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Rho1 Directs Formin-Mediated Actin Ring Assembly during Budding Yeast Cytokinesis

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Summary

In eukaryotic cells, dynamic rearrangement of the actin cytoskeleton is critical for cell division. In the yeast *Saccharomyces cerevisiae*, three main structures constitute the actin cytoskeleton: cortical actin patches, cytoplasmic actin cables, and the actin-based cytokinetic ring [1–4]. The conserved Arp2/3 complex and a WASP-family protein mediate actin patch formation, whereas the yeast formins (Bni1 and Bnr1) promote assembly of actin cables [5–9]. However, the mechanism of actin ring formation is currently unclear. Here, we show that actin filaments are required for cytokinesis in *S. cerevisiae*, and that the actin ring is a highly dynamic structure that undergoes constant turnover. Assembly of the actin ring requires the formin-like proteins and profilin, but is not Arp2/3-mediated. Furthermore, the formin-dependent actin ring assembly pathway is regulated by the Rho-type GTPase Rho1 but not Cdc42. Finally, we show that the formins are not required for localization of Cyk1/Iqg1, an IQGAP-like protein previously shown to be required for actin ring formation, suggesting that formin-like proteins and Cyk1 act synergistically but independently in assembly of the actin ring.

Results and Discussion

The actomyosin-based contractile ring is critical for cytokinesis in diverse eukaryotic organisms. Previous studies demonstrated that in budding yeast, a ring containing actin and myosin II assembles at the division site and exhibits a contraction-like size change during cytokinesis [3, 4]. However, the role and the mechanism of assembly of actin filaments (F-actin) within this ring remain unclear. In particular, it was reported that cells can undergo division in the presence of the actin polymerization inhibitor Latrunculin A (LAT-A), leading to the conclusion that F-actin is not required for cell division in budding yeast [4, 10]. However, it is not clear whether LAT-A has no effect or an incomplete effect on cytokinesis. To quantitatively assess the role of F-actin in cytokinesis, wild-type cells were arrested just prior to mitosis using nocodazole, when bud growth is complete but the F-actin ring has not yet assembled [3, 11]. After 3–4 hr, the culture was split into two and released into media containing either 100 μM LAT-A or the control solvent DMSO. Whereas cell division and normal rebudding were observed in the DMSO control culture, 90% of cells released into LAT-A-containing media arrested as large-budded cells with divided nuclei (data not shown). This result indicates a cell division defect in the presence of LAT-A. The effectiveness of LAT-A treatment was confirmed using rhodamine phalloidin staining to visualize F-actin (Figure 1A, right panels). To determine whether this defect was due to a failure in cytokinesis or in cell separation, cells fixed at various times after release from nocodazole arrest were treated with zymolyase to remove the cell wall and counted. After 6 hr, the cell number increased ~9 fold in the control culture, whereas no increase in cell number was observed in the presence of LAT-A and cells remained large-budded (Figure 1A). This result indicates that cells exhibit a failure in cytokinesis in the presence of LAT-A, suggesting that F-actin is required for cytokinesis in budding yeast.

Next, we wanted to understand how F-actin is assembled into the contractile ring. There are two potential mechanisms: (1) preformed actin filaments (e.g., those in actin cables or patches) are stabilized, recruited to the bud neck, and coalesced into a ring; or (2) F-actin is locally nucleated at the bud neck to form a ring. To help distinguish between these possibilities, we first tested if the actin filaments in the ring are dynamic. We took advantage of the fact that removal of Tem1, a small GTPase required for mitotic exit and cytokinesis, results in the arrest of cells in late mitosis with actin rings [12, 13]. An overnight culture in which the sole copy of *TEM1* is under the control of the *GAL1* promoter [14] was shifted from galactose-containing media to glucose-containing media to turn off the production of Tem1. After 4–5 hr, 95% of the cells were large-budded with divided nuclei and an actin ring at the bud neck. Addition of LAT-A, but not the control solvent DMSO, resulted in rapid disassembly of the actin rings (Figure 1B). After 1 min, only 8% of the cells had an actin ring, and no rings were observed after 5 min. This is comparable to the kinetics of actin filament turnover described previously for actin patches and cables [10, 15]. The effect of LAT-A was fully reversible, with 82% of cells having reassembled an actin ring within 60 min after LAT-A removal (Figure 1B). These results indicate that the actin ring is a dynamic structure in which filaments undergo rapid turnover, suggesting that activities that promote actin polymerization must also reside at the bud neck.

