Yeast Formins Bni1 and Bnr1 Utilize Different Modes of Cortical Interaction during the Assembly of Actin Cables[□] [▽]

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The budding yeast formins Bni1 and Bnr1 control the assembly of actin cables. These formins exhibit distinct patterns of localization and polymerize two different populations of cables: Bni1 in the bud and Bnr1 in the mother cell. We generated a functional Bni1-3GFP that improved the visualization of Bni1 in vivo at endogenous levels. Bni1 exists as speckles in the cytoplasm, some of which colocalize on actin cables. These Bni1 speckles display linear, retrograde-directed movements. Loss of polymerized actin or specifically actin cables abolished retrograde movement, and resulted in depletion of Bni1 speckles from the cytoplasm, with enhanced targeting of Bni1 to the bud tip. Mutations that impair the actin assembly activity of Bni1 abolished the movement of Bni1 speckles, even when actin cables were present. In contrast, Bnr1-GFP or 3GFP-Bnr1 did not detectably associate with actin cables and was not observed as cytoplasmic speckles. Finally, fluorescence recovery after photobleaching demonstrated that Bni1 was very dynamic, exchanging between polarized sites and the cytoplasm, whereas Bnr1 was confined to the bud neck and did not exchange with a cytoplasmic pool. In summary, our results indicate that formins can have distinct modes of cortical interaction during actin cable assembly.

INTRODUCTION

Actin cables are filamentous structures that assemble at the cell cortex, emanating from sites of polarized growth (Pruyne *et al.*, 2004b; Moseley and Goode, 2006). These cables guide the polarized movement of secretory vesicles and organelles and therefore reinforce the polarity established by bud site selection cues and the small G protein Cdc42 (Pruyne and Bretscher, 2000a,b). Once the bud site is selected, budding yeast actin cables are assembled by the formin proteins Bni1 and Bnr1 (Pruyne *et al.*, 2002; Sagot *et al.*, 2002a), directing Myo2-dependent movement toward the bud (Pruyne *et al.*, 2004a). Several components of actin cables have been identified, including the actin cross-linking proteins fimbrin (Sac6) and Abp140, and the tropomyosins Tpm1 and Tpm2 (reviewed in Pruyn *et al.*, 2004b; Moseley and Goode, 2006). Actin cables are highly dynamic structures that disassemble rapidly after treatment with latrunculin A, an actin monomer binding drug (Ayscough *et al.*, 1997; Karpova *et al.*, 1998). Electron microscopy in fission yeast demonstrated that actin cables are bundles of short actin filaments, primarily oriented with their fast growing, barbed ends toward the sites of assembly (Kamasaki *et al.*, 2005).

Formins are members of a highly conserved family of proteins characterized by the presence of the FH2 domain, which is necessary for actin filament assembly in vitro and in vivo (Evangelista *et al.*, 1997, 2002; Pruyn *et al.*, 2002; Sagot *et al.*, 2002a,b; reviewed in Faix and Grosse, 2006; Kovar, 2006). The FH2 domain nucleates actin filaments and enables barbed end elongation in the presence of capping protein (Zigmond *et al.*, 2003; Harris *et al.*, 2004; Moseley *et al.*, 2004; Kovar, 2006). Further, formins attach processively to the barbed end (Pruyn *et al.*, 2002; Higashida *et al.*, 2004; Kovar and Pollard, 2004; Romero *et al.*, 2004); thus they can generate linear arrays of actin filaments, such as actin cables or the cytokinetic actin ring (Kovar, 2006). Formins are also characterized by the presence of a proline-rich FH1 domain, which binds profilin (Chang *et al.*, 1997; Evangelista *et al.*, 1997; Watanabe *et al.*, 1997), a protein that facilitates actin subunit delivery to the barbed ends of formin-capped actin filaments (Sagot *et al.*, 2002b; Kovar, 2006).

Cells contain multiple formin isoforms; in budding yeast, the formins Bni1 and Bnr1 control the assembly of actin cables. Although deletion of either Bni1 or Bnr1 is not significantly deleterious to cell growth (Kohn *et al.*, 1996; Imamura *et al.*, 1997), loss of both genes is lethal (Ozaki-Kuroda *et al.*, 2001). Bni1 nucleates actin cables that originate in the bud and Bnr1 polymerizes actin cables at the bud neck (Pruyn *et al.*, 2004a). Budding yeast formins are also essential for cytokinetic actin ring assembly (Tolliday *et al.*, 2002; Yoshida *et al.*, 2006). In vitro actin assembly assays demonstrated that Bnr1 is ~10-fold more potent as an actin nucleator than Bni1. Second, Bnr1 has a greater processive capping activity than Bni1; Bnr1 can protect the barbed ends of actin filaments from the activity of capping protein at lower concentrations than Bni1. These differences in activity between Bnr1 and Bni1 suggest that Bnr1 has an increased affinity for actin filament ends. Finally, Bnr1 can bundle actin filaments in vitro, as judged by sedimentation and electron microscopy. Thus, Bnr1, at least at high concentra-

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Abbreviations used: CAR, cytokinetic actin ring.

tions, may also bind to the sides of actin filaments (Moseley and Goode, 2005). Therefore, Bni1 and Bnr1 have quantitatively distinguishable activities in vitro and nucleate different populations of cables in vivo. Hence, studying formins in budding yeast offers a unique advantage for correlating existing in vitro data with in vivo dynamics.

Cable assembly occurs in the vicinity of the bud cortex and bud neck (Yang and Pon, 2002; Pruyne *et al.*, 2004a). Because these sites of assembly coincide with the localization of the formins Bni1 and Bnr1 (Kikyo *et al.*, 1999; Ozaki-Kuroda *et al.*, 2001), one appealing idea has been that formins anchor actin cables at the cortex (Pruyne *et al.*, 2002, 2004b; Evangelista *et al.*, 2003; Zigmond, 2004). An implicit feature of this anchoring model is that formins would be relatively stably associated with the cell cortex; persisting at least for the lifetime of an actin cable. Alternatively, because actin cables are discontinuous filaments and because formins protect the barbed end from the activity of capping protein, Bni1 and/or Bnr1 may transiently associate with the cortex and could in fact incorporate into actin cables.

