absence of 14-3-3 function.

14-3-3 proteins are required for RAS/MAPK cascade signaling during pseudohyphal development

To test if *BMH1* and *BMH2* are required for RAS/MAPK cascade signaling during pseudohyphal development, we used the *STE12*-dependent *FG(TyA)-lacZ* transcriptional reporter. This construct reflects RAS/MAPK cascade activity during pseudohyphal development. Full expression of this reporter requires MAPK cascade components, and it is strongly induced by the activated $RAS2^{VAL19}$, $CDC42^{VAL12}$, and *STE11-4* alleles (Mosch et al., 1996). Overexpression of *BMH1* or *BMH2* from the GAL1-10 promoter does not significantly induce this reporter (Table 5.2). However, *bmh1 bmh2* strains show greatly reduced expression of *FG(TyA)-lacZ* relative to Bmh$^+$ (*BMH1/BMH1 BMH2/BMH2*) strains (Table 5.3). Moreover,

RAS2^{VAL19} and *CDC42*^{VAL12} do not induce *FG(TyA)-lacZ* in the *bmh1 bmh2* strain. In contrast, *STE11-4* and overexpression of *STE12* from the *GAL1-10* promoter (*GAL-STE12*) induce the *FG(TyA)-lacZ* reporter in both Bmh$^+$ and *bmh1 bmh2* strains (Table 5.3). Thus, the 14-3-3 proteins are strictly required for RAS/MAPK cascade signaling during pseudohyphal development.

Pseudohyphal development is a composite of several distinct morphogenetic events: cell elongation, filament formation, and agar invasion. We found that overexpression of *BMH1* or *BMH2*, like *RAS2*^{VAL19} and *CDC42*^{VAL12}, stimulates cell elongation and filament formation in a *STE20*-dependent manner (Figure 5.2). In contrast, *bmh1 bmh2* mutant diploid strains efficiently invade the agar, but they do not form long filaments or normal percentages of elongated cells (Figure 5.2, Table 5.4). Thus, the 14-3-3 proteins are required for proper filament formation and cell elongation but not agar penetration. We found that *RAS2*^{VAL19} and *CDC42*^{VAL12} do not stimulate cell elongation in *bmh1 bmh2* diploid strains (Figure 5.2, Table 5.4). *GAL-STE12* and *STE11-4* also failed to bypass the *bmh1 bmh2* cell elongation defect. These data indicate that the 14-3-3 proteins are essential for the induction of filament formation and cell elongation by *RAS2* and *CDC42*, and suggest that they control these processes downstream or independent of the pseudohyphal development MAPK cascade.

Figure 5.1

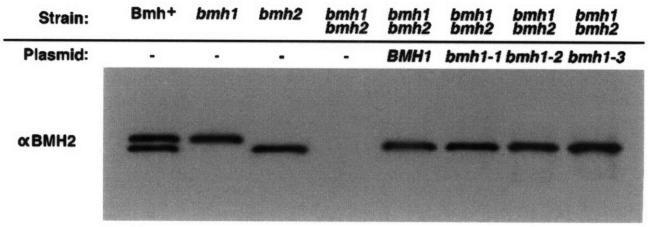

Figure 5.1

Detecting 14-3-3 proteins in *S. cerevisiae.*

Whole cell extracts were prepared from *Bmh+* (lane 1, at left)(RRY1045), *bmh1/bmh1* (lane 2)(RRY1064), *bmh2/bmh2* (lane 3)(RRY1276), and *bmh1/bmh1 bmh2/bmh2* (lanes 4, 5, 6, 7, 8)(RRY1257) strains containing no plasmid (lanes 1, 2, 3, 4), *BMH1* (lane 5)(B3455), *bmh1-1* (lane 6)(B3456), *bmh1-2* (lane 7)(B3457), or *bmh1-3* (lane 8)(B3458). 5.0 μg of protein from each extract was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal anti-Bmh2p antibody.

Figure 5.2

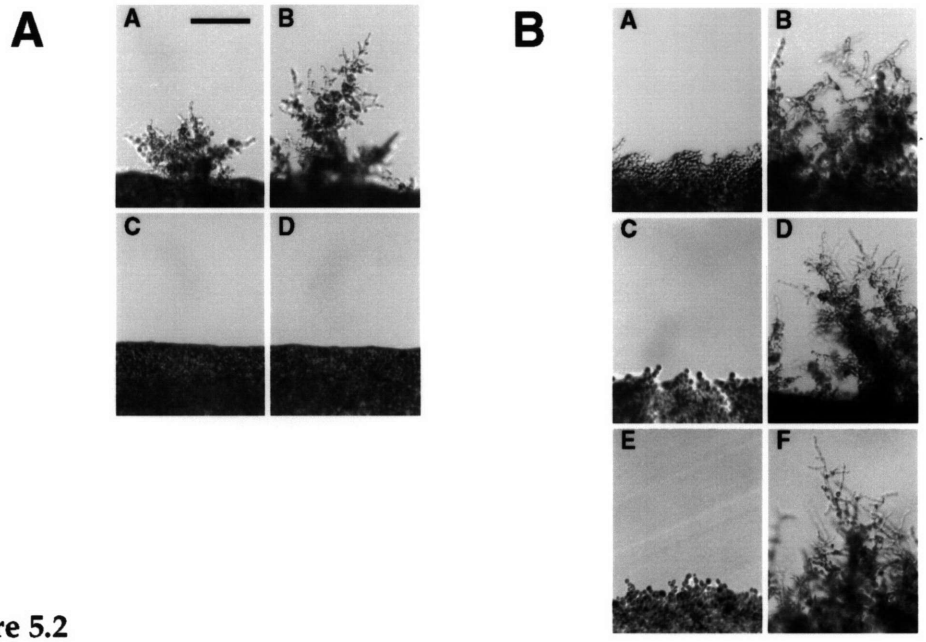

Figure 5.2

14-3-3 proteins are critical for RAS/MAPK signaling during pseudohyphal development.

(A) Diploid *Ste+, Bmh+* (A, B)(RRY1045), *ste20/ste20* (C, D)(L5624) strains containing the *FG(TyA)-lacZ* plasmid (B3161) and the control plasmid pRS316 (A, C)(B1820), or GAL-BMH1 (B, D) were assayed for pseudohyphal development after three days growth on SLAG.

(B) Diploid *bmh1/bmh1 bmh2/bmh2* (A, C, E,)(RRY1257) and *Ste+, Bmh+* (B, D, F)(L6160) strains containing the *FG(TyA)-lacZ* plasmid (B3160 or B3296) and plasmids encoding $RAS2^{VAL19}$ (A, B)(B2255), $CDC42^{VAL12}$ (C, D)(B3079), or *STE11-4* (E, F) (B2616) were assayed for pseudohyphal development after three days on SLAD (A, B, E, F) or SLAR (C, D). The pseudohyphal morphology of RRY1257 containing pRS316 (not shown) is similar to panels A, C, and E. The scale bar represents 30 μM.

Table 5.1

Strain	Genotype *	Source
RRY1	*MATa ura3-52 leu2*	Roberts et al 1994
RRY3	*MATa ura3-52 his3*	Roberts et al 1994
10560-4d	*MATa ura3-52 leu2 trp1*	This study
10560-4a	*MATa ura3-52 leu2 trp1 his3*	This study
RRY1063	*MATa bmh1::HIS3 ura3-52 his3 leu2*	This study
RRY1266	*MATa bmh2::HIS3 ura3-52 his3 leu2*	This study
RRY1216	*MATa bmh1::HIS3 bmh2::HIS3 ura3-52 his3 leu2*	This study
10560-6b	*MATα ura3-52 his3 leu2 trp1*	This study
RRY4	*MATα ura3-52 his3*	This study
RRY1198	*MATα bmh1::HIS3 ura3-52 his3*	This study
RRY1267	*MATα bmh2::HIS3 ura3-52 his3 trp1 leu2*	This study
RRY1217	*MATα bmh1::HIS3 bmh2::HIS3 ura3-52 his3 leu2 trp1*	This study
L5543	*MATa/α ura3-52 his3 trp1*	This study
L6160	*MATa/α ura3-52 leu2*	This study
RRY1045	*MATa/α ura3-52 his3*	This study
L5624	*MATa/α ste20::TRP1 ura3-52 trp1*	Liu et al 1993
RRY1576	*MATa/α ste5::LEU2 ura3-52 leu2*	This study
L5625	*MATa/α ste11::LEU2 ura3-52 leu2*	Liu et al 1993
L5626	*MATa/α ste7::LEU2 ura3-52 leu2*	Liu et al 1993
L5627	*MATa/α ste12::LEU2 ura3-52 leu2*	Liu et al 1993
RRY1064	*MATa/α bmh1::HIS3 ura3-52 his3*	This study
RRY1276	*MATa/α bmh2::HIS3 ura3-52 his3*	This study
RRY1257	*MATa/α bmh1::HIS3 bmh2::HIS3 ura3-52 his3 leu2*	This study

Table 5.1

Strains used in this study.

*The *his3*, *leu2*, and *trp1* alleles are hisG gene replacements. Diploid *MAT*a/α strains are homozygous for all loci indicated. All strains are congenic to the Σ1278b genetic background (Grenson et al., 1966; Liu et al., 1993).

Table 5.2

Strain	Control	*Gal-BMH1*	*GAL-BMH2*
Bmh+	147	159	157
Bmh+ Bmh+	25	26	35

Table 5.2

BMH1 or *BMH2* overexpression does not induce *FG(TyA)-lacZ* signaling.

The diploid (RRY1045)(Bmh+ Bmh+) and haploid (RRY3)(Bmh+) *Ste+ , Bmh+* strain containing the *FG(TyA)-lacZ* plasmid (B3161) and control plasmid pRS316 (B1820), pGAL-BMH1 (B3467) or pGAL-BMH2 (B3468) were starved for nitrogen and assayed for β-galactosidase activity normalized to total protein as described.

Table 5.3

Strain	Control	*RAS2^{VAL19}*	*CDC42^{VAL12}*	*STE11-4*	*GAL-STE12*
Bmh+	91	400	470	490	420
bmh1 bmh2 / *bmh1 bmh2*	11	11	11	20	47

Table 5.3

14-3-3 proteins are required for *FG(TyA)-lacZ* signaling.

Diploid *Ste+* , *Bmh+* (RRY1045 or L6160) and *bmh1/bmh1 bmh2/bmh2* (RRY1257) strains containing the *FG(TyA)-lacZ* plasmid (B3160 or B3161) and control plasmid pRS316 (B1820), *RAS2^{VAL19}* (B2255), *CDC42^{VAL12}* (B3079), *STE11-4* (B2616), or *GAL-STE12* (B2065) were starved for nitrogen and assayed for β-galactosidase activity normalized to total protein as described.

Table 5.4

Strain	Control	*RAS2^{VAL19}*	*CDC42^{VAL12}*	*STE11-4*	*GAL-STE12*
Bmh+	30	60	49	53	53
bmh1 bmh2 / *bmh1 bmh2*	3	3	3	3	3

Table 5.4

14-3-3 proteins are critical for cell elongation during pseudohyphal development.

Diploid *Ste+* , *Bmh+* (RRY1045 or L6160) and *bmh1/bmh1 bmh2/bmh2* (RRY1257) strains containing control plasmid pRS316 (B1820), *RAS2^{VAL19}* (B2255), *CDC42^{VAL12}* (B3079), *STE11-4* (B2616), or *GAL-STE12* (B2065) were starved for nitrogen and scored for the percent of elongated pseudohyphal cells (n = 200) as described.

14-3-3 proteins are not essential for MAPK cascade signaling during mating

We then wished to determine whether *BMH1* and *BMH2* are required for mating, which requires components of the pseudohyphal development MAPK cascade, including *STE20*, *STE11*, *STE7* and *STE12*. Mating consists of several measurable events: cell cycle arrest, induction of the mating-specific reporter *FUS1-lacZ*, the formation of mating projections, and formation of a diploid zygote. When a lawn of a *MAT***a** cells is exposed to a gradient of alpha pheromone, a zone of growth inhibition, or halo, is observed. The diameter of this halo reflects a strain's level of sensitivity to pheromone-mediated cell cycle arrest. The *bmh1 bmh2* mutant strains form halos of approximately the same diameter as isogenic *BMH1 BMH2* strains (Figure 5.3). In response to alpha pheromone, *MAT***a** strains induce the transcription of the mating-specific reporter *FUS1-lacZ*, and form mating projections. We found that pheromone induces *FUS1-lacZ* approximately 100 fold in both *BMH1 BMH2* and *bmh1 bmh2* mutant strains, even though the absolute level of *FUS1-lacZ* is lower in the *bmh1 bmh2* mutant (Table 5.5). In addition, both *BMH1 BMH2* and *bmh1 bmh2* haploid strains form some mating projections under these conditions (Figure 5.3). Finally, quantitative mating assays show that *bmh1 bmh2* strains mate as efficiently as *BMH1 BMH2* strains (98%). In contrast, mutations in components of the mating MAPK cascade (e.g. *ste7* (Zhou et al., 1993) reduce the mating efficiency approximately a million fold. Thus, *bmh1 bmh2* mutant strains do not behave like typical *ste* mutants with respect to pheromone induced growth arrest, induction of *FUS1-lacZ* transcription, formation of mating projections, and diploid formation. In summary, mating MAPK cascade signaling events do not strictly require the 14-3-3 proteins.

Figure 5.3

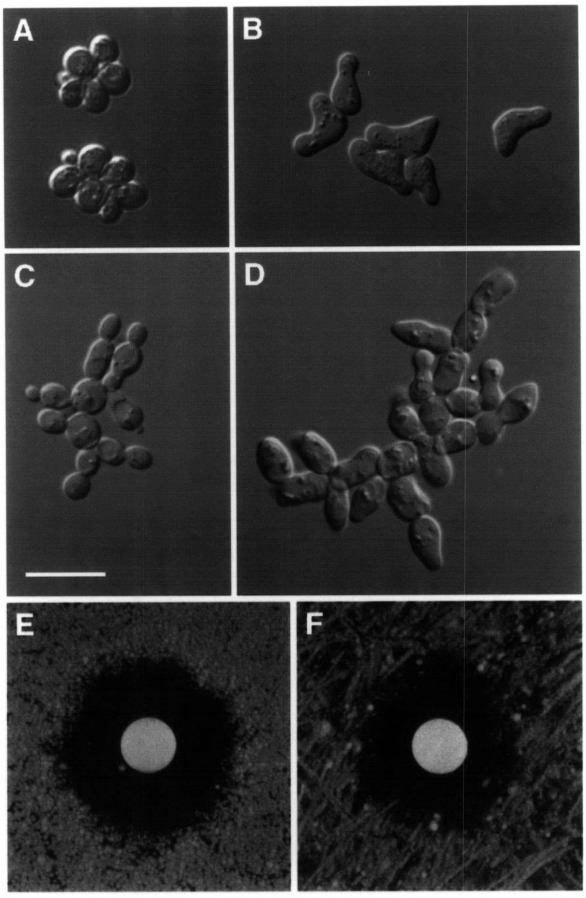

Figure 5.3

14-3-3 proteins are not essential for mating MAPK cascade signaling.

