Polarity establishment requires localized activation of Cdc42

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Establishment of cell polarity in animal and fungal cells involves localization of the conserved Rho-family guanosine triphosphatase, Cdc42, to the cortical region destined to become the “front” of the cell. The high local concentration of active Cdc42 promotes cytoskeletal polarization through various effectors. Cdc42 accumulation at the front is thought to involve positive feedback, and studies in the budding yeast Saccharomyces cerevisiae have suggested distinct positive feedback mechanisms. One class of mechanisms involves localized activation of Cdc42 at the front, whereas another class involves localized delivery of Cdc42 to the front. Here we show that Cdc42 activation must be localized for successful polarity establishment, supporting local activation rather than local delivery as the dominant mechanism in this system.

Introduction

The Rho-family GTPase, Cdc42, and its relatives are master regulators of cell polarity in most eukaryotes (Etienne-Manneville, 2004; Park and Bi, 2007; Wu et al., 2013). During polarity establishment, cells concentrate GTP-Cdc42 at a site on the cortex that then becomes the front of the cell (Ziman et al., 1993; Gulli et al., 2000). In budding yeast, there is consensus that polarity establishment involves positive feedback that can amplify small initial asymmetries in Cdc42 distribution to generate a highly concentrated patch of Cdc42. However, the mechanisms of positive feedback remain controversial.

Models of positive feedback via “local activation” posit that GTP-Cdc42 promotes GTP loading of neighboring GDP-Cdc42 at the plasma membrane by recruiting the guanine nucleotide exchange factor (GEF) Cdc24 (Goryachev and Pokhilko, 2008; Kozubowski et al., 2008; Johnson et al., 2011). Consistent with local activation, Cdc42 becomes co-concentrated with GTP-Cdc42 at the polarity site (Nern and Arkowitz, 1999; Toenjes et al., 1999). On the other hand, “local delivery” models posit that GTP-Cdc42 promotes targeted delivery of more Cdc42 (GDP or GTP bound) to the local vicinity from internal pools (Wedlich-Soldner et al., 2003; Slaughter et al., 2009, 2013). Local activation and local delivery are not mutually exclusive. However, findings from different laboratories have led to contradictory conclusions about their relative importance.

Support for the local activation model came from analyses of two proteins, Rsr1 and Bem1, which bind Cdc24 and concentrate it at the polarity site. Rsr1 is a Ras-family GTPase activated in the vicinity of “landmark” proteins inherited at specific sites by newborn cells. Rsr1-GTP can recruit Cdc24 from the cytoplasm, leading to Cdc24 activation near the landmarks (Howell and Lew, 2012). Bem1 is a scaffold protein that binds Cdc42-GTP and Cdc42 effectors in addition to Cdc24. These interactions allow GTP-Cdc42 to recruit Bem1-Cdc24 complexes from the cytoplasm, leading to GTP loading of neighboring Cdc42 in a positive feedback loop (Goryachev and Pokhilko, 2008; Kozubowski et al., 2008; Johnson et al., 2011). Cells lacking Rsr1 or Bem1 can polarize Cdc42, but cells lacking both cannot (Irazoqui et al., 2003). As Rsr1 and Bem1 act to localize Cdc24, these findings suggested that Cdc24 localization, and hence local activation of Cdc42, was critical for polarization.

A recent study (Smith et al., 2013) suggested that Rsr1-Cdc24 and Bem1-Cdc24 interactions are important primarily to activate Cdc24, not to localize it. In this view, Rsr1 and Bem1 simply enable sufficient GTP loading of Cdc42 to trigger positive feedback by local delivery: Localization of Cdc24 is not necessary, and as long as there is sufficient GEF activity it does not matter where the GTP loading of Cdc42 takes place. Here, we have directly tested this hypothesis. We demonstrate that local activation of Cdc42 is a key event in polarity establishment.

Results and discussion

Can polarization occur without RSR1 and BEM1?

