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Formin-dependent actin assembly is regulated by distinct modes of Rho signaling in yeast

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Formins are actin filament nucleators regulated by Rho-GTPases. In budding yeast, the formins Bni1p and Bnr1p direct the assembly of actin cables, which guide polarized secretion and growth. From the six yeast Rho proteins (Cdc42p and Rho1–5p), we have determined that four participate in the regulation of formin activity. We show that the essential function of Rho3p and Rho4p is to activate the formins Bni1p and Bnr1p, and that activated alleles of either formin are able to bypass the requirement for these Rho proteins. Through a separate signaling pathway, Rho1p is necessary for formin activation at elevated temperatures, acting through protein kinase C (Pkc1p), the major effector for Rho1p signaling to the actin cytoskeleton. Although Pkc1p also activates a MAPK pathway, this pathway does not function in formin activation. Formin-dependent cable assembly does not require Cdc42p, but in the absence of Cdc42p function, cable assembly is not properly organized during initiation of bud growth. These results show that formin function is under the control of three distinct, essential Rho signaling pathways.

Introduction

The determination of cell shape and organization requires coordination between signal transduction pathways, the cytoskeleton, and membrane traffic. In the budding yeast *Saccharomyces cerevisiae*, the actin cytoskeleton, without participation of microtubules, polarizes cell growth (Bretscher, 2003). During bud growth, actin filaments assemble into cortical patches that cluster in the expanding bud, and into cables that extend along the axis of growth (Adams and Pringle, 1984). Cortical patches are implicated in endocytic internalization (Munn, 2001), and their assembly depends upon the actin-nucleating Arp2/3 complex (Winter et al., 1999). Actin cables function as tracks that guide myosin-V–driven secretory vesicle, organelle, and mRNA transport and nonmyosin-driven mitochondrial transport into the growing bud (Schott et al., 2002). The assembly of actin cables is dependent on the functionally redundant formins Bni1p and Bnr1p (Evangelista et al., 2002; Sagot et al., 2002a). In contrast to the assembly of patches, cable assembly is Arp2/3 independent (Evangelista et al., 2002).

Like many formins from animals and fungi, yeast Bni1p and Bnr1p contain an NH_2-terminal Rho-binding domain (RBD),* a central proline-rich formin homology (FH) domain 1 that binds profilin, and a COOH-terminal FH2 domain (Kohno et al., 1996; Evangelista et al., 1997; Imamura et al., 1997) (Fig. 1, a and d). The isolated FH2 domain of Bni1p can serve as a nucleator for actin assembly in vitro (Pruyne et al., 2002; Sagot et al., 2002b). Because the FH2 domain has the novel capacity to remain bound to the barbed end of the assembling filament, Bni1p can potentially serve as a nucleator and filament anchor during actin cable assembly at the cell cortex (Pruyne et al., 2002).

Based on studies of the mammalian formins mDia1 and mDia2, the RBD is proposed to negatively regulate formin activity by binding a sequence COOH terminal to the FH2 domain termed the Dia autoregulatory domain (DAD) (Alberts, 2001; Palazzo et al., 2001). The association of GTP-bound Rho to the formin RBD relieves this interaction, thereby activating the formin. Consistent with this model, related DAD sequences are present in Bni1p and Bnr1p (Fig. 1), and Bni1p constructs lacking the RBD or DAD stimulate excessive filament assembly in vivo compared with full-length Bni1p (Evangelista et al., 2002; Sagot et al., 2002a).

Yeast contains six Rho-family GTPases (Cdc42p and Rho1–5p) that participate in multiple aspects of cell polarity, including septin organization (Holly and Blumer, 1999; Weiss et al., 2000; Gladfelter et al., 2002), the regulation of secretion (Adamo et al., 1999, 2001; Robinson et al., 1999; Guo et al., 2001; Zhang et al., 2001), and the stimulation of cell wall synthesis (Cabib et al., 1998). All six Rho proteins also have roles in regulating actin polarity. Cdc42p is re-

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*Abbreviations used in this paper: DAD, Dia autoregulatory domain; FH, formin homology; RBD, Rho-binding domain.

Key words: actin; polarity; Cdc42; PKC; MAPK

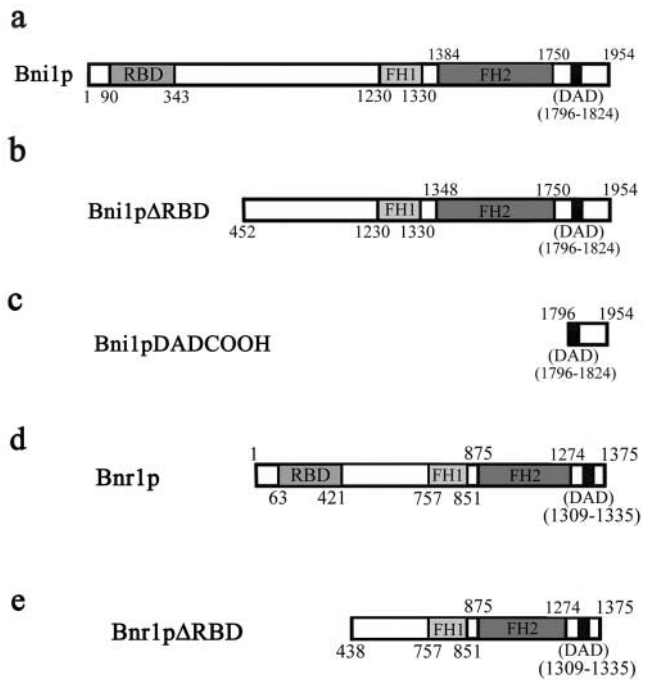


Figure 1. **Schematic representation of Bni1p- and Bnr1p-derived constructs.** (a) Full-length Bni1p, showing the RBD, FH1 and FH2, and DAD domains. (b) Truncated Bni1p lacking the RBD (Bni1pΔRBD), which is constitutively active in vivo (Evangelista et al., 2002). (c) Bni1pDADCOOH construct containing the Bni1p DAD and COOH-terminal sequences. (d) Full-length Bnr1p, showing the RBD, FH1 and FH2, and DAD domains. (e) Truncated Bnr1p lacking the RBD (Bnr1pΔRBD).

quired for actin polarization at bud emergence, with multiple effectors implicated in this process (Adams et al., 1990; Brown et al., 1997; Chen et al., 1997; Evangelista et al., 1997; Holly and Blumer, 1999; Bi et al., 2000; Lamson et al., 2002). Rho3p and Rho4p share a function in polarizing actin, though so far no physiologically relevant effectors have been defined (Matsui and Toh-e, 1992b; Imai et al., 1996). Rho1p functions through Pkc1p to regulate actin in a complex manner, driving MAPK-independent depolarization of the actin cytoskeleton in response to cell wall injuries and then stimulating a MAPK-dependent repolarization (Delley and Hall, 1999). Overexpressed Rho2p can substitute for the actin-polarizing activity of Rho1p (Marcoux et al., 2000), and Rho5p negatively regulates cell wall stress-induced actin depolarization (Schmitz et al., 2002), but effectors for these proteins are unknown.

The Bni1p and Bnr1p RBDs show two-hybrid interactions with multiple Rho proteins (Kohno et al., 1996; Evangelista et al., 1997; Imamura et al., 1997), but the contribution of each Rho protein to regulating formin-dependent actin cable assembly was not known. We find that multiple Rho-dependent pathways converge upon Bni1p and Bnr1p to regulate their activity, including the semiredundant Rho3p and Rho4p and the highly conserved homologues of RhoA (Rho1p) and CDC42 (Cdc42p). Thus, the formins are key targets for integrating signaling pathways in controlling actin polarity.

Results

Activation of full-length Bni1p requires Rho3p

To first investigate whether any of the nonessential Rho proteins are required to stimulate actin cable assembly, the actin cytoskeleton was examined in yeast lacking the Rho isoforms Rho2p, Rho3p, Rho4p, and Rho5p. The deletion strains *rho2*Δ, *rho4*Δ, and *rho5*Δ all grew as well as wild type, whereas the *rho3*Δ strain grew more slowly (unpublished data), and each deletion strain displayed normal actin cables when stained for either actin (Act1p) or tropomyosin (Tpm1p) (Fig. 2 a). Thus, none of these Rho proteins alone is required to stimulate both Bni1p and Bnr1p.

