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Introduction

Cdc42p, together with other polarity proteins, becomes polarized to a cap at the presumptive bud site and the tip of the emerging bud. The initial observation that Cdc42p is able to polarize, and remain polarized, in the complete absence of F-actin (Ayscough et al., 1997), has been confirmed repeatedly by many laboratories (including ours). These studies use Lat-A as a quick, effective method to completely depolymerize actin, and led to the conclusion that Cdc42p polarization is actin-independent. Polarized secretion and endocytic uptake require F-actin cables and patches, respectively (Karpova et al., 2000; Pruyne et al., 1998; Schott et al., 2002). When Lat-A is applied to yeast cells, neither actin patches nor cables are detectable, and all F-actin-dependent processes are disrupted (Ayscough et al., 1997). Thus, Cdc42p polarization occurs by a non-secretory pathway.

Is the polarized cap a stable, static structure? Current models of Cdc42p function propose that the cap acts as an organizing center for polarity establishment. Since this cap is associated with the plasma membrane, it may be a static scaffold acting as a site for polymerization of actin cables by formins, assembly of septins before bud emergence, signal transduction by effector kinases, etc. However, the observation that polarized Bni1p is a dynamic patch that oscillates back and forth on the bud cortex seemingly contradicts this widely held view of the cap (Ozaki-Kuroda et al., 2001).

Here we present further data that seem to indicate that the membrane-associated cap is in fact a dynamic structure. Our data reveal that the loss of actin cables, but not actin patches, leads to delocalization of Cdc42p. We propose that the initial polarization of Cdc42p is an actin-independent process. However, continued polarization is the result of two competing processes: endocytic removal of the plasma membrane, and a counterbalancing deposition process, dependent on the polarized secretion machinery.

Results

Lat-A vs. Lat-B

The latrunculins A and B were originally isolated from the Red Sea sponge *Latrunculia magnifica* (Spector et al., 1983). Apparently, what caught the attention of the researchers was a "reddish fluid" that exuded from the sponges when squeezed manually. This fluid "causes fish to retreat from its vicinity" and "causes agitation of fish in seconds, followed by hermorrhage, loss of balance, and, after 4 to 6 minutes, death" (Spector et al., 1983). The structures of the latrunculins were determined by NMR; they differ in that Latrunculin A has a 16-membered ring and Latrunculin B has a 14-membered ring (Fig 1.1).

In the course of independent experiments, we observed that wild type cells lost polarized Cdc42p when incubated with Lat-B, in contrast to Lat-A-treated cells (Fig 1.2A). When stained with Alexa-phalloidin to visualize F-actin, Lat-A-treated cells presented a diffuse, cytoplasmic background staining, indicating a complete depolymerization of F-actin. In contrast, Lat-B-treated cells presented a punctate staining similar in appearance to actin patches (Fig 1. 2A). No cables were detected, however. Thus, Lat-B appeared to be less potent than Lat-A at depolymerizing F-actin. Potentially, patch-dependent processes (such as endocytosis) may be at least partially functional in Lat-B treated cells. The depolarization of Cdc42p following Lat-B exposure occurred rapidly (Fig 1.2B). Unbudded cells and large-budded cells lost ~40% polarization within 15 min of Lat-B addition, whereas small-budded cells lost polarized Cdc42p over a longer incubation time.

Disruption of cables causes Cdc42p delocalization

As mentioned above, an obvious phenotype of Lat-B treated cells is the absence of detectable actin cables, while patches remain visible. Therefore, it is possible the loss of Cdc42p polarization is due to the loss of cables. Alternatively, Lat-B may be affecting some other process. To test whether selective loss of cables causes the depolarization of Cdc42p, we used a *tpm1-2 tpm2Δ* strain. This strain harbors a temperature-sensitive allele of tropomyosin as the only copy of tropomyosin. At restrictive temperature, actin cables rapidly disassemble in these cells, disrupting polarized secretion (Pruyne et al., 1998). In the *tpm2Δ* control, Cdc42p transiently delocalized upon shift from 24°C to 34.5°C, returning to polarized sites at about 40 min after shift. In contrast, the *tpm1-1 tpm2Δ* cells did not recover Cdc42p polarization even by 120 min (Fig 1. 3). Interestingly, unbudded cells lost polarization faster than small-budded cells, in both cases (data not shown). Thus, loss of cables (and, therefore, polarized secretion) causes loss of Cdc42p polarization.

Hyperosmotic shock causes transient Cdc42p depolarization

The transient depolarization of Cdc42p observed in the *tpm2Δ* controls suggested that Cdc42p depolarizes in response to heat shock, consistent with previous reports using wild-type cells (Ho and Bretscher, 2001). Given these observations, we wondered whether other stressful conditions that also depolarize actin, such as hyperosmotic shock (Chowdhury et al., 1992), would result in depolarization of Cdc42p. To test if hyperosmotic shock causes Cdc42p to delocalize, we incubated cells in 0.4 N NaCl. Wild type cells incubated in 0.4 N NaCl did, in effect, lose Cdc42p polarization rapidly (Fig 1. 4). Cdc42p re-polarized in small-budded cells starting at about 50 min after NaCl addition, in contrast to unbudded and large-budded cells, which failed to recover polarization by 120 min. Thus, two different stress conditions, i.e. heat shock (Ho and Bretscher, 2001) and hyperosmotic shock (Fig 1. 4), cause transient depolarization of Cdc42p. This response parallels previously published observations that the actin cytoskeleton transiently depolarizes in reaction to stressful conditions (Chowdhury et al., 1992).

The delocalization of Cdc42p does not require Rdi1p

These results suggest that an unknown mechanism is capable of removing polarized Cdc42p from the cap. When polarized secretion is compromised, the removed Cdc42p may not be replenished, leading to a loss of polarization. The unknown mechanism by which Cdc42p is removed from the cap may depend on Cdc42p-Rho-GDP dissociation inhibitor proteins (GDIs - Rdi1p in yeast). GDIs bind Rho proteins by their C-terminal prenylated domain, which fits in a hydrophobic pocket in the GDI. Binding to the GDI removes the G protein from the membrane and inhibits GDP release (Hoffman et al., 2000; Koch et al., 1997; Leonard et al., 1992; Masuda et al., 1994). If the re-insertion of Cdc42p depended on actin cables, then the absence of cables would result in Rdi1p-dependent delocalization of Cdc42p. To test whether Rdi1p was necessary for the delocalization of Cdc42p in the absence of actin cables, we incubated *rdi1Δ* cells with Lat-B. After 2 hr of incubation, the *rdi1Δ* cells had lost Cdc42p polarization, similar to wild type (Fig 1. 5). Thus, Cdc42p delocalization in cells lacking actin cables is unlikely to be Rdi1p-mediated.

Endocytosis removes Cdc42p from the cap

Alternatively, Cdc42p may be removed from the polarized cap *via* an endocytic process. In the absence of cables, recycling membranes removed from the cell cortex by endocytosis would be targeted by secretion to the entire cell surface, leading to the dispersal of the cap. If this were the case, then the result of simultaneous disruption of endocytosis and cable function may be analogous to that of Lat-A incubation: polarization of Cdc42p would be preserved. Lat-B is membrane-permeable; active endocytosis is not required for its effect. To test whether disruption of cables in endocytosis-defective cells leads to Cdc42p depolarization, we used *lcb1-1* and *end4-1* mutants. *lcb1-1* mutants are defective in sphingoid base biosynthesis, which causes a rapid loss of endocytosis at 37°C (Zanolari et al., 2000). *end4-1* mutants harbor a temperature-sensitive mutation in *SLA2*, which codes for an actin-associated protein required for internalization (Wesp et al., 1997). We shifted the mutants to 37°C for 1 hr to induce loss of endocytic uptake. Next, they were incubated in Lat-B medium for 15 min and 1 h at 37°C to induce cable depolymerization. It is expected that Cdc42p would depolarize in all these strains as a result of the heat shock. However, by 75 min (time of the first samples) of incubation at 37°C, most Cdc42p polarization returned, as expected. As shown in Fig 1. 6, wild type cells completely lost polarized Cdc42p when treated with Lat-B, as opposed to the DMSO-treated control. In contrast, the *end4* and *lcb1* mutants exhibited Cdc42p polarization despite treatment with Lat-B. Thus, the loss of actin cables causes the depolarization of Cdc42p only if the endocytic machinery is functional.

Discussion

Lat-B is less potent than Lat-A

Latrunculin is a very effective tool to study the actin cytoskeleton. In yeast, Lat-A is a more potent inhibitor of growth than Lat-B (Ayscough et al., 1997). This is consistent with evidence from fluorescence microscopy observations: patches are visible in Lat-B treated cells. Cables, however, cannot be detected, making it plausible that Lat-B disrupts cable function to a greater extent than patch function. Other evidence that cable function is disrupted is the depolarization of secretion. Cells treated with Lat-B become round and unbudded. At this time it is unknown whether the difference between Lat-A and Lat-B is a result of different affinities for G-actin, or if they are differentially membrane-permeable. It is feasible that Lat-B may simply reach lower intracellular levels than Lat-A at any given external concentration.

In any case, these observations seem to indicate that patches and cables are fundamentally different, beyond their obviously distinct morphologies. Presumably, actin filaments are the same in both structures. The differences between patches and cables may be due to actin-associated proteins that are

recruited differentially to either structure. It is possible that proteins found on the patches render those structures more resistant to the action of Lat-B. Since Latrunculin is thought to bind to actin monomers, preventing polymerization, it is possible that proteins associated with the patches may prevent depolymerization and thus prevent removal of actin monomers from the filaments in the patches, lowering the rate of depolymerization of F-actin. However, when cells were exposed to low doses of Lat-A, patches disassembled more quickly than cables(Karpova et al., 1998). Thus, it appears that the net rate of depolymerization in patches may be higher than the rate in cables. This lends more support to the view that perhaps some protein(s) localized preferentially to patches may compete with latrunculin B for the binding site on actin. If this were true, then overexpression of such protein might confer resistance to Latrunculin B but not Latrunculin A.

Cdc42p depolarizes in response to stress

It has been known for quite some time that heat shock and hyperosmotic shock cause the depolarization of actin, although the mechanisms involved are unclear. We now show that Cdc42p polarization is lost as well. It is possible that environmental insults result in depolarization by regulating Cdc42p localization through a signal transduction pathway. Depolarization (or inactivation) of Cdc42p would result in depolarization of actin, as shown by (Gladfelter et al., 2002). Alternatively, stress may lead to the initial loss of cable polarity, followed by Cdc42p delocalization. At this point, our data is insufficient to distinguish between these two possibilities, which are not necessarily mutually exclusive: some stresses may directly cause Cdc42p to delocalize, while others may act on Cdc42p indirectly through the actin cytoskeleton.

What benefit do cells derive from depolarizing in response to stress? During hyperosmotic and heat stresses, cell wall properties are affected. In the case of hyperosmotic shock, the cell wall may require remodeling as a countermeasure to ensuing plasmolysis; it is believed that intracellular turgor pressure must be preserved in order to be able to drive cell expansion and cell wall remodeling (Pruyne and Bretscher, 2000). In the case of heat shock, the cell wall may require reinforcement to prevent lysis, given the increased fluidity of the membrane at higher temperatures and the higher rates of cell growth at higher temperatures. Under both circumstances, the cell may benefit from relocalizing secretion and cell wall deposition to an isotropic config 1uration, in order to target remodeling factors and components to the entire cell surface. Later, as stress adaptation proceeds, the cytoskeleton is refocused for bud growth in a Hog1p-dependent manner (during osmotic shock)(Brewster and Gustin, 1994) and Ras2p-dependent manner (during heat shock)(Ho and Bretscher, 2001).

Unbalanced membrane trafficking causes depolarization

Endocytic removal of polarized Cdc42p causes depolarization when not counterbalanced by polarized secretion. This unexpected observation suggests that the protein complexes in the polarized cap, a structure assembled at the pre-bud site even in the absence of F-actin, are dynamic and can be transported on membranes. In wild type cells, the polarized cap may in fact be constantly renewed by localized deposition of secretory vesicles. Endocytic uptake may cycle the components through the cytosol before returning them to the polarized site, creating a steady state of polarization that is observable under the microscope.

It is conceivable that recycling on membranes allows for the disassembly of cap components on endosomal membranes in the cytoplasm, to be recruited by the cap structure at the cell cortex *via* an actin-independent mechanism. If polarized secretion is active, the cap remains in place and is competent to recruit the components that were released to the cytosol from endosomes. When polarized secretion is disrupted, the cap is removed from the bud tip by endocytosis. Recruitment of polarized cap protein complexes becomes delocalized and they disperse throughout the cortex. Thus, polarized secretion is required for maintenance of the cap structure, counterbalancing endocytic removal. This model can explain two seemingly opposite observations: a) Cdc42p and associated signaling proteins are able to polarize in absence of any preexisting asymmetries, including the lack of F-actin, and b) if an actin-dependent process (i.e., polarized secretion) is disrupted, polarization of Cdc42p and associated proteins is lost.

The physiological implications of this process are unknown. One possibility is that it provides the cell with a simple way to depolarize in response to stress; by disrupting cable-mediated vectorial delivery of secretory vesicles, it could acquire a depolarized state adequate to respond to the environmental insult. Alternatively, if the endocytosed cap components are released to the cytosol, this may be a step in the normal regulation of those proteins. It is also tempting to speculate that this may be a mechanism by which the apical-isotropic switch takes place, downstream of Clb1p-2p/Cdc28p activation at the S/G2

transition. By inhibiting polarized secretion, total depolarization can be enforced. If this is the case, there are a number of putative targets of Clb1p-2p/Cdc28p-regulation: Myo2p, tropomyosin, vesicle-specific Myo2p-receptors (analogous to the vacuole-specific receptor Vac17p (Ishikawa et al., 2003)), etc. It will be of great scientific value to understand this potential mode of regulation in more detail.

Table 4.I **Yeast strains used in this study.**

Strain	Relevant Genotype
DLY5	**a/α** *bar1*
RH1800	**a** *bar1*
RH3809	**a** *bar1 lcb1-100*
RVS152	**a** *bar1 end4-1*
DLY4051	**a/α** *tpm1-2::LEU2 tpm2::HIS3*
DLY4052	**a/α** *TPM1 tpm2::HIS3*
MOSY20	**a** *rdi1::TRP1*

Key research accomplishments

- Demonstration of the requirement of polarized secretion in the presence of active endocytosis for polarization of Cdc42p.
- Identification of polarized secretion as a means of regulating the polarity of upstream components.
- Manuscript in preparation describing the results presented in this report.

Reportable outcomes

- Publication showing the requirement of Bem1p for polarization in the absence of preexisting cues (Appendix 2 – paper accepted conditionally in Nature Cell Biology).
- Publication of results as a poster at the 2002 Yeast Genetics and Molecular Biology Meeting in Madison, Wisconsin.
- Publication of results as a poster at the 2002 American Association for Cell Biology Meeting in San Francisco, California.
- Publication of results as 2 posters at the XXI International Conference on Yeast Genetics and Molecular Biology in Gothenburg, Sweden, in July 2003.
- Degrees awarded by Duke University in May 2003: PhD in Molecular Cancer Biology, Certificate in Cell and Molecular Biology.
- Awarded the Jane Coffin Childs Postdoctoral Fellowship for continuing research in the laboratory of Dr. Frederick Ausubel, at the Harvard Medical School, focusing on the genetic and molecular dissection of the innate immune response to bacterial and fungal pathogens.

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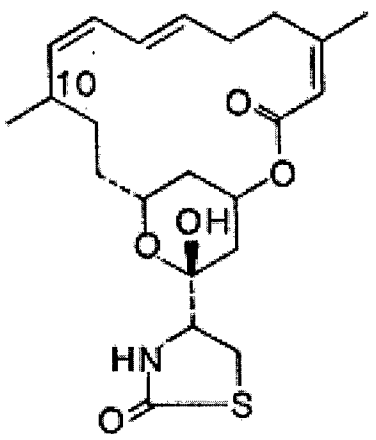
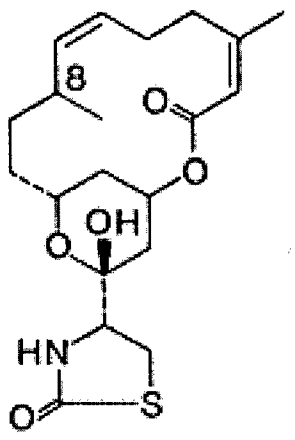
Appendices

1. **Figures**
2. **Paper conditionally accepted in Nature Cell Biology for publication.**



Latrunculin A
(LAT-A)



Latrunculin B
(LAT-B)