We previously reported that rsr1Δ bem1Δ mutants were viable in three different strain backgrounds, including S288C (Irazoqui et al., 2003). However, Smith et al. (2013) found that at 24°C, rsr1Δ bem1Δ mutants were viable in their version of S288C. By dissecting tetrads from diploid strains provided

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Abbreviations used in this paper: CCD, charge-coupled device; CSM, complete synthetic medium; dex, dextrose; DIC, differential interference contrast; FRB, FKBP12-rapamycin-binding; GEF, guanine nucleotide exchange factor; MT, membrane-targeted; YEP, yeast extract Bacto peptone.

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by S. Smith and R. Li (Johns Hopkins University School of Medicine, Baltimore, MD), we found that most rsr1Δ bem1Δ spores failed to yield viable colonies even at 24°C (Fig. 1 A); those that did contained many abnormally large and multinucleate cells (Fig. 1 B and Fig. S1). These data do support the existence of a mechanism that can drive bud emergence without Bem1 and Rsr1, whose nature remains to be determined. However, the process is clearly too weak to support polarization in a majority of cells.

Is Cdc24 activated by Bem1?
A membrane-targeted (MT) FRET-based Cdc42 biosensor reported higher GTP-Cdc42 levels in wild-type cells than in bem1Δ cells or bem1 point mutants that disrupt the Bem1-Cdc42 interaction (Smith et al., 2013), prompting the conclusion that Bem1–Cdc42 interaction stimulates Cdc24 GEF activity. However, because Bem1–Cdc42 interaction localizes Cdc24 to the polarity site at the cell cortex, that alone would increase access of Cdc24 to the membrane-localized Cdc42, enhancing overall GTP loading of Cdc42.

To ask whether Cdc24 activity is regulated by Bem1 interaction, we isolated Cdc24 from wild-type and bem1Δ mutant strains and compared its GEF activity in vitro. Both preparations were active (Fig. 1 C). Indirect experiments had suggested that the PB1 domain of Cdc24 was autoinhibitory and that Bem1 binding to that domain activated Cdc24 by relief of autoinhibition (Shimada et al., 2004). However, disrupting the Bem1–Cdc42 interaction by point mutation (Fig. 1 C) or deleting the PB1 domain (Fig. 1 D) had little effect on Cdc24 GEF activity. Because in vitro assays may not recapitulate in vivo conditions, we exploited previously characterized constructs in which Bem1–Cdc42 interaction (Smith et al., 2013), but previous work suggested that Bem1 localization occurs primarily via the second SH3 domain, which binds effectors of Cdc42 (Irazoqui et al., 2003; Kozubowska et al., 2008). Indeed, Bem1N253D–GFP polarized with similar timing to wild-type Bem1–GFP (Fig. 2 A), so the ability of Bem1N253D to function is unsurprising.

To determine whether Bem1 localization was necessary for Bem1 function, we exploited the chemical genetic “anchor away” system (Haruki et al., 2008) to generate a version of Bem1 that could be trapped in the cytoplasm (Fig. 2 B). In this system, rapamycin promotes interaction of Bem1 with ribosomes, rapidly sequestering Bem1 away from the polarity site (Fig. 2 C). Addition of rapamycin blocked bud emergence (Fig. 2 D and Video 1), indicating that Bem1 cannot function in the cytoplasm. Interestingly, this was true even in cells that contain Rsr1, where Bem1 itself is nonessential (Fig. S2). Our results are fully consistent with recent findings that optogenetic sequestration of Bem1 to the surface of mitochondria similarly blocked bud emergence (Jost and Weiner, 2015). Thus, Bem1 must be able to localize to the cortex to function.