We have previously shown that overexpression of full-length Bni1p causes the loss of extended actin cables in the mother cell and the appearance of excessive cable-like filaments in the bud. This shift from cables to cable-like filaments is thought to reflect excessive nucleation of filaments that depletes the cable-stabilizing protein tropomyosin (Evangelista et al., 2002). To evaluate whether Bni1p activity depends upon any of the nonessential Rho proteins, we expressed full-length myc-tagged Bni1p in the *rho2*Δ, *rho3*Δ, *rho4*Δ, and *rho5*Δ strains. Cable-like filaments accumulated in the buds of wild-type, *rho2*Δ, *rho4*Δ, and *rho5*Δ cells but not in *rho3*Δ yeast (Fig. 2, b and c). Western blotting showed that *rho3*Δ yeast produce Bni1p–myc to the same extent as wild-type cells (Fig. 2 d), and reintroduction of *RHO3* into the *rho3*Δ strain restored cable-like filaments with overexpression of Bni1p (Fig. 2, b and c), showing that overexpressed Bni1p requires Rho3p to assemble filaments in the bud.

As Bni1p and Bnr1p perform an essential function in the assembly of actin cables (Evangelista et al., 2002; Sagot et al., 2002a), we generated a strain lacking both *RHO3* and *BNR1* to examine whether Bni1p expressed at endogenous levels also requires *RHO3*. Consistent with a strong dependence of actin cable assembly by Bni1p upon Rho3p, the *rho3*Δ*bnr1*Δ cells grew very poorly and contained few actin cables (Fig. 3, a and b) but accumulated actin bars, a form of aggregated monomeric actin common to cytoskeletal mutants.

To explore whether the requirement of Rho3p is to activate full-length Bni1p or if it plays a different role in filament assembly, constitutively active Bni1p lacking the regulatory RBD (Bni1pΔRBD; Fig. 1 b) was overexpressed in wild-type and *rho* deletion strains. In all strains, including *rho3*Δ yeast, Bni1pΔRBD stimulated massive assembly of cable-like filaments (Fig. 2, e and f). Furthermore, Bni1pΔRBD expressed from the *BNI1* promoter was able to rescue the synthetic sick phenotype and restore normal actin cables to *bnr1*Δ*rho3*Δ yeast (Fig. 3, a and b). Thus, the full-length formin Bni1p requires Rho3p to stimulate filament assembly, but this requirement can be bypassed by a formin lacking the Rho-binding regulatory region.

Rho3p and Rho4p share an essential function in formin activation

The viability of the *rho3*Δ*bnr1*Δ strain suggested that Bni1p has some residual ability to assemble cables in the absence of Rho3p. One possible explanation is that other Rho proteins

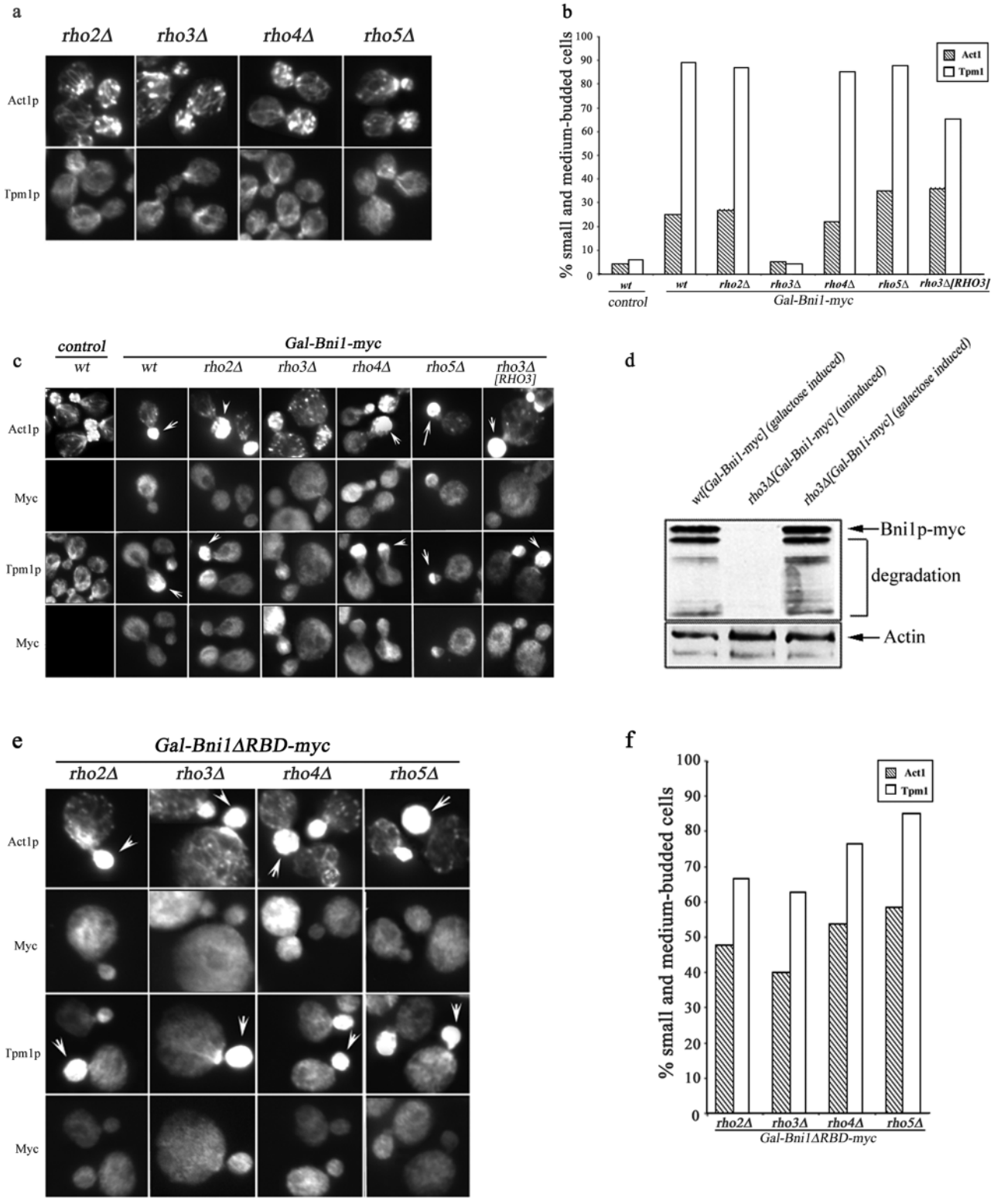


Figure 2. **Rho3p is required for the activation of full-length Bni1p.** (a) Visualization of actin (Act1p) and tropomyosin (Tpm1p) by immunofluorescence microscopy in the indicated cells. (b) Quantitation of the accumulation of cable-like filaments in wild-type or *rho* deletion cells overexpressing COOH-terminally myc-tagged full-length Bni1p. Cells were double labeled for Act1p and the myc epitope, or Tpm1p and myc, and the myc-positive cells were evaluated for a visible increase over wild-type controls of Act1p (gray) or Tpm1p (white) in the bud. (c) Examples of actin and tropomyosin localization in cells quantitated in b. Arrows indicate accumulation of cable-like filaments in the bud in *rho2*Δ, *rho4*Δ, and *rho5*Δ cells and in *rho3*Δ yeast containing plasmid-born *RHO3*. Myc staining indicates expression of Bni1p. (d) Western blot of Bni1p–myc and actin in wild-type and *rho3*Δ cells. (e) Actin and myc epitope, or tropomyosin and myc epitope, localization in *rho* deletion cells overexpressing COOH-terminally myc-tagged Bni1pΔRBD. Arrows indicate accumulation of cable-like filaments in the buds of *rho2*Δ, *rho3*Δ, *rho4*Δ, and *rho5*Δ cells. (f) Quantitation of cells showing a visible increase of actin (Act1p) and tropomyosin (Tpm1p) in cells overexpressing Bni1pΔRBD–myc.