Work in recent years has identified two major pathways for the nucleation of actin filaments in yeast. The Arp2/3 complex and the WASP-like protein Bee1/Las17 are required for the generation of actin patches [5–7], whereas the formin family proteins, Bni1 and Bnr1, are required for actin nucleation to form cables [8, 9, 16, 17]. We showed previously that yeast strains lacking subunits of the Arp2/3 complex can still form actin rings and exhibit no defect in cytokinesis [18]. This result was further confirmed using a temperature-sensitive *arp3* allele [6] (data not shown), and suggests that actin ring assembly is independent of the Arp2/3 complex. This prompted us to test if the formin-dependent actin assembly mechanism is required for actin ring formation.

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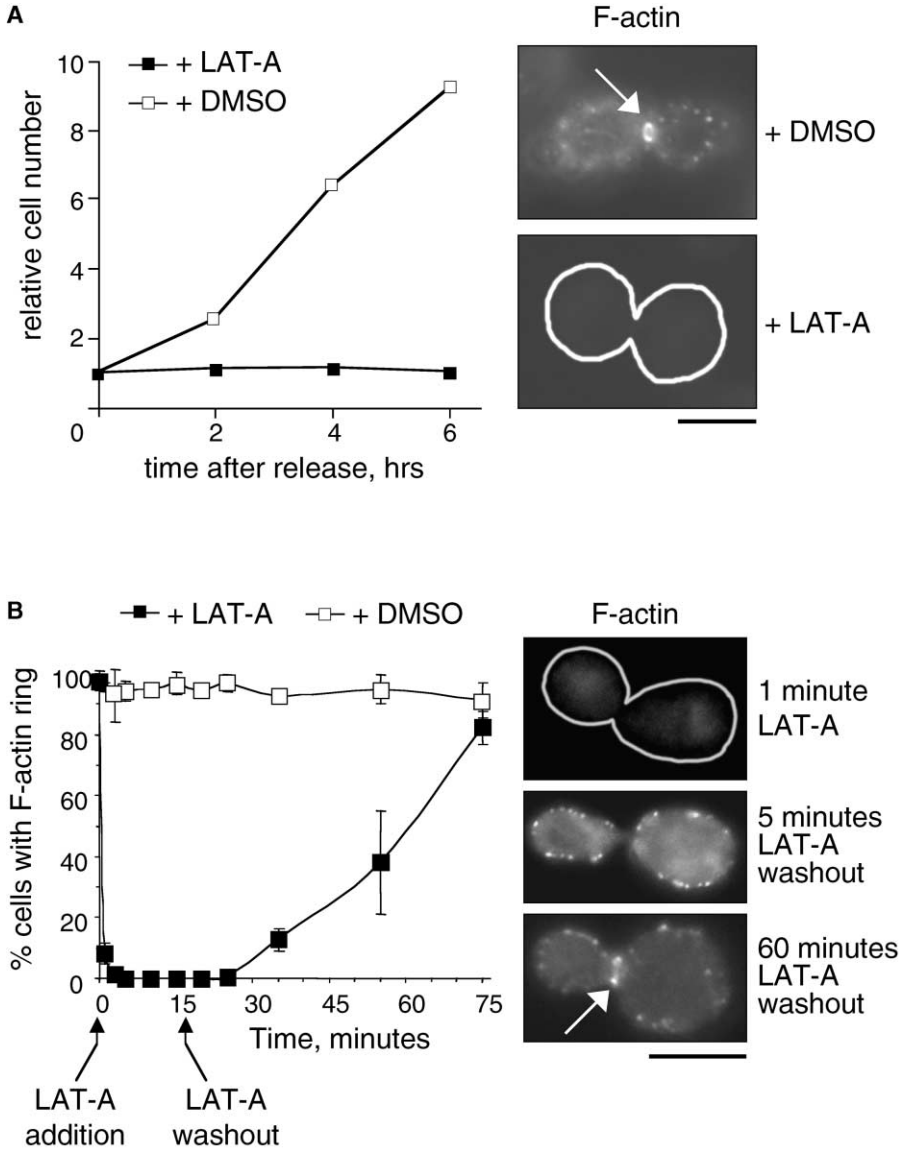

