Calcineurin, the Ca²⁺-dependent phosphatase, regulates Rga2, a Cdc42 GTPase-activating protein, to modulate pheromone signaling

Nina Ly and Martha S. Cyert*
Department of Biology, Stanford University, Stanford, CA 94305

ABSTRACT Calcineurin, the conserved Ca²⁺/calmodulin-activated phosphatase, is required for viability during prolonged exposure to pheromone and acts through multiple substrates to down-regulate yeast pheromone signaling. Calcineurin regulates Dig2 and Rod1/Art4 to inhibit mating-induced gene expression and activate receptor internalization, respectively. Recent systematic approaches identified Rga2, a GTPase-activating protein (GAP) for the Cdc42 Rho-type GTPase, as a calcineurin substrate. Here we establish a physiological context for this regulation and show that calcineurin dephosphorylates and positively regulates Rga2 during pheromone signaling. Mating factor activates the Fus3/MAPK kinase, whose substrates induce gene expression, cell cycle arrest, and formation of the mating projection. Our studies demonstrate that Fus3 also phosphorylates Rga2 at inhibitory S/TP sites, which are targeted by Cdks during the cell cycle, and that calcineurin opposes Fus3 to activate Rga2 and decrease Cdc42 signaling. Yeast expressing an Rga2 mutant that is defective for regulation by calcineurin display increased gene expression in response to pheromone. This work is the first to identify cross-talk between Ca²⁺/calcineurin and Cdc42 signaling and to demonstrate modulation of Cdc42 activity through a GAP during mating.

INTRODUCTION Calcineurin (CN), the Ca²⁺/calmodulin-activated phosphatase, translates Ca²⁺ signals into downstream regulatory events and is widely expressed in the animal and fungal kingdoms (Rusnak and Mertz, 2000). Although CN structure is highly conserved, its physiological functions have diverged significantly in fungi and mammals due to rapid rewiring of the CN signaling network through gain and loss of docking motifs in its substrates (Goldman et al., 2014). In mammals, CN is best known as the target of immunosuppressant drugs cyclosporin A and FK506, which inhibit CN-mediated dephosphorylation of NFAT transcription factors to block T-cell activation (Crabtree and Schreiber, 2009). In fungi, CN broadly regulates stress responses and is essential under stringent growth conditions (Cyert and Philpott, 2013). These include survival in a mammalian host for several pathogenic fungi, where CN is required for virulence (Juvvadi et al., 2014). Thus elucidation of fungal CN signaling pathways may identify novel targets for antifungal drug development.

In the budding yeast Saccharomyces cerevisiae, CN is dispensable for growth under standard laboratory conditions, when cytosolic Ca²⁺ levels are low, but essential under conditions that trigger Ca²⁺ signaling, that is, environmental stress, including ionic and cell wall stress, and pheromone signaling (Cyert and Philpott, 2013, Carbó et al., 2017). CN regulates distinct substrates and processes under different signaling conditions (Cyert and Philpott, 2013; Goldman et al., 2014; Arsenault et al., 2015; Guiney et al., 2015). In cells responding to pheromone, CN inhibits mating-induced gene expression and reduces levels of pheromone receptor at the cell surface by dephosphorylating Dig2 and Rod1/Art4, respectively (Cyert and Thorner, 1992; Alvaro et al., 2014, 2016; Goldman et al., 2014). This CN-mediated down-regulation of the pheromone response is essential because CN mutants lose viability during prolonged exposure to mating pheromone (Moser et al., 1996; Withee et al., 1997). A systematic screen uncovered >15 proteins and their associated processes as novel targets of CN (Goldman et al., 2014). One of these, Rga2, a GTPase-activating protein (GAP) for the Rho-type
GTPase Cdc42, suggested previously undescribed cross-talk between CN and Cdc42 signaling and was chosen for further investigation. Specifically, we sought to identify the effect of CN-dependent dephosphorylation on Rga2 function and the physiological context in which this regulation occurs.

Members of the Rho family of small GTPases, that is, Rho, Rac, and Cdc42, are master regulators that transduce extracellular signals into complex behaviors such as wound healing and chemotaxis by coordinately regulating cytoskeletal dynamics, vesicle trafficking, gene expression, and proliferation (Hall, 2012; Abreu-Blanco et al., 2014; Mócsai et al., 2015). Rho GTPases control diverse effectors and act as molecular switches; the active, GTP-bound state is promoted by guanine nucleotide exchange factors (GEFs) and inactivation is mediated by GAPs, which stimulate conversion to GDP-bound Rho (Hall, 2012). These Rho GEFs and GAPs allow Rho GTPase signaling to be regulated in a tissue- and signal-specific manner and precisely control the spatiotemporal pattern of Rho GTPase cycling in vivo (Fritz and Pertz, 2016). Perturbation of Rho GAPs causes pathologies including cancer, mental retardation, and kidney disease (Cherfils, 2014), and understanding their regulation provides critical insight into the complex control of Rho GTPase signaling in vivo (Barnards and Settleman, 2004).

In S. cerevisiae, the conserved Cdc42 GTPase is essential for asymmetric cell growth and critically regulates responses to pheromone, nutrient limitation, and osmotic stress (Chen and Thorner, 2007; Bi and Park, 2012). Cdc42 activity is controlled by a single essential GEF, Cdc24, and multiple GAPs, Bem3, Rga1, and Rga2 (Zheng et al., 1993, 1994; Stevenson et al., 1995; Chen et al., 1996; Smith et al., 2002), which serve both overlapping and unique functions (Smith et al., 2002; Cabiston et al., 2003; Tong et al., 2007). Bem3 and Rga2 undergo cell cycle–dependent phosphorylation (Knaus et al., 2007; McCusker et al., 2007; Sopko et al., 2007), and cyclin-dependent kinases (Cdks) target inhibitory S/TP sites in Rga2 to facilitate activation of Cdc42 during bud emergence in G1 (Sopko et al., 2007). During mating, pheromone-activated receptor recruits Cdc24 to the membrane, causing a local increase in Cdc42-GTP, which initiates a kinase cascade that culminates in activation of the Fus3 mitogen-activated protein kinase (MAPK) to induce cell cycle arrest, gene expression, and shmoo formation (Alvaro and Thorner, 2016). Although deletion of Cdc42 GAPs elevates both basal and induced levels of pheromone-dependent gene expression, whether these proteins are regulated during the mating response has not been investigated (Smith et al., 2002).