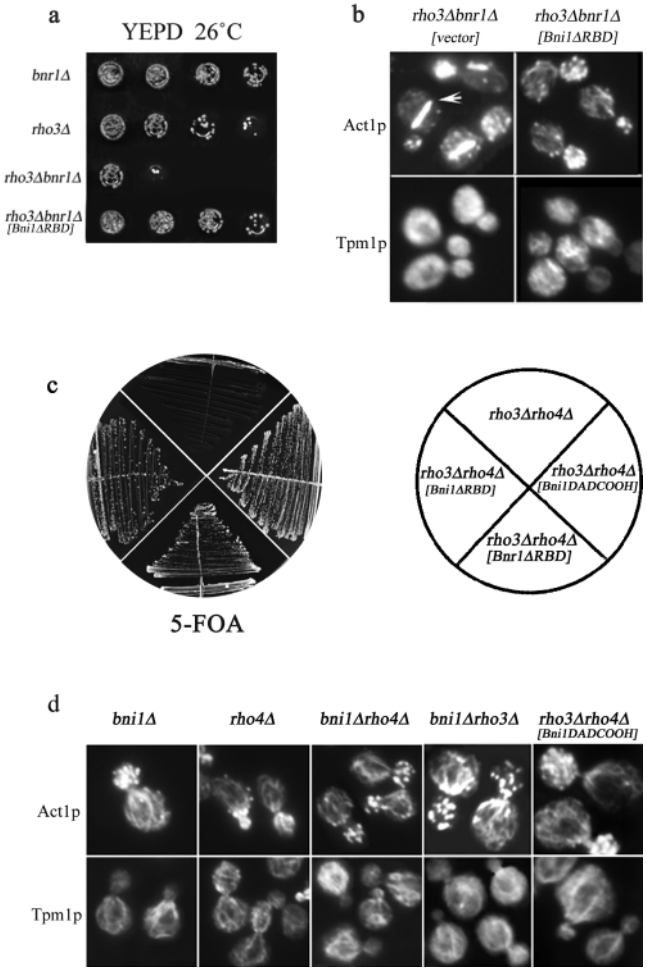


Figure 3. **The essential function of Rho3p and Rho4p is in formin activation.** (a) Growth of serial dilutions of strains *bnr1*Δ, *rho3*Δ, *rho3*Δ*bnr1*Δ, and *rho3*Δ*bnr1*Δ [*Bni1*Δ*RBD*] at 26°C for 2 d. (b) Localization of actin (Act1p) and tropomyosin (Tpm1p) in control *rho3*Δ*bnr1*Δ cells and cells expressing Bni1pΔRBD. Actin bar in *rho3*Δ *bnr1*Δ cells is indicated. (c) Bni1pΔRBD, Bnr1pΔRBD, and Bni1pDADCOOH can rescue the lethality of *rho3*Δ*rho4*Δ cells. *rho3*Δ*rho4*Δ [*pRS316-RHO3, pRS315*], *rho3*Δ*rho4*Δ [*pRS316-RHO3, pRS315-Bni1*Δ*RBD*], *rho3*Δ*rho4*Δ [*pRS316-RHO3, pRS315-Bnr1*Δ*RBD*], and *rho3*Δ*rho4*Δ [*pRS316-RHO3, pRS315-Bni1DADCOOH*] cells were grown on 5-FOA–containing medium to select for loss of the pRS316-*RHO3* plasmid. (d) Actin cables, visualized by staining for actin (Act1p) and tropomyosin (Tpm1p), are present in *bni1*Δ, *rho4*Δ, *bni1*Δ*rho4*Δ, *bni1*Δ*rho3*Δ, and *rho3*Δ*rho4*Δ [*Bni1DADCOOH*] cells.

play a role in this process. One likely candidate is Rho4p. Simultaneous deletion of *RHO3* and *RHO4* is lethal, and overexpression of Rho4p can suppress growth defects of *rho3*Δ cells (Matsui and Toh-e, 1992a), indicating that the two share some redundant function. Double conditional *rho3 rho4* mutants display severe cytoskeletal defects (Matsui and Toh-e, 1992b; Imai et al., 1996), yet the effectors for these Rho isoforms in organizing the cytoskeleton are unknown. If they share an essential role in formin activation, we predicted that expression of the constitutively active Bni1pΔRBD might bypass the requirement for these two Rho proteins. Indeed, expression of Bni1pΔRBD from the *BNI1* promoter rescued the viability of the double *rho3*Δ*rho4*Δ mutant (Fig. 3 c). An attractive explanation is that the normal function of Rho3p and Rho4p is to bind the Bni1p RBD to relieve the inhibitory interaction between the RBD and the DAD.

In mammalian cells, expression of the mDia DAD has been shown to also bypass the requirement for Rho activation of a formin by competing with the DAD of the endogenous full-length formin for binding the RBD (Alberts, 2001). To more specifically determine whether Rho3p and Rho4p play a role in regulating the interaction of the Bni1p RBD and DAD, we generated a construct containing the DAD of Bni1p (Fig. 1 c). Expression of this construct was able to rescue growth and permit actin cable assembly in *rho3*Δ*rho4*Δ yeast (Fig. 3, c and d). As expected, rescue by the Bni1p DAD was dependent upon full-length formins, because Bni1pDADCOOH was unable to rescue the lethality of *bni1*Δ*bnr1*Δ (unpublished data). These results suggest that Rho3p and, to a lesser extent, Rho4p share a role in regulating the interaction between the RBD and DAD of the yeast formin Bni1p.

Rho4p is the only Rho protein that binds the Bnr1p RBD, as analyzed by two-hybrid or in vitro binding assays (Imamura et al., 1997). To ascertain whether Rho4p is the preferred Rho activator for Bnr1p, the growth rates of wild-type, *rho4*Δ, *bni1*Δ, and *rho4*Δ*bni1*Δ strains were compared. No obvious growth defects were seen in any of these strains, and the actin cables of *rho4*Δ*bni1*Δ were normal, indicating that Bnr1p can function in the absence of Rho4p (Fig. 3 d).

The strong dependence of Bni1p upon Rho3p suggests that the actin cables present in *rho3*Δ yeast are dependent upon Bnr1p. Consistent with this, the growth rates of *rho3*Δ and *rho3*Δ*bni1*Δ strains were similar (unpublished data), and the actin cables of *rho3*Δ*bni1*Δ were normal (Fig. 3 d), suggesting that Bnr1p can also function in the absence of Rho3p. However, the lethality of *rho3*Δ*rho4*Δ yeast can be rescued by an activated version of Bnr1p (Bnr1pΔRBD) (Fig. 3 c), suggesting that yeast lacking Rho3p and Rho4p are deficient in all formin function. Also, the deletion of *RHO3* results in slow growth, but this can be rescued by the activated Bni1ΔRBD (unpublished data), suggesting that the slow growth of our *rho3*Δ strain results from diminished formin activity. Notably, loss of *BNI1* in our strain background does not show a slow growth phenotype, suggesting that the loss of *RHO3* also diminishes activation of Bnr1p. Thus, although Bnr1p-mediated filament assembly can occur in the presence of Rho3p or Rho4p, activation by Rho3p appears to be more important for normal growth. Our combined results indicate that the shared essential function of Rho3p and Rho4p is to activate the formins, specifically through disrupting the RBD–DAD interaction, and that Rho3p plays a more important role in this process.

Cdc42p is required for actin cable polarization at bud emergence

Loss of Cdc42p function causes multiple defects in polarized growth (Kozminski et al., 2000). However, many conditional alleles arrest as unbudded cells that grow isotropically (Adams et al., 1990; Miller and Johnson, 1997; Kozminski

