Functions for Cdc42p BEM adaptors in regulating a differentiation-type MAP kinase pathway

Sukanya Basu[a], Beatriz González[a], Boyang Li[a], Garrett Kimble[a], Keith G. Kozminski[b], and Paul J. Cullen[a,*]

[a]Department of Biological Sciences, University at Buffalo, Buffalo, NY 14260; [b]Departments of Biology and Cell Biology, University of Virginia, Charlottesville, VA 22904

ABSTRACT **Ras homology (Rho) GTPases regulate cell polarity and signal transduction pathways to control morphogenetic responses in different settings. In yeast, the Rho GTPase Cdc42p regulates cell polarity, and through the p21-activated kinase Ste20p, Cdc42p also regulates mitogen-activated protein kinase (MAPK) pathways (mating, filamentous growth or fMAPK, and HOG). Although much is known about how Cdc42p regulates cell polarity and the mating pathway, how Cdc42p regulates the fMAPK pathway is not clear. To address this question, Cdc42p-dependent MAPK pathways were compared in the filamentous (Σ1278b) strain background. Each MAPK pathway showed a unique activation profile, with the fMAPK pathway exhibiting slow activation kinetics compared with the mating and HOG pathways. A previously characterized version of Cdc42p, Cdc42p^{E100A}, that is specifically defective for fMAPK pathway signaling, was defective for interaction with Bem4p, the pathway-specific adaptor for the fMAPK pathway. Corresponding residues in Bem4p were identified that were required for interaction with Cdc42p and fMAPK pathway signaling. The polarity adaptor Bem1p also regulated the fMAPK pathway. Versions of Bem1p defective for recruitment of Ste20p to the plasma membrane, intramolecular interactions, and interaction with the GEF, Cdc24p, were defective for fMAPK pathway signaling. Bem1p also regulated effector pathways in different ways. In some pathways, multiple domains of the protein were required for its function, whereas in other pathways, a single domain or function was needed. Genetic suppression tests showed that Bem4p and Bem1p regulate the fMAPK pathway in an ordered sequence. Collectively, the study demonstrates unique and sequential functions for Rho GTPase adaptors in regulating MAPK pathways.**

Monitoring Editor
Patrick Brennwald
University of North Carolina, Chapel Hill

Received: Aug 13, 2019
Revised: Jan 9, 2020
Accepted: Jan 9, 2020

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E19-08-0441).

Author contributions: S.B. designed experiments, generated data, and wrote the paper; B.G. performed experiments and analyzed the data; B.L. and G.K. generated data; K.K. provided reagents, generated data, and analyzed the data; P.J.C. designed experiments and wrote the paper.

[AQ 1] The authors have no competing interests in the study.

*Address correspondence to: Paul J. Cullen (pjcullen@buffalo.edu).

Abbreviations used: ARM, Armadillo; BSA, bovine serum albumin; CFW, Calcofluor White; DIC, differential-interference contrast; EFF, effector domain; 5-FOA, 5-fluoroorotic acid; GAL, galactose; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; HOG, high osmolarity glycerol response; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; PDB, Protein Data Bank; PIP, phosphatidylinositol phosphate; PM, plasma membrane; PWA, plate washing assay; Rho, Ras homology; YEPD, yeast extract, peptone, and dextrose.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signaling modules that control cell proliferation, cell differentiation, and the response to stress in eukaryotes (Keshet and Seger, 2010). In the budding yeast *Saccharomyces cerevisiae*, MAPK pathways control cell differentiation to specific cell types, such as the mating cell type of spores, which is regulated by the pheromone response pathway, the filamentous cell type by the fMAPK pathway, and the formation of meiotic progeny by the sporulation pathway. MAPK pathways also control the response to stress, such as osmotic stress, which is regulated by the high osmolarity glycerol response (HOG) pathway, and the response to compromised cell integrity by the protein kinase C pathway. Like many signaling pathways, MAPK pathways in yeast can share components (Schwartz and Madhani, 2004; Bardwell, 2005; Chen and Thorner, 2007; Hohmann *et al.*, 2007; Saito, 2010; Levin, 2011). The Ras homology (Rho) GTPase Cdc42p regulates three MAPK pathways (mating, fMAPK, and

HOG) and is an essential protein that controls the establishment of cell polarity, polarized secretion, and protein trafficking (Park and Bi, 2007). How Cdc42p and other proteins induce a pathway-specific response in a particular setting is not clear. This question is relevant because in higher organisms, cross-talk and misregulation of GTPase pathways, like those requiring CDC42 and RAS, are leading causes of cancer and other diseases. Understanding how common proteins function in pathway-specific settings remains at the forefront of studies of signaling pathway regulation (Giehl, 2005; Dhillon *et al.*, 2007; Maik-Rachline and Seger, 2016; Rauch *et al.*, 2016).

In response to carbon or nitrogen limitation, yeast and other fungal species can undergo a microbial differentiation response called filamentous or invasive/pseudohyphal growth (Gimeno *et al.*, 1992; Cullen and Sprague, 2012). In some plant and animal pathogens, like the major human pathogen *Candida albicans*, filamentous growth is required for virulence (Lo *et al.*, 1997; Whiteway and Bachewich, 2007; Finkel and Mitchell, 2011; Desai *et al.*, 2014). In yeast, filamentous growth occurs in wild strain backgrounds (Σ1278b is used here) because the properties that are associated with filamentous growth have been lost due to genetic manipulation of laboratory strains (Liu *et al.*, 1996; Dowell *et al.*, 2010; Chin *et al.*, 2012; Ryan *et al.*, 2012). The Cdc42p-dependent fMAPK pathway is one of the major signaling pathways that regulates filamentous growth (Roberts and Fink, 1994; Cullen *et al.* 2004; Cullen and Sprague 2012). Studies of the fMAPK pathway have provided insights into how ERK-type MAPK pathways regulate eukaryotic cell differentiation and fungal pathogenesis.

The fMAPK pathway shares components with the HOG and mating pathways, yet each pathway induces a specific set of target genes and a unique response (Brewster *et al.*, 1993; Peter *et al.*, 1996; Leberer *et al.*, 1997; Roberts *et al.*, 2000; Lamson *et al.*, 2002; Schwartz and Madhani, 2004; Bardwell, 2005; Chen and Thorner, 2007; Cullen and Sprague, 2012). A core module composed of Cdc42p, the p21-activated kinase (PAK) Ste20p (Peter *et al.*, 1996; Leberer *et al.*, 1997), and the MAPKKK Ste11p regulates all three MAPK pathways. This core module regulates different MAP kinases for the three pathways. Kss1p is the main regulator of the fMAPK pathway (Madhani *et al.*, 1997). Kss1p regulates a suite of transcriptional activators (Ste12p, Tec1p, Msa1p, and Msa2p) and repressors (Dig1p and Dig2p) (Cook *et al.*, 1996, 1997; Madhani and Fink, 1997; Madhani *et al.*, 1997; Bardwell *et al.*, 1998a,b; Cullen and Sprague, 2000b; van der Felden *et al.*, 2014) to activate transcription at filamentation-specific promoters (Madhani and Fink, 1997; Zeitlinger *et al.*, 2003). Tec1p in particular functions exclusively in the fMAPK pathway and is degraded during the mating response (Bao *et al.*, 2004; Chou *et al.*, 2004).

Several proteins have been identified that function at or above the level of Cdc42p and regulate the fMAPK pathway. The mucin-type glycoprotein Msb2p interacts with the GTP-bound conformation of Cdc42p and is the main sensor that regulates the fMAPK pathway (Cullen *et al.*, 2004). Msb2p functions with two other transmembrane proteins, Sho1p and Opy2p (Wu *et al.*, 2006; Tatebayashi *et al.*, 2007; Ekiel *et al.*, 2009; Pitoniak *et al.*, 2009; Yang *et al.*, 2009; Yamamoto *et al.*, 2010, 2016; Karunanithi and Cullen, 2012; Tatebayashi *et al.*, 2015). Msb2p, Sho1p, and Opy2p regulate the fMAPK pathway and also the HOG pathway but do not control the activity of the mating pathway. Bem4p is a cytosolic adaptor and Cdc42p-interacting protein (Hirano *et al.*, 1996; Mack *et al.*, 1996) that regulates the fMAPK pathway but not the mating or HOG pathways (Pitoniak *et al.*, 2015). Bem4p associates with Cdc42p, the guanine nucleotide exchange factor (GEF) Cdc24p (Johnson, 1999; Erickson and Cerione, 2001; Etienne-Manneville, 2004), the MAPKKK Ste11p, and the MAPK Kss1p (Pitoniak *et al.*, 2015). Bem4p may direct Cdc42p, in some manner, to a pathway-specific complex containing Kss1p. We have also shown that bud-site-selection proteins, culminating at the Ras-type GTPase Rsr1p (Chant and Pringle, 1995; Bi and Park, 2012), also regulate the fMAPK pathway but not other Cdc42p-dependent MAPK pathways (Basu *et al.*, 2016). Therefore, Msb2p, Bem4p, and Rsr1p function at the level of Cdc42p and regulate the fMAPK pathway.

Despite the utility of studying MAPK pathways in a genetically amenable system, Cdc42p-dependent MAPK pathways have not been directly compared in yeast. One reason is that most studies of the mating and HOG pathways have been carried out in laboratory strains, which have lost the ability to undergo filamentous growth (Liu *et al.*, 1996; Chin *et al.*, 2012; Ryan *et al.*, 2012). Another reason is that the HOG pathway has redundant branches that converge on the MAPKK Pbs2p, bypassing Cdc42p. Only one branch is dependent on Cdc42p, the Ste11p branch (Posas *et al.*, 1996; Posas and Saito, 1997, 1998). Here, we developed a strain and assays that allowed us to directly compare Cdc42p-dependent MAPK pathways in yeast. By exploring this signaling landscape, we found that the pathways showed different kinetics, with the fMAPK pathway being activated more slowly than the mating and HOG pathways. We also found that the interaction between Bem4p and Cdc42p is critical for fMAPK pathway signaling. The major polarity adaptor for Cdc42p, Bem1p, also regulated the fMAPK pathway. Bem1p also regulates the mating (Leeuw *et al.*, 1995) and HOG pathways (Tanaka *et al.*, 2014), so its role in regulating the fMAPK pathway might be expected. What was surprising was that Bem1p regulated different effector pathways in different ways. We propose a model for how the Cdc42p module is activated in the context of the fMAPK pathway by interactions with Cdc24p (by Bem4p, Bem1p, and Rsr1p), Cdc42p (by Bem4p and Bem1p), and Ste20p (by Bem1p). Our study provides insights into how a Rho GTPase is directed to a differentiation-type MAPK pathway.

RESULTS

Signaling landscape of Cdc42p-dependent MAPK pathways

Three MAPK pathways in yeast (fMAPK, HOG, and mating) require common components yet induce different responses (Figure 1A; Roberts *et al.*, 2000; Maleri *et al.*, 2004; Bhattacharyya *et al.*, 2006). The Rho-type GTPase Cdc42p, PAK Ste20p, and MAPKKK Ste11p are central to the regulation of the three pathways (Figure 1A, gray box). To directly compare Cdc42p-dependent MAPK pathways, experiments were performed in filamentous (Σ1278b) strains lacking the Sln1p-branch of the HOG pathway (*ssk1*Δ). Growth of cells in the nonpreferred carbon source galactose (GAL) induced filamentous growth (Figure 1B, left) and phosphorylation of the MAP kinase Kss1p (Figure 1B, right, P~Kss1p and to a minor extent P~Fus3p). Osmotic shock (0.4 M KCl) did not induce a morphogenetic response (Figure 1C, left) but induced rapid phosphorylation of Hog1p (Figure 1C, right). The mating pheromone α-factor induced mating projections (Figure 1D, left) and phosphorylation of Fus3p and Kss1p (Figure 1D, right, note low levels of control protein at the 6-h time point). Kss1p is normally phosphorylated in response to α-factor and functions to modulate the mating response (Ma *et al.*, 1995; Sabbagh *et al.*, 2001).

The above experiments looked at phosphorylated MAP kinases as a readout of pathway activity. As an independent test, the expression of MAPK pathway reporter genes was evaluated. GAL induced expression of an fMAPK pathway-dependent reporter (Figure 1E, red, p*FRE-lacZ*). Osmotic shock induced expression of a HOG pathway reporter (Figure 1E, green, p*8XCRE-lacZ*), and α-factor induced

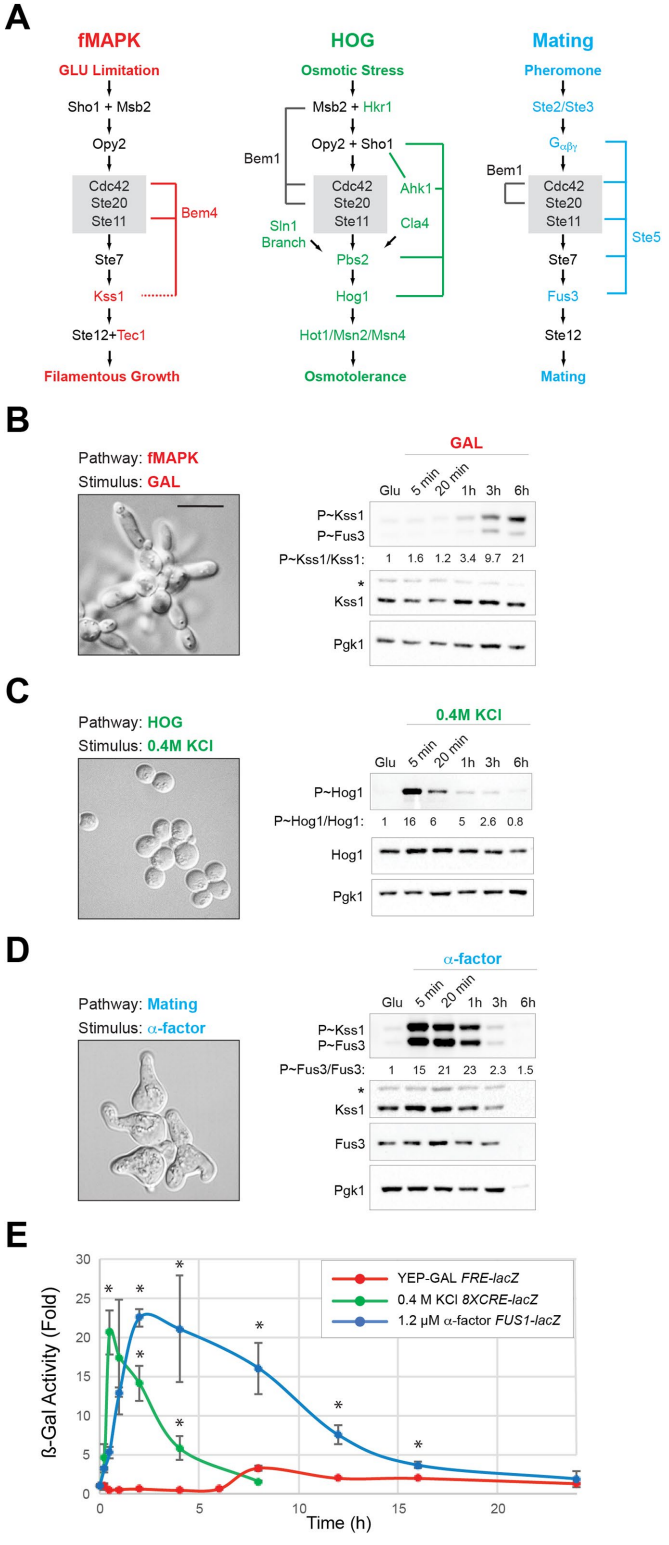

FIGURE 1: Comparison of MAP kinase pathways that require Cdc42p and other proteins. (A) Three Cdc42p-dependent MAP kinase pathways. Colored text refers to pathway-specific factors fMAPK (red), mating (blue), and HOG (green). Black text refers to common or shared factors. The gray box denotes factors that are common to all three pathways. (B) To assess fMAPK pathway activity, wild-type cells (PC6810) were grown for 8 h in YEP-GAL and photographed (left, DIC 100X, bar, 10 microns). At right, immunoblots were probed with antibodies (p44/p42) to detect P~Kss1p and P~Fus3p. Immunoblots were also probed with α-Kss1p and α-Pgk1p antibodies. (C) The same cells as in B were grown in YPED + 0.4 M KCl (HOG) for 8 h and photographed (left, DIC 100×, bar, 10 microns). At right, immunoblots were probed with antibodies to detect P~Hog1p (p38), Hog1p, and Pgk1p. (D) The same cells in B were grown for 8 h in YEPD + 6 μM α-factor to induce mating (left, DIC 100×, bar, 10 microns). At right, immunoblots were probed with α-p44/p42, α-Kss1p, α-Fus3p, and α-Pgk1p antibodies. In B–D, numbers indicate the relative band intensity of P~MAPK to total MAPK levels, which was set to 1 for basal conditions. Asterisk, background band. Phosphoblots were performed in multiple replicates ($n = 3$ experiments), and representative images are shown. (E) β-Galactosidase assays of MAPK pathway-dependent reporters. For the *x*-axis, values are expressed as fold induction compared with the uninduced condition (YEPD) for each reporter *y*-axis, time (h). Each data point is represents the average of three biological replicates ($n = 3$). Error bars show the SD between trials. *$p < 0.05$ for values compared between the *FUS1-lacZ* data points or the *8XCRE-lacZ* data points to the *FRE-lacZ* for the times indicated.

expression of a mating pathway reporter (Figure 1E, blue, p*FUS1-lacZ*). The phosphoblot analysis and transcriptional reporter assays showed that the fMAPK pathway was activated more slowly than the mating and HOG pathways. By P~blot analysis, the fMAPK pathway was maximally activated by 6 h, compared with 5 min for HOG, and 1 h for the mating pathway (Figure 1, B–D). The mating and fMAPK pathways were directly comparable because P~Fus3p and P~Kss1p are recognized by the same antibodies (Supplemental Figure S1). By transcriptional reporter assays, the fMAPK pathway showed maximal activity by 7.5 h compared with 5 min for the HOG and 20 min for the mating pathways (Figure 1E). The fMAPK pathway also showed less transcriptional reporter induction (fourfold for the *FRE-lacZ* reporter) than the HOG (20-fold) and mating pathways (20-fold). This observation is consistent with previous studies from our lab of the *FRE-lacZ* reporter (Chavel *et al.*, 2014) and other reporters (e.g., *KSS1, YLRO42c, MSB2*; Pitoniak *et al.*, 2009) that show moderate induction in GAL. Therefore, two separate tests show that the fMAPK pathway has slow activation kinetics compared with the HOG and mating pathways.