Figure1.1 **Structure of the latrunculins.**

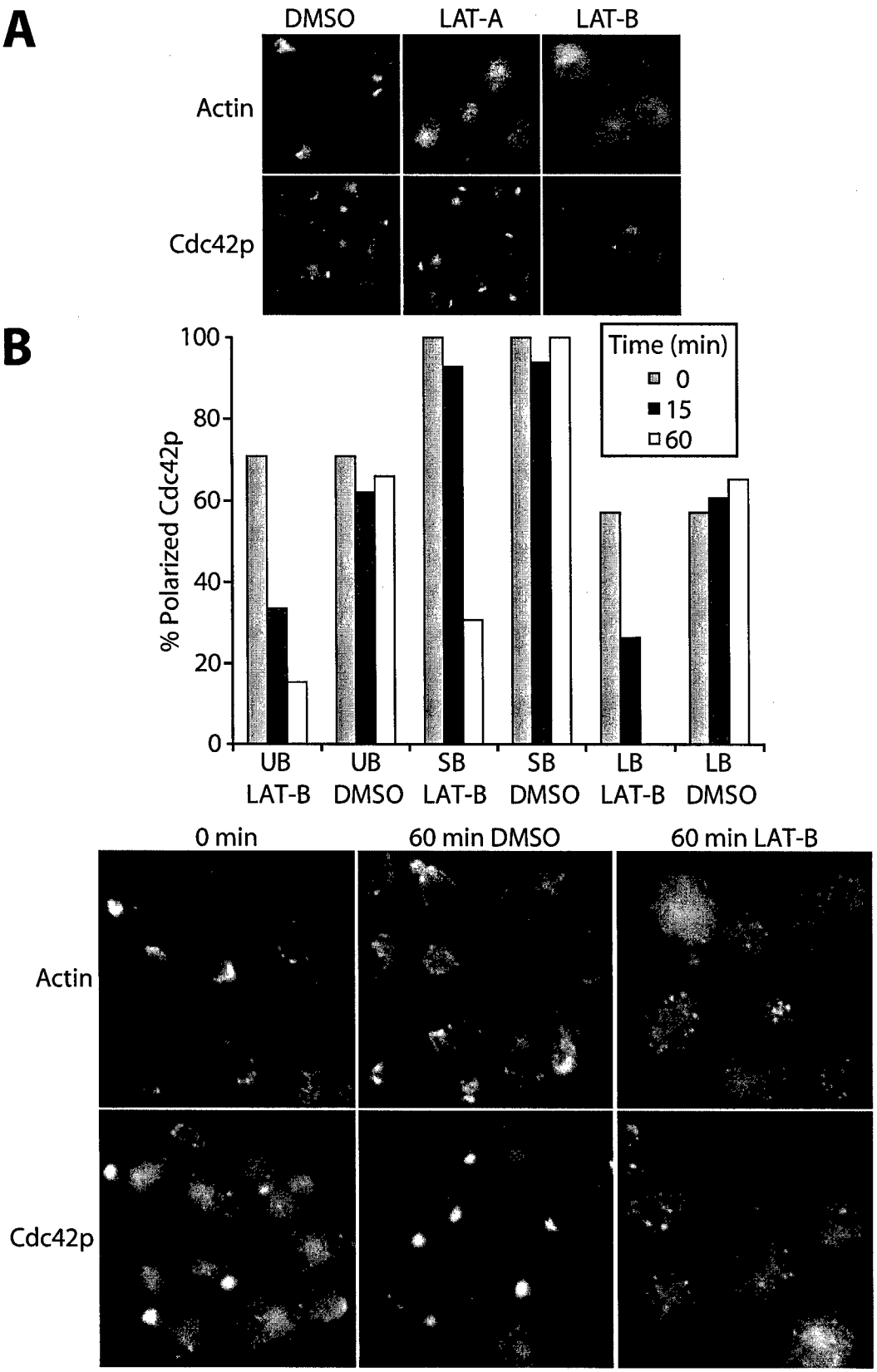
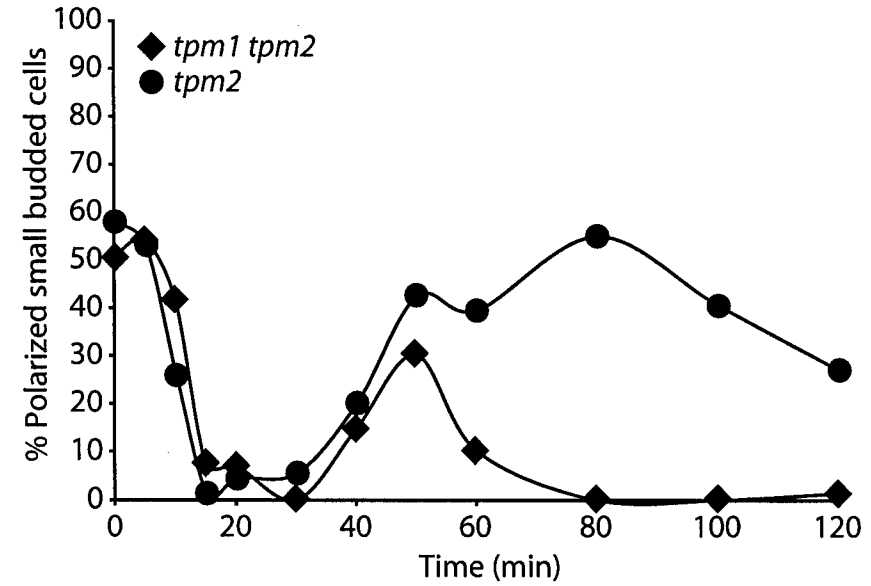


Figure1.2 **Latrunculin-B treatment causes Cdc42p to depolarize. A.** Wild type (DLY5) cells treated with 100 M Latrunculin -A, 200 M Latrunculin B or DMSO as control for 2 hr. **B.** Timecourse of loss of Cdc42p polarization. Wild type (DLY5) cells were treated with Latrunculin-B for 15 and 60 min. Quantitation of the fraction of unbudded cells (UB), small-budded cells (SB) and large-budded cells (LB) that have polarized Cdc42p. Actin was visualized by staining with Alexa-phalloidin, Cdc42p was visualized by immunofluorescence



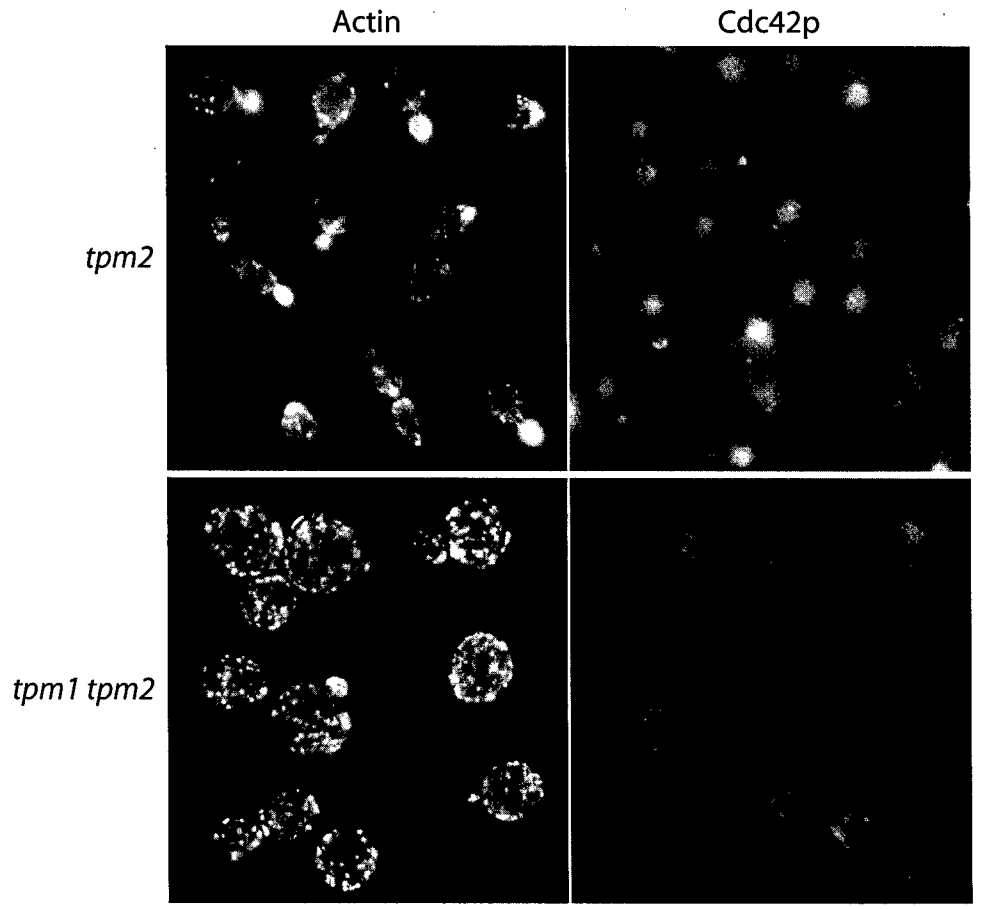


Figure1.3 **Loss of cables results in Cdc42p depolarization.** *tpm1-2 tpm2* cells and *tpm2* cells as control were grown at 24 °C until mid-log phase, then shifted to 34.5°C for the times indicated. Graph, percent small -budded cells with polarized Cdc42p vs. time of incubation at 34.5°C. In both cases, there is a transient repolar ization at approx. 50 min after shift; this is due to the induction of cytokinesis and the formation of rings at the mother -bud neck. Panels, micrographs of cells after 80 min of incubation at 34.5°C. Fluo rescence microscopy as in Fig. 1 .1.

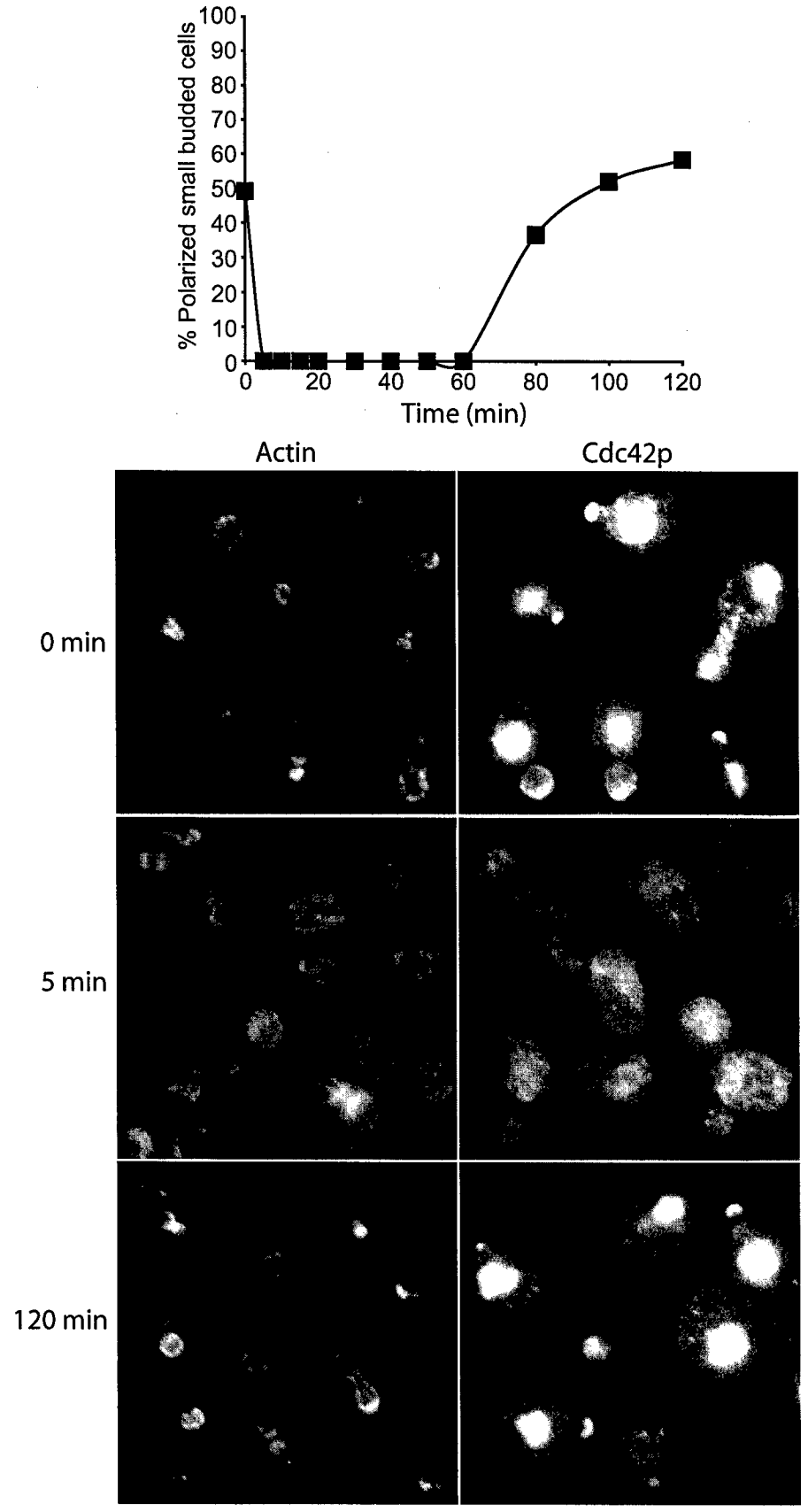


Figure1.4 **Osmotic shock results in Cdc42p depolarization.** Wild type cells (DLY5) were grown to mid-log phase previous to the addition of 0.4 N NaCl. Graph, percentage of small-budded cells that have polarized Cdc42p vs. time of incubation in 0.4 N NaCl. Below, micrographs of cells after 0, 5, and 120 min of incubation in 0.4 N NaCl. Fluorescence microscopy as in Fig. 1.1.

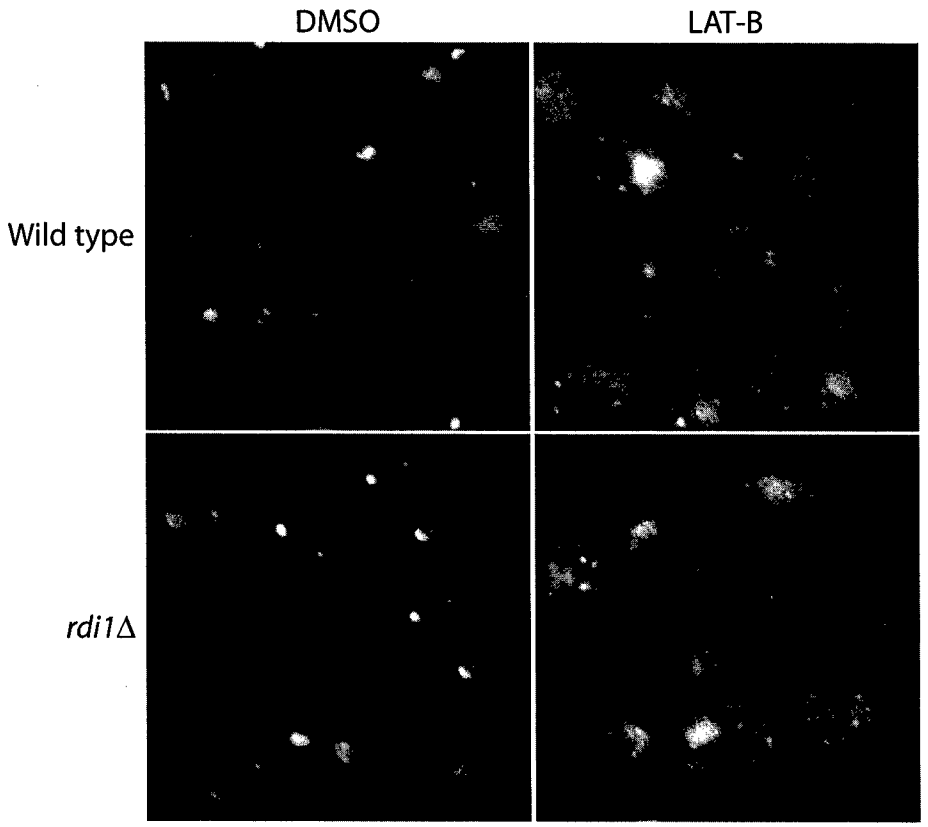


Figure1.5 **Rdi1p is not required for the depolarization of Cdc42p caused by Latrunculin-B.** Wild type cells (DLY5) or *rdi1* cells (MOSY20) were incubate d in 200 M Latrunculin B, or DMSO as control, for 2 hr. Fluorescence microscopy as in Figure1 .1.

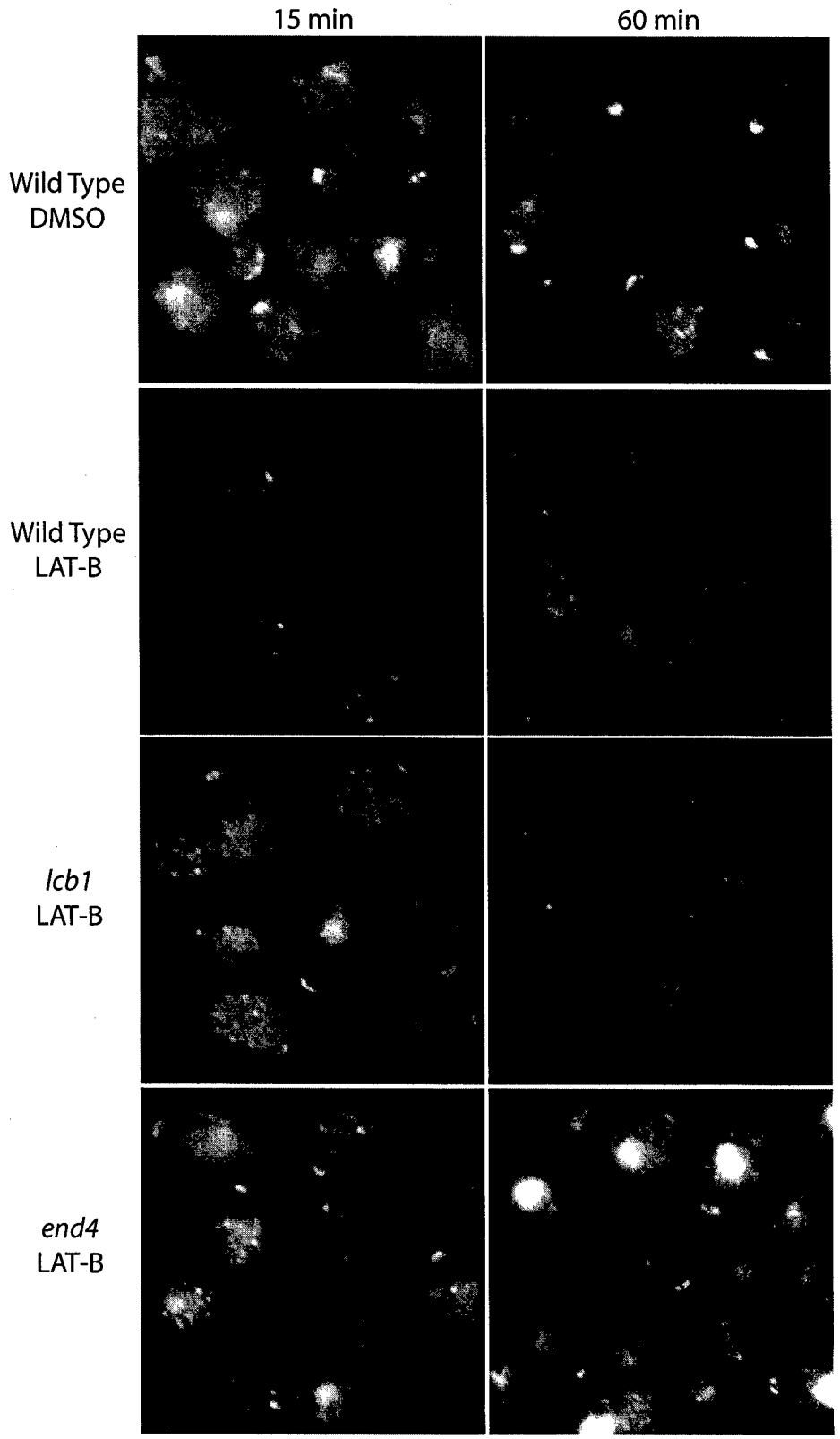


Figure1.6 **Endocytosis mutants do not depolarize Cdc42p in response to Latrunculin B treatment.** Wild type (RH1800), *lcb1-100* (RH3809) and *end4-1* (RVS152) cells were grown to mid-log phase at 24°C, shifted to 37°C for 1 hr and then treated with 200 M Latrunculin B or DMSO for 15 min and 60 min. *lcb1-100* mutants lose actin polarity on their own after 2 hr incubation at 37°C (Zanolari et al., 2000). Fluorescence microscopy as in Fig 1.1.

nature cell biology

Scaffold-mediated symmetry breaking by Cdc42p

Javier E. Irazoqui, Amy S. Gladfelter and Daniel J. Lew[1]

Cell polarization generally occurs along a single well-defined axis that is frequently determined by environmental cues such as chemoattractant gradients or cell–cell contacts, but polarization can also occur spontaneously in the apparent absence of such cues, through a process called symmetry breaking[1–5]. In *Saccharomyces cerevisiae*, cells are born with positional landmarks that mark the poles of the cell and guide subsequent polarization and bud emergence to those sites, but cells lacking such landmarks polarize towards a random cortical site and proliferate normally[6]. The landmarks employ a Ras-family GTPase, Rsr1p[7–9], to communicate with the conserved Rho-family GTPase Cdc42p, which is itself polarized and essential for cytoskeletal polarization[10,11]. We found that yeast Cdc42p was effectively polarized to a single random cortical site even in the combined absence of landmarks, microtubules and microfilaments. Among a panel of Cdc42p effectors and interacting proteins, we found that the scaffold protein Bem1p was uniquely required for this symmetry-breaking behaviour. Moreover, polarization was dependent on GTP hydrolysis by Cdc42p, suggesting that assembly of a polarization site involves cycling of Cdc42p between GTP- and GDP-bound forms, rather than functioning as a simple on/off switch.