In this work, we uncover a new role for Rga2 in regulating Cdc42 activity during pheromone signaling. Rga2 is phosphorylated by Fus3 on inhibitory S/TP sites and dephosphorylated by CN to dampen pheromone signaling. When exposed to mating factor, cells expressing an Rga2 mutant deficient for CN-dependent regulation show increased mating-induced gene expression. Thus we establish a novel mechanism, mediated by Ca²⁺ and CN, that regulates Cdc42 signaling, and identify Rga2 as an additional target through which calcineurin limits the pheromone response.

RESULTS AND DISCUSSION

CN interacts with and positively regulates Rga2 in vivo

Previous work showed that CN dephosphorylates Rga2 in vitro and in addition identified a sequence that lies downstream of the N-terminal LIM domains, PQVLVS (amino acids 192–197), as matching the consensus for a known CN-docking motif termed PxiIT (Figure 1A; Roy et al., 2007; Goldman et al., 2014). To examine the interaction of Rga2 with CN, we first showed that, when fused to glutathione S-transferase (GST), a 22–amino acid sequence from Rga2 containing PQVLVS copurified with recombinant hexahistidine (6xHis)-CN and that, consistent with PxiIT-dependent binding, mutation of key residues to alanine (AQALAA) disrupted the interaction (Figure 1B). To determine whether this putative PxiIT site is functional in the context of the full-length protein, we expressed wild-type (WT) Rga2 (Rga2WT) or PxiIT mutants (Rga2AQALAA or a deletion of PQVLVS, Rga2ΔPQVLVS) in Escherichia coli and tested them for copurification with GST-CN. Rga2WT but not Rga2AQALAA or Rga2ΔPQVLVS copurified with GST-CN, and none of the Rga2 proteins copurified with GST (Figure 1C). Thus the PQVLVS sequence mediates a specific interaction with CN. To examine whether, as for other substrates (Grigoriu et al., 2013; Alvaro et al., 2014; Goldman et al., 2014; Arsenault et al., 2015; Guiney et al., 2015), CN fails to regulate Rga2 PxiIT mutants, we examined the electrophoretic mobility of Rga2 in extracts of yeast in which CN was acutely activated by addition of 200 mM CaCl₂. A fast-migrating form of Rga2 was observed, whereas an additional, slower-migrating band appeared in cultures that were pretreated with the CN inhibitor FK506 (Figure 1D). Consistent with previous findings, this slower-migrating band reflects hyperphosphorylation (Sopko et al., 2007; Goldman et al., 2014) and confirms that Rga2 is dephosphorylated by CN in vivo. In contrast to Rga2WT, the Rga2 PxiIT mutants Rga2AQALAA and Rga2ΔPQVLVS were hyperphosphorylated under CN activating conditions, and phosphorylation levels were unaffected by FK506, demonstrating the abrogation of CN-dependent dephosphorylation. Thus Rga2 PxiIT mutants allow us to investigate the functional consequences of CN-mediated regulation of Rga2.

Because Cdc42 GAPs are functionally redundant, we examined Rga2 activity in a strain lacking three Cdc42 GAPs (bem3Δ rga1Δ rga2Δ), which displays elevated levels of pheromone-induced gene expression (Smith et al., 2002). In the presence of pheromone, high levels of FUS1-LacZ activity were observed in cells overexpressing vector or catalytically inactive Rga2 (K872A, Rga2ΔGAPmut), which were reduced in cells overexpressing Rga2WT (Figure 1E). In comparison, neither of the Rga2 PxiIT mutants was as effective as Rga2WT at reducing β-galactosidase activity (p < 0.01 and p < 0.05, Figure 1E), despite equivalent expression levels for all Rga2 variants (unpublished data). As a second measure of function, Rga2 was overexpressed in wild-type cells. As previously observed, overexpression of Rga2WT, but not Rga2ΔGAPmut or vector, decreased the growth rate (Figure 1F; Sopko et al. 2007). In contrast, neither of the Rga2 PxiIT mutants slowed growth to the same degree as Rga2WT (p < 0.001, Figure 1F). Together these findings show that loss of regulation by CN decreases Rga2 function in vivo and suggest that CN activates Rga2, presumably through dephosphorylation.

CN opposes Fus3 to regulate Rga2 during pheromone signaling

To determine when CN dephosphorylates Rga2 in vivo, we sought physiological conditions under which the phosphorylation status of Rga2WT and Rga2AQALAA differs (as in Figure 1D). Rga2 is expressed at very low levels from its native promoter (200–300 molecules/cell; Ghaemmaghami et al., 2003) and could not be reliably detected by immunoblot. Therefore we expressed FLAG-tagged Rga2 from a CEN plasmid under control of the GAL promoter in a wild-type strain (BEM3 RGA1 RGA2) carrying the GEV chimeric β-estriol–induced expression of galactose-regulated genes (Gao and Pinkham, 2000; Veatch et al., 2009). Cells were grown in the presence of low β-estradiol levels to allow detection of Rga2 without perturbing cell growth (Supplemental Figure S1, A and B). During G1 phase of the cell cycle, Cdks phosphorylate and inhibit Rga2, maximizing Cdc42 activation for bud emergence (Sopko et al., 2007).
The phosphatase(s) that counteract these phosphorylation events have not been identified. Because CN opposes Cdk1 regulation of these sites by Fus3 strongly suggests that it similarly inhibits Rga2 during pheromone signaling. The Fus3 MAPK was a prime candidate because it is activated during pheromone signaling and, like Cdks, targets S/TP sites. To test for Fus3-dependent phosphorylation of Rga2 in vivo, we incubated cells expressing an analogue-sensitive allele of Fus3 (Fus3-as1; Bishop et al., 2000; Mathews et al., 2004) with pheromone. Ten minutes after addition of inhibitor, Rga2 phosphorylation was reduced compared with cells treated with vehicle (Figure 2C), suggesting that Fus3 phosphorylates Rga2 during pheromone signaling. In vitro, recombinant WT Fus3 (Fus3WT) but not the kinase-dead form (Fus3K262R; Parnell et al., 2005) directly phosphorylated Rga2WT and Rga2AQALAA to similar degrees, confirming that differences in their phosphorylation states observed in vivo are due to differential dephosphorylation rather than phosphorylation (Figure 2D). Together these results newly identify Rga2 as a direct Fus3 substrate during the pheromone response.