The activity of the Cdc42p-dependent MAPK pathways was also examined in response to exposure to their noncognate stimuli (Supplemental Figures S1 and S2). With two exceptions, the pathways functioned in an insulated manner: 1) GAL induced the HOG pathway at a rate far less than that observed with KCl induction (Supplemental Figure S1A, p38 blot), which occurs during the filamentation response (Adhikari and Cullen, 2014), and 2) α-factor induced phosphorylation of Hog1p (Supplemental Figure S1A, p38 blot), which did not result in induction of the *8XCRE–lacZ* reporter (Supplemental Figure S2B). These results support the prevailing view that Cdc42p-dependent pathways induce unique transcriptional and morphogenetic responses to specific stimuli.

Characterization of mutant isoforms of Cdc42p with MAPK pathway-specific phenotypes

MAPK pathways were also assessed by tests that were less labor intensive than phosphoblot analysis and provided a first approximation of MAPK pathway activity. The plate-washing assay (PWA) measures invasive growth and the activity of the fMAPK pathway (Roberts and Fink, 1994). Salt sensitivity was used to measure the activity of the HOG pathway (Maeda *et al.*, 1994, 1995), and growth arrest by α-factor (halo assays) was used to measure the activity of the mating pathway (Sprague *et al.*, 1983). Data from the functional tests were quantified by ImageJ analysis and color coded to facilitate interpretation (Figure 2A). Mutants lacking an intact fMAPK

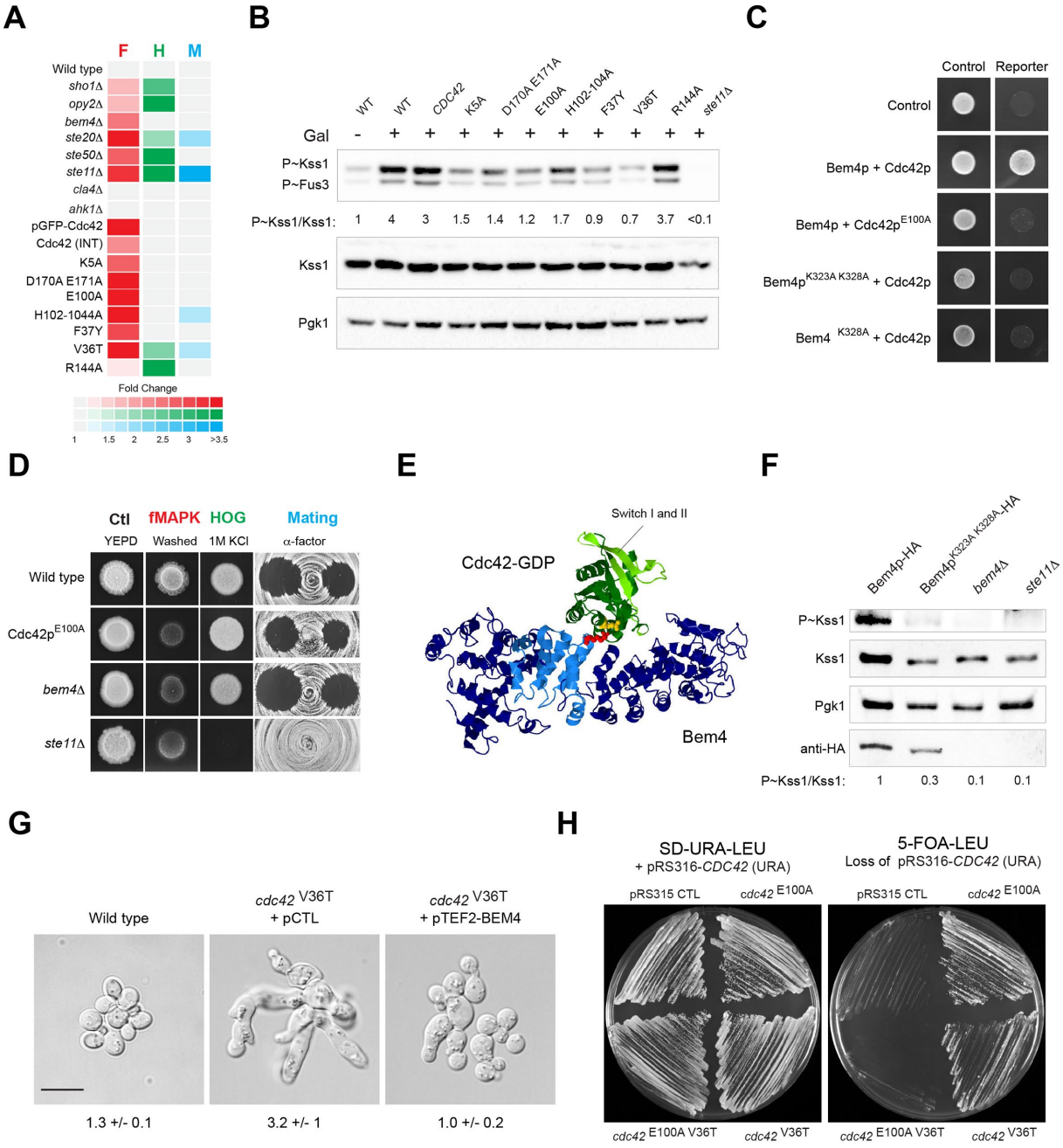

FIGURE 2: Interaction between Bem4p and Cdc42p is required for fMAPK pathway activity. (A) *CDC42* alleles that show phenotypes in MAP kinase-dependent assays (for raw data, see Supplemental Figures S3, S5, and S6). Color represents a phenotype in the PWA on YEPD (fMAPK, F, red), growth of cells on YEPD + 1 M KCl (HOG, H, green), or growth arrest by halo formation in response to α-factor (6 and 1.8 μM concentrations were tested; Mating, M, blue). Color intensity represents the severity of the defect based on quantitation by ImageJ analysis; gray, no difference from wild type. (B) Wild-type cells (PC6810), a collection of *cdc42* alleles, and the *ste11*Δ mutant control strain (PC6604), were grown in YEP-GAL for 5.5 h to examine P~Kss1p levels. See Figure 1B for details. (C) Two-hybrid analysis of the indicated versions of Cdc42p and Bem4p. Two-hybrid analysis was performed in multiple replicates ($n = 2$), and a representative images are shown. For the complete data set, see Supplemental Figure S8. (D) Phenotypes of Cdc42p^{E100A} and the *bem4*Δ mutant in tests for MAP kinase pathway activity. See panel A for details. (E) Ribbon diagram of the GDP-bound conformation of Cdc42p, based on the structure of the mammalian Cdc42p and DOCK9 complex (PDB ID#2wmn). The switch I and II regions are highlighted in light green (Johnson, 1999). The glutamate residue at position 100 in the α3 helix is shown in yellow. The K328 residue in Bem4p is shown in magenta. (F) P~Kss1p levels in strains carrying Bem4p$^{K323A\ K328A}$-HA and controls. Immunoblots were performed as in Figure 1B, except blots were also probed with α-HA antibodies to detect Bem4p-HA. (G) Wild-type cells (PC6810) and cells expressing *cdc42*^{V36T} (see Supplemental Table S1) containing pTEF2 (pCTL, PC6365) or pTEF2-*BEM4-GFP* (PC6367). Bar, 10 microns. Numbers indicate the circularity index (length-to-width ratio) of cells ($n > 100$) measured by ImageJ analysis; error, SD (H) The *cdc42::NAT* strain carrying the *pRS316-GFP-CDC42* plasmid and indicated *CDC42* alleles were examined for growth on SD-URA and 5-FOA media ($n = 2$).

pathway were examined by these tests. As expected, loss of common amplifiers of the fMAPK pathway showed defects in all three pathways (Figure 2A; Supplemental Figure S3A, *ste20*Δ and *ste11*Δ). Also as expected, several mutants were defective for two of the three pathways (Figure 2A; Supplemental Figure S3A, *sho1*Δ, *opy2*Δ, and *ste50*Δ). Ste50p interacts with Ste11p and is thought to regulate all three pathways (Wu *et al.*, 1999, 2006; Jansen *et al.*, 2001; Truckses *et al.*, 2006) but did not regulate the mating pathway in this context. We also found, in line with our previous work (Pitoniak *et al.*, 2015), that loss of the adaptor protein Bem4p only impacted in the fMAPK pathway (Figure 2A; Supplemental Figure S3A, *bem4*Δ). Two proteins that regulate the HOG pathway, the PAK Cla4p (Cvrckova *et al.*, 1995; Tatebayashi *et al.*, 2006) and the adaptor protein Ahk1p (Nishimura *et al.*, 2016), were also tested and were not found to regulate the HOG or fMAPK pathways (Figure 2A; Supplemental Figure S3, B–F). Thus, Ahk1p and Cla4p do not appear to regulate the fMAPK pathway. These results support and extend our understanding of the proteins that regulate the fMAPK pathway.

Despite the fact that Cdc42p binds to the same PAK, Ste20p, to regulate the three MAPK pathways, versions of Cdc42p have been identified with MAPK pathway-specific phenotypes (Mosch *et al.*, 2001; Tatebayashi *et al.*, 2006; Truckses *et al.*, 2006). An N-terminal green fluorescent protein (GFP) fusion to Cdc42p, expressed as the sole copy of *CDC42* in the cell, was defective in the fMAPK pathway but not the HOG or mating pathways (Figure 2A, pGFP-Cdc42; Supplemental Figure S4). To follow up on this observation, a collection of *cdc42* alleles that targeted charged, often evolutionarily conserved amino acid residues and functional domains (Kozminski *et al.*, 2000), was introduced into Σ1278b, which is capable of filamentous growth. Twenty-four nonlethal alleles at 25°C were viable and examined by functional tests (Supplemental Table S1; Supplemental Figures S5–S7). Most alleles of *CDC42* did not show a mutant phenotype (Supplemental Figures S5–S7). However, several alleles showed pathway-specific phenotypes. Five alleles were mainly defective in the fMAPK pathway based on invasive growth (Figure 2A, K5A, D170A E171A, E100A, H102-104A, and F37Y) and P~Kss1p analysis (Figure 2B). Note that the integrated version of *CDC42* (INT) had a defect in invasive growth. E100A was previously shown to be specifically defective for fMAPK pathway activity (Mosch *et al.*, 2001; Truckses *et al.*, 2006). Another allele of *CDC42* was identified that was primarily defective for growth on salt (Figure 2, A and B, R144A). Several alleles contained mutations in the Switch I domain, which binds PAKs and, as one might expect, were defective for all three pathways (Figure 2, A and B, V36T).

Interaction between Bem4p and Cdc42p stimulates fMAPK pathway signaling

Versions of Cdc42p that are specifically defective for fMAPK pathway activity might fail to interact with proteins that regulate the fMAPK pathway. In particular, we considered Bem4p because it is a pathway-specific regulator of the fMAPK pathway (Figure 2A; Pitoniak *et al.*, 2015) and a Cdc42p-interacting protein (Mack *et al.*, 1996; Drees *et al.*, 2001). Bem4p interacts with Cdc42p by two-hybrid analysis (Drees *et al.*, 2001; Pitoniak *et al.*, 2015). In this study, two-hybrid analysis was examined using a version that mimicked the GDP-bound conformation and did not associate with membranes (*CDC42*$^{D118A\ C188S}$). By two-hybrid analysis, Bem4p showed reduced interaction with Cdc42p^{E100A} (Figure 2C), which is present at the same level as wild-type Cdc42 (Supplemental Figure S4G). Bem4p was also partially defective for interaction with Cdc42p^{K5A} and Cdc42p$^{D170A\ E171A}$, but not for interaction with Cdc42p^{V36T}, Cdc42p$^{H102-104A}$, or Cdc42p^{R144A} (Supplemental Figure S8A). Thus, the signaling defect of Cdc42p^{E100A} in the fMAPK pathway might result from its inability to interact with Bem4p. Consistent with this possibility, the phenotypes of cells carrying Cdc42p^{E100A} resembled the phenotypes of the *bem4*Δ mutant. Like the *bem4*Δ mutant, Cdc42p^{E100A} was defective for invasive growth but not for growth on salt or halo formation (Figure 2D). Cdc42p^{E100A} was also defective for invasive growth by another commonly used test, the single cell invasive growth assay (Cullen and Sprague, 2000b), but not shmoo formation in response to α-factor (Supplemental Figure S7). Similar phenotypes have been reported for the *bem4*Δ mutant (Pitoniak *et al.*, 2015).

Like other monomeric GTPases, Cdc42p undergoes a conformational change upon GTP binding that allows the protein to interact with effector proteins. Most proteins that interact with Cdc42p bind to the active or GTP-bound conformation of the protein. An unusual feature of Bem4p is that it interacts with the GDP-bound (inactive) and GTP-bound (active) conformations of Cdc42p (Drees *et al.*, 2001; Pitoniak *et al.*, 2015). We modeled the yeast Cdc42p protein onto the crystal structure of human Cdc42p (Rittinger *et al.*, 1997; Nassar *et al.*, 1998; Hoffman *et al.*, 2000), which are >80% identical, and found that the glutamic acid at position 100, which is conserved between human and yeast Cdc42p, lies in the third alpha (α3) helix of the protein (Figure 2E, Cdc42 is in green, E100 is marked in yellow). The α3 helix is on the opposite side of the protein as the effector-binding domain (Figure 2E, Switch I and II) and is not thought to undergo a conformational change upon nucleotide binding. Therefore, Bem4p may interact with a region of Cdc42p that does not undergo a conformational change. This finding is consistent with Bem4's ability to associate with the GDP and GTP-bound conformations of Cdc42p (Pitoniak *et al.*, 2015).

We previously mapped a region of Bem4p from 300 to 400 amino acid residues as being required for interaction with Cdc42p (Pitoniak *et al.*, 2015). This region exhibits sequence similarity to members of the SmgGDS family of proteins. SmgGDS proteins contain Armadillo (ARM) repeats (Peifer *et al.*, 1994; Shimizu *et al.*, 2017) that mediate interactions with Rho GTPases (Vithalani *et al.*, 1998; Vikis *et al.*, 2002; Hamel *et al.*, 2011; Shimizu *et al.*, 2017, 2018; Jennings *et al.*, 2018) and other proteins. The predicted structure of Bem4p was determined by bioinformatics algorithms Phyre 2 and iTASSER, which generated similar structures with > 90% confidence and theoretical root mean square deviation values above cutoff. In both structures, Bem4p contained alpha helical motifs that resembled ARM repeats (Figure 2E, Bem4p is in blue, and the 300–400 region is marked in light blue).

Basic residues in the Bem4p$^{300-400}$ region might mediate electrostatic interactions with the glutamate at position 100 in Cdc42p. Ten basic residues are present in the Bem4p$^{300-400}$ region. Site-directed mutagenesis was used to generate versions of Bem4p that lacked basic residues based on the predicted minimal free energy between Cdc42p^{E100A} and Bem4p$^{300-400}$ using the protein-docking server Haddock (van Zundert *et al.*, 2016). Bem4p$^{K323A\ K328A\ K351A\ R352A}$, Bem4p$^{K323A,\ K328A}$, Bem4p$^{K351A\ R352A}$, Bem4p^{K323A}, and Bem4p^{K328A} were constructed and tested. Bem4p$^{K323A\ K328A\ K351A\ R352A}$, Bem4p$^{K323A\ K328A}$, and Bem4p^{K328A} were defective for interaction with Cdc42p (Figure 2C; Supplemental Figure S8). The Bem4p$^{300-400}$ region is not thought to be important for the stability of the protein (Pitoniak *et al.*, 2015). Bem4p$^{K351A\ R352A}$ was partially defective, and Bem4p^{K323A} was not defective (Supplemental Figure S8). The residue K328 in Bem4p had the lowest free energy based on Haddock and may be a key residue that mediates interaction with Cdc42p

(Figure 2E, red residue; Supplemental Movie S1). Compensatory mutations did not restore the interaction between Bem4p and Cdc42p (Supplemental Figure S8, Bem4p^{K328E} and Cdc42p^{E100A}), which indicates that, like for other Cdc42p-interacting proteins (Hoffman *et al.*, 2000), the interaction between Bem4p and Cdc42p may involve multiple electrostatic contacts. Therefore, basic residues in the ARM-type repeat region of Bem4p are required for interaction with Cdc42p.