To test whether Bem1 must concentrate at the polarity site or whether general membrane localization would suffice, we exploited previously characterized constructs in which Bem1 is fused to the transmembrane protein Snc2 (Howell et al., 2009). Snc2 is a v-SNARE that becomes polarized by delivery on exocytic vesicles and rapid endocytic recycling (Valdez-Taubas and Pelham, 2003). We showed that Bem1–Snc2 tethering specifically promoted budding (Howell et al., 2009). However, mutation of the Snc2 endocytosis signal caused Bem1–Snc2V39A,M42A to localize patchily all over the plasma membrane (Fig. 2 E). Introducing this construct into the anchor-away strain, we found that Bem1–Snc2V39A,M42A could not rescue budding upon addition of rapamycin (Fig. 2 F and Video 2). We conclude that general membrane localization of Bem1 cannot promote polarization, and that Bem1 polarization is essential for Bem1 function.
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Cdc24 polarization is necessary for Cdc24 function

A delocalized version of Cdc24 lacking the C-terminal PB1 domain, Cdc24ΔPB1, was reported to promote Cdc42 biosensor activation and perhaps aid in Cdc42 polarization, even though it could not bind Bem1 or concentrate at the polarity site (Smith et al., 2013). However, in agreement with earlier work (Kozubowski et al., 2008), we found that Cdc24ΔPB1 was unable to sustain viability in the absence of endogenous Cdc24 (Fig. 3A), suggesting that delocalized Cdc24 is not functional.

Cdc24ΔPB1 is primarily cytoplasmic, whereas its target, Cdc42, is associated with membranes. Thus, the inability of Cdc24ΔPB1 to function may simply reflect lack of access to the plasma membrane. To test this possibility, we fused Cdc24ΔPB1 (or full-length Cdc24 as control) to a 28-residue N-terminal peptide from Psr1, which confers plasma membrane localization via myristoylation and dual palmitoylation (Siniossoglou et al., 2000; Kuo et al., 2014). We refer to these as MT versions. Because previous experiments indicated that Cdc24 undergoes inhibitory phosphorylation at the plasma membrane (Kuo et al., 2014), we also generated versions that were nonphosphorylatable and hence could not be inhibited: MT-Cdc24S3A and MT-Cdc24A37AΔPB1. These proteins were expressed (Fig. 3B) and targeted to the plasma membrane (Fig. 3C), but MT-Cdc24S3A was polarized whereas MT-Cdc24A37AΔPB1 was not. MT-Cdc24S3A rescued the viability of cdc24Δ mutants, but MT-Cdc24A37AΔPB1 did not (Fig. 3D). In a temperature-sensitive cdc24-1 context, cells expressing MT-Cdc24S3A polarized and budded, but cells expressing MT-Cdc24A37AΔPB1 arrested as large, round, unbudded cells at restrictive temperature (Fig. 3E and F; and Video 3). Indeed, these cells were large even at permissive temperature, suggesting that unpolarized, active Cdc24A37AΔPB1 interferes with polarization. Similar results were obtained with MT-Cdc24 and MT-Cdc24ΔPB1 (Fig. S3). Thus, uniformly targeting Cdc24 to the plasma membrane is not sufficient to promote polarization, suggesting that localizing GEF activity is essential.

A remaining caveat is that truncation of the PB1 domain might render Cdc24 nonfunctional for reasons other than its localization. We consider this unlikely because Cdc24ΔPB1 retained normal GEF activity in vitro (Fig. 1D). However, as an additional test, we generated a Cdc24A37AΔPB1-Snc2 fusion analogous to the Bem1-Snc2 fusions discussed earlier. Fusion to Snc2 restored polarization (Fig. 4A) and function to the other
erwise nonfunctional Cdc24<sup>37A ΔPB1</sup> cells containing this construct as the sole source of Cdc24 proliferated successfully (Fig. 4 B). Similarly, fusion of Cdc24<sup>ΔPB1</sup> to the Cdc42 effector Cla4 (a p21-activated kinase) or to the SH3-2 domain of Bem1 (which binds to Cla4 and other Cdc42 effectors) restored both localization and function to Cdc24<sup>ΔPB1</sup> (Kozubowski et al., 2008). Thus, the functional deficit of a Cdc24 that lacks the PB1 domain can be rescued by linkage to a polarized protein, whether that protein polarizes by diffusion capture (Cla4) or vesicle recycling (Snc2).

