Pheromone-induced polarization is dependent on the Fus3p MAPK acting through the formin Bni1p

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During mating, budding yeast cells reorient growth toward the highest concentration of pheromone. Bni1p, a formin homologue, is required for this polarized growth by facilitating cortical actin cable assembly. Fus3p, a pheromone-activated MAP kinase, is required for pheromone signaling and cell fusion. We show that Fus3p phosphorylates Bni1p in vitro, and phosphorylation of Bni1p in vivo during the pheromone response is dependent on Fus3p. fus3 mutants exhibited multiple phenotypes similar to bni1 mutants, including defects in actin and cell polarization, as well as Kar9p and cytoplasmic microtubule localization. Disruption of the interaction between Fus3p and the receptor-associated Gα subunit caused similar mutant phenotypes. After pheromone treatment, Bni1p-GFP and Spa2p failed to localize to the cortex of fus3 mutants, and cell wall growth became completely unpolarized. Bni1p overexpression suppressed the actin assembly, cell polarization, and cell fusion defects. These data suggest a model wherein activated Fus3p is recruited back to the cortex, where it activates Bni1p to promote polarization and cell fusion.

Introduction

Saccharomyces cerevisiae reproduces sexually, through mating and meiosis (for review see Sprague and Thorner, 1994; Marsh and Rose, 1997). Haploid yeast cells exist in two mating types, MATα and MATα, which secrete mating-type–specific peptide pheromones that bind to specific transmembrane receptor proteins on the opposite cell type. When either cell is stimulated by pheromone, an intracellular signal transduction cascade is initiated that leads to a switch in the growth pattern from budding to mating. Stimulated cells polarize their growth toward each other, forming elongated projections following the pheromone gradient until cells have come into contact. Cells with mating projections are often referred to as “shmoo.” After contact, a stable junction is formed between the mating pair such that the intervening cell walls can be safely degraded and the plasma membranes can fuse. After cell fusion, the two haploid nuclei move toward each other and fuse to form a single diploid nucleus. The diploid zygotic cell then reenters the mitotic cell cycle.

Several proteins are required for cell fusion, including the MAPK, Fus3p. Although its specific role in cell fusion is unclear, Fus3p has several well-characterized functions in signal transduction and pheromone-induced cell cycle arrest (Elion et al., 1990; Fujimura, 1990). After pheromones bind to their receptor (Ste2p or Ste3p, depending on mating type), the associated trimeric G protein dissociates into Gα and Gβγ subunits (Gpa1p, Ste4p, and Ste18p, respectively). Free Gβγ interacts with several proteins, including Ste20p, a p21-activated protein kinase, and Ste5p, a scaffolding protein for the MAPK cascade comprised of Ste11p, Ste7p, and Fus3p. Ste20p phosphorylates Ste11p, which phosphorylates and activates Fus3p. Activated Fus3p then enters the nucleus where it phosphorylates Dig1p and Dig2p, negative regulators of the transcription factor Ste12p, leading to the transcription of genes required for cell and nuclear fusion (Cook et al., 1997; Tedford et al., 1997). In some strains, Fus3p is not essential for transcriptional activation because of the presence of a second partially redundant MAPK, Kss1p (Elion et al., 1991a,b). Activated Fus3p also phosphorylates Far1p (Elion et al., 1993), which acts as a cell cycle inhibitor to arrest the cell in G1. The requirement for Fus3p in cell cycle arrest can be suppressed by deletion of the G1 cyclin, CLN3 (Elion et al., 1990). Far1p plays a second role in determining the site of cell polarization (Vatz et al., 1995). In mitosis, Far1p is resident...
in the nucleus, where it sequesters Cdc24p, the exchange factor for the Rho-like G protein, Cdc42p. Cell cycle–dependent degradation of Far1p allows the release and recruitment of Cdc24p to the incipient bud (Shimada et al., 2000). However, during mating, a Far1p–Cdc24p complex exits the nucleus and interacts with GBY at the cortex, recruiting Cdc42p and Bem1p away from the bud site (Butty et al., 1998; Nern and Arkowitz, 1999). Mutants lacking Far1p still form shmoo, which are mislocalized at the site of bud emergence, rather than toward the mating partner. These results suggest that Far1p is required for orienting the shmoo projection, but not for the intrinsic mechanism of polarization. Mutants in which both the bud site and Far1p-dependent orientation have been inactivated show residual polarization, implying that there is another pathway responsible for pheromone-induced polarization (Nern and Arkowitz, 2000a).