We have generated new reagents to visualize Bni1 and Bnr1 at endogenous levels. In addition to localization at sites of polarized growth, Bni1-3GFP was visualized as speckles in the cytoplasm, which sometimes colocalized with actin cables. Strikingly, live-cell imaging demonstrated that Bni1 is highly dynamic: Bni1-3GFP speckles display linear, retrograde-directed movements, opposite to the direction of Myo2-dependent transport. Bni1-3GFP speckles moved at rates similar to the rate of cable extension. Further, we found that the retrograde movement of Bni1 depends on actin polymerization and the actin binding interaction of Bni1. Fluorescence recovery after photobleaching (FRAP) experiments showed that Bni1 turned over rapidly at both the bud tip and the bud neck. By contrast, Bnr1 is relatively static: Bnr1 does not exchange with a cytoplasmic pool and can only diffuse slowly within the bud neck region. In summary, our results indicate that formins can have distinct modes of cortical interaction during actin cable assembly.

MATERIALS AND METHODS

Strains and Plasmids

Media and genetic techniques were as previously described (Rose *et al.*, 1990). A Bni1 fragment consisting of the last 762 base pairs (bp) without the stop codon was cloned into the XhoI/BamHI sites of PB1963 or PB1964, which contain three tandem copies of green (GFP) or yellow fluorescent protein (YFP), respectively, in pRS305 or pRS306. Both the *BNI1-3GFP* and *BNI1-3YFP* plasmids were digested with BssHII to integrate the plasmid at the native *BNI1* locus. The Abp140 fluorescent constructs were generated in a similar manner, using the XhoI/PstI sites of PB1963 or PB1965. The *ABP140-3GFP* and *ABP140-3CFP* plasmids were digested with NdeI to integrate into the native *ABP140* locus. 3GFP-BNR1 was generated by the following method: 3GFP sequence was amplified by PCR from PB1543 and was inserted into the AflIII and NcoI sites of PB1683, which created a N terminal 3GFP tag under the control of the BNR1 promoter. *BUD6-RFP-dimer* was generated by inserting the last 963 bp of Bud6 (minus the stop codon) into the NotI/BamHI sites of PB2012. RFP-dimer in pRS306. *BNR1-GFP* and *MYO1-CFP* strains were generated as previously described (Longtine *et al.*, 1998) with a Gly-Gly-Ser-Gly-Gly-Ser linker.

Each construct was tested for complementation of the null phenotype, where appropriate. The *BNI1-3GFP* construct was functional, as it shows no defects in *bni1Δ* strains; this strain also has normal actin cable staining, suggesting that the construct is not hyperactivated. *BNR1-GFP* was functional as it supports viability in *bni1Δ* strains. None of these constructs affected growth rate or cell morphology. 3GFP-BNR1 was functional as it complements temperature-sensitive growth defect of *bni1-1 bnr1Δ*. This construct also did not show an apparent toxic effect on cell growth or actin cable organization. Transformants were screened for correct integration by PCR. See Supplementary Table 1 for complete details on strains and plasmids; additional details are available upon request.

The Bni1 FH2 domain mutant, Bni1^{K1601A}, was generated using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) with PB1032

serving as the template. After sequencing, the mutant construct was cloned into the XhoI/NheI sites of PB1993 generating PB2501. PB2501 was transformed into *BNR1/bnr1Δ* heterozygous diploid strains. The plasmid was digested with StuI to integrate the plasmid into the native *BNI1* locus. The correct integration was confirmed by PCR and the introduction of the FH2 domain mutation was verified by direct sequencing of genomic DNA spanning the integration site. Double mutants were then identified by tetrad analysis; the temperature-sensitive phenotype was observed in multiple double mutant strains.

Microscopy

Cells were imaged using a Zeiss 200M inverted or a Zeiss AX10 microscope (Carl Zeiss, Thornwood, NY) equipped with a MS-2000 stage (Applied Scientific Instrumentation, Eugene, OR), a Lambda LS 175 W Xenon light source (Sutter Instruments, Novato, CA), 63×, 100×, and 100× 1.45 Plan-Fluar objectives, and a five-position filter turret. Image acquisition was performed with a CoolSnap HQ camera (Roper Scientific, Tucson, AZ). The microscope and camera were controlled by SlideBook software (Intelligent Imaging Innovations, Denver, CO). The microscope was equipped with a complete set of zero-pixel shift Semrock GFP, YFP, CFP, and Cy3 filters. To ensure that the filters were aligned, we utilized Tetraspek Fluorescent Microspheres (Molecular Probes, Eugene, OR). Where appropriate, we used the iterative deconvolution algorithm in SlideBook, using a measured point spread function.

All live cell imaging was performed with log phase cells cultured in SC complete (SC-C) media supplemented with adenine. For latrunculin A (LatA) experiments, cells were cultured in SC-C, concentrated by centrifugation, and resuspended in a small volume of SC-C media. Just before imaging, LatA at twice the desired concentration in SC-C was added to an equal volume of concentrated cells. As a negative control, cells were treated with SC-C with DMSO. Strains bearing temperature-sensitive alleles were grown overnight and refreshed by changing the medium for 3–6 h at 24°C and then shifted to 34.5 or 37°C before observation. For live imaging of temperature-sensitive strains, cultures were visualized using a heated stage and objective (Applied Scientific Instrumentation, Eugene, OR) equilibrated to 34.5 or 37°C before imaging. Cell fixation was performed as previously described (Carvalho *et al.*, 2004). Staining of the actin cytoskeleton using phalloidin actin was performed as described (Sagot *et al.*, 2002a).

FRAP experiments and measurements were performed as previously described (Salmon and Wadsworth, 1986; Carvalho *et al.*, 2004). Briefly, the mean fluorescence intensity of a 4 × 2 (Bni1 bud tip and bud neck, Bnr1 bud neck) or 2 × 2 (Bnr1 partial bud neck) pixel area was measured at each time point for the bleached area. Intensity measurements were corrected for background and photobleaching (using the average of 5–7 unbleached cells). To determine the relative fluorescence intensity (RFI), we normalized the pre-bleach intensity to 1; for the experiments where half of the Bnr1-GFP signal was bleached, we normalized the postbleach intensity of the nonbleached region of the bud neck to 1. The $t_{1/2}$ values were calculated using Prism (GraphPad Software, San Diego, CA) software.

Analysis of Microscopy Data

All image manipulations and fluorescence intensity measurements were carried out using SlideBook software and exported to and analyzed in Microsoft Excel (Redmond, WA). The rate of Bni1 speckle movement was measured in images where Bni1 speckles could be followed for three or more consecutive frames, which we define as measurable speckles. The rate of Abp140 movement was determined in a similar manner by following the end of an extending cable.