To test whether Fus3 regulates similar sites as Cdks on Rga2, we used an Rga2 mutant in which eight experimentally verified Cdk-phosphorylated residues were changed to alanine (Rga2ΔAQALAA; Sopko et al., 2007). Phosphorylation of Rga2ΔA by Fus3 was drastically reduced in vivo and in vitro, suggesting that it targets one or more of the same sites as Cdks (Figure 2, E and F). Previous work established that phosphorylation of these sites by Cdks inhibits Rga2 function, thereby increasing active Cdc42 levels and promoting bud emergence; overexpression of a nonphosphorylatable mutant decreases Cdc42-GTP levels in vivo and causes accumulation of large, unbudded cells arrested in G1 (Sopko et al., 2007). Therefore phosphorylation of these sites by Fus3 strongly suggests that it similarly inhibits Rga2 during the pheromone response, when Cdk signaling is
Rga2 modulates the pheromone response

inhibited, to promote the sustained activation of Cdc42 required for downstream signaling events, that is, cell cycle arrest, expression of mating genes, and shmoo formation (Alvaro and Thorner, 2016). In contrast, we predict that, by dephosphorylating Rga2, CN down-regulates Cdc42 and pheromone signaling.

Next we examined the timing of Rga2 dephosphorylation by CN during the pheromone response. The electrophoretic mobility of Rga2WT and Rga2AQALAA (expressed at low levels as in Supplemental Figure S1) were compared in extracts of WT cells (BEM3 RGA1 RGA2) that were synchronized by release from hydroxyurea (HU)-triggered S-phase arrest and stimulated with mating factor before G1 (t = 0; Figure 3A). Dephosphorylation of Rga2WT was detected 30 min after pheromone addition, which coincided with entry into G1, the onset of pheromone signaling as detected by the appearance of active Fus3 (Fus3-P) and initial appearance of cells exhibiting early shmoo morphology (Figure 3, A–D). Compared to Rga2WT, Rga2AQALAA remained hyperphosphorylated throughout the time course, suggesting that CN was activated early and throughout the pheromone response to regulate Rga2 (Figure 3D). Consistent with this idea, studies of single yeast cells exposed to pheromone revealed that intracellular Ca2+ spikes initiate early during pheromone signaling and persist throughout the response, with the frequency and total number of Ca2+ signals increasing as a function of pheromone concentration (Carbó et al., 2017). However, these Ca2+ signals are asynchronous and heterogeneous in amplitude and duration, suggesting that each time point of our experiment included a variable subset of cells that were undergoing Ca2+/CN-activated

Under maximal signaling conditions, CN positively regulates Rga2 to suppress the pheromone response

FIGURE 2: Fus3-dependent phosphorylation of Rga2 during pheromone arrest. (A) Immunoblot showing enrichment of slow-migrating Rga2-FLAG band in extracts of pheromone-treated cells expressing Rga2AQALAA as compared with Rga2WT. (B) Shift in electrophoretic mobility is due to phosphorylation. Rga2WT or Rga2AQALAA was immunopurified from pheromone-treated cells, treated with λ-phosphatase (λP) in the presence or absence of phosphatase inhibitors (PPase I), and analyzed by immunoblot (anti-FLAG). (C) Rga2 phosphorylation in pheromone-treated cells is dependent on Fus3. Fus3-WT (NLY39 + MR4937) or Fus3-as1 (NLY39 + MR4938) cells expressing Rga2WT-FLAG were treated with or without 1Na-PP1 inhibitor. Representative blot shows Rga2 electrophoretic mobility (anti-FLAG). Graph shows quantification and SD from three biological replicates, *p < 0.05, Student's t test. (D) Rga2 is a substrate for Fus3 in vitro. Recombinant 6xHis-Rga2 and Fus3WT were incubated with [γ-32P]ATP, and 32P incorporation at 0, 8, 16, and 32 min was quantified by phosphoimager analysis. The 32P signal was adjusted for Rga2 protein level, normalized to maximal signal for Rga2WT, and relative phosphorylation level is displayed. (E, F) Fus3 and CDKs regulate similar phosphosites on Rga2. (E) During pheromone signaling, Rga28A is a poor substrate in vivo. Cells (NLY33) transformed with Rga2-FLAG plasmids were induced with 5 nM estradiol and incubated with 10 µM αF. (F) Rga28A phosphomutant is a poor substrate for Fus3 in vitro. Recombinant 6xHis-Rga2 and Fus3WT were incubated with [γ-32P]ATP and 32P incorporation at 0, 8, 16, and 32 min was quantified as described in D. For A–C and E, phosphorylation was assayed by electrophoretic mobility, with arrowheads indicating hyperphosphorylated (Rga2-P) and hypo-phosphorylated (Rga2) forms (anti-FLAG) and Hsc82 (anti-Hsc82) loading control. Percentage of total Rga2 signal represented by slower-migrating band is shown below each lane.
difference at higher pheromone concentrations (>5 µM), with Rga2\textsubscript{AQALAA} cells exhibiting higher levels of β-galactosidase activity than Rga2\textsubscript{WT} (Figure 4A, \(p < 0.01\) and \(p < 0.05\)). This difference between Rga2\textsubscript{WT} and Rga2\textsubscript{AQALAA} was consistent, as additional -says with a larger sample size (n ≥ 15) showed increased statistical significance (Figure 4B, \(p < 0.0001\) at 5 and 10 µM mating factor).

Thus, under conditions of sustained pheromone signaling, which trigger Ca\textsuperscript{2+} bursts, CN activates Rga2 to decrease pheromone-induced gene expression. Cdc42 signaling is regulated through its GAP, Rga2, during pheromone signaling

The yeast response to pheromone has been intensively studied and yet continues to yield new insights into the fundamental regulatory features of signaling pathways. Our findings provide the first evidence that a Rho GTPase-activating protein, Rga2, modulates the output of this response and is antagonistically regulated by Fus3, the pheromone-activated MAPK, and CN, the Ca\textsuperscript{2+}-activated phosphatase (Figure 5A).

During mating, Fus3 substrates induce cell cycle arrest, gene expression, actin polarization and cell fusion (Alvaro and Thorner, 2016). Fus3 promotes polarization by phosphorylating the Bni1 formin, which is also an effector of Cdc42 (Elion et al., 1993; Tedford et al., 1997; Matheos et al., 2004). Therefore our finding that Fus3 regulates Rga2 suggests that this MAPK elevates Cdc42 signaling to create positive feedback that reinforces activation of dephosphorylation of Rga2. Thus, in four independent experiments, we consistently observed that Rga2\textsubscript{AQALAA} was hyperphosphorylated compared with Rga2\textsubscript{WT} throughout the pheromone response, although the extent of hyperphosphorylation observed at each time point varied (Figure 3D and Supplemental Figure S1D, \(p < 0.01\)). Together these observations indicate that high levels of pheromone trigger Ca\textsuperscript{2+} signals that activate CN and suggest that CN and Fus3 oppose each other in regulating Rga2. By positively regulating Rga2, CN may inhibit the ability of Cdc42 to regulate cell morphology, cell cycle arrest, and/or gene transcription in response to pheromone (Alvaro and Thorner, 2016).