Mutations in fus3 cause a profound cell fusion defect (Elion et al., 1990), and several lines of evidence suggest that the cell fusion defect may be independent of defects in transcriptional activation and cell cycle arrest. If the transcriptional activation defect is suppressed by overexpression of STE12, or if the cell cycle arrest defect is suppressed by deletion of cln3 (Elion et al., 1991b; Fujimura, 1992), either singly (Elion et al., 1991b; Fujimura, 1992) or together (Matheos, 2003), fus3 mutants still exhibit a strong cell fusion defect. Therefore, it is likely that Fus3p has additional functions that are required for cell fusion.

Mutations in several genes involved in polarity establishment exhibit cell fusion defects. In particular, mutations affecting proteins in the “polarisome” (Bni1p, Spa2p, and Pea2p) cause strong cell fusion defects (Gehrung and Snyder, 1990; Chenevert et al., 1994; Dorer et al., 1997; Evangelista et al., 1997; Gammie et al., 1998). Of particular interest is the formin protein, Bni1p. Formins regulate actin and cell polarization in response to a variety of stimuli in a wide variety of eukaryotes. Recently, Bni1p has been shown to facilitate actin cable polymerization in vitro (Evangelista et al., 2002; Pryne et al., 2002; Sagot et al., 2002a,b). Bni1p interacts with Spa2p and Pea2p, and Spa2p is required for proper Bni1p localization to sites of polarized growth during mitosis (Fujiwara et al., 1998; Ozaki-Kuroda et al., 2001). Bni1p also interacts with and is regulated by a variety of rho-like G proteins (Dong et al., 2003). In particular, the small GTP-binding protein Cdc42p is known to regulate Bni1p function in both mitotic and mating cells to promote polarization (Evangelista et al., 1997). Because Cdc42p also interacts with GBY after release by the Gα subunit in response to pheromone (Butty et al., 1998; Nern and Arkowitz, 1999), it seems likely that this interaction plays a key role in the pathway by which cells polarize toward the pheromone gradient. Bni1p activated by Cdc42p near the site of pheromone response would nucleate actin cables, leading to polarized growth. Polarization is thought to be required during cell fusion to deliver proteins required for cell wall degradation and plasma membrane fusion (Gammie et al., 1998). In this paper, we provide evidence that one of Fus3p’s functions during mating is the activation and localization of Bni1p, to promote cell polarization and cell fusion.

Results

Fus3p phosphorylates Bni1p

To identify substrates of the MAPK Fus3p required for cell fusion, we screened proteins to see if any could be phosphorylated by Fus3p in vitro. Fus3p copurifies with other kinases, including Ste11p and Ste7p (Choi et al., 1994), and Fus3p must be phosphorylated in response to pheromone to be fully active (Gartner et al., 1992). To assay Fus3p’s protein kinase activity in the presence of other copurifying protein kinases, we used a form of the kinase engineered to use a novel ATP analogue, in addition to ATP. The bulky phenethyl-ATP is sterically hindered from binding to the acceptor pocket in the active site of most kinases. In previous work, Fus3p was engineered by introducing a single amino acid substitution (glutamine 93 to glycine) predicted to allow phenethyl-ATP to bind and serve as a phosphate donor (Shah et al., 1997; Liu et al., 1998; Bishop et al., 2000). Previously, we showed that Fus3pQ93G activity in vivo (but not that of wild-type Fus3p) is inhibited by the cognate analogue of a protein kinase inhibitor, 1-naphthyl PP1 (1-NA PP1), which has been modified with a similar bulky adduct (Bishop et al., 2000). To assay Fus3p activity, we partially purified either FLAG-tagged wild-type Fus3p or FLAG-tagged analogue-sensitive Fus3pQ93G from mitotic or pheromone-induced extracts. Addition of [32P]phenethyl-ATP to Fus3pQ93G purified from pheromone-induced cells lead to a high level of phosphorylation of Fus3p and copurifying proteins (Fig. 1 A). Very little activity was observed with Fus3pQ93G from uninduced mitotic cells or with wild-type Fus3p from either induced or mitotic cells. The residual activity observed with wild-type Fus3p from induced cells was effectively competed with excess ATP, whereas the activity observed with Fus3pQ93G was refractory to competition. In contrast, the activity of Fus3pQ93G was almost completely abolished by the addition of the analogue inhibitor 1-NA PP1, previously shown to inhibit the Fus3pQ93G activity in vivo (Bishop et al., 2000; Metodiev et al., 2002). Similar results were observed for the phosphorylation of an exogenous protein substrate, myelin basic protein (MBP; Fig. 1 B). Using [32P]phenethyl-ATP, phosphorylation was observed only with Fus3pQ93G and was inhibited by 1-NA PP1. Together, these results demonstrate that the protein kinase assay is specific for the pheromone-activated form of the analogue-sensitive Fus3pQ93G.