Figure 1. Actin Filaments Are Required for Cytokinesis and Are Dynamic within the Cytokinetic Ring

(A) Wild-type cells (RLY261) were cultured overnight in Yeast extract, Peptone, Dextrose (YPD) at 25°C. Cells were arrested with 10 μg/ml nocodazole for 3–4 hr, following which cells were washed 3 times with sterile water and resuspended in YPD. The cells were split into 2 cultures, to which either LAT-A (100 μm) or DMSO was added. Aliquots of cells were fixed with 5% formaldehyde at 0, 2, 4, and 6 hr after addition of LAT-A, treated with zymolyase (0.2 mg/ml), and counted on a hemocytometer. The resultant cell concentration at each time point was divided by that at time 0 to give relative cell number. The graph shown is a representative dataset from three independent experiments. Aliquots of zymolyase-treated cells from the 2 hr time point were also stained with rhodamine phalloidin to determine the effectiveness of LAT-A treatment (right panels). Note that the cell outline of the LAT-A treated cell has been drawn in for clarity.

(B) *GAL-TEM1 tem1*Δ cells (RLY635) were grown overnight at 30°C in galactose-containing media (YPGR) and arrested by switching to glucose-containing media (YPD). After 4–5 hr, the culture was split into two and grown at 30°C in the presence of either LAT-A (100 μm) or DMSO. Aliquots of cells were fixed at 0, 1, 3, 5, 10, and 15 min after the addition of LAT-A. Both cultures were then washed 3 times with sterile water, resuspended in YPD, and aliquots fixed at 5, 10, 20, 40, and 60 min after LAT-A washout. Fixed cells were processed for actin ring visualization as above. At least 200 large-budded cells at each time point were scored for the presence of an actin ring. Data shown are the average from three independent experiments. Representative images of rhodamine phalloidin stained cells are shown to the right of the graph. Note that the outline of the cell treated with LAT-A for 1 min has been drawn in for clarity. Scale bars: 5 μm. Arrow denotes the actin ring.

Because double deletions of the formin-like proteins, Bni1 and Bnr1, are lethal, we utilized a strain in which both formins have been deleted but viability is conferred by a temperature-sensitive allele of *BNI1* (*bni1-ts bnr1*Δ) [19]. Wild-type and *bni1-ts bnr1*Δ cells were arrested in M phase using nocodazole, just before the time of actin ring assembly. This ensures that the formin-dependent function in bud formation is complete. Cells were then shifted to the nonpermissive temperature for 1 hr and released from arrest at the nonpermissive temperature. Cells were fixed in formaldehyde at various times following release, and processed for visualization of F-actin