Cdc42p polarization is normally triggered by a signal from G1 cyclin–CDK complexes once cells are committed to entering the cell cycle[12]. Cdc42p polarization can also be induced artificially in G1-arrested cells through overexpression of a GTP-locked form of Cdc42p[13], and under these conditions polarization occurs towards one or more random sites and is independent of Rsr1p[14]. A recent investigation of this polarization concluded that a feedback loop involving Cdc42p-mediated F-actin nucleation and actin-mediated delivery of vesicles carrying Cdc42p could account for symmetry-breaking behaviour in yeast[14]. However, it remained unclear whether or not the polarization of overexpressed GTP-locked Cdc42p in G1-arrested cells was a faithful model for the polarization of endogenous Cdc42p in cells traversing the cell cycle, particularly as GTP-Cdc42p overexpression is lethal to the yeast cell.

To investigate the polarization of endogenous Cdc42p in cells traversing the cell cycle, we examined mutants that lacked Rsr1p and were thus unable to employ spatial landmarks. Previous studies showed that *rsr1*Δ mutants are mostly unable to interpret spatial cues, but that *rsr1*Δ daughter cells are still capable of identifying the distal pole[15]. This residual ability was eliminated after loss of the distal pole landmark Bud8p[16], so that *rsr1*Δ *bud8*Δ daughter cells polarized and formed buds randomly with regard to the poles (Fig. 1a). Unbudded wild-type and *rsr1*Δ *bud8*Δ daughter cells were isolated by centrifugal elutriation and incubated in fresh medium with or without nocodazole (to depolymerize microtubules) and Latrunculin A (to depolymerize actin). Control staining experiments confirmed the effectiveness of the drug treatments (Fig. 1b). Polarization of Cdc42p in *rsr1*Δ *bud8*Δ cells did not require microtubules or F-actin (Fig. 1b, c). Thus, under these more physiological conditions, establishment of Cdc42p polarization does not require F-actin, arguing against the hypothesis that symmetry breaking operates through a feedback loop involving actin-mediated vesicle delivery.

If the cytoskeleton is not required for symmetry-breaking Cdc42p polarization, we wondered what factors might be responsible for mediating this behaviour. We reasoned that proteins important for symmetry breaking would be essential for proliferation in strains lacking effective landmarks, but that they might be dispensable in strains using landmarks to guide polarization. To search for such proteins, non-lethal mutations affecting a panel of Cdc42p-associated effectors and scaffolds were tested to determine if they were lethal in strains lacking Rsr1p. Uniquely among this panel, *bem1*Δ mutants were inviable (synthetic lethal) when combined with *rsr1*Δ mutants (see Supplementary Information, Fig. S1a–c). Synthetic lethality was also observed for *bem1*Δ strains lacking the Rsr1p guanine nucleotide-exchange factor (GEF) Bud5p or GTPase-activating protein (GAP) Bud2p, which are also incapable of recognizing spatial landmarks[9,17–19] (see Supplementary Information, Fig. S1b), but not for strains lacking individual spatial landmarks, where the remaining landmarks can trigger polarization through Rsr1p[6,11] (see Supplementary Information, Fig. S1a). Consistently, an earlier screen for *cdc24*-like polarity mutants[20] yielded a strain containing two mutations required in combination for the *cdc24*-like phenotype; mutations that were later found to reside in *BEM1* and *BUD5* (ref. 18). Thus, Bem1p becomes essential in cells that cannot utilize the spatial cues that normally guide polarization.

BEM1 was originally isolated in genetic screens designed to identify components involved in bud emergence or in mating projection formation[18,21,22]. In our strain background, *bem1*Δ mutants were viable up to 39 °C, although *bem1*Δ cells displayed phenotypes indicative of impaired cell polarity, including a partial defect in Cdc42p polarization at elevated temperature (see Supplementary Information, Fig. S2). Bem1p binds directly to both the Cdc42p-directed GEF

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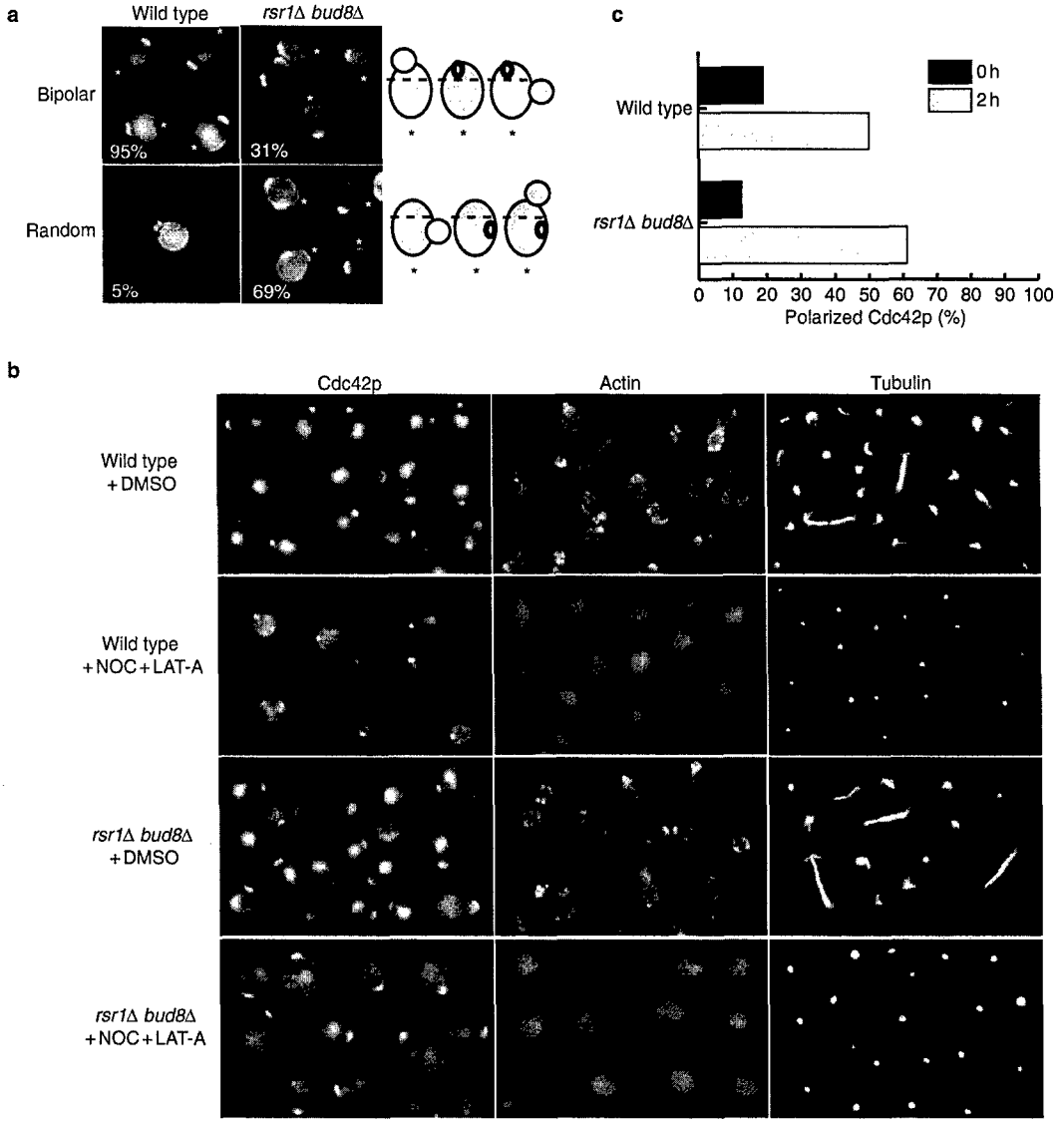

Figure 1 Landmark-independent Cdc42p polarization does not require polymerized actin or tubulin. (**a**) Diploid wild-type (DLY5077) and *rsr1Δ bud8Δ* (DLY5026) cells were grown to mid-log phase, stained with calcofluor to visualize chitin, and the position of the first bud or chitin ring was scored according to whether it was in the distal third or proximal two thirds of the cell (the proximal pole was identified through the position of the birth scar (asterisk). Wild-type daughter cells budded from the distal tip (95%), whereas the mutant cells budded randomly (31% distal third, 69% proximal two thirds). (**b**) Wild-type and *rsr1Δ bud8Δ* early-G1 daughter cells were isolated by centrifugal elutriation and incubated in rich medium with Latrunculin-A (100 μM) and nocodazole (15 μg ml^{-1}) or with DMSO (vehicle control), as indicated. After 2 h at 30 °C the localization of Cdc42p (left), F-actin (centre), and tubulin (right) was visualized by fluorescence microscopy of parallel samples. (**c**) The proportion of unbudded cells displaying polarized Cdc42p staining in the experiment above was determined in the elutriated population (0 h) and after incubation in medium containing Latrunculin A and nocodazole (2 h). At least 200 cells were scored in each case. Cells scored as polarized in the 0-h samples mostly included cells that retained a 'post-cytokinesis' Cdc42p localization from the previous cell cycle.

Cdc24p[23,24] (suggesting that Bem1p may function upstream of Cdc42p) and to GTP-Cdc42p, but not to GDP-Cdc42p[25] (suggesting that Bem1p may function downstream of Cdc42p). It was therefore essential to examine whether the observed synthetic lethality between *rsr1Δ* and *bem1Δ* mutants was caused by a defect in Cdc42p polarization or a defect in subsequent events.

To evaluate the basis for the death of *rsr1Δ bem1Δ* mutants, we used error-prone PCR mutagenesis to generate a temperature-sensitive *bem1* allele, *bem1-8*. The *bem1-8* mutant partially rescued the polarity defects of *bem1Δ* cells at 30 °C, but not at 39 °C, and *bem1-8 rsr1Δ* double mutants were viable at 30 °C but not at 39 °C (see Supplementary Information, Fig. S3). To assess whether cells lacking both Bem1p and Rsr1p function could polarize Cdc42p, unbudded *bem1-8 rsr1Δ* daughter cells were isolated by centrifugal elutriation and incubated at 39 °C. Similarly to control *BEM1 rsr1Δ* cells, *bem1-8 rsr1Δ* cells entered the cell cycle and formed spindles, as detected by anti-tubulin staining (Fig. 2a). However, they were unable to polarize Cdc42p (Fig. 2a) or actin (see Supplementary Information, Fig. S3), or to form buds at 39 °C, indicating that cells require either Bem1p or Rsr1p function to polarize Cdc42p.

Bem1p is a scaffold protein that contains several domains mediating interactions with other polarity establishment proteins[23–32] and

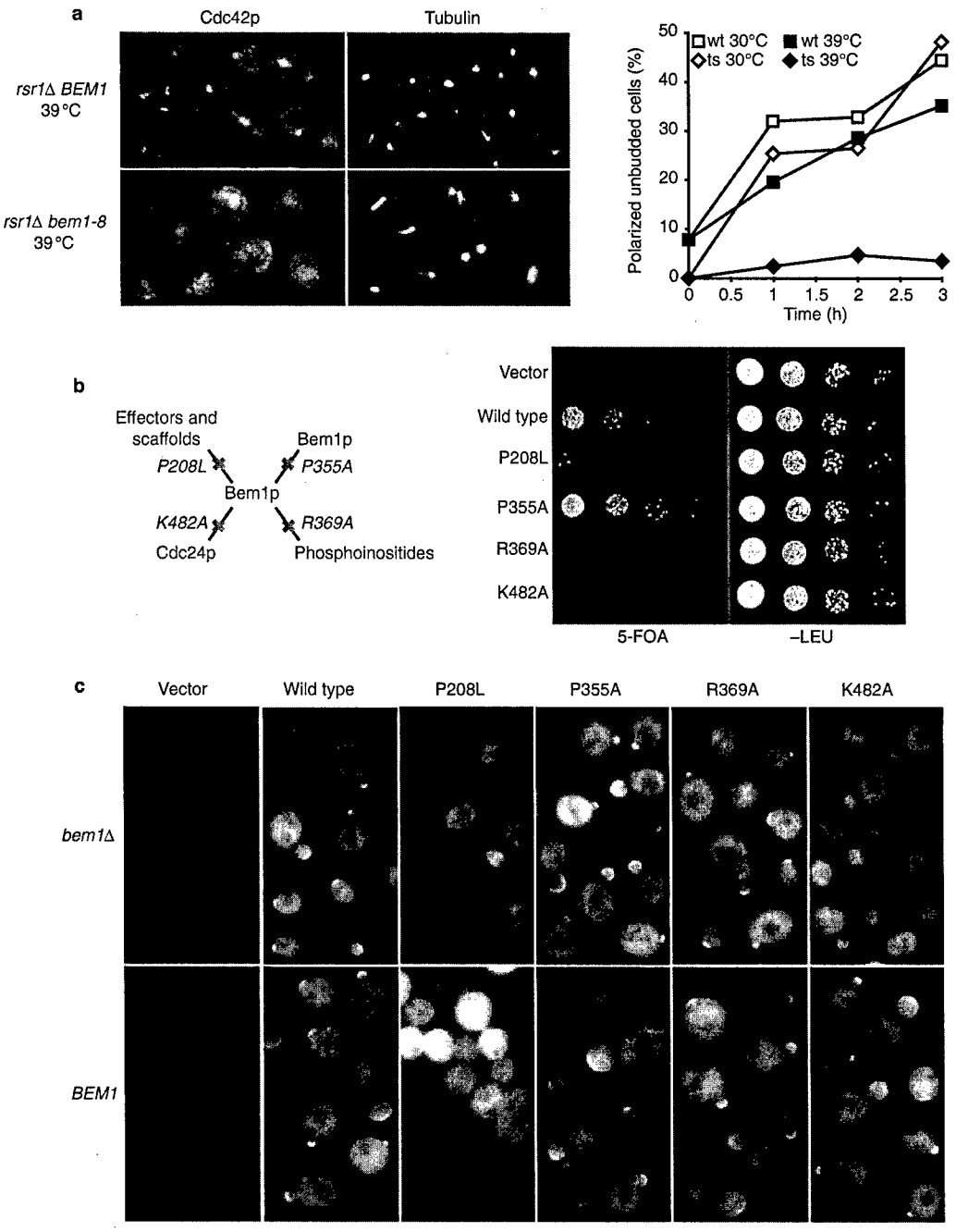

Figure 2 Landmark-independent Cdc42p polarization requires Bem1p. (**a**) Early G1 daughter cells were isolated by centrifugal elutriation from *bem1Δ rsr1Δ* strains carrying wild-type *BEM1* (DLY6146) or *bem1-8* (DLY6147) on a plasmid, and incubated in rich medium at 30 °C or 39 °C for 3 h. Cells were fixed and stained to visualize Cdc42p and tubulin localization at 1-h intervals. Representative cells from the 39 °C 3-h sample are shown (left), and the proportion of unbudded cells displaying polarized Cdc42p staining is plotted in the graph (right). At least 200 cells were scored for each time point. (**b**) Functional analysis of *bem1* mutants. Left panel shows a schematic representation of Bem1p interactions that would be abolished by the indicated mutations (see Supplementary Information, Fig. S4). Right panel shows *rsr1Δ bem1Δ* cells carrying a *URA3*-marked *BEM1* plasmid and high-copy *LEU2*-marked plasmids containing vector alone or the indicated alleles of *BEM1*. Cells were grown in medium containing uracil, but lacking leucine, to mid-log phase. Approximately 10^4, 10^3, 10^2 and 10^1 cells were spotted onto medium lacking leucine to examine viability of cells containing both plasmids (right) or complete medium with 5-FOA to eliminate cells that still contained the *URA3*-marked plasmid (left). Plates were incubated at 30 °C for 3 days. Only wild-type Bem1p and the P355A mutant could rescue the viability of *rsr1Δ bem1Δ* cells. Similar results were obtained from three independent experiments, and identical results were obtained using strains with low- and high-copy plasmids. (**c**) Subcellular localization of Bem1p variants. High-copy plasmids expressing Myc-tagged wild-type or mutant forms of Bem1p were transformed into *bem1Δ* (DLY5694) and *BEM1* (DLY5) cells. Bem1p localization was assessed by anti-Myc immunofluorescence analysis of log-phase cells. Variable staining probably results from different copy numbers of the 2-μm plasmids. Vector, YEpLac181.