Next, we examined the ability of Fus3pQ93G to phosphorylate proteins in a genomic library of GST fusion proteins (Martzen et al., 1999; Matheos, 2003). Known substrates of Fus3p (Dig1p and Dig2p) were among the proteins identified. We also examined the ability of Fus3pQ93G to phosphorylate HA epitope–tagged proteins known to have a role in cell fusion, including Bni1p, Rvs161p, and Fus2p. Of these, only Bni1p was phosphorylated by Fus3pQ93G in vitro (Fig. 1 C; unpublished data).

To validate the significance of the phosphorylation, we determined whether Bni1p is phosphorylated during mating in vivo, dependent upon Fus3p. Wild-type and fus3Δ cells were induced with pheromone and labeled with [32P]orthophosphate. HA epitope–tagged Bni1p was immunoprecipitated and examined for the incorporation of 32P (Fig. 1 C). In the wild type, a doublet of 32P-labeled Bni1p proteins was
observed with the top band containing a much higher level of incorporation. In the fus3/H9004 strain, incorporation of $^{32}$P into the top band was severely reduced. The residual incorporation of $^{32}$P into Bni1p in the fus3/H9004 strain is most likely due to mitotic phosphorylation (Goehring et al., 2003). In parallel control experiments, the total amount of protein purified from the wild-type and fus3/H9004 strains was found to be equal (Fig. 1 D). These data confirm that Bni1p is phosphorylated during the pheromone response in vivo, and that a portion of the phosphorylation is dependent on Fus3p.

**Figure 1.** Fus3p phosphorylates Bni1p both in vitro and in vivo. (A) Wild-type FLAG-Fus3p and analogue-sensitive FLAG-Fus3pQ93G were immunoprecipitated from mitotic and pheromone-induced extracts, and in vitro kinase assays were performed as described in the Materials and methods. Nonradioactive ATP was added to the indicated concentrations as a competitive inhibitor of endogenous kinases. The inhibitor analogue 1-Na PP1 was added to $5 \mu$M in indicated lanes. The Fus3p panel and the top Fus3pQ93G panel were exposed to x-ray film for equivalent times; the bottom Fus3pQ93G panel shows a shorter exposure to allow visualization of individual protein bands. (B) Wild-type FLAG-Fus3p or inhibitor-sensitive FLAG-Fus3pQ93G was immunoprecipitated from pheromone-induced extracts, and kinase assays were performed as described for A. Myelin basic protein (MBP) was added as an exogenous substrate. 1-Na PP1 was added to $10 \mu$M in indicated lanes. (C) In vitro phosphorylation of Bni1p by Fus3p. Top: HA-Bni1p was immunoprecipitated out of yeast and added to FLAG-Fus3pQ93G in the in vitro kinase assay. Bottom: Western blots were performed using anti-HA antibody (12CA5 at 1:2,500 dilution) to identify HA-Bni1p. In both cases, a strain containing no HA tagged proteins (EY699) was used as negative control. (D) In vivo phosphorylation of Bni1p by Fus3p. Top: wild-type (MY8195) and fus3Δ (MY8196) overexpressing HA-BNI1 were induced with α-factor and labeled with $^{32}$P orthophosphate before immunoprecipitation as described in the Materials and methods. Bottom: cells treated under the same conditions were processed for Western blot analysis to determine total amount of protein immunoprecipitated from each strain. EY699 was used as the negative control.

**fus3Δ mutants are defective in polarized morphogenesis and actin localization**

Bni1p has several well-characterized roles in polarizing the actin cytoskeleton. Bni1p and the related formin Bnr1p are required for bud emergence during mitosis (Imamura et al., 1997), and Bni1p is required for shmoo formation in response to pheromone (Evanistla et al., 1997). During mating, bni1Δ mutants fail to polarize, remain ellipsoidal, and contain delocalized cortical actin patches.

To determine whether Fus3p phosphorylation is required for Bni1p function, we examined fus3Δ mutants for bni1Δ-like phenotypes. First, we examined the morphology of cells responding to pheromone. Within 90 min of pheromone treatment, 99% of wild-type cells formed shmoo projections. In the fus3Δ strain, incorporation of $^{32}$P into the top band was severely reduced. The residual incorporation of $^{32}$P into Bni1p in the fus3Δ strain is most likely due to mitotic phosphorylation (Goehring et al., 2003). In parallel control experiments, the total amount of protein purified from the wild-type and fus3Δ strains was found to be equal (Fig. 1 D). These data confirm that Bni1p is phosphorylated during the pheromone response in vivo, and that a portion of the phosphorylation is dependent on Fus3p.