To assess the phenotypic consequences of Rga2 dephosphorylation, we constructed isogenic strains that contained all Cdc42 GAPs and differed only in the susceptibility of Rga2 to CN-mediated dephosphorylation. Replacement of genomic RGA2 through integration of isogenic, tagged RGA2\textsubscript{WT} or RGA2\textsubscript{AQALAA} alleles at the endogenous locus allowed for their expression at physiological levels. Characterization of these integrated Rga2\textsubscript{WT} or Rga2\textsubscript{AQALAA} strains through the pheromone response showed no detectable differences in the timing of cell cycle arrest and shmoo formation (Supplemental Figure S2, A and B) or in shmoo morphology (unpublished data). To test whether CN regulates pheromone-induced gene expression through Rga2, we assayed FUS1-LacZ activity in cells treated with a range of mating factor concentrations. Although Rga2\textsubscript{WT} and Rga2\textsubscript{AQALAA} responded similarly to low pheromone concentrations, there was a reproducible and statistically significant
Bni1 and of Fus3 itself, which is stimulated in a Cdc42-dependent manner.

The CN signaling network is essential for survival of yeast cells during prolonged exposure to high concentrations of pheromone and acts through multiple substrates to down-regulate signaling. Previous studies established that CN dephosphorylates and activates Dig2, an inhibitor of Ste12, to reduce mating-induced gene expression (Figure 5B). CN reverses Fus3-mediated phosphorylation of Dig2, and directly competes with Fus3 at overlapping CN- and MAPK-docking sites for Dig2 binding (Goldman et al., 2014). We speculate that CN signaling substantially decreases mating-induced gene expression through its combined regulation of Rga2 and Dig2. Furthermore, CN reduces signaling by activating Rod1/Art4-mediated receptor endocytosis (Alvaro et al., 2014, 2016) and may dampen other aspects of pheromone signaling through additional substrates. Candidates include the Elm1 kinase, which phosphorylates Gα (GPA1; Clement et al., 2013), proteins associated with polarized growth (Kic1, Boi2, Mlf3; Goldman et al., 2014), and the Crz1 transcription factor, which is activated by CN in response to pheromone (Stathopoulos et al., 1997). Given these multiple points of regulation, it is remarkable that disrupting CN-dependent regulation of a single target, Rga2, in cells that contain multiple Cdc42 GAPs and functional CN substrates noticeably increased pheromone-dependent gene expression.

Although pheromone-induced Ca2+ influx is well documented (Iida et al., 1990; Moser et al., 1996; Withee et al., 1997), new studies of Ca2+ dynamics in individual yeast cells revealed that pheromone induces asynchronous, heterogeneous Ca2+ bursts whose frequency increases with pheromone concentration (Carbó et al., 2017). Consistent with these findings, we observed partial dephosphorylation of Rga2 throughout the pheromone response. Furthermore, we identified increased expression of a mating gene in Rga2AQALAA-expressing cells specifically under maximal Bni1 and of Fus3 itself, which is stimulated in a Cdc42-dependent manner.

The CN signaling network is essential for survival of yeast cells during prolonged exposure to high concentrations of pheromone and acts through multiple substrates to down-regulate signaling. Previous studies established that CN dephosphorylates and activates Dig2, an inhibitor of Ste12, to reduce mating-induced gene expression (Figure 5B). CN reverses Fus3-mediated phosphorylation of Dig2, and directly competes with Fus3 at overlapping CN- and MAPK-docking sites for Dig2 binding (Goldman et al., 2014). We speculate that CN signaling substantially decreases mating-induced gene expression through its combined regulation of Rga2 and Dig2. Furthermore, CN reduces signaling by activating Rod1/Art4-mediated receptor endocytosis (Alvaro et al., 2014, 2016) and may dampen other aspects of pheromone signaling through additional substrates. Candidates include the Elm1 kinase, which phosphorylates Gα (GPA1; Clement et al., 2013), proteins associated with polarized growth (Kic1, Boi2, Mlf3; Goldman et al., 2014), and the Crz1 transcription factor, which is activated by CN in response to pheromone (Stathopoulos et al., 1997). Given these multiple points of regulation, it is remarkable that disrupting CN-dependent regulation of a single target, Rga2, in cells that contain multiple Cdc42 GAPs and functional CN substrates noticeably increased pheromone-dependent gene expression.

Although pheromone-induced Ca2+ influx is well documented (Iida et al., 1990; Moser et al., 1996; Withee et al., 1997), new studies of Ca2+ dynamics in individual yeast cells revealed that pheromone induces asynchronous, heterogeneous Ca2+ bursts whose frequency increases with pheromone concentration (Carbó et al., 2017). Consistent with these findings, we observed partial dephosphorylation of Rga2 throughout the pheromone response. Furthermore, we identified increased expression of a mating gene in Rga2AQALAA-expressing cells specifically under maximal

Bni1 and of Fus3 itself, which is stimulated in a Cdc42-dependent manner.

The CN signaling network is essential for survival of yeast cells during prolonged exposure to high concentrations of pheromone and acts through multiple substrates to down-regulate signaling. Previous studies established that CN dephosphorylates and activates Dig2, an inhibitor of Ste12, to reduce mating-induced gene expression (Figure 5B). CN reverses Fus3-mediated phosphorylation of Dig2, and directly competes with Fus3 at overlapping CN- and MAPK-docking sites for Dig2 binding (Goldman et al., 2014). We speculate that CN signaling substantially decreases mating-induced gene expression through its combined regulation of Rga2 and Dig2. Furthermore, CN reduces signaling by activating Rod1/Art4-mediated receptor endocytosis (Alvaro et al., 2014, 2016) and may dampen other aspects of pheromone signaling through additional substrates. Candidates include the Elm1 kinase, which phosphorylates Gα (GPA1; Clement et al., 2013), proteins associated with polarized growth (Kic1, Boi2, Mlf3; Goldman et al., 2014), and the Crz1 transcription factor, which is activated by CN in response to pheromone (Stathopoulos et al., 1997). Given these multiple points of regulation, it is remarkable that disrupting CN-dependent regulation of a single target, Rga2, in cells that contain multiple Cdc42 GAPs and functional CN substrates noticeably increased pheromone-dependent gene expression.

Although pheromone-induced Ca2+ influx is well documented (Iida et al., 1990; Moser et al., 1996; Withee et al., 1997), new studies of Ca2+ dynamics in individual yeast cells revealed that pheromone induces asynchronous, heterogeneous Ca2+ bursts whose frequency increases with pheromone concentration (Carbó et al., 2017). Consistent with these findings, we observed partial dephosphorylation of Rga2 throughout the pheromone response. Furthermore, we identified increased expression of a mating gene in Rga2AQALAA-expressing cells specifically under maximal

Bni1 and of Fus3 itself, which is stimulated in a Cdc42-dependent manner.