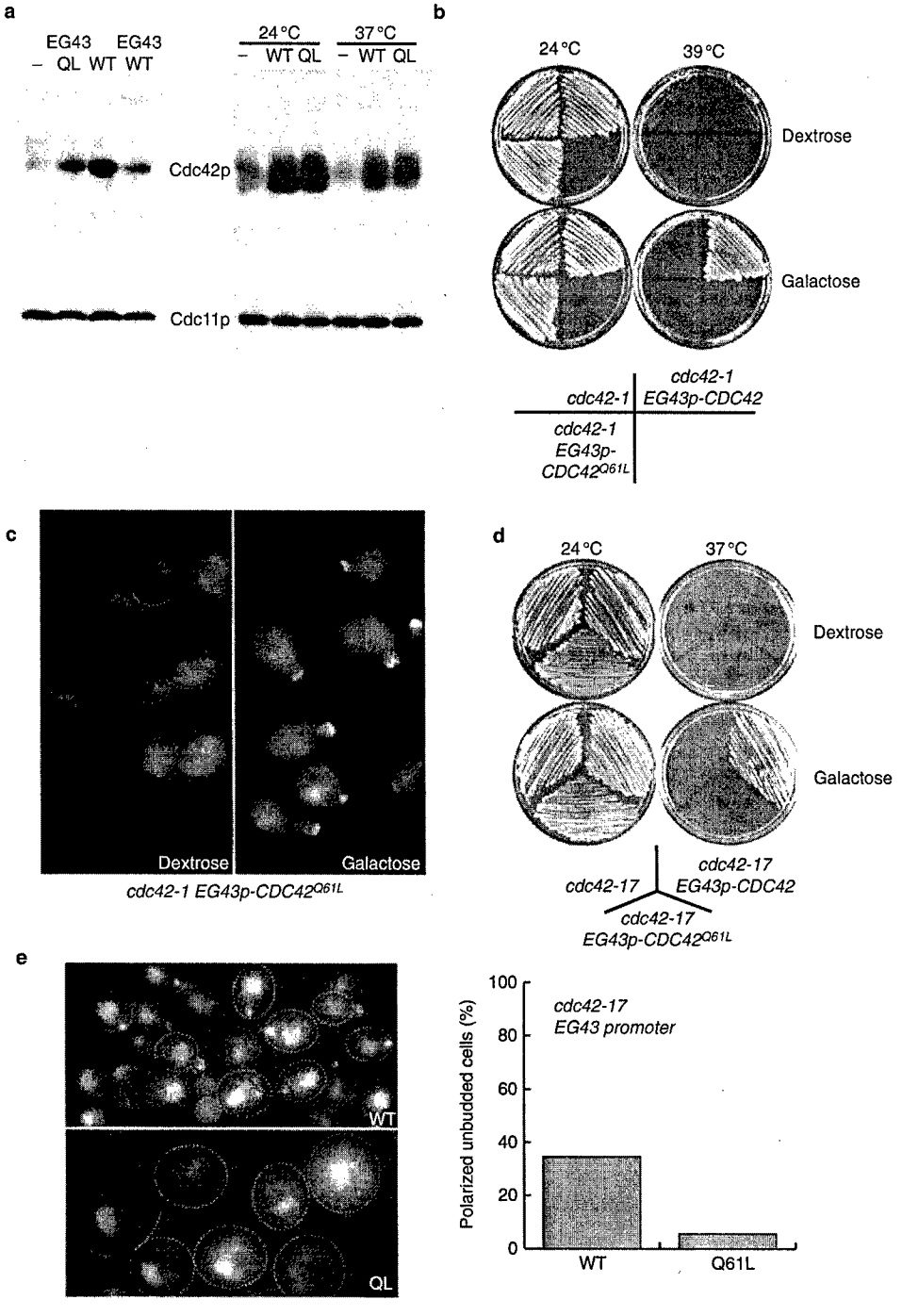

Figure 3 Cdc42p function requires GTP hydrolysis. **(a)** Cdc42p expression levels were determined by anti-Cdc42p immunoblotting of *cdc42-1* (DLY680,–), *cdc42-1 EG43p-CDC42*^{Q61L} (DLY5296, EG43 QL), *CDC42* (DLY1, WT) and *cdc42-1 EG43p-CDC42* (DLY5293, EG43 WT) cells grown in galactose medium to mid-log phase at 24 °C (left). Cdc42p expression levels were also determined in *cdc42-1* (DLY680,–), *cdc42-1 EG43p-CDC42* (DLY5293, WT) and *cdc42-1 EG43p-CDC42*^{Q61L} (DLY5296, QL) cells grown in galactose medium to mid-log phase at 24 °C, followed by incubation at 37 °C for 2 h. To control for gel loading and membrane transfer, the same membranes were probed with anti-Cdc11p. **(b)** *cdc42-1* (DLY680), *cdc42-1 EG43p-CDC42*^{Q61L} (DLY5296) and *cdc42-1 EG43p-CDC42* (DLY5293) cells were grown for 2 days at the indicated temperature on galactose or dextrose medium to induce or repress expression from the *EG43* promoter, respectively. **(c)** *cdc42-1 EG43p-CDC42*^{Q61L} cells (DLY5296) were grown to mid-log phase in dextrose (left) or galactose (right) medium at 24 °C. Cdc42p localization was visualized by immunofluorescence microscopy. **(d)** *cdc42-17* (MOSY122), *cdc42-17 EG43p-CDC42*^{Q61L} (DLY5301) and *cdc42-17 EG43p-CDC42* (DLY5299) cells were grown for 2 days at the indicated temperature on galactose or dextrose medium to induce or repress expression from the *EG43* promoter, respectively. **(e)** *cdc42-17* cells containing *EG43p-CDC42* (WT, DLY5299) or *EG43p-CDC42*^{Q61L} (Q61L, DLY5301) were grown in galactose medium at 24 °C to mid-log phase and then shifted to 37 °C for 2 h. Cdc42p localization was visualized by immunofluorescence microscopy (left) and the proportion of unbudded cells containing polarized Cdc42p was scored (right). At least 100 cells were scored in each strain.

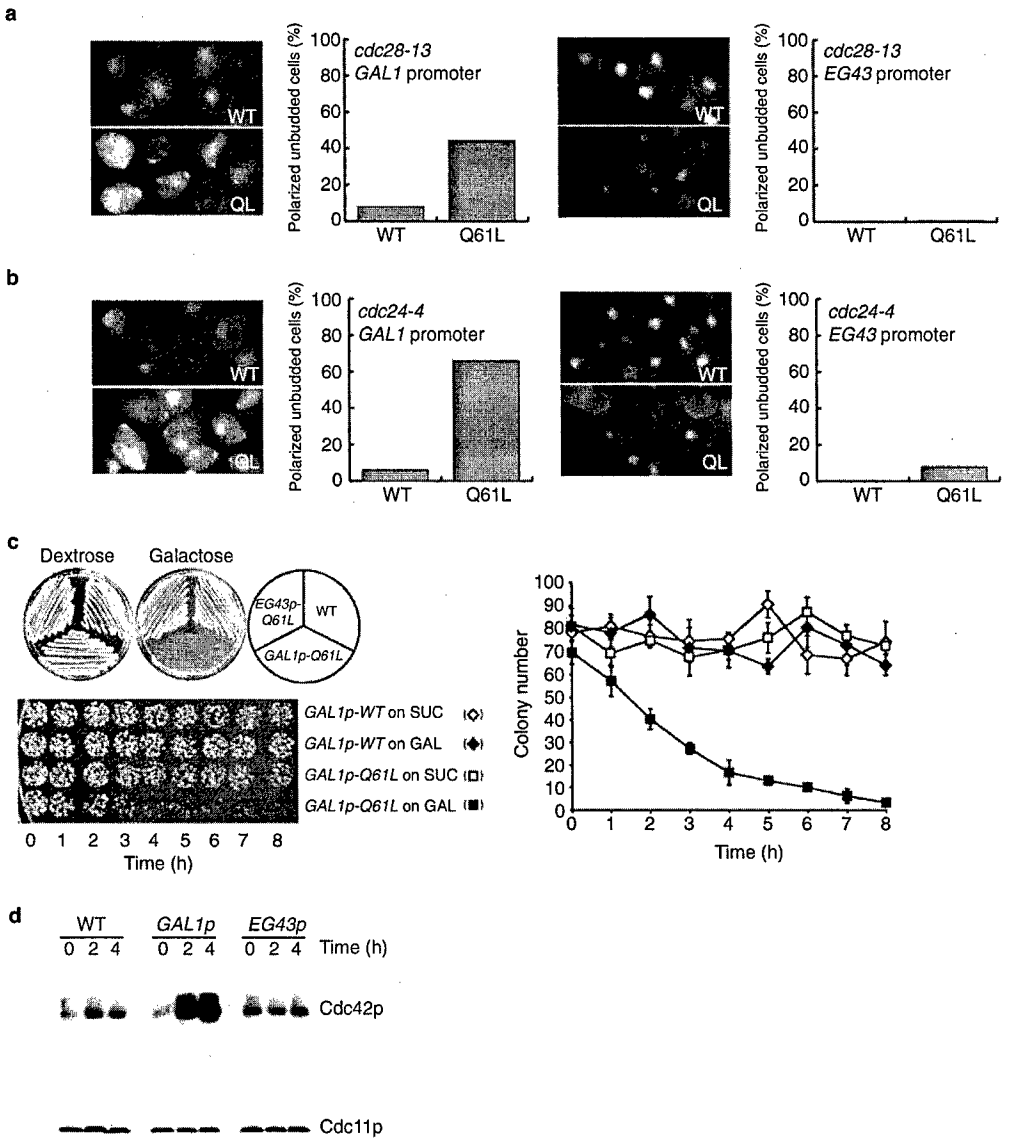

Figure 4 Spontaneous Cdc42p^{Q61L} polarization only occurs after overexpression to toxic levels. (**a**) *cdc28-13* cells expressing *CDC42* (WT) or *CDC42*^{Q61L} (QL) from the *GAL1* promoter (left; DLY2601 and DLY2603, respectively) or the *EG43* promoter (right; DLY5557 and DLY5562, respectively) were grown to mid-log phase in sucrose medium at 24 °C and then incubated in galactose medium at 37 °C for 2 h. Cdc42p localization was visualized by immunofluorescence microscopy and the proportion of unbudded cells displaying polarized Cdc42p staining was scored. At least 200 cells were scored in each case. (**b**) *cdc24-4* cells expressing *CDC42* (WT) or *CDC42*^{Q61L} (QL) from the *GAL1* promoter (left; DLY2605 and DLY2607 respectively) or the *EG43* promoter (right; DLY5555 and DLY5560, respectively) were analysed as in **a**. (**c**) Wild-type (DLY1, WT), *EG43p-CDC42*^{Q61L} (DLY5240, *EG43p-Q61L*) and *GAL1p-CDC42*^{Q61L} (DLY3003, *GAL1p-Q61L*) cells were grown for 2 days at 30 °C on galactose or dextrose medium to induce or repress expression from the *GAL1* and *EG43* promoters (top left). *GAL1p-CDC42* (DLY3005) and *GAL1p-CDC42*^{Q61L} (DLY3006) cells were also grown on sucrose medium (neutral) to mid-log phase then incubated for the indicated time at 30 °C in either galactose (GAL) or sucrose (SUC) medium, and ~100 cells per spot were plated onto dextrose (repressing) medium (bottom left). Quantifications of the number of colony-forming cells from three independent repeats of this experiment were plotted as mean viability (± standard deviation) versus time of incubation in galactose or sucrose medium. (**d**) Wild-type (WT, DLY1), *GAL1p-CDC42*^{Q61L} (*GAL1p*, DLY3003) and *EG43p-CDC42*^{Q61L} (*EG43p*, DLY5240) cells were grown in sucrose medium at 30 °C to mid-log phase. Galactose was added to 2% and Cdc42p levels of expression were determined by immunoblotting after the indicated times using the anti-Cdc42p antibody. To control for gel loading and membrane transfer, the same membrane was probed with anti-Cdc11p.

phosphoinositides[33–35] (see Supplementary Information, Fig. S4a). In addition, the *Schizosaccharomyces pombe* homologue of Bem1p is capable of assuming a 'closed' conformation through an intramolecular interaction (see Supplementary Information, Fig. S4a)[36]. To assess the roles of the various domains of Bem1p, we generated a panel of point mutations that impair interactions mediated by the individual domains (see Supplementary Information, Fig. S4a). Plasmids expressing wild-type or mutant Bem1p at expression levels comparable with wild type (see Supplementary Information, Fig. S4b) were tested for their ability to rescue the viability of *bem1Δ rsr1Δ* mutants (Fig. 2b). Bem1p^{P208L}, Bem1p^{R369A} and Bem1p^{K482A} were inactive in this assay, suggesting that interactions mediated by the SH3-2 domain,

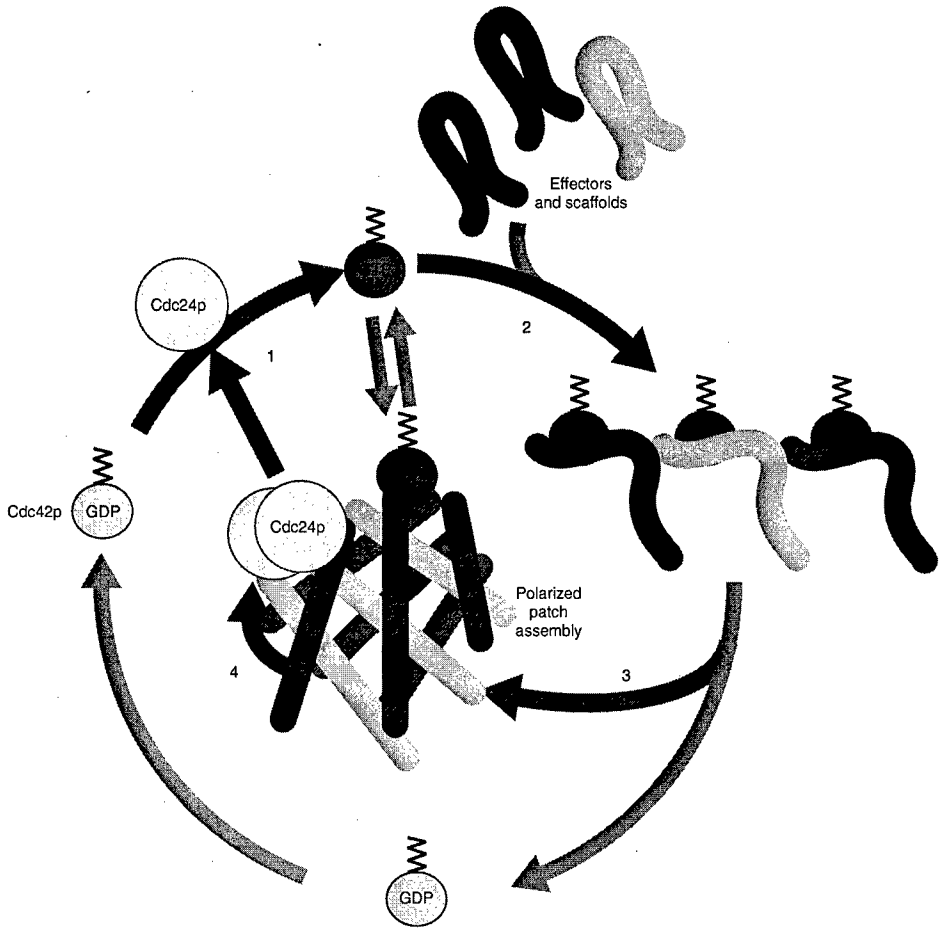

Figure 5 Cdc42p function in symmetry breaking and polarity establishment. Components involved in polarity establishment in yeast become highly concentrated in a 'polarization patch' after commitment to cell cycle progression. We suggest that assembly of this patch occurs through a local self-promoting feedback loop: GTP loading of Cdc42p is catalysed by the GEF Cdc24p (step 1), generating GTP-Cdc42p that interacts with auto-inhibited ('closed') effector and scaffold proteins and switches them to the active ('open') conformation (step 2). For scaffold proteins, this allows new interactions to occur, resulting in the assembly of complexes at the cortex. GTP hydrolysis by Cdc42p then triggers the release of bound Cdc42p, perhaps causing further conformational changes in the effectors and scaffolds, thereby permitting other interactions to occur and promoting the assembly of a nascent polarized patch of interlinked components (step 3). Cdc24p is recruited to this patch by the scaffold Bem1p and becomes phosphorylated within the nascent patch by effector kinases, promoting local generation of more GTP-Cdc42p in a positive feedback loop (step 4). Stochastic fluctuations in the local concentrations of the various components may allow initiation of the positive feedback loop when the local levels at a random cortical location exceed some threshold. Once initiated, the feedback loop would continue to activate and recruit more components into the polarization patch until the unassembled component concentrations became limiting. Rapid cycling through this assembly loop would ensure that such titration occurred before a second site was initiated, limiting polarization to a single site. Intriguingly, there is only one report of a mutant cell attempting to polarize to more than one site simultaneously, and that occurred in a *cdc42* mutant strain in which the encoded Cdc42p was thought to be slow in hydrolysing GTP[45]. Thus, the rate of Cdc42p GTP cycling may influence the rate of polarization patch assembly so that slow cycling provides sufficient time to assemble a second patch.

the PX domain and the PB1 domain are all essential for Bem1p function in symmetry breaking. In contrast, Bem1p^{P355A} was able to rescue *bem1*Δ *rsr1*Δ cell viability (Fig. 2b), suggesting that an intramolecular interaction analogous to that in *S. pombe*[36] is not required for symmetry breaking. Interestingly, Bem1p^{R369A}, but not Bem1p^{P208L} or Bem1p^{K482A}, retained significant ability to complement the bud emergence defect of *bem1*Δ single-mutant cells (see Supplementary Information, Fig. S4c), suggesting that its defect in binding phosphoinositides might specifically affect the symmetry-breaking function of Bem1p.

Similarly to Cdc42p, Bem1p is localized to the presumptive bud site in an actin-independent manner, as well as to the bud tip[13,37]. In *bem1*Δ cells, we found that Bem1p^{P355A} and Bem1p^{R369A} localized to the same sites as wild-type Bem1p, whereas Bem1p^{P208L} and Bem1p^{K482A} displayed a diffuse cytoplasmic localization (Fig. 2c). These results suggest that interactions mediated by the SH3-2 domain and the PB1 domain are important for Bem1p localization, whereas the putative intramolecular interaction and lipid binding by the PX domain are dispensable for Bem1p localization. Interestingly, Bem1p^{K482A} was localized normally in cells containing a wild-type endogenous copy of Bem1p (Fig. 2c), indicating that wild-type Bem1p could promote the localization of this mutant.

So, what is the role of Bem1p is in symmetry-breaking polarization? Symmetry-breaking behaviour is thought to require short-range positive feedback mechanisms whereby a small cluster of one or more key

polarity factors (arising at a random locations through stochastic fluctuations in component concentrations) promotes the recruitment of more such factors to the polarization site[4]. As a scaffold protein that binds to key polarity factors, Bem1p is an attractive candidate to participate in such a localized feedback mechanism. This hypothesis is supported by previous findings[25] that Bem1p can bring together the upstream GEF Cdc24p, GTP-Cdc42p and downstream effectors of Cdc42p, that assembly of such complexes promotes feedback phosphorylation of Cdc24p by effector kinases, and that (as shown here) all of the Bem1p domains involved in assembly of such complexes are required for Bem1p function in symmetry breaking. We suggest that Bem1p-containing complexes provide an actin-independent feedback mechanism to amplify stochastic fluctuations in Cdc42p concentration at random sites.

Cdc42p is generally thought to function as an 'on/off' molecular switch that is inactive in the GDP-bound state and active in the GTP-bound state, and whose binding to numerous effectors unleashes effector activity by disrupting auto-inhibitory intramolecular interactions[10]. However, some Cdc42p actions, including septin ring assembly in yeast and oncogenic transformation in mammalian cells, require GTP hydrolysis by Cdc42p, suggesting that GTP cycling of Cdc42p (rather than just GTP-loading) is important[38,39]. The question of whether a GTP-locked Cdc42p (that is, a mutant form unable to hydrolyse GTP, such as $Cdc42p^{Q61L}$) could substitute for endogenous Cdc42p in yeast has never been addressed because expression of $Cdc42p^{Q61L}$ from the *CDC42* promoter is lethal, even in otherwise wild-type cells[40]. To circumvent this problem, vectors expressing lower levels of $Cdc42p^{Q61L}$ were generated from a weak galactose-inducible promoter (*EG43*)[38] and introduced into temperature-sensitive *cdc42-17* and *cdc42-1* strains. *cdc42-17* is a very tight allele[38,41], whereas *cdc42-1* is a somewhat leaky allele that contains mutations in both the promoter and the coding region of *CDC42* resulting in greatly reduced Cdc42-1p expression even at the permissive temperature[42]. Thus, Cdc42-1p provides a low endogenous 'background' signal that allowed us to assess the abundance and localization of the introduced $Cdc42p^{Q61L}$. On galactose medium, cells containing *EG43-CDC42*^{Q61L} expressed levels of $Cdc42p^{Q61L}$ that were approximately 50% lower than those of total endogenous Cdc42p in wild-type cells (Fig. 3a), which is still likely to be higher than endogenous GTP-Cdc42p levels. *cdc42-1 EG43-CDC42*^{Q61L} cells proliferated on galactose medium at permissive temperature (Fig. 3b), indicating that $Cdc42p^{Q61L}$ no longer caused cell death at these levels (although it did cause defects in septin organization, as previously reported[38]). As shown previously[42,43], anti-Cdc42p staining was not sufficiently sensitive to detect the low-abundance Cdc42-1p, but clear Cdc42p polarization was detected in *cdc42-1 EG43-CDC42*^{Q61L} cells grown at permissive temperature on galactose medium (Fig. 3c), implying that $Cdc42p^{Q61L}$ was able to polarize in cells containing functional Cdc42-1p.