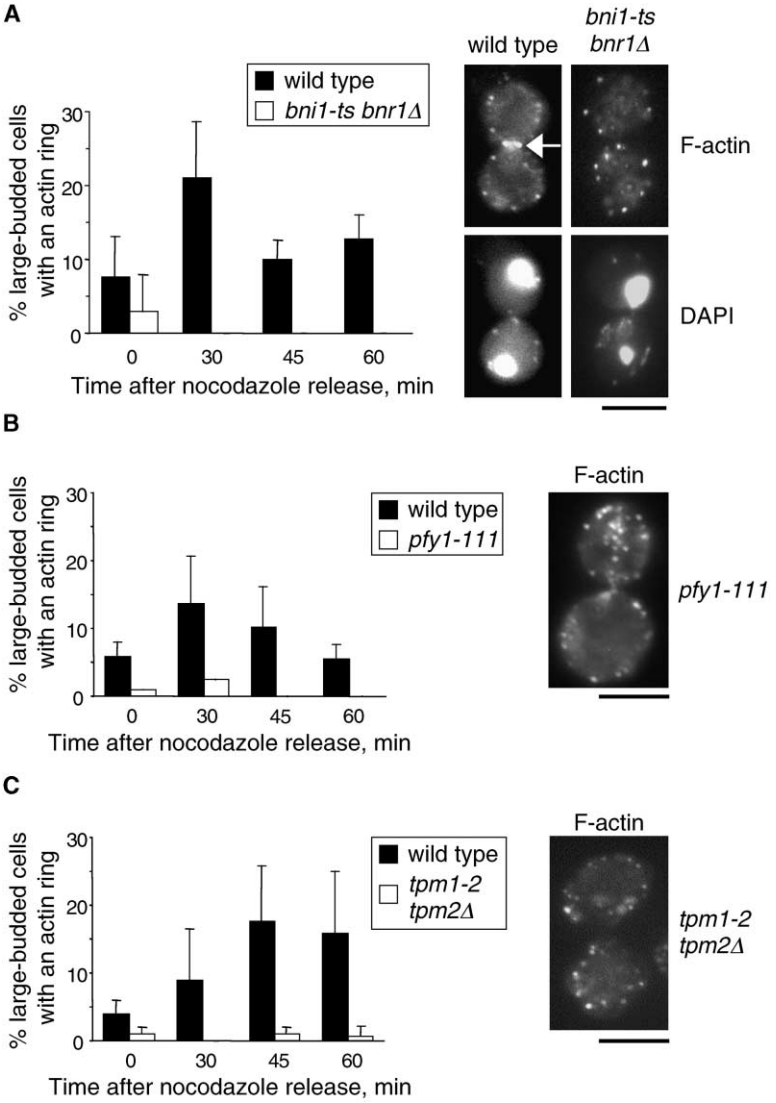

Figure 2. Components of the Formin-Mediated Actin Assembly Pathway Are Required for Actin Ring Formation

(A) Wild-type (RLY1670) and *bni1-ts bnr1*Δ (RLY1158) cells were grown overnight at 25°C and arrested with 10 μg/ml nocodazole. After 3–4 hr, cells were shifted to 37°C for 1 hr. Cells were washed 3 times with sterile water, resuspended in prewarmed YPD, and grown at 37°C. Aliquots of cells were fixed at 0, 30, 45, and 60 min after the release, and processed for actin ring visualization, as in Figure 1. Representative images of rhodamine phalloidin- and DAPI-stained cells from the 30 min time point are shown to the right of the graph.
(B) Wild-type (RLY211) and *pfy1-111* (RLY205) cells were grown and processed as in (A).
(C) *TPM1 tpm2*Δ (RLY966) and *tpm1-2 tpm2*Δ (RLY965) cells were grown and processed as in (A). In each case, at least 200 large-budded cells at each time point were scored for the presence of an actin ring. Data shown are the average from three independent experiments. Arrow denotes the actin ring. Scale bar: 5 μm.

structures using rhodamine-labeled phalloidin. Wild-type cells exhibited a peak of actin rings 30 min after release from nocodazole arrest (Figure 2A), with 21% of large-budded cells having an actin ring (the peak of actin ring formation is low because the actin ring is highly transient). In contrast, the peak of actin ring formation was not observed in *bni1-ts bnr1*Δ cells at the nonpermissive temperature. To ensure that the observed defect in actin ring formation was not a secondary consequence of spindle misalignment due to the *bni1* mutation [20, 21], cells were also stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize the chromosomes. Thirty minutes after release from arrest, both wild-type and *bni1-ts bnr1*Δ cells showed proper segregation of chromosomes between the mother and bud (Figure 2A), confirming correct alignment of the mitotic spindle. These data suggest that the formins are critical for actin ring assembly.