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**Figure 2.** fus3Δ mutants have mislocalized actin and fail to polarize in response to pheromone. (A) Cells were treated for 2.5 h with α-factor, fixed, and stained with rhodamine-phalloidin to examine actin localization by fluorescence microscopy as described in the Materials and methods. (B) WT (EY699), bni1Δ (MY8188), fus3Δ (EY700), and gpa1K21E R22E (MY8193) were scored for their ability to form shmooes after 1.5 h of exposure to pheromone ($n > 100$). (C) Cells were scored for their actin localization phenotype by fluorescence microscopy ($n > 100$).
peared to be buds that had begun to shmoo. Similar cells were not observed in the \textit{bni1} mutant. The shmoo buds suggest that in the absence of Fus3p, the lack of cell cycle arrest allows residual polarization to occur from the bud site.

Next, we examined actin localization (Fig. 2, A and C). Although most wild-type cells (96%) showed a heavy concentration of actin at the shmoo tip (Fig. 2 A), actin patches were delocalized in the \textit{fus3}\textsubscript{Δ} mutant cells (81%, Fig. 2 C). This defect was similar to that of the \textit{bni1} mutant, in which 95% of cells showed actin delocalization. Because only partially polarized cells were counted to determine actin localization, the overall actin localization defect was more severe than these data indicate.

Phosphorylated Fus3p binds to activated Gpa1p, and the \textit{gpa1}\textsuperscript{K21E R22E} form of G\textsubscript{a} is defective for binding to Fus3p in vivo (Metodiev et al., 2002). The \textit{gpa1}\textsuperscript{K21E R22E} mutant cells respond to pheromone, but their chemotropic response is compromised (Metodiev et al., 2002). We examined whether the interaction of Fus3p with Gpa1p might have a role in the activation of Bni1p. We found that the \textit{gpa1}\textsuperscript{K21E R22E} mutants were partly defective for actin polarization to the shmoo tip (21%) and for shmoo formation (27%; Fig. 2, B and C). These results suggest that the loss of the Gpa1p interaction may affect the efficiency of Fus3p regulation.

\textbf{\textit{fus3} mutants do not localize Kar9p to the shmoo tip properly}

Kar9p’s localization to the shmoo tip is dependent on Bni1p and actin (Lee et al., 1999; Miller et al., 1999), presumably through the association of Kar9p and Myo2p with actin cables (Yin et al., 2000; Hwang et al., 2003). Therefore, we tested whether a \textit{fus3} mutant would localize Kar9p properly (Fig. 3). Because shmoo-like cells are rare in the \textit{fus3}\textsubscript{Δ} mutant, we used 1-Na PP1 to inactivate Fus3p\textsubscript{Q93G} in vivo, after cells had begun to polarize in response to pheromone. The \textit{fus3}\textsubscript{Q93G} strain was induced with pheromone for 60 min, after which 1-Na PP1 was added and incubated for an additional 30 min. As a control, a parallel culture of the \textit{fus3}\textsubscript{Q93G} strain was mock treated with DMSO. As expected, in the control cells, GFP-Kar9p was localized most frequently as a single cortical dot, a line of localization, multiple dots near the shmoo tip, and dispersed dots elsewhere in the cell body, possibly on misoriented microtubules.

Through the association of Kar9p and Myo2p with actin cables, \textit{fus3} mutants have misaligned microtubules. WT (YE699), \textit{bni1}\textsubscript{Δ} (MY8189), fus3\textsubscript{Δ} (YE700), \textit{gpa1}\textsuperscript{K21E R22E} (MY8193), and fus3 \textit{pGAL-BNI1} (MY8196) cells were induced with pheromone for 3 h and processed for immunofluorescence. Microtubules were detected with YOL1/34 as the primary antibody and the nucleus was stained with DAPI (n > 100). Cells were scored as having (from left to right): a single bundle of cytoplasmic microtubules going to the shmoo tip, a single bundle of cytoplasmic microtubules going to the shmoo tip plus other additional bundles oriented away from the shmoo tip, a spray of microtubules going to the shmoo tip, no cytoplasmic microtubules going to the shmoo tip, and long nuclear microtubules with short or nonexistent cytoplasmic microtubules.

