Cortical actin dynamics driven by formins and myosin V

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Summary
Cell morphogenesis requires complex and rapid reorganization of the actin cytoskeleton. The budding yeast Saccharomyces cerevisiae is an invaluable model system for studying molecular mechanisms driving actin dynamics. Actin cables in yeast are formin-generated linear actin arrays that serve as tracks for directed intracellular transport by type V myosins. Cables are constantly reorganized throughout the cell cycle but the molecular basis for such dynamics remains poorly understood. By combining total internal reflection microscopy, quantitative image analyses and genetic manipulations we identify kinetically distinct subpopulations of cables that are differentially driven by formins and myosin. Bni1 drives elongation of randomly oriented actin cables in unpolarized cells, whereas both formins Bnr1 and Bni1 mediate slower polymerization of cables in polarized cells. Type V myosin Myo2 surprisingly acts as a motor for translational cable motility along the cell cortex. During polarization, cells change from fast to slow cable dynamics through spatio-temporal regulation of Bni1, Bnr1 and Myo2. In summary, we identify molecular mechanisms for the regulation of cable dynamics and suggest that fast actin reorganization is necessary for fidelity of cell polarization.

Key words: Actin dynamics, Cell polarity, Formin, MyoV

Introduction
Actin filaments can assemble into diverse structures such as lamellipodia, filopodia or stress fibers. These structures in turn provide protrusive and contractile forces during cell polarization, migration and division, and are central to many physiological and pathological processes such as differentiation, wound healing or malignant transformation. The driving force behind the often highly dynamic behavior of cortical actin is a combination of polymerization, depolymerization and myosin-dependent motility of actin filaments.

Polymerization of actin filaments relies on a variety of nucleation factors. Among the different types of nucleators, formins constitute the largest class. In addition to their nucleation activity, formins also have the ability to promote actin filament elongation. This dual function might explain why they have diverged so extensively (Chalkia et al., 2008). Formins support actin polymerization in vitro at rates of up to 10 subunits/second at 1 μM G-actin (Kovar and Pollard, 2004). By contrast, studies in a range of organisms indicate that formins facilitate in vivo rates of actin polymerization of more than 550 subunits/second or 1.5 μm/second (Higashida et al., 2007; Diez et al., 2005; Staiger et al., 2009). Importantly, although we understand formin biochemistry in greater detail (Kovar, 2006; Paul and Pollard, 2009), it has not been possible to link the elongation rate of a defined actin filament or bundle to the action of a particular formin in vivo.

Myosin motors also contribute to actin remodeling by moving filaments past one another or along fixed substrates. The generation and role of myosin-II-dependent contractile forces during muscle contraction (Yanagida, 2007) and cytokinesis (Wang, 2001; Wu et al., 2006; Clayton et al., 2010; Skau and Kovar, 2010) have been intensely studied. Myosin II has also been implicated in retrograde actin flow in budding yeast Saccharomyces cerevisiae (Huckaba et al., 2006). Although there are several reports suggesting that unconventional myosins could move actin filaments along the cell cortex, no direct evidence for such a function has yet been presented. Considering the ability of all myosins to move actin filaments in vitro gliding assays, and the association of many myosins with the plasma membrane (Zot and Pollard, 1993; Sousa and Cheney, 2005; McConnell and Tyska, 2007), this is quite remarkable.

The budding yeast S. cerevisiae has been an invaluable model system for the study of molecular components and mechanisms regulating formin-dependent actin assembly (Evangelista et al., 2002; Sagot et al., 2002; Moseley and Goode, 2006). Actin cables in S. cerevisiae are linear filamentous structures generated by the two formins, Bni1 and Bnr1. Bni1 localizes to the bud tip during polarized growth and nucleates short, bud-localized cables. During cytokinesis Bni1 relocates to the bud neck, where it supports assembly of cables and, possibly, the acto-myosin ring (Evangelista et al., 2002; Buttery et al., 2007). Bnr1 is immobilized at the bud tip during polarized growth and nucleates short, bud-localized cables. During cytokinesis Bnr1 relocates to the bud neck by interactions with septins, and nucleates cables that project into the mother cell (Buttery et al., 2007). Polarized cables are used as tracks for myosin-V-driven vectorial transport, which is essential for cell growth and homeostasis (Jansen et al., 1996; Pryune et al., 1998).