RESULTS

Bni1 Colocalizes with Actin Cables In Vivo

We generated *BNI1-3GFP* and *BNI1-3YFP* constructs, which consist of three tandem copies of GFP or YFP, to characterize Bni1 localization and dynamics at endogenous levels in vivo. In contrast with previous studies with Bni1-GFP, which was overexpressed exogenously and was only detectable in ~40% of cells (Ozaki-Kuroda *et al.*, 2001), a functional Bni1-3GFP fusion was detected in nearly 100% of cells. Endogenous level Bni1-3GFP was enriched at the bud tip or at the bud neck depending on the stage of the cell cycle; in addition, Bni1-3GFP was visualized as abundant speckles in the cytoplasm throughout the cell cycle (Figure 1A).

We determined if Bni1 cytoplasmic speckles colocalized with actin cables. A strain expressing both Bni1-3YFP and Abp140-3CFP (which marks actin cables) was generated. After fixation, we acquired 0.3-μm optical sections through the entire cell for both the cyan fluorescent protein (CFP)

Figure 1. Localization of Bni1 to actin cables. (A) Bni1-3GFP localization during the cell cycle. Shown are images of Bni1-3GFP in fixed cells. Note: Bni1 can be observed as speckles in the cytoplasm in addition to its enrichment at the bud tip and later in the cell cycle at the bud neck. Each panel is a maximum projection image generated from a series of 0.3- μ m stacks through the entire cell. (B and C) Colocalization of Bni1-3YFP with actin cables. Fixed cells labeled with Bni1-3YFP and Abp140-3CFP. Abp140 labels actin cables brightly as well as actin patches. Right, merged images. Arrows indicate colocalization (yellow) of Bni1 (red) on Abp140-labeled actin cables (green). Each panel is a single focal plane image ($n > 100$ cells). Scale bars, 2 μ m.

and YFP channels. As expected, Abp140 robustly decorates actin cables (Asakura *et al.*, 1998; Yang and Pon, 2002; Figure 1, B and C, left). Examination of single focal planes from cells at varying points in the cell cycle revealed that some Bni1 speckles colocalize on Abp140-labeled actin cables (Figure 1, B and C, right). In each cell examined, we found at least one Bni1 speckle present on an actin cable ($n = 100$ cells). However, the majority of Bni1 speckles do not colocalize with actin cables. Dual-color imaging of Bni1 and Abp140 in live cells could not be performed because of the rapid movement and low signal intensity of both Bni1 speckles and Abp140.

Bni1 Exhibits Retrograde-directed Movement In Vivo

We used live-cell imaging to verify that Bni1 was associated with actin cables. Single focal plane time-lapse imaging of Bni1-3GFP revealed retrograde-directed linear movements from the bud tip to the mother cell (Figure 2A; Supplementary Movie S1) in small- to medium-budded cells. The direction of Bni1-3GFP speckle movement throughout the cell cycle is consistent with the direction of actin cable growth (Yang and Pon, 2002; Pruyne *et al.*, 2004a). For instance, during cytokinesis Myo2 movement is directed toward the bud neck (Pruyne *et al.*, 2004a), and actin cables elongate away from the bud neck and toward the mother or daughter cell (Yang and Pon, 2002). When Bni1 is localized at the bud neck, Bni1-3GFP speckles were also observed moving from the bud neck either to the daughter cell (Figure 2B; Supplementary Movie S2) or the mother cell.

The mean rate of Bni1 linear retrograde movement in the wild-type background is 0.48 ± 0.22 μ m/s ($n = 72$; Figure 2C). The direction and rate of actin cable extension was assayed using GFP-labeled Abp140, an actin-bundling protein, as a marker for cables in live cells (Yang and Pon, 2002). Actin cable elongation rates (0.29 ± 0.08 μ m/s or >100 actin monomers/s) are on the same order of magnitude as the rate at which formins allow barbed end elongation in vitro (Yang and Pon, 2002; Zigmond *et al.*, 2003). Under culture conditions similar to those used for Bni1-3GFP, Abp140-3GFP moved at 0.36 ± 0.13 μ m/s ($n = 68$; Figure 2C). Some Bni1-3GFP speckles could be followed across the distance of the bud (2 μ m) and up to half the distance of the mother (2 μ m) in a single focal plane. The true maximum distance traveled by Bni1 speckles is difficult to determine as actin cables move in and out of the plane of focus because of the curved surface of budding yeast. The rapid movement and low signal intensity of Bni1-3GFP speckles prevented us from using 4D imaging.

Bni1 Speckle Movement Depends on Actively Polymerizing Actin Cables

If the retrograde movement of Bni1 speckles represents the incorporation of Bni1 into the actin cable, then linear Bni1 speckle movement should be abolished by disassembly of actin cables. Actin structures are sensitive to LatA, an actin-monomer binding compound that inhibits actin polymerization and induces disassembly of actin cables and patches within 1–5 min (Ayscough *et al.*, 1997; Karpova *et al.*, 1998). We treated cells expressing Bni1-3GFP with LatA. Within 1 min after 100 or 400 μ M LatA treatment, actin cables are disassembled and the linear movement of Bni1-3GFP speckles was indeed abolished (observation of 38 cells; 250 s total filming). Imaging over a period of 10 min revealed a significant accumulation of Bni1-3GFP at the bud cortex or at the bud neck and accompanying depletion of Bni1-3GFP fluorescent speckles in the cytoplasm in live cells (Figure 3; Supplementary Movies S3 and S4). We observed a similar trend when we fixed cells after treatment with LatA for 15 min. Similar results have been reported when strains overexpressing GFP-Bni1 were treated with LatA (Ozaki-Kuroda *et al.*, 2001).

We compared the fluorescence intensity of Bni1-3GFP in the bud and the mother cell over this 10-min time period. Both concentrations of LatA led to an approximately two-fold increase in the fluorescence intensity of the bud tip signal relative to the signal in the mother cell cytoplasm over time (Figure 3C). DMSO-treated controls showed no change in the relative amount of Bni1 signal at the cortex over the same time frame (Figure 3C). In contrast, treatment with LatA did not affect the localization of Bud6 (Supplementary Figure S1A; observation of 27 cells) or Bnr1 (Supplementary Figure S1B; observation of 13 cells) over a 10-min time period. Actin cables are not detectable in the absence of profilin Pfy1 (Haarer *et al.*, 1990), and we did not observe Bni1-3GFP linear movement in the *pfy1* Δ strain (observation of 29 cells; 300 s total filming). Moreover, Bni1-3GFP is enriched at the bud tip in *pfy1* Δ strains and cytoplasmic speckles are depleted (Supplementary Figure S2). Thus, actin cables are required for Bni1 speckle movement and for the release of Bni1 from the polarized sites to the cytoplasm. Because LatA treatment depleted most Bni1-3GFP speckles from the cytoplasm, these cytoplasmic speckles are dependent on actin filament assembly. Further, these experiments demonstrate that polymerized actin is not essential for localization of Bni1 to the bud tip or the bud neck.