To assess whether the formins are also required for cytokinesis, aliquots of the fixed cells described above were treated with zymolyase to remove the cell wall, and cells were scored according to bud morphology (large-budded, unbudded, or small-budded). After two hours following release from nocodazole arrest, the number of large-budded wild-type cells had decreased by 46%, with a concomitant increase in unbudded and small-budded cells, indicating successful completion of cytokinesis (Table 1). In contrast, only a 4% decrease in large-budded cells was observed with *bni1-ts bnr1*Δ cells, indicating that the formins are required for completion of cytokinesis.

Formin-mediated actin cable assembly also requires profilin and tropomyosin [8, 9]. Profilin (Pfy1) is a regulator of actin polymerization and binds directly to Bni1 [22]. Tropomyosins (Tpm1 and Tpm2) are F-actin binding proteins that are required for actin cable stability, and localize to the bud neck in large-budded cells [15, 23]. To determine whether these factors are also required for actin ring formation, we repeated the experiment described above using temperature-sensitive alleles of profilin (*pfy1-111*) [24] and tropomyosin (*tpm1-2 tpm2*Δ) [15]. Cells deficient in either tropomyosin or pro-

Table 1. Effects of Various Mutations on the Completion of Cytokinesis

Strain	Large-budded (% Decrease)	Unbudded (% Increase)	Small-budded (% Increase)
BNI1	46 ± 6	43 ± 15	8 ± 2
bni1 ts bnr1Δ	4 ± 4	2 ± 2	3 ± 1
RHO1	54 ± 12	41 ± 6	22 ± 8
rho1-2	10 ± 5	14 ± 4	−4 ± 0.9[a]
CDC42	43 ± 6	22 ± 3	20 ± 6
cdc42-1	34 ± 5	32 ± 0.4	3 ± 3
RHO1 + BNI1	50 ± 0.4	27 ± 12	23 ± 12
rho1-2 + BNI1	49 ± 10	26 ± 18	23 ± 8

BNI1 (RLY1670), *bni1-ts bnr1Δ* (RLY1158), *RHO1* (RLY1602), *rho1-2* (RLY1603), *CDC42* (RLY1), *cdc42-1* (RLY171), *RHO1 GFP-BNI1* (RLY1718), and *rho1-2 GFP-BNI1* (RLY1719) cells were arrested and released as described in Figure 2. Aliquots of cells were fixed at 0, 1, and 2 hours after the release, treated with zymolyase (0.2 mg/ml) to remove the cell wall, and scored for bud morphology (large-budded, unbudded, and small-budded). The percentage of each population at the 2 hr timepoint was subtracted from that at the 0 timepoint to determine the percent decrease or increase in each population. In each case, at least 200 cells at each time point were counted. Data shown are the average from three independent experiments.

[a] A decrease in this population is seen instead of an increase.

filin also displayed significant defects in actin ring assembly (Figures 2B and 2C). Whereas wild-type cells displayed a peak of actin rings 30–45 min after release from nocodazole arrest (*PFY1*: 14% of large-budded cells had an actin ring; *TPM1 tpm2Δ*: 18%), only 2.5% of *pfy1-111* and 1% of *tpm1-2 tpm2Δ* cells showed actin rings. Together, these results indicate that components of the formin-mediated actin assembly pathway are required for actin ring formation.

Formins have been implicated as downstream effectors of Rho-type GTPases that link cellular signaling pathways to changes in the actin cytoskeleton [25]. Bni1 has been shown to interact with two Rho-type GTPases, Rho1 and Cdc42 [22, 26]. To determine if either GTPase is required for actin ring formation, temperature-sensitive alleles of *RHO1* (*rho1-2*) [27] and *CDC42* (*cdc42-1*) [28] were subjected to the temperature-shift actin ring assembly assay described above. Cells lacking functional Rho1 were deficient in actin ring formation, as compared to cells expressing the wild-type protein (Figure 3A). A peak in actin ring formation was seen in wild-type cells 30 min after release from nocodazole arrest, with 15% of large-budded cells showing actin rings, whereas only 3.5% of *rho1-2* large-budded cells assembled actin rings at the restrictive temperature. Both *RHO1* and *rho1-2* cells were also assayed for completion of cytokinesis, as described above. *rho1-2* cells were unable to complete cytokinesis (Table 1): after 2 hr, only a 10% decrease was observed in the number of large-budded *rho1-2* cells, whereas the number of large-budded wild-type cells decreased by 54%. These results indicate that Rho1 is required for actin ring assembly and the completion of cytokinesis. In contrast, cells with disrupted Cdc42 did not appear to have significant defects in actin ring formation (Figure 3B) or completion of cytokinesis (Table 1). Both wild-type and *cdc42-1* cells showed a peak in actin ring formation at 30 min after release from nocodazole arrest, with 17% of wild-type cells and 12% of *cdc42-1* cells showing an actin ring. Similar results were also obtained using a dominant-negative allele of Cdc42 ($cdc42^{D118A}$) under control of the *GAL1* promoter [19] (our unpublished data).