Although actin cables are highly dynamic (Ayscough et al., 1997; Karpova et al., 1998), previous studies have focused on relatively slow actin dynamics displayed by polarized cells (Yang and Pon, 2002; Huckaba et al., 2006). Dynamics of cables in unpolarized cells and during cell polarization remained uncharacterized. Furthermore, the molecular mechanisms driving cable dynamics remain unknown. Here, we use total internal reflection microscopy (TIRFM) to quantify and compare cable dynamics in unpolarized and polarized cells. Combining quantitative image analyses with genetic manipulations, we probe the molecular basis for the observed dynamics.
Results
Visualizing cortical actin cables by TIRFM
To visualize actin cables, we fused the endogenous copy of ABP140 to green fluorescent protein (GFP) as previously reported (Yang and Pon, 2002). By employing TIRFM actin, structures labeled by Abp140–GFP could be visualized with improved sensitivity (Fig. 1A). Due to the limited illumination depth of TIRFM (Axelrod et al., 1983), we had to ensure that actin cables were associated with the cell cortex as previously reported (Amberg, 1998). We therefore analyzed Z-stacks of Abp140–GFP-labeled yeast cells using wide-field microscopy (Fig. 1B), and counted an average of 6.6±2.1 (mean ± s.d., n=51) cables for each mother cell or unbudded cell analyzed. Cables in small and medium-sized buds were hardly visible and were therefore not included for quantitative analyses. Importantly, nearly all cables (n=336) were found restricted to the cortical planes with only 1.2% extending into the interior of cells (Fig. 1B). Our results indicate that TIRFM is a reliable technique for monitoring actin cable dynamics in S. cerevisiae cells.

Actin cable dynamics are tightly regulated during cell polarization
To characterize cable dynamics in detail we followed individual cables moving through the field of view. Actin cables were rarely static and often either exhibited elongation (Fig. 2A; supplementary material Movie 1) or translational motility (Fig. 2B; supplementary material Movie 2). Due to the illumination limit of TIRFM and the oval shape of S. cerevisiae cells, only 50–70% of the surface area of a cellular hemisphere could be visualized (field of view with 2–5 μm diameter). However, most actin cables extended beyond the field of view at one or both ends and it was often impossible to directly distinguish elongation from translational motility. Following reliable fiduciary marks on cables was not feasible because Abp140 has very low affinity to actin filaments and exchanges on cables within less than 200 milliseconds (supplementary material Fig. S1A) (Riedl et al., 2008). We therefore

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**Fig. 1. Cortical actin cable network in S. cerevisiae cells.** (A) Comparison of wide-field and TIRF microscopy for visualization of Abp140–GFP labeled actin in wild-type yeast cells (strain JYY141). Cell periphery is marked by dotted lines. Time is given in seconds. (B) Example of a Z-stack used for cable counts. Relative z-position given in μm. Arrowheads indicate cables close to the cell surface, asterisks indicate one cable found away from the cell surface. Scale bars: 2 μm.

**Fig. 2. Actin cable dynamics during cell polarization.** (A) Cable elongation (arrowheads show leading end; supplementary material Movie 1). The kymograph was generated along the dotted lines. (B) Cable translational motility (arrowheads show leading end and asterisks the lagging end; supplementary material Movie 2). Scale bars: 2 μm. (C) Probability density distributions of cable movement rates in JYY141 wild-type unpolarized (unpol) and polarized (pol) cells (see Materials and Methods for details). (D) Stacked bar graphs of cable movement rates in wild-type cells. (E,F) Cable movements in synchronized cells. Abp140–GFP-expressing cells (JYY207) were arrested for 3 hours and released into the cell cycle through washout of methionine. Cable movements were measured during arrest, 40–50 minutes after release, and 80–90 minutes after release. Insets in E: typical cell morphology in G1 arrest (a), 40-50 minutes after release (b) and 80–90 minutes after release (c). (G) Cable number (N) per cell and per minute in wild-type (n=20) and arrest strains (n>10). **P<0.01, ***P<0.0001 compared with unpolarized cells. Bar graphs show means ± s.e.m.
defined all extensions of leading cable ends as ‘cable movement’ and attempted to separate kinetic subpopulations of cables by careful quantification and statistics. To this end, we analyzed the measured velocities by kernel density estimation and plotted their probability density functions. This approach allows a smoothed representation of the raw data distribution and is considered superior to histograms (see Materials and Methods for details). In addition, we recorded the number of cables moving through the field of view as a measure for cable abundance and turnover in a cell.

In unbudded cells with no observable polarized cable arrays (referred to hereafter as unpolarized cells; supplementary material Fig. S1B) we observed rapid cable movement with velocities up to 5 μm/second with probability densities peaking around 1.2 μm/second and 2.2 μm/second (Fig. 2C; supplementary material Fig. S2A). In small or medium-budded cells (bud diameter less than half of mother diameter, referred to hereafter as polarized cells; supplementary material Fig. S1C), cable movement was overall slower with a single peak around 1 μm/second (Fig. 2C; supplementary material Fig. S2A). For simplicity, cable movement measurements were separated into three velocity categories: slow, below 1 μm/second; intermediate, between 1 and 2 μm/second; and fast, above 2 μm/second. These were plotted into stacked bar graphs and used to supplement the kernel density analyses. In polarized cells, a reduction of fast movements and increase in slow movements was clearly observed (Fig. 2D). In addition, the number of emerging cables was significantly reduced in polarized cells (10.7±3.5 cables/minute, mean ± s.d., n=20) compared to unpolarized cells (24.5±5.8 cables/minute, n=20; Fig. 2G). These data suggested a decrease in cable dynamics upon cell polarization.