As discussed earlier, Bem1-Snc2 was able to rescue polarization in <sup>rsr1Δbem1Δ</sup> even though the fusion protein was polarized by a vesicular mechanism. We showed previously that such “re-wired” cells used an artificial positive feedback mechanism to polarize and occasionally generate two buds at the same time (Howell et al., 2009). If the only essential role for Bem1 and Rsr1 is to localize Cdc24 to the polarity site, then cells containing Cdc24-Snc2 derivatives (which also polarize by a vesicular mechanism) should no longer require Bem1 or Rsr1. Indeed, cells containing Cdc24<sup>38A</sup>-Snc2 or Cdc24<sup>37A ΔPB1</sup>-Snc2 as the sole source of Cdc24 could polarize (Fig. S3) and proliferate in the absence of Rsr1 and Bem1 (Fig. 4 C). As with Bem1-Snc2, occasional cells had two buds (Fig. S3). The finding that heterologous localization of Cdc24 (with or without the PB1 domain) to the polarity site rescues <sup>rsr1Δbem1Δ</sup> synthetic lethality indicates that the essential role of Bem1 and Rsr1 is to localize the GEF.

Previous studies showed that overexpression of MT-Cdc24 blocked polarization even if cells also had an endogenous wild-type Cdc24 (Shimada et al., 2004; Kuo et al., 2014). Unlike MT-Cdc24 expressed at endogenous levels, which became concentrated at the polarity site (Fig. 3 C), overexpressed MT-Cdc24 accumulated uniformly all over the membrane (Kuo et al., 2014). The dominant lethality of overexpressed MT-Cdc24 could be due to excessive Cdc42 activation or to a lack of localized GTP loading of Cdc42. Co-overexpression of Bem1 rescued the lethality of overexpressed MT-Cdc24 (Fig. 4 D), allowing cells to bud and in many cases to concentrate MT-Cdc24 in the bud (Fig. 4 E). This result is not consistent with the view that lethality is attributable to excess GTP-Cdc42 because extra Bem1 should (if anything) increase GTP loading even further. Instead, we conclude that the lethality of overexpressed MT-Cdc24 stems from the failure to localize GTP loading of Cdc42. The effect is dominant because the overexpressed MT-Cdc24 titrates Bem1 away from the endogenous Cdc24, but that can be ameliorated by co-overexpression of Bem1. In summary, our findings show that blocking Cdc24 accumulation at the polarity site by any of several strategies (deletion of the PB1 domain, blocking Bem1 localization, or overexpression of MT-Cdc24) blocks polarization and budding.
**Conclusions**

A key requirement of the local activation model is that GEF activity be targeted to the polarity site. Here we show that de-localized Cdc24 GEF activity is unable to support polarization. A major pathway for localizing Cdc24 is mediated by interaction with Bem1, and we found that, as for Cdc24, localization of Bem1 was essential for polarization. The finding that Bem1 and Cdc24 must polarize to function supports the local activation model in which localized GTP loading of Cdc42 underlies polarity establishment.