Figure 2. Retrograde movement of Bni1-3GFP. (A) Bni1-3GFP speckles move from the bud tip toward the bud neck. Shown are time-lapse images of two different Bni1-3GFP speckles (first speckle indicated by arrow; second by arrowhead) that move linearly in the same G2/M phase cell. Time is in milliseconds. (B) Time-lapse images of Bni1-3GFP speckles that move from the bud neck into the daughter in an anaphase cell (arrow). (C) Histogram comparing the rate of Bni1-3GFP movement and the rate of actin cable extension, as measured by Abp140-3GFP movement. $n = 71$ for Bni1 speckles; $n = 66$ for actin cables marked with Abp140-3GFP. Scale bar, 2 μm .

The budding yeast tropomyosins Tpm1 and Tpm2 localize to actin cables and are essential for cable integrity (Liu and Bretscher, 1989; Drees *et al.*, 1995). We visualized Bni1-3GFP dynamics in *tpm1-2 tpm2Δ* at 34.5°C using a temperature-controlled stage. At the restrictive temperature, actin cables disassembled rapidly (Pruyne *et al.*, 1998), and Bni1 speckles did not display retrograde movement (observation of 20 cells; 175 s total filming), whereas movement was obvious in the wild-type strain at this temperature (observation of 18 cells; 125 s total filming). Furthermore, after the temperature shift, there was a marked accumulation of Bni1 speckles at the bud tip and a concomitant decrease in the presence of cytoplasmic speckles (observation of 20 cells); this phenotype was apparent after a 5-min shift and was most dramatic after 1 h at 34.5°C (Supplementary Figure S2; observation of 21 cells). Actin cables are poorly detected in *tpm1Δ* strains (Drees *et al.*, 1995). Furthermore, we were unable to observe linear movement of Bni1-3GFP in *tpm1Δ* strains (observation of 17 cells; 250 s total filming), and Bni1 speckles were diminished from the cytoplasm and enriched at the bud cortex (Supplementary Figure S2). By contrast, actin cables in *tpm2Δ* strains appear normal (Drees *et al.*, 1995) and the rate of Abp140-3GFP and Bni1-3GFP movement in *tpm2Δ*

strains is not different than that of wild type (Table 1). Thus, linear retrograde movement of Bni1 requires actin cables, and the cytoplasmic pool of Bni1 speckles likely originates, at least in part, from actin cables that subsequently disassemble. We note that we did not observe that *tpm2Δ* strains have a twofold increase in actin cable elongation rate as reported in Huckaba *et al.* (2006). At present we do not know the basis for this difference; however, different strain backgrounds are one possible explanation.

To determine whether actin patches affect the movement of Bni1-3GFP speckles, we imaged Bni1-3GFP in a strain lacking the WASp homologue Las17/Bee1, which results in disorganized actin patches (Li, 1997). However, we still observed the linear movement of Bni1 speckles (observation of 10 cells; 75 s total filming) as in wild type. Additionally, we introduced the *BNI1-3GFP* construct in a temperature-sensitive mutant of the Arp2/3 complex, *arp2-1*. After 2 h at the restrictive temperature actin patches disassemble in the *arp2-1* strain (Moreau *et al.*, 1997), but Bni1-3GFP linear movement is still detectable (observation of 25 cells; 200 s total filming). Thus, actin patches are not essential for the linear, retrograde movement of Bni1 speckles.

Figure 3. Actin disassembly results in an enrichment of Bni1-3GFP at the bud tip with depletion of Bni1-3GFP speckles from the cytoplasm. Time-lapse images of Bni1-3GFP after treatment with 100 μ M (A) or 400 μ M (B) LatA for 1 min. Numbers indicate the time from the start of filming. (C) Ratio of the fluorescence intensity (fl. int. ratio) of Bni1-3GFP in the bud to the intensity in the mother at the time points in the images in A (■) and B (●). Control sample is treated with DMSO (▲). Note that fluorescence intensity ratio is normalized to 1 at time point 0. Scale bar, 2 μ m.

Formin Regulators Are Not Associated with Cytoplasmic Bni1 Speckles

Strains lacking Bud6 demonstrated a clear depolarization of actin patches, as previously described (Amberg *et al.*, 1997), as well as a mild defect in actin cables. However, this defect in actin cable structure did not alter the rate of actin cable extension or the rate of Bni1 speckle movement compared with wild-type strains (Table 1). However, Bni1 speckle

movement was less frequent and, when observed, was of a shorter duration, as judged by the number of measurable speckles. In *bud6* Δ strains we found 30 measurable speckles in 1800 s of filming, and in wild-type strains we observed 59 measurable speckles in 1000 s of filming. Strains lacking Spa2 have normal cable morphology (Sheu *et al.*, 1998; Shih *et al.*, 2005), but fail to concentrate Bni1 to the bud tip (Fujiwara *et al.*, 1998). However, the linear movement of Bni1 speckles was not altered in *spa2* Δ strains (Table 1).

We generated a strain expressing Bni1-3GFP and Bud6-RFP. As described previously, Bud6 labels the bud tip and the bud neck, depending on the stage of the cell cycle (Amberg *et al.*, 1997). Additionally, we observed Bud6 spots in the cytoplasm (Figure 4). Bni1 and Bud6 colocalize at sites of polarized growth, but they do not appear to coincide within the cytoplasm or on actin cables (Figure 4, bottom). Bud6 does not exhibit linear, retrograde movement (observation of 87 cells; 1400 s of filming). Imaging of Bud6-RFP with Abp140-3GFP revealed that Bud6 speckles occasionally associated with actin cables (observation of 53 cells), most likely due to the interaction of Bud6 with vesicles (Jin and Amberg, 2000). We were unable to perform two-color live imaging of Bni1-3GFP and Bud6-RFP, because of the rapid movement of Bni1 and the exposure time required to visualize both Bni1 and Bud6.