Versions of Bem4p that showed reduced interaction with Cdc42p were tested for fMAPK pathway activity. Bem4p-HA$^{K323A\ K328A}$ was expressed from the *BEM4* locus at levels similar to the Bem4p-HA protein (Figure 2F, anti-HA blot). Bem4p-HA$^{K323A\ K328A}$ was defective for fMAPK pathway activity based on P~Kss1p levels (Figure 2F). Bem4p-HA$^{K323A\ K328A}$ was also defective for invasive growth and expression of the *FUS1-HIS3* reporter (Supplemental Figure S9), which in strains lacking an intact mating pathway (*ste4*Δ) provides a readout of fMAPK pathway activity (Cullen *et al.*, 2004; Chavel *et al.*, 2010; Adhikari *et al.*, 2015b; Pitoniak *et al.*, 2015; Basu *et al.*, 2016). Bem4p-HA^{K328A} was also tested but did not show the same defects in MAPK signaling and invasive growth as Bem4p-HA$^{K323A\ K328A}$. Thus, both residues of Bem4p are probably important for the regulation of Cdc42p in the fMAPK pathway.

Bem4p can also regulate cell polarity. In particular, high-copy plasmids carrying *BEM4* can suppress the temperature-sensitive growth defects of *cdc42* alleles (Mack *et al.*, 1996). A high-copy plasmid expressing *BEM4* (pTEF2-*BEM4*) suppressed the morphological defect of cells carrying a version of Cdc42p with a single amino acid change in its effector binding domain (EFF), Cdc42p^{V36T} (Figure 2G). To test whether suppression occurred through the interaction between Bem4p and Cdc42p, the E100A mutation was introduced into *cdc42*^{V36T} by site-directed mutagenesis. Based on the above results, the E100A mutation would be expected to interfere with the interaction between Bem4p and Cdc42p. Cells expressing Cdc42p$^{V36T\ E100A}$ had a viability defect compared with cells carrying versions of Cdc42p with either single change, based on plasmid-loss with the drug 5-fluoroorotic acid (Figure 2H, 5-FOA; Boeke *et al.*, 1984). Thus, Cdc42p^{V36T} and Cdc42p^{E100A} may function synergistically to regulate viability. Therefore, the interaction between Bem4p and Cdc42p may also be required for viability when Cdc42p interaction with effector proteins and its cell polarity function is compromised.

The polarity adaptor Bem1p also regulates the fMAPK pathway

Bem4p is a pathway-specific adaptor for the fMAPK pathway. Bem1p is another adaptor for Cdc42p, which functions to regulate the establishment of cell polarity (Bender and Pringle, 1991; Zheng *et al.*, 1995; Park *et al.*, 1997). Bem1p is critical for symmetry breaking by generating positive feedback (Butty *et al.*, 1998; Bose *et al.*, 2001; Gladfelter *et al.*, 2001; Yamaguchi *et al.*, 2007). Bem1p also regulates the mating (Leeuw *et al.*, 1995; Lyons *et al.*, 1996) and HOG pathways (Tanaka *et al.*, 2014). Bem1p has not been tested for a role in regulating the fMAPK pathway because it is essential for viability in the Σ1278b background but is not essential in laboratory strains like S288c (Dowell *et al.*, 2010).

To determine whether Bem1p regulates the fMAPK pathway, the *BEM1* gene was deleted in a strain containing the *BEM1* gene on a plasmid (p*BEM1*, provided by D. Lew). Plasmid-loss experiments with 5-FOA showed that the *bem1*Δ mutant was viable under some conditions (YEP-GAL media at 30°C and synthetic media) but not others (yeast extract, peptone, and dextrose [YEPD] media at 30°C or YEP-GAL media at 25°C). The conditional viability of the *bem1*Δ mutant allowed assessment of Bem1p's role in regulating fMAPK. The *bem1*Δ mutant showed reduced fMAPK pathway activity based on phosphorylation of Kss1p (Figure 3A). The signaling defect of the *bem1*Δ mutant was less severe than that of the *ste11*Δ mutant and resembled the phenotype of cells lacking other upstream pathway components like Msb2p (Cullen *et al.*, 2004), Sho1p (Adhikari *et al.*, 2015a), and Rsr1p (Basu *et al.*, 2016). The *bem1*Δ mutant was also defective for invasive growth by the PWA (Figure 3B, washed). The *bem1*Δ mutant had a defect in colony ruffling (Figure 3B,

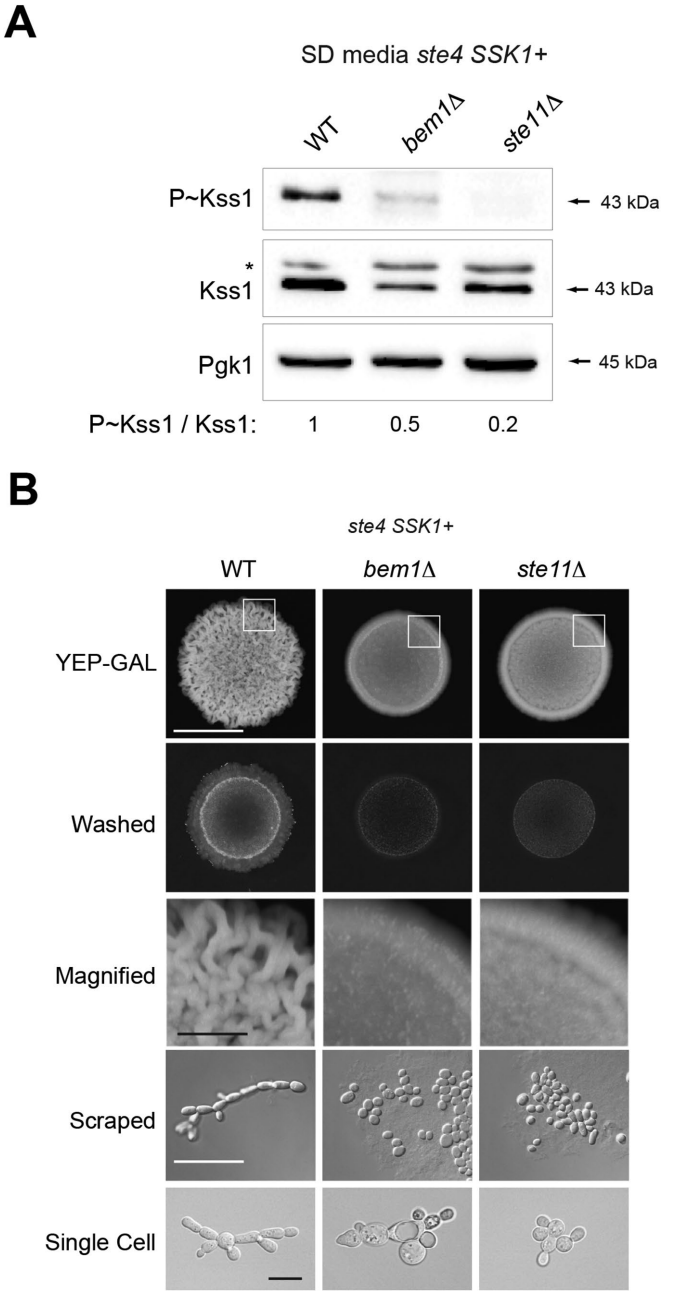

FIGURE 3: Bem1p regulates the fMAPK pathway. (A) P~Kss1p levels in wild-type cells (PC538), the *bem1*Δ mutant (PC6509), and the *ste11*Δ mutant (PC3861) grown for 5.5 h in SD + AA media. See Figure 1B for details. (B) Filamentous growth and colony patterning of the same strains as in A grown in YP-GAL for 3 d. Bar, 20 microns. The experiment was performed in multiple biological replicates ($n = 3$). Representative images are shown.

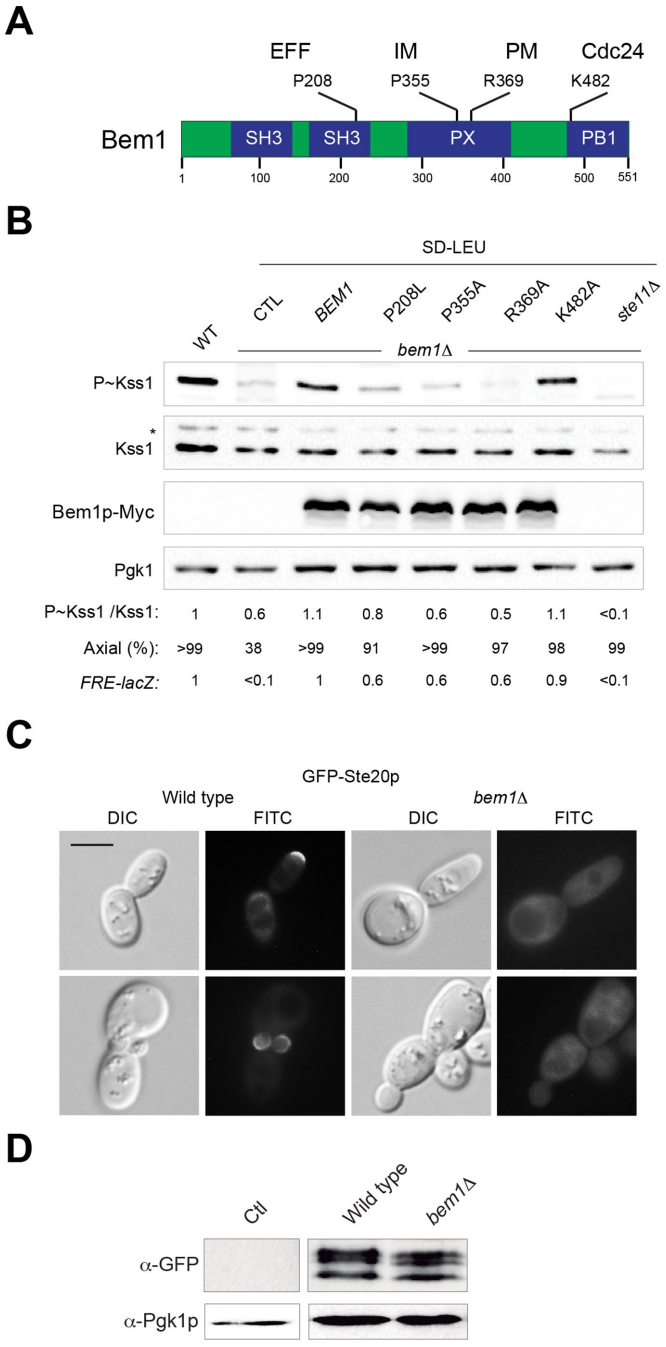

FIGURE 4: Role of Bem1p in regulating the fMAPK pathway. (A) Diagram of the Bem1p protein, which shows the EFF, intramolecular binding domain (IM), plasma membrane (PM) binding domain, and Cdc24p-interacting domain (Cdc24). Alleles used in the study come from Irazoqui *et al.* (2003). (B) Immunoblot analysis of wild-type cells (PC6810) and the *bem1*Δ mutant (PC6680) containing wild-type *BEM1* (PC6514), a control plasmid pRS315 (CTL), or *BEM1* alleles on plasmids (PC6515-6518), and the *ste11*Δ mutant (PC6604) grown on SD-LEU media. Immunoblots were performed as in Figure 1B, except blots were also probed with α–Myc antibodies to detect Bem1p-Myc. Axial budding (%) was determined for cells grown to mid-log phase in SD-LEU medium. Budding pattern was determined by CFW staining ($n > 200$). *FRE-lacZ* assays were performed under the same conditions ($n = 3$) and the average values are shown. A <10% difference by SD was observed between trials. (C) Localization of GFP-Ste20p expressed from plasmid (PC4394) described in (Leberer *et al.*, 1997) in wild-type cells (PC6810) and the *bem1*Δ mutant (PC6680). Cells were grown in S-GAL-URA medium to mid log phase (4 h). Bar, 10 microns. (D) Immunoblot of GFP-Ste20p in the indicated strains grown under the same conditons. Antibodies to Pgk1p were used as a control for protein levels.

Magnified), which occurs due to cell-to-cell contacts during biofilm/mat formation, a microbial process that is also controlled by the fMAPK pathway (Reynolds and Fink, 2001; Granek and Magwene, 2010). The *bem1*Δ mutant was defective in the formation of filamentous chains of cells (Figure 3B, Scraped; Supplemental Figure S10). By the single cell assay, the *bem1*Δ mutant exhibited polarity and bud-site-selection defects (Figure 3B, Single cell; Supplemental Figure S10). Therefore, Bem1p regulates the fMAPK pathway and is required for invasive growth and biofilm/mat formation.

Bem1p regulates the fMAPK pathway by recruitment of Ste20p to the plasma membrane (PM)

Bem1p is composed of domains that perform specific functions during polarity establishment (Park *et al.*, 1997; Endo *et al.*, 2003; Irazoqui *et al.*, 2003). These include two SH3 domains, a PX domain, and a PB1 domain (Figure 4A). Point mutations in these domains selectively cripple individual aspects of Bem1p function (Irazoqui *et al.*, 2003). Versions of Bem1p lacking several of these functional domains were tested to ascertain Bem1p's role in regulating the fMAPK pathway. The fMAPK pathway operates in basal (Figure 4B, SD-LEU) and activated states (Supplemental Figure S11A, YEP-GAL) depending on the condition (Cullen *et al.*, 2004; Adhikari *et al.*, 2015a; Basu *et al.*, 2016). Bem1p^{P208L}, which is defective for binding to effector proteins like Ste20p (Leeuw *et al.*, 1995; Bose *et al.*, 2001; Winters and Pryciak, 2005; Takaku *et al.*, 2010), was defective for fMAPK pathway signaling (Figure 4B). Bem1p^{P355A}, which prevents an intramolecular interaction that results in a constitutively open conformation of the protein (Irazoqui *et al.*, 2003), was also defective for fMAPK pathway signaling (Figure 4B). Bem1p^{R369A}, which is defective for localization to the PM and binding to the phosphatidyl inositol polyphosphate (PIP) PI(4,5)P_2 at the PM (Ago *et al.*, 2001; Yu and Lemmon, 2001; Irazoqui *et al.*, 2003), was also defective for fMAPK pathway activity (Figure 4B). Bem1p^{K482A}, which is defective for interaction with Cdc24p (Peterson *et al.*, 1994; Zheng *et al.*, 1995; Park *et al.*, 1997; Toenjes *et al.*, 1999; Elion, 2000; Gulli *et al.*, 2000; Nern and Arkowitz, 2000; Shimada *et al.*, 2000; Ito *et al.*, 2001; Butty *et al.*, 2002), did not show a defect in fMAPK signaling (Figure 4B) However, a minor defect was seen in YEP-GAL (Supplemental Figure S11A). One interpretation of this result is that Bem1p interacts with effector proteins and Cdc24p at the PM to regulate the fMAPK pathway. Bem1p may also require cycling between its open and closed conformation to regulate the fMAPK pathway.