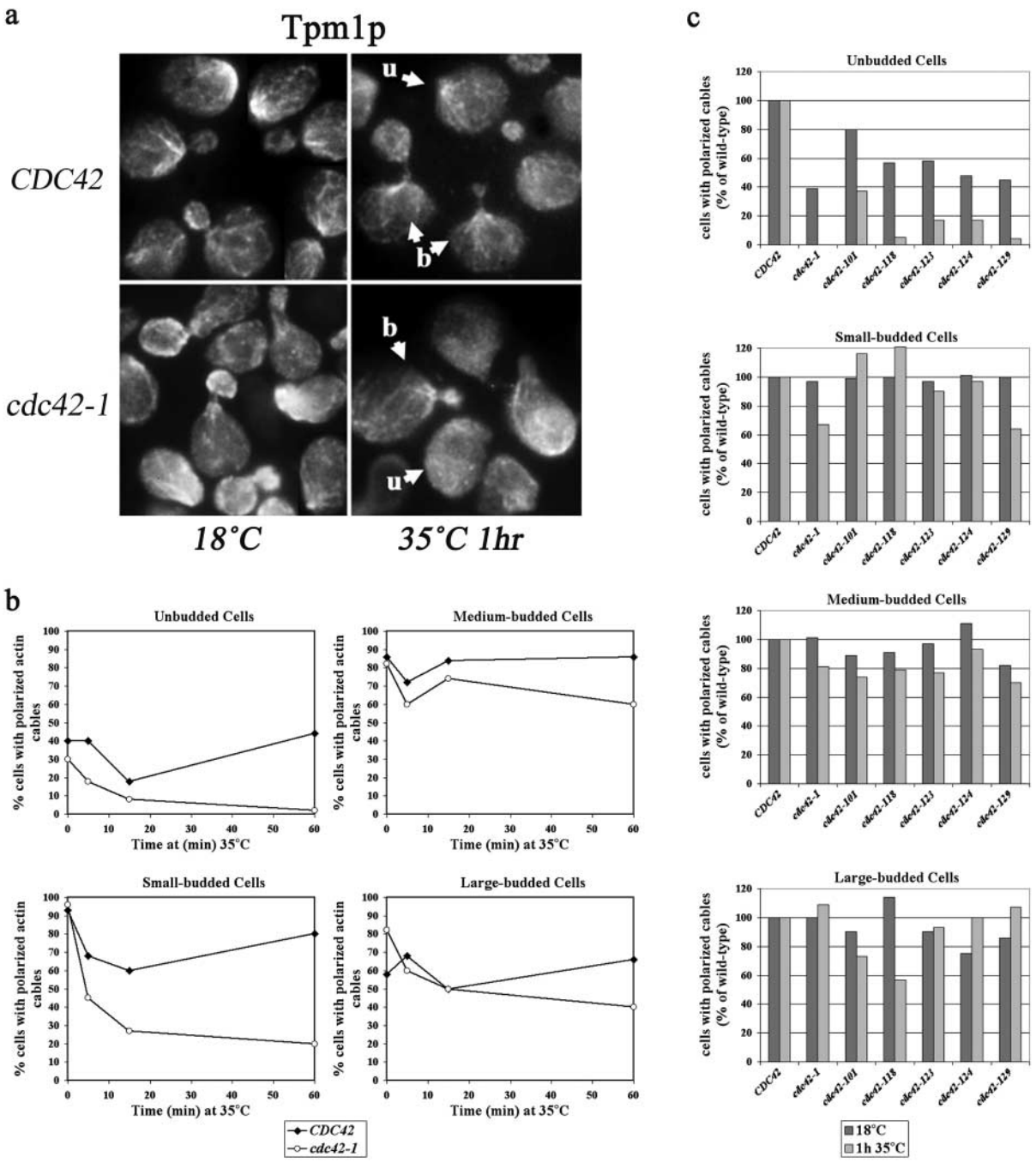


Figure 4. **Cdc42p function is required for actin cable organization but not assembly in unbudded cells only.** (a) Wild-type and conditional *cdc42-1* mutant cells were subjected to the indicated temperature shifts and prepared for immunofluorescence using Tpm1p antibodies. Unbudded *cdc42-1* cells (u) lacking polarized actin cables and budded *cdc42-1* cells (b) retaining polarized actin cables at 35°C are shown. (b) Quantitation of polarized cables in wild type (filled diamonds) and conditional mutant *cdc42-1* (open circles) after shifting to 35°C. (c) Unbudded, small-budded, medium-budded, and large-budded cells from asynchronous cultures of wild type and six conditional *cdc42* mutants were scored for the presence of polarized actin cables before (dark gray) and after (light gray) a 1-h shift to 35°C.

et al., 2000), similar to the terminal phenotype of double conditional *bni1bnr1* mutants (Evangelista et al., 2002). To determine whether Cdc42p regulates Bni1p or Bnr1p activity, we examined whether actin cables are present in a temperature-sensitive *cdc42-1* strain. When *cdc42-1* yeast were shifted to 35°C, polarized actin cables were lost from unbudded cells but, to a large extent, were retained in budded cells (Fig. 4, a and b). The defect in the unbudded *cdc42-1* cells did not appear to be a loss of cables but an inability to properly organize them. The unbudded *cdc42-1* cells retained actin cables to a similar extent as wild-type cells but in a disorganized distribution.

To determine whether these phenotypes were specific to the *cdc42-1* allele or were a general phenotype for loss of Cdc42p function, five conditional alleles (*cdc42-101*, *cdc42-118*, *cdc42-123*, *cdc42-124*, and *cdc42-129*) isolated from a

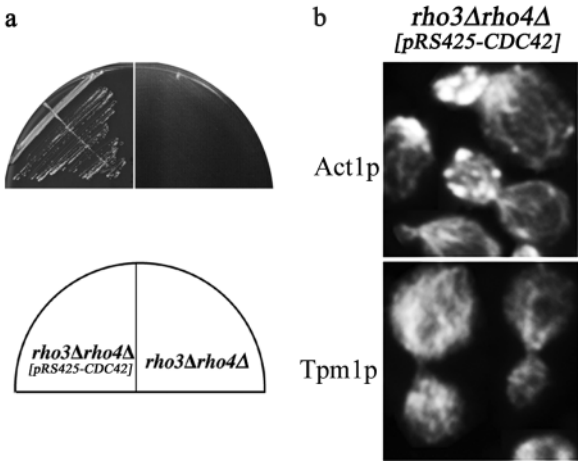
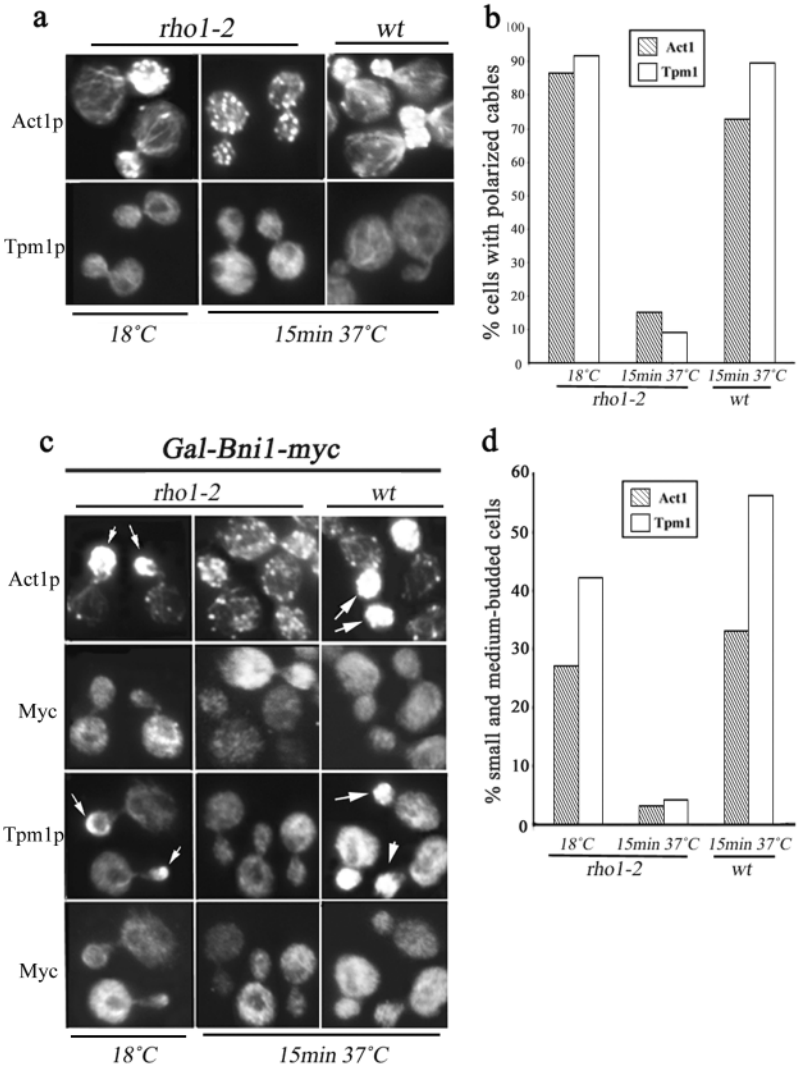


Figure 5. **Overexpression of Cdc42p can rescue the synthetic lethality of *rho3*Δ*rho4*Δ cells.** (a) Growth of *rho3*Δ*rho4*Δ [*pRS316-RHO3, pRS425*] and *rho3*Δ*rho4*Δ [*pRS316-RHO3, pRS425-CDC42*] cells on 5-FOA–containing medium to select for loss of the pRS316-*RHO3* plasmid. (b) Normal actin cables are present in *rho3*Δ*rho4*Δ [*pRS425-CDC42*] cells.

collection of 37 site-directed *cdc42* mutants (Kozminski et al., 2000) were examined for actin cable defects under restrictive conditions. Similar to *cdc42-1*, all five alleles showed a profound loss in polarized actin cables in unbudded cells yet retained a polarized network of cables in budded cells (Fig. 4 c). Also consistent with *cdc42-1*, all the *cdc42* mutants retained actin cables in unbudded cells in a disorganized distribution (unpublished data). Because Bni1p and Bnr1p are required for actin cable assembly throughout the cell cycle (Evangelista et al., 2002; Sagot et al., 2002a), we conclude that Cdc42p is not essential for formin activation. Consistent with this, the expression of the activated formin construct Bni1pΔRBD was unable to rescue any of the defects of the *cdc42-1* mutant; actin cables still became disorganized in unbudded cells at the restrictive temperature, and the mutants arrested as large and unbudded cells (unpublished data). Thus, Cdc42p appears to be important for the proper organization of cables during bud emergence but not for the bulk assembly of cables that occurs in unbudded cells.