The CN signaling network is essential for survival of yeast cells during prolonged exposure to high concentrations of pheromone and acts through multiple substrates to down-regulate signaling. Previous studies established that CN dephosphorylates and activates Dig2, an inhibitor of Ste12, to reduce mating-induced gene expression (Figure 5B). CN reverses Fus3-mediated phosphorylation of Dig2, and directly competes with Fus3 at overlapping CN- and MAPK-docking sites for Dig2 binding (Goldman et al., 2014). We speculate that CN signaling substantially decreases mating-induced gene expression through its combined regulation of Rga2 and Dig2. Furthermore, CN reduces signaling by activating Rod1/Art4-mediated receptor endocytosis (Alvaro et al., 2014, 2016) and may dampen other aspects of pheromone signaling through additional substrates. Candidates include the Elm1 kinase, which phosphorylates Gα (GPA1; Clement et al., 2013), proteins associated with polarized growth (Kic1, Boi2, Mlf3; Goldman et al., 2014), and the Crz1 transcription factor, which is activated by CN in response to pheromone (Stathopoulos et al., 1997). Given these multiple points of regulation, it is remarkable that disrupting CN-dependent regulation of a single target, Rga2, in cells that contain multiple Cdc42 GAPs and functional CN substrates noticeably increased pheromone-dependent gene expression.

Although pheromone-induced Ca2+ influx is well documented (Iida et al., 1990; Moser et al., 1996; Withee et al., 1997), new studies of Ca2+ dynamics in individual yeast cells revealed that pheromone induces asynchronous, heterogeneous Ca2+ bursts whose frequency increases with pheromone concentration (Carbó et al., 2017). Consistent with these findings, we observed partial dephosphorylation of Rga2 throughout the pheromone response. Furthermore, we identified increased expression of a mating gene in Rga2AQALAA-expressing cells specifically under maximal

Bni1 and of Fus3 itself, which is stimulated in a Cdc42-dependent manner.

The CN signaling network is essential for survival of yeast cells during prolonged exposure to high concentrations of pheromone and acts through multiple substrates to down-regulate signaling. Previous studies established that CN dephosphorylates and activates Dig2, an inhibitor of Ste12, to reduce mating-induced gene expression (Figure 5B). CN reverses Fus3-mediated phosphorylation of Dig2, and directly competes with Fus3 at overlapping CN- and MAPK-docking sites for Dig2 binding (Goldman et al., 2014). We speculate that CN signaling substantially decreases mating-induced gene expression through its combined regulation of Rga2 and Dig2. Furthermore, CN reduces signaling by activating Rod1/Art4-mediated receptor endocytosis (Alvaro et al., 2014, 2016) and may dampen other aspects of pheromone signaling through additional substrates. Candidates include the Elm1 kinase, which phosphorylates Gα (GPA1; Clement et al., 2013), proteins associated with polarized growth (Kic1, Boi2, Mlf3; Goldman et al., 2014), and the Crz1 transcription factor, which is activated by CN in response to pheromone (Stathopoulos et al., 1997). Given these multiple points of regulation, it is remarkable that disrupting CN-dependent regulation of a single target, Rga2, in cells that contain multiple Cdc42 GAPs and functional CN substrates noticeably increased pheromone-dependent gene expression.

Although pheromone-induced Ca2+ influx is well documented (Iida et al., 1990; Moser et al., 1996; Withee et al., 1997), new studies of Ca2+ dynamics in individual yeast cells revealed that pheromone induces asynchronous, heterogeneous Ca2+ bursts whose frequency increases with pheromone concentration (Carbó et al., 2017). Consistent with these findings, we observed partial dephosphorylation of Rga2 throughout the pheromone response. Furthermore, we identified increased expression of a mating gene in Rga2AQALAA-expressing cells specifically under maximal
pheromone signaling conditions, when Ca\(^{2+}\) signaling is most intense. The Ca\(^{2+}\)/CN-dependent regulation of pheromone signaling is known only to promote cell survival under conditions of prolonged cell cycle arrest (Withee et al., 1997). However, in many cell types, such as fungal hyphae, pollen tubes, neuronal axons, and mast cells, localized Ca\(^{2+}\) gradients regulate Rho GTPase signaling to direct polarized growth and migration (Brand et al., 2014; Sutherland et al., 2014; Chen et al., 2015; Holowka et al., 2016). Thus Ca\(^{2+}\)/calcineurin-dependent regulation of Cdc42 signaling may play undiscovered roles in directional aspects of pheromone signaling, such as chemotrophic growth (McClure et al., 2015; Ismael et al., 2016).

Are other Cdc42 GAPs, Rga1 and Bem3, modulated during pheromone signaling? Single deletion or mutation of these GAPs has little or no effect on the pheromone response (Stevenson et al., 1995; Bidlingmaier and Snyder, 2004). Furthermore, Rga2 is the only Cdc42 GAP that harbors a CN-docking site (Goldman et al., 2014) and forms a complex with Cdc24, Bem1, Boi1, and Boi2 (another CN substrate). Proposed to regulate Cdk1-dependent expansion of the yeast cell surface during the cell cycle (McCusker et al., 2007), this complex might also function during pheromone signaling, as most of its components contribute to shmoo formation or localize to the shmoo tip (Chenevert et al., 1992, 1994; Nern and Arkowitz, 1999; Narayanaswamy et al., 2009). Further analyses are required to determine whether Fus3 phosphorylates additional GAPs and examine possible regulation of Rga2 by other MAPKs, including Hog1, whose activation is regulated by Cdc42 during the yeast response to hypertrophic stress, a condition under which CN is also active (Chen and Thorner, 2007; Guiney et al., 2015).

Regulation of Rho GAP activity is a fundamental mechanism to spatially and temporally regulate the dynamics of Rho GTPase signaling. In a fungal human pathogen, Candida albicans, phosphorylation and inhibition of Rga2 by a hyphal-specific Cdk are required for the morphogenetic switch from yeast to hyphal growth, which promotes virulence (Zheng et al., 2007). Unidentified phosphatases may therefore prevent pathogenesis by maintaining the fungi in the yeast growth phase. In humans, epidermal growth factor promotes virulence (Zheng et al., 2007). Unidentified phosphatases whose activation is regulated by Cdc42 during the yeast response to hyperosmotic stress, a condition under which CN is also active, is known only to promote cell survival under conditions of prolonged cell cycle arrest (Withee et al., 1997). However, in many cell types, such as fungal hyphae, pollen tubes, neuronal axons, and mast cells, localized Ca\(^{2+}\) gradients regulate Rho GTPase signaling to direct polarized growth and migration (Brand et al., 2014; Sutherland et al., 2014; Chen et al., 2015; Holowka et al., 2016). Thus Ca\(^{2+}\)/calcineurin-dependent regulation of Cdc42 signaling may play undiscovered roles in directional aspects of pheromone signaling, such as chemotrophic growth (McClure et al., 2015; Ismael et al., 2016).