To test whether Bem1p is required for the recruitment of Ste20p to the PM, the localization of GFP-Ste20p was examined. In wild-type cells, GFP-Ste20p localized to the cell cortex in small buds and the distal pole in large cells (Figure 4C, seen in 5–10% of cells). In cells lacking Bem1p, GFP-Ste20p showed a diffuse pattern (Figure 4C, seen in >200 cells examined). By immunoblot analysis, the level of GFP-Ste20p was the same in wild-type and *bem1*Δ mutant cells (Figure 4D). These data fit with the idea that Ste20p is the major effector of Cdc42p in the fMAPK pathway (Peter *et al.*, 1996; Leberer *et al.*, 1997; Truckses *et al.*, 2006) and with the idea that Bem1p recruits Ste20p to GTP-Cdc42p at the PM during mating (Peter *et al.*, 1996; Leberer *et al.*, 1997; Winters and Pryciak, 2005; Truckses *et al.*, 2006) and is required for plasma-membrane

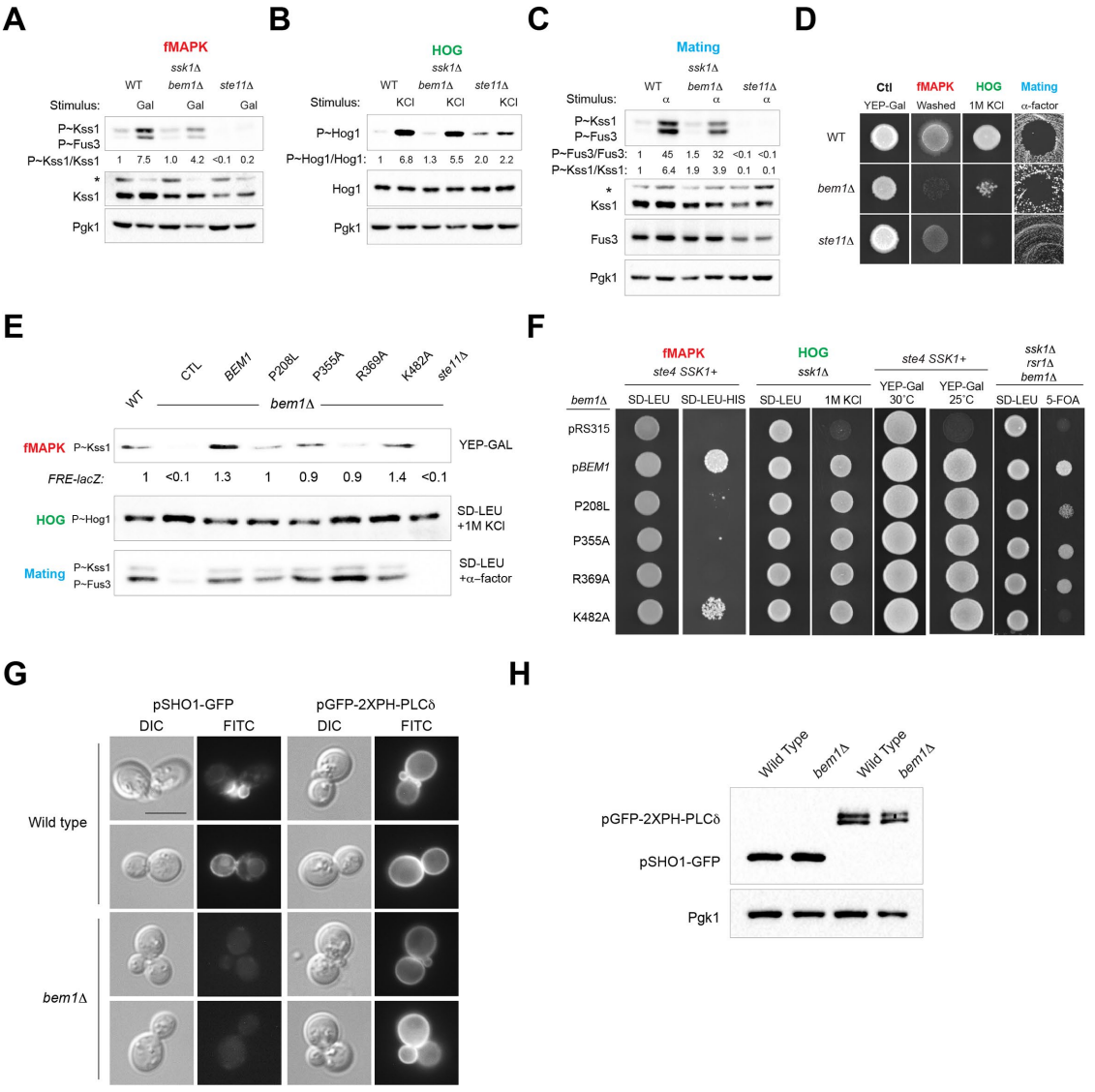

FIGURE 5: Bem1p regulates different pathways in different ways. (A) Wild-type cells (PC6810), the *bem1*Δ mutant (PC6680), and the *ste11*Δ mutant (PC6604) were grown for 5.5 h in YEP-GAL media and extracts were probed by immunoblot analysis as described in Figure 1B. (B) Cells in A were grown in SD + AA containing 0.4 M KCl (5 min). Immunoblots were performed as described in Figure 1C. Cells in A were also grown in SD + AA media with 6 μM of α-factor. (D) Cells in A were examined by phenotypic tests. (E) Cells in Figure 4B were examined by phosphoblot analysis under fMAPK pathway-activating conditions (YEP-GAL). *FRE-lacZ* assays were performed in YEP-GAL ($n = 3$). Average values are reported with <10% SD between trials. Phosphoblots were also performed as indicated during growth on SD-LEU + 0.4 M KCl (to evaluate P~Hog1p) and on SD-LEU + 6 μM α-factor (to evaluate P~Fus3p). (F) Growth of *bem1*Δ mutants in the *ste4 SSK1+* (PC6509) or *ssk1*Δ background (PC6680), and the *ssk1*Δ *rsr1*Δ *bem1*Δ triple mutant (PC7343) containing the indicated plasmids was assessed by spotting cells onto the indicated media. On SD-LEU-HIS, the *FUS1-HIS3* growth reporter was used to examine the fMAPK pathway activity. (G) The localization of Sho1p-GFP and pGFP-2XPH-PLCδ in wild-type cells and the *bem1*Δ mutant. Two representative examples are shown ($n > 50$). (H) Immunoblot analysis of pGFP-2XPH-PLCδ and Sho1p-GFP in wild-type cells and the *bem1*Δ mutant. Blots for α-GFP and α-Pgk1p as a control for protein loading.

[AQ 3]

localization of Ste20p during filamentous growth (Mosch *et al.*, 2001). Therefore, Bem1p is required for the PM localization of Ste20p in the fMAPK pathway. This may be due to a direct interaction between Bem1p and Ste20p or involve other proteins.

Bem1p regulates effector pathways by different mechanisms

To compare Bem1p function in Cdc42p-dependent MAPK pathways, phosphoblot and functional assays were performed. As shown above (Figures 3 and 4), Bem1p regulated the fMAPK pathway (Figure 5, A and D). Bem1p did not play a major role in regulating the HOG pathway (Figure 5B, at 5 min) although the *bem1*Δ *ssk1*Δ mutant was sensitive to growth on high-osmolarity medium (Figure 5D, KCl). Bem1p also regulated the mating pathway (Figure 5, C and D). Therefore, Bem1p is a general regulator of Cdc42p-dependent MAPK pathways in yeast.

Does Bem1p regulate Cdc42p-dependent MAPK pathways in the same way? As shown above for basal conditions (Figure 4), all of

the domains of Bem1p tested (P208L, P355A, R369A, and K482A) were required for Bem1p to regulate the fMAPK pathway under activating conditions (Figure 5E; for the blot plus controls, see Supplemental Figure S11A). A different pattern was seen in response to salt or pheromone (Figure 5E; Supplemental Figure S11, B and C). Therefore, Bem1p regulates Cdc42p-dependent MAPK pathways by different mechanisms. In line with this idea, *bem1* alleles showed different patterns of fMAPK pathway reporter activity (Figure 5F, SD-HIS) and salt sensitivity (Figure 5F, KCl). The requirement of Bem1p alleles in production of P~Fus3p levels did not match its requirement for shmoo formation (Supplemental Figure S11D), which might be explained because Bem1p has multiple functions in mating that include direction sensing (Moore *et al.*, 2013) and the formation of mating projections (Ito *et al.*, 2001; Butty *et al.*, 2002).

Bem1p is required for viability in the Σ1278b background under some conditions (Dowell *et al.*, 2010). The viability defect of the *bem1*Δ mutant was rescued by all of the versions of Bem1p tested (Figure 5F, YEP-GAL 25°C). The *bem1*Δ mutant also had a bud-site-selection defect that was rescued by all of the versions of Bem1p tested (see Figure 4B, Axial %). Versions of Bem1p that rescued the bud-site-selection defect failed to rescue the fMAPK pathway signaling defect (see Figure 4B), which addressed the potential concern that Bem1p regulates the fMAPK pathway by its role in regulating bud-site selection (Basu *et al.*, 2016). In some strains, Bem1p requires Rsr1p for viability, which defines a function for Bem1p in symmetry breaking during polarity establishment (Irazoqui *et al.*, 2003). Bem1p also required Rsr1p for viability in the Σ1278b background (Figure 5F, 5-FOA). Here, a unique pattern of rescue by *bem1* alleles was seen. The pattern we saw matched the pattern shown in Irazoqui *et al.* (2003), except that R369A rescued the viability defect in this study but not in Irazoqui *et al.* (2003). The fact that *bem1* alleles have different phenotypes in symmetry breaking in the two studies might be explained by differences in strain backgrounds. Bem1p is required for viability in some strain backgrounds but not others.

Bem1p was also required for the localization of Sho1p-GFP to the PM (Figure 5G). This was not due to a change in the levels of the Sho1p protein (Figure 5H). Sho1p is an integral membrane tetraspan protein that regulates the fMAPK and HOG pathways (Maeda *et al.*, 1995; O'Rourke and Herskowitz, 1998; Cullen *et al.*, 2004; Tatebayashi *et al.*, 2015). At first, we thought this phenotype might explain how Bem1p regulates the fMAPK pathway. However, all versions of Bem1p tested rescued the localization defect of Sho1p (Supplemental Figure S12A). Like Cdc42p (Zhang *et al.*, 2001; Menon, 2007), Bem1p regulates exocytosis, the polarized delivery of vesicles to the PM (Zajac *et al.*, 2005; France *et al.*, 2006b). Bem1p was not required for regulating the distribution of PIPs at the PM, which might also impact protein localization, based on the localization (Figure 5G) and levels of a PI(4,5)P_2 binding reporter protein (Figure 5H, pGFP-2XPH-PLCδ; Stefan *et al.*, 2002). The localization defect of Sho1p-GFP in the *bem1*Δ mutant might result from a defect in exocytosis. Indeed, mutants defective for exocytosis were also defective for the plasma-membrane distribution of Sho1p-GFP (Supplemental Figure S*12B*). Therefore, Bem1p regulates different effector pathways in different ways. In some settings, Bem1p acts as a multifunctional adaptor where one or more domain(s) is required for function. In other settings, a single domain of Bem1p is required.

Ordering the activation sequence of the Cdc42p module in the fMAPK pathway

Bem4p and Bem1p are two adaptor proteins that function at the level of Cdc42p and regulate the fMAPK pathway. As their names suggest (Bud emergence defect), *BEM1* (Bender and Pringle, 1991; Chant *et al.*, 1991) and *BEM4* (Hirano *et al.*, 1996; Mack *et al.*, 1996) were initially identified as high-copy suppressors of the growth defects of conditional alleles of *cdc24* and/or *cdc42*. Bem1p and Bem4p are not homologues, and the proteins have unrelated amino acid sequences and protein-interaction domains. Loss of either protein results in a defect in the fMAPK pathway (e.g., Figures 2A and 3–5). Thus, Bem1p and Bem4p play nonredundant roles in regulating the fMAPK pathway. In support of this idea, a high-copy plasmid containing *BEM1*, which rescues the signaling defect of the *bem1*Δ mutant, did not rescue the fMAPK signaling defect of the *bem4*Δ mutant (Supplemental Figure S*13*). Likewise, a high-copy plasmid containing Bem4p, which rescues the signaling defect of the *bem4*Δ mutant, did not rescue the signaling defect of the *bem1*Δ mutant (Supplemental Figure S13). Therefore, Bem4p and Bem1p have nonoverlapping functions in regulating the fMAPK pathway.

As adaptor proteins, Bem1p and Bem4p interact with multiple proteins, particularly those that function at the level of the Cdc42p module. Bem1p and Bem4p both interact with Rsr1p, Cdc24p, and Cdc42p (Peterson *et al.*, 1994; Zheng *et al.*, 1995; Hirano *et al.*, 1996; Drees *et al.*, 2001). Rsr1p is the Ras-type GTPase that regulates bud-site selection (Park *et al.*, 1997) and the fMAPK pathway (Basu *et al.*, 2016). Rsr1p can also interact with Cdc42p (Kozminski *et al.*, 2003). To define how Rsr1p, Bem4p, and Bem1p regulate the fMAPK pathway, genetic suppression analysis was used. Genetic suppression analysis allows the ordering of components in a pathway by gain- and loss-of-function alleles. GFP-Msb2p (Adhikari *et al.*, 2015b), Sho1p^{P120L} (Vadaie *et al.*, 2008a), and Ste11-4p (Stevenson *et al.*, 1992) hyperactivate the fMAPK pathway and were tested for bypass of the signaling defects of the *rsr1*Δ, *bem4*Δ, and *bem1*Δ mutants. Hyperactive versions of Msb2p, Sho1p, and Ste11p partially rescued the fMAPK signaling defect of the *rsr1*Δ mutant (Figure 6A). This finding is consistent with previous results (Basu *et al.*, 2016), which suggest that Msb2p/Sho1p and Rsr1p converge on the Cdc42p module. For the *bem4*Δ mutant, a hyperactive version of Msb2p did not bypass the signaling defect, whereas hyperactive versions of Sho1p and Ste11p did (Figure 6B). This result indicates that Bem4p functions between Msb2p and Sho1p in the fMAPK pathway. For the *bem1*Δ mutant, hyperactive versions of Msb2p and Sho1p did not bypass the signaling defect, whereas a hyperactive version of Ste11p did (Figure 6C). This result indicates that Bem1p functions between Sho1p and Ste11p in the fMAPK pathway. Collectively, the data indicate that Bem4p functions upstream of Bem1p in the fMAPK pathway. This conclusion is probably an oversimplification, because the proteins exist in multi-protein complexes and have multiple functions in regulating the fMAPK pathway. However, this order is supported by the fact that Bem4p associates with the GDP-bound conformation of Cdc42p (Drees *et al.*, 2001; Pitoniak *et al.*, 2015), whereas Bem1p preferentially associates with the GTP-bound conformation of Cdc42p (Butty *et al.*, 1998; Kozminski *et al.*, 2003). Two-hybrid analysis showed that the interaction between Bem1p and GDP-Cdc42p required Rsr1p (Figure 6D, Cdc42 refers to *CDC42*$^{D118A\ C188S}$), whereas the interaction between Bem4p and GDP-Cdc42p did not require Rsr1p or Bem1p (Figure 6E).

The above results led us to ask how the activation of the fMAPK pathway is initiated at the level of Cdc24p. To address this question, we turned to the mating pathway, which is known to recruit Cdc24p by Ste4p (Zhao *et al.*, 1995). By two-hybrid analysis, Ste4p, which regulates the mating pathway and binds the CH domain of Cdc24p, bound to full-length Cdc24p, whereas Rsr1p, which regulates the fMAPK pathway and also binds the CH domain of Cdc24p (Shimada *et al.*, 2004), did not (Figure 6F). Rather, Rsr1p bound to a version of

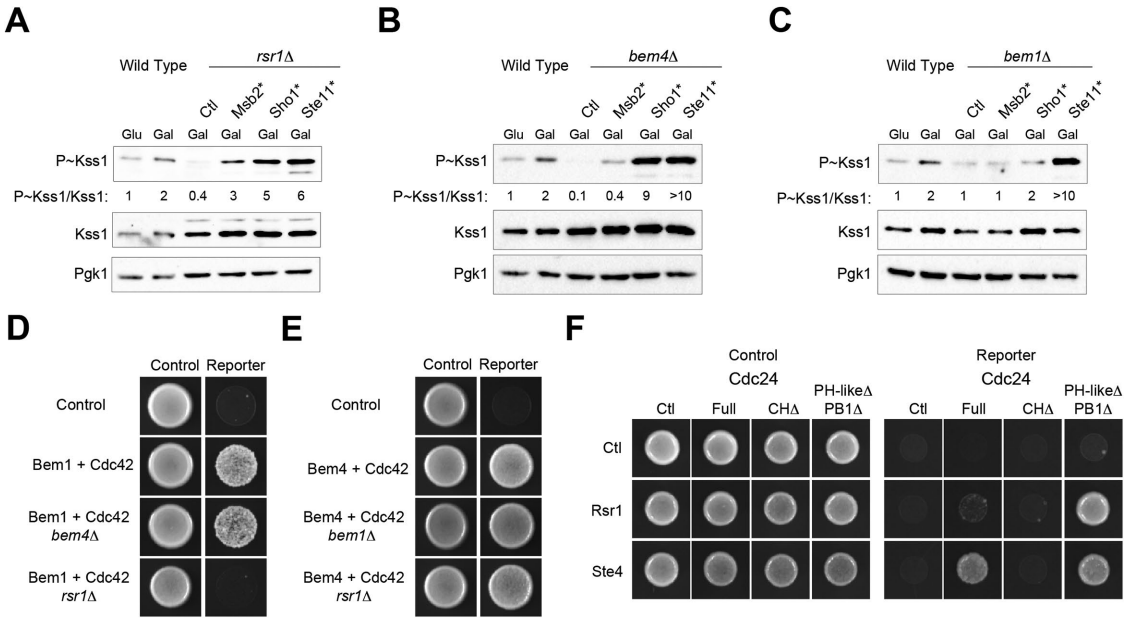

FIGURE 6: Bem1p and Bem4p regulate the fMAPK pathway at different points in the signaling cascade. (A) Wild-type cells (PC538) and the *rsr1*Δ mutant (PC4256) were examined in YEPD (Glu) or YEP-GAL (Gal) media by immunoblot analysis. The *rsr1*Δ mutant contained either a control plasmid (pRS316, Ctl) or plasmids harboring hyperactive versions of Msb2p (Msb2*, pGFP-MSB2; Sho1*, pSHO1-P120L-GFP, or Ste11*, pSTE11-4, see Table 2 for details). Cells were grown in YEPD or YEP-GAL media for 5.5 h. See Figure 1B for details. (B) Same as in A, except the *bem4*Δ mutant (PC3551) was examined. (C) Same as in A, except the *bem1*Δ mutant (PC6509) was examined. (D) Two-hybrid analysis of Bem1p and Cdc42p in wild-type cells and the indicated mutants. (E) Two-hybrid analysis of Bem4p and Cdc42p in wild-type cells and the indicated mutants. (F) Two-hybrid analysis of the interactions of Rsr1p and Ste4p with full-length Cdc24p and versions that lack the CH (CHΔ) or PH-like and PB1 (PH-likeΔ PB1Δ) domains. See Table 2 for details about plasmids.