Formation of mating projections: haploid *MAT**a** BMH1 BMH2* (A, B)(RRY3) and *bmh1 bmh2* (C, D)(RRY1216) strains were incubated in the absence (A, C) or presence (B, D) of 5 μM alpha pheromone and photographed. The scale bar for panels A-D represents 10 μM. Pheromone-induced cell cycle arrest: haploid *MAT**a** BMH1 BMH2* (E)(RRY3) and *bmh1 bmh2* (F)(RRY1216) strains were incubated for four days with a filter containing 10 nanomoles of alpha pheromone and then photographed.

Table 5.5

Strain	- pheromone	+ pheromone
BMH1 BMH2	**8.6**	**880**
bmh1 bmh2	**1.5**	**150**

Table 5.5

14-3-3 proteins are not essential for *FUS1-lacZ* induction during mating.

Haploid *BMH1 BMH2* (RRY3) and *bmh1 bmh2* (RRY1216) *MAT**a*** strains containing the *FUS1-lacZ* plasmid pJB207 (B2146) were incubated in the absence (-) or presence (+) of 5 μM alpha pheromone. Extracts were prepared and assayed for β-galactosidase activity normalized to total protein as described.

Strains lacking *BMH1* and *BMH2* are sensitive to several environmental stresses

In addition to its role in MAPK cascade signaling during pseudohyphal development, *RAS2* independently regulates the accumulation of the storage carbohydrate glycogen via cAMP and PKA. We assayed the glycogen accumulation patterns of our mutant strains and found that *bmh1 bmh2* strains accumulate abnormally high levels of glycogen. The overaccumulation of glycogen by *bmh1 bmh2* strains is suppressed by $RAS2^{VAL19}$ and overexpression of *TPK1* (Data not shown), suggesting that the 14-3-3 proteins are important for RAS/PKA signaling.

Although *bmh1 bmh2* double mutant strains grow at 30°C, they exhibit many phenotypes. They fail to grow at 34°C, 36°C, or 37°C and this reduced viability is ameliorated by the addition of 1.0 M sorbitol (Figure 5.4). In addition, *bmh1 bmh2* strains are sensitive to 5 mM caffeine, high osmolarity (1.5 M sorbitol), and growth on galactose medium (Figure 5.4). After long incubations, *bmh1 bmh2* strains form small colonies on 2% galactose and 1.5 M sorbitol, but they do not form colonies on 5 mM caffeine or at 34°C at all. Furthermore, *bmh1 bmh2* diploid strains have a defect in sporulation: they fail to form tetrads on sporulation medium (0/200 versus 65/200 for Bmh^+). Finally, *bmh1 bmh2* haploid cells form buds on the free (distal) end of each new daughter cell instead of the birth (proximal) end (Figure 5.3a, c). Nevertheless, *bmh1 bmh2* strains grow on glucose, raffinose, glycerol and minimal medium. We found that *bmh1* and *bmh2* single mutant strains do not exhibit any detectable phenotypic differences from Bmh^+ strains, indicating that *BMH1* and *BMH2* are functionally redundant. In summary, *bmh1 bmh2* strains are sensitive to several environmental stresses.

Alleles of *BMH1* defective in *FG(TyA)-lacZ* signaling

The fact that *bmh1 bmh2* double mutant strains are defective in some signaling pathways but not others suggests some specificity to the function of 14-3-3 proteins. In agreement with this, both *STE11-4* and *GAL-STE12* suppress the *bmh1 bmh2 FG(TyA)-lacZ* signaling defect but none of the other *bmh1 bmh2* phenotypes (Table 5.3, data not shown). Similarly, $RAS2^{VAL19}$ and overexpression of *TPK1* suppress the *bmh1 bmh2* glycogen accumulation defect, but neither one suppresses any other *bmh1 bmh2* phenotypes (data not shown). These data suggest the 14-3-3 proteins may control each of these processes independently.

To test explicitly whether different 14-3-3 functions are independent of one another, we screened for alleles of *BMH1* specifically defective in *FG(TyA)-lacZ* signaling. We introduced a mutagenized library of *BMH1* plasmids into a *bmh1 bmh2* diploid strain carrying the *FG(TyA)-lacZ* reporter. A screen of the transformants identified three *bmh1* alleles, *bmh1-1*, *bmh1-2*, and *bmh1-3*, that grew at 36°C and showed reduced *FG(TyA)-lacZ* expression (Figure 5.4). All three mutant alleles complement the *bmh1 bmh2* sensitivity to caffeine, 1.5 M sorbitol, and defects in cell elongation, glycogen storage, budding, and growth on galactose (Figure 5.4). Moreover, these alleles show basal and induced levels of *FUS1-lacZ* activity comparable to their *BMH1* counterparts. Specifically, strains carrying *bmh1-1* are indistinguishable from the *BMH1* control in both basal and induced levels of *FUS1-lacZ* while strains carrying *bmh1-2* and *bmh1-3* show slightly reduced *FUS1-lacZ* activity (Table 5.6).

Western analysis reveals that Bmh1p, bmh1-1p, bmh1-2p, and bmh1-3p are all abundantly expressed *in vivo* (Figure 5.1). Bmh^+ strains carrying these alleles do not show any phenotypic differences from Bmh^+ strains, indicating that these alleles are recessive. Taken together, these data suggest that the reduced *FG(TyA)-lacZ* signaling of *bmh1 bmh2* strains is a result of a specific defect in RAS/MAPK cascade signaling and not a more general defect.

Figure 5.4

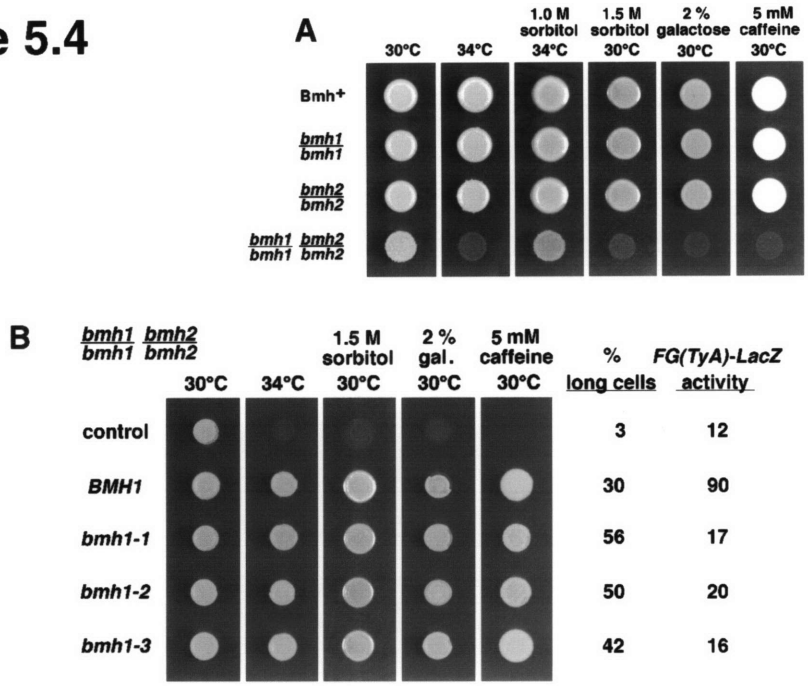

Figure 5.4

Dissecting the functions of 14-3-3 proteins.

(A) Diploid *Bmh+* (row 1, top)(RRY1045), *bmh1/bmh1* (row 2)(RRY1064), *bmh2/bmh2* (row 3)(RRY1276), and *bmh1/bmh1 bmh2/bmh2* (row 4)(RRY1257) strains were photographed after 48 hours of growth on plates containing YPD (column 1 (left), 2) YPD + 1.0 M sorbitol (column 3), YPD + 1.5 M sorbitol (column 4), YP 2% galactose (column 5), or YPD + 5 mM caffeine (column 6) at 30°C (columns 1, 3, 4, 5, 6) or 34°C (columns 2, 3).

(B) Diploid *bmh1/bmh1 bmh2/bmh2* (RRY1257) strain carrying the *FG(TyA)-lacZ* reporter plasmid (B3296) and control plasmid pRS316 (row 1, top)(B1820), *BMH1* (row2)(B3455), *bmh1-1* (row 3)(B3456), *bmh1-2* (row 4)(B3457), or *bmh1-3* (row 5)(B3458) was photographed after 48 hours of growth on plates containing YPD (column 1 (left), 2), YPD + 1.5 M sorbitol (column 3), YP 2% galactose (column 4), or YPD + 5 mM caffeine (column 5) at 30°C (columns 1, 3, 4, 5) or 34°C (column 2). The same strains were assayed for the percent of long pseudohyphal cells (column 6)(% long cells)(n = 200) and for *FG(TyA)-lacZ* activity (column 7)(*FG(TyA)-lacZ* activity) under conditions of nitrogen starvation as described.

Figure 5.5

Figure 5.5

BMH1 mutant allele sequence changes.

(A) Residues encompassing helix 3 of *BMH1* (39 to 73) and the positions of the *bmh1-2* and *bmh1-3* mutations are shown along with their alignments to other 14-3-3 homologs.

(B) The region surrounding helix 9 of *BMH1* (212 to 241) and the position of the *bmh1-1* mutation are shown along with their alignments to other 14-3-3 homologs. Periods denote amino acids identical to those of *BMH1*.

Figure 5.6

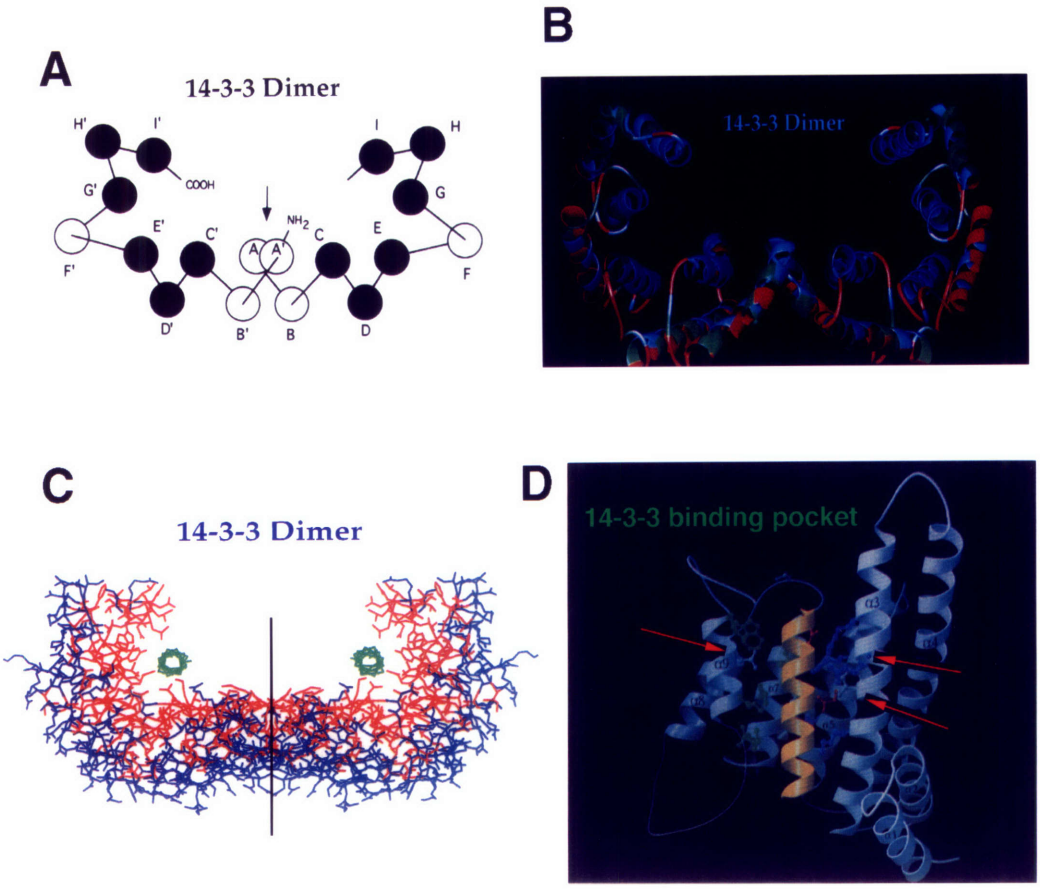

Figure 5.6

Mapping *bmh1* mutations on the 14-3-3 crystal structure.

The side view of the 14-3-3 dimer crystal structure is shown with the nine alpha helices and two-fold axis of symmetry indicated (Arrow)(A, B, C). A putative alpha helix of a substrate is shown docked into the cleft (C = green) (D = yellow). The putative substrate binding cleft is shown (D) with the amino acids thought to contact the substrate indicated in green. The positions of *bmh1* mutant alleles described in this study are indicated by red arrows (D). Adapted from Liu et al., 1995 and Xiao et al., 1995.

Table 5.6

Allele	- pheromone	+ pheromone
BMH1	**3.0**	**145**
bmh1-1	**2.9**	**164**
bmh1-2	**1.8**	**65**
bmh1-3	**1.3**	**99**

Table 5.6

FUS1-lacZ expression in strains carrying mutant *bmh1* alleles.

The diploid *bmh1/bmh1 bmh2/bmh2* (RRY1257) strain carrying the *FUS1-lacZ* reporter plasmid (B2146) and*BMH1* (row1)(B3455), *bmh1-1* (row 2)(B3456), *bmh1-2* (row 3)(B3457), or *bmh1-3* (row 4)(B3458) was incubated in the presence or absence of alpha factor mating pheromone and assayed for β-galactosidase activity normalized to total protein as described.