For Rga2 full-length binding assays, His-Rga2 constructs (pNL143, 145,146) were transformed in BL21 DE3 cells (Invitrogen), and GST-CNA1 trunc + CNB1 was transformed in BL21 DE3 pLys cells (Invitrogen). Single colonies were grown in Luria broth supplemented with 50 µg/ml ampicillin overnight with shaking at 37°C. Overnight cultures were backdiluted to OD\(_{600}\) = 0.01 and grown with shaking at 37°C until OD\(_{600}\) = 0.5. Cultures were grown for an additional 1 h at 25°C and then induced with 1 mM IPTG at 18°C for 20 h. Cultures were harvested by centrifugation (5 min, 5000 rpm at 4°C using a Sorvall GS-3 rotor), flash-frozen in liquid nitrogen, and stored at −80°C. His-Rga2 pellets were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol [β-ME], protease inhibitors) and sonicated 5 × 30 s at 30% output with 1 min breaks. Cell debris was pelleted (5 min, 10,000 rpm at 4°C in a Sorvall SS-34 rotor) and then clarified twice (20 min, 17,000 rpm at 4°C in a Sorvall SS-34 rotor). Total protein concentration of extracts was determined by Bradford assay, and extracts were aliquoted and flash-frozen in liquid nitrogen and stored at −80°C.

For GST-peptide binding assays, plasmids for 6xHis-CN (CNA1 trunc-p11) and Cnb1 (pET15b-Cnb1) were transformed into BL21 DE3 cells (Invitrogen). Single colonies were grown overnight in Luria broth supplemented with 50 µg/ml ampicillin with shaking at 37°C. Overnight cultures were diluted to OD\(_{600}\) = 0.01 in Luria broth supplemented with 100 µg/ml carbencillin and 100 µg/ml kanamycin and grown with shaking at 37°C until OD\(_{600}\) = 0.5. Culture was grown for an additional 1 h with shaking at 25°C and then protein expression induced with 1 mM IPTG with shaking at 18°C for 20 h. Cultures were harvested by centrifugation (5 min, 5000 rpm at 4°C with a Sorvall GS-3 rotor), and pellets were washed in cold water and frozen at −80°C. Pellets were resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM β-ME, and protease inhibitors) and lysed by sonication: 5 × 30 s at 30% output with 1 min breaks. Cell debris was pelleted (5 min, 10,000 rpm at 4°C in a Sorvall SS-34 rotor) and further clarified twice (20 min, 17,000 rpm at 4°C in a Sorvall SS-34 rotor). Pellets were washed in cold water and stored at −80°C. Pellets were resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM β-ME, and protease inhibitors) and lysed by sonication: 5 × 30 s at 30% output with 1 min breaks. Cell debris was pelleted (5 min, 10,000 rpm at 4°C in a Sorvall SS-34 rotor) and further clarified twice (20 min, 17,000 rpm at 4°C in a Sorvall SS-34 rotor). Total protein concentration of extracts was determined by Bradford assay, and extracts were aliquoted and flash-frozen in liquid nitrogen and stored at −80°C.

Purification of constitutively active truncated yeast CN Plasmids for 6xHis-CN (CNA1 trunc-p11) and Cnb1 (pET15b-Cnb1) were transformed into BL21 DE3 cells (Invitrogen). Single colonies were grown overnight in Luria broth supplemented with 50 µg/ml ampicillin with shaking at 37°C. Overnight cultures were diluted to OD\(_{600}\) = 0.01 in Luria broth supplemented with 100 µg/ml carbencillin and grown with shaking at 37°C until OD\(_{600}\) = 0.5. Culture was grown for an additional 1 h with shaking at 25°C and then protein expression induced with 1 mM IPTG with shaking at 18°C for 20 h. Cultures were harvested by centrifugation (5 min, 5000 rpm at 4°C with a Sorvall GS-3 rotor), and pellets were washed in cold water and frozen at −80°C. Pellets were resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM β-ME, and protease inhibitors) and lysed by sonication: 5 × 30 s at 30% output with 1 min breaks. Cell debris was pelleted (5 min, 10,000 rpm at 4°C in a Sorvall SS-34 rotor) and further clarified twice (20 min, 17,000 rpm at 4°C in a Sorvall SS-34 rotor). CN complex was purified over a nickel–nitriloacetic acid (Ni-NTA) agarose column (FP240000; 5 Prime) according to manufacturer’s specifications. Purified protein was eluted in fractions with 150 mM imidazole and assayed by SDS–PAGE, pooled, and dialyzed into 50 mM Tris-HCl, pH 7.5, plus 100 mM NaCl buffer and stored at −80°C with 10% glycerol.

GST-peptide and CN trunc binding assay To test the putative CN-docking site on Rga2, 50 µg of E. coli extract containing GST or GST-tagged peptide was mixed with 10 µg of purified 6xHis-CN trunc in binding buffer (50 mM Tris-HCl, pH 7.5,
150 mM NaCl, 0.1% Triton X-100, 1 mM β-ME, 5 mM imidazole) for 1 h at 4°C with end-over-end mixing. A 25-µl amount of washed Ni-NTA agarose beads (FP2400000; 5 Prime) was added to the binding mixture and incubated for another 2 h. Beads were washed three times in binding buffer containing 15 mM imidazole, and bound proteins were eluted by boiling in Laemmli buffer for 5 min. From 5 to 20 µl of sample was resolved by SDS–PAGE and immunoblotted with primary antibodies to GST (MMS112R500, mouse monoclonal; Covance/BERKELEY Antibody Co.) and histidine (34660, mouse monoclonal; Qiagen). Primary antibodies were detected on film by chemiluminescence with secondary antibody α-mouse conjugated to horseradish peroxidase (NA931-1ML; GE Healthcare).