Cdc24p lacking an auto-inhibitory domain (PH-likeΔ-PB1Δ). Given that Bem4p binds to the PH-like domain of Cdc24p to relieve auto-inhibition, it is possible that Ste4p initiates recruitment of Cdc24p in the mating pathway, whereas in the fMAPK pathway, Bem4p may bind Cdc24p to relieve auto-inhibition (Pitoniak *et al.*, 2015), which permits subsequent binding by Rsr1p. Bem1p binds the PB1 domain of Cdc24p and may also initiate recruitment of Cdc24p in the fMAPK pathway. However, Bem1p acts at a later step in the pathway than Bem4p and does not require binding to Cdc24p to activate the fMAPK pathway under some conditions, which argues against this possibility.

Exploring the roles of Bem4p and Bem1p on fMAPK pathway kinetics

Given that the fMAPK pathway has a unique induction profile, we became interested in understanding the relative contribution of the Bem adaptors in regulating the kinetics of fMAPK pathway signaling. Phosphorylation of the MAP kinase Kss1p was examined in cells carrying the Cdc42^{E100A} allele, and in the *bem1*Δ and *bem4*Δ mutants over the time. Similar to the results shown in Figure 1, the phosphorylation of Kss1p was induced by Gal after 1 h and peaked around 6 h (Figure 7). The Cdc42^{E100A} allele showed a similar activation profile but levels of P~Kss1p were reduced compared with wild type (Figure 7A). This result indicates that this residue of Cdc42p is involved in induction of the fMAPK pathway. E100 is in a region of Cdc42p that does not undergo a conformation change, suggesting that this is not due to a defective interaction with the effector PAK Ste20p. A similar pattern was observed in cells lacking Bem4p (Figure 7B), except that the levels of P~Kss1p were even lower than seen in cells with Cdc42^{E100A}. These data support the idea that the signaling defect of Cdc42^{E100A} is through a defect in interaction with Bem4p. In cells lacking Bem1p, no induction of P~Kss1p was observed (Figure 7C, compare 3- and 6-h time points), even when P~Kss1p levels were monitored for extended periods (8 h). This result indicates that Bem1p is critical for induction of the fMAPK pathway, since the lack of this protein completely ablated pathway activity under this condition. These results support the idea that Bem4p and Bem1p regulate the fMAPK pathway by different mechanisms.

DISCUSSION

Rho GTPases are key regulators of cell polarity and signal transduction in eukaryotes. Many proteins interact with Rho GTPases and impact their functions and activities, including adaptor proteins that link GTPases to their activators and effectors. By examining two adaptors of Cdc42p in yeast, Bem4p and Bem1p, we further define how Cdc42p regulates one of the many pathways in which it functions. Bem1p and Bem4p, as well as Rsr1p, the GTPase that regulates bud-site-selection and the fMAPK pathway, exhibit some degree of pathway selectivity. Bem4p and Rsr1p regulate the fMAPK pathway but not the mating or HOG pathways, while Bem1p regulates the fMAPK pathway by a specific mechanism.

Cdc42p-dependent MAPK pathways show different kinetic profiles

The way that signaling pathways are activated can shape the output response, as well as signal specificity. MAPK pathway dynamics can be complex, involving fast and slow responses (English *et al.*, 2015), oscillations (Hilioti *et al.*, 2008), and feedback control (Madhani and Fink, 1997; Breitkreutz *et al.*, 2001). Moreover, activation of one

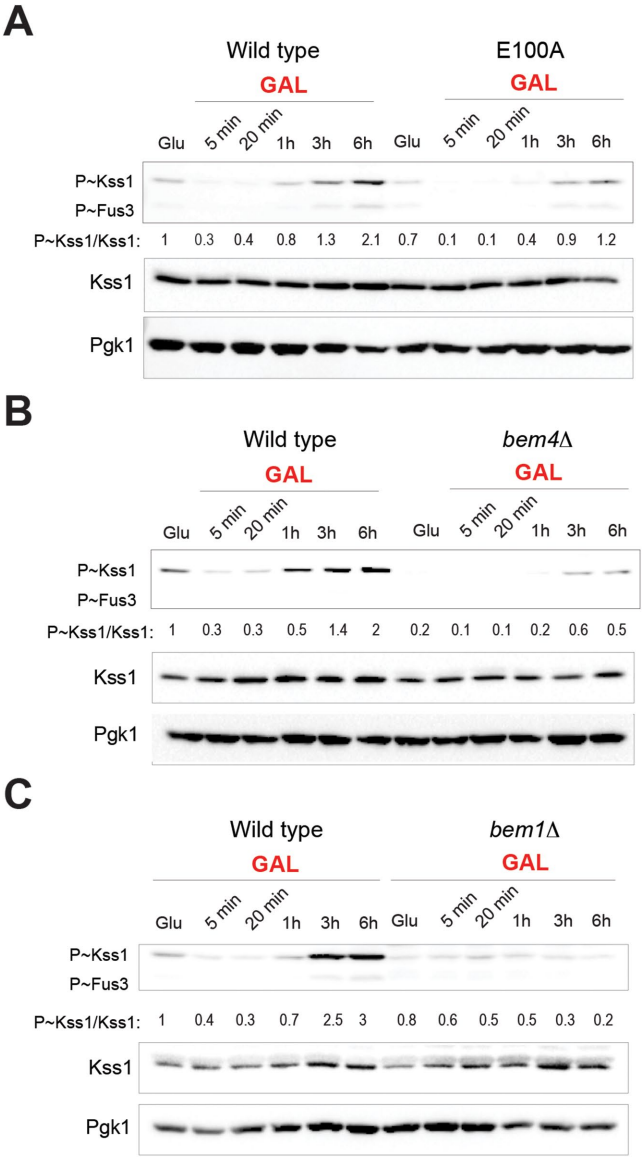

FIGURE 7: Time-course phosphoblot analysis of P~Kss1 induction in response to growth in (GAL). (A) Wild-type cells (PC6810) were compared with the *cdc42*^{E100A} mutant (Supplemental Table S1). Cells were grown in YEP-GAL media to induce fMAPK activity at the time points indicated. See Figure 1B for details. (B) Wild-type cells (PC538) were compared with the *bem4*Δ mutant (PC3551). Cells were grown as described in A. See Figure 1B for details. (C) Wild-type cells (PC6810) were compared with the *bem1*Δ mutant (PC6680). Cells were grown as described in A. See Figure 1B for details.

pathway before another can stimulate or attenuate the activation of the other pathway (Baltanas *et al.*, 2013). It is also known that filamentation morphogenetic responses do not always correlate with MAP-kinase cascade-dependent transcriptional activation (Mosch *et al.*, 1999). By directly comparing Cdc42p-dependent MAPK pathways in yeast, we show that the MAPK pathways have different activation profiles. The mating and HOG pathways are rapidly induced, whereas the fMAPK pathway has slow activation kinetics. The slow activation kinetics of the fMAPK pathway might mean that gene expression is required for MAPK pathway activation. Given that the fMAPK pathway is induced under nutrient-limiting conditions, one possibility is that derepression of glucose-repressed

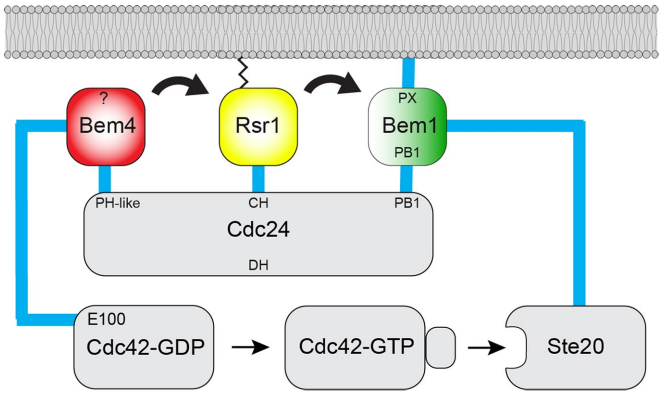

FIGURE 8: Model for proteins that regulate the Cdc42p module in the fMAPK pathway. Bem4p interacts with the PH-like domain of Cdc24p (Pitoniak *et al.*, 2015) and the α3 helix of Cdc42p (this study) and may initiate recruitment of Cdc24p to the fMAPK pathway. Rsr1p interacts with the CH domain of Cdc24p to regulate the fMAPK pathway (Basu *et al.*, 2016), and Bem1p interacts with the PB1 domain of Cdc24p and with Ste20p to regulate the fMAPK pathway (this study). Each protein is associated with the PM: Rsr1p and Cdc42p by lipid modification (not shown for Cdc42p), Bem1p by the PX domain, and Bem4p in an unknown manner (question mark). Black arrows refer to the order for recruitment and activation of the GTPase module into the fMAPK pathway, which is based on genetic suppression analysis.

genes may be required for fMAPK function (Olson, 1999). In a related study from our lab (Prabhakar, Chow, Cullen, unpublished data), we show that the fMAPK pathway is regulated throughout the cell cycle and peaks in G_2/M phase. Cell synchronization experiments also showed that the fMAPK pathway is activated (in G_2/M) to the same levels as the mating and HOG pathways. Future studies are required to determine whether the different activation kinetics of Cdc42p-dependent MAPK pathways contributes to pathway-specific responses.

Interaction between Bem4p and Cdc42p is critical for fMAPK pathway activity

One challenge in studying scaffold-type adaptors that interact with multiple proteins is to define which protein interactions are critical for pathway activity. Bem4p interacts with Cdc24p, Cdc42p, Ste11p, and Kss1p (Pitoniak *et al.*, 2015), yet the critical interactions relevant for Bem4p function have not been explored. Here, we identify a version of Cdc42p (E100A) that is defective for interaction with Bem4p and the fMAPK pathway. Similarly, specific amino acid residues on Bem4p were identified that are defective for interaction with Cdc42p and fMAPK pathway activity. Together these results define the interaction between Cdc42p and Bem4p as being critical for fMAPK pathway activity. This may be the first example in yeast where a protein binds to Cdc42p to impart pathway selectivity.

A model for Cdc42p regulation in the fMAPK pathway

We also show that the major polarity adaptor of Cdc42p, Bem1p, regulates the fMAPK pathway. Bem1p is required for the localization of Ste20p, the effector of Cdc42p in the fMAPK pathway, to the PM. Bem1p regulates the mating pathway through a similar mechanism (Yamaguchi *et al.*, 2007). This conclusion addresses a longstanding problem of how Ste20p is recruited to the PM during filamentous growth. Recently, Bem1p has been shown to interact with Msb2p in the HOG pathway (Tanaka *et al.*, 2014). Like Bem1p, Msb2p preferentially associates with the GTP-bound conformation of

Cdc42p (Cullen *et al.*, 2004). Therefore, Bem1p may function in a protein complex that connects Msb2p, the Cdc42p module, and Ste20p.

[AQ 4] In this study, together with previous work, four proteins interact with the Cdc42p module and regulate the fMAPK pathway: Msb2p (Cullen *et al.*, 2004), Bem4p (Pitoniak *et al.*, 2015), Rsr1p (Basu *et al.*, 2016), and Bem1p (this study). Each of these proteins has a nonredundant function in the fMAPK pathway. A current challenge is to define the specific functions for each of these proteins in regulating the GTPase module. We have previously shown that Msb2p associates with the GTP-bound conformation of Cdc42p (Cullen *et al.*, 2004) and Rsr1p regulates the fMAPK pathway by interacting with the CH domain of Cdc24p (Basu *et al.*, 2016). Here, we show that Bem4p interacts with Cdc42p to regulate the fMAPK pathway, and Bem1p is required for recruitment of Ste20p to the PM to GTP-Cdc42p. Collectively, these findings may begin to explain how [AQ 5] Cdc42p is directed to the fMAPK pathway (Figure 8). Bem4p and Bem1p may affect each other's localization and/or stability, and future experiments are needed to explore this issue.

One way to order proteins in a pathway is by genetic suppression analysis. Genetic suppression analysis using hyperactive versions of fMAPK pathway components showed that Bem4p functions above Bem1p in the fMAPK pathway. This conclusion is consistent with the fact that Bem4p can interact with the inactive (GDP-bound) conformation of Cdc42p, whereas Bem1p primarily interacts with the active (GTP-bound) conformation of the protein. Our results further indicate that Rsr1p does not initiate Cdc24p recruitment to the pathway, because it does not interact with the full-length version of Cdc24p. Bem4p or Bem1p may initiate the recruitment of Cdc24p; however, because the interaction between Bem1p and Cdc24p is not necessary under all conditions, Bem4p may initiate the recruitment of Cdc24p to the fMAPK pathway.

It will be interesting to define whether the mechanism surrounding Cdc42p regulation in the fMAPK pathway postulated here is conserved among fungal species. Experiments were performed in a haploid *S. cerevisiae* of the Σ1278b background. Future work is needed to determine how broadly our findings overlap with other cell types and systems. It has previously been shown that Cdc42p and its regulators, Rsr1p and Bem1p, contribute to hyphal formation and virulence in the major human fungal pathogen *C. albicans* (Bassilana *et al.*, 2003). Perhaps Rsr1p, Bem1p, and Bem4p regulate the orthologous MAP kinase pathway in *C. albicans* and other pathogens.

Comparison of Cdc42p regulation in the mating and fMAPK pathways

Proteins that regulate Cdc42p in the fMAPK pathway characterized in this study can be compared with proteins that regulate Cdc42p in the mating pathway, which has been characterized previously (Sprague *et al.*, 1983, 1991; Errede *et al.*, 1995). A potentially analogous activation mechanism comes from comparing Ste4p, the ß-subunit of the heterotrimeric G-protein in the mating pathway, to Rsr1p in the fMAPK pathway. Both proteins interact with the CH domain of Cdc24p (Zhao *et al.*, 1995; Basu *et al.*, 2016), and both proteins are tied to the PM. Ste4p is anchored to the PM by Ste18p, which is a palmitoylated and farnesylated protein (Zhao *et al.*, 1995; Butty *et al.*, 1998; Nern and Arkowitz, 1998, 1999; Pryciak and Huntress, 1998; Wiget *et al.*, 2004), and Rsr1p is modified by a lipid moiety (Park *et al.*, 2002). Thus, Cdc24p may be activated by Ste4p in the mating pathway and by Rsr1p in the fMAPK pathway. Ste4p can bind to full-length Cdc24p, perhaps to initiate signaling, whereas Rsr1p binds to versions of Cdc24p that lack auto-inhibitory domains. During mating, Ste4p may be critical for initiating recruitment of Cdc24p into the mating pathway, whereas during filamentous growth, Rsr1p is not likely to initiate Cdc24p recruitment to the fMAPK pathway.

During mating, the interaction between Ste4p and Cdc24p is stabilized by Far1p, an adaptor protein in the mating pathway that is also required for cell-cycle arrest in response to pheromone (Simon *et al.*, 1995; Zhao *et al.*, 1995; Nern and Arkowitz, 1998). Bem4p may play an analogous role in the fMAPK pathway. Bem4p interacts with Rsr1p (Drees *et al.*, 2001) and Cdc24p (Chavel *et al.*, 2014) and might stabilize the Rsr1p–Cdc24p interaction. Bem4p also interacts with Cdc42p to regulate the fMAPK pathway. Finally, Bem1p regulates the mating and fMAPK pathways by plasma-membrane recruitment of Ste20p (Figure 7), though this may be [AQ 6] an oversimplification, as there are differences in the ways that Bem1p regulates the two pathways. Therefore, the mating and fMAPK pathways utilize different proteins to regulate the same GTPase module.

Bem1p regulates different pathways in different ways

We also show that Bem1p regulates different effector pathways in different ways. Bem1p regulates multiple Cdc42p-dependent processes, including polarity establishment, exocytosis, and MAPK signaling. Remarkably, different domains of Bem1p play different roles in regulating these different pathways. In some pathways, Bem1p acted as a multifunctional adaptor because multiple domains were required for Bem1p function. In other pathways, only one or none of the functional domains of Bem1p were required. Two general conclusions can be reached from these observations. First, context is critically important for studying adaptor protein function. Assessing Bem1p function was sensitive to the particular strain, assay, and pathway being tested. The second conclusion is that adaptors may operate in different ways in different contexts.

MATERIALS AND METHODS

Media and growth conditions

Experiments were performed at 30°C unless otherwise indicated. YEPD (2% glucose [D]), YEP-GAL (2% GAL), and synthetic complete media containing yeast nitrogen and 2% glucose (SD) or 2% galactose (S-GAL) were used. Amino acids were added to the synthetic media as required. For some experiments, 0.2% glucose was used as indicated.

Strains and plasmids

Strains are listed in Table 1 and plasmids in Table 2. *Escherichia coli* and *S. cerevisiae* strains were manipulated using standard methods (Rose *et al.*, 1990). Gene disruptions and *GAL1* promoter fusions were made by PCR-based methods (Longtine *et al.*, 1998). Some gene deletions were constructed using cassettes that contained antibiotic resistance markers (Goldstein *et al.*, 1999). Gene disruptions were confirmed by PCR Southern analysis and phenotype.