To determine whether non-lethal levels of $Cdc42p^{Q61L}$ could substitute for wild-type Cdc42p, we tested whether *EG43-CDC42*^{Q61L} could provide Cdc42p function in *cdc42-17* cells at the restrictive temperature. On galactose medium, wild-type Cdc42p rescued the temperature sensitivity of *cdc42-17*, but $Cdc42p^{Q61L}$ did not (Fig. 3d; similar results were obtained with the leakier *cdc42-1* allele, as shown in Fig. 3b). After a shift to restrictive temperature, no polarization of $Cdc42p^{Q61L}$ was observed (Fig. 3e), even though similar amounts of $Cdc42p^{Q61L}$ were expressed at restrictive and permissive temperature (Fig. 3a). Thus, Cdc42p function requires its ability to hydrolyse GTP, and GTP-locked Cdc42p cannot polarize in the absence of functional endogenous Cdc42p.

Our findings indicate that GTP hydrolysis by Cdc42p is essential for its ability to establish a polarization site. In apparent contradiction to this conclusion, overexpression of $Cdc42p^{Q61L}$ from the *GAL1* promoter induces 'spontaneous' polarization in G1-arrested cells[13,14]. Similarly, we observed polarization of overexpressed $Cdc42p^{Q61L}$ (Fig. 4a) and F-actin (data not shown) in G1-arrested cells and found that such polarization was independent of the GEF Cdc24p (Fig. 4b). As described previously[13,14,44], polarization sometimes occurred towards more than one site (Fig. 4b) and was accompanied by cell lysis (data not shown). Thus, overexpressed $Cdc42p^{Q61L}$ causes polarization in cells lacking the normal triggers for polarization. However, *GAL1-CDC42*^{Q61L} expression also caused a rapid loss of viability even in otherwise wild-type cells (Fig. 4c) and resulted in accumulation of $Cdc42p^{Q61L}$ to levels much higher than those of the total endogenous Cdc42p (Fig. 4d). This raised the concern that the observed polarization might reflect a pathological event rather than a physiological polarization. When $Cdc42p^{Q61L}$ was expressed at lower levels from the *EG43* promoter (Fig. 4d) in G1-arrested cells (Fig. 4a) or *cdc24-4* cells (Fig. 4b), no polarization was observed. Thus, the previously documented polarization of overexpressed GTP-Cdc42p does not occur at more physiologically relevant levels of expression and seems unlikely to reflect the normal mechanism of polarization. We conclude that the physiological mechanism for establishing polarity in yeast requires that Cdc42p can hydrolyse GTP.

These findings raised the question of how GTP hydrolysis contributes to polarization. One possibility is that GTP hydrolysis is needed to reduce the GTP-Cdc42p concentration at sites other than the polarization site, to generate or maintain a sufficiently steep spatial gradient of GTP-Cdc42p. However, this hypothesis seems inconsistent with our observations that GTP-locked Cdc42p was itself highly polarized in cells containing functional endogenous Cdc42p, and that GTP-locked Cdc42p did not disrupt polarization triggered by the much less abundant endogenous Cdc42-1p at permissive temperature. A more attractive possibility is that the Cdc24p (GEF)–Cdc42p interaction, which normally triggers GTP loading on Cdc42p, is also important for localizing and/or targeting the GTP-Cdc42p to specific interactors. By skipping the Cdc24p-catalysed step, GTP-locked Cdc42p fails to be localized/targeted in this manner and cannot function in establishing polarity. This hypothesis is entirely consistent with our data, but seems to be at odds with a recent study that identified a *cdc42* allele capable of polarizing (albeit rather poorly) in the absence of Cdc24p[45]. Another possibility, analogous to the way in which translation elongation factors promote protein synthesis[46], is that GTP hydrolysis by Cdc42p bound to effector complexes alters interactions among the other members of the complex, promoting a step in the assembly of a polarization site.

Polarization of Cdc42p involves its concentration at a small patch of the cell cortex, together with scaffold and effector proteins that bind to GTP-Cdc42p and display additional interactions among themselves[47,48]. A speculative model for how such a patch might be assembled in the absence of effective landmarks or cytoskeletal elements is presented in Fig. 5. We suggest that a local increase in GTP-Cdc42p concentration caused by stochastic fluctuations results in the 'opening' of local auto-inhibited scaffold and effector proteins, including Bem1p. Interactions between Bem1p, Cdc24p and effectors would then stimulate further local generation of GTP-Cdc42p and opening of more scaffolds and effectors, producing a larger patch of interacting polarity factors in a self-enhancing process that continues until the concentrations of unassembled polarity factors become limiting (Fig. 5). Once it has assembled, such a patch would polarize actin cables towards the site, leading to polarized delivery of vesicles and other factors, resulting in polarized growth. □

METHODS

Yeast strains, plasmids and PCR manipulations. Standard media and methods were used for plasmid manipulations[49] and yeast genetic manipulations[50]. The yeast strains used are listed in Supplementary Information, Table 1; plasmids are listed in Supplementary Information, Table 2; oligonucleotides are listed in Supplementary Information, Table 3). The crippled *GAL* promoter *EG43* was described previously[38].

pDLB1974 was generated by digesting pDLB678 with *Eco*RI and *Nsi*I. The 3.5-kb fragment, which contains the 3′ sequence of *KTR4* (the gene upstream of *BEM1*), the 500-bp intergenic sequence, the *BEM1* ORF and downstream sequence was ligated with YEpLac195 (high-copy *URA3*) previously digested with *Eco*RI and *Pst*I.

For synthetic lethality analysis, *bem1::kan*^{R} was amplified by PCR using genomic DNA from DLY5631 and primers BEM1-744 and MACBEM1-2, and incorporated by transformation into the homozygous deletion strains as indicated in Supplementary Information, Table 1. Alternatively, *bem1::URA3* was generated as previously described[22]. After sporulation and tetrad dissection, viability was evaluated on rich medium and the nutritional markers were followed on the appropriate selective media. DLY5489, DLY5694 and DLY5681 were generated by PCR amplification of the *rsr1::kan*^{R} locus, using genomic DNA from DLY5631 as a template and oligonucleotides RSR1-163 and RSR1+1443 as primers, and transformation of DLY5 or DLY5077. Correct integration was confirmed by PCR and the haploid strains were generated by sporulation and tetrad dissection. Synthetic lethality was evaluated by crossing these with the haploid strains of the opposite mating type harbouring the mutations described in Supplementary Information, Table 1, followed by sporulation and tetrad analysis.

For deletion of *BOI1* and *BOI2*, *HIS2* from YIpGAP2 was cloned into *BOI1* (removing the *Mlu*I–*Nhe*I fragment) and *BOI2* (removing the *Bst*EII–*Nru*I fragment) in pCRII-BOI1 and pCRII-BOI2, respectively, to generate pCRII-BOI1::HIS2 and pCRII-BOI2::HIS2. Digestion of pCRII-BOI1::HIS2 with *Xba*I and *Nhe*I, and pCRII-BOI2::HIS2 with *Not*I and *Bgl*II, followed by transformation, creates a truncated ORF at the *BOI1* (5′ 457 bp) and *BOI2* loci (5′ 800 bp) followed by *HIS2*. Correct integration was confirmed by PCR. Plasmid details are available on request.

For expression of Bem1p–Myc at single copy, we amplified a genomic fragment from DLY4000 corresponding to upstream sequence, *BEM1*ORF, Myc tag and *SWE1* terminator. As previously described[25], DLY4000 contains a genomic insertion at the *BEM1* locus that expresses wild-type *BEM1* with a carboxy-terminal 12×Myc tag, followed by a terminator sequence from the *SWE1* locus. pDLB2226 was generated by amplifying 480 bp upstream of *BEM1* (*BEM1p*), the *BEM1* ORF, the 12×Myc tag and the *SWE1* terminator (*SWE1t*) from the chromosomal locus in DLY4000, using primers BEM1-480 and SWE1TERM. The 2.9-kb amplification product was digested with *Sac*I and *Xho*I and cloned into the respective sites in pRS315 (low-copy *LEU2*).

For generation of temperature-sensitive *BEM1* alleles, DLY5023 was transformed with the PCR product obtained from amplification of the *bem1::kan*^{R} construct at the *BEM1* locus in DLY5631 using oligonucleotides BEM1-744 and MACBEM1-2. After transformation and growth on G418 medium, proper integration was confirmed by PCR. This strain was then transformed with pDLB1974, grown on medium lacking uracil for plasmid selection and induced to sporulate. After tetrad dissection, a *bem1::kan*^{R} *rsr1::HIS* p*BEM1*strains (DLY5452) were identified by the corresponding nutritional markers and mating-type tests.

Random mutagenesis of *BEM1* was performed using error-prone PCR as described previously[51], using pDLB2226 as template and primers BEM1-480 and SWE1TERM. The PCR product was cotransformed into DLY5452 together with pDLB2226 previously digested with *Hin*dIII and *Sal*I before gel purification to isolate the ~8-kb vector backbone. This linear backbone contains *BEM1p* and the 5′ 300 bp of *BEM1* separated from the 12×Myc tag and *SWE1t* by a gap. Homologous recombination thus regenerated plasmids containing the mutagenized alleles plus the sequences required for their expression. The transformed cells were grown at 24 °C on selective dextrose medium lacking leucine and replica-plated at the same temperature onto dextrose medium containing 5-FOA to force the loss of pDLB1974 and select against null alleles of *BEM1*. Colonies were then replica-plated onto four sets of plates of dextrose medium lacking leucine and incubated at 24 °C, 30 °C, 37 °C and 39 °C. Colonies that grew at 24 °C, but not at 37 °C or 39 °C, were analysed further to identify mutants that would have normal morphology at 24 °C. pDLB2261, harbouring *bem1-8*, was isolated by plasmid rescue and retransformed into DLY5452 to verify the phenotype.

The P355A and R369A alleles of *BEM1* were generated by site-directed mutagenesis using pDLB2226, the primers listed in Supplementary Information Table 3, and the QuickChange PCR kit (Stratagene, La Jolla, CA) in accordance with the manufacturer's instructions. The P208L mutation was amplified from pDLB1822 using primers MOSKOW1.2 and DOWNSTREAM. The ~1.6-kb product was cut with *Bam*HI and *Pst*I and the 0.6-kb fragment cloned into the corresponding sites in pDLB2226, replacing the wild-type sequence. The K482A mutation was amplified from pDLB2278 using primers MOSKOW1.4 and DOWNSTREAM. The ~1 kb product was cut with *Sal*I and cloned into the corresponding site in pDLB2226, replacing the wild-type sequence. The resulting plasmids were sequenced and confirmed to have no other mutations in the *BEM1* ORF and regulatory sequences than the intended ones.

Wild-type and mutant *BEM1* alleles were transferred to YEpLac181 (high-copy *LEU2*), excising the *BEM1p-BEM1-12×Myc-SWE1t* fragment from the pRS315 backbone by cutting with *Sac*I and *Xho*I, followed with ligation into YEpLac181 digested with *Sal*I and *Sac*I.

For the generation of DLY5293, DLY5296, DLY5299, DLY5301, DLY5555, DLY5557, DLY5560 and DLY5562, plasmids pDLB659 and pDLB664 were transformed into the corresponding backgrounds as described previously[38].

The generation of the *leu2::GAL1p-CDC42::LEU2* and *leu2::GAL1p-CDC42*^{Q61L}*::LEU2* alleles was described previously[41].

Fluorescence staining and microscopy. Cells were stained with 4,6-diamidino-2-phenylindole (Sigma, St. Louis, MO) to visualize DNA[52], with 10 mg ml^{-1} Calcofluor (Sigma) to visualize chitin[52], or with rhodamine–phalloidin (Molecular Probes, Eugene, OR) to visualize F-actin[53]. To localize Cdc42p, cells were fixed for a total of 3 h in 3.6% formaldehyde, as previously described[54]. Fixed cells were incubated with 0.5% SDS and processed for immunofluorescence microscopy, as previously described[55]. The anti-Cdc42p antibody (used at 1:200 dilution) was generously provided by P. Brennwald (UNC-Chapel Hill). The anti-Cdc42p antibody somtimes stains the nuclear region, as well as the polarization site, but this background nuclear staining was not reproducible and it is not clear whether it represents a pool of Cdc42p or a cross-reacting antigen. To visualize Myc-tagged Bem1p, cells were fixed and treated as for Cdc42p immunofluorescence microscopy. The monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:200 dilution. Cy3-conjugated goat anti-mouse IgG and Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) were added sequentially at 1:200 dilutions after 1-h incubations. Cells were examined using a Zeiss Axioskop (Carl Zeiss, Thornwood, NY). Images were captured using an ORCA cooled charge-coupled device camera (Hamamatsu, Bridgewater, NJ), interfaced with MetaMorph software (Universal Imaging, Silver Spring, MD). Images were processed for presentation using Photoshop (Adobe Systems, San Jose, CA).

Preparation of protein extracts and western blotting. Procedures for harvesting and lysis of yeast cells, SDS–PAGE and immunoblotting were as previously described[25]. The Anti-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology) was used at 1:1000 dilution. Anti-Cdc42p polyclonal antibody was used at a 1:500 dilution. Anti-Cdc11p polyclonal antibody (Santa Cruz Biotechnology) was used at a 1:5,000 dilution.

Analysis of cell viability. To evaluate the viability of cells overexpressing Cdc42p^{Q61L}, cells were grown to mid-log phase in sucrose-rich medium at 30 °C. At the start of the timecourse, galactose was added to a final concentration of 2% (w/v). Samples were taken at 1-h intervals. Cell number was determined using a haemocytometer and the samples were diluted to a final cell count of 2×10^4 cells ml^{-1}. 5 μl (~100 cells) were then spotted onto triplicate YEPD plates. Colony number in each spot was determined 2 days later and plotted as mean ± standard deviation.

To evaluate the functionality of the point mutant alleles of *BEM1*, cells were grown to mid-log phase in dextrose medium lacking leucine. The cell density of the cultures was determined and diluted to a final cell count of 2×10^6 cells

ml^{-1}. Subsequent tenfold serial dilutions were performed, and 5 µl (~10^4, 10^3, 10^2 and 10^1 cells) of each dilution was spotted on YEPD, dextrose medium plus 5-FOA and dextrose medium lacking leucine. The plates were incubated at 30 °C for 2 days and colony numbers were determined in each spot.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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Opposing roles for actin in Cdc42p polarization

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Abstract

In animal and fungal cells, the monomeric GTPase Cdc42p is a key regulator of cell polarity that itself exhibits a polarized distribution in asymmetric cells. Previous work showed that in budding yeast, Cdc42p polarization is unaffected by depolymerization of the actin cytoskeleton (Ayscough et al., *J. Cell Biol.* **137,** 399-416, 1997). Surprisingly, we now report that unlike complete actin depolymerization, partial actin depolymerization leads to the dispersal of Cdc42p from the polarization site in unbudded cells. We provide evidence that dispersal is due to endocytosis associated with cortical actin patches, and that actin cables are required to counteract the dispersal and maintain Cdc42p polarity. Thus, although Cdc42p is initially polarized in an actin-independent manner, maintaining that polarity may involve a reinforcing feedback between Cdc42p and polarized actin cables to counteract the dispersing effects of actin-dependent endocytosis. In addition, we report that once a bud has formed, polarized Cdc42p becomes more resistant to dispersal, revealing an unexpected difference between unbudded and budded cells in the organization of the polarization site.

Introduction

The Rho-family GTPase Cdc42p is critical for cell polarity in both yeast and mammalian cells (Pringle *et al.*, 1995; Etienne-Manneville, 2004). In *Saccharomyces cerevisiae*, cell cycle commitment in late G1 triggers cell polarization in preparation for bud formation (Lew and Reed, 1993). This process involves the concentration of Cdc42p together with many other proteins in a "cap" at the presumptive bud site, the polarization of actin cables toward that site, the clustering of cortical actin patches (thought to be sites of endocytosis (Kaksonen *et al.*, 2003)) at that site, and the assembly of a ring of septin filaments surrounding the Cdc42p cap (Pruyne and Bretscher, 2000). Cdc42p is essential for polarization of cytoskeletal filaments (Adams *et al.*, 1990), and it is believed that the concentration of Cdc42p at the presumptive bud site is critical for effective cell polarization. Consistent with this view, experiments in mammalian cells demonstrated that whereas activated, membrane-targeted Cdc42p distributed throughout the cell cortex did not induce cytoskeletal changes, spatial clustering of that Cdc42p sufficed to induce dramatic actin polarization (Castellano *et al.*, 1999). Thus, the molecular basis for Cdc42p polarization is of fundamental importance to polarity establishment.

Once a cell is polarized, many cortical proteins are asymmetrically distributed. Three general classes of models have been invoked to explain such polarization (Fig. 1). In the first model (Fig. 1A), a pre-existing stably polarized "anchor" interacts (directly or indirectly) with the protein of interest, thereby increasing its local concentration. In yeast, a subset of "bud site selection" proteins are integral membrane proteins with large extracellular domains which are thought to interact with the rigid cell wall in a manner that renders them immobile in the plane of the membrane (Harkins *et al.*, 2001). These proteins are deposited at the poles of the cell during bud formation, allowing them to serve as "landmarks" that can anchor proteins to those sites in

the following cell cycle (Schenkman *et al.*, 2002). In the second model (Fig. 1B), a "fence" of membrane-associated filaments forms a diffusion barrier such that proteins delivered to one compartment cannot cross the fence to another compartment. In yeast, the septin filament system is thought to act as a fence between the cortex of the bud and that of the mother, maintaining the asymmetric distributions of cortical proteins delivered to only one side (Barral *et al.*, 2000; Takizawa *et al.*, 2000). In the third model (Fig. 1C), asymmetric distribution arises through a dynamic process in which the polarized cytoskeleton delivers cortical proteins to the "front" of the cell, and endocytic retrieval of the proteins occurs before they can diffuse too far from that site. Recycling of the endocytosed proteins to the front of the cell maintains the polarized distribution. In yeast, endocytosis of integral membrane SNARE proteins was required for their asymmetric distribution, indicating that they are polarized by this mechanism (Valdez-Taubas and Pelham, 2003). In all cases, a polarized cytoskeleton is needed to set up the asymmetry: the anchors and fences must be deposited or assembled at the right location, and polarized delivery to right side of the fence or the front of the cell is key for the latter two models.

Which of these models accounts for Cdc42p polarization to the presumptive bud site? In a classic study, Ayscough et al. (1997) demonstrated that whereas F-actin was required for polarization of many proteins, depolymerization of all F-actin did not prevent Cdc42p polarization, suggesting that polarized delivery of Cdc42p or of other factors is not required for polarization of Cdc42p. The simplest explanation for that result is that Cdc42p is polarized via interactions with previously deposited anchors, and indeed the bud site selection landmarks are known to influence the location to which Cdc42p becomes polarized (Pringle *et al.*, 1995). However, mutations that eliminate the landmarks themselves or other factors required for

recognition of the landmarks do not prevent polarization, but only randomize the site at which polarization takes place (Chant, 1999). We recently showed that even in cells lacking effective landmarks to serve as anchors, Cdc42p could become polarized in the complete absence of F-actin or microtubules (Irazoqui *et al.*, 2003). This result indicated that Cdc42p polarization could occur without pre-existing anchors and without the (actin-mediated) polarized delivery required by the fence and recycling models, indicating that this key regulator of polarity was itself polarized by a novel mechanism. Other data suggested that, together with interacting scaffolds and effectors including Bem1p, Cdc42p triggered the cooperative assembly of an anchor-like patch *de novo* (Irazoqui *et al.*, 2003) (Fig.1A').