**His-Rga2 and CN trunc binding assay**

To test whether the CN-docking site is functional within the context of the full-length protein, 2.5 µg of *E. coli* extract containing 6×His-tagged Rga2 was mixed with 30 µg of GST extract or 450 µg of GST-CNA1 trunc extract in binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM β-ME, and protease inhibitors) for 1 h at 4°C with end-over-end mixing. A 25-µl amount of washed glutathione–Sepharose 4B beads (17075601; GE Healthcare) was added to the binding mixture and incubated for another 2 h. Beads were washed three times in binding buffer, and bound proteins were eluted by boiling in Laemmli buffer for 5 min. A 20-µl amount of sample was resolved by 10% acrylamide SDS–PAGE gel and immunoblotted with primary antibodies to GST (MMS112R500, mouse monoclonal; Covance) and histidine (34660, mouse monoclonal; Qiagen). Primary antibodies were detected by infrared (IR) fluorescence with secondary antibody anti-mouse conjugated to Alexa 680 (A-21058; Invitrogen) and visualized with an Odyssey scanner (Li-Cor Biosciences, Lincoln, NE).

**Yeast extracts**

For in vivo dephosphorylation under Ca²⁺-activating conditions, cells at OD₆₀₀ = 0.6 were treated with FK506 (1 µg/ml final concentration) or vehicle (90% ethanol and 10% Tween-20) for 30 min before protein induction with 2% galactose for 4 h. CN was then activated with 200 mM CaCl₂ for 20 min and then harvested with pellets stored at −80°C. Yeast pellets were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors) for 1 h at 4°C with end-over-end mixing. A 25-µl amount of sample was resolved by 10% acrylamide SDS–PAGE gel and immunoblotted with primary antibodies to GST (MMS112R500, mouse monoclonal; Covance) and histidine (34660, mouse monoclonal; Qiagen). Primary antibodies were detected by infrared (IR) fluorescence with secondary antibody anti-mouse conjugated to Alexa 680 (A-21058; Invitrogen) and visualized with an Odyssey scanner (Li-Cor Biosciences, Lincoln, NE).

**β-Galactosidase assay**

YG557 transformed with plasmids (pBA2059, pNL110-112) was grown in SC-LEU plus 2% raffinose to mid log phase, OD₆₀₀ = 0.4. Rga2 expression was induced by adding 2% final galactose for 2 h. Cells were then backdiluted to OD₆₀₀ = 0.5 and treated for 90 min with 5 µM α-factor (αF; RP01002; GenScript). A 100-µl amount of pheromone-treated cells was transferred to 96-well plates for FUS1-lacZ analysis, and 5-mL cultures were set aside to assay Rga2 protein expression in yeast extracts as described. FUS1-lacZ activity was determined using substrate FDG (F-1179; Invitrogen) as previously described (Hoffman et al., 2002), and fluorescence emission at 530 nm was detected using a fluorescence plate reader (BioTek, Winooski, VT). For FUS1-lacZ in the integrated strain, cells (NLY52 and 54) were grown in SCD to mid log phase, OD₆₀₀ = 0.3, and treated for 90 min at indicated αF conditions. For Figure 4B, 15 or 16 biological samples over the course of 2 d for each Rga2 WT and Rga2AAALAA were assayed for FUS1-lacZ activity. FUS1-lacZ activity was determined using as substrate fluorescein di-β-o-galactopyranoside and normalized to OD₆₀₀.

**Yeast liquid growth assay**

Yeast strain (NLY33) transformed with plasmids (pNL126-128, pNL135, or pEGKT) was grown in SCD-URA to mid log phase. Cells were then backdiluted to a starting OD₆₀₀ = 0.01 in a 96-well plate and protein expression induced with 20 µM estradiol (E1024-1G; Sigma-Aldrich). Absorbance at 600 nm was read every 5 min for 24 h, and a growth rate of doubling/hour was calculated from at least three technical replicates. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey’s correction for multiple comparisons.

**λ-Phosphatase treatment of Rga2 WT and PxlxIT mutant**

Cells (NLY33) transformed with plasmids (pBA2059 or pNL111) were grown in SCD-LEU plus 5 mM estradiol to mid log phase. At OD₆₀₀ = 0.35, cells were arrested in G1 with 10 µM ect for 2 h and then harvested. Yeast extracts were made in RIPA buffer as detailed earlier with 500 µg of total protein used to purify Rga2-FLAG by anti-FLAG (F3165, mouse monoclonal; Sigma-Aldrich). For λ- phosphatase assay, yeast pellets were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors) and homogenized by glass-bead lysis on a vortexer, 5 × 2 min with 1-min breaks. Lysate was separated from beads by needle-puncture spin collection and further clarified at 14,000 rpm for 20 min. Total protein concentration of extracts was determined by Bradford assay. Samples were analyzed by 5.5% acrylamide SDS–PAGE gel and immunoblotted with primary antibodies: anti-FLAG (sc-807, rabbit polyclonal; Santa Cruz Biotechnology); anti-p44/42 MAPK (9101S, rabbit polyclonal; Cell Signaling Technologies), anti-Fus3 (sc-28548, rabbit polyclonal; Santa Cruz Biotechnology), and loading control, anti-Hsc82 (ab30920, rabbit polyclonal; Abcam). Primary antibodies were detected by IR fluorescence with secondary antibody anti-rabbit conjugated to Alexa 790 (A11369; Invitrogen) or anti-mouse conjugated to Alexa 680 (A-21058; Invitrogen) and visualized with an Odyssey scanner.

**β-Galactosidase assay**

YG557 transformed with plasmids (pBA2059, pNL110-112) was grown in SC-LEU plus 2% raffinose to mid log phase, OD₆₀₀ = 0.4. Rga2 expression was induced by adding 2% final galactose for 2 h. Cells were then backdiluted to OD₆₀₀ = 0.5 and treated for 90 min with 5 µM α-factor (αF; RP01002; GenScript). A 100-µl amount of pheromone-treated cells was transferred to 96-well plates for FUS1-lacZ analysis, and 5-mL cultures were set aside to assay Rga2 protein expression in yeast extracts as described. FUS1-lacZ activity was determined using substrate FDG (F-1179; Invitrogen) as previously described (Hoffman et al., 2002), and fluorescence emission at 530 nm was detected using a fluorescence plate reader (BioTEK, Winooski, VT). For FUS1-lacZ in the integrated strain, cells (NLY52 and 54) were grown in SCD to mid log phase, OD₆₀₀ = 0.3, and treated for 90 min at indicated αF conditions. For Figure 4B, 15 or 16 biological samples over the course of 2 d for each Rga2 WT and Rga2AAALAA were assayed for FUS1-lacZ activity. FUS1-lacZ activity was determined using as substrate fluorescein di-β-o-galactopyranoside and normalized to OD₆₀₀.
flouride (10 mM), sodium metavanadate (0.4 mM), sodium orthovanadate (0.4 mM), and 3-glycerol-phosphate (0.1 mM) was used. Proteins were eluted from beads by boiling in Laemmli buffer for 5 min. Samples were resolved by electrophoretic mobility in 5.5% acrylamide SDS–PAGE gel, and phosphorylation was assayed by immunoblot (anti-FLAG, rabbit) and Hsc82 loading control (anti-Hsc82). Primary antibody was detected by IR fluorescence with secondary antibody anti-rabbit conjugated to Alexa 790 (A11369; Invitrogen) and visualized with an Odyssey scanner. Percentage of total Rga2 signal represented by slower-migrating band was quantified by Image Studio (Li-Cor Biosciences).