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& & & & & \\
\textbf{WT} & 84 & 10 & 6 & \\
\textbf{\textit{bni1}\textsubscript{Δ}} & 28 & 20 & 12 & 40 & \\
\textbf{\textit{FUS3}}\textsubscript{Q93G} & 78 & 13 & 9 & \\
\textbf{\textit{FUS3}}\textsubscript{Q93G} & 78 & 13 & 9 & \\
\textbf{+ 1-Na PP1} & 13 & 31 & 20 & 36 & \\
\textbf{\textit{gpa1}}\textsuperscript{K21E R22E} & 9 & 11 & 30 & 50 & \\
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\caption{Both \textit{fus3} mutants and \textit{gpa1}\textsuperscript{K21E R22E} mutants mislocalize Kar9p. Cells containing \textit{pgAL-GFP-KAR9} were pregrown in raffinose, induced for 2 h by addition of galactose, and then pheromone was added for another 3 h. After brief fixation, WT (MY8189), \textit{bni1}\textsubscript{Δ} (MY), \textit{fus3}\textsubscript{Q93G} (MY7494), and \textit{gpa1}\textsuperscript{K21E R22E} (MY8194) cells were scored for GFP-Kar9p localization (n > 100). To inactivate Fus3p\textsubscript{Q93G} in vivo, 10 \textmu{}M 1-Na PP1 was added after 60 min of pheromone induction. An equivalent amount of DMSO was added to a separate culture as a control. Cells were scored as having (from left to right): a single cortical dot, a line of localization, multiple dots near the shmoo tip, and dispersed dots elsewhere in the cell body, possibly on misoriented microtubules.}
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& & & & & \\
\textbf{WT} & 91 & 4 & 5 & 0 & 0 & \\
\textbf{\textit{bni1}\textsubscript{Δ}} & 14 & 23 & 23 & 37 & 6 & \\
\textbf{\textit{fus3}}\textsubscript{Δ} & 35 & 2 & 8 & 56 & 0 & \\
\textbf{\textit{gpa1}}\textsuperscript{K21E R22E} & 44 & 16 & 18 & 12 & 10 & \\
\textbf{\textit{fus3}}\textsubscript{Δ} & 43 & 17 & 9 & 24 & 6 & \\
\hline
\end{tabular}
\caption{Both \textit{fus3} and \textit{gpa1}\textsuperscript{K21E R22E} mutants have misaligned microtubules. WT (YE699), \textit{bni1}\textsubscript{Δ} (MY8188), \textit{fus3}\textsubscript{Δ} (YE700), \textit{gpa1}\textsuperscript{K21E R22E} (MY8193), and fus3 \textit{pGAL-BNI1} (MY8196) cells were induced with pheromone for 3 h and processed for immunofluorescence. Microtubules were detected with YOL1/34 as the primary antibody and the nucleus was stained with DAPI (n > 100). Cells were scored as having (from left to right): a single bundle of cytoplasmic microtubules going to the shmoo tip, a single bundle of cytoplasmic microtubules going to the shmoo tip plus other additional bundles oriented away from the shmoo tip, a spray of microtubules going to the shmoo tip, no cytoplasmic microtubules going to the shmoo tip, and long nuclear microtubules with short or nonexistent cytoplasmic microtubules.}
\end{table}
cells showed microtubule orientation defects, with only 37% of cells having a single bundle of microtubules going into the shmoo tip. There was a significant increase in the number of cells with splayed microtubules (23%) or severely misoriented microtubules (37%) that were not oriented near the shmoo tip. A similar result was seen for the fus3 mutant cells, except that the class with completely misoriented microtubules (37%) that were not oriented near the shmoo tip. A significant increase in the number of cells with splayed microtubules (23%) or severely misaligned microtubules. Therefore, these three mutants have similar effects in cytoplasmic microtubule orientation, which may result from the inability to localize Kar9p properly to the shmoo tip.

**Fus3p is required for Bni1p localization to the shmoo tip**

Next, we examined whether Fus3p phosphorylation is required for Bni1p localization. For this purpose, we expressed a fully functional HA-Bni1p-GFP construct in wild-type, fus3Δ, and gpa1K21E R22E cells (Evangelista et al., 1997). Bni1p colocalizes with actin at cortical sites where cell growth and secretion occur. As previously observed, in both wild-type and fus3Δ mitotic cells, Bni1p was localized to the incipient bud site, the tip of the growing bud, and the mother-bud neck at cytokinesis. Bni1p was found at the shmoo tip in 84% of wild-type cells treated with pheromone (Fig. 5 A). In contrast, Bni1p was localized in only 10% of the fus3Δ cells treated with pheromone. HA-Bni1p-GFP localization was not detected either by direct visualization or by indirect immunofluorescence (Fig. 5, A and B). HA-Bni1p-GFP localized normally in a strain deleted for KSS1 (unpublished data), indicating that the defect is specific to fus3. The gpa1K21E R22E mutant also showed a partial loss of Bni1p localization at the shmoo tip (26%), consistent with the partial defect in actin polarization and shmoo formation. Loss of localization was not due to defects in the expression or stability of Bni1p (Fig. 5 C).

