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 MATa, 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 “shmoos.” 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 Ste7p, 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 (Valtz 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 GβY 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 shmooes, which are mislocalized at the site of bud emergence, rather than toward the mating partner. These results suggest that Far1p is required for orienting 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 “polarosome” (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 (Fujitaya 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 GβY 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 orient 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.
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.

fus3 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 (Evangelista et al., 1997). During mating, bni1 mutants fail to polarize, remain ellipsoidal, and contain delocalized cortical actin patches.

To determine whether Fus3p phosphorylation is required for Bni1p function, we examined fus3 mutants for bni1-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 same time, 76% of bni1 mutant cells were unpolarized and remained ellipsoidal. Likewise, 78% of the fus3 mutant cells were unpolarized (Fig. 2 B). Although most cells were rounded, a few partially polarized shmoo-like cells were observed among the fus3 cells, suggesting that hypophosphorylated Bni1p retains some function. Interestingly, most cells with shmoo-like morphology ap-
fus3-Q93G were delocalized in the concentration of actin at the shmoo tip (Fig. 2 A), actin patches 

Although most wild-type cells (96%) showed a heavy concentration, the overall actin localization defect was more severe than these data indicate.

Phosphorylated Fus3p binds to activated Gpa1p, and the gpa1<sup>K21E R22E</sup> form of G<sub>a</sub> is defective for binding to Fus3p in vivo (Metodiev et al., 2002). The gpa1<sup>K21E R22E</sup> mutant cells respond to pheromone, but their chemotropoic 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 gpa1<sup>K21E R22E</sup> 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.

**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 fus3 mutant would localize Kar9p properly (Fig. 3). Because shmoo-like cells are rare in the fus3Δ mutant, we used 1-Na PP1 to inactivate Fus3p<sup>Q93G</sup> after cells had begun to polarize in response to pheromone. The fus3<sup>Q93G</sup> 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 fus3<sup>Q93G</sup> 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.

**Figure 3. Both fus3 mutants and gpa1<sup>K21E R22E</sup> mutants mislocalize Kar9p.** Cells containing 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), bni1Δ (MY), fus3<sup>Q93G</sup> (MY7494), and gpa1<sup>K21E R22E</sup> (MY8194) cells were scored for GFP-Kar9p localization (n > 100). To inactivate Fus3p<sup>Q93G</sup> in vivo, 10 µM 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.
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 increased to 56% (Fig. 4). The gpa1K21E R22E mutant cells also exhibited an increase in the number of cells with splayed microtubules in the shmoo tip and with 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 Bni1p-GFP in the fus3-Q93G mutant. By 90 min of pheromone induction, almost all (>90%) of the cells formed shmoos and Bni1p-GFP was properly localized (86%). Subsequent treatment with 1-Na PPI (60 min) caused mislocalization of Bni1p-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 Bni1p-GFP was mislocalized in most (73%), similar to the fus3Δ mutant. In contrast, almost all (>90%) of wild-type cells formed shmoos, and Bni1p-GFP was properly localized in most (79%). Because the level of Fus3p-Q93G is unaffected by 1-Na PPI (Matheos, 2003), these results support the hypothesis that Fus3p’s kinase activity is required to localize Bni1p.

Because Bni1p interacts with components of the polarisome (Fujisawa 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 shmoo, 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 shmoos 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/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/H9004 cln3/H9004 double mutant to suppress the cell cycle arrest defect (Elion et al., 1991b). Both the wild-type and cln3/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/H9004 cln3/H9004 mutant and the bni1/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.

Discussion

Here, we report that the formin protein Bni1p is a substrate of the MAPK Fus3p in vitro, and the phosphorylation of
Fus3p-dependent polarization during mating

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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 (cyc24-m1) and defective for the cortical bud site cue (bud1/H9004) 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 functions downstream of Fus3p to promote these processes. 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 GBγ 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 GBγ 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 (cyc24-m1) and defective for the cortical bud site cue (bud1/H9004) 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 functions 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-
In the idea that Gpa1p helps localize active Fus3p at the shmoo tip.

We have previously shown that Fus3p is dependent on the G protein, while Gpa1p (Metodiev et al., 2002), consistent with a model in which Gα 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.