In vivo kinase inhibition

Fus3-WT (NLY39+MR4937) or Fus3-as1 (NLY39+MR4938) was transformed with Rga2WT–FLAG (pBA2059). Strains were grown in SCD-URA-LEU plus 5 mM estradiol at 30°C to OD600 = 0.3 before addition of 10 µM αf pheromone for 2 h, followed by addition of 10 µM 1Na-PP1 (10954; Cayman Chemical Company) or vehicle (dimethyl sulfoxide) for 10 min. Cells were harvested and processed as described in Hughes Hallett et al. (2014), and 30 µg of total protein was loaded to detect electrophoretic mobility by immunoblot. Samples were resolved by electrophoretic mobility in 5.5% acrylamide SDS–PAGE gel, and phosphorylation was assayed by immunoblot (anti-FLAG, mouse) and Hsc82 loading control (anti-Hsc82). Primary antibody was detected by IR fluorescence with secondary antibody anti-rabbit conjugated to Alexa 790 (A11369; Invitrogen) and visualized with an Odyssey. Percentage of total Rga2 signal represented by the slower-migrating band was quantified by Image Studio.

In vitro kinase assay

GST-Fus3 kinase was purified and assayed for in vitro activity as described in Parnell et al. (2014). Briefly, BL21 DE3 cells expressing pGEX-2T6-FUS3 or pGEX-2T6-FUS3 K42R were purified by glutathione–Sepharose (17075601, GE Healthcare). Rga2 constructs were purified by Ni-NTA in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM β-ME, protease inhibitors) from E. coli extracts prepared as described earlier. Approximately 100 nM GST-Fus3WT or GST-Fus3K42R was activated with 300 µM ATP in kinase buffer (25 mM HEPES, pH 7.2, 15 mM MgCl2, 5 mM EGTA, 1 mM MnCl2, 3.5 mM DTT, and protease inhibitors) before being incubated with 450 nM 6xHis-Rga2, 6xHis-Rga2K42A, or 6xHis-Rga2K42A supplemented with 5 µCi of [γ-32P]ATP (PerkinElmer Cetus). Reaction was carried out at room temperature. Samples were collected at indicated time points and reaction terminated with addition of Laemmli buffer and boiling for 5 min. Samples were resolved in 7.5% SDS–PAGE, stained with Instant Blue Stain (Expegen Protein Solutions), dried, and exposed to phosphoimager screen. The 32P incorporation was detected by Typhoon scanner, and storage phosphor count signal was quantified using ImageJ (Schneider et al., 2012) and normalized to Coomassie signal.

Pheromone time course

Cells (NLY33) transformed with plasmids (pBA2059 or pNL111) were grown in SCD-LEU plus 5 nM estradiol to mid log phase. At OD600 = 0.35, cells were treated with 0.2 M HU for 3 h and then washed and released into SCD-LEU plus 5 nM estradiol at OD600 = 0.5 and allowed to recover for 20 min before 10 µM αf was added at time 0. The 10-min time points were taken every 5 min for fluorescence-activated cell sorting (FACS) analysis, microscopy, and electrophoretic mobility from yeast extracts prepared as described. A 35-µg amount of total protein was analyzed for electrophoretic mobility in 5.5% acrylamide SDS–PAGE gel, and phosphorylation was assayed by immunoblot (anti-FLAG, rabbit) and Hsc82 loading control (anti-Hsc82). Primary antibody was detected by IR fluorescence with secondary antibody anti-rabbit conjugated to Alexa 790 (A11369; Invitrogen) and visualized with an Odyssey scanner. Percentage of total Rga2 signal represented by the slower-migrating band was quantified by Image Studio.

Samples for Fus3 activation (phospho-Fus3) blot were resolved on 12% acrylamide SDS–PAGE gel and immunoblotted with primary antibodies anti-p44/42 MAPK and anti-Fus3. Primary antibodies were detected by IR fluorescence with secondary antibody anti-rabbit conjugated to Alexa 790 (A11369; Invitrogen) and visualized with an Odyssey scanner. Percentage of total Rga2 signal represented by the slower-migrating band was quantified by Image Studio.

FACS

A 1-ml amount of harvested yeast cells was fixed in 70% ethanol overnight at 4°C. Cells were washed in 50 mM sodium citrate, pH 7.4, sonicated briefly, and treated with 0.25 mg/ml RNase A for 2 h at 50°C and then 0.8 mg/ml Proteinase K for another 2 h at 50°C. Cells were washed in 50 mM sodium citrate before DNA was stained with 16 µg/ml propidium iodide in sodium citrate and analyzed on a BD FACS Calibur (BD Biosciences, San Jose, CA).

Microscopy

A 1-ml amount of harvested yeast cells was fixed in 70% ethanol overnight at 4°C. Cells were washed in 50 mM sodium citrate, pH 7.4, sonicated briefly, and stained with 24 mg/ml fluorescein isothiocyanate (FITC)–concanavalin A (C7642-2MG; Sigma-Aldrich) in P-buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.2) at room temperature for 5 min. Cells were washed in P-buffer and sonicated briefly before imaging with Zeiss Axio Imager M1 microscope (Carl Zeiss, Jena, Germany) with an LED lamp with a 100×/1.30 numerical aperture oil immersion objective. A 474/40-nm excitation and a 530/50-nm emission filter were used for FITC–concanavalin A imaging. Images were captured on a Hamamatsu Orca-ER digital camera (Bridgewater, NJ) coupled to Openlab Software 4.0.4 (PerkinElmer-Cetus).

ACKNOWLEDGMENTS

We thank Brenda Andrews, Henrik Dohlman, and Mark Rose for re-agents and the members of the Cyert lab for helpful discussions and critical reading of the manuscript. We are grateful to Mardo Kõivomägi for his experimental guidance and support. We thank Pablo Aguilar for sharing unpublished data and for insightful discussion. Research reported here was supported by the National Institute of General Medical Services of the National Institutes of Health under Awards 5T32GM008412 and 2T32GM007276 to N.L. and R01GM48728 to M.S.C. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Institutes of Health.

REFERENCES


584 | N. Ly and M. S. Cyert