Overexpression of Cdc42p is reported to bypass the requirement for Rho3p and Rho4p (Matsui and Toh-e,

Figure 6. **Rho1p function is required for formin activation at 37°C.** (a) Localization of actin (Act1p) and tropomyosin (Tpm1p) in *rho1-2* and wild-type cells before and after a shift to 37°C. (b) Quantitation of the presence of actin cables in cells stained for actin (gray) or tropomyosin (white). (c) Double label actin and myc epitope, and tropomyosin and myc epitope, localization at 18°C or 37°C in *rho1-2* or wild-type cells overexpressing full-length Bni1–myc. Arrows indicate enhanced cable-like filament accumulation in the bud. (d) Quantitation of cells from c, scored for an increase over wild-type controls of cable-like actin (gray) and tropomyosin (white) in the bud.



1992b). To examine the ability of the various Rho isoforms to replace Rho3p and Rho4p function, we introduced *CDC42*, *RHO1*, *RHO2*, or *RHO5* on high-copy plasmids into *rho3*Δ*rho4*Δ yeast grown in the presence of pRS316-*RHO3* and then tested each strain for its ability to lose the *RHO3* plasmid. Consistent with Matsui and Toh-e (1992b), *CDC42* was able to rescue the lethality of *rho3*Δ*rho4*Δ, and the rescued cells displayed normal actin cables (Fig. 5, a and b), indicating that at least one yeast formin was active under these conditions. However, none of the other three Rho proteins was able to rescue the lethality of *rho3*Δ*rho4*Δ (unpublished data). Cdc42p was also able to bypass the synthetic sickness and actin cable defects of *rho3*Δ*bnr1*Δ yeast (unpublished data), indicating that when overexpressed, Cdc42p can relieve the dependence of Bni1p activation upon Rho3p.

Rho1p signaling through Pkc1p is required for formin activation at elevated temperatures

The essential functions of Rho1p in cell wall synthesis and in actin organization have been dissected using conditional mutations specifically defective for one function or the other (Saka et al., 2001). One mutation, *rho1-2*, was shown to have a depolarized actin cytoskeleton after 5 h at the restrictive temperature (Helliwell et al., 1998). We found that when shifted to 37°C, *rho1-2,* but not *RHO1,* cells lost actin cables after 15 min, suggesting that Rho1p might also be important for Bni1p- and Bnr1p-stimulated filament assembly (Fig. 6, a and b). In support of this, when full-length Bni1p was overexpressed in *rho1-2* cells, cable-like filaments accumulated in the bud at the permissive temperature, but after 15 min at 37°C, these cable-like filaments were no longer present (Fig. 6, c and d). To determine if Rho1p is required for the activation of Bni1p, we expressed Bni1pΔRBD from the *BNI1* promoter in *rho1-2* cells. When shifted to 37°C, actin cables now remained in the *rho1-2* cells (Fig. 7, a and b), although viability was not rescued, suggesting that Rho1p is required for the activation of Bni1p and Bnr1p, but failure in formin activation is not the only defect of *rho1-2* cells.

Rho1p is able to bind several putative effectors, but the growth and polarity defects of *rho1-2* mutants are specifically suppressed by an activated form of the Rho1p effector protein kinase C (Pkc1p*) (Helliwell et al., 1998). Expression of Pkc1p* from the *PKC1* promoter restored normal actin cables to *rho1-2* cells at 37°C (Fig. 8 a), and when Pkc1p* was coexpressed with full-length Bni1p in *rho1-2* cells, the activated kinase permitted cable-like filament assembly at 37°C (Fig. 8, b and c). Thus, activated Pkc1p can provide the Rho1p-dependent signal for activation of full-length Bni1p.

Signaling from Rho1p to the actin cytoskeleton bifurcates at the level of Pkc1p (Delley and Hall, 1999). One signaling pathway functions through the cell wall stress response MAPK cascade composed of Bck1p (MAPK kinase kinase), Mkk1/2p (MAPK kinases), and Slt2p (MAPK). Pkc1p targets of the second pathway are not known. Depending upon strain background, deletions of the Slt2p MAPK cascade can have either severe or mild effects upon the cell, resulting either in slow growth, cell lysis, and temperature sensitivity, or in normal growth but temperature sensitivity (Mazzoni et al., 1993; Delley and Hall, 1999). To determine whether the MAPK cascade is involved in formin activation, we examined mutants lacking components of the cascade from both types of backgrounds. In both a *bck1*Δ strain with severe growth defects and a *slt2*Δ strain with mild defects, actin cables were present at room temperature and throughout a 1-h shift to 37°C (unpublished data). Expression of Slt2p from a 2μ high-copy plasmid can rescue the polarity of cortical actin patches in *rho1-2* yeast but cannot rescue viability (Helliwell et al., 1998). However, overexpression of Slt2p was unable to restore actin cables in *rho1-2* yeast at 37°C and was also unable to allow overexpressed Bni1p to generate cable-like filaments in *rho1-2* yeast (unpublished data), suggesting that the Slt2p MAPK cascade is not involved in Rho1p-stimulated activation of the formins.

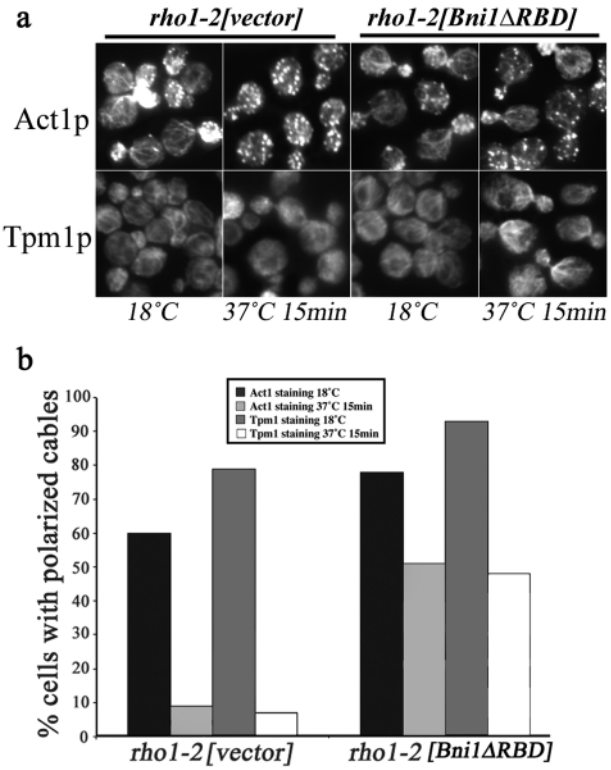


Figure 7. **Bni1pΔRBD induces actin cable assembly in *rho1-2* cells at 37°C.** (a) *rho1-2* or *rho1-2* [*Bni1ΔRBD*] cells grown at 18°C or after shifting to 37°C for 15 min were stained for actin or tropomyosin. (b) The percentage of small- and medium-budded cells with visible actin cables was determined.