Table 1. Mean rate of Abp140 and Bni1 movement in various deletion strains

Strain	Abp140-3GFP (μ m/s)	Bni1-3GFP (μ m/s)
WT	0.35 $\pm$ 0.13 (66)	0.48 $\pm$ 0.22 (71)
<i>pfy1</i> Δ	No cables detected	No linear movement detected
<i>tpm1</i> Δ	No cables detected	No linear movement detected
<i>tpm2</i> Δ	0.45 $\pm$ 0.17 (32)	0.38 $\pm$ 0.12 (30)
<i>bud6</i> Δ	0.33 $\pm$ 0.12 (32)	0.44 $\pm$ 0.17 (30)
<i>spa2</i> Δ	Not measured	0.43 $\pm$ 0.14 (31)
<i>bnr1</i> Δ	0.40 $\pm$ 0.09 (22)	0.44 $\pm$ 0.19 (35)

Entries are the mean $\pm$ SD for the number of samples measured (in parentheses).

Figure 4. Bud6 and Bni1 colocalize at sites of polarized growth, but not on actin cables or in cytoplasmic speckles. Colocalization (yellow) of Bni1-3GFP (green) with Bud6-RFP (red). Note: although there is colocalization at the bud tip, there is little if any colocalization of cytoplasmic speckles. Each panel is a maximum projection image generated from a series of deconvolved 0.3- μ m stacks. Scale bar, 2 μ m.

The Rho type GTPases, Rho1, Rho3, and Cdc42, are implicated in Bni1 activation (Evangelista *et al.*, 1997; Tolliday *et al.*, 2002; Dong *et al.*, 2003). It is also unlikely that these Rho GTPases colocalize with Bni1 in the cytoplasm because Rho is active only when membrane bound (Hall, 2005). Consistent with this, Rho1, Rho3, and Cdc42 localized to the plasma membrane and endomembranes and were not detected as speckles in the cytoplasm (Robinson *et al.*, 1999; Richman *et al.*, 2002; Abe *et al.*, 2003; our unpublished data). In summary, we did not detect known Bni1 regulators associated with Bni1 speckles on actin cables or in the cytoplasm.

Bni1 Movement Depends on the Actin-binding Activity of Bni1

The FH2 domain of Bni1 is required for its actin polymerization activity in vivo and in vitro (Evangelista *et al.*, 2002;

Pruyne *et al.*, 2002; Sagot *et al.*, 2002a,b). To test the requirement for the FH2 domain on Bni1 speckle movement, we generated a construct lacking the FH2 domain. As expected, Bni1- Δ FH2-3GFP did not display detectable movement even in the presence of actin cables (observation of 30 cells; 240 s of filming). This suggests that the retrograde movement of Bni1 speckles may be dependent on the actin-binding activity of Bni1. Similarly, the FH1 domain of Bni1 is required for actin filament assembly by Bni1 in vivo (Evangelista *et al.*, 2002; Sagot *et al.*, 2002b). Consistent with this data, Bni1- Δ FH1-3GFP speckles did not display linear movement (observation of 32 cells; 210 s of filming). Both Bni1- Δ FH1 and Bni1- Δ FH2-3GFP showed localization similar to Bni1-3GFP: at the bud tip or the bud neck. Imaging these constructs in fixed cells revealed that Bni1-3GFP constructs lacking either the FH1 or FH2 domain had an increased fluorescence intensity at the bud tip or bud neck with a decrease in the signal of cytoplasmic speckles. The full-length Bni1-3GFP construct appeared similar to our integrated Bni1-3GFP in wild-type strains (Supplementary Figure S3). These results suggest that both the FH1 and FH2 domains are required for Bni1 speckle movement and subsequent localization in the cytoplasm. Moreover, these data are consistent with previously published reports, which suggest that neither the FH1 or FH2 domain is required for localization of Bni1 (Ozaki-Kuroda *et al.*, 2001).

Next, we took advantage of our previously described temperature-sensitive Bni1 FH2 domain mutant (*bni1-1*), which is inactive in vitro and fails to nucleate actin cables at 34.5°C in vivo (Sagot *et al.*, 2002a). In the *bni1-1-3GFP bnr1 Δ* cells, Bni1-1-3GFP speckles did display retrograde movement (observation of 31 cells; 75 s total filming) at 24°C. However, we did not detect Bni1-1-3GFP speckle movement at the restrictive temperature (Supplementary Movie S5; observation of 52 cells; 275 s total filming). We also observed an increase in the intensity of Bni1-1-3GFP at the bud tip and a decrease in cytoplasmic speckles, which was more pronounced after 20 min or more at the restrictive temperature (observation of 20 cells). Bni1-3GFP movement was apparent in *bnr1 Δ* strains at both 24°C (Table 1) and 34.5°C (observation of 30 cells; 200 s total filming).

These results are consistent with the finding that Bni1 speckles require actin cables to move. To test whether this inactive Bni1-1 speckle can move in the presence of actin cables, we introduced a wild-type copy of Bni1 in the *bni1-1-3GFP bnr1 Δ* strain. Strikingly, transformation with the Bni1 plasmid restores the assembly of actin cables, but did not rescue the movement of Bni1-1-3GFP speckles at the restrictive temperature (Supplementary Movie S6; observation of 16 cells; 225 s total filming). Therefore, even when actin cables are present, Bni1-1-3GFP speckles cannot move at the restrictive temperature. These results suggest that Bni1 retrograde movement may be coupled with actin assembly induced by Bni1 itself.

To further test this idea, we generated an FH2 domain mutation, which alters the actin-binding interface of Bni1 (Xu *et al.*, 2004). This Bni1 FH2 domain mutant (K1601A) is inactive for actin nucleation in vitro and fails to protect the barbed end from the activity of capping protein, suggesting that this mutation prevents or dramatically reduces the binding of Bni1 to actin (Xu *et al.*, 2004; Otomo *et al.*, 2005). When overexpressed in vivo, this mutation decreases the association of Bni1 with actin filaments (Tcheperegine *et al.*, 2005).