BMH1 mutant allele sequence changes lie in the putative substrate binding pocket

To determine the molecular defects of our *bmh1-1*, *bmh1-2*, and *bmh1-3* alleles, we isolated each of the plasmids and sequenced the entire open reading frame and several hundred base pairs of flanking DNA. Each mutant had only a single guanine to adenine substitution. The *bmh1-1* mutation is predicted to change leucine 232 to serine in helix 9. The *bmh1-2* mutation is predicted to change glycine 55 to aspartate in helix 3. Both of these residues are absolutely conserved (Figure 5.5). The *bmh1-3* mutation is predicted to change alanine 59 to threonine in helix 3. The only two amino acids normally found at this position are alanine and serine (Figure 5.5). When these mutations are mapped on the 14-3-3 dimer crystal structure, all three fall in the cleft of the putative alpha helix binding pocket, even though these amino acids lie in distant parts of the primary sequence (Liu et al., 1995; Xiao et al., 1995)(Figure 5.6). Furthermore, all three amino acids side chains could project directly into this binding pocket. These data suggest that these mutant proteins may have altered binding affinities for different 14-3-3 substrates.

Bmh1p and Bmh2p associate with GST-STE20 *in vivo*

As *bmh1 bmh2* strains are defective in *RAS2* and *CDC42* signaling to the *FG(TyA)-lacZ* reporter, and this defect was bypassed by *STE11-4* and overexpression of *STE12*, we suspected that the 14-3-3 proteins act downstream of *CDC42* and upstream of *STE11* at the level of *STE20*. To test if the 14-3-3 proteins associate with Ste20p, we constructed an in-frame fusion between GST and *STE20* that complements *STE20* functions for both mating and pseudohyphal development. Vectors containing either GST-STE20, GST-STE20ΔN, GST-SEC23, or GST alone under the control of the GAL1-10 promoter were introduced into yeast strains.

Fusion proteins were induced and purified with glutathione agarose to isolate each fusion and any associated proteins. Proteins bound to glutathione agarose were denatured and probed with an anti-Bmh2p polyclonal antibody or an anti-GST polyclonal antibody. Western analysis shows that both Bmh1p and Bmh2p copurify with GST-STE20 but not with the GST-SEC23 or GST controls (Figure 5.7a). We obtained similar results in both haploid and diploid strains. Moreover, the association of the 14-3-3 proteins with GST-STE20 does not depend on *STE11*, *STE5*, *STE7*, or *STE12* (Figure 5.7b). Bmh1p and Bmh2p associate with GST-STE20ΔN, which lacks the N-terminal 493 amino acids of *STE20* including the *CDC42* binding domain (Figure 5.7a). Thus, both Bmh1p and Bmh2p associate specifically with GST-STE20 *in vivo*, and this is independent of other MAPK cascade components and the N-terminal portion of *STE20*.

Mutant *bmh1* proteins associate weakly with GST-STE20

To test whether our mutant *bmh1* proteins associate with GST-STE20, we transformed a *bmh1 bmh2* diploid strain with a plasmid carrying *BMH1*, *bmh1-1*, *bmh1-2* or *bmh1-3* and a plasmid containing either GST-STE20 or GST alone. We induced the fusion proteins and purified them using glutathione agarose. Bound proteins were denatured and subjected to western analyses with an anti-GST polyclonal antibody and an anti-Bmh2p polyclonal antibody. Bmh1p associates strongly with GST-STE20 but not with GST alone (Figure 5.7c). Significantly less of the bmh1-1p, bmh1-2p, and bmh1-3p mutant proteins copurifies with GST-STE20 relative to Bmh1p, although some of each mutant protein was still detectable (Figure 5.7c). These data demonstrate that the bmh1-1p, bmh1-2p, and bmh1-3p mutant proteins have a reduced capacity to associate with GST-STE20 *in vivo*.

Figure 5.7

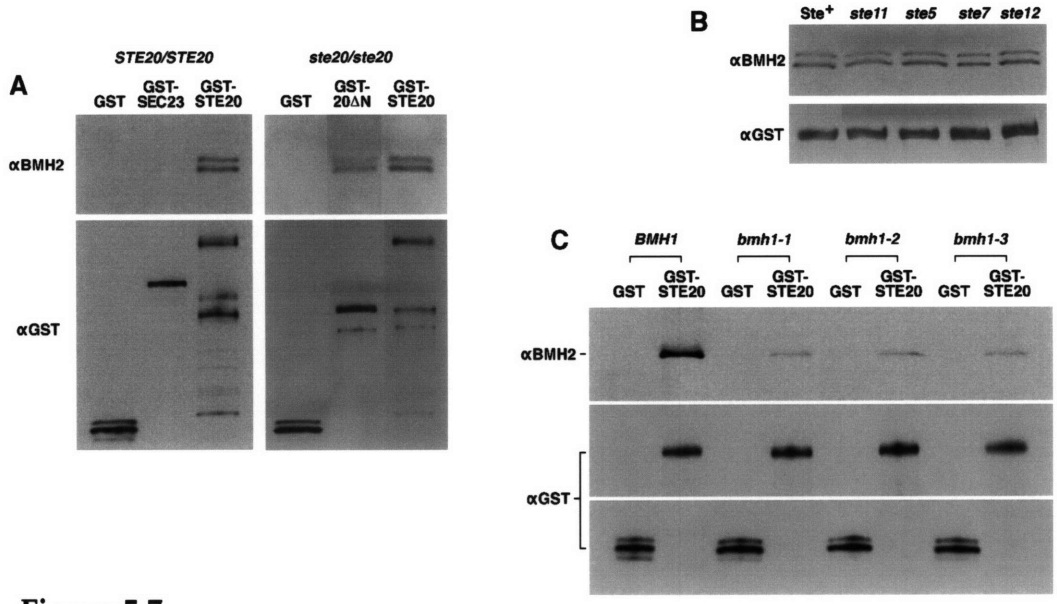

Figure 5.7

Bmh1p and Bmh2p associate with GST-STE20 in vivo.

(A) Diploid *Bmh+ Ste+* (L5543)(*STE20/STE20*) and *Bmh+ ste20* (L5624)(*ste20/ste20*) strains carrying plasmids encoding GST alone (B3298), GST-SEC23 (B3554), GST-STE20ΔN (B3538) or GST-STE20 (B3553).

(B) Diploid *Ste+, Bmh+* (L5543), *ste11/ste11* (L5625), *ste7/ste7* (L5626), *ste5/ste5* (RRY1576), and *ste12/ste12* (L5627) strains carrying a plasmid encoding GST-STE20 (B3553).

(C) Diploid *bmh1/bmh1 bmh2/bmh2* (RRY1257) strains carrying *BMH1* (B3469), *bmh1-1* (B3470), *bmh1-2* (B3471), or *bmh1-3* (B3472) and plasmids encoding GST alone (B3298) or GST-STE20 (B3553). Extracts were prepared and fusion proteins were purified as described. Equivalent amounts of each sample were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal anti-Bmh2p antibody (upper panel) or a polyclonal anti-GST antibody (lower panels).

Figure 5.8

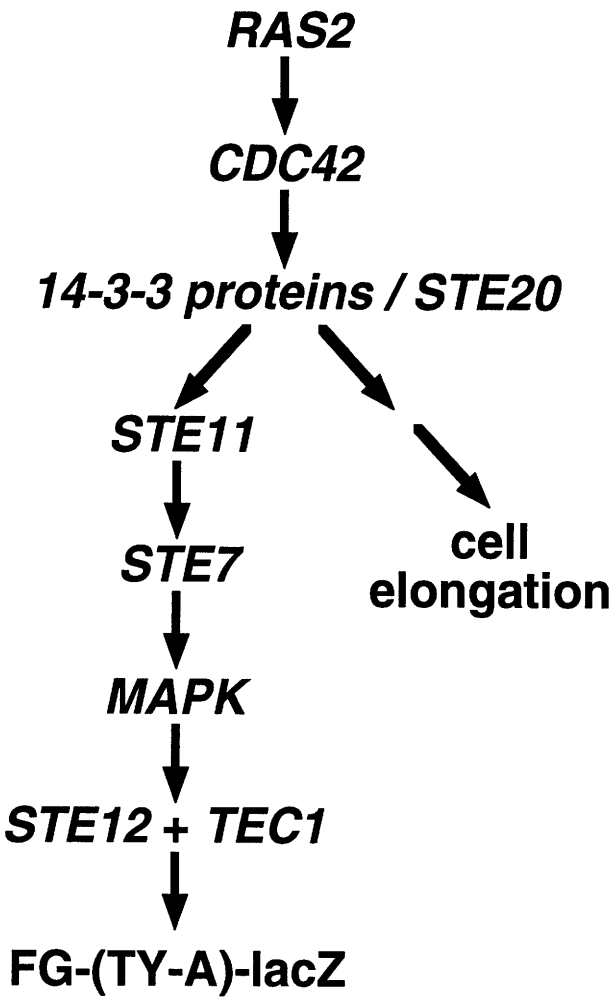

Figure 5.8

Model for the role of 14-3-3 proteins in RAS/MAPK cascade signaling during pseudohyphal development.

Discussion

The 14-3-3 proteins are highly conserved, ubiquitous proteins that have been implicated in a variety of processes, including protein kinase C regulation, tryptophan hydroxylase activation, Ca^{2+}-dependent exocytosis, and Ras/Raf/MAPK cascade signaling (Aitken et al., 1992; Chamberlain et al., 1995; Fantl et al., 1994; Ichimura et al., 1995; Li et al., 1995; Toker et al., 1992). In none of these cases has loss of 14-3-3 function been shown to affect the relevant process *in vivo*. Human Raf expressed in yeast can be activated by RAS in a *BMH1*-dependent manner to signal via the mating MAPK cascade (Irie et al., 1994). However, the interpretation of this experiment is complicated by the requirement for RAS overexpression and the activated $STE7^{P368}$ allele, neither of which is involved in endogenous signaling via the mating MAPK cascade. In order to test directly whether the 14-3-3 proteins are required for RAS/MAPK cascade signaling in *S. cerevisiae in vivo*, we deleted the genes encoding them (*BMH1* and *BMH2*). Double mutant *bmh1 bmh2* strains in the dimorphic Σ1278b strain background are viable, in contrast to *S. pombe* and other *S. cerevisiae* strains that lack 14-3-3 function (Ford et al., 1994; Gelperin et al., 1995; van Heusden et al., 1996). These viable *bmh1 bmh2* strains enable us to study RAS/MAPK cascade signaling in the absence of 14-3-3 protein function.

Although *bmh1 bmh2* double mutant strains are viable, they are defective in RAS/MAPK cascade signaling during pseudohyphal development. The *STE12*-dependent transcriptional reporter *FG(TyA)-lacZ* provides an accurate reflection of pseudohyphal development MAPK cascade signaling (Mosch et al., 1996). This reporter is strongly induced by the activated alleles $RAS2^{VAL19}$, $CDC42^{VAL12}$, and *STE11-4*, and by overexpression of *STE12*. We found that *bmh1 bmh2* mutant strains show greatly reduced *FG(TyA)-lacZ* expression relative to Bmh^+ strains, and $RAS2^{VAL19}$ and $CDC42^{VAL12}$ completely fail to bypass this defect. In contrast, *STE11-4*

and overexpression of *STE12* both induce *FG(TyA)-lacZ* in these strains, suggesting that the reduced *FG(TyA)-lacZ* activity of the *bmh1 bmh2* double mutant reflects a specific signaling defect and not a general defect in transcriptional induction. Thus, the 14-3-3 proteins are required for RAS/MAPK cascade signaling *in vivo*.

The 14-3-3 proteins are not essential for signaling via the mating-MAPK cascade, which shares common components with the pseudohyphal development MAPK cascade. Specifically, *bmh1 bmh2* mutant strains arrest growth, form mating projections, and undergo cell and nuclear fusion to form normal proportions of diploid cells. The basal and induced levels of *FUS1-lacZ* are lower in *bmh1 bmh2* strains than in *BMH1 BMH2* strains, which may reflect slight MAPK signaling attenuation or a general reduction in basal transcription. Nevertheless, alpha pheromone induces the mating-specific reporter *FUS1-lacZ* 100 fold in both *BMH1 BMH2* and *bmh1 bmh2* mutant strains, indicating that 14-3-3 proteins are not essential for mating MAPK cascade-mediated transcriptional induction. These data suggest that the *FG(TyA)-lacZ* signaling defect of *bmh1 bmh2* strains reflects a specific defect in RAS/MAPK cascade signaling rather than a more general defect.

Pseudohyphal development is a composite of several distinct morphological events: cell elongation, filament formation, and agar invasion. Mutations in *STE20*, *STE11*, *STE7*, *STE12*, and *TEC1* affect all of these processes. In addition, downstream genes have been identified that only affect subsets of these processes. For example, tropomyosin (*TPM1*) is required for cell elongation and filament formation but not agar invasion, whereas *BUD1* and *BUD8* are required for filament formation but not cell elongation or agar invasion (Mosch and Fink, 1997; Roberts, 1994). Like *TPM1*, *BMH1* and *BMH2* are required for filament formation and cell elongation but not agar invasion. Though their primary effect on morphogenesis may be independent of the pseudohyphal development MAPK cascade, the 14-3-3 proteins are critical for *RAS2* and *CDC42* induction of cell elongation.

In addition to their defects in RAS/MAPK cascade signaling, *bmh1 bmh2* double mutant strains are sensitive to several environmental stresses. They accumulate abnormally high levels of the storage carbohydrate glycogen, and this is suppressed by $RAS2^{VAL19}$ or overexpression of the PKA kinase *TPK1*. This result suggests that the 14-3-3 proteins are required for RAS signaling via the PKA pathway. The *bmh1 bmh2* strains also exhibit osmo-remediated temperature sensitivity, sensitivity to caffeine, sensitivity to 1.5 M sorbitol, and defects in sporulation. Interestingly, the *bmh1* and *bmh2* single mutant strains do not manifest any of these phenotypes. Thus, *BMH1* and *BMH2* are functionally interchangeable and 14-3-3 protein homo- and heterodimer species do not appear to have distinct functions in yeast, as has been proposed for other organisms (Jones et al., 1995).

Despite the multiplicity of phenotypes of *bmh1 bmh2* mutant strains, our data suggest that these phenotypes are not interdependent. Specifically, $RAS2^{VAL19}$ and overexpression of *TPK1* suppress the glycogen storage defects of *bmh1 bmh2* mutant strains but do not suppress any other *bmh1 bmh2* phenotypes. Similarly, *STE11-4* and overexpression of *STE12* suppress the *FG(TyA)-lacZ* signaling defects of *bmh1 bmh2* mutant strains but do not suppress any other *bmh1 bmh2* phenotypes. Three recessive alleles of *BMH1*, *bmh1-1*, *bmh1-2* and *bmh1-3*, encode proteins defective in *FG(TyA)-lacZ* signaling. Importantly, these mutant proteins rescue other *bmh1 bmh2* phenotypes: temperature sensitivity, sorbitol sensitivity, caffeine sensitivity, glycogen accumulation, cell elongation, and galactose growth defects. Moreover, these alleles show basal and induced levels of *FUS1-lacZ* activity comparable to their *BMH1* counterparts. Thus, the reduced *FG(TyA)-lacZ* expression of strains carrying these *bmh1* alleles is probably is not caused by a general defect in *lacZ* expression and function in these strains. In summary, it is possible to destroy the function of *BMH1* in the pseudohyphal development MAPK

cascade without simultaneously abolishing its other functions. These data indicate that the *FG(TyA)-lacZ* signaling defect of *bmh1 bmh2* strains is not a consequence of a general growth defect.