To verify this in a synchronous cell population undergoing polarization, we introduced Abp140–GFP into a strain that could be arrested in the G1 phase of the cell cycle by cyclin depletion (Richardson et al., 1989; Wedlich-Soldner et al., 2004). Cable dynamics were then measured before and after synchronous release of cells from the G1 cell cycle arrest. Confirming our previous measurements, cable movement velocities and cable numbers were reduced as cells became polarized (Fig. 2E,F). This effect became most pronounced after 80 minutes when most cells had already established polarity (Wedlich-Soldner et al., 2004) and formed small buds (Fig. 2E, insets).

Cortical Bni1 drives rapid assembly of unpolarized actin cables

Actin cables in S. cerevisiae were reported to depend on formin activity and we confirmed by TIRFM that cables were absent in cells lacking both formins (bnr1Δ bni1-11; supplementary material Fig. S3A) (Evangelista et al., 2002) or the essential formin cofactor profilin (pfy1-4; supplementary material Fig. S3A) (Wolven et al., 2000). We then studied cable dynamics in individual formin knockout mutants. In unpolarized bni1Δ cells, cable numbers were reduced by 90% (Fig. 3A). Interestingly, we also observed a selective reduction of cable movement at intermediate velocities (Fig. 3B,C). On the other hand, in unpolarized bnr1Δ cells, cable numbers were only reduced by 24% (Fig. 3A) and cable movement velocities remained largely unchanged with a slight reduction of the slow population (Fig. 3B,C). These results suggest that Bni1 generates most of the cables in unpolarized cells and regulates cable movement at

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**Fig. 3.** Cable dynamics in unpolarized cells of formin mutants. (A) Cable numbers in formin mutants (n=20); **P<0.01, ***P<0.0001 compared with control cells. Bar graphs show means ± s.e.m. (B,C) Cable movement in control (identical to ‘unpol’ in Fig. 2C,D) with bni1Δ (JYY22) and bnr1Δ (JYY24) mutants. (D,E) Cable movement in bnr1Δ cells expressing Bni1-4PPT (JYY183, control) and bni1-1PPT (JYY189). (F) Dual-color TIRFM showing an Abp140-(1-214)-mRFPRuby (Abp140RFP)-labeled actin cable (arrowheads) originating from a cortical Bni1–GFP patch (asterisks). Images were acquired from strain JYY144, and were deconvolved to improve visualization. Scale bar: 1 μm. (G,H) Cable movement rates in bnr1Δ cells treated with indicated concentrations of LatB.

intermediate velocities between 1 and 2 \( \mu \text{m/second} \). Bnr1, by contrast, plays a minor role at this stage and might be associated with slow movement below 1 \( \mu \text{m/second} \).

To mechanistically understand cable movement we wanted to directly link cable movements at intermediate velocities to Bni1-driven actin polymerization. Both Bni1 and Bnr1 contain formin homology 1 (FH1) and FH2 domains (Evangelista et al., 2002). FH2 binds to the barbed end of the actin filament, whereas FH1 recruits profilin-bound actin via polyproline tracks (PPT) (Kovar, 2006). It was previously reported that the number of PPTs in truncated constructs determined the rate of filament elongation by Bni1 in vitro (Paul and Pollard, 2008). We attempted to reproduce this result in cells. Therefore, the genomic copy of \textit{BNII} was modified in a \textit{bnr1A} background to either contain four (wild type) or only one PPT. This modification did not affect cable numbers (Fig. 3A), in accordance with in vitro results where nucleation efficiency was unaffected (Paul and Pollard, 2008). However, compared to cells expressing Bni1–4PPT, we found a clear and selective shift of the velocity population associated with Bni1 in cells expressing \textit{bnr1A} (Fig. 3D, peak from 1.4 \( \mu \text{m/second} \) to 1 \( \mu \text{m/second} \); Fig. 3E). This result confirms the dependence of intermediate cable movement velocities on Bni1 and also corroborated the reduced polymerization rates with fewer PPTs in vitro (Paul and Pollard, 2008).