In a *BNR1* background, *bni1^{K1601A}*-3GFP cells had a morphology similar to that of *bni1 Δ* cells (rounded buds and wide bud necks), even at 24°C (Figure 5). We constructed a

Figure 5. A mutation disrupting actin binding (*Bni1*-K1601A) depletes cytoplasmic speckles and disrupts actin cables. (A) *Bni1^{K1601A}* shows a temperature-sensitive growth defect in *bnr1Δ* strains. Dilution of cells spotted onto YPD plates, grown at 37°C for 1 d. (B) *Bni1^{K1601A}*-3GFP is more enriched at the bud tip. Images of the *Bni1*-3GFP and *Bni1^{K1601A}*-3GFP in strains lacking *Bnr1*. (C) Actin cable assembly is impaired in *Bni1^{K1601A}*-3GFP *bnr1Δ* strains. Phalloidin-labeled *Bni1^{K1601A}*-3GFP *bnr1Δ* strains contain a few actin cables at 24°C (arrow). After 1 h at 37°C, the cells lack cables (C, right). Images on the left show the phalloidin staining of *Bni1*-3GFP in *bnr1Δ*. Pictures in B are single focal planes images; C shows deconvolved, maximum intensity projections generated from 0.2-μm stacks. Scale bar, 2 μm.

bni1^{K1601A}-3GFP *bnr1Δ* strain (see Materials and Methods). The double mutant strain exhibited a temperature-sensitive growth defect (Figure 5A). Therefore, the K1601A mutation, although inactive *in vitro*, appears to have a low level activity *in vivo* sufficient to support viability. At 24°C, *Bni1^{K1601A}*-3GFP speckles display linear retrograde movement in *bnr1Δ* strain (Supplementary Movie S7; observation of 48 cells; 350 s total filming). Moreover, we observed an increase in the fluorescence intensity of *Bni1^{K1601A}*-3GFP at the bud tip, with a decrease in the intensity of cytoplasmic signal (Figure 5B). Staining the actin cytoskeleton with Alexa568-conjugated phalloidin demonstrated that 60% of cells contain actin cables at 24°C ($n = 200$); after 1 h at 37°C, only 3.5% contain actin cables ($n = 200$; Figure 5C). Consistent with the actin cable defect, *Bni1^{K1601A}*-3GFP retrograde movement is abolished after the 1-h shift to 37°C (Supplementary Movie S8; observation of 25 cells; 300 s total filming).

Bni1 Displays Rapid Turnover at the Bud Tip and the Bud Neck

Next, we characterized the stability of the interaction of *Bni1* and *Bnr1* with the cell cortex by FRAP. We photobleached the region containing *Bni1*-3GFP at the bud tip of small-budded cells. The recovery of *Bni1*-3GFP was imaged by

rapid single focal plane time lapses. We observed that *Bni1*-3GFP signal at the bud tip is highly dynamic (Figure 6A); the turnover of *Bni1* at the bud tip was highly variable. This variability, although providing strong evidence of *Bni1* dynamics, precluded a precise calculation of the *Bni1* turnover rate. The average maximal recovery was 85%, and the average time to reach maximal recovery was 16 s (range: 6–32 s; $n = 4$). Thus, we estimate that the $t_{1/2}$ is <8 s. This estimation is similar to the $t_{1/2}$ of Spa2 at the bud tip (6.7 s; van Drogen and Peter, 2002 and 11.0 s; Dobbelaere and Barral, 2004).

As previously reported, *Bni1* localizes to the bud neck at the very late stage of the cell cycle (Ozaki-Kuroda *et al.*, 2001; Pruyne *et al.*, 2004a). Dual-color imaging of *Bni1*-3YFP and CFP-Tub1 showed that *Bni1* localizes at the bud neck just after anaphase spindle disassembly (Supplementary Figure S4) and coincides with the onset of cytokinetic actin ring (CAR) constriction as judged by colocalization with Myo1-CFP, the only type II myosin in budding yeast. *Bni1*-3YFP did not accumulate at the bud neck in cells where Myo1-CFP had not constricted (~ 1 μm in diameter, $n = 21$). Conversely, cells in which Myo1-CFP is constricting (<0.7 μm in diameter, $n = 6$) always had *Bni1*-3YFP signal at the bud neck (Supplementary Figure S4). FRAP assays revealed that the turnover of *Bni1*-3GFP at the bud neck during cytokinesis was rapid ($t_{1/2} = 8.0 \pm 0.8$ s, range 2.1–15.4 s; average maximal recovery 76.3%, $n = 4$; Figure 6B). The $t_{1/2}$ of *Bni1* at the bud neck is similar to previously published data on the recovery of Spa2 at the bud neck during cytokinesis ($t_{1/2} = 13.0$ s; Dobbelaere and Barral, 2004).

Bnr1 Is Relatively Static at the Bud Neck

The general pattern of localization of *Bnr1*-GFP is consistent with that described in previous reports: *Bnr1* localizes to the bud neck at bud emergence and remains at this site until the start of cytokinesis (Kamei *et al.*, 1998; Pruyne *et al.*, 2004a). With the same exposure time used for *Bni1*-3GFP, we did not detect *Bnr1*-GFP speckles in the cytoplasm and did not detect linear movement of *Bnr1*-GFP. Moreover, we did not observe localization of *Bnr1* on actin cables (as judged by colocalization of *Bnr1*-GFP with phalloidin labeled actin or Abp140-3YFP), suggesting that *Bnr1* is localized exclusively to the bud neck. Additionally, we generated a 3GFP-*BNR1*-*CEN* construct. Again, we did not detect *Bnr1* speckles in the cytoplasm in live cells in either wild-type strains (observation of 25 cells, 250 s of filming; Supplementary Movie S9) or strains lacking *Bni1* (observation of 25 cells, 250 s of filming). Moreover, in fixed cells *Bnr1* was localized solely at the bud neck (Figure 7). Further, localization of *Bnr1* at the bud neck was not enhanced after LatA treatment (Supplementary Figure S1B). Dual-color imaging with *Bnr1*-GFP and Myo1-CFP demonstrated that *Bnr1*-GFP is recruited to the bud neck early in the cell cycle and remains there until anaphase. Interestingly, *Bnr1*-GFP is lost from the bud neck before CAR constriction (Figure 8). Consistent with the loss of *Bnr1* before cytokinesis, we did not find any defects in CAR assembly or in cytokinesis in *bnr1Δ* cells (data not shown).

The dynamics of *Bnr1* were also strikingly different from that of *Bni1*. FRAP of *Bnr1*-GFP at the bud neck in small-budded cells revealed that there was little, if any, signal recovery (average maximal recovery 27.2%, $n = 6$; Figure 6C). This suggests that *Bnr1* at the bud neck either does not exchange with a cytoplasmic pool or that there is little soluble *Bnr1* available for exchange. Next, we bleached half of the *Bnr1*-GFP signal at the bud neck in medium-budded cells. In this case, the half-time of recovery was 57.7 ± 6.6 s (range = 8.2–115.9 s; average maximal recovery 55.1%, $n =$

Figure 6. Bni1-3GFP speckles turn over rapidly at the bud neck and bud tip, whereas Bnr1 is relatively static at the bud neck. (A) Four examples of FRAP experiments to characterize the turnover of Bni1-3GFP after photobleaching at the bud tip. Although turnover is always rapid, these examples illustrate the variability in recovery rates. Time is in seconds. (B) Rapid turnover of Bni1-3GFP at the bud neck. Left, time-lapse images from one experiment; below each image an enlargement of the neck region is presented. Right, average RFI after photobleaching for Bni1-3GFP at the bud neck (n = 4). (C) Lack of turnover of Bnr1-GFP at the neck. Left, images of a cell from one experiment; below each image an enlargement of the neck region is presented. Right, average RFI after photobleaching for Bnr1-GFP at the bud neck (n = 6). (D) Lateral diffusion of Bnr1 at the bud neck. Bleaching of half of the Bnr1-GFP bud neck signal enables slow recovery of signal from the bleached region with concomitant loss of signal from the unbleached region. Left, images from one experiment; below each image an enlargement of the neck region is presented. Right, average RFI after photobleaching for Bnr1-GFP at the bud neck for the bleached half (■) versus the unbleached half (▲; n = 4). For all images, the zero time point is the first postbleach image; subsequent times (in seconds) are indicated. Scale bar, 5 μm .