To confirm the requirement for Fus3p’s kinase activity, we examined the localization of Bni1-GFP in the fus3-Q93G mutant. By 90 min of pheromone induction, almost all (>90%) of the cells formed shmoos and Bni1-GFP was properly localized (86%). Subsequent treatment with 1-Na PPI (60 min) caused mislocalization of Bni1-GFP in the majority of cells (58%), and the localized Bni1p-GFP was substantially dimmer. 1-Na PPI had no effect in Fus3 wild-type cells. When 1-Na PPI was added with pheromone, by 2.5 h very few (16%) of the fus3-Q93G mutants formed shmoos, and Bni1-GFP was mislocalized in most (73%), similar to the fus3Δ mutant. In contrast, almost all (>90%) of wild-type cells formed shmoos, and Bni1-GFP was properly localized in most (79%). Because the level of Fus3p-Q93G is unaffected by 1-Na PPI (Mathesos, 2003), these results support the hypothesis that Fus3p’s kinase activity is required to localize Bni1p.

Because Bni1p interacts with components of the polarisome (Fujwara et al., 1998; Sheu et al., 2000), we next examined the localization of GFP-Spa2p. Normally, Spa2p localizes to the shmoo tip (Fig. 6 A). In contrast, in both bni1 and fus3 mutant cells, 92% and 88% of cells, respectively, showed no localization of Spa2p at the shmoo tip. These results show that Fus3p is required for the polarized localization of the polarisome during mating.

**Overexpression of Bni1p suppresses a fus3Δ mutant strain**

If Fus3p’s role in polarization and cell fusion is through Bni1p, then Fus3p-independent activation of Bni1p should partially suppress the requirement for Fus3p. As predicted, overexpression of Bni1p-GFP from the GAL1 promoter partially suppressed the polarization defect. Although only 22% of fus3Δ mutants were able to shmoos, upon Bni1p overexpression 69% of cells polarized in response to pheromone (Fig. 7 A and C). Furthermore, although only 18% of fus3Δ shmoos had polarized actin, overexpression of Bni1p-GFP caused 50% of cells to localize actin to the shmoo tip (Fig. 7 B). Consistent with these data, the overexpression of Bni1p-GFP also partially suppressed misorientation of the cytoplasmic microtubules (Fig. 4).

Although actin was polarized in these cells, its localization was often noticeably different from the wild type. Wild-type shmoo invariably contained a single projection with actin concentrated at the cortex. Although wild-type cells exposed to mating pheromone for longer times produce additional shmoo projections, only one projection will contain cortical actin patches. In contrast, in 25% of the fus3Δ cells overexpressing Bni1p-GFP, multiple actin-containing projections were observed (Fig. 7 C). Multi-projection shmoos were never observed in the wild-type strain overexpressing Bni1p.
When Bni1p was overexpressed, the cell fusion defect was suppressed more than fivefold; 32% of the zygotes completed cell fusion. Thus, at least part of the cell fusion defect of a fus3\textsuperscript{H9004} mutant is associated with the defect in Bni1p-dependent polarization.

Simultaneous ablation of the preexisting bud site as well as the Far1p-dependent chemotropic pathway also results in cells that do not form projections (Nern and Arkowitz, 2000a). Nevertheless, such cells do show polarized growth, suggesting that a third pathway is responsible for cell polarization (Nern and Arkowitz, 2000a). To explore the contribution of Fus3p to polarized growth, we used differentially labeled Con A to distinguish the sites of cell wall growth (Nern and Arkowitz, 2000a). Mitotic cells were first labeled with FITC-Con A to mark the preexisting cell wall, then treated with pheromone, and finally labeled with TRITC-Con A to mark the sites of new growth (Fig. 9). We used a fus3\textsuperscript{H9004} cln3\textsuperscript{H9004} double mutant to suppress the cell cycle arrest defect (Elion et al., 1991b). Both the wild-type and cln3\textsuperscript{H9004} mutant cells showed prominent polarized surface growth with new cell wall deposition occurring in one region with little overlap between the new and the old cell surfaces. In contrast, both the fus3\textsuperscript{H9004} cln3\textsuperscript{H9004} mutant and the bni1\textsuperscript{H9004} mutant showed completely overlapping TRITC and FITC signals with no single site of new cell surface growth. We conclude that Fus3p, like Bni1p, is required for polarized growth in response to pheromone.

**Figure 6.** Spa2p does not localize to the shmoo tips in fus3 and bni1 mutants. (A) WT (EY699), bni1\textsuperscript{MY8188}, and fus3\textsuperscript{EY700} cells transformed with p426S2G (expressing Spa2p-GFP) were induced with pheromone for 2 h, fixed with formaldehyde, and processed for immunofluorescence. Rabbit polyclonal anti-GFP antibody (from CLONTECH Laboratories, Inc.) was used at 1:25 dilution in PBS-BSA. (B) Cells were scored for GFP-Spa2p localization at the shmoo tip (\(n > 100\)).