Rho family GTPases are thought to regulate the formin-family proteins by converting the formins from an autoinhibited conformation to an active conformation, through binding to an N-terminal region of the formins [29]. To test if the defect in actin ring formation observed in *rho1-2* cells was due to disruption of Bni1 function, we asked whether the *rho1-2* defect could be rescued by overproduction of Bni1. *RHO1* and *rho1-2* cells were transformed with a functional plasmid bearing full-length Bni1 N-terminally tagged with green fluorescent protein (GFP) under control of the *ACT1* promoter [30]. This tagged protein, when expressed in cells, is thought to be weakly activated [8]. *RHO1* and *rho1-2* cells with or without GFP-Bni1 were assayed as above for actin ring formation. As shown in Figure 3C, the Bni1 construct can rescue the actin ring formation defects observed in *rho1-2* cells. *RHO1*, *RHO1 + BNI1*, and *rho1-2 + BNI1* cells, but not *rho1-2* cells, showed a peak of actin ring formation at 30 min after release from nocodazole arrest, with an average of 15% of large-budded cells with actin rings in both *RHO1 + BNI1* and *rho1-2 + BNI1* cultures. Similarly, *RHO1 + BNI1* and *rho1-2 + BNI1* cells were both capable of completing cytokinesis at comparable rates (Table 1). Together, these results demonstrate that Bni1 functions downstream of Rho1 to induce assembly of the actin ring during cytokinesis.

The above data strongly suggest that the Rho1-Bni1 pathway is critical for assembly of actin filaments in the contractile ring in budding yeast. We and others have previously shown that an IQGAP like protein, Cyk1, localizes to the bud neck immediately prior to and is required for actin ring formation [3, 11]. Therefore, we tested whether the *bni1-ts* mutation prevents actin ring formation by affecting Cyk1 localization. Wild-type and *bni1-ts bnr1Δ* cells carrying Cyk1-GFP were arrested with nocodazole, before the time of Cyk1 localization to the bud neck. Upon release from arrest, cells were fixed at various times and the presence of Cyk1-GFP at the bud neck was determined using fluorescence microscopy. Additionally, cells were processed for visualization of both Cyk1-GFP and F-actin using α-GFP indirect immunofluorescence and rhodamine phalloidin staining. Wild-type and *bni1-ts bnr1Δ* cells exhibited a peak of Cyk1 rings 30 min after release from arrest (Figure 4),