Plasmids pRS315 and pRS316 have been described (Sikorski and Hieter, 1989). Plasmid pRL116 CEN/URA4 pGFP-Ste20 has been described and was provided by P. Pryciak (Leberer *et al.*, 1997). Plasmids carrying *BEM1p-BEM1-12XMYC-SWE1t* (pDLB2374), *BEM1p-bem1*^{P208L}*-12XMYC-SWE1t*(pDLB2375), *BEM1p-bem1*^{P355A}*-12XMYC-SWE1t* (pDLB2377), *BEM1p-bem1*^{R369A}*-12XMYC-SWE1t* (pDLB2378), and *BEM1p-bem1*^{K482A}*-12XMYC-SWE1t* (pDLB2379) have been described (Irazoqui *et al.*, 2003). These plasmids and pRS316-*BEM1* were generously provided by Daniel Lew at Duke University School of Medicine.

Name	Genotype[a,b]	Reference
PC313	MATa ura3-52	Liu *et al.*, 1993
PC344	MATa ura3-52/MATα ura3-52	Cullen and Sprague, 2000a
PC538	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52	Cullen *et al.*, 2004
PC859[c]	MATα ura3-52 ura3- his3	Cullen and Sprague, 2002
PC1660	MATa ura3-52 sec10-2	Grote *et al.*, 2000
PC1661	MATa ura3-52 sec15-1	Grote *et al.*, 2000
PC2738	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bem4::URA3	Pitoniak *et al.*, 2015
PC3861	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::NAT	Karunanithi *et al.*, 2010
PC4256	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 rsr1::NAT	Basu *et al.*, 2016
PC4988[d]	PJ69-4a MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA LYS2::GALl-HIS3 GAL2-ADE2 met2::GAL7-lacZ	James *et al.*, 1996
PC5024	MATa ura3-52 ste11::NAT	Pitoniak *et al.*, 2015
PC6472	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 cyk3::KlURA	This study
PC6509	MATa ura3-52 leu2 bem1::NAT pRS316 BEM1	This study
PC6539	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 cdc42::NAT pRS316-GFP-linker-CDC42	This study
PC6545	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 cdc42::NAT pRS316-GFP-linker-CDC42 rga1::HYG	This study
PC6548	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 cdc42::NAT pRS316-GFP-linker-CDC42 rsr1::HYG	This study
PC6555	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ssk1 cdc42:: HYG pRS316-GFP-linker-CDC42	This study
PC6557	MATa ura3-52 cdc42::HYG pRS316-GFP-linker-CDC42	This study
PC6591	MATa ura3-5 leu2	This study
PC6810	MATa ura3-52 leu2 ssk1	This study
PC6604	MATa ura3-52 leu2 ssk1 ste11::NAT	This study
PC6614	MATa ura3-5 leu2 ahk1::NAT	This study
PC6622	MATa ura3-52 cdc42:: HYG pRS316-GFP-linker-CDC42 rdi1::NAT	This study
PC6624	MATa ura3-52 leu2 ssk1 cla4::NAT	This study
PC6630	MATa ura3-52 GAL rga1::NAT	This study
PC6632	MATa ura3-52 cdc42:: HYG pRS316-GFP-linker-CDC42 rga1::NAT	This study
PC6634	MATa ura3-52 GAL-RDI1:: KanMX6	This study
PC6636	MATa ura3-52 cdc42:: HYG pRS316-GFP-linker-CDC42 GAL RDI1:: KanMX6	This study
PC6680	MATa ura3-52 ssk1 bem1::NAT pRS316-BEM1	This study
PC6685	MATa ura3-52 ssk1 cdc42::NAT pGFP-Cdc42	This study
PC6701	MATa ura3-52 leu2 ssk1 GAL RDI1:: KanMX6	This study
PC6724	MATa ura3-52 leu2 ssk1 HOG1-GFP	This study
PC6726	MATa ura3-52 leu2 ssk1 KSS1-GFP	This study
PC6729	MATa ura3-52 leu2 ssk1 FUS3-GFP	This study
PC6883[d]	PJ69-4a MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA LYS2::GALl-HIS3 GAL2-ADE2 met2::GAL7-lacZ bem4::KlURA3	This study
PC7064	MATa ura3-52 leu2 ssk1 bem4::KlURA3	This study
PC7085[d]	PJ69-4a MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18O LYS2::GALl-HIS3 GAL2-ADE2 met2::GAL7-lacZ rsr1:: KlURA3	This study
PC7159[d]	PJ69-4a MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18O LYS2::GALl-HIS3 GAL2-ADE2 met2::GAL7-lacZ bem1:: KlURA3	This study
PC7309	MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 BEM4-HA K323A 328A:: KanMX6	This study
PC7343	MATa ura3-52 leu2 ssk1 bem1 rsr1::KanMX6 pRs315-BEM1	This study

[a]All strains are in the Σ1278b background unless otherwise indicated.
[b]Strains containing 26 alleles were also constructed in the Σ1278b ssk1 background (Supplemental Table S1).
[c]Strains designated ura3- were generated by selection for resistance to 5-FOA.
[AQ 7] [d]Strain from M. Johnston lab.

TABLE 1: Yeast strains used in the study.

Name	Description	Reference
PC1405	pFRE-lacZ	Madhani *et al.*, 1997
PC1422	pRS315	Sikorski and Hieter, 1989
PC1441	YCp50-STE11-4	Stevenson *et al.* 1992
PC1601	pRS316-SHO1-GFP	Marles *et al.*, 2004
PC1614	pRS316-SHO1(D16H)-GFP	Marles *et al.*, 2004
PC1696	pGFP-MSB2	Vadaie *et al.*, 2008b
PC1715	pRS316-SHO1(P120L)-GFP	Vadaie *et al.*, 2008b
PC1882	pRS316-GAL-SHO1(D16H)-GFP::KanMX6	Vadaie *et al.*, 2008b
PC2205	pNAT	Goldstein and McCusker, 1999
PC2206	pHYG	Goldstein and McCusker, 1999
PC2207	pRS316	Sikorski and Hieter, 1989
PC2560	pRS426 GFP-2XPH-PLC	Stefan *et al.*, 2002
PC4190	pOAD-BEM4	McCraith *et al.*, 2000
PC4205	pOAD	McCraith *et al.*, 2000
PC4206	pOBD-2	McCraith *et al.*, 2000
PC4232	pOBD-2 CDC42(D57Y, C188S)	Pitoniak *et al.*, 2015
PC4394	pRL116 CEN/URA3 GFP-Ste20	Leberer *et al.*, 1997
PC4590	pOAD-RSR1	This study
PC4878	pV84-FUS1-lacZ	This study
PC6025	pYM111 pRS416 8XCRE-LacZ Amp Ura	Yamamoto *et al.*, 2010
PC6365	pYEp352-TEF2	52
PC6367	pYEp352-TEF2-BEM4-GFP	Pitoniak *et al.*, 2015
PC6455	pRS306-GFP-linker-CDC42 (pDLB3609)	Irazoqui *et al.*, 2003
PC6454	pRS316-GFP-linker-CDC42	This study
PC6514	BEM1p-BEM1-12XMYC-SWE1t (pDLB2374)	Irazoqui *et al.*, 2003
PC6515	BEM1 p-bem1P208L-12XMYC-SWE1t (pDLB2375)	Irazoqui *et al.*, 2003
PC6516	BEM1 p-bem1P355A-12XMYC-SWE1t (pDLB2377)	Irazoqui *et al.*, 2003
PC6517	BEM1p-bem1R369A-12XMYC-SWE1t (pDLB2378)	Irazoqui *et al.*, 2003
PC6518	BEM1p-bem1K482A-12XMYC-SWE1t (pDLB2379)	Irazoqui *et al.*, 2003
PC6519	pRS316-BEM1	D. Lew
PC6892	2μ SEC4: URA3; pNB142	France *et al.*, 2006a
PC6893	2μ SEC15: URA3; pNB192	France *et al.*, 2006a
PC6894	EXO70, 2u, URA3	France *et al.*, 2006a
PC6797	pOBD-2-CDC42(K5A, D57Y, C188S)	This study
PC6798	pOBD-2-CDC42(R144A, D57Y, C188S)	This study
PC6799	pOBD-2-CDC42(E100A, D57Y, C188S)	This study
PC6800	pOBD-2-CDC42(V36T, D57Y, C188S)	This study
PC6802	pOBD-2-CDC42(H102A, H103A, H104A D57Y, C188S)	This study
PC6805	pOBD2-CDC42(F37Y, D57Y, C188S)	This study
PC6831	pOAD-BEM1	This study
PC6833	pOBD-2 CDC42(C188S)	This study
PC6834	pOBD-2 CDC42(C188S, G12V)	This study
PC6835	pOBD-2 CDC42(C188S, T17N)	This study
PC6836	pOBD-2 CDC42(C188S, D118A)	This study
PC7025	pRS316-HIS-CDC42	This study

TABLE 2: Plasmids used in the study.

Continues

Name	Description	Reference
PC7028	pYIp352-TEF2-RDI1	This study
PC7096	pOAD-BEM4(K323A, K328A, K351A, R352A)	This study
PC7097	pOAD-BEM4(K351A, R352A)	This study
PC7112	pOAD-BEM4(K323A, K328A)	This study
PC7160	pOAD-BEM4(K323A)	This study
PC7161	pOAD-BEM4(K328A)	This study
PC7162	pOAD-BEM4(K328E)	This study
PC7163	pOBD-CDC42(E100K, D57Y, C188S)	This study

TABLE 2: Plasmids used in the study. Continued

To generate PC6810, the *LEU2* gene was disrupted by *NAT* in PC313 by homologous recombination. The NAT gene was replaced with *URA3* cassette (HA-URA3-HA). The strain was treated on 5-FOA to force out the *URA3* selection marker. In this strain, the *SSK1* gene was disrupted using the *NAT* gene, which was again replaced with *URA3* cassette. The resulting strain PC6810 is a derivative of PC313, which is *MAT*a *ura3-52* carrying unmarked *ssk1* and *leu2* gene deletions.

To generate pRS316-GFP-Linker-Cdc42p, the insert was subcloned from pDLB3609 (Irazoqui *et al.*, 2003) using *Eco*R1 (NEB Cat #R0101, Ipswich, MA) and *Sal*1 (NEB Cat #R3138, Ipswich, MA). To generate pRS315-GFP-Linker-Cdc42p, the insert was subcloned from pDLB 3609 (Irazoqui *et al.*, 2003) using *Pst*I (NEB Cat #R3140, Ipswich, MA) and *Sal*I (NEB Cat #R3138, Ipswich, MA). To generate pYEp352-*TEF2-RDI1* (PC7028), the *RDI1* gene was amplified from the genomic DNA cloned in plasmid carrying *TEF2* promoter using *Xba*1 (NEB Cat #R0145, Ipswich, MA) and *Sal*1 (NEB Cat #R3138, Ipswich, MA). To create the Bem4p alleles for two-hybrid analysis PC4190 with pOAD *BEM4* was utilized. pV84-*FUS1-lacZ* was constructed in plasmid V84 by homologous recombination.

Functional tests to evaluate budding pattern and MAP kinase activity

The budding pattern was determined as described (Basu *et al.*, 2016). Budding pattern was determined by bud position and bud scar position to the proximal, equatorial, or distal parts of the cell. For some experiments, cells were grown to mid-log phase and resuspended in 1 ml of water. Cells were stained with Fluorescent brightener 28 (Calcofluor White [CFW], Sigma-Aldrich Life Science and Biochemicals, St. Louis, MO) using a final concentration of 0.01% for 10 min at 25°C. Cells were washed one time in water and examined by microscopy.

The PWA was performed as described (Roberts and Fink, 1994). The single cell invasive growth assay was performed as described (Cullen and Sprague, 2000b). To evaluate the HOG pathway, cells were spotted onto media supplemented with 1 M KCl. Halo assays were performed as described (Sprague *et al.*, 1983). Cells were grown for 16 h to saturation. Cell density was measured by adding 20 µl overnight culture to 180 µl water (OD_{600nm} ~ 0.2). Approximately 5 µl overnight culture was added to 300 µl water and top spread on agar plates; 3 µl (1.8 µM) and 10 µl (6 µM) alpha factor (1 mg/ml) was added on the dried plates and incubated at 30°C for 2 d. ImageJ analysis was used to quantitate colony growth or the degree of agar invasion by measuring signal intensity compared with background.

Experiments to evaluate the morphogenetic response to pheromone (shmoos) were performed by microscopy. The *FUS1-HIS3* reporter in cells lacking *STE4* was used to evaluate the activity of the fMAPK pathway (Cullen *et al.*, 2004; Chavel *et al.*, 2010; Adhikari *et al.*, 2015b; Pitoniak *et al.*, 2015; Basu *et al.*, 2016) and was measured by spotting cells onto SD-HIS or SD-HIS medium with 3-amino-1,2,4 triazole. Tests for viability were performed by plasmid loss experiments of the *URA3* gene on 5-FOA (Boeke *et al.*, 1984).

Construction and evaluation of a collection of cdc42 alleles in the Σ1278b background

A collection of *CDC42* alleles that was previously generated (Kozminski *et al.*, 2000) was introduced into a filamentous (Σ1278b) strain lacking *SSK1* (PC6810). Plasmids from *E. coli* strains harboring *CDC42* alleles linked to the *LEU2* selection marker and flanking sequence required for homologous recombination were digested with *Ban*II (NEB Cat #R0119S, Ipswich, MA) and *Xba*1 (NEB#R0145S, Ipswich, MA) and transformed into yeast lacking a genomic copy of *CDC42* (cdc42::*NAT*) and containing *pRS316 GFP-CDC42* (PC6454). Selection for *LEU2+/NAT–* colonies favored integration of the alleles at the *CDC42* locus. Selection on 5-FOA was used to force out the p*GFP-CDC42* plasmid and determine whether the alleles were viable. To confirm the DNA sequence of each allele, genomic DNA was extracted. The *CDC42* gene and flanking region was amplified by PCR. PCR products were purified and the entire *CDC42* gene was evaluated by DNA sequencing analysis (Roswell Park Cancer Research Center, Buffalo, NY). In addition to the mutations mentioned in Supplemental Table S1, the library also carries a polymorphic variation at A190T. Twenty-four of 40 alleles were obtained by this method that were viable without the cover plasmid (*pRS316-GFP-CDC42*). Seven alleles were unable to lose the p*GFP-CDC42* plasmid, indicating that they produced versions of Cdc42p that compromised its essential function, and one allele was not obtained. Introduction of the wild-type version of *CDC42* resulted in an invasive growth defect that was taken into account when examining the other alleles.

β-Galactosidase assays

Wild-type cells (PC313), or cells lacking the redundant branch of the HOG pathway (*ssk1*Δ PC6810), were transformed with plasmids *pFRE-lacZ/URA3* 2µ AMP (PC1405) provided by H. Madhani (Madhani *et al.*, 1997), pRS416 *8XCRE-LacZ* AMP URA3 (pYM111 or PC6025) provided by Haruo Saito (Tatebayashi *et al.*, 2006), or pV84-*FUS1-lacZ* (PC4878, this study). Cells harboring plasmids were grown to saturation at 30°C in SD-URA medium to maintain selection for the plasmids. Cells in mid-log phase in YEPD were collected, washed three times in distilled water, and added to YEP-GAL media, YEPD medium supplemented with 0.4 M KCl, or YEPD medium supplemented with 1.2 µM alpha-factor (5 µl of 1 mg/ml α-factor

[0.6 μM] in 5 ml) or 6 μM alpha-factor unless specified. One milliliter of culture aliquots was collected at the indicated time points, assessed for cell density at A_{600}, and stored at –80°C.

β-Galactosidase assays were performed as described (Cullen *et al.*, 2004). Cells were resuspended in 100 μl Z-buffer (44.32 ml H_2O with 5 ml phosphate buffer [0.6 M Na_2HPO_4 + 0.4 M NaH_2PO_4], 0.5 ml 1 M KCl, 50 μl 1 M $MgSO_4$, 135 μl ß-mercaptoethanol) containing 2 μl of 5% sarkosyl and 2 μl toluene. Tubes were incubated at 37°C for 30 min. Z-buffer (650 μl) containing *ortho*-nitrophenyl-β-galactoside was added to each tube. Reactions were stopped with 250 μl of 1 M Na_2CO_3, and the time was recorded. Cell extracts were removed by centrifugation (13,000 rpm for 3 min), and 200 μl of the supernatant was used to determine optical density at A_{420}. Miller units were calculated as 1000 × A_{420}/(A_{600} X time). Experiments were performed in three independent replicates. The two-tailed paired Student's *t* test was used to measure the statistical significance between samples from multiple trials using ProStat (Poly Software International).

Phosphoblot and immunoblot analysis

Cells were induced for immunoblots according to standard conditions and protocols. For basal conditions, cells grown for 16 h (750 μl) were inoculated into fresh YEPD media (15 ml) and incubated for 3 h to mid-log phase. For inductions, mid-log phase cultures were harvested, washed three times with water, and resuspended in YEP-Gal (15 ml), YEPD + 0.4 M KCl (15 ml), or YEPD + α-factor (15 ml) media over a designated time series.

