There was an error published in *J. Cell Sci.* **118**, 2837-2848 (2005).

Throughout the article and in the supplementary figures, the plasmid GFP-Ste5 was incorrectly referred to as Ste5-GFP.

The authors apologise for this error.
Introduction
Eukaryotic cells frequently respond to external stimuli with signal transduction pathways that are activated by plasma membrane receptor-linked G proteins and GTPases (Leof, 2000). Often, scaffold proteins mediate the linkage of a G protein or GTPase to effectors and their targets. This general regulatory device is used by cells to respond to a wide variety of extracellular stimuli and is particularly crucial for cell polarity and migration (Palmieri and Haarer, 1998; Garrington and Johnson, 1999; Henrique and Schweisguth, 2003).

Scaffold proteins play important roles in the activation of mitogen-activated protein kinase (MAPK) cascades by G proteins and GTPases and have been speculated to specify and intensify the activation of kinases and assemble them at sites of stimulation (Elion, 1995; Burack and Shaw, 2000; Garrington and Johnson, 1999; Morrison and Davis, 2003). A number of MAPK scaffolds have been identified, including Ste5, Pbs2, the JIPs (JIP-1, JIP-2, JIP3, JSAP1), KSR and β-arrestin (Elion, 1995; Morrison and Davis, 2003). MAPK scaffolds can be asymmetrically enriched at plasma membrane sites, suggesting their localization may be sensitive to spatial cues of cell polarity and they, in turn, may influence cell polarity (Mahanty et al., 1999; Kelkar et al., 2000; Muller et al., 2001; Ge et al., 2003).

Many stimuli that activate MAPK cascades also stimulate changes in cell morphology and motility through GTPase-induced changes in the actin and microtubule cytoskeletons. For example, Rho-type GTPases, including Cdc42, play a central role in mediating cell polarity by establishing asymmetry by binding a wide variety of effector proteins that nucleate actin filaments and localize microtubules at leading edges in eukaryotic cells (Erickson and Cerione, 2001). Key downstream effectors of Rho-type GTPases include WASP family proteins and formin homology (FH) proteins that nucleate higher-order actin structures (Takenawa and Miki, 2001; Zigmond, 2004). Many intersections between regulators of the actin cytoskeleton and signaling pathways also exist. For example, Cdc42 anchors p21-activated kinases (PAKs) that directly phosphorylate mitogen-activated protein kinase kinase (MAPKKKs) (Bokoch, 2003), and binds to the Par6-Par3 complex and activates atypical protein kinase C (Etienne-Manneville and Hall, 2003). In mammalian T cells, the actin cytoskeleton may function as a scaffold for signaling components such as PKC-θ (Dustin and Cooper, 2000). The complexity of these interactions makes it difficult to establish the order of events.

The response to mating pheromone in budding yeast provides a system to study the relationship between signal transduction and the actin cytoskeleton. Cells respond to mating pheromone through a MAPK cascade that is activated by a serpentine receptor coupled to a heterotrimeric G protein, which releases an inhibitory Go subunit from a stimulatory βγ-subunit.
The localization of the Ste5 scaffold at the cell cortex is also polarized and requires prior nuclear shuttling, which is also required for activation of Fus3 (Mahanty et al., 1999). In the absence of pheromone, a small pool of Ste5 accumulates at cortical sites in G2/M and G1 phase cells through a process that involves Cdc42, Cdc24 and Bem1, and is independent of Ste4 and the actin cytoskeleton (Wang et al., 2005). Cdc24 shuttles through the nucleus (Gulli and Peter, 2001), binds to Ste5 and promotes nuclear accumulation and recruitment of Ste5 (Wang et al., 2005). These, and other findings, suggest that Ste5 is basally recruited by internal cues set up by Bem1, Cdc42 and Cdc24, which may shuttle through the nucleus and be recruited with Ste5 as a complex. During pheromone stimulation, polarized recruitment of Ste5 becomes dependent upon the actin cytoskeleton in addition to Ste4 (Wang et al., 2005). Ste5 is detected at the cortex of G1 phase cells within minutes after α factor addition and is found at the tips of emerging shmoo (Pyczak and Huntress, 1998; Mahanty et al., 1999). Fus3 also localizes at the shmoo tip, but fluorescence recovery after photobleaching (FRAP) analysis suggests its residency has a much shorter half-life than that of Ste5 (van Drogen et al., 2001). Here, we show that during pheromone stimulation, the formin Bni1 is essential for Ste5 scaffold recruitment at cortical sites and controls Ste5 recruitment through actin cables. Bni1 is also required for cortical recruitment of Cdc24, efficient activation of Fus3 and stable recruitment of Fus3 at the cell cortex. Myo2 is required for cortical localization of Ste5 and is found in Ste5 immune complexes during pheromone-stimulation, raising the possibility that a pool of Ste5 may translocate along actin cables via Myo2.

**Materials and Methods**

**Yeast strains and plasmid construction**

Details can be found in supplementary material.

**Quantitative mating and pheromone response assays**

Mating assays were performed as described previously (Sprague, 1991). The percentages of unbudded and shmooed cells were quantitated after a 2-hour exposure to 50 mM α factor. **FUS1** transcription was assayed with a **FUS1-lacZ** reporter gene maintained in a 2μ plasmid. β-galactosidase activity was quantitated as described previously (Lyons et al., 1996). Halo assays were performed as described previously (Lyons et al., 1996) using 5 μl of 2 mM or 0.1 mM α factor in dimethylsulfoxide, depending on whether strains were **sst1** or **sst1-1**, Ste5Δ is a null allele of **STE5**.

**Yeast whole cell extracts and MAPK activation assay**

Yeast strains were grown at 30°C in selective SC medium containing 2% dextrose to an A₆₀₀ of ~0.6. Cells were treated with α factor or induced with 2% galactose. Whole cell extracts (WCE) were prepared as described previously (Elion et al., 1993) except that a modified H buffer contains 250 mM NaCl. Protein concentrations were determined with the Bio-Rad protein assay. Fus3 kinase assays were done as described previously (Elion et al., 1993). To detect MAPK phosphorylation, 200 μg total protein was separated by 10% SDS-PAGE, then transferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, then in the same buffer containing rabbit anti-phospho-p44/p42 antibody (1:1000; Cell Signaling, Beverly, MA, USA), followed by washing and incubation in the same buffer containing hors eradish pero xidase-coupled goat anti-rabbit antibody (1:10000, Bio-Rad). After the signal was visualized with ECL (Amersham), the membrane was stripped and reprobed with anti-Tcm1 monoclonal antibody (from J. Warner, Albert Einstein College of Medicine, Bronx, NY).