4; Figure 6D). Notably, recovery in the bleached half was accompanied by a proportional decrease in fluorescence intensity in the unbleached half (Supplementary Movie S10). Thus, unlike Bni1, Bnr1 is restricted to a small cortical domain within which it appears to undergo relatively slow lateral diffusion.

On the basis of the differences in the dynamics and localization of Bni1 and Bnr1, we examined the actin cables nucleated by each formin. Staining the actin cytoskeleton with Alexa568-conjugated phalloidin in diploid cells, we found that both Bni1- and Bnr1-nucleated actin cables show variations in the fluorescence intensity of actin along the

Figure 7. Bni1-3GFP was visualized as fluorescent speckles in the cytoplasm, whereas 3GFP-Bnr1 brightly labels the bud neck, but does not produce cytoplasmic speckles. Images are maximum intensity projections generated from 0.3- μ m stacks of fixed cells of the indicated genotype. Note: we observe some fluorescent signal in the cytoplasm of both 3GFP-BNR1 and control 3GFP-CEN strains. This signal is not dynamic, does not colocalize with actin, and was readily distinguished from cytoplasmic Bni1 speckles. Scale bar, 5 μ m.

length of the cable (Figure 9), which indicates that both populations of cables are composed of bundles of discontinuous actin filaments (Ayscough *et al.*, 1997; Karpova *et al.*, 1998; Kamasaki *et al.*, 2005). To confirm that these variations in fluorescence intensity are not due to actin patches, we labeled actin patches with Cap1-GFP in wild type, *bni1* Δ , and *bnr1* Δ and stained the actin cytoskeleton with Alexa phalloidin (Supplementary Figure S5). We found that all three strains showed similar variations in fluorescence intensity along the length of the cable; some, but not all, of these spots of high fluorescence intensity correlated with the presence of actin patches. Therefore, although the cortical dynamics of Bni1 and Bnr1 may differ, the cables nucleated by each formin appear to be constructed in a similar manner.

DISCUSSION

In this study we have characterized the *in vivo* dynamics of the two budding yeast formins Bni1 and Bnr1. Our study reveals that these proteins have strikingly distinct modes of interaction at the cell cortex that nonetheless allow common functions in actin cable assembly. Below we discuss the potential implications of these findings for actin cable assembly in budding yeast and for the *in vivo* function of the large number of formin proteins found in other eukaryotes.

The Dynamic Life Cycle of the Budding Yeast Formin Bni1

Our experiments demonstrate that budding yeast Bni1 is highly dynamic: transiting between the cell cortex, actin

cables, and a cytoplasmic pool. Bni1, originating either from the bud cortex or the bud neck, can be incorporated into actin cables, and, once associated with actin cables, displays linear, retrograde movement. The retrograde movement of Bni1 proceeds at a similar velocity to actin cable extension (Yang and Pon, 2002) and requires actin polymerization, and the integrity and the actin binding activity of the Bni1 FH2 domain, and the presence of the FH1 domain. These findings strongly suggest that after initiating the assembly of actin filaments destined to be incorporated into actin cables, Bni1 can itself be incorporated into the actin cable. While this manuscript was in preparation, Martin and Chang (2006) reported dynamic behavior of the *Schizosaccharomyces pombe* formin for3 that is similar to our findings with Bni1. Thus, Bni1 and for3 likely illustrate one general pattern for the dynamic behavior of formin proteins.

In principal, Bni1 could interact with actin filaments within the actin cable in at least three ways: 1) lateral binding to the actin cable, 2) binding to the barbed end of an actin filament and actively promoting actin polymerization within the cable, or 3) binding to the barbed end of an actin filament but in an inactive “capping” mode. Although several formins have actin bundling or actin severing activities that indicate an ability to bind to the side of actin filaments (Harris *et al.*, 2004, 2006; Moseley and Goode, 2005), Bni1 does not appear to share these properties (Moseley and Goode, 2005; Harris *et al.*, 2006). Furthermore, *in vitro* Bni1 predominantly binds to the barbed end of the actin filament (Pruyne *et al.*, 2002). Finally, Bni1-1-3GFP speckles do not

Figure 8. Bnr1 is lost from the bud neck during cytokinesis. (A) Dual-color imaging of Bnr1-GFP and Myo1-CFP. Arrows indicate colocalization (yellow) of Bnr1 (red) with Myo1 (green) in cells with small- to medium-sized buds before cytokinesis. Scale bar, 2 μ m (B) Time-lapse images of cells labeled with Bnr1-GFP (red) and Myo1-CFP (green). Note that Bnr1 is lost from the bud neck during CAR constriction. Each frame is 1 min. Each panel is a single focal plane image. Scale bar, 5 μ m.

Figure 9. Bni1- and Bnr1-nucleated cables display similar variations in fluorescence intensity. Phalloidin-labeled cells with the indicated genotypes in homozygous diploid strains. Arrows indicate discontinuous regions of increased fluorescence intensity along the actin cable. Images were deconvolved and maximum intensity projections were generated from 0.3-μm stacks. The fluorescence intensity (fl. int.) in arbitrary units versus the length of the cable from top to bottom was measured for each of the pseudocolored cables in the cell above each graph. Scale bar, 2 μm.

move at the restrictive temperature, even when actin cables are present. These results therefore make it unlikely that in vivo Bni1 associates with actin cables by binding the sides of actin filaments.