A central finding supporting anchor models (Fig.1A,A') for Cdc42p polarization was the ability of Cdc42p to polarize in the complete absence of F-actin. However, we now report that unlike loss of all F-actin, selective loss of actin cables causes the dispersal of Cdc42p from the pre-bud site in unbudded cells. Such dispersal is reduced in an endocytosis mutant, suggesting that endocytic internalization of polarity factors by actin patches must be counteracted by polarized delivery along actin cables to maintain polarization of Cdc42p. Intriguingly, the polarized cap of Cdc42p in cells that have already formed a bud is much less sensitive to actin cable perturbation, suggesting that there are significant and previously unsuspected differences in the organization of the Cdc42p cap in budded and unbudded cells.

Materials and Methods

Yeast strains, Elutriation, and Lat treatment.

Standard media were used for yeast growth (Guthrie and Fink, 1991). The yeast strains used are listed in Table I. Lat A and Lat B were purchased from Molecular Probes, Eugene, OR, and kept as 200x (Lat A) and 100x (Lat B) stocks in DMSO at –20°C. Centrifugal elutriation to isolate early G1 cells was performed as described previously (Lew and Reed, 1993).

Fluorescence staining and Microscopy.

To visualize Cdc42p and Sec4p, cells were fixed for a total of 3 h in 3.6% formaldehyde, permeabilized with 0.5% SDS, and processed for immunofluorescence as previously described (Redding *et al.*, 1991; Lehman *et al.*, 1999). Anti-Cdc42p antibody (used at 1:200 dilution) and anti-Sec4p antibody (used at 1:100 dilution) were generously provided by Patrick Brennwald (UNC Chapel Hill). To visualize F-actin, cells were fixed as above, treated with acetone, and processed for staining with alexafluor488-phalloidin as previously described for rhodamine-phalloidin (Pruyne *et al.*, 1998). Cells were examined using a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) with a 100x oil immersion objective. Images were captured using an ORCA cooled charge-coupled device camera (Hamamatsu Corp., Bridgewater, NJ), interfaced with MetaMorph software (Universal Imaging, Silver Spring, MD). Images were processed for presentation using Photoshop (Adobe Systems, Inc., San Jose, CA). Individual cells or groups of cells in the correct focal plane were grouped together from one or more fields to assemble the figures.

Results

Partial actin depolymerization results in loss of Cdc42p polarity.

The role of F-actin in Cdc42p polarization has been addressed using Latrunculin A (Lat A), a G-actin binding compound isolated from the Red Sea sponge *Latrunculia magnifica* (Spector *et al.*, 1983). Treatment of yeast cells with 100 μM (or more) Lat A results in the rapid disassembly of all detectable F-actin structures (Fig. 2A), blocking both polarized growth and endocytosis (Ayscough *et al.*, 1997; Karpova *et al.*, 2000). Because of the high cost of Lat A, we sought to extend our findings using its less expensive relative, Lat B. Lat B is not as potent as Lat A, and although detectable cables are lost following exposure to 100 μM Lat B, some F-actin patches remain (Fig. 2A) (McMillan *et al.*, 1999). We were surprised to find that unlike Lat A, treatment of cells with Lat B led to a dispersal of Cdc42p from the pre-bud site (Fig. 2B), with most unbudded cells losing polarized Cdc42p staining within an hour of Lat B addition.

One interpretation of these findings is that Lat B has another target in addition to actin, and that this second target causes dispersal of Cdc42p. However, we found that actin mutants previously shown to be resistant to Lat A (Ayscough *et al.*, 1997) were also resistant to Lat B (Fig. 2C), suggesting that Lat B exerts its effects through actin. Consistent with this view, lower doses of Lat A also caused some Cdc42p dispersal (see supplementary Figure 1).

It is not clear from our data whether the dispersed Cdc42p remains at the cortex or redistributes to intracellular membranes, as immunofluorescence protocols do not reveal detectable unpolarized pools. Studies using GFP-Cdc42p, in contrast, detect abundant fluorescence throughout the cortex as well as on intracellular membranes (Richman *et al.*, 2002; Wedlich-Soldner *et al.*, 2004), but in that case the polarized signal is only a small fraction of the total, and redistribution would be hard to detect.

Selective loss of actin cables results in loss of Cdc42p polarity.

Why would partial F-actin depolymerization disperse Cdc42p when full depolymerization does not? As actin cables appear to be more sensitive to Lat B than cortical patches, one possibility was that selective disruption of cables by Lat B was responsible for dispersing Cdc42p. To test this hypothesis we made use of temperature-sensitive tropomyosin mutants, which selectively lose actin cables but not patches upon shift to restrictive temperature, leading to the dispersal of polarized Sec4p (a secretory vesicle marker) within a minute after shift (Fig. 2E) (Pruyne *et al.*, 1998). We found that polarized Cdc42p was dispersed from the pre-bud site within ten minutes upon shift of tropomyosin mutant cells to restrictive temperature (Fig. 2D). A complication with this experiment is that even wild-type cells transiently disperse polarized Cdc42p upon temperature shift (Fig. 2D)(Ho and Bretscher, 2001). However, dispersal of Cdc42p from the pre-bud site was reproducibly more rapid in tropomyosin mutants than in wild-type cells. Indeed, considerable Cdc42p dispersal had occurred after only one minute at restrictive temperature in the tropomyosin mutant cells, and most of the cells still displaying polarized staining for Cdc42p had fainter or more diffuse staining compared to the bright Cdc42p spots in the wild-type cells (Fig. 2D, inset). Thus, selective loss of actin cables led to rapid dispersal of Cdc42p from the polarization site in unbudded cells. Moreover, unbudded wild-type cells recovered polarized Cdc42p staining by 50 min following temperature shift, but the unbudded tropomyosin mutant cells never recovered polarized Cdc42p (Fig. 2E), indicating that cables are required for regaining and/or maintaining polarity.

To ask whether loss of actin cables prevented the establishment as well as the maintenance of Cdc42p polarity, we isolated early G1 wild-type and tropomyosin mutant cells from asynchronous populations using centrifugal elutriation, incubated them at restrictive

temperature, and examined Cdc42p localization as cells progressed through the cell cycle. The 50 min temperature shift recovery period in such cells occurs prior to cell cycle commitment and polarization. Wild-type and tropomyosin mutant cells both initially polarized Cdc42p at 80 min, but whereas wild-type cells retained polarized Cdc42p and went on to bud, the tropomyosin mutants lost polarized Cdc42p staining within 20 min and never formed a bud (Fig. 3A,B). Thus, Cdc42p polarization to the presumptive bud site can occur in the absence of actin cables, but it is not maintained.

Only 20% of tropomyosin-mutant cells showed a clear Cdc42p polarization at 80 min, as compared with 43% of control WT cells. Thus, the Cdc42p dispersal mechanism may be sufficiently effective to completely block Cdc42p polarization in many cells. Alternatively, all cells may be able to establish an initial Cdc42p polarity, which is then rapidly dispersed. As polarization of Cdc42p is triggered by a cell cycle cue and the population of cells was not perfectly synchronous with respect to cell cycle stage, we might only detect a robust Cdc42p cap in the 20% of cells that were in just the right cell cycle stage in this experiment.

One way that actin cables could contribute to Cdc42p polarity is by promoting polarized trafficking of cargo by myosin motors. The type V myosin Myo2p transports secretory vesicles and other cargo along actin cables (Schott *et al.*, 1999; Schott *et al.*, 2002). To test whether Myo2p-mediated trafficking was important for maintaining Cdc42p polarization at the pre-bud site, we repeated the synchrony experiment using temperature-sensitive *myo2-16* mutants. As shown in Fig. 3C,D, *myo2-16* cells initially polarized Cdc42p at the same time as wild-type controls, but then lost polarized Cdc42p staining and failed to form buds. A significantly higher fraction of cells were observed to polarize Cdc42p in the myosin mutants (Fig. 3C,D) as compared with the tropomyosin mutants (Fig. 3A,B). This observation may reflect the presence

of residual Myo2p activity in the mutant, or perhaps a contribution from additional actin cable-mediated pathways (e.g. the related myosin Myo4p). Regardless, these experiments indicate that Myo2p-mediated polarized traffic along actin cables is required for maintainance (though not for initial establishment) of Cdc42p polarity at the pre-bud site.

Dispersal of Cdc42p involves endocytosis.

Cdc42p is prenylated and peripherally associated with cellular membranes, but significant cytoplasmic pools also exist (Ziman *et al.*, 1993). It is thought that the guanine nucleotide dissociation inhibitor (GDI) Rdi1p removes Cdc42p from the membrane to the cytosolic pool (Masuda *et al.*, 1994; Koch *et al.*, 1997). Thus, the Cdc42p dispersal following selective elimination of actin cables could occur via direct displacement of Cdc42p from the membrane by Rdi1p, or by endocytic uptake of Cdc42p (or of anchoring factors for Cdc42p) from the presumptive bud site. We found that dispersal of Cdc42p in response to Lat B was unaffected by deletion of *RDI1* (Fig. 4A,B). To test whether Cdc42p dispersal required endocytosis, we used temperature-sensitive *sla2* mutants (also called *end4*) that have a defect in the internalization step of endocytosis (Raths *et al.*, 1993). Sla2p is a homolog of the mammalian huntingtin interacting protein (HIP1)(Henry *et al.*, 2002) and is localized to actin patches (Yang *et al.*, 1999). Sla2p binds to clathrin and is thought to be a conserved endocytic adaptor protein (Henry *et al.*, 2002; Kaksonen *et al.*, 2003). Wild-type and *sla2* mutant cells were shifted to restrictive temperature for 1 h to inactivate Sla2p (in the mutant) and allow recovery of Cdc42p polarity after the shift. Lat B was then added for 15 or 60 min and Cdc42p polarity was examined (Fig. 4C,D). Dispersal of Cdc42p from the pre-bud site was significantly reduced in *sla2* mutants compared to the controls, at both time points (Fig. 4C,D). Thus, Cdc42p dispersal occurs primarily by endocytosis, and F-actin cables are required to counteract this

effect. As endocytosis is also dependent on F-actin (Ayscough *et al.*, 1997), complete actin depolymerization by Lat A eliminates the dispersal mechanism and hence obviates the need for actin cables in maintaining Cdc42p polarization.

Cdc42p polarization becomes more resistant to dispersal following bud emergence.

The studies described above focused on Cdc42p polarization to the presumptive bud site in unbudded cells. Following bud emergence, Cdc42p remains polarized to the bud tip until the activation of Cdc28p by mitotic cyclins triggers the apical-isotropic switch in bud growth, after which Cdc42p is dispersed until cytokinesis, when it accumulates at the mother-bud neck (Pruyne and Bretscher, 2000; Richman *et al.*, 2002). Like unbudded cells, small-budded cells also lost polarized Cdc42p staining following exposure to Lat B (data not shown) or prolonged shift of tropomyosin mutants to restrictive temperature (Fig. 5A). However, in the tropomyosin mutants this loss was significantly slower than the depolarization of Cdc42p in unbudded cells (compare Fig. 5A with Fig. 2F): Cdc42p initially depolarized due to temperature shift at the same rate in wild-type and tropomyosin mutant cells, then repolarized 50 minutes later, but was subsequently dispersed in the tropomyosin mutants. Although some of this dispersal likely reflects progress of the cell cycle to a point where Cdc42p normally becomes dispersed, recent evidence suggests that at least some of the budded tropomyosin mutants arrest the cell cycle prior to that stage (Pruyne *et al.*, 2004). Thus, loss of actin cables leads to a delayed loss of Cdc42p polarization in budded cells.

To directly test whether budded cells at a cell cycle stage prior to the apical-isotropic switch would disperse Cdc42p upon loss of cable-directed traffic, we arrested cells by overexpressing the mitotic cyclin/Cdc28p inhibitor Swe1p (Booher *et al.*, 1993) and monitored Cdc42p localization following treatment with Lat B or shift-up of *myo2-16* mutants. As shown

in Fig. 5B,C, most Swe1p-arrested cells treated with Lat B retained polarized Cdc42p staining. In *myo2-16* mutants, Cdc42p polarity was initially lost and then recovered following temperature shift as in wild-type cells. However, the degree of recovery was impaired in *myo2-16* cells compared to wild-type controls (Fig. 5D,E), indicating that myosin-mediated traffic contributes to Cdc42p polarity in budded cells. In summary, treatments that all caused dispersal of Cdc42p in unbudded cells had differing effects in budded cells, causing complete (tropomyosin mutant), partial (myosin mutant) or little (Lat B) dispersal. Even when dispersal did occur, it was much slower than in unbudded cells. Thus, the polarized Cdc42p in budded cells is significantly more resistant than that in unbudded cells to treatments that selectively disrupt actin cable-mediated traffic.

Discussion

Opposing effects of actin cables and actin patches on Cdc42p polarity

There are two clearly distinct types of F-actin structures in polarized yeast cells. Actin cables are polarized towards the pre-bud site and the bud tip, and serve as tracks for myosin-mediated traffic to those sites (Schott *et al.*, 2002). Actin patches cluster at the pre-bud site and within the bud (Adams and Pringle, 1984), and are thought to mark sites of endocytosis as well as endocytic vesicles which have just been internalized (Kaksonen *et al.*, 2003). F-actin is essential for both polarized traffic and endocytosis (Ayscough *et al.*, 1997). Previous studies documented the ability of Cdc42p to become polarized and to maintain polarity in the complete absence of F-actin in yeast (Ayscough *et al.*, 1997; Irazoqui *et al.*, 2003). However, we now report that selective impairment of actin cables caused dispersal of Cdc42p from the pre-bud site, and that such dispersal was dependent on actin patch-mediated endocytosis. Our results suggest that actin patches disperse Cdc42p through endocytosis, whereas oriented actin cables help to

maintain Cdc42p polarization through myosin-mediated traffic. Thus, following its initial actin-independent polarization, subsequent maintenance of Cdc42p polarization at the pre-bud site involves an actin-mediated trafficking cycle.

We used three different perturbations that selectively disrupt actin cable function. Tropomyosin mutants eliminate cables without reducing the number or brightness of the patches (Pruyne *et al.*, 1998). *myo2-16* mutants affect the major (though not the only) myosin that delivers cargo along actin cables, and do not affect actin patches (Schott *et al.*, 1999). Lat B binds to G-actin (Ayscough *et al.*, 1997), and appears to be more effective at sequestering the actin from cable nucleators than from patch nucleators, although it clearly does reduce the amount of F-actin in patches as well. These perturbations all dispersed Cdc42p from the pre-bud site, although there were differences in the rapidity and effectiveness of the dispersal (fast and complete in tropomyosin mutants, slower and incomplete upon Lat B treatment). Tropomyosin mutants were also more effective than Lat B at dispersing Cdc42p from the bud tip in budded cells, although in all cases dispersal was slower and less complete in budded than in unbudded cells (see below). These differences are consistent with the hypothesis that dispersal is mediated by the actin patches, whose function is partly impaired upon Lat B treatment but not in tropomyosin mutants. Dispersal of Cdc42p from the pre-bud site was greatly reduced upon inactivation of the actin patch component Sla2p, which is required for endocytosis. In summary, our data indicate that if unopposed, actin-dependent endocytosis disperses Cdc42p. Endocytosed factors are normally recycled to the polarization site by vesicles trafficking on oriented actin cables, thereby maintaining Cdc42p polarity. As both endocytosis and polarized delivery are actin-dependent, actin perturbations exhibit different effects depending on the degree to which actin's opposing roles are affected by the perturbation. Thus, maintenance of Cdc42p polarization

at the presumptive bud site involves a dynamic and antagonistic interplay between distinct F-actin structures.

We also confirmed previous work (Ho and Bretscher, 2001) showing that mild temperature shift of wild-type cells causes a transient, reversible dispersal of Cdc42p from polarization sites. Interestingly, temperature shift also induces a transient and partial actin depolymerization associated with selective loss of actin cables (Lillie and Brown, 1994), which may contribute to the dispersal of Cdc42p. However, temperature-shift-induced dispersal was also observed in *sla2* mutants (data not shown), indicating that it does not require endocytosis. Actin patches and glucan synthase complexes are also transiently dispersed upon temperature shift, and that dispersal has been ascribed to a Pkc1p-dependent stress signaling pathway (Delley and Hall, 1999), which may also act on Cdc42p.

Establishment versus maintenance of Cdc42p polarization

Once it is polarized, Cdc42p directs the orientation of actin cables towards the polarization site, most likely through formin proteins that are thought to be Cdc42p effectors as well as actin cable nucleators (Evangelista *et al.*, 1997; Evangelista *et al.*, 2002; Sagot *et al.*, 2002). As we found that actin cables, in turn, contribute to maintaining Cdc42p polarity, there is potential for a positive feedback loop in which clustering of Cdc42p promotes actin cable orientation which reinforces Cdc42p clustering. Does this "actin-feedback" pathway contribute to the initial polarization of Cdc42p?

Wedlich-Soldner and colleagues have proposed that delivery of secretory vesicles carrying Cdc42p along actin cables concentrates Cdc42p at the polarization site, and that such actin-mediated positive feedback contributes to polarity establishment when bud site selection cues are absent (Wedlich-Soldner *et al.*, 2003; Wedlich-Soldner *et al.*, 2004). Initial support for

this model came from the demonstration that polarization of an overexpressed GTP-locked mutant form of Cdc42p was absolutely dependent on F-actin, Myo2p, and secretory function, and that the GTP-locked Cdc42p was present on secretory vesicles (Wedlich-Soldner *et al.*, 2003). However, wild-type Cdc42p can polarize in the absence of F-actin (Ayscough *et al.*, 1997), even in the absence of bud site selection cues (Irazoqui *et al.*, 2003), indicating that if such a feedback loop does operate, it is not absolutely required for polarization.

In a more recent study monitoring wild-type GFP-Cdc42p localization, Wedlich-Soldner at al. found that whereas most cells established and maintained robust Cdc42p polarization upon treatment with Lat A, a subset of cells established a "flickering" Cdc42p polarity that was subsequently lost (Wedlich-Soldner *et al.*, 2004). This observation suggests, consistent with our results, that actin can help to maintain Cdc42p polarization, making the polarization site more stable. In addition, they showed that in the absence of the scaffold protein Bem1p, F-actin was absolutely essential for any detectable polarization (Wedlich-Soldner *et al.*, 2004). These observations suggested that the initial polarization of Cdc42p could occur either through an actin-independent pathway involving Bem1p (Irazoqui *et al.*, 2003) or through an actin-dependent pathway. Distinguishing whether this actin-dependent pathway involves the proposed actin-Cdc42p feedback loop will require identification of the trafficking cargo relevant to Cdc42p polarization.

How do actin cables promote Cdc42p polarity?