**Immunoprecipitation**

One mg of WCE was mixed with 3 µg 12CA5 antibody (ascites provided by Harvard University antibody facility) in modified H buffer with 150 mM NaCl and incubated on ice for 45 minutes. 50 µl protein A-Sepharose beads (Pharmacia) were added and samples were rotated at 4°C for 2 hours. The beads were washed three times with modified H buffer and then boiled in protein loading buffer. Samples were separated with 8% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was then probed with 9E10 (ascites from Harvard University antibody facility) or 12CA5 monoclonal antibody.

**Visualization of Ste5-Myc9 and GFP-tagged protein localization**

Ste5, Ste20, Cdc24, Fus3 and Bni1 tagged with GFP were observed with direct fluorescence. Cells were treated with α factor then fixed in 3.7% formaldehyde for 45 minutes. After washing with PBS and brief sonication, cells were mounted in Vectashield medium (Vector Laboratories). Ste5 tagged with Myc9 was visualized by indirect immunofluorescence as described previously (Mahanty et al., 1999) with some modifications. Cells were fixed with 3.7% formaldehyde for 45 minutes at room temperature, spermioplasted for 30 minutes in
PBS containing 100 μg/ml lyticase, 3 μg/ml phenylmethylsulphonyl fluoride and 2 μl/ml β-mercaptoethanol. Cells were then fixed in –20°C methanol for 5 minutes followed by 20 seconds in –20°C acetone. After being air-dried, cells were permeabilized with 0.5% Triton X-100 for 5 minutes, washed with PBS three times, and then blocked with 2% BSA at room temperature for 1 hour. To detect Myc-tagged Ste5, samples were incubated in a 1:1000 dilution of 9E10 ascites fluid at room temperature for 1 hour or at 4°C overnight. After washing with PBS, samples were exposed to a CY3-conjugated secondary antibody (1:1000, Zymed Laboratories, South San Francisco, CA, USA) and incubated in the dark for 1 hour. After four washes with PBS, the slides were mounted with Vectashield medium. All samples were observed on an Axioskop 2 microscope (Carl Zeiss, Thornwood, NY, USA) linked to a digital camera (C4742-95; Hamamatsu, Bridgewater, NJ, USA).

Temperature shift experiments, latrunculin A treatment and actin staining

Cells were grown at room temperature then shifted to the nonpermissive temperature as follows: bni1Δ/Δtpm2Δ cells were shifted to media that were pre-warmed to 34°C and 34.5°C, respectively, and 5 μM α factor was added to the culture at the same time. Cells were incubated at the nonpermissive temperature for 10 minutes, then fixed for immunostaining, rho1-2 and rho1-104 cells were first shifted to 37°C for 1 hour before being treated with 5 μM α factor for 30 minutes and myo2-66 cells were pre-cultured at 37°C for 2 hours, then both were stimulated with 5 μM α factor for 30 minutes before being fixed. Strains harboring GAL1-MYO2DN were grown in 2% galactose for 1 hour and then incubated with 5 μM α factor for 15 minutes.

Latrunculin A (Molecular Probes) was dissolved in dimethylsulfoxide (DMSO) at 5 mM and the stock solution was stored at –20°C. To treat the cells, latrunculin A was added to the medium at a final concentration of 100 mM and DMSO was used as control.

To visualize actin, cells were fixed with 4% formaldehyde for 45 minutes and then stained with Rhodamine-phalloidin (Molecular Probes; final concentration 5 U/ml) in the dark for 1 hour. Cells were washed twice in PBS and resuspended in Vectashield mounting medium. Actin was examined using an Axioskop 2 microscope.

Results

Bni1 is required for efficient pheromone-induced MAPK activation

We identified BNI1 in a targeted screen for cytoskeletal and cell polarity genes required for efficient G1 arrest induced by α mating pheromone (C.M. and E.E., unpublished). Although Bni1 was known to be required for mating pheromone-induced actin reorganization and cell polarization, it has not been thought to play a direct role in signal transduction (Evangelista et al., 1997; Buehrer and Errede, 1997). Since G1 arrest requires the activation of MAPK Fus3, we investigated a possible relationship between actin cytoskeleton reorganization by Bni1 and MAPK signal transduction. We used a previously published bni1 null mutant in the W303-1a background (Evangelista et al., 1997) in which mating pathway functions are strictly dependent upon the MAPKs Fus3 and Kss1 (Ellion et al., 1991). The bni1Δ mutant was severely defective in α factor-induced shmoo formation (Fig. 1B) and mated at reduced frequency (Fig. 1D) as previously shown. In addition, the strain also underwent less efficient G1 arrest in a halo assay (Fig. 1C), accumulated fewer unbudded cells after treatment with α factor (Fig. 1D) and expressed a FUS1-lacZ reporter gene for the pathway less efficiently than the WT strain (Fig. 1E). We note that the previous analysis of a
Fig. 2. bni1Δ cells are defective in α factor-induced Fus3 activation. (A) Dose dependence. WT (EY957) and bni1Δ (EYL917) cells harboring FUS3-HA (PYEE1102) were treated with different concentrations of α factor for 15 minutes and frozen at –80°C. (B) Time course. Cells were treated with 25 nM α factor for 15 minutes. WT (EY957) and bni1Δ (EYL917) cells harboring FUS3-HA (PYEE1102) were treated with different concentrations of α factor for 15 minutes and frozen at –80°C. (B) Time course. Cells were treated with 25 nM α factor for 15 minutes.

**Bni1 acts at, or upstream of, Ste5 in mating pathway signaling**

Bni1 localizes at sites of polarized growth in dividing cells and in pheromone-stimulated cells (Evangelista et al., 1997; Ozaki-Kuroda et al., 2001), suggesting that it was likely to affect an aspect of Fus3 activation that takes place at the plasma membrane, near the receptor, G protein or Ste20 steps of the mating MAPK cascade (Fig. 1A). To determine whether Bni1 acts upstream or downstream of Ste5, we tested whether the bni1Δ mutation interferes with activation of Fus3 by a constitutively active form of ERK1 and ERK2 in mammalian cells and cross-reacts with active Fus3 and Kss1 (Andersson et al., 2004). In the cells expressing wild-type BNI1, a stronger signal of active Fus3 than Kss1 was detected after α factor stimulation (Fig. 2C, lanes 3, 4). Strikingly, the level of active Fus3 was lower at all time points in the bni1Δ strain, compared to a much larger reduction in the level of active Fus3 (Fig. 2C, lanes 1, 2). Thus, Bni1 is specifically required for high-level activation of Fus3.