Although *PCK1* is essential, a deletion can be rescued by a dominant active allele of the MAPK kinase kinase *BCK1* (*BCK1-20*) (Lee and Levin, 1992; Levin and Bartlett-Heubusch, 1992). To determine whether Pkc1p is required for Rho1p-dependent activation of the formins, *pkc1*Δ *BCK1-20* cells were examined at several temperatures. At room temperature, actin cables were present (unpublished data), but when shifted to 37°C, actin cables disassembled in the *pck1*Δ *BCK1-20* cells within 15 min, just like the *rho1-2* mutants (Fig. 8, d and e). A temperature-sensitive *PKC1* allele (*pkc1-2ts*) (Lee and Levin, 1992) yielded similar results

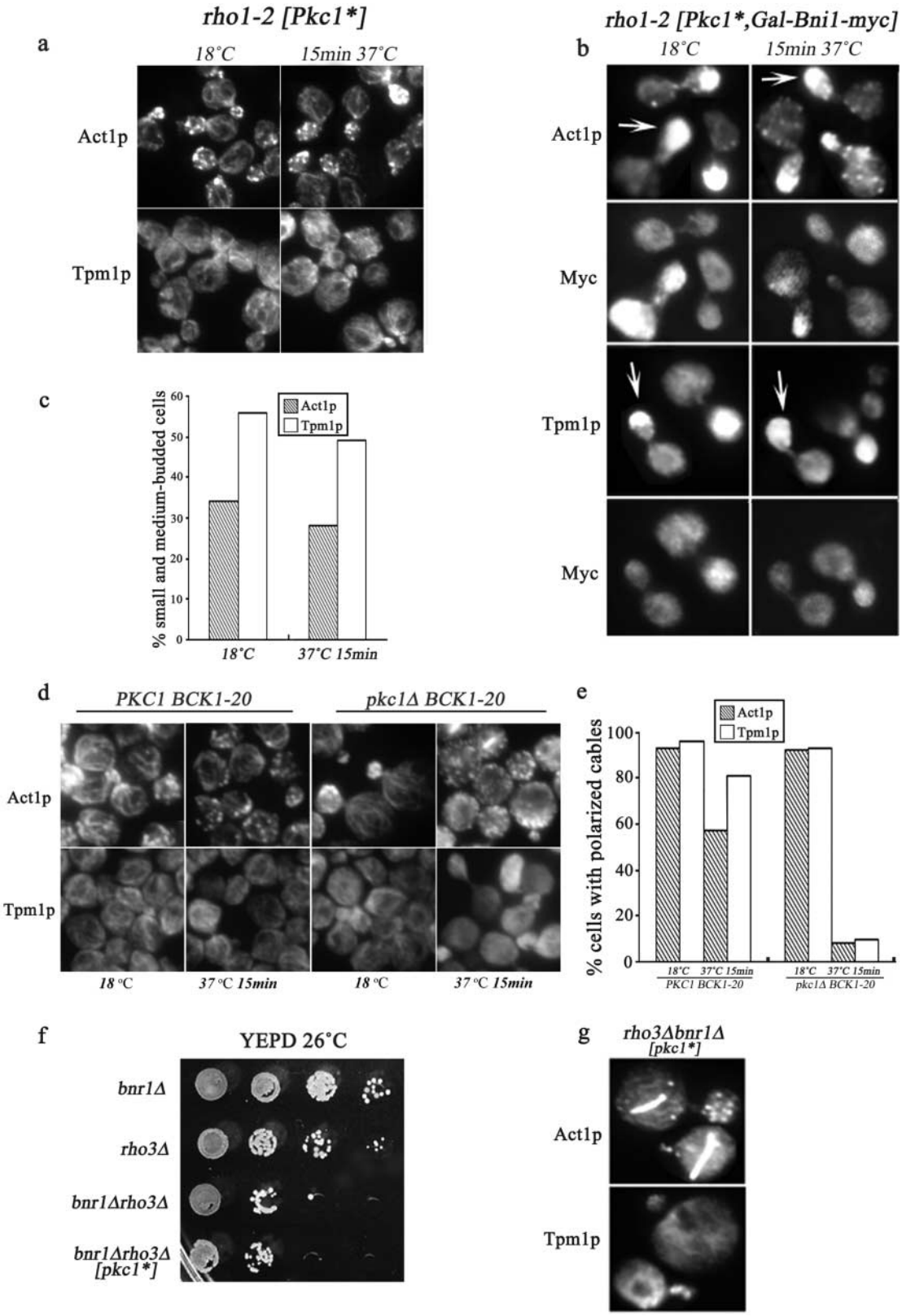


Figure 8. **Rho1p signals through Pkc1p to activate the formins.** (a) Localization of actin (Act1p) and tropomyosin (Tpm1p) at 18°C or after a 15-min shift to 37°C in *rho1-2* cells expressing constitutively activated Pkc1p* from its own promoter. (b) Montages showing actin and myc, or tropomyosin and myc, localization at 18°C or 37°C in *rho1-2* cells coexpressing Pkc1p* and full-length Bni1p–myc. Arrows indicate the accumulation of cable-like filaments in the bud. Myc staining indicates expression level of full-length Bni1p. (c) Quantitation of cells from b, scored for a visible increase over wild-type controls of cable-like actin (gray) and tropomyosin (white) in the bud. (d) *pkc1*Δ *BCK1-20* and control *PKC1 BCK1-20* cells were stained for actin and tropomyosin at 18°C and after a 15-min shift to 37°C. (e) Cells from d were quantitated for the presence of visible actin cables based on actin (gray) and tropomyosin (white) stain. (f) Growth of serial dilutions of *bnr1*Δ, *rho3*Δ, *bnr1*Δ*rho3*Δ, and *bnr1*Δ*rho3*Δ [*pkc1**] cells on rich media after 2 d. (g) *rho3*Δ*bnr1*Δ cells bearing Pkc1p* were prepared for immunofluorescence with Act1p and Tpm1p antibodies.

(unpublished data), whereas wild-type *PKC1* or *PKC1 BCK1-20* controls showed no loss of actin cables. Thus, Rho1p works through its effector Pkc1p to activate the formins in a MAPK-independent manner, though this signaling appears to be required only at elevated temperatures.

To determine whether Pkc1p signaling can bypass the requirement for Rho3p-dependent activation of Bni1p, Pkc1p* was expressed in *rho3*Δ*bnr1*Δ yeast. The presence of the activated kinase was unable to rescue the slow growth of this strain (Fig. 8 f) or restore actin cables and eliminate actin bars (Fig. 8 g), suggesting that Pkc1p cannot bypass the requirement for Rho3p to activate Bni1p. To determine whether Rho3p can bypass the need for Rho1p/Pkc1p signaling in activating Bni1p, we coexpressed full-length Bni1p with an activated GTPase-deficient allele of *RHO3* (*RHO3-V25*) (Adamo et al., 1999) from a CEN plasmid in *rho1-2* yeast. The presence of activated Rho3p was unable to bypass the requirement of Bni1p-stimulated filament assembly for Rho1p (unpublished data), indicating that both Rho1p/Pkc1p and Rho3p are required for efficient activation of Bni1p at 37°C. Similarly, overexpression of *CDC42* was unable to suppress the *rho1-2* mutant at 37°, either with respect to growth or the actin cable defect. Based on the loss of all cables in *rho1-2* yeast, Rho1p/Pkc1p is likely to also be required for the activation of Bnr1p.

Discussion

The formins provide a crucial target for regulating cell polarity in yeast. As nucleators of actin filaments, the formins define the spatial organization of the actin cable array, which in turn guides polarized secretion, organelle segregation, and mitotic spindle orientation (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002a,b). We have found that four of yeast's six Rho proteins are involved in regulating the formins Bni1p and Bnr1p in several distinct ways.

Rho3p and Rho4p were previously known to share an essential role in yeast growth (Matsui and Toh-e, 1992a), and we find here that this function appears to be regulation of the inhibitory interaction between the RBD and DAD of the yeast formins. Thus, these two Rho proteins become dispensable in cells expressing Bni1p or Bnr1p from which the RBD has been deleted, or when the RBD–DAD interaction is disrupted by overexpression of exogenous DAD sequence. By several criteria, Rho3p appears to be the more important of the two GTPases. Loss of Rho3p almost completely eliminates Bni1p-dependent filament assembly. Furthermore, *rho3*Δ yeast grow slowly, but this slow growth can be bypassed by an activated Bni1p, suggesting that the growth defect is related to a loss of signaling to the formins. As *bni1*Δ does not cause a similar decrease in growth rate in our strain background, we suggest that the loss of Rho3p eliminates a significant component of Bnr1p activation.

An attractive model would have Rho3p and Rho4p activate the formins directly. GTP-bound forms of multiple Rho proteins show two-hybrid interactions with the RBD of the formins, although the reported interactions do not completely coincide with our in vivo analysis; Bni1p RBD interacts with GTP-Cdc42p, -Rho1p, -Rho3p, and -Rho4p (Kohno et al., 1996; Evangelista et al., 1997), but Bnr1p interacts only with GTP-Rho4p (Imamura et al., 1997). Our results suggest that the most physiologically important interaction for Bni1p is with Rho3p. However, with no reported interaction between Bnr1p and Rho3p, it is possible that signaling between these two molecules is indirect.