Our findings do not rule out the possibility that Cdc42 is also delivered to the polarity site. Indeed, a recent study suggested that Cdc42 undergoes endocytosis and recycling to the cortex on secretory vesicles (Watson et al., 2014). However, the Cdc42 concentration on secretory vesicles was estimated to be approximately threefold lower than that at the polarity site (Watson et al., 2014). From top: DLY17405, DLY17402, DLY19606, DLY19604, DLY18430, and DLY18603. (C) Heterologous polarization of Cdc24 bypasses the need for Rsr1 and Bem1 in polarity establishment. Rsr1Δ bem1Δ cells containing the indicated Cdc24-Snc2 fusions were tested as the only source of Cdc24 and carrying a URA3-marked BEM1 plasmid were grown to mid-log phase, spotted [10⁴, 10³, and 10² cells] onto medium with or without 5-fluoroorotic acid (to select for plasmid loss), and incubated for 2 d at 30°C. From top: DLY19826, DLY19773, and DLY19774. (D) Overexpression of Bem1 restores budding to cells overexpressing MT-Cdc24. Inverted, maximum projection images of cells incubated for 4 h in 5 nM β-estradiol medium to induce overexpression of MT-Cdc24 alone (DLY15297) or MT-Cdc24 and Bem1 (DLY15311) and incubated for 2 d at 24°C. (E) Overexpression of Bem1 restores budding to cells overexpressing MT-Cdc24. Inverted, maximum projection images of cells incubated for 4 h in 5 nM β-estradiol medium to induce overexpression of MT-Cdc24 alone (DLY15297) or MT-Cdc24 and Bem1 (DLY15311). MT-Cdc24 itself becomes enriched in most buds of cells coexpressing Bem1. Bar, 5 μm.

**Materials and methods**

**Yeast strains**

Yeast strains used in this study are listed in Table S1. Experiments assessing the function of Bem1 derivatives were performed in strains...
GFP-CDC24, the codons encoding first 28 residues from the N terminus of the PSR1 bem1-GFP construct and integrated it at the endogenous locus. Functional GFP-SEC4 (Chen et al., 2012) was integrated at the URA3 locus as previously described. CDC42-mCherry was based on the Cdc42 probe first reported in S. pombe (Bendezzi et al., 2015), and integrated at the URA3 locus. To generate Cdc24V39A-GFP-Snc2 or Cdc24D47AΔPB1-GFP-Snc2, we constructed a new vector based on the PCR-based C-terminal tagging method (Longtine et al., 1998). BEM1-GFP-SNC2Δ482 (Howell et al., 2009) was integrated at the URA3 locus as previously described.

To replace the endogenous BEM1 with bem1N253D-GFP, we deleted one copy of BEM1 in a diploid strain with URA3. An integrating bem1N253D-GFP-TRP1 plasmid (DLB4214) was subcloning the 1.8 kbp Smal-XcmI fragment containing the N253D mutated one copy of URA3 resuspended in sonication buffer containing 10 mM glutathione, and GST-Cdc42 was purified in sonication buffer containing 10 mM glutathione. The eluate was dialyzed against diethyl sulfate buffer (20 mM Tris, 5 mM MgCl2, 1 mM EDTA, 5 μM GDP, 2 mM DTT, and 50% glycerol) overnight at 4°C.

Immunoprecipitation and GEF assay
Yeast lysates were prepared by high-speed vortexing with glass beads in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5% NP-40, 4 mM β-glycerophosphate, 4 mM NaF, 4 mM Na3VO4, 4 mM NaP2O7, 2 mM DTT, and 1x protease inhibitor cocktail). Lysates were clarified by centrifugation at 10,000 g for 10 min at 4°C. For immunoprecipitation, 10-μl antibody-coupled beads (monoclonal anti-HA agarose, Sigma-Aldrich) were mixed with lysis containing 2.5 mg protein that was diluted to 1 ml using lysis buffer without NP-40. After incubation at 4°C for 2 h, beads were pelleted and washed twice with lysis buffer and once with GEF buffer (20 mM Tris, 5 mM MgCl2, 1 mM EDTA, 5 μM GDP, and 2 mM DTT). Beads were then incubated with 40 pmol GST-Cdc42 and 2.5 μCi GTPγS in 45 μl GEF buffer for 15 min at RT, with gentle mixing every 3 min. Reactions were stopped with 0.5 μl ice-cold GEF buffer, after which beads were pelleted by centrifugation and supernatants were filtered through nitrocellulose filters (Protran BA 85, Whatman; GE Healthcare) to trap and assay protein-bound radioactivity. The beads containing immunoprecipitated protein were then incubated with SDS sample buffer (2% SDS, 2 mM β-mercaptoethanol, 4% glycerol, 40 mM Tris-HCl, pH 6.8, and 0.01% bromophenol blue) at 95°C, and Cdc42 levels were analyzed by immunoblot.