Requirement for Bni1 in the pheromone response pathway (Evangelista et al., 1997) was done in a strain that had an sst2Δ mutation in the RGS protein that downregulates Gα (Gpa1) and has unnaturally high levels of free Gβγ (Siekhaus and Drubin, 2003). Our findings show that Bni1 compromises multiple outputs of the mating MAPK cascade, suggesting it interferes with MAPK activation.

Dose–response curves and fus3 mutant analysis suggest that increased Fus3 activation is required for transcriptional activation, G1 arrest and shmoo formation (Farley et al., 1999). Therefore, the partial defects in FUS1-lacZ expression and G1 arrest in a bni1Δ strain could be explained by a reduction in the level of Fus3 activation. Fus3 kinase activity was monitored in wild-type (WT) and bni1Δ strains that lack the SST1/BAR1 protease, which degrades α factor, allowing the use of much lower amounts of α factor and bypassing the downregulatory effects of pheromone-induced expression of the Sst1/Bar1 protease. The bni1Δ mutation still caused a reduction in G1 arrest and FUS1-lacZ expression in the sst1Δ background, however a slightly smaller effect on FUS1-lacZ expression was detected (data not shown). A comparison of Fus3 activity in these cells confirmed that bni1Δ cells do not efficiently activate the mating MAPK cascade. Fus3 activity was lower in bni1Δ mutant cells than in WT cells at all α factor concentrations in a dose–response experiment, including saturating concentrations of α factor (25 nM and 125 nM; Fig. 2A). Furthermore, a time-course experiment showed that the reduced Fus3 activity was not the result of a delay in signal transduction. Fus3 was activated with similar kinetics in WT and bni1Δ cells, however, the overall level of activation was lower at all time points in the bni1Δ cells (Fig. 2B). Quantitation of multiple experiments by densitometry revealed an approximate fourfold reduction in Fus3 activity at various time points and α factor concentrations (Fig. S1 in supplementary material). Therefore, a bni1Δ mutant is intrinsically defective in α factor-induced MAPK activation.

We determined whether Bni1 was also required to activate Kss1, which does not need to be bound to Ste5 to be activated by mating pheromone (Andersson et al., 2004). The activation of endogenous Fus3 and Kss1 in whole cell extracts was monitored with an antiphospho p42p44 antibody that recognizes the active form of ERK1 and ERK2 in mammalian cells and cross-reacts with active Fus3 and Kss1 (Andersson et al., 2004). In the cells expressing wild-type BNI1, a stronger signal of active Fus3 than Kss1 was detected after α factor stimulation (Fig. 2C, lanes 3, 4). Strikingly, the level of active Kss1 was only slightly reduced in the bni1Δ strain, compared to a much larger reduction in the level of active Fus3 (Fig. 2C, lanes 1, 2). Thus, Bni1 is specifically required for high-level activation of Fus3.
that the levels of Fus3 activation in the \textit{bni1}\Delta mutant approached that of the WT strain, suggesting that Bni1 functions near the Ste11 step. Ste5 was a probable target point because full activity of Ste11-4 is dependent upon recruitment of Ste5 (Andersson et al., 2004).

We therefore asked whether Bni1 regulates Fus3 at or upstream of the Ste5 recruitment step that is essential for Ste11 activation, by testing whether artificial recruitment of Ste5 to the plasma membrane bypasses the \textit{bni1}\Delta defect in Fus3 activation. This was done with Ste5-CTM, which localizes to the plasma membrane through a carboxyl-terminal transmembrane sequence and constitutively activates the mating MAPKs through a process that requires activation of Ste11 by Ste20, but bypasses the need for binding of Ste5 to the G protein (Pryciak and Huntress, 1998). This phenotype is dominant and detected in the presence of wild-type Ste5. WT and \textit{bni1}\Delta cells were shifted to galactose medium to induce the expression of Ste5-CTM and Fus3 kinase activity was monitored. Ste5-CTM-activated Fus3 nearly identically in \textit{bni1}\Delta and WT cells (Fig. 3B). Therefore, Bni1 regulates Fus3 activation at or upstream of Ste5 recruitment.

\textbf{Bni1 is required for cortical recruitment of Ste5 during pheromone stimulation}

We determined whether \textit{bni1}\Delta mutants were defective in localizing Ste5 to cortical sites on the plasma membrane during \textit{a} factor stimulation in a time-course experiment. Cortical recruitment of Ste5 was analyzed in live cells with a functional Ste5-GFP fusion expressed at the same level as wild-type Ste5 (Mahanty et al., 1999). The recruitment of Ste5-GFP was severely defective in \textit{bni1}\Delta cells at all time points after \textit{a} factor addition, including the 15 minute time point that is well before shmoo formation (Fig. 3A,C). In WT cells after 15 minutes of \textit{a} factor treatment, Ste5-GFP could be detected at the plasma membrane (rim staining) in ~12% of the population (Fig. 3C). These cells had not undergone shmoo formation and were still round. As the length of \textit{a} factor exposure increased, the number of cells that exhibited Ste5 rim staining also increased, with more intense staining at emergent shmoo tips. After 2 hours of exposure to \textit{a} factor, ~90% of the cells formed shmoos, and strong Ste5 rim staining was detected in 67% of cells. In contrast, Ste5 recruitment was greatly reduced in \textit{bni1}\Delta cells at all time points. Similar results were found by indirect immunofluorescence of a functional Ste5-Myc9 fusion in fixed cells (Fig. 3B,D). Ste5 was recruited to wild-type levels in a \textit{bnr1}\Delta strain lacking Bn1, the homolog of Bni1 (data not shown) and in \textit{she} mutant strains (including \textit{she5}, which is allelic to \textit{bni1}) defective in polarized localization of specific mRNAs (Fig. S2 in supplementary material). Thus, Bni1 plays a critical role in Ste5 recruitment that is not performed by Bn1 and is distinct from proteins involved in polarized mRNA localization.

\textbf{Bni1 is still required for recruitment of Ste5 after the Fus3 signaling defect is suppressed}

Ste5 relocalizes from the nucleus to the plasma membrane in response to \textit{a} factor stimulation of the associated MAPK cascade (Mahanty et al., 1999). Therefore, it was possible that the defect in Ste5 localization in the \textit{bni1}\Delta mutant was a secondary consequence of a defect in MAPK activation. To test this possibility, we restored wild-type levels of Fus3 activation in a \textit{bni1}\Delta mutant by expressing Ste5-CTM from the \textit{GAL1} promoter that is induced in the presence of galactose, and examined whether Ste5-Myc9 could now be recruited to the plasma membrane. In WT cells, the expression of Ste5-CTM was sufficient to induce translocation of Ste5-Myc9 from the nucleus to the emerging shmoo tip (Fig. 4F,G, percentage nuclear accumulation and percentage rim staining), demonstrating that activation of the MAPK cascade is sufficient to induce Ste5 relocalization. (Note that it is easier to see the nuclear pool of Ste5 when cells are grown in galactose containing medium than in glucose containing medium; P. Maslo, R. McCully, E.A.E., unpublished data.) Ste5-CTM did not induce recruitment of Ste5-Myc9 in \textit{bni1}\Delta cells; Ste5-Myc9 remained predominantly nuclear despite nearly wild-type levels of Fus3 activation (Fig. 4E-G). Thus, Bni1 regulates recruitment of Ste5 by a mechanism that is independent of MAPK activation. Furthermore, the retention of Ste5 in nuclei of the \textit{bni1}\Delta cells indicates that Bni1 also promotes redistribution of Ste5 from the nucleus to the cytoplasm and cell cortex.