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 Gα 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 (Dorer et al., 1997). fus3 mutants show defects in both the default and the chemotropic mating pathways (Schrick et al., 1997). The fus3 chemotropic defect is weaker than that seen for mutations affecting the receptor and Gβγ, consistent with a model in which Gβγ recruitment plays a primary role in determining the site of polarization. Presumably, the fus3 chemotropic defect results from the inability of Bni1p to localize; the failure to polarize at a unique site might allow fus3 cells to mate with nonsignaling partners via adventitious contacts.

The gpa1K21E R22E mutation also causes a defect in chemotropic mating (Metodiev et al., 2002), consistent with the idea that Gpa1p helps localize active Fus3p at the shmoo tip. In the gpa1K21E R22E mutant, the localization of Fus3p at the shmoo tip was altered but not abolished (Metodiev et al., 2002). Active Fus3p would still be present to activate Bni1p, although possibly with reduced efficiency, consistent with the relatively mild defects of the gpa1K21E R22E mutant in actin polarization, shmoo formation, and Bni1p localization. The stronger defect in Kar9p localization and microtubule orientation may indicate that Gpa1p interacts with other proteins affecting microtubule function independent of Bni1p and/or Fus3p.

Several mutations cause defects in both cell polarity and cell fusion. Before cell fusion, vesicles localize to the shmoo tip, and these vesicles are thought to carry cargo required for the localized degradation of the cell walls and subsequent membrane fusion (Gammie et al., 1998). Accordingly, we expect defects in actin polarization and localized secretion to cause defects in cell fusion. Bni1p overexpression partially suppressed the fus3 mutant polarization and cell fusion phenotypes. Most likely, suppression occurs because, through mass action, overexpressed Bni1p assembles at sites of activated receptor where there are other proteins with which it interacts, and which also may be needed for Bni1p 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 1 and Table II. To construct the gpa1Δ::URA3, pDSB138 was digested with EcoRI and was cotransformed.
DeltaVision microscope workstation (Applied Precision) based on a micro-

For cell surface growth experiments, images were acquired using a

tured to computer disk with an image capture board (Scion Corporation).

section), processed using an Omnex Image processing unit (Imagen), and cap-

were acquired with a video camera (C2400-08 SIT; Hamamatsu Corpora-

Plasmids were from the laboratories of M.D. Rose, D. Stone, or as indicated.

UH7

ura3

gpa1K21E R22E TRP1 CEN4 ARS1 AMPR

DSB138

gpa1

TRP1 CEN4 ARS1 AMPR

ura3::LEU2 KAN^R AMP^R

plasts (25 µg/ml Zymolyase 100,000T [ICN Biomedicals] and 25 mM

l PBS. 50

l of cells

Matings

Microscopic analysis of zygotes was performed as described previously

(Rose et al., 1990). Briefly, MATa and MATa cells in early log phase of

growth were mixed on 0.45-µm nitrocellulose filter discs (Millipore), trans-

ferred to a YEPD plate, and incubated for 6 h. Cells were rinsed off the fil-

shop® was used to optimize image contrast.

Immunochemical techniques

Actin was visualized using rhodamine-phalloidin (Molecular Probes, Inc.)
as described previously (Rose et al., 1990). Briefly, 5 ml of cells were in-
duced with pheromone and fixed with 4% formaldehyde for 30 min at

30°C. Cells were washed and resuspended in 100 µl PBS. 50 µl of cells were added to 25 µl rhodamine-phalloidin and incubated at RT for 5 min.

Cells were washed three times in PBS.

For visualizing GFP-tagged proteins by immunofluorescence, cells were

fixed with 4% formaldehyde (for 1 h at 30°C) and converted to spher-

oplasts (25 µg/ml Zymolyase 100,000T [ICN Biomedicals] and 25 mM

β-mercaptoethanol for 45 min at 30°C) as described previously (Rose et al., 1990). Polyclonal anti-GFP antibody (CLONTECH Laboratories, Inc.)

was used at 1:25 dilution in 10 µg/ml PBS-BSA. Fluorescein-conjugated
goat anti–rat antiserum (Boehringer) was used at 1:25 dilution.