Immunoblots were performed as described (Basu *et al.*, 2016) with the following exceptions in antibody dilution and blocking buffer. The p44/p42 antibodies (Cell Signaling Technology, Danvers, MA; Cat #4370) and Phospho p38 antibodies (Cell Signaling Technology, Danvers MA; #9211) were used to detect P~MAP kinases and diluted 1:10,000 in 5% bovine serum albumin (BSA). Anti-Kss1p antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; Cat #6775) and anti-Hog1p antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; #yC-20) were used at dilutions 1:10000 and 1:5000, respectively, in 5% nonfat dried milk. Anti-Fus3p antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; #6773) was used at a 1:10,000 dilution in 5% BSA. Anti-Pgk1p antibodies (Life Technologies; Camarillo, CA; Cat #459250) were used at 1:10,000 dilution. Secondary antibodies goat anti-mouse IgG–HRP (Bio-Rad Laboratories, Hercules, CA; Cat #170-6516), goat anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA; Cat #111-035-144), and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA; Cat #sc-2020) were used. Quantitation of band intensities for immunoblot analysis was performed with Image Lab Software (Bio-Rad) at an exposure with no saturated pixel in any lane. Background subtraction was performed according to the software user guide provided by the manufacturer. Band intensities of phosphoproteins were measured against total protein levels based on Pgk1p band intensity.

Site-directed mutagenesis

To insert point mutations in plasmids carrying the *CDC42* or *BEM4* genes, the GeneArt Site-Directed Mutagenesis Kit was used (Thermo Fisher Cat #A13282, Grand Island, NY) according to the manufacturer's protocol. Briefly, an overlapping ~40 nucleotide primer set was designed with the desired point mutations designed in the center of the primers. The template was amplified using AccuPrime Pfx DNA polymerase (Thermo Fisher Cat #12344024, Grand Island, NY). The template was methylated by adding DNA methylase and S-adenosyl methionine in the PCR, which was provided by the manufacturer. Following PCR, the free ends of the PCR product were recombined in vitro by the addition of recombinase enzyme mix. The recombined PCR product was transformed into *E. coli* cells (One Shot MAX Efficiency DH5α-T1^R). The specially designed host *E. coli* strain that contains *Mcr*Bc endonuclease digested the methylated template, leaving the nonmethylated PCR amplified mutated product intact.

Differential interference contrast (DIC) and fluorescence microscopy

DIC and fluorescence microscopy of cells were performed using DIC and fluorescence filter sets on an Axioplan 2 fluorescence microscope (Zeiss) with a PLAN-APOCHROMAT 100×/1.4 (oil) objective (N.A. 0.17) (Zeiss). For most experiments, proteins were visualized by resuspending cells in water at 25°C. Digital images were obtained with the Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition and analysis.

Protein modeling

To visualize the putative structure of Bem4p, two web-based protein fold recognition and function prediction servers were utilized: Phyre2 (www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and iTASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Phyre2 is designed to predict structure based on primary amino acid sequence (Kelley *et al.*, 2015) and was used to model the structure of yeast Cdc42p and alleles using information from the crystal structure of *Homo sapiens Cdc42p* in Protein Data Bank (PDB; www.rcsb.org/pdb/home/home.do). The protein-docking server Haddock (van Zundert *et al.*, 2016) was also used and is publicly available (http://milou.science.uu.nl/services/HADDOCK2.2/).

ACKNOWLEDGMENTS

Thanks to David Thomas (McGill University), Daniel Lew (Duke University), Rong Li (Johns Hopkins University), John Pringle (Stanford University), Haruo Saito (University of Tokyo, Japan), David Drubin (University of California, Berkeley), Peter Novick (University of California, San Diego), Stan Fields (University of Washington), Scott Emr (Cornell University), Hiten Madhani (University of California, San Francisco), P. Pryciak (UMASS Medical School, Worcester, MA), and D. Thomas (McGill University) for reagents. Thanks to Lawrence Kelley (Imperial College, London) for help with modeling predictions. Nadia Vadaie, Lauren Caccamise, and Colin Chavel helped with experiments. Atindra Pujari provided editorial assistance with the manuscript. The work was supported by a grant from the National Institutes of Health (GM098629).

REFERENCES

Adhikari H, Caccamise LM, Pande T, Cullen PJ (2015a). Comparative analysis of transmembrane regulators of the filamentous growth mitogen-activated protein kinase pathway uncovers functional and regulatory differences. Eukaryot Cell 14, 868–883.

Adhikari H, Cullen PJ (2014). Metabolic respiration induces AMPK- and Ire1p-dependent activation of the p38-type HOG MAPK pathway. PLoS Genet 10, e1004734.

Adhikari H, Vadaie N, Chow J, Caccamise LM, Chavel CA, Li B, Bowitch A, Stefan CJ, Cullen PJ (2015b). Role of the unfolded protein response in regulating the mucin-dependent filamentous-growth mitogen-activated protein kinase pathway. Mol Cell Biol 35, 1414–1432.

Ago T, Takeya R, Hiroaki H, Kuribayashi F, Ito T, Kohda D, Sumimoto H (2001). The PX domain as a novel phosphoinositide-binding module. Biochem Biophys Res Commun 287, 733–738.

Baltanas R, Bush A, Couto A, Durrieu L, Hohmann S, Colman-Lerner A (2013). Pheromone-induced morphogenesis improves osmoadaptation capacity by activating the HOG MAPK pathway. Sci Signal 6, ra26.

Bao MZ, Schwartz MA, Cantin GT, Yates JR 3rd, Madhani HD (2004). Pheromone-dependent destruction of the Tec1 transcription factor

is required for MAP kinase signaling specificity in yeast. Cell 119, 991–1000.
Bardwell L (2005). A walk-through of the yeast mating pheromone response pathway. Peptides 26, 339–350.
Bardwell L, Cook JG, Voora D, Baggott DM, Martinez AR, Thorner J (1998a). Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK. Genes Dev 12, 2887–2898.
Bardwell L, Cook JG, Zhu-Shimoni JX, Voora D, Thorner J (1998b). Differential regulation of transcription: repression by unactivated mitogen-activated protein kinase Kss1 requires the Dig1 and Dig2 proteins. Proc Natl Acad Sci USA 95, 15400–15405.
Bassilana M, Blyth J, Arkowitz RA (2003). Cdc24, the GDP-GTP exchange factor for Cdc42, is required for invasive hyphal growth of Candida albicans. Eukaryot Cell 2, 9–18.
Basu S, Vadaie N, Prabhakar A, Li B, Adhikari H, Pitoniak A, Chow J, Chavel CA, Cullen PJ (2016). Spatial landmarks regulate a Cdc42-dependent MAPK pathway to control differentiation and the response to positional compromise. Proc Natl Acad Sci USA 113, E2019–E2028.
Bender A, Pringle JR (1991). Use of a screen for synthetic lethal and multicopy suppressee mutants to identify two new genes involved in morphogenesis in Saccharomyces cerevisiae. Mol Cell Biol 11, 1295–1305.
Bhattacharyya RP, Remenyi A, Good MC, Bashor CJ, Falick AM, Lim WA (2006). The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. Science 311, 822–826.
Bi E, Park HO (2012). Cell polarization and cytokinesis in budding yeast. Genetics 191, 347–387.
Boeke JD, LaCroute F, Fink GR (1984). A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet 197, 345–346.
Bose I, Irazoqui JE, Moskow JJ, Bardes ES, Zyla TR, Lew DJ (2001). Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. J Biol Chem 276, 7176–7186.
Breitkreutz A, Boucher L, Tyers M (2001). MAPK specificity in the yeast pheromone response independent of transcriptional activation. Curr Biol 11, 1266–1271.
Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993). An osmosensing signal transduction pathway in yeast. Science 259, 1760–1763.
Butty AC, Perrinjaquet N, Petit A, Jaquenoud M, Segall JE, Hofmann K, Zwahlen C, Peter M (2002). A positive feedback loop stabilizes the guanine-nucleotide exchange factor Cdc24 at sites of polarization. EMBO J 21, 1565–1576.
Butty AC, Pryciak PM, Huang LS, Herskowitz I, Peter M (1998). The role of Far1p in linking the heterotrimeric G protein to polarity establishment proteins during yeast mating. Science 282, 1511–1516.
Carlson M (1999). Glucose repression in yeast. Curr Opin Microbiol 2, 202–207.
Chant J, Corrado K, Pringle JR, Herskowitz I (1991). Yeast BUD5, encoding a putative GDP-GTP exchange factor, is necessary for bud site selection and interacts with bud formation gene BEM1. Cell 65, 1213–1224.
Chant J, Pringle JR (1995). Patterns of bud-site selection in the yeast Saccharomyces cerevisiae. J Cell Biol 129, 751–765.
Chavel CA, Caccamise LM, Li B, Cullen PJ (2014). Global regulation of a differentiation MAPK pathway in yeast. Genetics 198, 1309–1328.
Chavel CA, Dionne HM, Birkaya B, Joshi J, Cullen PJ (2010). Multiple signals converge on a differentiation MAPK pathway. PLoS Genet 6, e1000883.
Chen RE, Thorner J (2007). Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. Biochim Biophys Acta 1773, 1311–1340.
Chin BL, Ryan O, Lewitter F, Boone C, Fink GR (2012). Genetic variation in Saccharomyces cerevisiae: circuit diversification in a signal transduction network. Genetics 192, 1523–1532.
Chou S, Huang L, Liu H (2004). Fus3-regulated Tec1 degradation through SCFCdc4 determines MAPK signaling specificity during mating in yeast. Cell 119, 981–990.
Cook JG, Bardwell L, Kron SJ, Thorner J (1996). Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast Saccharomyces cerevisiae. Genes Dev 10, 2831–2848.
Cook JG, Bardwell L, Thorner J (1997). Inhibitory and activating functions for MAPK Kss1 in the S. cerevisiae filamentous-growth signalling pathway. Nature 390, 85–88.
Cullen PJ, Sabbagh W Jr, Graham E, Irick MM, van Olden EK, Neal C, Delrow J, Bardwell L, Sprague GF Jr (2004). A signaling mucin at the head of the Cdc42- and MAPK-dependent filamentous growth pathway in yeast. Genes Dev 18, 1695–1708.
Cullen PJ, Sprague GF (2000a). Glucose depletion causes haploid invasive growth in yeast. Proc Natl Acad Sci USA 97, 13619.
Cullen PJ, Sprague GF Jr (2000b). Glucose depletion causes haploid invasive growth in yeast. Proc Natl Acad Sci USA 97, 13619–13624.
Cullen PJ, Sprague GF Jr (2002). The roles of bud-site-selection proteins during haploid invasive growth in yeast. Mol Biol Cell 13, 2990–3004.
Cullen PJ, Sprague GF Jr (2012). The regulation of filamentous growth in yeast. Genetics 190, 23–49.
Cvrckova F, De Virgilio C, Manser E, Pringle JR, Nasmyth K (1995). Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. Genes Dev 9, 1817–1830.
Desai JV, Mitchell AP, Andes DR (2014). Fungal biofilms, drug resistance, and recurrent infection. Cold Spring Harb Perspect Med 4, a019729.
Dhillon AS, Hagan S, Rath O, Kolch W (2007). MAP kinase signalling pathways in cancer. Oncogene 26, 3279–3290.
Dowell RD, Ryan O, Jansen A, Cheung D, Agarwala S, Danford T, Bernstein DA, Rolfe PA, Heisler LE, Chin B, *et al*. (2010). Genotype to phenotype: a complex problem. Science 328, 469.
Drees BL, Sundin B, Brazeau E, Caviston JP, Chen GC, Guo W, Kozminski KG, Lau MW, Moskow JJ, Tong A, *et al*. (2001). A protein interaction map for cell polarity development. J Cell Biol 154, 549–571.
Ekiel I, Sulea T, Jansen G, Kowalik M, Minailiuc O, Cheng J, Harcus D, Cygler M, Whiteway M, Wu C (2009). Binding the atypical RA domain of Ste50p to the unfolded Opy2p cytoplasmic tail is essential for the high-osmolarity glycerol pathway. Mol Biol Cell 20, 5117–5126.
Elion EA (2000). Pheromone response, mating and cell biology. Curr Opin Microbiol 3, 573–581.
Endo M, Shirouzu M, Yokoyama S (2003). The Cdc42 binding and scaffolding activities of the fission yeast adaptor protein Scd2. J Biol Chem 278, 843–852.
English JG, Shellhammer JP, Malahe M, McCarter PC, Elston TC, Dohlman HG (2015). MAPK feedback encodes a switch and timer for tunable stress adaptation in yeast. Sci Signal 8, ra5.
Erickson JW, Cerione RA (2001). Multiple roles for Cdc42 in cell regulation. Curr Opin Cell Biol 13, 153–157.
Errede B, Cade RM, Yashar BM, Kamada Y, Levin DE, Irie K, Matsumoto K (1995). Dynamics and organization of MAP kinase signal pathways. Mol Reprod Dev 42, 477–485.
Etienne-Manneville S (2004). Cdc42-the centre of polarity. J Cell Sci 117, 1291–1300.
Finkel JS, Mitchell AP (2011). Genetic control of Candida albicans biofilm development. Nat Rev Microbiol 9, 109–118.
France YE, Boyd C, Coleman J, Novick PJ (2006a). The polarity-establishment component Bem1p interacts with the exocyst complex through the Sec15p subunit. J Cell Sci 119, 876.
France YE, Boyd C, Coleman J, Novick PJ (2006b). The polarity-establishment component Bem1p interacts with the exocyst complex through the Sec15p subunit. J Cell Sci 119, 876–888.
Giehl K (2005). Oncogenic Ras in tumour progression and metastasis. Biol Chem 386, 193–205.
Gimeno CJ, Ljungdahl PO, Styles CA, Fink GR (1992). Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and RAS. Cell 68, 1077–1090.
Gladfelter AS, Moskow JJ, Zyla TR, Lew DJ (2001). Isolation and characterization of effector-loop mutants of CDC42 in yeast. Mol Biol Cell 12, 1239–1255.
Goldstein AL, McCusker JH (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 15, 1541–1553.
Goldstein AL, Pan X, McCusker JH (1999). Heterologous URA3MX cassettes for gene replacement in Saccharomyces cerevisiae. Yeast 15, 507–511.
Granek JA, Magwene PM (2010). Environmental and genetic determinants of colony morphology in yeast. PLoS Genet 6, e1000823.
Grote E, Carr CM, Novick PJ (2000). Ordering the final events in yeast exocytosis. J Cell Biol 151, 439–452.
Gulli MP, Jaquenoud M, Shimada Y, Niederhauser G, Wiget P, Peter M (2000). Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. Mol Cell 6, 1155–1167.
Hamel B, Monaghan-Benson E, Rojas RJ, Temple BR, Marston DJ, Burridge K, Sondek J (2011). SmgGDS is a guanine nucleotide exchange factor that specifically activates RhoA and RhoC. J Biol Chem 286, 12141–12148.