Spot assay
For cell viability analysis, cells were grown overnight in yeast extract Bacto peptone (YPE; 2% Bacto peptone, 1% yeast extract, 0.001% uracil and adenosine) medium (BD Biosciences) with 2% dextrose (dex) liquid media and diluted to ~3 × 106 cells/ml. 10-fold serial dilutions (105, 104, 103, 102, and 101 cells) were spotted onto YEP + dex agar plates or complete synthetic medium (CSM; MP Biomedicals) + dex agar plates lacking uracil. For cell viability analysis on 5-fluoroorotic acid + dex agar plates, cells were diluted 2 × 107 cells/ml and spotted onto YEP + dex agar plates containing the indicated concentrations of β-estradiol. Rapamycin experiments were performed in TOR1-1 prl1Δ strains as described (Haruki et al., 2008). To use the ribosome “anchor,” two tandem copies of FKBP12 and an HA tag were fused to the C terminus of the endogenous Rpl13a using the “pop-in/pop-out” strategy and confirmed by sequencing. Expression was assessed by Western blot using α-HA antibodies. Rapamycin binds to FKBP12, creating an interaction surface for the FKBP12-rapamycin–binding (FRB) domain for human mammalian target of rapamycin (Chen et al., 1995). We generated a BEM1-2xFRB-HA-GFP construct and integrated it at the BEM1 locus, with correct integration confirmed by sequencing. Expression was checked by Western blot and microscopy.

Cdc42 purification
GST-Cdc42 was expressed in Escherichia coli BL21(DE3) as described elsewhere (Bose et al., 2001). Cell pellets were resuspended in sonication buffer (50 mM phosphate buffer, pH 7.6, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 10% glycerol, 5 μM GDP, and 1x protease inhibitor cocktail [Complete, EDTA-free tablet; Roche]) and then disrupted by sonication. Lysates were clarified by centrifugation at 10,000 g for 15 min at 4°C and incubated for 2 h with Glutathione Sepharose 4B (GE Healthcare) at 4°C. Beads were washed twice in sonication buffer containing 1 mM glutathione, and GST-Cdc42 was purified in sonication buffer containing 10 mM glutathione. The eluate was dialyzed against dialysis buffer (20 mM Tris, 5 mM MgCl2, 1 mM EDTA, 5 μM GDP, 2 mM DTT, and 50% glycerol) overnight at 4°C.
rose (Denville Scientific Inc.), and sealed with petroleum jelly. Cells were imaged at 24°C unless otherwise noted. Also unless otherwise noted, images were acquired by using an Andor Revolution XD spinning-disk confocal microscope (Olympus) with a Yokogawa CSU-X1 5,000 pmp disc unit, 100x/1.4 UPlanSapo oil-immersion objective, and were captured with an Andor Ixon3 897 512 EM charge-coupled device (CCD) camera using MetaMorph software (Universal Imaging).

Fig. S1 shows additional examples of cells depicted in Fig. 1B. Fig. S2 shows the results of sequestering Bem1 in the cytoplasm in Rsr1Δ cells. Video 1 shows the results of sequestering Bem1 in the cytoplasm in Rsr1Δ cells expressed additional Bem1 targeted to the plasma membrane (Bem1-GFP-Snc2V39A,M42A). Video 3 shows cdc24-1 cells expressing MT-Cdc24Δ GFP shifted to restrictive temperature. Table S1 shows yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201506108/DC1.

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