Double mutant *bmh1 bmh2* strains are defective in *RAS2* and *CDC42* signaling to the *FG(TyA)-lacZ* reporter, and this defect is suppressed by *STE11-4* and overexpression of *STE12*, suggesting a role for the 14-3-3 proteins downstream of *CDC42* and upstream of *STE11*. Because *STE20* acts at this step, we suspected that the 14-3-3 proteins might associate with Ste20p. Both Bmh1p and Bmh2p associate with GST-STE20 *in vivo* but do not associate with GST alone or GST-SEC23, a yeast protein used as a control. Though we do not know if Bmh1p binds Ste20p directly, the association of these proteins does not depend on other MAPK cascade components: Ste11p, Ste5p, Ste7p or Ste12p. Moreover, Bmh1p and Bmh2p associate with GST-STE20ΔN, a protein that lacks the N-terminal 493 amino acids and the Cdc42p binding domain of Ste20p. These data indicate that the Bmhp/Ste20p association does not require the Cdc42p binding domain of Ste20p. The implication is that Ste20p/Bmhp association does not require the activation or binding of Ste20p by Cdc42p. Similarly, the binding of 14-3-3 isoforms to the vertebrate RAF kinase does not require kinase activation or binding by RAS (Freed et al., 1994; Muslin et al., 1996). Although the 14-3-3 proteins may also bind other pertinant substrates, our finding that Bmh1p and Bmh2p associate with Ste20p is consistent with our genetic data that these proteins act between Cdc42p and Ste11p.

All three of our mutant *bmh1* proteins show a much weaker association with Ste20p *in vivo* relative to the Bmh1p control. Strikingly, the *bmh1-1*, *bmh1-2*, and *bmh1-3* mutations all fall in the amphipathic groove that lines the putative substrate binding pocket, and the side chains of all three amino acids project directly into this pocket. Thus, amino acids in the putative 14-3-3 substrate binding pocket are critical for the association between Bmh1p and Ste20p. Although these data

suggest that a Bmhp/Ste20p association is essential for *FG(TyA)-lacZ* signaling, *bmh1 bmh2* mutant diploid strains show lower *FG(TyA)-lacZ* signaling (13-18% of wild-type) than *ste20/ste20* strains (50% of wild-type)(Table 5.3, Figure 5.4b and (Mosch et al., 1996)). This difference raises the possibility that the 14-3-3 proteins may interact with an additional protein required for *FG(TyA)-lacZ* signaling. Cla4p and YOL113w are good candidates for such proteins as they both show high homology to Ste20p and bind Cdc42p (Peter, 1996). Nevertheless, our data suggest that *BMH1* and *BMH2* act downstream of *RAS2* and *CDC42*, at the level of *STE20*, to control cell elongation and *FG(TyA)-lacZ* signaling (Figure 5.8).

Our data reveal a disparity between *FG(TyA)-lacZ* signaling and cell elongation. The three *BMH1* alleles we isolated show reduced *FG(TyA)-lacZ* signaling yet form high percentages of long cells. Furthermore, *STE11-4* and *GAL-STE12* fail to suppress the *bmh1 bmh2* cell elongation defect, even though they suppress the *bmh1 bmh2* defect in *FG(TyA)-lacZ* signaling. One possibility is that *STE20* has an output independent of *STE11*, *STE7*, *STE12*, and *TEC1* important for cell elongation (Figure 5.8). In support of this model, mutations in *STE11*, *STE7* and *STE12* completely block $RAS2^{VAL19}$ induction of *FG(TyA)-lacZ* but not its induction of cell elongation (See (Mosch et al., 1996). Moreover, the *ste20/ste20* mutant makes fewer long cells than other MAPK cascade mutants and this defect is only partially bypassed by *STE11-4*. Together, these data suggest that *STE20* has an important role in morphogenesis independent of the MAPK cascade. According to this model, our *bmh1* mutant alleles are defective in *FG(TyA)-lacZ* signaling via *STE11*, *STE7* and *STE12* but retain cell elongation functions independent of the MAPK cascade. By analogy, mammalian RAC mutations differentially affect the JNK MAPK signaling and actin morphogenesis outputs of this G protein (Lamarche, 1996). This disparity between the MAPK cascade signaling and morphogenetic outputs of small G proteins is an emerging theme in eukaryotic signal transduction.

Several lines of evidence suggest that the reduced *FG(TyA)-lacZ* signaling of *bmh1 bmh2* strains reflects a specific defect in RAS/MAPK cascade signaling and not a more general defect of these strains. Most importantly, there are *BMH1* alleles defective in *FG(TyA)-lacZ* signaling that complement the other *bmh1 bmh2* phenotypes. Moreover, $RAS2^{VAL19}$ and $CDC42^{VAL12}$ fail to induce *FG(TyA)-lacZ* in *bmh1 bmh2* mutant strains, whereas *STE11-4* and *GAL-STE12* induce the reporter but do not suppress any other *bmh1 bmh2* phenotypes. In addition, alpha pheromone strongly induces the mating-specific transcriptional reporter *FUS1-lacZ* in both *BMH1 BMH2* and *bmh1 bmh2* strains, indicating that the 14-3-3 proteins are not essential for mating MAPK cascade-dependent transcriptional induction. Taken together, these data strongly suggest that the *bmh1 bmh2* defect in RAS/MAPK cascade signaling during pseudohyphal development reflects a specific signaling defect. Because the 14-3-3 proteins and other elements of the pseudohyphal development RAS/MAPK cascade are highly conserved in evolution, we expect that our studies in *S. cerevisiae* will ultimately provide a paradigm for understanding their functions in all eukaryotes.

Materials and Methods

Yeast strains and growth conditions

Standard yeast culture medium was prepared as described (Sherman et al., 1986). Except where noted, all yeast strains were incubated at 30°C. Synthetic low ammonia medium used to induce pseudohyphal development was prepared with different carbon sources (SLAD = 2% glucose)(SLAR = 3% raffinose)(SLAG = 2% glucose) as described (Gimeno et al., 1992). Where indicated, plates were supplemented with 2% galactose, 1.0 or 1.5 M sorbitol, or 5 mM caffeine. Sporulation was induced by incubating strains for two days on a GNA plate and

transferring them to a SPOR plate (Sherman et al., 1986). Samples were scored for the frequency of four spore tetrads. Scoring growth on plates: patches of strains from plates were resuspended in 100 µl medium in a 96 well plate. Samples were diluted in a five fold series and gently stamped onto each target plate using a sterile multi-pronged device. Dilutions with comparable numbers of colony forming units were compared.

All yeast strains used in this study are congenic to the Σ1278b genetic background (Table 1)(Grenson et al., 1966; Liu et al., 1993). Strains carrying *bmh1::HIS3* and *bmh2::HIS3* deletions were constructed by transforming the RRY1045 parent strain with EcoRI+HincII cut B3453 and B3454 plasmids respectively. Southern analysis confirmed that the open reading frames for *BMH1* and *BMH2* were replaced by *HIS3* sequences. All *bmh1 bmh2* mutant spores showed identical phenotypes that were rescued by *BMH1* (B3455) or *BMH2* (B3475).

Plasmids

Deletion plasmids: (i) DNA fragments flanking the *BMH1* and *BMH2* open reading frames were amplified from genomic DNA using polymerase chain reaction (PCR). The *BMH1* 5′ flanking region was amplified using GAGATCTGCTTTTTCTTCTCACTTGCTTGCG and GGCCGGGGCGCGGGGGGACGAGG, while the *BMH1* 3′ flanking region was amplified using CAGATCTCCAAAGTAAGTATTCTGATAAATC and CTAAAGTTGCTTCTCGCTATACC. Primers AATATTCATCATCAAAGGTTACG and GAGATCTGAGTATTAACCTTCTGTCCAAG were used to amplify the *BMH2* 5′ region while GAGATCTCCAGCTGAACAAACTCAAGGTG and GATATTATCTCTTCCCCTAAAAAAAG were used to amplify the *BMH2* 3′ region. PCR fragments were cloned separately into oligo-dT primed pBluescriptKS (Strategene) at the EcoRV site. (ii) 5′ and 3′ fragments were subcloned together using

BglII (in primers) and XhoI restriction sites. (iii) a 1.8kb *HIS3* BamHI fragment was then cloned into the BglII site to yield pbmh1::HIS3 (B3453) and pbmh2::HIS3 (B3454).

Plasmid pYGEX-STE20 (B3553), which expresses GST-STE20 from the GAL1-10 promoter: (i) insertion of a 360 bp BglII-XbaI STE20 fragment into the BamHI and XbaI sites of plasmid pYGEX-2T (Schlenstedt et al., 1995). The 360 bp BglII-XbaI STE20 fragment was obtained by PCR using the primer GGCCGAAGATCTAGCAATGATCCATCTGCTGT and primer CTGTAGAATCTGTTAACGACAG, which was subsequently digested with BglII and XbaI. (ii) a genomic 3 kb XbaI *STE20* fragment from plasmid pSTE20-5 was then inserted into the XbaI site of construct (i) to yield plasmid pYGEX-STE20 (B3553) (Leberer, 1992). Plasmid GST-STE20ΔN (B3538), with the C-terminal 494 amino acids of *STE20* fused to GST, was constructed by cloning an EcoRI-KpnI fragment from pYGEX-STE20 into an EcoRI-KpnI cut derivative of pRD56.

RAS2^{VAL19} was expressed from YCp50-RAS2^{VAL19} (B2255). *CDC42^{VAL12}* was expressed from the GAL1-10 promoter on plasmid YCpCDC42^{VAL12} (B3079). *STE11-4* was expressed from pSL1509 (B2616) (Cairns et al., 1992). *STE12* was overexpressed from the GAL1-10 promoter on pNC252 (B2065)(Dolan and Fields, 1990). *TPK1* was overexpressed on the 2μ plasmid YEpTPK1 (B2258)(Toda, 1987). pRS316 was used as a control plasmid (B1820). The *FG(TyA)-lacZ* reporter was expressed from plasmids with the *URA3* (B3160), *LEU2* (B3296), and *HIS3* (B3161) markers (Mosch et al., 1996). GST fusions were expressed from the GAL1-10 promoter: pRD56 (GST alone = B3298) or pPE119 (GST-SEC23 = B3554)(Espenshade et al., 1995). Plasmids expressing *BMH1* (B3467) and *BMH2* (B3468) from the GAL1-10 promoter were isolated from a pRS316-based cDNA library. Plasmids carrying the genomic copies of *BMH1* (B3455) and *BMH2* (B3475) genes were isolated from a pCT3 genomic library. B3455 grown in mutagenic XL1-red cells (Stratagene) yielded *bmh1-1* (B3456), *bmh1-2* (B3457), and

bmh1-3 (B3458). SacI-XhoI fragments from these plasmids were subcloned to SacI-XhoI cut pRS315 (B1819) to yield LEU2-marked plasmids containing *BMH1* (B3469), *bmh1-1* (B3470), *bmh1-2* (B3471), and *bmh1-3* (B3472). The *FUS1-lacZ* reporter was expressed from plasmid pJB207 (B2146). Southern analysis: genomic DNA was prepared (Sherman et al., 1986), digested with restriction endonucleases, run on an agarose gel, transferred to Hybond N+ nitrocellulose (Hybond), and probed with fragments of *BMH1* and *BMH2* in Church buffer at 65°C. For low stringency, blots were washed at room temperature in 2x SSC, 0.5 % SDS. For high stringency, blots were washed at 65°C in 2x SSC, 0.5 % SDS and 0.2x SSC, 0.5 % SDS. Xomat X-AR film (Kodak) was used to expose blots.

Pseudohyphal development assays

Pseudohyphal development: samples were photographed with a Zeiss WL light microscope with a 35mm camera using Technical Pan film (Kodak) after three days on SLAD, SLAR, or SLAG medium. Cell elongation: pseudohyphal filaments were isolated from strains grown on SLAD, SLAR, or SLAG medium for four days and the percentage of pseudohyphal cells (n = 200) observed in each strain was determined by microscopic examination (Mosch et al., 1996). Pseudohyphal cells were defined as those cells with a length to width ratio greater than two.

β-galactosidase assays

FG(TyA)-lacZ activity: extracts from strains starved for nitrogen were prepared and assayed for β-galactosidase activity (Mosch et al., 1996). β-galactosidase activity was normalized to the total protein in each extract. Assays were performed on three independent transformants and the mean value is presented. *FUS1-lacZ* activity: strains were grown to approximately OD_{600} 0.6 at 30°C and exposed to 5 μM

alpha factor for two hours at 30°C. Crude extracts were prepared and assayed as described.

Mating assays

Quantitative matings: 200 µl of overnight cultures of *MAT***a** (RRY1216 or RRY1) and *MAT*α (RRY4) strains were mixed in 1.0 ml YPD, gently pelleted, incubated six hours at 30°C, and plated. Mating efficiency was defined as the number of diploid colonies divided by the number of colonies formed by the haploid *MAT***a** parent. *BMH1 BMH2* and *bmh1 bmh2* haploid strains form clumps that contain 3-10 cells that are not readily dispersed. Therefore, this assay will not detect small reductions in mating efficiency. Formation of mating projections: haploid *MAT***a** strains were grown to OD_{600} 0.6, incubated in YPD for four hours in the absence or presence of 5 µM alpha pheromone and photographed with a Zeiss axioscope using Technical Pan film (Kodak). Halo assay: dilute samples of overnight cultures were spread on a YPD plate and allowed to dry. A sterile disc with 5 µl of a 2 mM of alpha pheromone/DMSO stock was added to the center of the plate. Plates were incubated four days at room temperature and photographed with Technical Pan film (Kodak). Plates with *MAT*α cells or DMSO alone did not produce halos.