\textbf{Bni1 is required for cortical recruitment of Fus3 and Cdc24, but not Ste20}

In parallel experiments, we looked at the localization of Fus3, whose recruitment is dependent on Ste5. In WT cells, Fus3-GFP was nuclear and cytoplasmic and accumulated at shmoo
tips only after long-term treatment with α factor. In contrast, no cortical recruitment of Fus3-GFP was detected at all in the bni1Δ cells (Fig. 5A middle panels, B). Consistent with these findings, we found that the cortical pool of Ste5 always co-localizes with Bni1, with ectopic expression of Bni1 modestly enhancing basal and pheromone-induced cortical recruitment of Ste5 and both basal and pheromone-induced activation of Fus3 (data not shown). Thus, Bni1 is critical for recruitment of Fus3 in addition to Ste5.

We assessed whether Ste20 was still recruited in bni1Δ cells to determine whether loss of Bni1 globally interferes with cortical localization during the pheromone response. Ste20 accumulates at the cell cortex by binding to Cdc42 through a CRIB motif (Cdc42-Rac interactive binding motif) (Lamson et al., 2002). The bni1Δ mutation did not block cortical recruitment of Ste20-GFP, which localized to the cortex in a polarized manner in bni1Δ cells that had been treated with α factor to the same degree as WT cells, even though the cells were blocked in shmoo formation and round (Fig. 5A lower panels, 5B). Therefore, Cdc42-mediated asymmetry still exists in bni1Δ cells that have lost polarization of the actin cytoskeleton and cannot undergo polarized growth.

The guanine exchange factor Cdc24 also binds Cdc42 in addition to Ste5 and is required for polarized recruitment of Ste5. In contrast to Ste20, cortical recruitment of Cdc24 was greatly inhibited in the bni1Δ mutant during pheromone stimulation, although it was still recruited during mitotic growth (Fig. 5C,D). Similar results were found for Bem1 (data not shown), which has previously been shown to be dependent on the actin cytoskeleton for cortical recruitment during pheromone stimulation (Ayscough and Drubin, 1998) and forms complexes with both Ste5 and Cdc24 (reviewed by Wang et al., 2005). Thus, Bni1 is required for cortical recruitment of Cdc24 during pheromone stimulation, consistent with the possibility that Cdc24 and Ste5 are recruited as a complex (Wang et al., 2005).

The Rho1-binding domain and FH domains in Bni1 regulate MAPK activation

Bni1 is a large protein with many domains that bind directly or indirectly to a variety of proteins including Rho GTPases (i.e. Rho1, Rho3 and Rho4), Cdc42, Spa2, profilin, actin, EF1α and Bud6 (Fig. 6A) (Evangelista et al., 1997; Evangelista et al., 2002; Sagot et al., 2002). During mitotic cell division, Cdc42 regulates cortical recruitment of Bni1 potentially with
multiple Rho proteins (Jacquenoud and Peter, 2001; Dong et al., 2003). The control by Cdc42 is indirect compared to that of Rho1, which is sufficient to stimulate plasma membrane recruitment of Bni1 (Jacquenoud and Peter, 2000). Bni1 promotes actin cable formation through the FH1 and FH2 domains and is thought to exist in an autoinhibited form via interactions between the Rho-binding domain (RBD) and a carboxy-terminal domain (DAD), by analogy to Diaphanous-related formin (Zigmond, 2004). The binding of Rho GTPase is thought to relieve the autoinhibitory interaction and permit the FH2 domain to nucleate actin polymerization (Evangelista et al., 2002; Sagot et al., 2002).

We deleted these key functional domains of Bni1 and assessed their role in Fus3 activation (Fig. 2C) and mating outputs (data not shown). As summarized in Table 1, deletion of the DAD that binds the RBD and Bud6 did not block mating responses, whereas removing the RBD did. Loss of the RBD also reduced Fus3 activation to the same degree as a bni1 null strain (Fig. 2C) and blocked shmoo formation (percentage shmoos after 2 hours in 50 nM α factor was 80% for WT, 0.01% for bni1Δ, 0.03% for bni1ΔRBD; n=300 cells; see also Fig. 6B). Furthermore, deletions that removed the FH1 and FH2 domains also inhibited Fus3 activation (Fig. 2C) and mating outputs. These findings suggest that Bni1 regulation of Ste5 recruitment requires a Rho GTPase and the formation of actin cables.

To test this hypothesis, we determined whether the RBD was required for cortical localization of Bni1. The RBD (amino acid residues 93-286) has been defined as a region that binds Rho1-GTP directly in vitro and in two-hybrid assays (Kohno et al., 1996). This region overlaps the region of Bni1 with homology to the Diaphanous GTPase binding domain (amino acids 93-286) (Marchler-Bauer et al., 2003). Bni1 lacking the RBD (bni1ΔRBD-GFP) did not localize to bud tip and bud neck during vegetative growth and was not recruited to the cell cortex during response to mating pheromone, even in cells that expressed wild-type Bni1 (Fig. 6B). Consistent with the absence of cortical recruitment, bni1ΔRBD-GFP failed to support shmoo formation in bni1Δ cells (Fig. 6B), or mediate cortical recruitment of Ste5 (data not shown).