Our results also suggest that it is unlikely that the Bni1 speckles associated with actin cables are actively polymerizing actin filaments. If this were the case, Bni1 speckles should move slowly relative to the rate of extension of actin cables. However, we observed that the retrograde movement of Bni1 speckles is at rates very similar to actin cable elongation; thus the Bni1 speckle is likely to be static on the actin cable. Ideally, dual-color imaging of Bni1 and Abp140 in live cells would be used to confirm this conclusion; however, we could not perform this experiment because of the rapid movement and low signal intensity of both Bni1 speckles and Abp140. Because of the findings described above, we favor the idea that Bni1 binds to the barbed ends of actin filaments within the cables but, once incorporated into the cable, is inactive. Further support for this conclusion comes from our characterization of various Bni1 activators. Cdc42, Rho1, Rho3, and Bud6 colocalize with Bni1 only at

sites of polarized growth near the plasma membrane and were not detected together with Bni1 speckles in the cytoplasm or on cables.

How does Bni1 get to the cell cortex and what is the relationship between this cortical pool and the large cytoplasmic pool revealed by our experiments? We found that disassembly of actin resulted in a marked accumulation of Bni1 at the bud tip with a concomitant decrease in cytoplasmic speckles. Furthermore, cortical accumulation and cytoplasmic depletion was also observed in mutants that specifically compromise actin cable assembly, e.g., *pfy1Δ*, *tpm1Δ*. Thus, removal of Bni1 from the cortex requires actin cable assembly, whereas recruitment of Bni1 to the cortex is largely independent of actin cables. This suggests that the cytoplasmic pool of Bni1 originates, at least in part, from Bni1 speckles dissociated from recently disassembled cables. These speckles probably return to the cortex by diffusion rather than myosin-dependent transport. Thus, the rate of return of Bni1 to the cell cortex appears to be slower than the rate of Bni1 movement to the cytoplasm, which is dependent on the actin cable elongation rate and the rate of actin cable

disassembly. Given the rapid turnover of actin cables (Ayscough *et al.*, 1997), Bni1 associated with actin cables is expected to be transient. We have examined the stability of Bni1- and Bnr1-derived actin cables to low doses of LatA, but were unable to detect any difference in their rates of disassembly. Based on our data, we hypothesize that Bni1 incorporation into actin cables represents protection from dimeric actin-capping protein, which usually localizes to actin patches, not actin cables (Amatruda and Cooper, 1992; Amatruda *et al.*, 1992). However, we did not detect capping protein on actin cables irrespective of whether the strains expressed Bni1. Thus, it is not clear at this point whether the Bni1 associated with actin cables contributes to their structural integrity.

The Static Life Cycle of the Budding Yeast Formin Bnr1

Localization of Bnr1 to the bud neck requires amino acids 35–500, which includes the Rho-binding domain of Bnr1. However, the Rho-binding domain is not sufficient for the bud neck localization of Bnr1 (Kikyo *et al.*, 1999). Moreover, localization of Bnr1 to the bud neck and Bnr1-dependent actin cable assembly are dependent on septins (Pruyne *et al.*, 2004a). Our FRAP experiments demonstrate that Bnr1 has a strikingly different mode of interaction with the cell cortex than Bni1. Unlike Bni1, Bnr1 localizes exclusively to the bud neck. Although Bni1 turns over rapidly at the bud tip and bud neck, Bnr1 is relatively static. Bnr1 does not exchange with a cytoplasmic pool but was confined to the bud neck. In vitro, Bni1 and Bnr1 both promote actin assembly and protect the barbed end from capping protein (Moseley and Goode, 2005). Although qualitatively similar, there are significant quantitative differences: Bnr1 is 10-fold more potent in stimulating actin assembly (Moseley and Goode, 2005). In addition, Bnr1 can bundle actin filaments whereas Bni1 does not (Moseley and Goode, 2005). Spa2 and Bud6 specifically interact with Bni1 and not Bnr1 (Fujiwara *et al.*, 1998; Moseley and Goode, 2005). However, the in vivo significance of these observations is not yet fully understood.

Finally, we found that Bni1 accumulates to the bud neck at the onset of CAR constriction, whereas Bnr1 is lost from the bud neck just before the CAR contraction. These results are consistent with the genetic evidence that Bni1 but not Bnr1 is essential for the Myo1-dependent cytokinesis pathway (Vallen *et al.*, 2000).

Yeast Formins Have Different Modes of Cortical Interaction But Similar Functional Roles in Actin Cable Assembly

Altogether, our findings support a new view of the biogenesis of actin cables by formins in budding yeast. Both in vivo and in vitro formins can remain attached to the barbed ends of elongating actin filaments (Zigmond *et al.*, 2003; Higashida *et al.*, 2004; Moseley *et al.*, 2004; Romero *et al.*, 2004; Kovar *et al.*, 2006). Intriguingly, Bni1-attached filaments can elongate in vitro at velocities comparable to the rate of actin cable extension in vivo (Yang and Pon, 2002; Pruyne *et al.*, 2004b). These observations have led to the proposal that Bni1 might anchor filaments at the bud cortex while driving cable elongation, coupling actin assembly to cortical attachment (Evangelista *et al.*, 2002; Pruyne *et al.*, 2002, 2004b; Zigmond, 2004). However, our findings demonstrate that Bni1 is highly dynamic and thus is unlikely to stably anchor actin cables at the cortex.

Actin cables are assembled as bundles of short linear actin filaments. This was inferred initially from the variation in fluorescence intensity of phalloidin-labeled actin cables (Ayscough *et al.*, 1997; Karpova *et al.*, 1998) and subse-

quently directly demonstrated in fission yeast by electron microscopy (Kamasaki *et al.*, 2005). Our experiments reveal that actin cables assembled from Bni1- or Bnr1-derived filaments are discontinuous and thus likely to be similarly constructed from bundles of short filaments. This also argues against the hypothesis that Bnr1 serves as a static anchor for the barbed ends of actin cables, as was initially considered for Bni1 (Evangelista *et al.*, 2002; Pruyne *et al.*, 2002, 2004b; Zigmond, 2004). Rather we propose that the localization of formins in budding yeast serves mainly to facilitate having a local source of actin filaments that can then assemble into actin cables that subsequently elongate by an interesting but as yet poorly understood mechanism. Presumably these short filaments detach from Bnr1 before cable assembly, whereas Bni1, at least some fraction of the time, can remain on the filaments and become incorporated into the actin cable. Our model leaves open the subsequent molecular steps in actin cable assembly and whether or not there is a barbed end anchoring mechanism.

Genome sequencing has revealed a large number of formin proteins with diverse sequence features (Higgs, 2005; Higgs and Peterson, 2005). Our study demonstrates that formins with strikingly different mechanisms for interacting with the cell cortex can fulfill similar cellular functions. This will be important to consider when evaluating the cellular roles of other formin proteins.

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