Protein analysis

Whole cell extracts: strains were grown to OD_{600} 0.6 in 2% glucose. Cells were chilled, washed in buffer B (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA), lysed with glass beads in buffer B + PIM (1 mM PMSF, and 5 µg/ml each of aprotinin, antipain, leupeptin, and pepstatin), added to more buffer B + Triton (0.08% Triton X-100) + PIM and spun at 2000 rpm. Glycerol was added to 10% and aliquots were frozen at -80°C. Purification of GST-fusions: strains were grown to

OD_{600} 0.6 in 2% glucose, spun, and incubated in 2% galactose for three hours to induce the GST fusions. Extracts were prepared as described above except they were never frozen. Instead, they were incubated with glutathione agarose overnight at 4°C in 1.0 ml buffer B + Triton + PIM. Glutathione agarose was repeatedly washed and collected to purify the GST fusions and any associated proteins. Samples were denatured by boiling them for five minutes in SDS loading dye.

Western blotting: samples were subjected to SDS-PAGE electrophoresis (Biorad), transferred to nitrocellulose (Hybond), and probed with an anti-Bmh2p polyclonal antibody or an anti-GST antibody. The anti-Bmh2p antibody was used at a 1/10,000 dilution, a polyclonal anti-GST antibody (Santa Cruz Biotechnologies) was used at a 1/400 dilution, and an HRP-conjugated donkey anti-rabbit IgG antibody (Amersham) was used at a 1/1850 dilution. Chemiluminescent detection of HRP was performed by mixing 10 mls buffer 1 (100 mM Tris-HCl pH 8.5, 5.4 mM H_2O_2) with 10 mls buffer 2 (100 mM Tris-HCl pH 8.5, 2.5 mM luminol, 400 µl paracoumaric acid), filtering the mixture, and immersing the blot for sixty seconds. Biomax MR film was used for detection (Kodak). Luminol (Fluka) and paracoumaric acid (Sigma) were stored in DMSO in 250 mM and 90 mM stock solutions respectively.

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Chapter six

Perspective and future directions

Radclyffe L. Roberts and Gerald R. Fink

Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology. Cambridge, Massachusetts 02142

All of the ideas described in this chapter were conceived and written by RLR.

Developmental decisions in the life cycle of *Saccharomyces cerevisiae.*

Saccharomyces cerevisiae diploid strains starved for nitrogen enter a pseudohyphal growth mode, forming filaments of elongated cells that invade the growth substrate. After extended incubation on rich medium, haploid strains enter an invasive growth mode with many similarities to pseudohyphal development, including filament formation and agar penetration. Both of these developmental events require *STE20*, *STE11*, *STE7* and *STE12*, elements of a conserved mitogen-activated protein kinase (MAPK) cascade required for mating in haploid cells. Thus, a single MAPK cascade controls two distinct developmental programs in the same cell type: mating and invasive growth.

How do cells distinguish between two different stimuli that activate the same MAPK cascade and execute the proper developmental program in each case? To help address this we developed a transcriptional reporter for the filamentous growth MAPK cascade: *FG(TyA)-lacZ*. Regulation of *FG(TyA)-lacZ* and *FUS1-lacZ*, a mating specific reporter, indicates that distinct transcriptional targets are induced during mating and filamentous growth. Specifically, mating pheromones induce *FUS1-lacZ* but not *FG(TyA)-lacZ* while conditions that stimulate filamentous growth induce *FG(TyA)-lacZ* but not *FUS1-lacZ*. These data indicate that the bifunctional MAPK cascade is carefully regulated and maintains a high level of signal specificity. We have strived to gain insight into the mechanisms underlying this signal specificity.

Determining the specificity of the bifunctional MAPK cascade.

A model for the generation, maintenance, and execution of MAPK cascade specificity is emerging (Figure 6.2). During mating, a pheromone receptor (Ste2p or

Ste3p) and heterotrimeric G protein (Ste4p, Ste18p and Gpa1p) are triggered, while filamentous growth is stimulated by Ras2p, Cdc42p, and the 14-3-3 proteins. These upstream signaling components differentially activate the MAPK cascade via Ste20p and Ste11p. Once the MAPK cascade has been activated, scaffold molecules like Ste5p help maintain signal specificity. In turn, MAPKs (Fus3p or Kss1p) help specify pathway outputs by differentially activating the transcription factors Ste12p and Tec1p. Finally, distinct Ste12p-Ste12p and Ste12p-Tec1p complexes may determine the spectrum of genes regulated by this bifunctional MAPK cascade. Because elements of this bifunctional *S. cerevisiae* MAPK cascade are highly conserved in evolution, the model system we have developed should provide a conceptual framework for understanding signal transduction in all eukaryotes. Although we have gained insights into this system, there are many questions that still remain. I will discuss a few of them and describe experiments that could address each one.

Figure 6.1

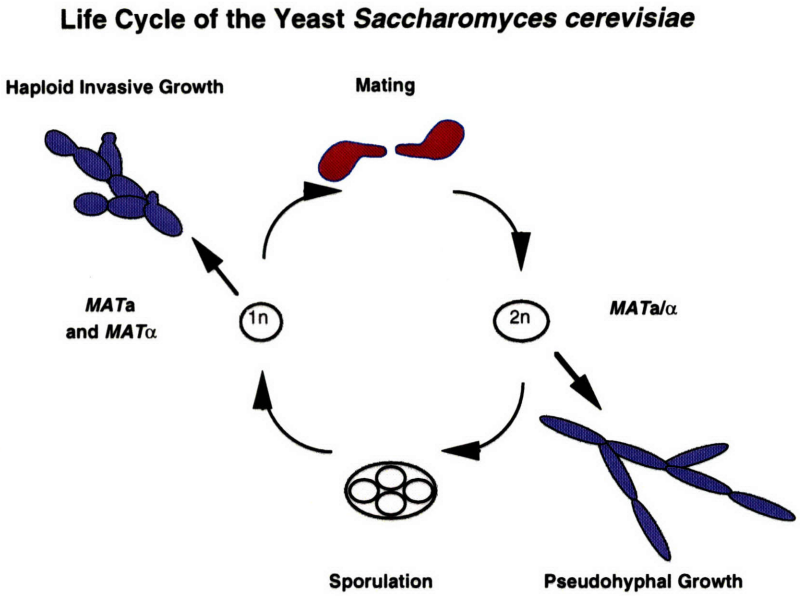

Figure 6.1

Life cycle of the yeast *Saccharomyces cerevisiae*.

S. cerevisiae can can exist in either a haploid (n) or diploid (2n) state. Haploid cells have two sexes: *MAT**a*** and *MAT*α. When cells of opposite mating type encounter one another they mate to form a diploid (2n) zygote. When diploid strains are starved for nitrogen and carbon they enter meiosis and begin sporulation, generating four haploid spores. In contrast, when diploid strains are starved for nitrogen in the presence of a fermentable carbon source they enter a pseudohyphal growth mode, forming filaments of elongated cells that penetrate the growth substrate. Haploid strains have a similar invasive filamentous growth mode that occurs after prolonged incubations on rich medium: haploid invasive growth.

Figure 6.2

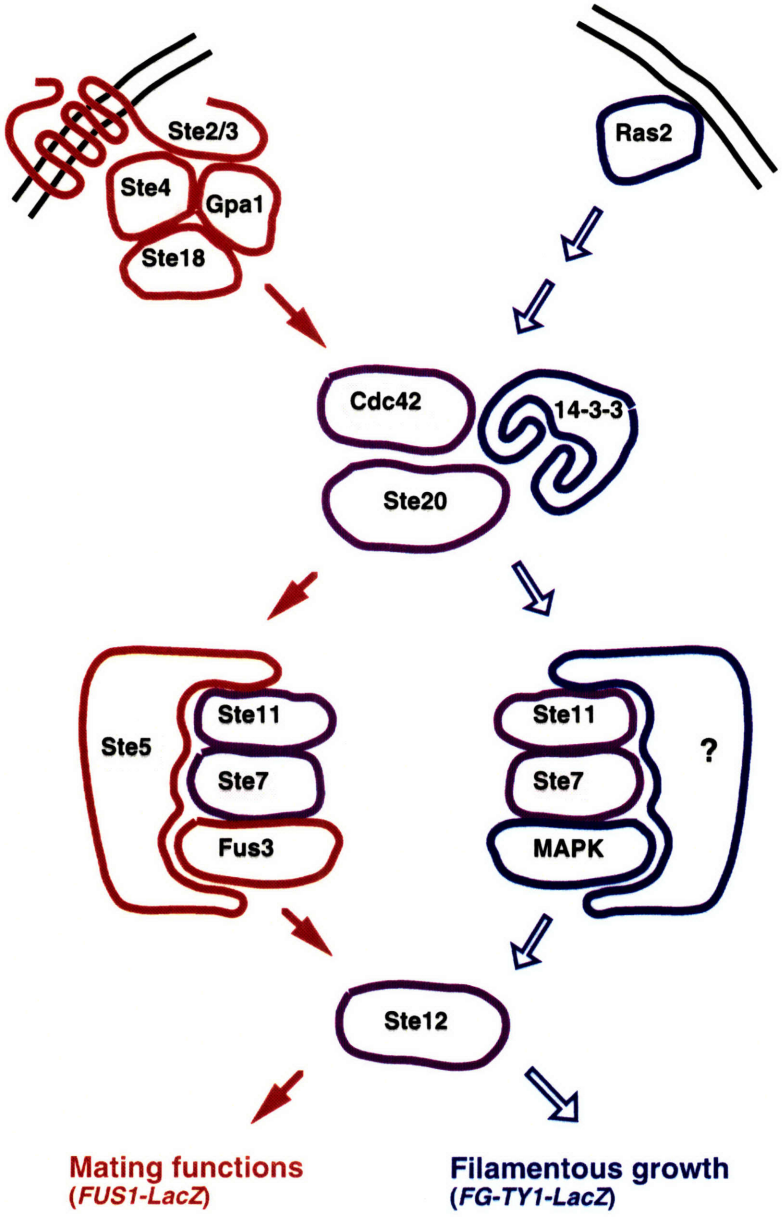

Figure 6.2
A Bifunctional MAPK Cascade.

Mating specific components are red. Filamentous growth specific components are blue. Shared components are purple.

A scaffold molecule for filamentous growth.

Is there a scaffold molecule analogous to *STE5* specific to the filamentous growth MAPK cascade? Two approaches could be taken to address this question. First, biochemical studies similar to those performed for *STE5* (Choi et al., 1994; Marcus et al., 1994) could be undertaken in diploid strains, which do not express *STE5*, to determine if Ste11p, Ste7p and Kss1p associate in a large complex. Immunoprecipitation and/or purification of such a complex might lead to the identification of its constituents, including a hypothetical novel scaffold molecule. An alternative approach is to screen for a mutant that blocks stimulation of filamentous growth and *FG(TyA)-lacZ* by $RAS2^{Val19}$ but is bypassed by *Gal-STE12*. Among the mutants isolated in such a screen, one that abolishes Ste11p-Ste7p-Kss1p complex formation would be an excellent candidate for the scaffold molecule.

Subcellular localization of signaling events.

Where do all of these signaling events take place? Ras2p, Cdc42p, the mating pheromone receptors and the heterotrimeric G protein all reside in the plasma membrane while Ste12p resides in the nucleus. Clearly one or more signaling components must travel between these different subcellular compartments in order to transmit information between these proteins. In mammalian cells ERK1, translocates into the nucleus during prolonged exposure to growth factors (Traverse et al., 1992). In yeast the STE5/MAPK protein complex might enter the nucleus. Immuno-localization of Ste5p, Ste11p, Ste7p, Kss1p and Fus3p during mating, yeast form growth, and filamentous growth should provide some insights into this issue.

Primary signaling events: intercellular signaling during filamentous growth?

What is the primary signal that activates RAS and the filamentous growth MAPK cascade? Nutrient limitation is a good candidate for this signal as it is critical for diploid pseudohyphal development (Gimeno et al., 1992). However, strains carrying mutations that stimulate the filamentous growth MAPK cascade, such as *RAS2*^{Val19} and *STE11-4*, still require nitrogen starvation for pseudohyphal development. Moreover, the effects of *STE11-4* and nitrogen starvation on the *FG(TyA)-lacZ* reporter are approximately additive, suggesting that these factors act independently. Thus, the filamentous growth MAPK cascade is probably not the primary conduit for the nitrogen starvation signal.

Density dependent signaling is important in bacterial development and it may be important for pseudohyphal development. Both nutritional signals and intercellular signaling are important in the multi-cellular fruiting body development of *M. xanthus* (Hagen et al., 1978; Kim and Kaiser, 1990). In the same vein, intercellular signaling events between different populations of cells in a yeast colony might occur during filamentous growth in *S. cerevisiae*. Under conditions of nitrogen starvation, cells form colonies with filaments of elongated cells that penetrate the agar substrate. However, only a fraction of the total cells in a pseudohyphal colony differentiate into elongated cells (less than 1%). Thus, different subpopulations within a single pseudohyphal colony adopt different cell fates. These different fates might arise from distinct micro-environments. For example, the vast majority of pseudohyphal filaments lie underneath the bulk of the colony and are therefore in a different environment than the cells above them. This suggests that cells in a starved yeast colony adopt different cell fates depending on their local nutrient and/or cell signaling environment.

By analogy to the yeast mating MAPK cascade and mammalian growth factors, one might expect that an extracellular signal binds a cell surface receptor and triggers filamentous growth. To date no such ligand/receptor pair has been identified in *S. cerevisiae*. In the corn smut *Ustilago maydis*, filamentous growth and pathogenesis require autocrine signaling via peptide pheromones and their receptors (Bolker et al., 1992). In *S. cerevisiae*, the mating pheromones and their receptors are not required for pseudohyphal development, but another signaling system might be. To detect intercellular signaling, one could incubate yeast form cells with conditioned medium isolated from strains undergoing filamentous growth. This approach has been employed successfully to dissect the intercellular signaling pathways of *M. xanthus* and *B. subtilis* (Hagen et al., 1978; Kim and Kaiser, 1990; Magnuson et al., 1994). In yeast, conditioned medium could be incubated with non-induced wild-type strains or with non-filamentous mutants. One could monitor *FG(TyA)-lacZ* activity and/or filamentous growth as a functional assay for this experiment. If stimulatory activity were detected, one could fractionate the conditioned medium to purify the putative signaling molecule(s). Moreover, mutants whose filamentous growth is rescued by the addition of conditioned medium might define genes important for signal production. These experiments could reveal intercellular signaling events that have thus far remained elusive.

Activation of the STE20/MAPK cascade by *RAS2* and *CDC42*.