In addition to formins, Rho3p and Rho4p can bind to several proteins involved in polarized secretion, including the exocyst proteins Sec3p and Exo70p and (for Rho3p) the myosin-V Myo2p (Adamo et al., 1999; Robinson et al., 1999; Guo et al., 2001). Furthermore, mutants for *RHO3* show defects in exocytosis (Adamo et al., 1999), but the contributions of the Rho proteins to secretion and to actin organization seem to be distinct. Many suppressors of *rho3*Δ growth defects encode proteins involved in the secretory pathway, but these are not able to rescue *rho3*Δ*rho4*Δ lethality (Matsui and Toh-e, 1992b; Imai et al., 1996; Kagami et al., 1997). Also, defects in the function of Sec3p or Myo2p do not directly affect the organization of actin cables (Haarer et al., 1996; Schott et al., 1999). Finally, *rho3* alleles specifically defective in secretion retain a normal actin cytoskeleton (Adamo et al., 1999), suggesting that they are not defective in activating the formins. Despite the importance of Rho3p for exocytosis, the growth defects of our *rho3*Δ mutants appear to be limited to formin activation, as they can be rescued by an activated allele of Bni1p. This discrepancy might reflect a difference in strain background or a difference in growth conditions, such that the requirement for Rho3p in exocytosis does not impact the growth rate under the conditions used in this study.

A previous screen identified two high-copy suppressors of *rho3*Δ*rho4*Δ lethality, the highly conserved Rho family member Cdc42p and the Cdc42p-binding scaffold protein Bem1p (Matsui and Toh-e, 1992b). We found that overexpressed Cdc42p could activate the formins to generate actin cables in *rho3*Δ*rho4*Δ yeast and could specifically activate Bni1p in *rho3*Δ*bnr1*Δ yeast. The ability of Cdc42p to associate with the Bni1p RBD (Evangelista et al., 1997; Imamura et al., 1997) supports the possibility that Cdc42p might directly activate Bni1p.

The need to overexpress Cdc42p to rescue the *rho3*Δ*rho4*Δ lethality suggests that the normal role of Cdc42p is more restricted. Previous work demonstrated that Cdc42p plays an important role in organizing actin during bud emergence (Adams et al., 1990), and we found that the loss of Cdc42p function in six conditional *cdc42* alleles specifically compromised actin cable organization in unbudded cells, but cables appeared to be unperturbed in budded cells. Notably, the levels of actin cables did not appear to be diminished in the unbudded cells, suggesting that the formins were still activated, presumably by Rho3p and Rho4p. This suggests that Cdc42p plays a role in properly recruiting the active formins to the nascent bud site. In fact, previous results have demonstrated that Bni1p is unable to localize in the absence of Cdc42p function (Jaquenoud and Peter, 2000; Ozaki-Kuroda et al., 2001). The inability of *cdc42-ts* strains to organize actin cables in unbudded cells can account for two other phenotypes of the arrested cells, isotropic growth and depolarized cortical patches, because both of these phenotypes can also arise as a secondary consequence of loss of transport along actin cables (Pruyne et al., 1998;

Schott et al., 1999; Evangelista et al., 2002; Sagot et al., 2002a). The ability of Cdc42p to replace Rho3p and Rho4p when overexpressed may reflect a subtle role for Cdc42p in regulating formin activity for cable polarization under normal conditions.

The conserved RhoA homologue, Rho1p, makes an additional, independent contribution to the activation of the formins. Loss of Rho1p function at 37°C in *rho1-2* yeast eliminates both actin cable assembly by endogenous formins and cable-like filament accumulation stimulated by exogenous Bni1p. The expression of the activated Bni1pΔRBD construct restored cables to *rho1-2* yeast at the restrictive temperature, though the viability of the *rho1-2* yeast was not rescued. Despite the ability of Rho1p to bind the Bni1p RBD in two-hybrid assays (Kohno et al., 1996), our evidence suggests that Rho1p does not directly regulate the formin RBD–DAD interaction but acts indirectly through the Rho1p effector, Pkc1p. Yeast without functional Pkc1p also lost actin cables at 37°C, whereas expression of an activated kinase Pkc1p* restored cables to *rho1-2* yeast and permitted overexpressed Bni1p to generate ectopic filaments in *rho1-2* yeast. The Pkc1p/Rho1p dependence of formin activation was only observable at elevated temperatures, suggesting that some other change in the cell status under these conditions impinges on the formins such that a Pkc1p-dependent signal is required to maintain their activity.

The Pkc1p-dependent signaling did not appear to act through the downstream MAPK cascade, as cells lacking MAPK components retained actin cables even at 37°C, and overexpression of the MAPK Slt2p was unable to restore cables in *rho1-2* yeast or restore Bni1p function in *rho1-2* yeast. As further confirmation that formin function and MAPK signaling are distinct events, defects in formin function (e.g., *bni1*Δ) and loss of MAPK signaling (e.g., *slt2*Δ) have additive deleterious effects (Fujiwara et al., 1998). While this paper was in preparation, it was reported that Rho1p and formins are necessary for the assembly of the contractile ring at elevated temperatures (Tolliday et al., 2002). These findings are consistent with our results and suggest that the role of Rho1p in that process might also be indirect through Pkc1p.

Thus, we find that three distinct Rho-dependent signals regulate the proper function of the formins. Rho3p and Rho4p share a critical role in activating the formins. This activity cannot be replaced by Rho1p/Pkc1p signaling, either through Rho1p overexpression or through activation of Pkc1p. Cdc42p can replace Rho3p/Rho4p when overexpressed, but its role appears to normally be restricted to organizing the formins for bud emergence. Similarly, the requirements for Rho1p and Pkc1p appear to be restricted to conditions that trigger a cell wall stress response (i.e., 37°C). Thus, regulation of the formins may require multiple events, for example, recruitment of the formin, a specific phosphorylation of the RBD, and the binding of a Rho protein to the RBD. A similar phenomenon is seen with Pkc1p, where binding of Rho1 and phosphorylation by the redundant kinases Pkh1/2p contribute to Pkc1p activation (Inagaki et al., 1999). It will be interesting to determine whether the animal Rho1p homologue, RhoA, regulates formins in a similarly indirect manner through its Pkc1p-related kinase effectors, the PRKs (Amano et al., 1996; Watanabe et al., 1996; Vincent and Settleman, 1997).

The multiple inputs to Bni1p and Bnr1p activation link formin-mediated actin assembly into multiple essential regulatory pathways. Undoubtedly, other inputs contribute to the regulation of formin activity. For example, osmotic stabilization can also rescue viability of *rho3*Δ*rho4*Δ (Matsui and Toh-e, 1992b), suggesting that high osmolarity activates an alternative signaling pathway to bypass the requirement for Rho3p/Rho4p. The conservation of formins and their roles in regulating cytoskeletal organization suggests that these principles of multiple activating inputs are likely to be conserved across the eukaryotes.

Materials and methods

Yeast strains

All yeast strains used in this study are described in Table I. Single *RHO* deletion strains were obtained from American Type Culture Collection except the *rho3*Δ strains. The *rho3*Δ strains were generated by transformation of the diploid strain ABY500 with a PCR-derived kanamycin resistance knockout construct generated from template pUG6 (Guldener et al., 1996) using primers 5′-CTTTATAACAACAAGTTTTTCATTAAAATCCATAGTAGCACAAGGTAAAGGGAAACAAACCAGCTGAAGCTTCGT-3′ and 5′-TTTGTTTAGTCTCTCTTTTTGGGTTCTATTCTTTTGTGTGTGCGTCCGTGTCTTATTATAGCATAGGCCACTAGT-39, followed by sporulation and isolation of kanamycin-resistant segregants. The deletion was confirmed by PCR amplification of the *RHO3* locus using primers outside the deleted region.