Which cargo transported on actin cables is responsible for helping to maintain Cdc42p polarization? The simplest hypothesis would be that trafficking of Cdc42p itself is important: GTP-locked Cdc42p was found in a vesicle fraction (Wedlich-Soldner *et al.*, 2003), and it seems likely that wild-type Cdc42p can also associate with vesicles. However, it appears that wild-type

Cdc42p exchanges between membrane and cytosolic pools far more rapidly that GTP-locked Cdc42p (Wedlich-Soldner *et al.*, 2004). In addition, polarization by the "actin-feedback" membrane recycling pathway illustrated in Fig. 1C will only be effective if diffusion is slow relative to endocytic recycling, as has been shown for some integral membrane proteins (Valdez-Taubas and Pelham, 2003), but fluorescence recovery after photobleaching experiments suggest that Cdc42p diffuses much more rapidly (Wedlich-Soldner *et al.*, 2004). Thus, it is not clear whether vesicle trafficking of Cdc42p itself would be a major contributor to Cdc42p polarization. Many integral and peripheral membrane proteins co-localize with Cdc42p (Irazoqui and Lew, 2004), and it seems likely that several of these proteins undergo both endocytosis and cable-mediated delivery. Thus, endocytosis may disperse Cdc42p by removing not only Cdc42p itself but several of its interacting factors from the polarization site. But because most known polarized factors are peripheral (not integral) membrane proteins, it is not clear whether they would need to be recycled to the polarization site via actin cables or whether they could return by diffusion through the cytoplasm. A recent study reported that in tropomyosin mutants, budded cells lost not only Cdc42p but also Rho1p polarization (as well as the ability to nucleate cables at the bud tip) after one hour without cables at the restrictive temperature (Pruyne *et al.*, 2004). However, other peripheral components including Spa2p and the formin Bni1p were still largely polarized under those conditions, suggesting that these components either escape endocytosis or are able to return to the polarization site in a cable-independent manner.

Maturation of the polarization site following bud emergence

To all appearances, the Cdc42p cap at the presumptive bud site and the cap at the tip of budded cells are very similar and share numerous components. However, we found that the cap in budded cells was considerably more resistant to dispersal upon selective loss of cable function

than the cap in unbudded cells. In principle, this could be explained either by a difference in endocytic recycling or a difference in the cap itself. At present we cannot distinguish between these possibilities, but because extensive research into endocytosis has never (to our knowledge) revealed any differences between budded and unbudded cells, we favor the hypothesis that the organization of the Cdc42p cap changes during or shortly after bud emergence. The nature and function of any such change remain to be discovered.

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Figure Legends

Figure 1. Mechanisms underlying polarized protein distribution. A protein of interest (squares) displaying an asymmetric distribution at the cell cortex can attain that polarization via one or more of the indicated mechanisms. A) A pre-existing asymmetric distribution of an anchored interacting factor can concentrate the protein of interest. Here, the interacting factor is shown connected to an extracellular anchor that might be a site of cell-cell contact, or an immobilizing extracellular matrix, or the yeast cell wall. A variant of the anchor model (A') is that a dynamic patch of interacting proteins is assembled by a symmetry-breaking process and serves to localize the protein of interest. Like the anchor model, this does not require input from the cytoskeleton. However, the anchor-like patch is not fixed but dynamic, and can assemble "de novo" at random locations. B) A diffusion barrier (stop signs indicate blocked movement of the squares), such as the claudin-dependent tight junction, or the yeast septin-dependent neck cortex, can keep a cortical protein from crossing the fence (thick double black line). For this to produce a polarized distribution, the protein must be delivered to only one side of the fence (indicated by the blue arrow), along polarized cytoskeletal elements (red lines). C) Polarized delivery (blue arrow and red lines as in B) can yield a polarized distribution in the absence of a diffusion barrier if active recycling of the protein (here indicated by endocytic internalization, passage through a recycling endosome, and re-export along the polarized cytoskeleton) is fast enough to counter diffusion.

Figure 2. Loss of Cdc42p polarization upon partial actin depolymerization. A) Wild-type cells (DLY5) were grown to exponential phase in YEPD at 30°C and treated with DMSO (control), Lat A (100 μM), or Lat B (100 μM) for 2 h. Cells were fixed and processed to visualize F-actin with alexafluor488-phalloidin. B) Cells treated as above except in this case with 200 μM Lat B were processed to visualize Cdc42p by indirect immunofluorescence. C) Lawns of wild-type

(*ACT1*: DDY354) and latrunculin-resistant actin mutant (*act1-117*: DDY345) cells were spread on YEPD plates, sterile paper discs spotted with 10 µl DMSO (control), Lat A (2 mM), or Lat B (4 mM) were placed on the agar, and the plates were incubated for 2 days at 30°C. Growth inhibition by Lat A and (less potently) Lat B produced a clear zone or halo around the discs, but *act1-117* mutants were completely resistant to both Lat A and Lat B. Identical results were obtained with *act1-113* mutants (DDY342). D,E) Tropomyosin mutant (*tpm1-2 tpm2*Δ: ABY971) and control (*tpm2*Δ: ABY973) cells were grown to exponential phase in YEPD at 24°C and shifted to 34.5°C at t=0. Rapid temperature shift was performed as described by Pruyne et al. (1998). Samples were fixed and double-stained to visualize Cdc42p (D) and Sec4p (E) by indirect immunofluorescence. >190 unbudded cells were scored for the presence of polarized signal at each time point. In the case of Cdc42p the polarized signal, though present, was diminished in the tropomyosin mutant after 1 min of shift: the inset in D indicates the proportion of cells with a bright polarized patch (black bar) versus a faint patch or extended crescent (white bar) at this time. F) Cdc42p staining to a bright patch was quantitated in >100 unbudded cells from a separate experiment extending the temperature-shift to longer times.

Figure 3. Selective loss of actin cables or Myo2p function impairs maintenance but not establishment of Cdc42p polarization. A,B) Tropomyosin mutant (*tpm1-2 tpm2*Δ: ABY971) and wild-type (ABY551) cells were grown to exponential phase in synthetic complete medium without histidine at 24°C, and small daughter cells were isolated by centrifugal elutriation, harvested by centrifugation, and resuspended in pre-warmed YEPD at 35°C (t=0). Samples were fixed at 20 min intervals and processed to visualize Cdc42p by indirect immunofluorescence. A) Quantitation: >100 cells were scored for the presence or absence of Cdc42p polarization. B) Representative cells from the indicated timepoints. C,D) Myosin mutant (*myo2-16*: ABY553)

and wild-type (ABY551) cells were treated as above except that the restrictive temperature was 37°C. C) Quantitation: >100 cells were scored for the presence or absence of Cdc42p polarization. D) Representative cells from the indicated timepoints.

Figure 4. Cdc42p dispersal involves endocytosis but not Rdi1p. A,B) Wild-type (DLY1) and *rdi1*Δ (GPY10) cells were grown to exponential phase in YEPD at 30°C and treated with DMSO (control) or Lat B (100 μM) for 1 h, and then fixed and processed to visualize Cdc42p by indirect immunofluorescence. A) Representative cells. Bar, 5 μM. B) Quantitation: >100 cells were scored in each sample. C,D) Wild-type (RH1800) and temperature-sensitive *sla2* (*sla2-41=end4-1*: DDY595) cells were grown to exponential phase in YEPD at 24°C, and shifted to 37°C (restrictive temperature) for 1 h, after which Cdc42p polarity had largely recovered from the temperature shift. Lat B (200 μM) was added for an additional 15 or 60 min at 37°C, as indicated, leading to dispersal of Cdc42p in the wild-type but not in the *sla2* cells. C) Representative cells. Bar, 5 μM. D) Quantitation: >100 cells were scored in each sample.

Figure 5. Cdc42p in budded cells is more resistant to dispersal by actin perturbation. A) Tropomyosin mutant (*tpm1-2 tpm2*Δ: ABY971) and control (*tpm2*Δ: ABY973) cells from the same experiment as in Fig. 2F above were scored to quantitate the proportion of small-budded cells displaying a tight patch of polarized Cdc42p. B, C) Wild-type cells containing a *GAL1*-regulated *SWE1* gene (DLY3466) were grown to exponential phase in YEP-sucrose at 30°C and induced to express Swe1p by addition of galactose to 2% final concentration. After 1 h 40 min, many cells displayed large elongated buds characteristic of Swe1p-mediated G2 arrest (Lew and Reed, 1993), and at that time ("Swe1p Arrest") either DMSO or 100 μM Lat B was added. After 90 min, control (DMSO) cells continued to elongate their buds, whereas Lat B-treated cells ceased polarized growth and grew uniformly larger, although most did not disperse tip-localized

Cdc42p. B) Representative cells from the indicated samples. C) Quantitation: >100 cells were scored for the presence or absence of Cdc42p polarization. D,E) Wild-type and *myo2-16* cells containing a *GAL1*-regulated *SWE1* gene (DLY7543 and DLY7544, respectively) were grown to exponential phase in YEP-raffinose at 24°C and induced to express Swe1p by addition of galactose to 2% final concentration. After 2 h ("Swe1p Arrest"), the cells were shifted to 37°C, samples were fixed at 15 min intervals and processed to visualize Cdc42p. D) Representative cells from the indicated samples. E) Quantitation: >100 cells were scored for the presence or absence of Cdc42p polarization.

Supplementary Figure 1. Wild-type cells (DLY5) were grown to exponential phase in YEPD at 30°C and treated with the indicated doses of Lat A or Lat B for 2 h. Separate aliquots were processed to visualize F-actin or Cdc42p. A) Representative cells from each sample, photographed using the same exposure times. B) Quantitation of Cdc42p polarization, showing that in this experiment 100 μM Lat B and 30 μM Lat A promoted similar degrees of Cdc42p dispersal, while higher and lower doses of Lat A allowed greater maintenance of Cdc42p polarization. C) Actin organization in cells treated with 100 μM Lat B and 30 μM or 100 μM Lat A (and untreated controls), photographed using different exposures optimized to detect the remaining actin structures. Only cortical patches (or their remnants) are detected in the treated cells. Surprisingly, there was reproducibly much less total F-actin in cells treated with 30 μM Lat A (the dose at which Cdc42p was most dispersed) than in cells treated with 100 μM Lat B (where Cdc42p was also dispersed). Both samples lacked detectable cables, but the remaining patches were much brighter in the Lat B-treated cells, suggesting that patch components can compete effectively with Lat B, but not with Lat A, for actin monomers.

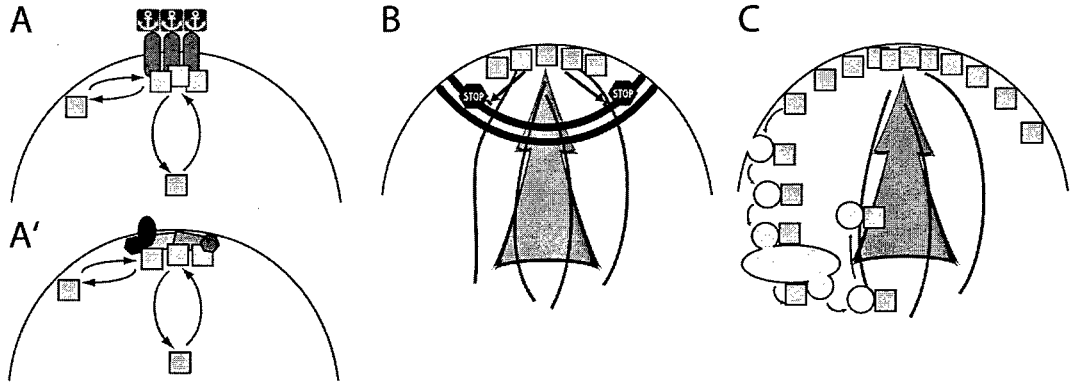
Table I. Yeast strains used in this study.

Strain	Relevant Genotype	Reference/Source
ABY551	**a**/α *MYO2::HIS3/MYO2::HIS3*	(Schott *et al.*, 1999)
ABY553	**a**/α *myo2-16::HIS3/myo2-16::HIS3*	(Schott *et al.*, 1999)
ABY971	**a**/α *tpm1-2::LEU2/tpm1-2::LEU2 tpm2Δ::HIS3/tpm2Δ::HIS3*	(Pruyne *et al.*, 1998)
ABY973	**a**/α *tpm2::HIS3/tpm2::HIS3*	(Pruyne *et al.*, 1998)
DDY342	α *act1-113::HIS3*	(Ayscough *et al.*, 1997)
DDY345	α *act1-117::HIS3*	(Ayscough *et al.*, 1997)
DDY354	**a** *ACT1::HIS3*	(Ayscough *et al.*, 1997)
DDY595	**a** *bar1 sla2-41*	(Wesp *et al.*, 1997)
DLY5[1]	**a**/α *bar1/BAR1*	(Lew and Reed, 1993)
DLY3644[1]	**a** *his3 HIS2 GAL1p-SWE1::LEU2*	This work
DLY7543[2]	**a**/α *MYO2::HIS3/MYO2::HIS3 GAL1p-SWE1myc::URA3*	This work
DLY7544[3]	**a**/α *myo2-16::HIS3/myo2-16::HIS3 GAL1p-SWE1-myc::URA3*	This work
GPY10[1]	**a** *bar1 rdi1::URA3*	David Stone
RH1800	**a** *bar1*	(Friant *et al.*, 2000)
RH3809	**a** *bar1 lcb1-100*	(Friant *et al.*, 2000)

[1] Strains in the BF264-15Du background (*ura3Δns leu2-3,112 trp1-1a ade1 his2*).

[2] Generated by transforming ABY551 with pRS306-GAL1-SWE1myc (McMillan *et al.*, 1998).