Because actin cables were localized predominantly at the cell cortex (Fig. 1B), Bni1 had to also localize to the cell cortex to regulate cable dynamics. Indeed, previous data suggested that formins were activated by membrane association (Dong et al., 2003). However, only cytosolic Bni1 dots had been observed in unpolarized cells so far (Buttery et al., 2007). No clear evidence of cortical localization of Bni1 in these cells was reported. Taking advantage of the increased sensitivity of our setup we now monitored Bni1–GFP localization in unpolarized cells by TIRFM. We found that Bni1–GFP transiently accumulated in cortical patches that were distinct from endocytic actin patches (supplementary material Fig. S3B,C). Importantly, using dual-color TIRFM with Bni1–GFP and Abp140–RFP we observed short actin cables originating from cortical Bni1 patches (Fig. 3F). In summary, we propose that cortical Bni1 contributes to cable dynamics in unpolarized cells by driving filament elongation at intermediate velocities of 1–2 \( \mu \text{m/second} \).

\textbf{Cortical Myo2 drives rapid cable motility in unpolarized cells}

Remarkably, none of the formin mutations resulted in a reduction of the cable population moving faster than 2 \( \mu \text{m/second} \) (Fig. 3B–E), suggesting that these movements might occur independently of actin polymerization. To verify this, we treated \textit{bnr1A} cells with latrunculin B (LatB), which specifically inhibits actin polymerization by sequestering actin monomers (Ayscough et al., 1997). At increasing LatB concentrations, cables became diminished until they disappeared completely in 16 \( \mu \text{M} \) LatB (supplementary material Fig. S3D). In cells treated with 2–8 \( \mu \text{M} \) LatB, actin cables became shorter and underwent rapid translational motility (supplementary material Movie 3). This movement mostly occurred at rates above 2 \( \mu \text{m/second} \) (Fig. 3G,H). These results strongly suggest that translational cable motility was mechanistically distinct from formin-driven cable elongation and occurred at higher speed. The initial double-peak distribution (Fig. 2C) could have resulted from the combination of these mechanisms.

In \textit{S. cerevisiae} the only previously reported process occurring at velocities above 2 \( \mu \text{m/second} \) is intracellular transport by the type V myosin Myo2 (Schott et al., 2002; Sheltzer and Rose, 2009). Therefore, we monitored cable dynamics in unpolarized cells of various myo2 mutants. The temperature-sensitive mutant myo2-66 is defective in actin binding (Johnston et al., 1991), whereas the myo2-16 mutant (Schott et al., 1999) has a defect in cargo binding. At restrictive temperature (36°C), cable numbers in cells of either mutant were not affected (Fig. 4A). Strikingly, populations of cables moving above 2 \( \mu \text{m/second} \) were strongly reduced for both mutants (Fig. 4B–E). This became especially apparent in \textit{bni1A myo2-16} double mutant cells. In these cells, the cable number was reduced by 73% (Fig. 4A), similar to the \textit{bnl1A} mutant (Fig. 3A). Notably, over 70% of the remaining cables now moved with velocities below 1 \( \mu \text{m/second} \) (Fig. 4D,E), consistent with the loss of both Bni1-mediated (1–2 \( \mu \text{m/second} \)) and Myo2-mediated (above 2 \( \mu \text{m/second} \)) movements. Moreover, cables in myo2 mutant cells frequently became immobile for extended periods (Fig. 4H), compared to rapid continuous motility in the control strain (Fig. 2B, supplementary material Movie 3). Disruption of any other \textit{S. cerevisiae} myosin (Myo4, Myo1, Myo3+5) did not cause any reduction of cable movement in unpolarized cells (supplementary material Fig. S4A,B) indicating a specific role of Myo2 in this process.

Both myo2-16 and myo2-66 alleles lead to cell lethality at the restrictive temperature (Johnston et al., 1991; Schott et al., 1999). To rule out secondary physiological defects as the cause of changes in cable dynamics, we wanted to directly link the motor activity of Myo2 to cable motility. To this end, we measured cable dynamics in Myo2 mutants containing different numbers of IQ motifs in their neck domain. Previous study showed that stepwise reduction of IQ motifs from six (wild type) to zero might reduce myosin step size, causing progressive slow-down of Myo2-dependent transport of secretory vesicles (Schott et al., 2002). Consistently, after reduction of IQ motifs, the population of cables moving at velocities above 2 \( \mu \text{m/second} \) was progressively reduced in our TIRFM assay (Fig. 4F,G).

Because actin cables localized predominantly close to the plasma membrane (Fig. 1B) (Amberg, 1998), Myo2 had to be anchored at the cell cortex to mediate cable movement. Using dual-color TIRFM we showed that Myo2–GFP formed relatively static cortical cables in myo2-16 and myo2-66 mutants. Whereas the \textit{myo2-16} mutant showed clear and consistent movement mostly above 1 \( \mu \text{m/second} \) (Fig. 4G), consistent with previous reports (Schott et al., 2003; Sheltzer and Rose, 2009). In contrast, the \textit{myo2-66} mutant showed reduced cable mobility and fewer active cables (Fig. 4F). This suggests that the Myo2-mediated cable dynamics are not sufficient in the absence of IQ motifs to maintain cable motility.