How is *STE20* differentially activated by the mating and filamentous growth upstream components? *In vitro*, Cdc42p binds Ste20p and stimulates its kinase activity (Zhao et al., 1995). Notably, the *CDC42* binding domain of *STE20* is necessary for filamentous growth but not mating (Leberer, 1997; Peter, 1996). The 14-3-3 proteins may also regulate the activity of Ste20p (Roberts, 1997). One possibility is

that oligomerization of Ste20p is essential for its activation, as has been suggested for Raf1 (Farrar et al., 1996; Luo et al., 1996), and that the 14-3-3 proteins are vital for this event. Ste20p is capable of forming oligomers *in vivo*, but the functional significance of these complexes has not been ascertained (Roberts unpublished). One way to determine whether this oligomerization is important is to co-express two alleles of *STE20* defective in filamentous growth (ie: one with the *CDC42* binding domain deleted and the other with the catalytic lysine mutated to an arginine) and determine whether these alleles complement each other. Interallelic complementation would suggest that oligomerization is important for *STE20* function. Using *bmh1 bmh2* mutant strains, it would then be possible to test whether complementation and oligomerization depend on the 14-3-3 proteins. These studies should shed light into the differential activation of *STE20*, which may be critical in generating MAPK cascade signal specificity.

Morphogenetic outputs of *STE20*.

It is important to remember that *FG(TyA)-lacZ* and *FUS1-lacZ* are not the only outputs of this bifunctional MAPK cascade. Distinct morphological events are initiated during mating and filamentous growth. Moreover, the pathway for pseudohyphal development may not be a single unbranched sequence from *RAS2* to *STE12*. Although mutations in *STE11*, *STE7*, and *STE12* completely block *FG(TyA)-lacZ* induction by $RAS2^{VAL19}$, they do not completely block induction of cell elongation, suggesting that *RAS2* has outputs important for cell elongation independent of the MAPK cascade (Mosch and Fink, 1997; Roberts, 1997). This bifurcation may occur at the level of *STE20*. Notably, *ste20* mutants exhibit much stronger defects in haploid invasive growth and diploid pseudohyphal development than these other MAPK cascade mutants (Roberts and Fink, 1994; Liu

et al., 1993). The pseudohyphal defects of *ste20/ste20* mutant strains are only partially bypassed by *STE11-4* (Roberts, 1997). In addition, when the Cdc42p binding site of Ste20p is deleted, pseudohyphal development is blocked but mating MAPK cascade signaling via Ste11p, Ste7p and Ste12p is unaffected (Leberer, 1997; Peter, 1996). Thus, there may be a signaling bifurcation at the level of Ste20p that separates MAPK cascade signaling from aspects of cellular morphogenesis. Studies in mammalian cells suggest that G proteins and $p65^{PAK}$ homologs have morphological outputs independent of their downstream MAPK cascade (Lamarche, 1996).

All of the experiments in these studies utilized a *TRP1* insertion in *STE20* (*ste20::TRP1*), instead of a clean deletion (*ste20::HIS3*). The *STE20/STE20* and *ste20::HIS3/STE20* strains form equally vigorous pseudohyphae whereas the *ste20::TRP1/STE20* strain forms less vigorous pseudohyphae (Mosch personal communication). Moreover, the *ste20::TRP1* allele is not fully complemented by a single copy (CEN) of *STE20*. Nevertheless, the pseudohyphal and *FG(TyA)-lacZ* phenotypes, and epistasis profiles, for strains carrying the *ste20::HIS3* clean deletion and *ste20::TRP1* insertion were indistinguishable (data not shown).

The targets of *STE20* important for cellular morphogenesis have not been clearly defined. Overexpression or ectopic activation of *STE20* results in actin depolymerization and death independent of MAPK cascade signaling (Leberer et al., 1997; Ramer and Davis, 1993). By suppressing this lethality it might be possible to identify the proteins that link *STE20* to the actin cytoskeleton and morphogenesis. This signal bifurcation at the level of *RAS/CDC42/STE20* may be a general theme, suggesting that new components identified by this genetic screen might have homologs in a wide array of eukaryotes.

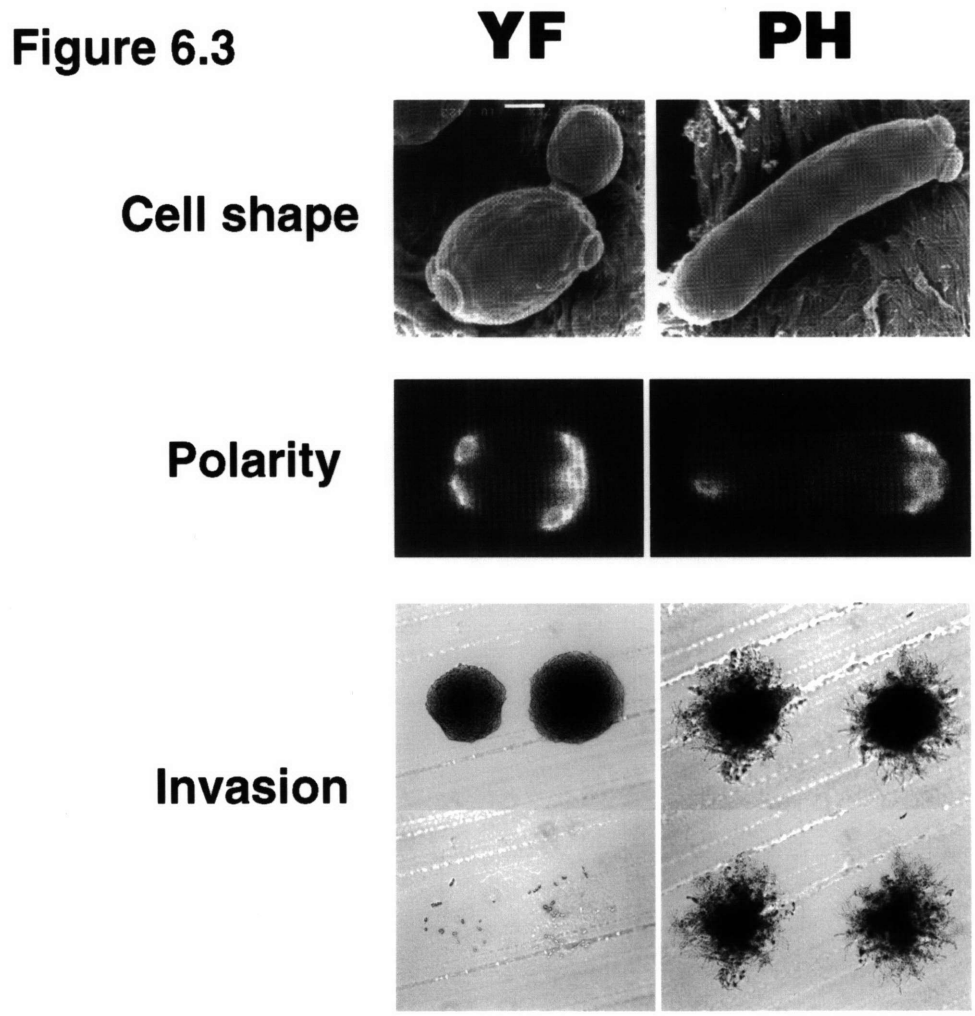

Figure 6.3

Morphological features of pseudohyphal development.

Pseudohyphal development is a composite of several distinct morphological events. 1 - Pseudohyphal cells (PH) are significantly longer than yeast form (YF) cells (Cell shape). 2 - Pseudohyphal cells bud in a unipolar pattern where all new buds emerge from the distal end of the cell, directing new growth away from the mother cell in a series of polar divisions leading to filament formation (polarity). 3 - Pseudohyphal filaments penetrate the substrate on which they are grown (Invasion). This is revealed by washing the cells off the surface (A) to reveal a population of invading cells (B).

Dissecting the morphological events of pseudohyphal development

Pseudohyphal development is a composite of several distinct morphological events: cell elongation, filament formation, and agar invasion (Figure 6.3). Some signaling components, such as *STE20*, control all of these aspects of pseudohyphal development. However, mutations in downstream genes demonstrate that these morphological outputs are largely independent of one another. For example, mutations in *BUD1*, that cause cells to choose bud sites randomly over their surface instead of within a restricted apical domain, abolish filament formation but do not alter a strain's capacity to form elongated cells that penetrate the agar substrate (Gimeno et al., 1992; Roberts, 1994). Similarly tropomyosin (*TPM1*) is required for cell elongation and filament formation but not agar invasion (Mosch and Fink, 1997). By screening for mutants defective in only one morphological process (cell elongation, filament formation, or agar penetration), it should be possible to better define the molecular mechanisms underlying each process.

Other regulators of pseudohyphal development.

Although MAPK cascade components are clearly important regulators of fungal dimorphism, filamentous growth is not completely abolished in strains lacking these components (Liu et al., 1993; Lo, 1997; Mosch and Fink, 1997; Roberts, 1994). Several genes have been identified that may regulate filamentous growth independent of the pseudohyphal development MAPK cascade (Mosch and Fink, 1997). For example, the putative transcription factor *PHD1* stimulates pseudohyphal development when overexpressed (Gimeno and Fink, 1994) and *phd1 ste12* double mutant strains exhibit a much stronger pseudohyphal defect that *ste12* single mutant strains (Lo, 1997). A few negative regulators of filamentous growth have

been identified, including *ELM1* and *GRR1* (Barral and Mann, 1995; Blacketer et al., 1993). The mechanisms by which these elements control pseudohyphal development remain uncertain. Based on these results and the observation that as many as a hundred genes are required for haploid invasive growth, there are probably several pathways that regulate filamentous growth.

Functional genomics.

During my five years at MIT, genetic analysis of *Saccharomyces cerevisiae* has vaulted into the post-genomic era. With the completion of the yeast genome sequencing project, the first complete DNA sequence of any eukaryotic organism, the entire coding capacity of this organism is known. The complicated business of assigning functions to each of these genes, "functional genomics", is the next frontier. The powerful tools of molecular genetics and biochemistry available for *S. cerevisiae* will help realize this ambitous objective.

In *S. cerevisiae,* one of the first steps in characterizing a gene is to knock it out using the endogenous homologous recombination machinery. This is typically performed in one of two ways. 1 - Delete the complete open reading frame and surrounding regulatory regions. 2 - Insert foreign DNA sequences into the middle of the gene, disrupting it at the point of insertion. Because the remaining N-terminal portion of a gene may retain some function, the first method is more trustworthy. Regardless of the method, it is presumed that the knock-out is a null allele and that the function of the gene knocked out has been removed from the cell. By studying the phenotype of the resulting "knock-out" strain, researchers draw inferences about the normal function of the gene knocked-out. This technology, the cornerstone of modern genetics, provides valuable information about gene function.

For multifunctional proteins, inferences drawn from knock-out phenotypes are often incomplete and specific alleles of these proteins are critical for dissecting these functions. The tenet that one gene encodes one protein that executes one function was a fundamental concept that became part of the dogma for biologists. Although the first link seems to stand the test of time there are a myriad of examples of proteins with multiple functions. For example, as both *RAS* and *CDC42* are essential in yeast, the knock-out phenotypes of these proteins do not reveal their functions in the filamentous growth MAPK cascade. Dominant active and dominant negative alleles of these G proteins are vital in helping define their functions. Although these alleles are important reagents, they affect pathways independent of the filamentous growth MAPK cascade (protein kinase A for *RAS* and bud emergence for *CDC42*). Alleles that dissect these different functions would be very valuable. In this vein, the alleles of *BMH1* defective in *FG(TyA)-lacZ* signaling that retain other *BMH1* functions help define the role of *BMH1* in the filamentous growth MAPK cascade (Roberts, 1997). Similar approaches might help define the functions of *RAS2* and *CDC42*. In addition to providing strong evidence for the specificity of these G proteins, this approach could help identify the domains of each molecule that interact with components of different pathways. In summary, knock-out phenotypes are important, but if they were the only genetic method used to assign functions to genes, our vision of biology would be very narrow.

Extending the paradigm to other fungi

The morphological features of pseudohyphal development and haploid invasive growth greatly enhance the capacity of a yeast strain to explore its local environment, presumably to search for nutrients. Foraging behaviors are important for all organisms, but understanding them is particularly important in

the study of microbial pathogens. In addition to serving as a model to study the dynamics of MAPK cascades in higher eukaryotes, filamentous growth in *S. cerevisiae* is a powerful model for studying dimorphism and pathogenesis of other fungi. *Ustilago maydis* is a dimorphic fungus that exhibits filamentous growth and infects corn plants. *U. maydis* strains mutant for *FUZ7*, a *STE7* homolog, show defective diploid filamentous growth and are avirulent (Banuett and Herskowitz, 1994). The major fungal pathogen of rice, *Magnaporthe grisea*, forms hyphae and a specialized appresorium structure to penetrate the cell wall of its host during infection. *PMK1*, a MAPK homolog functionally and structurally related to the *FUS3* and *KSS1* MAPKs of *S. cerevisiae*, is essential for *M. grisea* appresorium development and virulence (Xu and Hamer, 1996). *Candida albicans*, the most prevalent human fungal pathogen, readily interconverts between yeast form and hyphal growth. *C. albicans* strains carrying deletions of *CST20*, *HST7*, or *CPH1* (*STE20*, *STE7* and *STE12* homologs respectively) fail to produce normal hyphae under certain conditions. However, they still form hyphae in response to serum and are virulent in a mouse model (Kohler and Fink, 1996; Liu et al., 1994 and data not published). In contrast, *C. albicans* strains carrying deletions of both *CPH1* and *EFG1*, a *PHD1* homolog, fail to form hyphae in response to serum and are avirulent (Lo, 1997). Importantly, *CST20*, *HST7*, *CPH1*, and *EFG1* all function in *S. cerevisiae*. Together, these results demonstrate that genes homologous to elements of the *S. cerevisiae* filamentous growth MAPK cascade control filamentous growth and pathogenesis in a wide range of fungi. Moreover, many of these genes can function in *S. cerevisiae*. This evolutionary conservation suggests that by expressing genes from other pathogenic fungi in *S. cerevisiae*, it may be possible to elucidate their functions and develop novel anti-fungal compounds. Thus, in addition to providing a paradigm for eukaryotic signal transduction, our studies of the *S. cerevisiae* filamentous growth MAPK cascade should provide a conceptual and

experimental model for understanding the control of filamentous growth and pathogenesis in all fungi.

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Appendix A

Defective invasive growth screen (DIG).

Radclyffe L. Roberts and Gerald R. Fink

All of the experiments described in this chapter were designed and executed by RLR.