The requirement for the RBD for Bni1 localization and pheromone responses suggested that Bni1 is activated during mating by a Rho GTPase. Five Rho GTPases (Rho1-Rho5) are implicated in the regulation of Bni1, with varying roles during the cell cycle and under different environmental conditions (Dong et al., 2003). Although early work with a temperature sensitive rho1-2 mutant suggested that Rho1 is not involved in localization of Bni1 (Ozaki-Kuroda et al., 2001), other work has shown that Rho1 regulates the actin cytoskeleton during G1 phase and shmoo formation (Kohno et al., 1996; Drgonova et al., 1999), and binds to multiple effector proteins including Bni1, glucan synthase and protein kinase C (Evangelista et al., 1997; Saka et al., 2001). We compared Bni1 and Ste5 localization in rho1-2 (E45V in Switch I) (Saka et al., 2001) and rho1-104 (D72N; C164Y) (Yamochi et al., 1994) temperature sensitive mutants that have different actin cytoskeleton phenotypes and abilities to polarize myosin. Bni1-GFP was not recruited in a polarized manner to the cortex of dividing and pheromone-induced rho1-2 and rho1-104 cells at nonpermissive temperature (Fig. 6C). To determine whether Rho1 was also required for Ste5 localization we used TagNLSK128T-Ste5-Myc9, which is more efficiently reimported to the nucleus and recruited to the plasma membrane than Ste5-Myc9, allowing its detection in a greater...
number of cells after brief treatment with α factor (Mahanty et al., 1999). Strikingly, the rho1-2 and rho1-104 mutations both decreased Ste5 recruitment at nonpermissive temperature (Fig. 6D). By contrast, Bni1 and Ste5 were still recruited to the site of polarized growth in rho2, rho3, rho4 and rho5 null mutants that had been treated with α factor, and these cells were able to form shmoos (Fig. S3 in supplementary material). Therefore, Rh1 specifically recruits Bni1 to cortical sites during mating and positively regulates recruitment of Ste5 through control of Bni1.

Bni1-induced actin cables are required for Ste5 recruitment

To more stringently test for a primary role of Bni1-induced cables in Ste5 recruitment, we used a bni1TS temperature-sensitive mutation within conserved residues of the FH2 domain (R1528A and R1530A) that blocks actin cable formation (Sagot et al., 2002). Previous work has demonstrated that inactivation of bni1TS after a brief 10-minute shift to 34°C selectively disrupts actin cables while polarized actin patches are maintained, although the effect is not complete. Because of partial functional overlap between Bni1 and Bnr1, the bni1TS mutation was assessed in a bnr1Δ mutant as described (Evangelista et al., 2002; Sagot et al., 2002). At the nonrestrictive temperature, TAgNL5K128T-Ste5-Myc9 was recruited to the plasma membrane in ~30% of WT and bni1TS bnr1Δ mutant cells after α factor stimulation (Fig. 7A). Shifting the temperature to 34°C greatly inhibited the recruitment of TAgNL5K128T-Ste5-Myc9 in the bni1TSbnr1Δ mutant, but did not reduce it in the WT control strain (Fig. 7A), consistent with a primary role for actin cables in Ste5 recruitment.

To further confirm that actin cables regulate Ste5 recruitment, we checked the effect of disrupting the TPM1 and TPM2 genes encoding tropomyosins, which bind to and stabilize actin cables and are not a component of actin patches (Pruyne et al., 1998). To assess the immediate importance of

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WT, wild type. bni1Δ cells were transformed with empty vector, or vector expressing different forms of the BNI1 gene. Sensitivity to α factor was monitored by Fus3 kinase assay and halo assay.
tropomyosin in Ste5 recruitment, we used the same rapid temperature shift conditions used for the bni1<sup>TS</sup> mutant. Although TAgNLStr<sup>128T</sup>-Ste5-Myc9 was recruited to the cortex in <i>tpm1<sup>TS</sup> tpm2Δ</i> cells at nonrestrictive temperature, shifting to nonpermissive temperature blocked its recruitment within a few minutes (Fig. 7B). Thus, actin cables generated by Bni1 and stabilized by tropomyosin are required for recruitment of Ste5 to polarized sites at the plasma membrane.

Myo2 is required for recruitment of Ste5

All of the known transport events along actin cables in <i>S. cerevisiae</i> require either of the type V myosin motor proteins, Myo2 and Myo4. Myo2 has been implicated in the transport of proteins (i.e. Kar9, Smy1) and organelles (i.e. secretory vesicles and vacuoles) along cytoplasmic actin cables, whereas Myo4 is involved in transport of mRNAs to sites of polarized growth (Bretscher, 2003; Vale, 2003). We used two approaches to test whether Myo2 is involved in Ste5 recruitment, a myo2-66 temperature-sensitive point mutation that maps to the actin-binding region of the motor domain of Myo2 (Lillie and Brown, 1994) and a dominant negative tail fragment of Myo2, TAgNLStr<sup>128T</sup>-Myo9 that displaces Myo2 from actin cables (Reck-Peterson et al., 1999). Shifting the myo2-66 strain to 37°C for 2 hours blocked recruitment of Ste5 (Fig. 7C). Since the myo2-66 mutation also induces actin depolarization in addition to inactivating Myo2, we examined the effect of TAgNLStr<sup>128T</sup> under conditions of short-term overexpression from the GAL1 promoter, which does not disrupt the actin cytoskeleton (Reck-Peterson et al., 1999; Karpova et al., 2000). Shifting cells from noninducing raffinose medium to inducing galactose medium for 1 hour resulted in a fourfold inhibition of recruitment of TAgNLStr<sup>128T</sup>-Ste5-Myc9 in the presence of α factor (Fig. 7D). Thus, Ste5 recruitment was blocked to nearly as great an extent by TAgNLStr<sup>128T</sup> as it was by myo2-66. A comparative analysis of Ste5 recruitment in strains harboring mutations in the remaining myosin genes, <i>MYO1, MYO3, MYO4</i> and <i>MYO5</i> (of which <i>MYO3</i> and <i>MYO5</i> are functional homologs (Bretscher, 2003; Vale, 2003)) was also done. Deletion of <i>MYO4</i>, in either a WT or myo2-66 background did not interfere with Ste5 recruitment (Fig. S4A in supplementary material). Furthermore, null mutations in <i>MYO1, MYO3, MYO5</i> and double null mutations in <i>MYO3 MYO5</i> had no significant effect on Ste5 recruitment (Fig. S4B,C in supplementary material). Thus, Myo2 is specifically required for cortical recruitment of Ste5.