\textbf{Bnr1 and Bni1 govern actin cable dynamics in polarized mother cells}

We identified Bni1 and Myo2 as molecules driving cable movement above 1 \( \mu \text{m/second} \) in unpolarized cells. What then mediated slow cable movement below 1 \( \mu \text{m/second} \)? Our measurements showed only a few cables in this range in unpolarized cells, and lack of Bnr1 specifically abolished these cables (Fig. 3B,C). By contrast, slower cable movements were much more prominent in polarized mother cells, with half of the cables moving below 1 \( \mu \text{m/second} \) (Fig. 2C–F). The activity profile of Bnr1 closely correlated with the observed differences in cable movement [high activity in polarized cells (Pruyne et al., 2004a), low activity in unpolarized cells (Paul and Pollard, 2008)] (Fig. 4H). This suggests that Bnr1 is required for efficient cable motility in unpolarized cells, whereas Myo2 is sufficient to drive rapid cable movement in both unpolarized and polarized cells. In conclusion, our results suggest that Bnr1 and Myo2 govern actin cable dynamics in polarized mother cells by controlling cable motility and stability, respectively.
cells (Chesarone et al., 2009). We therefore suspected that Bnr1 mediated cable movement below 1 μm/second.

To test our hypothesis, we studied cable dynamics in polarized mother cells of formin mutants. Deletion of Bni1 caused no change in cable numbers (Fig. 5A) but nearly completely abolished cable movement above 1 μm/second (Fig. 5B,C). Almost all cables were now polarized towards the bud neck (Fig. 5D), where Bnr1 was reportedly localized (Fig. 5E) (Buttery et al., 2007). Surprisingly, cable number and movement remained largely unaffected by deletion of Bnr1 (Fig. 5A–C), but more than 40% of the cables were now depolarized (Fig. 4D). These randomly oriented cables moved significantly faster than polarized cables (1.4±0.74 μm/second vs 0.94±0.6 μm/second, P<0.001; Fig. 4F,G). Notably, rapid cable motility above 2 μm/second was absent from all polarized cells (Fig. 5B,C) indicating a negligible role of Myo2-driven cable movement. In agreement with this, Myo2–GFP was mostly found at the bud tips of polarized cells (Fig. 5H) (Lillie and Brown, 1994), whereas cortical density of Myo2 patches was significantly reduced in unpolarized cells (Fig. 5I; P<0.0005).

In summary, our results suggest that cable dynamics in polarized cells are predominantly mediated by the two formins Bni1 and Bnr1. Bud-neck-confined Bnr1 was associated with elongation of polarized cables at rates below 1 μm/second. Bni1 appeared to drive slow elongation of polarized cables (<1 μm/second) as well as elongation of unpolarized cables at intermediate speeds (1–2 μm/second). This model is consistent with the concentration of Bni1 at bud tips and necks of polarized cells (Fig. 5J) (Pruyne et al., 2004a) as well as transient localization of Bni1 on the mother cell cortex (Fig. S3B) (Buttery et al., 2007). Myo2-driven rapid cable motility (>2 μm) plays almost no role in cable dynamics at this stage.
Cable dynamics are important for correct cell polarization

Polarized transport into the bud requires stable arrays of actin cables (Pruyne et al., 2004b). Here, we established that such arrays could be generated by oriented assembly and immobilization of actin cables. However, the advantage of maintaining rapidly moving and randomly oriented cables in unpolarized cells is less obvious because this high-energy-consuming process occurs before any polarized growth. Our results suggested that cells switch between fast and slow cable dynamics by activating Bnr1 at the bud neck. We therefore decided to observe cells that are forced to exclusively rely on Bnr1-driven slow cable dynamics by either deleting the other formin or by removing the Bnr1 inhibitor Bud14 (Chesarone et al., 2009).

One possible role of rapid and random cable dynamics is to enable efficient cell polarity establishment, a self-organizing process that depends on rapid recycling of polarity regulators such as Rho-GTPase Cdc42 (Wedlich-Soldner and Li, 2004). Recycling of Cdc42 is mediated by a combination of actin- and guanine nucleotide dissociation inhibitor (GDI)-dependent pathways (Slaughter et al., 2009). We assayed cell polarization during bud formation by monitoring the localization of GFP–Cdc42 in synchronized cell populations released from G1 cell cycle arrest (Wedlich-Soldner et al., 2004). To better discern defects in actin-mediated recycling, we deleted the S. cerevisiae GDI Rdi1, which can extract Cdc42 from membranes (Tiedje et al., 2008). We then tested the effect of Bni1 deletion on polarization. Although deletion of either Rdi1 or Bni1 led to no obvious defects, polarization was significantly delayed in the double mutant (Fig. 6A). In addition, 5% of all cells exhibited a striking phenotype with split caps or bifurcated buds (Fig. 6B).