Summary

Haploid cells have an invasive growth mode on rich medium that shares several features with pseudohyphal development (Roberts, 1994). We observed that the capacity of a strain to form pseudohyphae correlates well with the ability of haploid colonies to grow invasively into an agar substrate. Based on this phenotype, we developed a novel genetic screen to isolate mutants defective in invasive growth (*dig*). As expected, we isolated mutations in the MAPK cascade components required for filamentous growth (*STE20*, *STE11*, *STE7*, and *STE12*), in addition to many other genes. Many non-sterile recessive *dig* mutants were identified that are required for both haploid invasive growth and diploid pseudohyphal development. A subset of these are bypassed by the dominant active *STE11-4* kinase while the majority are not. These bypassed *dig* mutants may define primary signaling components upstream of *STE11*. We cloned one of these and found it to be allelic to *PTC1*, a serine/threonine phosphatase thought to regulate signaling in the HOG MAPK cascade. In summary, the DIG screen is a powerful tool for isolating mutations in genes required for yeast filamentous growth.

Results

The capacity of a strain to form pseudohyphae correlates well with the ability of haploid colonies to grow invasively into an agar substrate. Based on this phenotype, we developed a novel genetic screen to isolate mutants defective in invasive growth (*dig*) (Figure A.1). Haploid *MATa* and *MATα* strains from the dimorphic Σ1278b genetic background were mutagenized, treated with zymolyase to disperse clumps of cells, and plated on YPD. Colonies were allowed to grow three days at 30°C and two days at room temperature before they were replica plated and washed. Washed and unwashed plates were aligned to identify mutants with defective invasive growth (Figure A.1). Approximately 60,000 mutants were screened and a total of 268 *MATα dig* mutants and 254 *MATa dig* mutants were collected (Figure A.2).

Mutants were subdivided according to the severity of their invasive growth defect. One hundred thirty two mutants showed a completely non-invasive phenotype similar to that exhibited by *flo8* mutants (Liu et al., 1996). Two hundred fifty six mutants showed strong invasive growth defects. In addition, we found hyper-invasive mutants. Four mutants were extremely hyper-invasive and formed filaments of worm-like cells that were extremely clumpy and invasive. Two of these hyper-invasive *dig* mutants failed to complement *elm1* mutants. *ELM1* is a negative regulator of filamentous growth, cell elongation and invasion (Blacketer et al., 1993). Eighteen mutants showed a less dramatic hyper-invasive phenotype. These data suggest that the number of genes that negatively regulate invasive growth is much smaller than the number of genes that promote it.

Figure A.1

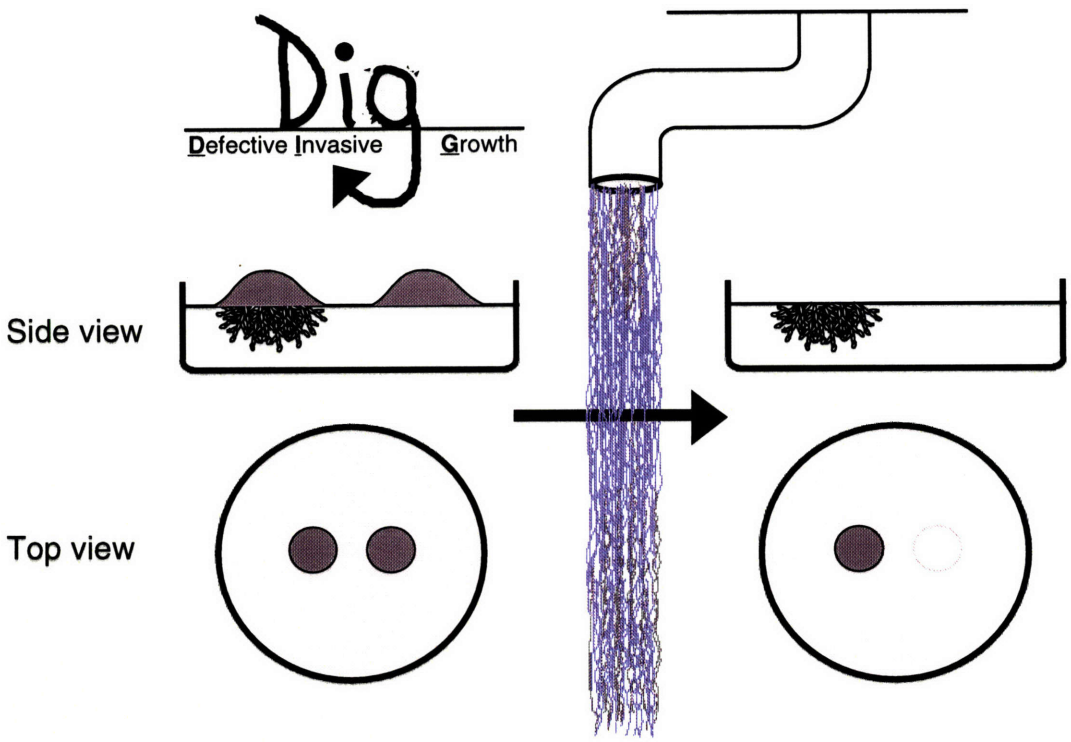

Figure A.1

Defective invasive growth screen: assay

Haploid strains grown on rich medium invade the agar substrate (left colony). The population of invading cells is revealed by washing the main body of the colony off the agar surface with water. Mutants defective in invasive growth (*dig*)(right colony) were identified by plating mutagenized cells on YPD, allowing these cells to form colonies and invade (left), replica plating the colonies to a new plate, washing the original plate, and aligning the unwashed (left) and washed (right) plates.

Figure A.2

Defective Invasive Growth (dig)

dig mutant frequency

Spontaneous *dig* mutant frequency	< .02%
Viability after mutagenesis	20%
Number of colonies screened	60,000
Number of *dig* mutants collected	522
dig mutant frequency after mutagenesis	1%

Gene	# new alleles	Secondary phenotype
STE20	1	mating defect
STE11	3	mating defect
STE7	1	mating defect
STE12	3	mating defect
CUD3	2	calcofluor resistant
CUD4	3	calcofluor resistant
CUD5	2	calcofluor resistant
CUD6	1	calcofluor resistant
CUD7	4	calcofluor resistant
CUD8	1	calcofluor resistant
ELM1	2	hyper-invasive

Figure A.2

Defective invasive growth screen: summary and secondary phenotypes.

Figure A.3

Pseudohyphal phenotypes of *dig* mutants

Class	Description	Number
Off	Very few or no filaments	83
Abnormal	Filaments with morphological defects	25
Enhanced	more florid/frequent pseudohyphae	8
Normal	no detectible pseudohyphal phenotype	36

	Percent colonies with filaments		
diploid strain	vector	*STE11-4*	*Gal-STE12*
wild-type	95	100	100
ste20/ste20	0	37	9
dig1/dig1	1	95	62
dig3/dig3	4	48	52
dig4/dig4	3	75	62
dig5/dig5	36	89	94
dig6/dig6	13	83	57
dig7dig7	20	56	51
cud9/cud9	9	82	20

Figure A.3

Pseudohyphal phenotypes of *dig* mutant diploid strains.

Homozygous *dig/dig* mutant diploids were assayed for the ability to form pseudohyphae on low ammonium plates (SLAD) The percent of colonies forming filaments was determined for each strain. Mutants showing defective pseudohyphal development were transformed with a control plasmid (vector) or plasmids carrying *STE11-4* or *Gal-STE12*. A subset of mutants showed enhanced pseudohyphal development with these plasmids.

Standard complementation analysis was not feasible as invasive growth on rich medium is very poor in diploid strains. To help identify alleles of known genes, we scored *dig* mutants for secondary phenotypes (Figure A.2). A subset of *dig* mutants were found to exhibit mating defects. These were transformed with plasmids containing *STE20*, *STE11-4*, *STE7*, or *STE12*. Based on complementation of their mating phenotypes, we determined that one *dig* mutant was allelic to *STE20*, three to *STE11*, one to *STE7*, and three to *STE12*. Because *kss1* mutants are not sterile in the Σ1278b background, it is not surprising that we did not identify alleles of *KSS1* by these methods, even though this gene is required for haploid invasive growth (Roberts, 1994). These data confirm the utility of this screen to identify components of the filamentous growth MAPK cascade.

Haploid *dig* mutants were scored for resistance to calcofluor white (See appendix B). Sixty six calcofluor resistant *dig* mutants were identified, and these were crossed to *cud* mutant strains (See appendix B) for complementation analysis. This revealed that 13 *dig* mutants are allelic to *cud* mutants (Figure A.2). These data suggest that the *cud* screen was not exhaustive. Because only one to four alleles of *STE*, *CUD*, and *ELM* genes were identified in over five hundred *dig* mutants, there may be as many as a hundred genes required for haploid invasive growth.

We crossed many *dig* mutants to a *DIG+* parent strain of the opposite mating type. The resultant diploid strains were sporulated and subjected to tetrad analysis. In almost every case, two non-invasive and two invasive spores were recovered from each tetrad, consistent with a single *dig* mutation. Homozygous *dig* mutant diploids were constructed by crossing non-invasive strains from these tetrads, and these diploid strains were assayed for the ability to form pseudohyphae on low ammonium medium (SLAD). Many of these diploids were defective in pseudohyphal development (Figure A.3).

The pheromone receptors and G protein involved in activating the MAPK cascade during mating are not required for haploid invasive growth or diploid pseudohyphal growth. What are the upstream elements that control MAPK cascade activation during these processes? Novel *dig* mutants were transformed with a dominant allele of the *STE11* kinase (*STE11-4*), which bypasses the requirement for upstream signaling events in both mating and filamentous growth. This allowed us to identify several new mutants that may act upstream of *STE11* (Figure A.3). These mutants exhibit defective invasive growth and pseudohyphal development, but retain a normal haploid pheromone response, suggesting that they may define primary signaling components specific to fungal dimorphism. The *dig1* mutant was cloned by transforming a genomic library into a haploid *dig1* strain and screening for colonies with restored invasive growth. Mapping and sequence analysis reveal that *dig1* is an allele of *PTC1*, a serine/threonine phosphatase thought to regulate signaling in the HOG MAPK cascade (Maeda et al., 1993).

To determine whether invasive growth depends on mitochondrial function, we grew strains (RRY2 and RRY3) in YPD + ethidium bromide for two days to make them rho-. Resultant rho- segregants grow slowly on YPD, but are quite invasive. Thus, mitochondrial function is not required for haploid invasive growth.

FLO8, a gene important for flocculation and cell adhesion, is required for both pseudohyphal development and haploid invasive growth. Are flocculation and cell adhesion the most important elements of haploid invasive growth? Many hyper-invasive mutants, including *elm1* and *fus3*, form extremely sticky and clumpy colonies. In contrast, cells within a *flo8* mutant colony are well dispersed and non-invasive. We find that mutations in *STE20*, *STE11*, *STE7* and *STE12* greatly reduce haploid invasive growth, but they do not affect the amount of cell-adhesion exhibited in liquid cultures (Data not shown). Thus, flocculation and adhesion are not the only factors important for invasive growth.

We observed that there is an excellent correlation between the invasive growth capacity of a strain and the consistency and texture of colonies of that strain growing on a YPD plate. Specifically, wild-type strains are moderately invasive and form colonies with rough surfaces. In contrast, haploid *dig* mutant colonies with strong defects in invasive growth are all smooth and shiny, whereas mutant colonies that are hyper-invasive are all wrinkled and dull. For example, hyper-invasive *elm1* and *fus3*, form very wrinkled colonies. In contrast, non-invasive *flo8* mutants are completely smooth and shiny. Strains carrying mutations in the filamentous growth MAPK cascade, which show slightly more invasive growth than *flo8* mutant strains, are smooth but not as shiny as *flo8* mutants. Importantly, we find that for every mutant we tested these colony morphology and invasive growth phenotypes were completely linked in tetrad analysis. Although these two phenotypes correlate extremely well, no causal relationship has been established between these processes. The physiologic basis of colony morphology phenotypes is unclear, but these are useful phenotypes to follow nonetheless.

Discussion

Pseudohyphal development is a diploid specific process. For this reason it is relatively intractable to standard yeast genetics. Haploid invasive growth shares many features with diploid pseudohyphal development. As the capacity of a diploid strain to form pseudohyphae correlates well with the ability of haploid colonies to grow invasively into an agar substrate, haploid invasive growth provides a powerful genetic tool to dissect yeast filamentous growth. To exploit this, we developed a novel genetic screen for mutants defective in invasive growth (*dig*). Alleles of filamentous growth MAPK csacade components were isolated, confirming the utility of the dig screen in studying this pathway. Many non-sterile recessive *dig*

mutants were identified that are required for both haploid invasive growth and diploid pseudohyphal development. A subset of these are bypassed by the dominant active *STE11-4* kinase while the majority are not. Those *dig* mutants bypassed by *STE11-4* may define primary signaling components upstream of *STE11*. We cloned one of these and found it to be an allele of *PTC1*, a serine/threonine phosphatase thought to regulate signaling in the HOG MAPK cascade. Overexpression of *PTC1* suppresses the lethality caused by constitutive HOG pathway activation in a *sln1* mutant (Maeda et al., 1994). The mechanisms underlying haploid invasive growth (cell adhesion and/or enzymatic substrate degradation?) are poorly understood and this phenotype has a narrow phenotypic range assayed by the wash assay. Nevertheless, the DIG screen is a powerful tool for isolating mutations in genes required for yeast filamentous growth. As many of these genes are conserved in other fungi, we expect that this phenotype will prove very useful in identifying genes important for invasive growth and pathogenesis in those other fungi.

Materials and Methods

Strains were assayed for invasive growth as described (Roberts, 1994). The frequency of *dig* mutants in the unmutagenized parent strain was less than one in 10,000. Haploid strains in the Σ1278b background form clumps of three to ten cells that are not easily dispersed(Roberts, 1994). In screens for recessive mutants, it is critical to separate these clumps of cells because otherwise the remaining non-mutant cells will mask the phenotype of the mutant. Several treatments were used to separate cells. The most effective treatment was a one hour incubation in 1 mg/ml zymolyase T100. This was somewhat toxic to the cells, but it was much more effective than sonication, vortexing, EDTA treament or pushing cells through a 24 guage needle, none of which had much effect.

1.5 mls of overnight cultures of RRY2 and RRY3 strains were rinsed twice with water and resuspended in EMS buffer. 30 µl of EMS was added and samples were incubated at room temperature for forty minutes. Samples were rinsed twice with sodium thiolsulfate and twice with water and resuspended in 200ml 1mg/ml zymolyase T100. These were incubated at room temperature for 60 minutes and diluted into 10 mls YPD containing 1 M sorbitol. Samples were rinsed twice more with YPD + 1 M sorbitol and allowed to stand at room temperature for four hours before they were plated on YPD plates at a density of about 300 colonies per plate. This procedure killed about 80% of the potential colony forming units. Plates were incubated three days at 30°C and two days at room temperature. They were then gently replica plated to a fresh YPD plate and the original plate was washed as described. Colonies with defective invasive growth (*dig*) were identified by aligning the washed and unwashed plates. The frequence of mutants defective in invasive growth was approximately 0.8%.