The actin cables and Myo2 could regulate Ste5 recruitment indirectly by creating a polarized site of factors that capture and stabilize Ste5. Alternatively, it is possible that Myo2 played a more direct role through translocation of Ste5 along actin cables. To test the possibility that Myo2 might regulate translocation of Ste5 we determined whether Ste5 and Myo2 form complexes in vivo in a co-immunoprecipitation assay. Ste5-Myc9 and Myo2-HA were co-expressed in a <i>ste5Δ</i> strain at physiological levels using low copy centromeric plasmids and Ste5 and Myo2 promoters to drive the expression of <i>STE5-MYC9</i> and <i>MYO2-HA</i> genes, respectively. Strikingly, Myo2-HA associated with Ste5-Myc9 in cells that had been
mutant cells expressing TAgNLSK128T-Ste5-Myc9 were treated with \( \alpha \) factor for 30 minutes at room temperature, then shifted to 34°C for 10 minutes and 20 minutes. The treatment with \( \alpha \) factor induced Ste5 recruitment in 50% of the cells (Fig. 8A). The subsequent shift to 34°C blocked a further increase in recruitment of Ste5 to the polarization sites, but did not decrease the pool of Ste5 that had accumulated prior to the temperature shift (Fig. 8A). Therefore, once Ste5 is recruited to the plasma membrane, it no longer needs the FH2 domain of Bni1. The addition of latrunculin A prior to \( \alpha \) factor induction completely blocks the formation of actin patches and cables and \( \alpha \) factor-induced actin polarization as well as recruitment of Ste5 to the plasma membrane (Wang et al., 2005) (Fig. 8B). In sharp contrast, addition of latrunculin A to \( \alpha \) factor-induced cells did not abolish the previously established Ste5-Myc9 recruitment (Fig. 8C), although it did disrupt the actin cytoskeleton, further demonstrating that the actin cytoskeleton is not required to maintain Ste5 at the plasma membrane. Therefore, Bni1-induced actin cables are not required to maintain Ste5 at the plasma membrane.

**Discussion**

While it is known that regulators of the actin cytoskeleton are required for signal transduction during MAPK signaling, specific functions of actin in signaling have not been delineated. Here we present the first evidence of a specific role for a formin and Rho protein in assembly of a scaffold-kinase signal transduction cascade at the plasma membrane in response to an external stimulus. This conclusion is based on the selective requirement for Bni1 in high-level activation of MAPK Fus3, but not MAPK Kss1 (Fig. 2), together with the critical role for Bni1 in cortical recruitment of Ste5 and Fus3 (Figs 4 and 5). Previous work suggests that Fus3 must be activated while bound to Ste5 (Andersson et al., 2004) but Kss1 cannot be activated from the scaffold by upstream kinases (Andersson et al., 2004; Maleri et al., 2004). In addition, plasma-membrane-bound Ste5-CTM does not recruit Kss1 to the plasma membrane although it does recruit Fus3 (van Drogen et al., 2001). Thus, Bni1 and cortical localization of Ste5 only play a significant role in the MAPK that co-localizes with Ste5. Although Bni1 and actin cables are required for polarized recruitment of Ste5 (Figs 4 and 7), they are not required for maintenance of Ste5 at the plasma membrane (Fig. 8). This finding is consistent with the expectation that once recruited, Ste5 is able to bind to free Gβ (Ste4) at the plasma membrane.

Our findings also reveal that Rho1 plays a specific role in mediating the recruitment of Bni1 and Ste5, acting through the Rho Binding Domain of Bni1 (Fig. 6). This conclusion is consistent with the ability of Rho1-GTP to bind to a Bni1 fragment that overlaps the Rho binding domain (Kohno et al., 1996) and the lack of evidence of functional overlap from the other Rho proteins (Rho2, Rho3, Rho4, Rho5, Fig. S3 in supplementary material), which may regulate Bni1 during the budding cycle (Dong et al., 2003). During the mitotic cycle, Rho1 also regulates the actin cytoskeleton indirectly through the protein kinase C pathway (Dong et al., 2003), raising the possibility of an indirect link between Rho1 and Bni1 during mating pheromone stimulation. However, the protein kinase C pathway is activated by \( \alpha \) factor much later than the mating

**Ste5 does not need the Bni1 FH2 domain or actin to stay at the plasma membrane**

We determined whether Bni1 or actin cables were required to maintain Ste5 at the cell cortex once it has been recruited to the cell cortex was assessed. \( bni1^{TS} \) and \( bnr1 \) are impaired in cell cycle progression, so we used a temperature-sensitive mutant of either (\( bni1^{TS} \)) or (\( bnr1 \)), with the wild-type parent and the \( bni1^{TS} \) mutant expressing TAgNLSK128T-Ste5-Myc9. As in the non-immortalized cell line, the addition of latrunculin A prior to \( \alpha \) factor induction completely blocked the formation of actin patches and cables and \( \alpha \) factor-induced actin polarization as well as recruitment of Ste5 to the plasma membrane (Wang et al., 2005) (Fig. 8B). In sharp contrast, addition of latrunculin A to \( \alpha \) factor-induced cells did not abolish the previously established Ste5-Myc9 recruitment (Fig. 8C), although it did disrupt the actin cytoskeleton, further demonstrating that the actin cytoskeleton is not required to maintain Ste5 at the plasma membrane. Therefore, Bni1-induced actin cables are not required to maintain Ste5 at the plasma membrane.

**Fig. 8.** Actin is not required to keep Ste5 at the plasma membrane. (A) Dynamics of Ste5 localization in \( bni1^{TS} \) mutants. WT (EY1765) and \( bni1^{TS}bnr1 \) cells (EY1748) expressing TAgNLSK128T-Ste5-Myc9 were treated with 100 \( \mu \)M \( \alpha \) factor but not in vegetatively growing cells (Fig. 7E), consistent with the dependence of polarized recruitment of Ste5 on \( \alpha \) factor stimulation. The ability to co-immunoprecipitate Ste5 and Myo2 was reproducible and dependent on expressing Myo2 at native levels, with no interaction detected when Myo2 was overexpressed (data not shown). Collectively, these findings open the possibility that Ste5 translocates along actin cables through the action of Myo2.

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**Discussion**

While it is known that regulators of the actin cytoskeleton are required for signal transduction during MAPK signaling, specific functions of actin in signaling have not been delineated. Here we present the first evidence of a specific role for a formin and Rho protein in assembly of a scaffold-kinase signal transduction cascade at the plasma membrane in response to an external stimulus. This conclusion is based on the selective requirement for Bni1 in high-level activation of MAPK Fus3, but not MAPK Kss1 (Fig. 2), together with the critical role for Bni1 in cortical recruitment of Ste5 and Fus3 (Figs 4 and 5). Previous work suggests that Fus3 must be activated while bound to Ste5 (Andersson et al., 2004) but Kss1 cannot be activated from the scaffold by upstream kinases (Andersson et al., 2004; Maleri et al., 2004). In addition, plasma-membrane-bound Ste5-CTM does not recruit Kss1 to the plasma membrane although it does recruit Fus3 (van Drogen et al., 2001). Thus, Bni1 and cortical localization of Ste5 only play a significant role in the MAPK that co-localizes with Ste5. Although Bni1 and actin cables are required for polarized recruitment of Ste5 (Figs 4 and 7), they are not required for maintenance of Ste5 at the plasma membrane (Fig. 8). This finding is consistent with the expectation that once recruited, Ste5 is able to bind to free Gβ (Ste4) at the plasma membrane.