Bud14 was first identified as a factor regulating various aspect of cell morphogenesis during vegetative growth and mating. Deletion of Bud14 led to defects in cell growth and to elongated cells that undergo pseudohyphal growth (Cullen and Sprague, 2002). It was recently shown that Bud14 regulates Bnr1 by displacing it from the barbed ends of actin filaments (Chesarone et al., 2009). Without Bud14, Bnr1 was expected to remain attached to newly generated actin cables. Here we show that, strikingly, Bnr1–GFP, which could not be detected on the cortex of control unpolarized cells, formed polarized caps and dot-like cortical structures in unbudded bud14A cells (Fig. 6C). This abnormal localization was also reflected in reduced rates of cable movement, consistent with the predicted Bnr1-dependent slow cable dynamics (Fig. 6D,E). These results indicate that cells with constantly activated Bnr1 and, hence, abnormally slow actin dynamics are not able to become depolarized. These constitutively polarized cells can only grow in a polarized manner throughout the cell cycle, resulting in abnormal cell morphology or pseudohyphal growth.

Discussion

In this study, we provide insights into the mechanisms underlying actin cable organization and dynamics via spatio-temporal patterning of formins and myosin. In unpolarized G1 cells, actin cables are randomly oriented and their polymerization is largely mediated by the cortical formin Bni1 (Fig. 6F). The second formin, Bnr1, plays only a minor role in unpolarized cells and is supposedly kept inactive by Bud14. Cortical type V myosin Myo2 contributes to actin dynamics by mediating rapid sliding of actin cables along the plasma membrane. In polarized cells cable dynamics are significantly slowed down. Cables become mostly oriented towards the bud neck, where they are polymerized by bud-neck-confined Bnr1. At the same time, Bni1 is recruited to the bud tip and bud neck, where it also participates in the generation of polarized cable array. Myo2 is depleted from the cell cortex and targeted to the bud neck, where it also participates in the generation of polarized cable array (Pruyne et al., 2004b). Here, we established quantitatively that such arrays could be generated by oriented formation and immobilization of actin cables (Pruyne et al., 2004b). Here, we established quantitatively that such arrays could be generated by oriented formation and immobilization of actin cables (Pruyne et al., 2004b). Here, we established quantitatively that such arrays could be generated by oriented formation and immobilization of actin cables (Pruyne et al., 2004b). Here, we established quantitatively that such arrays could be generated by oriented formation and immobilization of actin cables (Pruyne et al., 2004b). Here, we established quantitatively that such arrays could be generated by oriented formation and immobilization of actin cables (Pruyne et al., 2004b). 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Fig. 5. Formins Bnr1 and Bni1 govern cable dynamics in polarized cells. (A) Cable numbers in control, bni1Δ (JYY22) and bnr1Δ (JYY24) polarized mother cells. (B,E) Cable movement rates in control (equal to ‘pol’ in Fig. 2C,D), bni1Δ and bnr1Δ polarized mother cells. (D) Proportion of randomly oriented cables that do not originate from the bud neck in polarized mother cells (control n=20; bni1Δ n=18; bnr1Δ n=20). *P<0.05, **P<0.0005 compared with control cells. (E) Bud neck localization of Bnr1–GFP (JYY7). (F,G) Movement rates of randomly oriented vs polarized cables in polarized cells of bnr1Δ. (H) Concentration of Myo2–GFP at the bud tip of a medium-budded cell. Image was acquired in strain JYY8. (I) Density of Myo2–GFP patches on the cell cortex in unpolarized (unpol) and polarized mother (pol) cells of control strain JYY8. Patch density was calculated using TIRFM by dividing number of Myo2–GFP patches emerged per minute in a cell (unpol n=21, pol n=19). Patch number was then divided by the area of the field of view (in μm²) to yield the density (*P<0.005 compared with unpolarized cells). (J) Concentration of Bni1–GFP at both the bud tip and the bud neck of small and medium-budded cells (JYY6). Images in E, H and J were acquired using wide-field optics. Bar graphs show means ± s.e.m. Scale bars: 2 μm.
Also, the use of a highly sensitive EMCCD camera enabled us to achieve higher temporal resolution in our sequences. These allowed us to study cables moving faster than 1 \( \mu \)m/second, which were probably underrepresented in previous studies.