GFP. Presumably, multiple sites of polarization arise because the Bni1p-dependent actin localization is uncoupled from the spatial signal defined by the site of receptor activation.

Although Bni1p-GFP overexpression suppressed the fus3\textsuperscript{H9004} polarization defect, in these cells, Bni1-GFP was not detected at the shmoo tips. Possibly, the small amount of Bni1p-GFP at the ends of the actin cables was too faint to be detected above the background fluorescence. Presumably, increasing the level of Bni1p raises it above a critical concentration for actin cable assembly at the shmoo tip, whereupon secondary interactions lead to actin cable clustering.

Next, we determined whether overexpression of Bni1p suppressed the fus3\textsuperscript{H9004} cell fusion defect. To measure cell fusion, we performed matings with a fus1 fus2 double mutant and determined the number of defective zygotes (residual septum between the cells and two nuclei; Fig. 8). Under these conditions, fus3\textsuperscript{H9004} mutants exhibited a very strong mating defect; only 6% of mating pairs completed cell fusion. In contrast, 89% of zygotes formed by mating the fus1 fus2 double mutant with the wild type completed cell fusion.

**Discussion**

Here, we report that the formin protein Bni1p is a substrate of the MAPK Fus3p in vitro, and the phosphorylation of
Bni1p is dependent on Fus3p in vivo, during the pheromone response. Moreover, during the pheromone response, the phenotypes of fus3Δ and bni1Δ mutants were similar with respect to actin and cell polarization, Kar9p and Spa2p localization, and microtubule alignment. Overexpression of Bni1p partially suppressed the polarization phenotypes of a fus3 mutant, suggesting that Bni1p functions downstream of Fus3p to promote these processes. Overexpression of Bni1p also partially suppressed the fus3 cell fusion defect, suggesting that activation of Bni1p is one of the primary functions of Fus3p in cell fusion. Finally, a mutant form of Gpa1p that is defective for binding Fus3p conferred similar phenotypes, albeit weaker, suggesting that Fus3p regulation of Bni1p during pheromone signaling is partially dependent on the interaction between Fus3p and Gpa1p.

In the current model for cell polarization during mating, the interaction of Far1p with free Gβγ subunit at the cortex is thought to be the key signal for polarity establishment. By binding to Far1p, Cdc24p (and thus Cdc42p) would be targeted to the free Gβγ subunit, overriding the preexisting cortical cue for polarization at the bud site (Butty et al., 1998; Nern and Arkowitz, 1999, 2000b; Shimada et al., 2000). However, this model does not entirely explain the basis of pheromone-induced polarization. First, Far1p is not required for polarization; in the absence of Far1p, projections do form at the preexisting bud site. Second, although strains simultaneously containing a mutant form of Cdc24p unable to bind to Far1p (cdc24-m1) and defective for the cortical bud site cue (bud1Δ) are unable to form shmoo projections, they are still able to undergo polarized growth (Nern and Arkowitz, 2000a). Proteins required for polarized growth, such as Spa2p, still localize to the growth region, although their localization is unstable. These data imply that another signal establishes cell polarization in response to pheromone. We found that fus3 mutants were unable to establish residual polarized growth during pheromone signaling, suggesting that Fus3p signaling is required to establish or maintain polarized cell growth in response to pheromone.

Our finding that Bni1p was mislocalized in the fus3 mutant and that Bni1p overexpression suppressed the fus3 defects supports a model in which Bni1p acts downstream of Fus3p for cell polarization. Given the in vitro phosphorylation data, it is most likely that Bni1p is directly phosphorylated by Fus3p in vivo. However, it remains formally possible that a downstream event is responsible for activation. Regardless of mechanism, activation of Bni1p would facilitate its ability to nucleate actin assembly at the site of the incipient shmoo projection. Bni1p might be either more stably associated with the cortex at that site, be more active for actin assembly, or interact more strongly with other proteins required for assembly. One possible target for regulation is the FH3 domain, which has been implicated in the localization of the Fus1 formin (as well as other formins) in the fission yeast Schizosaccharomyces pombe (Petersen et al., 1998).