For the FITC- and TRITC-Con A (Sigma-Aldrich) labeling experiments
(Nern and Arkowitz, 2000a), cells were grown in the SC complete media

Table I. Yeast strains

| Strain  | Genotype/description                                                                 |
|---------|----------------------------------------------------------------------------------------|
| EY699   | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+                           |
| EY700   | MATa fus3-6Δ::LEU2 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+            |
| MY5801  | MATa fus3-6Δ::LEU2 cln3::ura3FOA' ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ |
| MY5802  | MATa cln3::ura3FOA' ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+            |
| MY8188  | MATa bni1Δ::URA3 ura3 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+                |
| MY8193  | MATa gpa1Δ::URA3 ura3 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (MDB107)       |
| JY429   | MATa fus3-6Δ::LEU2 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (pMR3465) |
| MY8189  | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (pMR5049) (pMR3465)      |
| MY7494  | MATa fus-6Δ::URA3 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+              |
| MY8194  | MATa gpa1Δ::URA3 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (MDB107) (pMR3465) |
| MY8195  | MATa ura-3 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (p1955)                     |
| MY8196  | MATa fus-6Δ::LEU2 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+              |
| MY8351  | MATa gpa1Δ::ura3::HIS3 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+         |
| MY8352  | MATa gpa1Δ::HIS3 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (MDB107) (p1955) |
| MY8341  | MATa fus-6Δ::LEU2 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+              |
| MY8342  | MATa fus-6Δ::LEU2 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (pMR4937)     |
| MY8343  | MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (p426S2G)                  |
| MY8344  | MATa fus-6Δ::LEU2 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL+ (p426S2G)    |

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

| Strain | Genotype/description                        | Source                  |
|--------|---------------------------------------------|-------------------------|
| pMR4937 | FLAG-FUS3 URA3 CEN3 ARS1 AMP^E            | Dr. C. Boone, University of Toronto, Toronto, Canada |
| pMR4938 | FLAG-FUS3^Q93G2 URA3 CEN3 ARS1 AMP^E      | Dr. C. Boone            |
| pMR5049 | FLAG-FUS3^Q93G2 HIS3 CEN3 ARS1 AMP^E       | Dr. R. Arkowitz, CNRS, Nice, France |
| pMR3465 | pCAG^I::GFP-KAR9 LEU2 CEN4 ARS1 AMP^E       |                        |
| p321   | BNI1 disruption construct                   |                        |
| p1955  | 2µ pCAG^I::HA-BNI1-GFP URA3 CEN4 ARS1 AMP^E | Dr. F. Cross, The Rockefeller University, New York, NY |
| p426S2G | 2µ SPA2-GFP URA3 AR51 AMP^E                | Dr. R. Arkowitz, CNRS, Nice, France |
| DSB138 | GPA1 disruption construct                   |                        |
| MDB107 | gpa1::FUS3^Q93G2 TRP1 CEN4 ARS1 AMP^E       |                        |
| UH7    | ura3::LEU2 KAN^R AMP^E                     |                        |

Plasmids were from the laboratories of M.D. Rose, D. Stone, or as indicated.
In vitro kinase assay

For FLAG-Fus3p, 200-ml cultures of early logarithmic cells were induced for 45 min with a-factor. For HA-Bni1p, 150 ml of early logarithmic cells was used. In both cases, extracts were prepared in 1 ml lysis buffer and tagged proteins were bound to the affinity gel. Bound proteins were resuspended in kinase buffer. Each reaction contained 5 μl Fus3p and Bni1p were resuspended in 20 μl kinase buffer. Each reaction contained 5 μl Fus3p, 10 μM ATP, and 5 μM γ[32P]ATP. γ[32P]ATP was prepared from phenethyl-ADP (provided by K. Shah, Novartis, San Diego, CA) using nucleoside diphosphate kinase by the following method. A 200-μl slurry of Protein G Plus Agarose (Santa Cruz Biotechnology, Inc.) was added and cells were vortexed for five minutes for 1 min followed by 1 min on ice. Unbroken cells were removed by 3,000-rpm centrifugation for 10 min. Supernatants were cleared by centrifugation (15 min at 11,000 rpm). For anti-FLAG IPs, 30 μl of anti-FLAG M2 Affinity Gel (Sigma-Aldrich) was used. For anti-HA IPs, a 1:150 dilution of 12CA5 antibody was used with a 30-μl slurry of Protein G Plus Agarose (Santa Cruz Biotechnology, Inc.).

Metabolic cell labeling

For in vivo 32P labeling, 100-ml cultures were grown to 1.2 × 10^8 cells/ml in minimal media, transferred to growth in low phosphate media with galactose for 2 h, and then collected and resuspended in no-phosphate media containing galactose and α-factor. After 60 min, 1 mM carrier-free 32Porthophosphate (ICN Biomedical) was added and cells were grown for an additional 30 min. Cells were harvested, and extracts and immuno-precipitation were performed as described above.

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