It was previously shown that the two yeast formins, Bni1 and Bnr1, differ in their interaction partners and localization patterns (Dong et al., 2003; Moseley and Goode, 2005; Buttery et al., 2007; Chesarone et al., 2009). We now present evidence suggesting that they also assemble kinetically separable cable populations in a cell cycle-dependent manner. Bni1 was the dominant player in cable assembly in unpolarized G1 cells (Fig. 3A–F), whereas Bnr1 only became localized and/or activated during bud formation (Fig. 5A–E). Importantly, altered cable dynamics in several formin mutants suggested that Bni1 might polymerize actin cables twice as fast as Bnr1. This notion was supported by the velocity peaks at 1.2–1.5 \( \mu \)m/second in cells containing Bni1 as the only formin (Fig. 3B,D,G), and the peaks appearing faithfully at 0.5–0.7 \( \mu \)m/second in bni1Δ or Bnr1 hyperactivation strains (Fig. 3B, Fig. 4D, Fig. 6D). Additionally, it was observed that Bnr1-generated cables were uniformly polarized towards the bud neck – the place of their origin, whereas even in polarized cells the Bni1-nucleated cables were frequently randomly oriented (Fig. 5D). This could be explained by a higher affinity of Bnr1 to actin barbed ends, consistent with a tenfold higher affinity of Bnr1 in biochemical experiments (Moseley and Goode, 2005) as well as the presence of Bni1 outside polarized sites (supplementary material Fig. S3B) (Buttery et al., 2007).

In summary, we identified the switch between the two yeast formins as a major mechanism for regulation of cable dynamics.

Our TIRFM-based cable dynamics measurements were sensitive enough to detect the effect of specific manipulation of the Bni1-FH1 domain on actin polymerization in vivo. Previously, Paul and Pollard demonstrated that by reducing the number of PPT from four to one, polymerization rate by Bni1 is reduced by about 50% (Paul and Pollard, 2008). We introduced these FH1 mutations into endogenously expressed Bni1 and found a clear shift of Bni1-dependent cable movements from around 1.4 \( \mu \)m/second to around 1.0 \( \mu \)m/second, with no major changes in Myo2-driven rapid motility (Fig. 3D,E). Therefore the trend of the in vitro measurements could be reproduced faithfully, indicating that TIRFM-based observations of in vivo cable kinetics could be used to directly study the biochemical mechanisms of actin polymerization.

Type V myosins are well known for their role in polarized transport of various cargos (Pruyne et al., 2004b; Trybus, 2008). We describe here a surprising novel role for Myo2 in moving actin filaments along the cell cortex. In unpolarized cells, sliding of cables by Myo2 allowed continuous movement and mixing of cables along the periphery of the cell. To our knowledge, this is also the first demonstration of a myosin moving actin filaments at the cortex. In polarized cells, cables formed polarized arrays that were probably anchored to the bud neck and could not be moved by Myo2 (Fig. 5D). In this scenario, we expect myosin molecules...
to be removed from the cortex (Fig. 5I) and move along the stable cables to the bud, where they can support polarized organelle transport (Pruyne et al., 2004b). This dual function of Myo2 as a motor that moves both cargos and tracks provides an efficient mechanism of adaptation to cell cycle-specific requirements.

Assembly and rapid reorganization of the actin cytoskeleton is clearly required for migration of amoeba and mammalian cells (Bretscher et al., 2002; Lammermann et al., 2008). It is less clear why S. cerevisiae, a unicellular fungus enclosed in a cell wall, spends so much effort on keeping actin cables highly dynamic in unpolarized cells. We have shown here that reduction of cable motility can have serious consequences for polarity establishment. In cells (bni1Δ) that rely on slower cable assembly driven by Bnr1, polarization after G1 release was significantly delayed (Fig. 6A) and these cells often could not properly focus secretion on a single polarization site (Fig. 6B). One possible explanation for this phenotype is that in bni1Δ cells all cables generated during polarization became stably anchored to the septin ring by Bnr1 (Pruyne et al., 2004a) and served as fixed vectors for delivery of Cdc42-containing vesicles (Wediich-Soldner et al., 2003). In cases where cables happened to lie on opposite sides of the septin ring, separate Cdc42 caps would be formed, giving rise to the observed split-bud phenotype. Deleting Bud14, a negative regulator of Bnr1, caused a severe slow-down of cable dynamics as well as untimely polarization of cells in G1 (Fig. 6C–E). This illustrates the problems associated with overly stable actin cables. Rapid reorganization of cables might be necessary to enable a precise response during highly dynamic processes such as polarity establishment (Wediich-Soldner and Li, 2004; Brandman et al., 2005). Cells might not even be able to attain a non-polarized state without Bnr1-inactivation and, hence, rapid cable reorganization.