Hilioti Z, Sabbagh W Jr, Paliwal S, Bergmann A, Goncalves MD, Bardwell L, Levchenko A (2008). Oscillatory phosphorylation of yeast Fus3 MAP kinase controls periodic gene expression and morphogenesis. Curr Biol 18, 1700–1706.
Hirano H, Tanaka K, Ozaki K, Imamura H, Kohno H, Hihara T, Kameyama T, Hotta K, Arisawa M, Watanabe T, *et al*. (1996). ROM7/BEM4 encodes a novel protein that interacts with the Rho1p small GTP-binding protein in Saccharomyces cerevisiae. Mol Cell Biol 16, 4396–4403.
Hoffman GR, Nassar N, Cerione RA (2000). Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. Cell 100, 345–356.
Hohmann S, Krantz M, Nordlander B (2007). Yeast osmoregulation. Methods Enzymol 428, 29–45.
Irazoqui JE, Gladfelter AS, Lew DJ (2003). Scaffold-mediated symmetry breaking by Cdc42p. Nat Cell Biol 5, 1062–1070.
Ito T, Matsui Y, Ago T, Ota K, Sumimoto H (2001). Novel modular domain PB1 recognizes PC motif to mediate functional protein-protein interactions. EMBO J 20, 3938–3946.
James P, Halladay J, Craig EA (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425–1436.
Jansen G, Buhring F, Hollenberg CP, Ramezani Rad M (2001). Mutations in the SAM domain of STE50 differentially influence the MAPK-mediated pathways for mating, filamentous growth and osmotolerance in Saccharomyces cerevisiae. Mol Genet Genomics 265, 102–117.
Jennings BC, Lawton AJ, Rizk Z, Fierke CA (2018). SmgGDS-607 Regulation of RhoA GTPase prenylation is nucleotide-dependent. Biochemistry 57, 4289–4298.
Johnson DI (1999). Cdc42: an essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiol Mol Biol Rev 63, 54–105.
Karunanithi S, Cullen PJ (2012). The filamentous growth MAPK pathway responds to glucose starvation through the Mig1/2 transcriptional repressors in Saccharomyces cerevisiae. Genetics 192, 869–887.
Karunanithi S, Vadaie N, Chavel CA, Birkaya B, Joshi J, Grell L, Cullen PJ (2010). Shedding of the mucin-like flocculin Flo11p reveals a new aspect of fungal adhesion regulation. Curr Biol 20, 1389–1395.
Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015). The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 10, 845–858.
Keshet Y, Seger R (2010). The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Methods Mol Biol 661, 3–38.
Kozminski KG, Beven L, Angerman E, Tong AH, Boone C, Park HO (2003). Interaction between a Ras and a Rho GTPase couples selection of a growth site to the development of cell polarity in yeast. Mol Biol Cell 14, 4958–4970.
Kozminski KG, Chen AJ, Rodal AA, Drubin DG (2000). Functions and functional domains of the GTPase Cdc42p. Mol Biol Cell 11, 339–354.
Lamson RE, Winters MJ, Pryciak PM (2002). Cdc42 regulation of kinase activity and signaling by the yeast p21-activated kinase Ste20. Mol Cell Biol 22, 2939–2951.
Leberer E, Wu C, Leeuw T, Fourest-Lieuvin A, Segall JE, Thomas DY (1997). Functional characterization of the Cdc42p binding domain of yeast Ste20p protein kinase. EMBO J 16, 83–97.
Leeuw T, Fourest-Lieuvin A, Wu C, Chenevert J, Clark K, Whiteway M, Thomas DY, Leberer E (1995). Pheromone response in yeast: association of Bem1p with proteins of the MAP kinase cascade and actin. Science 270, 1210–1213.
Levin DE (2011). Regulation of cell wall biogenesis in Saccharomyces cerevisiae: the cell wall integrity signaling pathway. Genetics 189, 1145–1175.
Liu H, Styles CA, Fink GR (1993). Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science 262, 1741–1744.
Liu H, Styles CA, Fink GR (1996). Saccharomyces cerevisiae S288C has a mutation in FLO8, a gene required for filamentous growth. Genetics 144, 967–978.
Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997). Nonfilamentous C. albicans mutants are avirulent. Cell 90, 939–949.
Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14, 953–961.
Lyons DM, Mahanty SK, Choi KY, Manandhar M, Elion EA (1996). The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in Saccharomyces cerevisiae. Mol Cell Biol 16, 4095–4106.
Ma D, Cook JG, Thorner J (1995). Phosphorylation and localization of Kss1, a MAP kinase of the Saccharomyces cerevisiae pheromone response pathway. Mol Biol Cell 6, 889–909.
Mack D, Nishimura K, Dennehey BK, Arbogast T, Parkinson J, Toh-e A, Pringle JR, Bender A, Matsui Y (1996). Identification of the bud emergence gene BEM4 and its interactions with rho-type GTPases in Saccharomyces cerevisiae. Mol Cell Biol 16, 4387–4395.
Madhani HD, Fink GR (1997). Combinatorial control required for the specificity of yeast MAPK signaling. Science 275, 1314–1317.
Madhani HD, Styles CA, Fink GR (1997). MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell 91, 673–684.
Maeda T, Takekawa M, Saito H (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science 269, 554–558.
Maeda T, Wurgler-Murphy SM, Saito H (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369, 242–245.
Maik-Rachline G, Seger R (2016). The ERK cascade inhibitors: towards overcoming resistance. Drug Resist Updat 25, 1–12.
Maleri S, Ge Q, Hackett EA, Wang Y, Dohlman HG, Errede B (2004). Persistent activation by constitutive Ste7 promotes Kss1-mediated invasive growth but fails to support Fus3-dependent mating in yeast. Mol Cell Biol 24, 9221–9238.
Marles JA, Dahesh S, Haynes J, Andrews BJ, Davidson AR (2004). Protein-protein interaction affinity plays a crucial role in controlling the Sho1p-mediated signal transduction pathway in yeast. Mol Cell 14, 813–823.
McCraith S, Holtzman T, Moss B, Fields S (2000). Genome-wide analysis of vaccinia virus protein-protein interactions. Proc Natl Acad Sci USA 97, 4879–4884.
Menon AK (2007). A flip-flop switch in polarity signaling. Dev Cell 13, 607–608.
Moore TI, Tanaka H, Kim HJ, Jeon NL, Yi TM (2013). Yeast G-proteins mediate directional sensing and polarization behaviors in response to changes in pheromone gradient direction. Mol Biol Cell 24, 521–534.
Mosch HU, Kohler T, Braus GH (2001). Different domains of the essential GTPase Cdc42p required for growth and development of Saccharomyces cerevisiae. Mol Cell Biol 21, 235–248.
Mosch HU, Kubler E, Krappmann S, Fink GR, Braus GH (1999). Crosstalk between the Ras2p-controlled mitogen-activated protein kinase and cAMP pathways during invasive growth of Saccharomyces cerevisiae. Mol Biol Cell 10, 1325–1335.
Nassar N, Hoffman GR, Manor D, Clardy JC, Cerione RA (1998). Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP. Nat Struct Biol 5, 1047–1052.
Nern A, Arkowitz RA (1998). A GTP-exchange factor required for cell orientation. Nature 391, 195–198.
Nern A, Arkowitz RA (1999). A Cdc24p-Far1p-Gbetagamma protein complex required for yeast orientation during mating. J Cell Biol 144, 1187–1202.
Nern A, Arkowitz RA (2000). Nucleocytoplasmic shuttling of the Cdc42p exchange factor Cdc24p. J Cell Biol 148, 1115–1122.
Nishimura A, Yamamoto K, Oyama M, Kozuka-Hata H, Saito H, Tatebayashi K (2016). Scaffold protein Ahk1, which associates with Hkr1, Sho1, Ste11, and Pbs2, inhibits cross talk signaling from the Hkr1 osmosensor to the Kss1 mitogen-activated protein kinase. Mol Cell Biol 36, 1109–1123.
O'Rourke SM, Herskowitz I (1998). The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in Saccharomyces cerevisiae. Genes Dev 12, 2874–2886.
Park HO, Bi E (2007). Central roles of small GTPases in the development of cell polarity in yeast and beyond. Microbiol Mol Biol Rev 71, 48–96.
Park HO, Bi E, Pringle JR, Herskowitz I (1997). Two active states of the Ras-related Bud1/Rsr1 protein bind to different effectors to determine yeast cell polarity. Proc Natl Acad Sci USA 94, 4463–4468.
Park HO, Kang PJ, Rachfal AW (2002). Localization of the Rsr1/Bud1 GTPase involved in selection of a proper growth site in yeast. J Biol Chem 277, 26721–26724.
Peifer M, Berg S, Reynolds AB (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. Cell 76, 789–791.

Peter M, Neiman AM, Park HO, van Lohuizen M, Herskowitz I (1996). Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast. EMBO J 15, 7046–7059.
Peterson J, Zheng Y, Bender L, Myers A, Cerione R, Bender A (1994). Interactions between the bud emergence proteins Bem1p and Bem2p and Rho-type GTPases in yeast. J Cell Biol 127, 1395–1406.
Pitoniak A, Birkaya B, Dionne HM, Vadaie N, Cullen PJ (2009). The signaling mucins Msb2 and Hkr1 differentially regulate the filamentation mitogen-activated protein kinase pathway and contribute to a multimodal response. Mol Biol Cell 20, 3101–3114.
Pitoniak A, Chavel CA, Chow J, Smith J, Camara D, Karunanithi S, Li B, Wolfe KH, Cullen PJ (2015). Cdc42p-interacting protein bem4p regulates the filamentous-growth mitogen-activated protein kinase pathway. Mol Cell Biol 35, 417–436.
Posas F, Saito H (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. Science 276, 1702–1705.
Posas F, Saito H (1998). Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. EMBO J 17, 1385–1394.
Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell 86, 865–875.
Pryciak PM, Huntress FA (1998). Membrane recruitment of the kinase cascade scaffold protein Ste5 by the Gbetagamma complex underlies activation of the yeast pheromone response pathway. Genes Dev 12, 2684–2697.
Rauch N, Rukhlenko OS, Kolch W, Kholodenko BN (2016). MAPK kinase signalling dynamics regulate cell fate decisions and drug resistance. Curr Opin Struct Biol 41, 151–158.
Reynolds TB, Fink GR (2001). Bakers' yeast, a model for fungal biofilm formation. Science 291, 878–881.
Rittinger K, Walker PA, Eccleston JF, Nurmahomed K, Owen D, Laue E, Gamblin SJ, Smerdon SJ (1997). Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. Nature 388, 693–697.
Roberts RL, Fink GR (1994). Elements of a single MAP kinase cascade in Saccharomyces cerevisiae mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev 8, 2974–2985.
Roberts CJ, Nelson B, Marton MJ, Stoughton R, Meyer MR, Bennett HA, He YD, Dai H, Walker WL, Hughes TR, *et al.* (2000). Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. Science 287, 873–880.
Rose MD, Winston F, Hieter P (1990). Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Ryan O, Shapiro RS, Kurat CF, Mayhew D, Baryshnikova A, Chin B, Lin ZY, Cox MJ, Vizeacoumar F, Cheung D, *et al.* (2012). Global gene deletion analysis exploring yeast filamentous growth. Science 337, 1353–1356.
Sabbagh W, Flatauer LJ, Bardwell AJ, Bardwell L (2001). Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation. Mol Cell 8, 683–691.
Saito H (2010). Regulation of cross-talk in yeast MAPK signaling pathways. Curr Opin Microbiol 13, 677–683.
Schwartz MA, Madhani HD (2004). Principles of map kinase signaling specificity in Saccharomyces cerevisiae. Annu Rev Genet 38, 725–748.
Shimada Y, Gulli MP, Peter M (2000). Nuclear sequestration of the exchange factor Cdc24 by Far1 regulates cell polarity during yeast mating. Nat Cell Biol 2, 117–124.
Shimada Y, Wiget P, Gulli MP, Bi E, Peter M (2004). The nucleotide exchange factor Cdc24p may be regulated by auto-inhibition. EMBO J 23, 1051–1062.
Shimizu H, Toma-Fukai S, Kontani K, Katada T, Shimizu T (2018). GEF mechanism revealed by the structure of SmgGDS-558 and farnesylated RhoA complex and its implication for a chaperone mechanism. Proc Natl Acad Sci USA 115, 9563–9568.
Shimizu H, Toma-Fukai S, Saijo S, Shimizu N, Kontani K, Katada T, Shimizu T (2017). Structure-based analysis of the guanine nucleotide exchange factor SmgGDS reveals armadillo-repeat motifs and key regions for activity and GTPase binding. J Biol Chem 292, 13441–13448.
Sikorski RS, Hieter P (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27.
Simon MN, De Virgilio C, Souza B, Pringle JR, Abo A, Reed SI (1995). Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway. Nature 376, 702–705.
Sprague GF Jr (1991). Signal transduction in yeast mating: receptors, transcription factors, and the kinase connection. Trends Genet 7, 393–398.
Sprague GF Jr, Blair LC, Thorner J (1983). Cell interactions and regulation of cell type in the yeast Saccharomyces cerevisiae. Annu Rev Microbiol 37, 623–660.
Stefan CJ, Audhya A, Emr SD (2002). The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. Mol Biol Cell 13, 542–557.
Stevenson BJ, Rhodes N, Errede B, Sprague GF Jr (1992). Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein. Genes Dev 6, 1293–1304.
Takaku T, Ogura K, Kumeta H, Yoshida N, Inagaki F (2010). Solution structure of a novel Cdc42 binding module of Bem1 and its interaction with Ste20 and Cdc42. J Biol Chem 285, 19346–19353.
Tanaka K, Tatebayashi K, Nishimura A, Yamamoto K, Yang HY, Saito H (2014). Yeast osmosensors Hkr1 and Msb2 activate the Hog1 MAPK cascade by different mechanisms. Sci Signal 7, ra21.
Tatebayashi K, Tanaka K, Yang HY, Yamamoto K, Matsushita Y, Tomida T, Imai M, Saito H (2007). Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. EMBO J 26, 3521–3533.
Tatebayashi K, Yamamoto K, Nagoya M, Takayama T, Nishimura A, Sakurai M, Momma T, Saito H (2015). Osmosensing and scaffolding functions of the oligomeric four-transmembrane domain osmosensor Sho1. Nat Commun 6, 6975.
Tatebayashi K, Yamamoto K, Tanaka K, Tomida T, Maruoka T, Kasukawa E, Saito H (2006). Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway. EMBO J 25, 3033–3044.
Toenjes KA, Sawyer MM, Johnson DI (1999). The guanine-nucleotide-exchange factor Cdc24p is targeted to the nucleus and polarized growth sites. Curr Biol 9, 1183–1186.
Truckses DM, Bloomekatz JE, Thorner J (2006). The RA domain of Ste50 adaptor protein is required for delivery of Ste11 to the plasma membrane in the filamentous growth signaling pathway of the yeast Saccharomyces cerevisiae. Mol Cell Biol 26, 912–928.
Vadaie N, Dionne H, Akajagbor DS, Nickerson SR, Krysan DJ, Cullen PJ (2008a). Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast. J Cell Biol 181, 1073–1081.
Vadaie N, Dionne H, Akajagbor DS, Nickerson SR, Krysan DJ, Cullen PJ (2008b). Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast. J Cell Biol 181, 1073–1081.
van der Felden J, Weisser S, Bruckner S, Lenz P, Mosch HU (2014). The transcription factors Tec1 and Ste12 interact with coregulators Msa1 and Msa2 to activate adhesion and multicellular development. Mol Cell Biol 34, 2283–2293.
van Zundert GCP, Rodrigues J, Trellet M, Schmitz C, Kastritis PL, Karaca E, Melquiond ASJ, van Dijk M, de Vries SJ, Bonvin A (2016). The HADDOCK2.2 Web Server: User-friendly integrative modeling of biomolecular complexes. J Mol Biol 428, 720–725.
Vikis HG, Stewart S, Guan KL (2002). SmgGDS displays differential binding and exchange activity towards different Ras isoforms. Oncogene 21, 2425–2432.
Vithalani KK, Parent CA, Thorn EM, Penn M, Larochelle DA, Devreotes PN, De Lozanne A (1998). Identification of darlin, a Dictyostelium protein with Armadillo-like repeats that binds to small GTPases and is important for the proper aggregation of developing cells. Mol Biol Cell 9, 3095–3106.
Whiteway M, Bachewich C (2007). Morphogenesis in Candida albicans. Annu Rev Microbiol 61, 529–553.
Wiget P, Shimada Y, Butty AC, Bi E, Peter M (2004). Site-specific regulation of the GEF Cdc24p by the scaffold protein Far1p during yeast mating. EMBO J 23, 1063–1074.
Winters MJ, Pryciak PM (2005). Interaction with the SH3 domain protein Bem1 regulates signaling by the Saccharomyces cerevisiae p21-activated kinase Ste20. Mol Cell Biol 25, 2177–2190.
Wu C, Jansen G, Zhang J, Thomas DY, Whiteway M (2006). Adaptor protein Ste50p links the Ste11p MEKK to the HOG pathway through plasma membrane association. Genes Dev 20, 734–746.

Wu C, Leberer E, Thomas DY, Whiteway M (1999). Functional characterization of the interaction of Ste50p with Ste11p MAPKKK in Saccharomyces cerevisiae. Mol Biol Cell 10, 2425–2440.

Yamaguchi Y, Ota K, Ito T (2007). A novel Cdc42-interacting domain of the yeast polarity establishment protein Bem1. Implications for modulation of mating pheromone signaling. J Biol Chem 282, 29–38.

Yamamoto K, Tatebayashi K, Saito H (2016). Binding of the extracellular eight-cysteine motif of Opy2 to the putative osmosensor Msb2 is essential for activation of the yeast high-osmolarity glycerol pathway. Mol [AQ 8] Cell Biol 36, 475–487.

Yamamoto K, Tatebayashi K, Tanaka K, Saito H (2010). Dynamic control of yeast MAP kinase network by induced association and dissociation between the Ste50 scaffold and the Opy2 membrane anchor. Mol Cell 40, 87–98.

Yang HY, Tatebayashi K, Yamamoto K, Saito H (2009). Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK. EMBO J 28, 1380–1391.

Yu JW, Lemmon MA (2001). All phox homology (PX) domains from Saccharomyces cerevisiae specifically recognize phosphatidylinositol 3-phosphate. J Biol Chem 276, 44179–44184.

Zajac A, Sun X, Zhang J, Guo W (2005). Cyclical regulation of the exocyst and cell polarity determinants for polarized cell growth. Mol Biol Cell 16, 1500–1512.

Zeitlinger J, Simon I, Harbison CT, Hannett NM, Volkert TL, Fink GR, Young RA (2003). Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling. Cell 113, 395–404.

Zhang X, Bi E, Novick P, Du L, Kozminski KG, Lipschutz JH, Guo W (2001). Cdc42 interacts with the exocyst and regulates polarized secretion. J Biol Chem 276, 46745–46750.

Zhao ZS, Leung T, Manser E, Lim L (1995). Pheromone signalling in Saccharomyces cerevisiae requires the small GTP-binding protein Cdc42p and its activator CDC24. Mol Cell Biol 15, 5246–5257.

Zheng Y, Bender A, Cerione RA (1995). Interactions among proteins involved in bud-site selection and bud-site assembly in Saccharomyces cerevisiae. J Biol Chem 270, 626–630.

ETOC:

[AQ 9] Rho GTPase adaptors play unique and sequential roles in the regulation of MAPK pathways.

Author Queries

[AQ 1]: Impact Sentence and highlights not included with MBoC articles so have been omitted here.
[AQ 2]: Change micron to μm throughout?
[AQ 3]: "also" added OK?
[AQ 4]: OK as revised?
[AQ 5]: Figure 8, correct? If not, cite Figure 8.
[AQ 6]: Figure 8?
[AQ 7]: Give institution for lab.
[AQ 8]: Duplicate (2015) reference deleted.
[AQ 9]: Please add a sentence or two specific to your study reported here.