Plasmids

p2529 (*CEN URA3 GAL1-BNI1-13myc*) and p2528 (*CEN URA3 GAL1-BNI1ΔRBD-13myc*) were as previously described (Evangelista et al., 2002). pPKC1* (YCp50-*PKC1*^{R398P}) was a gift from M. Hall (University of Basel, Basel, Switzerland) (Helliwell et al., 1998). pRS316-*RHO3V25* was a gift from P. Brennwald (University of North Carolina at Chapel Hill, Chapel Hill, NC) (Adamo et al., 1999). pRS313-*RHO3* (*CEN HIS3 RHO3*) was constructed by introduction of a 1.19-kb PCR-amplified fragment, including the *RHO3* open reading frame and 290-bp upstream and 200-bp downstream sequences, inserted between XbaI and SacI sites of pRS313 (Sikorski and Hieter, 1989). PCR was performed with primers 5′-GCTCTAGACATCCACTCCCAGG-3′ and 5′-TCGAGCTCACATGCTGGAGG-3′. To generate pYD518 (*CEN TRP1 GAL1-BNI1-13myc*), the *GAL1-10* promoter from p2529 was ligated between the EcoRI and BamHI sites of pRS314 (Sikorski and Hieter, 1989), and the 6.4-kb *BNI1-13myc* fragment from p2529 between the BamHI and NotI sites. To generate plasmids pYD125 (*CEN HIS3 BNI1ΔRBD-myc*) and pYD126 (*CEN LEU2 BNI1ΔRBD-myc*) for expression of *BNI1ΔRBD* behind the *BNI1* promoter, a 450-bp fragment of the *BNI1* promoter region was amplified by PCR with primers and cloned into the XhoI and BamHI sites of pRS313 (for pYD125) or pRS315 (for pYD126). Subsequently, a 5.1-kb *BNI1ΔRBD-13myc* fragment from p2528 was ligated between the BamHI and NotI sites. To generate pYD127 (*CEN LEU2 BNI1-DAD-C-terminus-myc*) for expression of the *BNI1* DAD and COOH terminus from the *BNI1* promoter, a PCR fragment encompassing the 480 3′-terminal bases of *BNI1* plus ~500 bp of additional *myc* and downstream sequences was amplified from p2529 using primers 5′-CGGGATCCATGGATAGGCGCGCTGTTAT-3′ and T7 and then cloned between the BamHI and NotI sites of pYD126, replacing *BNI1ΔRBD*. To generate pYD128 (*CEN LEU2 BNR1ΔRBD*) for expression of *BNR1ΔRBD* behind the *BNI1* promoter, a 2.9-kb *BNR1ΔRBD* fragment with EcoRI and XbaI sites was amplified by PCR with primers 5′-CGGAATTCATGAAGGCAAAAGATAGTCCCG-3′ and 5′-GCTCTAGAATCTGTCCATCTCCAAATC-3′, and the *BNI1* promoter fragment with XhoI and EcoRI sites was amplified by PCR with primers 5′-CCCTCGAGTTTTTGACGTGGAAGAG-3′ and 5′-CGGAATTCTTCCTTTCCTTCTCTTCC-3′, and then a three-piece ligation was performed to clone the *BNI1* promoter and *BNR1ΔRBD* between the XhoI and XbaI sites of pRS315. To construct pRS425-*CDC42* (*2μ LEU2 CDC42*), a 1.18-kb fragment, including the *CDC42* open reading frame and 380-bp upstream and 220-bp downstream sequences, was amplified by PCR with primers 5′-TCGAGCTCGCATTAAAGATGTCTTC-3′ and 5′-GCTCTAGAGCGTTGAAGAACATGG-3′ and ligated between the SacI and XbaI sites of pRS425 (Christianson et al., 1992). All PCR-derived constructs were verified by sequencing.

Table I. **Yeast strains used**

Name	Genotype	Source
ABY 500	*MATa/α ade2-101/− his3-Δ200/− leu2-3,112/− lys2-801/− ura3-52/−*	T. Huffaker[a]
ABY 165	*MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52*	T. Huffaker
ABY1854	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rho2Δ::KanR*	Research Genetics
ABY1590	*MATa ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52 rho3Δ::KanR*	This study
ABY1849	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rho4Δ::KanR*	Research Genetics
ABY1862	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rho5Δ::KanR*	Research Genetics
ABY1802	*MATa his3Δ1 met15Δ0 leu2Δ0 lys2Δ0 ura3Δ0 bni1Δ::KanR*	Research Genetics
ABY1680	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bnr1Δ::KanR*	Research Genetics
ABY1910	*MATa his3 leu2 lys2 ura3 rho3Δ::KanR rho4Δ::KanR [RHO3 URA3CEN]*	This study
ABY1913	*MATa his3 leu2 lys2 ura3 rho3Δ::KanR bnr1Δ::KanR*	This study
ABY1916	*MATa his3 leu2 ura3 rho3Δ::KanR bni1Δ::KanR*	This study
DDY1300	*MATa his3-Δ200 leu2-3,112 lys2-801am ura3-52 CDC42::LEU2*	K. Kozminski[b]
DDY1304	*MATa his3-Δ200 leu2-3,112 lys2-801am ura3-52 cdc42-101::LEU2*	K. Kozminski
DDY1326	*MATa his3-Δ200 leu2-3,112 lys2-801am ura3-52 cdc42-118::LEU2*	K. Kozminski
DDY1334	*MATa his3-Δ200 leu2-3,112 lys2-801am ura3-52 cdc42-123::LEU2*	K. Kozminski
DDY1338	*MATa his3-Δ200 leu2-3,112 lys2-801am ura3-52 cdc42-124::LEU2*	K. Kozminski
DDY1344	*MATa his3-Δ200 leu2-3,112 lys2-801am ura3-52 cdc42-129::LEU2*	K. Kozminski
ABY371	*MATα his4 ura3 gal2 cdc42-1*	C. Chan[c]
ABY1656	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics
ABY1588	*MATa ade2 his3 leu2 lys2 trp1 rho1::HIS3 ade3::RHO1 LEU2*	M. Hall
ABY1589	*MATa ade2 his3 leu2 lys2 trp1 rho1::HIS3 ade3::rho1-2 LEU2*	M. Hall
ABY1930	*MATα his3-Δ200 ura3-52 slt2Δ::KanR*	R. Collins[a]
ABY1933	*MATα leu2-3,112 trp1-1 ura3-52 his4 bck1Δ::URA3*	S. Henry[a]
DL123	*MATa his4 leu2-3,112 trp1-1 ura3-52 can1R pkc1Δ::LEU2 BCK1-20*	D. Levin[d]
DL245	*MATa his4 leu2-3,112 trp1-1 ura3-52 can1R BCK1-20*	D. Levin
DL102	*MATa/α his4/− leu2-3,112/− trp1-1/− ura3-52/− can1R/−*	D. Levin
DL519	*MATa/α his4/− leu2-3,112/− trp1-1/− ura3-52/− can1R/− pkc1Δ::LEU2/−*	D. Levin

[a]Cornell University.
[b]University of Virginia, Charlottesville, VA.
[c]University of Texas at Austin, Austin, TX.
[d]The Johns Hopkins University, Baltimore, MD.

Immunofluorescence microscopy

Cells were prepared, fixed, and stained using antibodies to actin, tropomyosin, and the myc epitope as previously described (Pruyne et al., 1998; Evangelista et al., 2002). Cells were categorized as unbudded, small budded (bud lengths <1/3 the length of the mother cell), medium budded (bud lengths <2/3 the length of the mother cell but ≥1/3 the length of the mother cell), or large budded (bud length ≥2/3 the length of the mother cell). For each assay, 100 cells of the indicated categories from asynchronous cultures were scored. When scoring for the presence of actin cables, cells of all categories were scored positive if cables were visible by eye, and negative if not. When scoring for the presence of polarized actin cables, cells of all categories were scored as positive if the majority of cables present in a cell were aligned along the axis of the cell or emanated from a nascent bud site. Cells were scored as negative for polarized actin cables if no cables were detectable or if half or more of the cables present in a cell were not aligned along the growth axis or associated with a bud site. When scoring for the accumulation of cable-like filaments in the bud, small- and medium-budded cells were scored positive if the stain in the bud showed a clearly visible increase in fluorescence beyond that seen in wild-type cells. Note that for the images shown in this paper, the intensity has been digitally reduced to allow dimmer portions of the displayed cells to be visible. The increased intensity of stain due to formin-stimulated accumulation of filaments is clearly discernible by eye when compared with controls. For the galactose induction experiments, midlog phase cultures grown in defined raffinose medium were induced by the addition of 2% galactose for 2 h.

Western blotting

Cells were grown up to midlog phase in defined raffinose medium and induced by the addition of 2% galactose for 2 h. Samples were equalized based on OD_{600}, and extracts were isolated as previously described (Horvath and Riezman, 1994) and resolved by SDS-PAGE. Blots were probed with 9E10 (anti-myc) or B28 (anti–yeast actin).

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