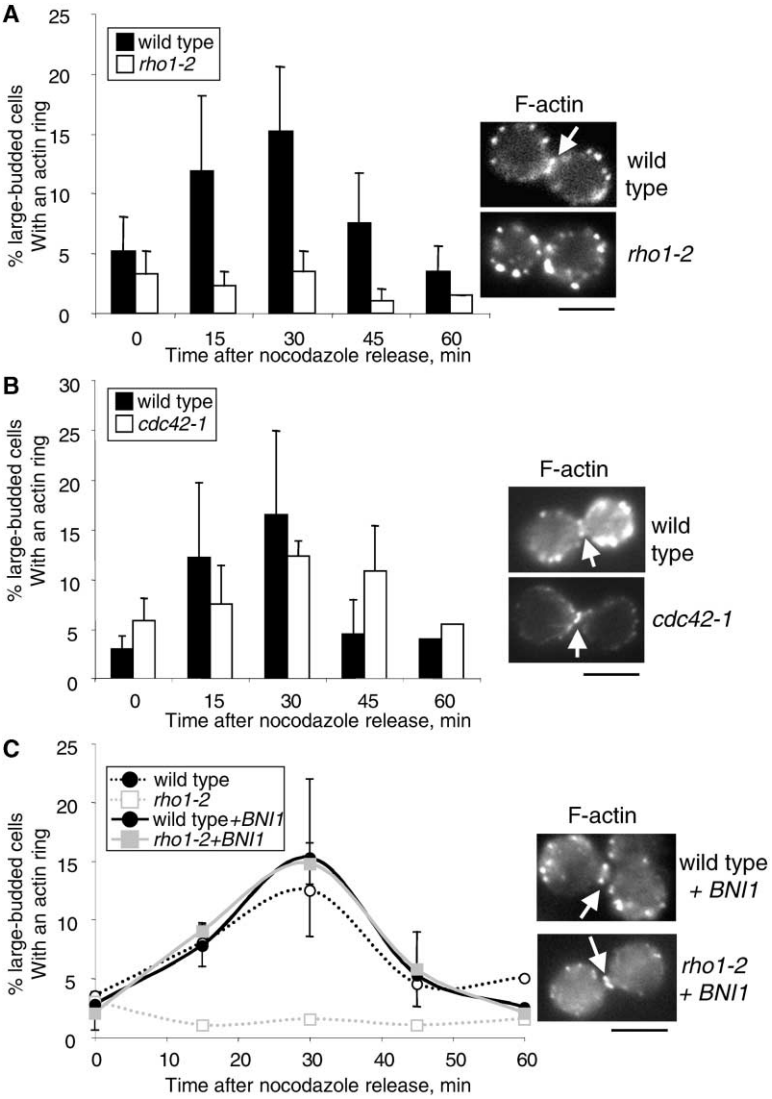

Figure 3. Rho1 Is an Upstream Regulator of Bni1 Required for Actin Ring Formation

(A) Wild-type (RLY1602) and *rho1-2* (RLY1603) cells were grown overnight at 25°C and arrested with 10 μg/ml nocodazole. After 3–4 hr, cells were shifted to 37°C for 1 hr. Cells were washed 3 times with sterile water, resuspended in prewarmed YPD, and grown at 37°C. Aliquots of cells were fixed at 0, 15, 30, 45, and 60 min after the release, and processed for actin ring visualization, as in Figure 1. Representative images of rhodamine phalloidin stained cells from the 30 min time point are shown to the right of the graph.

(B) Wild-type (RLY1) and *cdc42-1* (RLY171) cells were grown and processed as in (A).

(C) Wild-type (RLY1602), *rho1-2* (RLY1603), wild-type + *GFP-BNI1* (RLY1718), and *rho1-2 GFP-BNI1* (RLY1719) cells were grown and processed as in (A). In each case, at least 200 large-budded cells at each time point were scored for the presence of an actin ring. Data shown are the average from five (A), three (B), or two (C) independent experiments. Arrow denotes the actin ring. Scale bar: 5 μm.

with ~23% of large-budded cells showing Cyk1-GFP at the bud neck, suggesting that the localization of Cyk1 is unaffected by the *bni1-ts* mutation.

The above results suggest that Bni1 functions in a pathway parallel to, but not redundant with, Cyk1 in actin ring formation. Three additional observations suggest that the role for Bni1 and its associated proteins in actin ring formation is direct. First, Bni1 is localized at the bud neck during anaphase [30]. Second, recent work showed that Bni1 can nucleate actin filaments in vitro [8, 16]. Third, we have found that bud neck-directed actin cables are not visible in ~97% of the cells that contain an actin ring, and that the peak of bud neck-directed cables occurs at 45–60 min after mitotic release in wild-type cells, whereas a peak of actin rings is observed 30 min after release. Furthermore, actin ring assembly is not affected by the *myo2-66* mutation [31] (data not shown). These findings argue against the possibilities that actin ring formation results from either coalescence of actin cables at the bud neck, as described in fission yeast [32], or transport of actin ring components along the cables, both of which would be consistent with the requirement for formins in actin ring formation.