It is thought that small differences in pheromone receptor activity are amplified and reinforced to generate the asym-
Figure 10. A model of Fus3p-dependent Bni1p activation. Upon pheromone binding to its receptor, a heterotrimeric G protein dissociates. The Gβγ subunit, comprised of Ste4p and Ste18p, respectively, is required for activation of the MAPK module and for recruitment of Far1p, Cdc42p, and Cdc24p, which establish the site for the future shmoo projection. Free Gpa1p recruits phosphorylated active Fus3p back to the site of receptor activation, facilitating the local Fus3p-dependent phosphorylation of Bni1p and leading to the stable localization of Bni1p at the incipient shmoo site. Bni1p then nucleates actin assembly and other functions required for polarization of the cell toward the pheromone gradient.

The cortical site of receptor activation has been thought to be marked solely by the appearance of free Gβγ subunit. However, phosphorylated Fus3p also interacts with the Gα subunit, Gpa1p (Metodiev et al., 2002). Therefore, the recruitment of activated Fus3p by Gpa1p may also contribute to recognition of the cortical site of receptor activation. Active Fus3p recruited to the cortex near the active receptor would lead to local activation of Bni1p, facilitating localized actin assembly and polarized growth. Thus, one model for the Gpa1p–Fus3p–Bni1p interaction is that it serves as an additional cortical cue in determining the site of shmoo formation.

In this view, there would be two "pathways" for cell polarization in response to pheromone (Fig. 10). In one path, the Gβγ interaction with Far1p recruits Cdc24p, Cdc42p, and Bem1p to the incipient shmoo site, away from the bud site. In the second path, activated Fus3p, presumably in association with Gpa1p, would localize Bni1p and the polarisome complex to the incipient shmoo site. Cells would need both pathways to respond correctly to a pheromone gradient. However, the two pathways need not be mutually exclusive, and other pathways may also contribute.

During chemotropic mating, cells orient growth along pheromone gradients (Schrick et al., 1997). During "default mating," cells cannot sense the gradient (as happens in an excess of pheromone) and instead use the bud site to initiate polarized growth. However, Gβγ, which interacts with the transmembrane receptor and which also may be needed for Bni1p localization, is required for yet more functions in mating, in addition to activation (e.g., Cdc42p). There are several possible explanations for incomplete suppression. First, overexpression of Bni1p mutant may not restore stable localization to the shmoo tip. Second, overexpression led to the appearance of abnormal shmoos with multiple projections. Possibly, part of the requirement for two pathways in cell polarization is to ensure that only a single site is chosen for polarization. Finally, although these results strongly imply that Fus3p acts through Bni1p for cell fusion, it is also likely that Fus3p is required for yet more functions in mating, in addition to the known roles.

Materials and methods

General yeast techniques

Yeast media and general techniques were described previously (Rose et al., 1990). In all cases, yeast strains were grown at 30°C. When inducing with galactose, cells were first grown overnight in synthetic complete (SC) media containing 2% raffinose, and then grown to early logarithmic phase in 2% raffinose + 2% galactose. Synthetic a factor (Syn/Seq Facility, Princeton University, Princeton, NJ) was added to 10 μg/ml. Transformations were done as described previously (Gietz and Woods, 2002). Yeast strains and bacterial plasmids are listed in Table I and Table II. To construct the gpa1Δ::URA3, pDSB138 was digested with EcoRI and was cotransformed...
Fus3p-dependent polarization during mating  | Matheos et al. 107

Table I. Yeast strains

<table>
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<th>Strain</th>
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<tr>
<td>EY699</td>
<td>MAT(\text{A}) ura3(\text{a})-1 his3(\text{a})-11,15 leu2(\text{a})-3,112 trp1(\text{a})-1 ade2(\text{a})-1 can1(\text{a})-100 GAL(\text{a})+</td>
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Strains EY699, EY700, and JY429 are from the laboratory of G. Fink (Massachusetts Institute of Technology, Cambridge, MA). Otherwise, strains were constructed for this work.

Table II. Plasmids and bacterial strains

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<tr>
<td>pMR4937</td>
<td>pGAL-FUS3 URA3 CEN3 ARS1 AMP(\text{a})+</td>
<td>Dr. C. Boone, University of Toronto, Toronto, Canada</td>
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Plasmids were from the laboratories of M.D. Rose, D. Stone, or as indicated.
cases, the inhibitor 1-Na PP1 was added to 10
phorylation, 10
added to a mini-column constructed from a 1-ml pipette tip. 200
(Sigma-Aldrich) and HBS (150 mM NaCl and 10 mM Hepes, pH 7.4) was
and washed twice with 1 ml HBS
protein kinase, in polarisome activation.

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scriptional activation of the mating pheromone response pathway in Saccha-

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Bni1p at the bud site and regulation of the actin cytoskeleton in Saccha-

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romyces cerevisiae requires tyrosine and threonine phosphorylation of FUS3

Sprague, Jr. 2003. Synthetic lethal analysis implicates Ste20p, a p21-activated

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