Acknowledgments

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Appendix B

Calcofluor resistant mutants defective in pseudohyphal growth.

Radclyffe L. Roberts and Gerald R. Fink.

All of the experiments described in this chapter were designed and executed by RLR.

Summary

Chitin is a cell wall polysaccharide in *Saccharomyces cerevisiae* that provides structural support to the yeast cell. It is localized to the bud site and comprises the several layers of septa that ultimately separate mother and daughter cells. Chitin synthesis is induced during the morphological changes accompanying mating. In *Neurospora crassa* disruption of CHS1, a chitin synthase gene, greatly reduces the capacity of this organism to make hyphae. These and other findings suggest that the control of chitin synthesis and deposition may be important for the morphological changes associated with pseudohyphal development in *S. cerevisiae.*

The vital dye calcofluor binds chitin and kills yeast cells. In this study we developed a screen for mutants resistant to calcofluor. These mutants define fourteen complementation groups. Mutants from seven of these groups, designated *CUD* for calcofluor unresponsive defective pseudohyphae, are defective for diploid pseudohyphal growth and haploid invasive growth, two filamentous growth modes. These results suggest that regulation of chitin deposition may play an important role in filamentous growth in *S. cerevisiae.*

Introduction

Chitin is a cell wall polysaccharide in Saccharomyces cerevisiae that provides structural support to the yeast cell. It is localized to the bud site and comprises the several layers of septa that ultimately separate mother and daughter cells(Cabib et al., 1993). Chitin synthesis is induced during the morphological changes accompanying mating. In *Neurospora crassa*, disruption of *CHS1*, a chitin synthase gene, compromises the ability of this organism to make hyphae (Yarden and Yanofsky, 1991). These and other findings suggested that the control of chitin

synthesis and deposition may be important for the morphological changes associated with pseudohyphal development. The vital dye calcofluor white (fluorescent brightener #28) binds to chitin and prevents the oligomerization of chitin strands into fibrils (Bartnicki-Garcia et al., 1994). Cells grown in the presence of calcofluor exhibit gross morphological abnormalities and fail to produce macro-colonies. Despite many years of research, the mechanisms by which calcofluor kills cells are not understood (Valdivieso et al., 1991).

Three distinct chitin synthase enzymes have been characterized in *S. cerevisiae* (Cabib et al., 1993). *CHS1* and *CHS2* encode chitin synthase one and two respectively (Bulawa, 1992; Cabib et al., 1993). Chitin synthase three is responsible for about ninety percent of the total chitin synthesis in the cell. A screen for mutants defective in chitin synthesis (CSD) identified three genes: *CSD2*, *CSD3* and *CSD4* (Bulawa, 1992). Mutations in any of these three genes abolish chitin synthase three activity and confer resistance to calcofluor (Bulawa, 1992). *CSD2* has homology to *CHS1* and *CHS2*, suggesting that it may encode the catalytic subunit of the chitin synthase three enzyme. Double mutant combinations between the *CSD2*, *CHS1* and *CHS2* genes are viable whereas the triple mutant is inviable (Bulawa, 1992).

Results

We developed a screen for mutants calcofluor uninhibited, defective in pseudohyphal development (CUD). Spontaneous mutants resistant to calcofluor (fluorscent brightener 28, Sigma) were selected on YPD plates containing 100 μg/ml calcofluor. At this concentration, the wild-type parental strains divide six or seven times but fail to form macro-colonies, producing grotesque chains of wormy cells. Eighty two recessive mutants representing fourteen complementation groups were isolated at a frequency of 1 in 10^5 (10 mutants per 10^6 cells per plate) and

characterized further (Table B.1). By sporulating non-complementing diploid strains we confirmed that the two parental *cud* mutations were linked in every case. Mutations in the *CUD1* and *CUD2* complementation groups were determined to be alleles of *CSD2* and *CSD3* respectively (Bulawa, 1992). These are the only two complementation groups resistant to 1 mg/ml calcofluor. A *csd2-8::LEU2* disruption we constructed in Σ1278b and alleles of *CUD1* show identical phenotypes.

Homozygous diploid mutants from seven complementation (*CUD3-9*) groups fail to form pseudohyphae when starved for nitrogen. Mutant *cud3-9* strains are also defective in haploid invasive growth and form shiny, smooth colonies on YPD (Table B.1). Homozygous *cud6* and *cud9* mutants form high percentages of elongated cells even though they fail to form long filaments or invade agar (Table B.1). Mutant *cud6* and *cud9* strains are also defective in sporulation.

Diploid *cud* mutant strains were stained with calcofluor to determine whether any abnormal chitin deposition could be detected. Mutant *cud3*, *cud4*, *cud5*, *cud7*, and *cud8* strains show a staining pattern comparable to the wild-type *CUD+* strain. In contrast, *cud6* and *cud9* mutants show an unusual staining pattern (Data not shown). The significance of this staining pattern is uncertain.

Table B.1

Calcofluor Uninhibited Defective pseudohyphae (CUD)
Summary of *cud* mutant phenotypes

Gene	MATα strains	MATa strains	1	2	3	4	5	6	7	8	9	10	
CUD+	RRY2	RRY3		D	67-85	99	+	+	-	-	+	+	
cud1 =csd2	MP, NZ, OZ, PK, PA, OU	A, G, AS, AU, AY, BV BY, BR, BN, BK, BM	sd	D	80 83	97	+	+	+	+			
cud2 =csd3	OA, OL, OY	AA, BU, I	sd	D	85 76		+	+	+	+			
cud3	NE, NF, PB, NS, MQ, ME	Q, BW, AL	r	S	2 2, 7	25	-	+	+	+/-	+	+	
cud4	NV, NC, ML	BS, AH	r	S	0 0, 0	27	-	+	+	+/-	+	+	
cud5	OX, NI	M, K, E	r	S	1 * 11	71	-	+	+	+/-	+	+	
cud6	MX, ND	BC	r	S	62** 89**	76	-	+ POOR	+	+/-	- none	ND	
cud7	NG	BQ, AJ, BA	r	S	2	22	-	+	+	+/-	+	+	
cud8	NT	AC, Y	r	S	0	21	-	+	+	+/-	+	+	
cud9	MM	D, Z, AG	r	S	32** 45**	89	-	+	+	+/-	poor	OK	
cud10	PH, PM	AN	r	D			+		+	+/-			
cud11	PI	R	r	D			+		+	+/-			
cud12	PJ	BI	r	D			+		+	+/-			
cud13	OG, PL, OT	BJ	r	D	88		+		+	+/-			
cud14	PG	F	r	D	81		+		+	+/-			

* Makes good pseudohyphae in broken agar.

** Many long cells but no long filaments.
Many dead cells. Makes OK Psh in broken agar.

Table B.1
Phenotypes of calcofluor resistant mutants (*cud*).

Genes are listed (Gene)(*CUD+* is wild-type). Each letter pair denotes an independent *cud* mutant (*MATα* strain, *MATa* strain). Mutations were scored as recessive (r), dominant (d), or semi-dominant (sd)(1). Haploid *cud* strains were scored for the ability to form dull (D) or shiny (S) colonies (2), invade agar (5), grow on glycerol plates (6), grow on 100 μg/ml calcofluor plates (7), and grow on 1 mg/ml calcofluor plates (8). Diploid *cud/cud* strains were scored for the percent of colonies forming pseudohyphae on SLAD in the absence (3) or presence (4) of *PHD1* 2 μ, and for the ability to form tetrads on sporulation medium (9), and form viable spores (10). Where more than one number is shown, multiple strains were scored.

Discussion

Despite several screens for calcofluor resistant and hypersensitive mutants, the mechanisms by which calcofluor kills yeast cells are still uncertain. By using low doses of calcofluor (100 µg/ml) we isolated greater numbers of mutants than those originally isolated using 1 mg/ml calcofluor (Valdivieso et al., 1991). Moreover, a subset of these mutants are defective in both haploid invasive growth and diploid pseudohyphal development. These data suggest that the regulation of chitin synthesis and deposition is important for filamentous growth and that the CUD screen is useful for isolating genes that regulate yeast filamentous growth.

Materials and Methods

Resistance to calcofluor is most reliably scored by streaking strains for single colonies on a plate and scoring their growth after twenty four hours at 30°C. High cell density, including replica plating and frogging, and prolonged incubations confound the assay. Calcofluor white (fluorescent brightener 28, Sigma) is stored in a water in a 50 mg/ml stock at 4°C in the dark. A small aliquot is diluted and sterile filtered immediately prior to use. Add 1.2 mls of this stock to 600mls media + agar for a final concentration of 100 µg/ml. Calcofluor precipitates at low pH so YPD is preferable to unbuffered synthetic medium. Plates containing calcofluor may accumulate spider-like spots. These are thought to be precipitating calcofluor and not contamination.

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Appendix C

Over the kitchen wall: quotes from HIS lunch.

Gerald R. Fink and others....

Compiled by Radclyffe L. Roberts .

RLR collected all of these quotes between peals of laughter.

Summary

HIS lunches, as the Fink group meetings are called, have always been intellectually stimulating and entertaining. Part of the entertainment derives from the witticisms bounced back and forth between our revered mentor and the gallery. I have endeavored to capture some of the more colorful phrases expressed behind closed doors. This is a task I inherited from Julie Brill and now hope to pass on to Laura Robertson and Anya Goodman.

Career guidance

GRF to AS - Hold on. Don't write your epitaph so quickly.

GRF to JL- We need to air the sheets on nucleoporins in public.

GRF to JB- I'm not saying you should turn off your slides and run into the lab, but...

GRF to HL- You can't work with DNA that doesn't exist.

GRF to KDH - I cannot provide psychaitric help.

GRF to KDH - What you see depends on what you look at. Tell me what you did.

GRF to KDH - It's cheaper than TAQ to make a phone call.

GRF to KDH - Why do you want them in Kyle's pants? Let them stay in yours!

GRF to AC- Autoclave that slide.

GRF to HUM - You could be cloning moron genes.

HUM to GRF - bcy1 cells are life sensitive.

GRF to HUM - The days of bingo are over except in churches in the midwest.

GRF to BB- The question is how many nails do you want to put in this coffin? After a while you start splitting the wood and the corpse comes out.

GRF to PH - This requires some psychiatric treatment, not genetics.

PH to GRF - (PHD1) may keep the background down.
GRF to PH - But it may keep your backside exposed.

Truisms

CAS to GRF - It happened to the best of us: me.

JK - Don't eat eight grams of Candida albicans.

JK - Getting anything out of Candida is like trying to milk a byson.

GRF to HJL - It's like your relatives: they have varying amounts of similar genetic material but they're probably all still worth knowing.

GRF to KDH - It depends on whether you read the new testament or the old testament (on whether yeast is closer to plants or humans).

GRF to JK- There's nothing closer to Saccharomyces than Saccharomyces.

AD - It's helpful, but they're meaningless. (genetic distances and physical mapping).

PH to GRF - It's statistically dead.
GRF to PH - Most of us are.

GRF to JK - If I didn't put it that extreme you'd spend more of my money.

RG - This is just a marijuana dream.

From the illustrious Carlos Gimeno:

SJK to CJG - Do you know if sigma is a phd1 mutant?
CJG to SJK- No, I also don't know if Lee Harvey Oswald killed Kennedy.

CJG - The point is, we did the experiment right.

CJG - I blame the readers for not being careful readers.

CJG - It's all in my paper, Cora, coming out next month.

CJG - I'm just presenting data. Posterity will put it into context.

Communication breakdown

DK to GRF- The interpretation is not straightforward.
GRF to DK- But it would be nice if it were straightforward.

GRF to AC - The only mystery is why you think there is a mystery.
AC to GRF - I don't think there is a mystery.

GRF to AD- You think I'm just flapping my lips, but I wouldn't be here if I understood it.

GRF to HUM- You WILL agree with me.

GRF to HJL- I know you said it, but I want to rub it in.
HJL to GRF - EMPHASIZE it.

GRF to SR- Is this the end of group meeting?
SR to GRF - Yes.
GRF to SR - No.

PH to AD - Are there other things that affect root growth?
AD to PH - Concrete.

GRF to SJK - Could you tell us what you are telling us?

The affable, inimitable Amir Sherman:

AS - I couldn't find what the auxin transport inhibitors do, but they inhibit the transport of auxin.

AS - It's almost on the edge of imagination.

AS - Getting DNA from yeast you just have to spit on them.

AS - It's just the two of us: the bench and me.

AS - Me and the bench can't get to other places.

GRF to AS- You still shouldn't sleep well.
AS to GRF - Don't worry, I'm not sleeping well all the time.

AS to GRF- I am willing to try anything, because I've tried a lot of things worse than this.

AS - I want to get some assurance that we are in the right place and not running around our tails.

AS - If you get a mutant in the plant you are on the horse.

Is that tape rolling?

PH to GRF - I'm going to bore you into submission.

GRF to PH- The only thing that was worth showing was him eating crow.

HDM to all- I definitely see what I see.

HDM to RG - THIS is what you are asking...

GRF to TM- I just don't believe in recreational phosphorylation.

GRF to HJL - For five bucks I deserve a result.

GRF to RG - It's only wild-type in Vienna (about W303).

GRF to all - Argue amonst yourselves (on being called out).

GRF to all - Somebody should play a funeral dirge.

CL to GRF- There's not so much to tell about mutS.

GRF to CL- That's ok, we have a short attention span.

GRF to DK- I have a short memory about what is going through my mind.

GRF to SR- You don't have to excuse it. I will forget anyhow.

GRF to DK - This guy had a cranial rectal inclusion.

GRF to HUM- All you want to do is put the real data down, but what we're going to say is...

GRF - I don't care about complexity, I just want to know what it all means.

Cast of characters:

BB	Bonnie Bartel
JB	Judy Bender
AC	Amy Chang
AD	Andrew Diener
GRF	If you have to ask you'll never know.
RG	Roberto Gaxiola
CJG	Carlos Gimeno
PH	Peter Hecht
KDH	Kendal Hircschi
JK	Julia Kohler
DK	Daniel Kornitzer
SJK	Steve Kron
HL	Haoping Liu
HJL	Hsiu-Jung Lo
JL	Jonathon Loeb
CL	Christian Luschnig

HDM	Hiten Madhani
TM	Todd Milne
HUM	Hans Mosch
SR	Steffen Rupp
AS	Amir Sherman
CAS	Cora Styles

Materials and methods

Listen. Put pencil on paper.

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