Actin nucleation by the formin-dependent machinery could result in the creation of a pool of short actin filaments at the bud neck that can form either the actin ring or actin cables, depending upon differing accessory proteins. Whereas a transport function requires that the actin cables contain parallel bundles of actin filaments, contractile ring actin should in theory be aligned in an antiparallel configuration. It is possible that the presence of Cyk1, the mammalian homologs of which possess an actin crosslinking activity [33, 34], as a ring at the bud neck is critical for formation of the contractile actin ring.

Important roles for the formin and Rho families of proteins in cytokinesis have been reported for diverse organisms [35, 36]. In particular, Cdc12, a formin-like protein, was shown to be required for actin ring formation in fission yeast [37]. It is thought that a particle that contains Cdc12 moves along actin and microtubules to the site of cell division and spreads out into a ring. Upstream regulators of Cdc12 in this function have not been reported. In *C. elegans* and mammalian cells, Rho

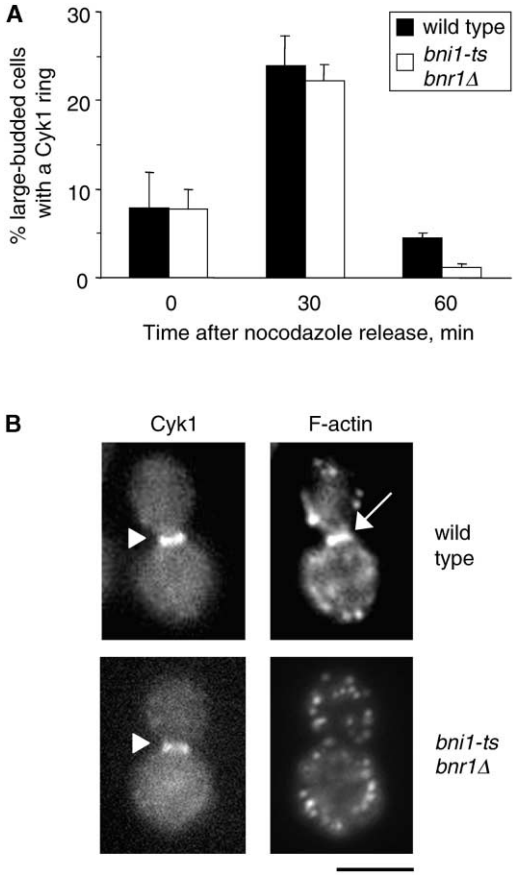

Figure 4. Cyk1 Localization Is Independent of the Formins

Wild-type (RLY1756) and *bni1-ts bnr1*Δ (RLY1757) cells in which Cyk1 is C-terminally tagged with GFP at the endogenous locus were grown in YPD at 25°C overnight. Cells were arrested with 10 μg/ml nocodazole for 3–4 hr, and then shifted to 37°C for 1 hr. Cells were washed 3 times with sterile water, resuspended in prewarmed YPD, and grown at 37°C. Aliquots of cells were fixed at 0, 30, and 60 min after the release, and large-budded cells were scored visually for Cyk1-GFP fluorescence at the bud neck (A). At least 200 large-budded cells at each time point were scored, and data shown are the average from three independent experiments. (B) Aliquots of fixed cells were zymolyase-treated and double-stained for Cyk1-GFP and F-actin, using α-GFP indirect immunofluorescence and rhodamine phalloidin, respectively. Representative images are shown. Arrow denotes actin ring; arrowhead denotes Cyk1 ring. Scale bar: 5 μm.

is known to function in the regulation of myosin contractility through effector kinases [38–41]. An additional requirement of Rho in actomyosin ring formation was first suggested by the phenotype of a Rho exchange factor mutant in *Drosophila* [42]. The data presented in this work provide direct evidence that Rho stimulates actin polymerization in the contractile ring through regulation of formin-family proteins. It is highly likely that this pathway of actin ring assembly is conserved in many eukaryotes.

Supplementary Material

Supplementary Material is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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