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We determined whether Bni1 or actin cables were required to maintain Ste5 at the cell cortex once it has been recruited to the cell cortex was assessed. \( bni1^{TS}bnr1 \)
MAPK cascade, as a result of polarized growth (Buehrer and Errede, 1997; Zarzov et al., 1996), arguing against this possibility. Interestingly, it was recently reported that Rho1 associates with Ste4 in co-immunoprecipitates (Bar et al., 2003), raising the possibility that Gβγ recruits Rho1 to the site of polarized growth during mating. Taken together with our findings, we speculate that Gβγ directs polarized growth through dual recruitment of a Rho1-GTP-Bni1 complex, which facilitates polarized growth directly through actin cable formation, as well as through recruitment of Ste5.

A major question that still remains is how Ste5 is recruited in a polarized manner. One possibility is that Ste5 is exported from the nucleus either alone or in a complex with Cdc42 and simply diffuses to the cortex where it is captured by cell polarity factors such as Cdc42 and Bem1 whose localization is also dependent on Bni1. The position of these factors could be determined by the asymmetry that is established from the receptor and activated G protein and polarized actin that assembles in response to the external pheromone stimulus. Such asymmetry could be built through an interaction between Ste4 and Rh1, which binds to Bni1. Potential direct or indirect interactions between Bni1 and Cdc42 (Evangelista et al., 1997; Jaquenoud and Peter, 2000) might serve to link the Bni1 complex to Ste5-Cdc42. This possibility is consistent with our ability to co-immunoprecipitate Bni1 with Bem1 (M.Q. and E.A.E., data not shown).

Another interesting possibility that is consistent with our findings is that a pool of Ste5, either alone or with Cdc24, translocates along actin cables once it is exported from the nucleus. This possibility is consistent with the requirement for the cargo domain of Myo2 in Ste5 recruitment and the ability to co-immunoprecipitate Ste5 with Myo2. While it is possible that the interaction between Myo2 and Ste5 is indirect, it is noteworthy that we can detect it, in light of the fact that we are unable to co-immunoprecipitate Bni1 with Ste5. Additional support for the physiological relevance of this interaction comes from the observation that the interaction is not detected when Myo2 is overexpressed (data not shown) and that it is dependent upon pheromone signaling. Prior work has implicated Myo2 in the translocation of secretory vesicles, vacuoles and several cytoskeleton-associated proteins (i.e. Kar9 and Smy1) (Vale, 2003) but not the translocation of a cytoplasmic protein. Additionally, the possibility that Ste5 translocates along actin cables with Myo2 is still consistent with a model in which Ste5 is captured at the cell cortex by cell polarity proteins. While further work is needed to know whether Ste5 is translocating along actin cables, this interpretation is attractive in that it provides a physical link between nuclear export and events at a specific site at the cell cortex. Interestingly, concentration of JIP scaffolds at nerve terminals requires kinesin, suggesting they translocate along microtubules via a direct interaction with kinesin (Verhey et al., 2001), although the reason for this phenomenon has not been established.

Finally, previous work has shown that Fus3 is required for cortical recruitment of Bni1 (Mathews et al., 2004). Our unpublished findings are consistent with Fus3 being required for cortical recruitment of Ste5 in G1 phase of the cell cycle (M.Q. and E.A.E., unpublished). This finding raises the possibility that the formation of a stable signaling complex is a downstream event that occurs after initial pathway activation by mechanisms that are dependent on the Bni1-induced actin cytoskeleton. Further work is needed to clarify the relative position of MAPK activation with formation of a stable Ste5 scaffold signaling complex and whether polarized recruitment of Ste5 plays a specific role in events that take place at the shmoo tip, including polarized growth.

We are extremely grateful to: C. Moon, B. N. Lee and S. Mahanty, who made the initial observations that bni1Δ mutant is defective in G1 arrest, Fus3 activation and Ste5 recruitment, respectively. We thank C. Boone, D. Pellman, A. Bretscher, R. Li, L. Weisman and A. Levchenko for yeast strains and plasmids used in this study. We thank D. Pellman and D. Lew for comments on the manuscript. This research was supported by National Institutes of Health grant GM46962, a Taplin Funds for Discovery Award, and American Heart Grant 0150175N to E.A.E.

References


Fig. S1. *bnil* deletion reduces mating MAPK activation

Fus3 kinase activity in the experiments shown in Fig. 2A and 2B was quantified. Films were scanned and bands were analyzed with Scion Image software. The entire lane (including bands of both endogenous and exogenous substrates) of individual kinase assays was quantified and normalized to the level of Fus3 protein. Relative values (wild type before treatment was set to 1) are shown. (A) Dose response; (B) Time course. Values represent average ± STD of two independent experiments.
Fig. S2. Ste5 recruitment in she mutants

WT (EYL1684), she1 (EYL1685), she4 (EYL1688) and she5 (EYL1689) cells expressing Ste5-Myc9 (EBL453) were treated with 5 μM α factor for 0, 15, 45, and 120 minutes. Indirect immunofluorescence was done to detect Ste5-Myc as in Fig. 3B.
Fig. S3. Bni1-GFP and Ste5-Myc9 recruitment in rho2~5 mutants

WT, rho2, rho3, rho4 and rho5 null mutant cells expressing Bni1-GFP or Ste5-Myc9 were treated with 5 μM α factor for 2 hours. Bni1-GFP was visualized by direct fluorescence microscopy and Ste5-Myc9 was visualized by indirect immunofluorescence.
Fig. S4. Ste5-Myc9 recruitment in myo mutants

(A) Ste5-Myc9 localization myo2-66/myo4 cells. TAgNLSK128TSte5-Myc9 was expressed in myo2-66 (QMY630), myo4 (QMY631) and myo2-66/myo4 (QMY632) strains. The cells were treated as in (Fig. 7A).

(B) Ste5 localization in myo1 mutants. WT (EY699) and myo1 (EYL1098) cells expressing Ste5-Myc9 (EYL453) were treated with 5 μM α factor for 30 and 60 minutes, then fixed and treated for indirect immunofluorescence to observe Ste5-Myc9. Ste5-Myc9 rim staining was tallied in over 300 cells.

(C) Ste5-Myc9 recruitment in myo3 and myo5 mutants. WT (QMY550), myo3D (QMY483), myo5(QMY484), or myo3/myo5 (QMY485) cells expressing Ste5-Myc9 (EBL453) were treated with 5 μM α factor for 60 minutes.
Supplementary material

Yeast strains
Strains were grown in rich medium or synthetic medium as described (Guthrie and Fink, 1991).