Materials and Methods

Cell culture and yeast strains

Unless otherwise stated, all cells were haploid, grown at room temperature (24°C) and analyzed from logarithmically growing cultures. Standard media and genomic manipulation procedures (Janke et al., 2004) were used. To generate bni1-1 IPTP, a fragment of BNI1 (1927–5862) was cloned into plasmid pRS15. The FH1 domain (3687–4056) was then replaced by a fragment encoding IPTP as described (Pual and Pollard, 2008). Plasmids with intact FH1 (40PT) or IPTP were linearized and integrated into the BNI1 locus of the bni1Δ strain. All strains used in this study are listed in supplementary material Table S1.

Plasmid construction for actin cable markers

To label actin cables with RFP we used the construct pRS315-Abp140(1-214)-mRFPruby, based on the pRS315 (CEN, LEU2) plasmid. It contains the Abp140 promoter followed by the coding region for the N-terminal 214 amino acids of Abp140, amplified with primers 5'-GGATCCGGGTGCTGGTGCTGGTGC-3' and 5'-GGCGGCCTTAAGCGCCTGT-3'. Expression of Abp140 (1-214)-mRFPruby from this plasmid labels actin more strongly than endogenous Abp140, allowing the detection of cables in two color experiments.

Microscopy

All images were acquired on a custom TIRFM setup (Till Photonics, Gräfelfing, Germany) based on an automated iMic-stand and an Olympus 1.45 NA 100x objective. Lasers at 488 nm (Coherent Sapphire 75 mW) and 561 nm (Cobolt Jive 1540 mW) were coupled through an AOTF and a galvanometer-driven scan head to adjust laser power. All images were acquired in TIRF mode at 5 Hz. Cable ends were traced over at least two frames. Distance of cable movement was divided by elapsed time to obtain velocities.

Drug treatment and G1 cell cycle arrest

Cells subjected to LatB treatment were grown in drug-containing media at room temperature for 30–45 minutes prior to imaging. For G1 arrest assay, cln-1Δ arrest cells (Richardson et al., 1989) were grown to OD 0.5–0.8 and arrested in synthetic dropout medium containing 3 mM methionine for 2–3 hours. Cells were then released by washing out the methionine. Cable dynamics were imaged before and after release in at least 10 cells per time point. Cell polarization was monitored by determining the percentage of cells exhibiting concentration polar cap of GFP-Cdc42 fluorescence on the plasma membrane.

Measurement of cable movement

Images were acquired in TIRF mode at 5 Hz. Cable ends were traced over at least two frames. Rapid reorganization of cables might be necessary to enable a precise response during highly dynamic processes such as polarity establishment (Wediich-Soldner and Li, 2004; Brandman et al., 2005). Cells might not even be able to attain a non-polarized state without Bnr1-inactivation and, hence, rapid cable reorganization.

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Measurement of cable movement

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Kernel density estimation

This technique is a non-parametric way of estimating the probability density function of a random variable and allows smoothing of a finite data set. It avoids the bias inherent in histograms when selecting bin size and endpoints. Kernel estimators smooth out the contribution of each observed data point over a local neighborhood of that data point. Here raw measurements of cable velocities were processed and plotted using the Gaussian kernel density function in MATLAB (The MathWorks), given as:

\[
p(x) = \frac{1}{nh} \sum_{i=1}^{n} \left( \frac{1}{\sqrt{2\pi}h} \right) \exp\left( -\frac{(x-x_i)^2}{2h^2} \right),
\]

where \( p(x) \) is the probability density of events at a given velocity, \( n \) is the sample size and \( h \) is the bandwidth of the kernel. The bandwidth was estimated for each dataset as:

\[
h = 1.06 \min\left( \frac{\sigma}{1.34}, \frac{\sigma}{Q_{25}} \right),
\]

where \( \sigma \) is the estimated standard deviation of the dataset and \( Q_{25} \) and \( Q_{75} \) are its 25% and 75% quartiles, respectively.

Image processing

Unless specified otherwise, images are raw or inverted for visualization. For smoothing, images were convolved with a Gaussian kernel (1 3 1; 3 7 3; 1 3 1). Iterative deconvolution was performed in Huygens (Scientific Volume Imaging, Hilversum, The Netherlands). Point spread functions were calculated separately for 488 nm and 561 nm lasers, using 40 nm yellow–green fluorescent microbeads (Invitrogen).

Statistics

All averages are given as geometric mean ± s.e.m. or s.d. \( p \)-values were calculated using the \( t \)-test (Mann-Whitney) in Prism (GraphPad).

We thank Anthony Bretscher (Cornell University, Ithaca, NY), Liza Pon (Columbia University Medical Center, New York, NY) and Rong Li (Stowers Institute for Medical Research, Kansas City, MO) for plasmids and strains, S. Wedlich, S. Oliferenko, T. Soldati and members of the Wedlich-Soldner group for comments and discussion on the manuscript, and G. Beck for technical support. This work was supported by the Max-Planck Society.

Supplementary material available online at http://jes.biologists.org/cgi/content/full/124/9/1533/DC1

References


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