Table S1. Yeast strains

<table>
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<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
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<td>EY492</td>
<td>( \text{MAT}^{\alpha} \text{ura}3-1 \text{leu}2-3,112 \text{trp}1-1 \text{his}3-11 \text{ade}2-1 \text{can}1-100 \text{Gal}^{+} )</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>EY699</td>
<td>( \text{ura}3-1 \text{leu}2-3,112 \text{trp}1-1 \text{his}3-11 \text{ade}2-1 \text{can}1-100 \text{Gal}^{+} )</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>EY700</td>
<td>EY699 ( \text{fus}3-6::\text{LEU}2 )</td>
<td>E. Elion</td>
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<tr>
<td>EY957</td>
<td>EY699 ( \text{sst}1 )</td>
<td>E. Elion</td>
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<td>EY957 ( \text{ste}5::\text{TRP}3 )</td>
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<td>EYL300</td>
<td>( \text{MAT}^{\alpha} \text{leu}2-3 \text{ura}3-52 )</td>
<td>R. Li</td>
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<td>EYL302</td>
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<td>EYL427</td>
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<td>EYL1807</td>
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<td>D. Pellman</td>
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<td>QMY27</td>
<td>( \text{sst}1::\text{LEU}2 \text{bni}1::\text{kanR} \text{FUS}3\text{GFP}::\text{HIS}3 \text{ura}3-1 \text{leu}3-3,112 \text{trp}1-1 \text{his}3-11 \text{ade}2-1 \text{can}1-100 )</td>
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<td>QMY458</td>
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QMY483  myo3::HIS3 ura3 leu2 trp1 his3 ade2  R. Li
QMY484  myo5::TRP1 ura3 leu2 trp1 his3 ade2  R. Li
QMY485  myo3::HIS myo5::TRP1 ura3 leu2 trp1 his3 ade2  R. Li
QMY550  ura3 leu2 trp1 his3 ade2  R. Li
QMY551  rho1-104 ura3 leu2 trp1 his3 ade2  R. Li
QMY553  rho1::HIS3 rho1-2 LEU2 ura3 leu2 trp1 his3 ade2  R. Li
QMY574  rho3::KAN his3ΔI leu2Δ0 met15Δ0 ura3Δ0  This study
QMY576  rho5::KAN his3ΔI leu2Δ0 met15Δ0 ura3Δ0  This study
QMY630  myo2-66::HIS3  This study
QMY631  myo4::KAN  This study
QMY632  myo2-66::HIS3 myo4::KAN  This study
5126  rho4::KAN his3ΔI leu2Δ0 met15Δ0 ura3Δ0  Res Gene
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22032  MATa  rho5::KAN his3ΔI leu2Δ0 met15Δ0 ura3Δ0  Res Gene
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22277  MATα  rho3::KAN his3ΔI leu2Δ0 met15Δ0 ura3Δ0

All strains except EY492, EYL428, 22032 and 22277 are MATa.

QMY27 was produced by cross of EYL428 with EYL1096. QMY630-632 were created by crossing EYL1685 with EYL1807 (she1 is myo4). QMY574 and QMY576 were made by sporulation of 22277 and 22032.

**Plasmid construction**

All BNI1 plasmids were made from a pRS316-based vector (PB1025, former name p182, see Evangelista et al., 1997) containing the BNI1 coding sequence with 800 base pairs (bp) upstream and 1600 bp downstream sequence. The Rho-binding domain deletion mutant, bni1ΔRBD (QMB16, deletes aa 90-343), was made from two PCR amplified fragments of BNI1 using the following primers: -788–268 fragment: 5′-ACGCGGATCC-TTCACCGCTTTCGCACCTACTT-3′ (BamHI site at 5′ terminus), 5′-TCCCCCGGCGGATTATTTTATCTAACAAGGCCT-3′ (SacII site at 3′ terminus) and 1031–2472 fragment: 5′-CTACCGGCGGATCTCTGCGCAGAACAAACTT-3′ (SacII site at 5′ terminus), 5′-TGCAATGCTCAGTCTCATACCTGTGGTGCCTTC-3′. The PCR products were cut with BamHI/SacII and SacII/XhoI respectively, then performed a 3-way ligation with BamHI/XhoI vector fragment of PB1025 to produce QMB16. The BamHI/XhoI fragment of QMB16 containing bni1ΔRBD mutation was inserted into EBL334 to produce QMB79 (bni1ΔRBD-GFP). The bni1ΔFH1FH2 mutant (QB5M, deletes
aa 1230-1748) was made by cloning the BNI1 gene into BamHI-EagI sites of pBR322 to make QMB3. A BNI1 fragment from the XhoI site to the FH1 domain (nucleotides 3572-4791) was PCR amplified with primers: 5′-GAACTCGAGCCTAATTTCTTCAG-3′ and 5′-AGATCCGCGGCAGTAGAGAGATCTTCTTGCG-3′ (SacII site at 5′ terminus). This PCR product was used to replace the XhoI-SacII fragment of BNI1 gene in QMB3 to make QMB4. Then the BamHI-EagI fragment of QMB4 was cloned into pRS316 to make QMB5. To make bni1ΔFH1 (QMB21, deletes aa 1230-1328), fragment from XhoI site to FH1 domain (3572-4791) was PCR amplified by the same way as for FH domain deletion, but added a SphI site instead of SacII site to the second primers: 5′-GATGCATGCAGTAGAGAGATCTTCTTGCG-3′. Fragment from the end of FH1 to SacII site (5086-6352) were PCR amplified with 5′-CATGCATGCAGCATCGCAAATCAAATCAGCT-3′ (SphI site at 5′ terminus) and 3′ primer: 5′-CTTCCCGCGCTAGATTTTGCGCTT-3′. The first and second PCR products were digested with XhoI/SphI and SphI/SacII respectively, then used in a 3-way ligation with the XhoI/SacII fragment of QMB3 to make QMB19. The BamHI-EagI fragments of QMB19 was cloned into pRS316 to make QMB21. The same strategy was used to make bni1ΔFH2 (QMB22, deletes aa 1492-1640), except a different 3′ primer (5′-TGTAGCATGCCCTCACGCCCTCCCAGTCTG-3′) for the first PCR, which amplifies fragment from XhoI site to FH2 domain (3572-5577), and a different 5′ primers (5′-CGATGCATGCCTCCATTGAGCAGTTAGTTAA-3′) for the second PCR, which amplifies fragment from the end of FH2 domain to SacII site (6021-6352). QMB34 (bni1ΔCT, deletes aa 1749-1953) was made by cloning BamHI-EagI fragment of PB1046 (Lee et al., 1999) into pRS315.

**Table S2. Plasmids used in this study**

<table>
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<tr>
<th>Plasmids</th>
<th>Description</th>
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**Supplemental References**