[3] Generated by transforming ABY553 with pRS306-GAL1-SWE1myc (McMillan *et al.*, 1998).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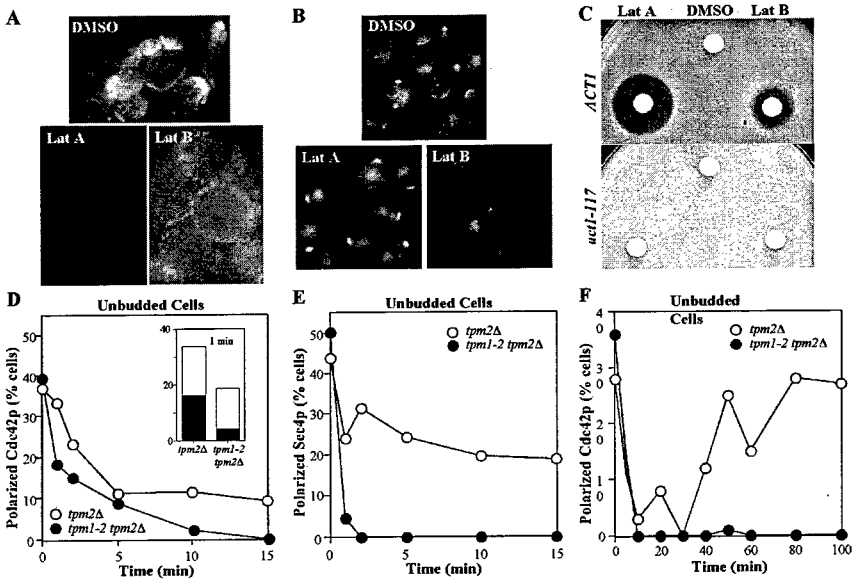
STOP
C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Lat B
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Lat B
ACT1
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Time (min)
1 min
tpm2Δ
tpm1-2 tpm2Δ
E
Unbudded Cells
Polarized Sec4p (% cells)
Time (min)
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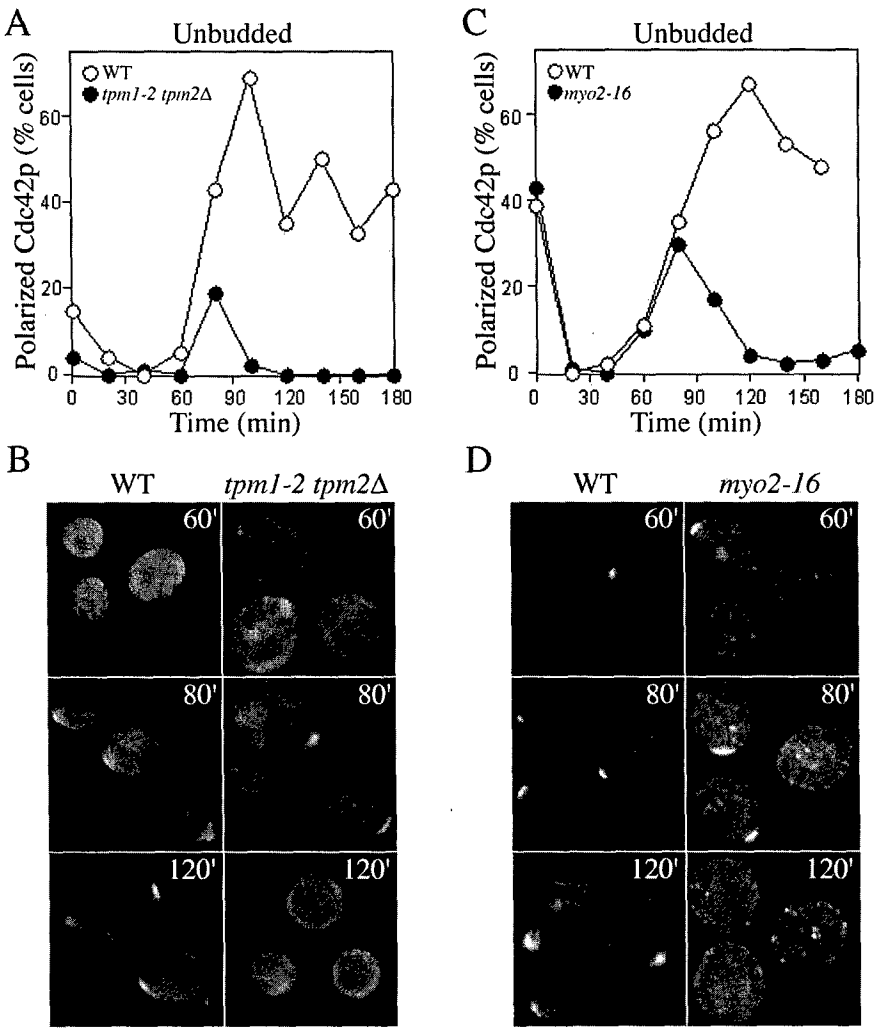
A
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tpm1-2 tpm2Δ
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20
40
60
0 30 60 90 120 150 180
Time (min)
C
Unbudded
WT
myo2-16
Polarized Cdc42p (% cells)
0
20
40
60
0 30 60 90 120 150 180
Time (min)
B
WT
tpm1-2 tpm2Δ
60'
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80'
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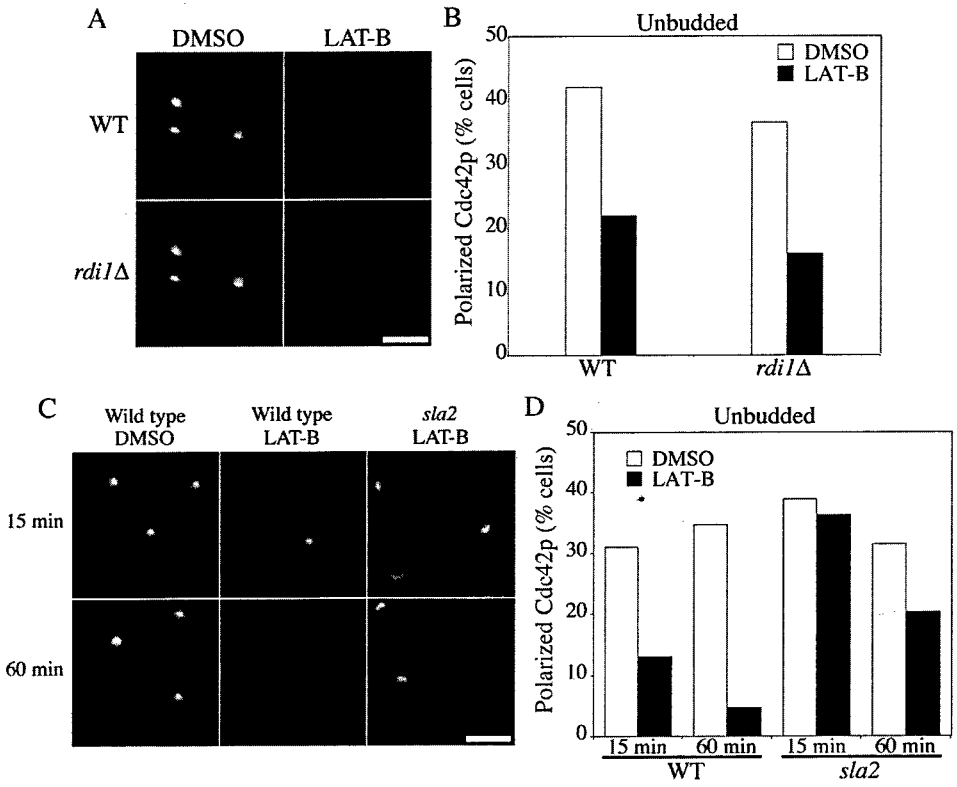
120'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rdi1Δ
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LAT-B
Polarized Cdc42p (% cells)
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40
30
20
10
0
WT
rdi1Δ
C
Wild type
DMSO
Wild type
LAT-B
sla2
LAT-B
15 min
60 min
D
Unbudded
DMSO
LAT-B
Polarized Cdc42p (% cells)
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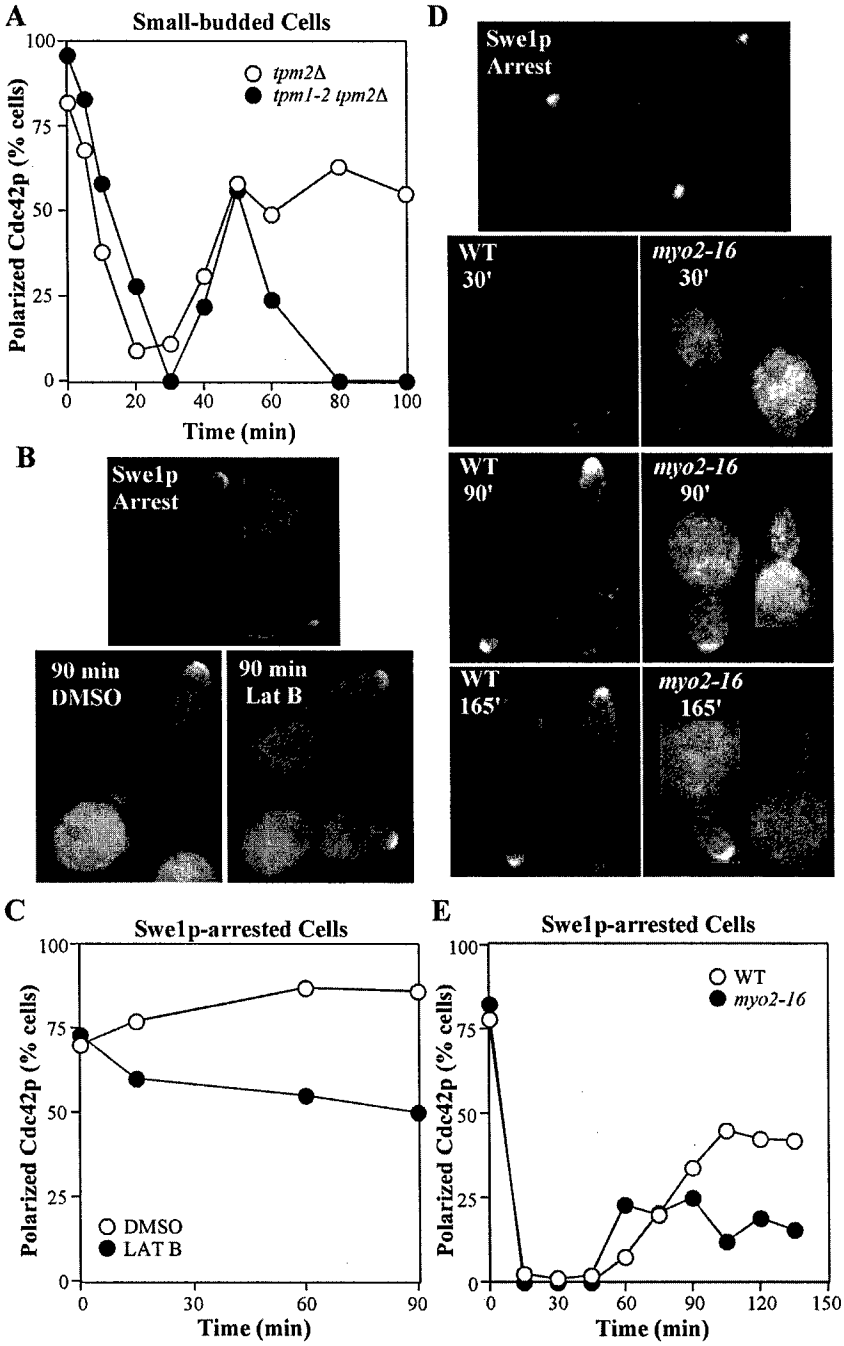
40
30
20
10
0
15 min
60 min
15 min
60 min
WT
sla2


A
Small-budded Cells
Polarized Cdc42p (% cells)
Time (min)
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tpm1-2 tpm2Δ
B
Swe1p Arrest
90 min DMSO
90 min Lat B
C
Swe1p-arrested Cells
Polarized Cdc42p (% cells)
Time (min)
DMSO
LAT B
D
Swe1p Arrest
WT 30'
myo2-16 30'
WT 90'
myo2-16 90'
WT 165'
myo2-16 165'
E
Swe1p-arrested Cells
Polarized Cdc42p (% cells)
Time (min)
WT
myo2-16

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Extra Views

Cdc42p, GTP Hydrolysis, and the Cell's Sense of Direction

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KEY WORDS

Cdc42, GTP hydrolysis, cell polarity, Cdc24, Bem1, Scaffold

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ABSTRACT

The GTPase Cdc42p is essential for polarity establishment in animals and fungi.[1] Human Cdc42p can functionally replace yeast Cdc42p,[2] indicating a high degree of evolutionary conservation. Current models of Cdc42p action generally follow the signaling paradigm established for Ras, in which receptors responding to an initiating stimulus cause guanine nucleotide exchange factors (GEFs) to trigger GTP-loading of Ras, leading to engagement of downstream effectors and ensuing cell proliferation. Key support for the Ras paradigm came from the finding that oncogenic forms of Ras, unable to hydrolyze GTP and therefore constitutively GTP-bound, mimicked the effect of constitutive signaling by the upstream receptors even in the absence of stimuli. Attempts to assess whether or not this paradigm is valid for Cdc42p-induced polarization of yeast cells have yielded conflicting results.[3-6] Here, we discuss the available information on this issue and conclude that unlike Ras signaling, Cdc42p directed polarity establishment additionally requires cycling between GTP- and GDP-bound forms. We suggest that such cycling is critical for a little-studied "function" of Cdc42p: its ability to designate a unique portion of the cell cortex to become the polarization site, and to become concentrated at that site.

GTP HYDROLYSIS BY Cdc42p IS ESSENTIAL FOR PHYSIOLOGICAL POLARIZATION

In proliferating yeast cells, the physiological stimulus for cell polarization comes from the activation of G_1 cyclin/CDK complexes upon commitment to a new cell cycle.[7] Expression of a constitutively GTP-bound mutant of Cdc42p, $Cdc42p^{Q61L}$, induced cytoskeletal polarization in G_1-arrested cells in which G_1 cyclins[3,8] or the CDK Cdc28p [5] had been inactivated, suggesting that GTP-bound Cdc42p was sufficient to promote polarity and that the role of the cell cycle stimulus was simply to promote GTP-loading of Cdc42p. As might be expected, polarization induced by $Cdc42p^{Q61L}$ did not require the Cdc42p-directed GEF, Cdc24p.[5] Independent experiments identified a series of other mutants, exemplified by $Cdc42p^{G60D}$, which could also promote polarization (and even, albeit poorly, proliferation) in the absence of the GEF.[4] The proximity of the G60 residue to Q61, as well as biochemical evidence, suggested that $Cdc42p^{G60D}$ was slow to hydrolyze GTP and therefore predominantly GTP-bound.[4] These findings supported the idea that GTP-bound Cdc42p was sufficient to promote polarity, and that the role of the GEF and the cell cycle cue was simply to promote GTP-loading of Cdc42p.

A concern in the interpretation of these experiments is whether or not the observed polarization accurately reflects the physiological process. Prior work had shown that expression of $Cdc42p^{Q61L}$, even from the endogenous *CDC42* promoter, was lethal to yeast cells,[9] and most experiments cited above were performed using strong conditional promoters yielding substantially higher levels of expression. Moreover, polarization did not occur immediately following synthesis of $Cdc42p^{Q61L}$ but only after a long delay, and unlike polarization triggered by endogenous Cdc42p, which is always restricted to one site on the cell cortex, polarization triggered by the overexpressed $Cdc42p^{Q61L}$ sometimes occurred at two or even more sites.[3,5,8] Some of these features were also observed in the $Cdc42p^{G60D}$ experiments, including unexpectedly high levels of $Cdc42p^{G60D}$ (even though the endogenous promoter was used), polarization to more than one site, and frequent cell death.[4] Thus, although GTP-Cdc42p can clearly promote some form of polarity, the possibility remained that such polarity might be occurring through mechanisms distinct from those which mediate polarization during the normal cell cycle.

To assess whether expression of more physiologically relevant levels of $Cdc42p^{Q61L}$ could promote polarization without deleterious side-effects, we tested various promoters

and found that expression of Cdc42p^{Q61L} at levels comparable to 30–50% of endogenous Cdc42p was non-toxic. As only a small fraction of endogenous Cdc42p is likely to be GTP-bound at any one time, this level of constitutively GTP-bound Cdc42p^{Q61L} is still likely to be supraphysiological. However, at those levels, Cdc42p^{Q61L} was no longer able to promote polarization in cells lacking either CDK (Cdc28p) activity or GEF (Cdc24p) activity.[5] Moreover, Cdc42p^{Q61L} was unable to replace endogenous Cdc42p, indicating that at these more physiological levels, GTP-locked Cdc42p cannot cause polarization even when the cell cycle cue and the GEF are intact.[5] We concluded that the physiological mechanism of polarization requires that Cdc42p be able to hydrolyze GTP, and that simply loading Cdc42p with GTP could not explain how the cell cycle cue induces polarization.

In cells containing both wild-type Cdc42p and Cdc42p^{Q61L} (expressed at non-lethal levels), the cells polarize normally (i.e. to a single site, immediately following G_1 cyclin/CDK activation).[5] Similarly, cells containing both wild-type Cdc42p and Cdc42p^{G60D} polarize normally.[4] Thus, the ability of Cdc42p^{G60D} to polarize to multiple sites is recessive, which is certainly not predicted by the Ras paradigm, in which oncogenic, constitutively GTP-bound Ras is fully dominant to wild-type Ras. Based on this observation, Caviston et al. also concluded that GTP hydrolysis by Cdc42p was critical for the normal polarization mechanism, and that slowed GTP hydrolysis by Cdc42p^{G60D} resulted in a more feeble polarization capacity, albeit one that could still function without the GEF.[4] These various studies are all inconsistent with the simple view that GTP-loading of Cdc42p suffices for polarization. Instead, these data suggest that the physiological polarization mechanism involves an essential role for GTP hydrolysis by Cdc42p, and that the cell cycle cue must do more than just stimulate overall GTP-loading of Cdc42p.

POLARIZATION OF Cdc42p ITSELF: TWO WAYS TO POLARIZE

What is the role of GTP hydrolysis by Cdc42p? During the polarization process, Cdc42p itself becomes concentrated at the polarization site, and it is believed that clustering of the Cdc42p is responsible for orienting the cytoskeleton towards that site. In cells containing low levels of Cdc42p^{Q61L} but no endogenous wild-type Cdc42p, the Cdc42p^{Q61L} itself fails to polarize upon G_1 cyclin/CDK activation, and instead remains diffusely distributed.[5] Thus, GTP hydrolysis appears to be essential for generating a localized pool of Cdc42p.

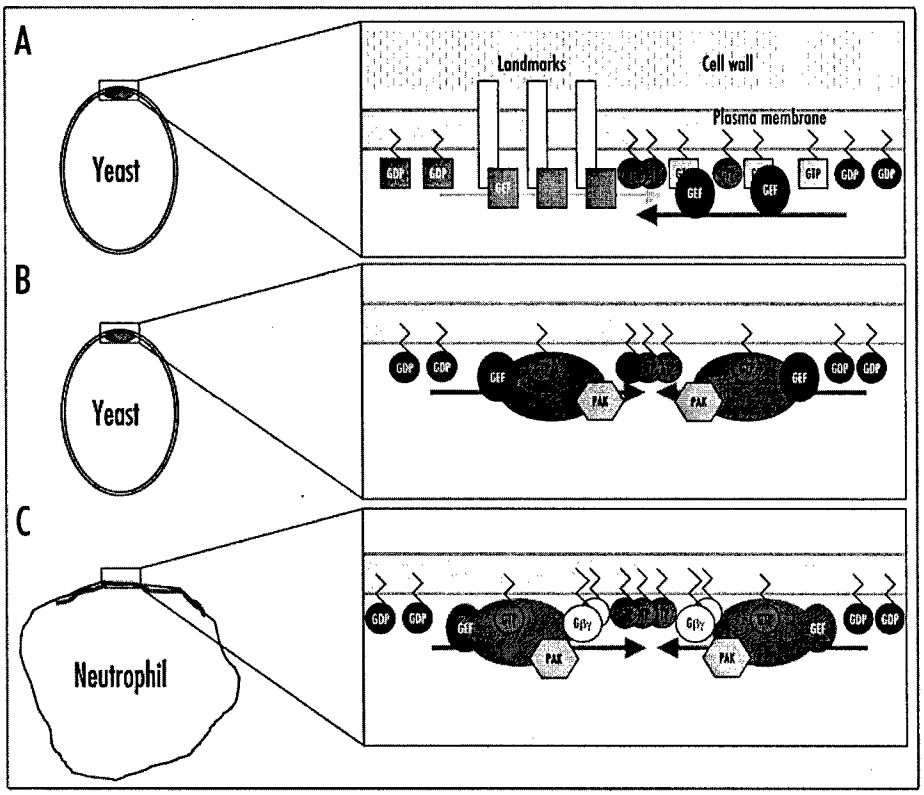

Figure 1. Polarization of Cdc42p. (A) Landmark proteins, thought to be anchored to the rigid cell wall, are present at the poles of newborn yeast cells. Cdc42p is diffusely distributed throughout the plasma membrane and in intracellular membranes until a cell cycle cue triggers concentration of Cdc42p at a site near one of the landmarks.[28] The landmarks interact with a GEF (dark green rectangle) for Rsr1p,[12] generating a localized pool of Rsr1p-GTP (light green), which in turn binds to both Cdc24p (brown oval) and GDP-Cdc42p (red).[14,15] These interactions are thought to promote localization and GTP-loading of Cdc42p near the landmarks. It is unclear where in this pathway the cell cycle cue acts to stimulate Cdc42p polarization. How only one site is chosen from the two marked poles is also unclear. (B) Bem1p (blue scaffold) binds directly to the GEF Cdc24p (brown oval), to GTP-bound Cdc42p (red), and to Cla4p (PAK: orange), and within such complexes Cla4p phosphorylates multiple sites on Cdc24p.[19] In vivo, these Cdc24p phosphorylations occur at about the time of polarization during the cell cycle and they are absolutely dependent on both Bem1p and Cdc42p.[19] The effect of Cdc24p phosphorylation on its activity has not yet been determined, but we suggest that it may activate Cdc24p to promote enhanced generation of local Cdc42p-GTP, which could promote the assembly of more scaffolds and effectors in the vicinity of the complex. As there are multiple and redundant interactions between Cdc42p and its scaffolds and effectors, this process would yield a large and self-sustaining "cap" of multiply-interacting proteins, constituting the polarization site. The various effectors in the cap would then orient the actin cytoskeleton towards the site, and actin-mediated trafficking could re-inforce the cap by delivering membrane-bound cap constituents to that site. A number of mutant alleles encoding versions of Cdc42p impaired for different functions (actin organization, septin organization, exocytosis) have been isolated, and genetic screens for suppressors have identified a variety of downstream elements in those pathways.[29-31] Astonishingly, Bem1p and Cla4p have emerged as "universal suppressors" whose overexpression can ameliorate many different kinds of Cdc42p-related defects.[20,29,31] Although it is possible that Bem1p and Cla4p act as important effectors in multiple distinct pathways, we consider it more likely that by participating in the feedback loop described above, they play a key role in clustering of Cdc42p, which is central to all of its functions in cell polarity. (C) The mammalian Cdc42p-directed GEF PIX1α (blue) merges features of Cdc24p (DH and PH domains) and Bem1p (GTP-Cdc42p binding and PAK binding via an SH3 domain).[32] In neutrophils exposed to chemoattractant, receptor-mediated release of Gbγ (light blue) allows it to bind and recruit PAK to the site of receptor activation, along with associated PIX1α.[27] By analogy to the Bem1p-scaffolded complex in yeast, this complex may act to amplify local GTP-Cdc42p production and clustering.

Two basic strategies can be envisioned to explain how a global signal like G_1 cyclin/CDK activation leads to unidirectional polarization of Cdc42p. In the first, some cellular component is already asymmetrically distributed within the cell, and the cell cycle signal simply allows Cdc42p to recognize that pre-existing asymmetry and concentrate at the specified site. In the second, the asymmetry is

generated de novo in a process called symmetry-breaking. Yeast cells can apparently use either strategy.

Certain "landmark proteins" are localized to cell poles in newborn early G_1 cells, and can influence the location of subsequent Cdc42p clustering.[10] The landmark proteins are thought to promote localized activation of the Ras-like protein Rsr1p,[11-13] which in its GTP-bound form can bind directly to the GEF Cdc24p [14] as well as to GDP-bound Cdc42p.[15] Rsr1p binding may activate Cdc24p catalytic activity,[6] and these interactions presumably serve to promote GTP-loading of Cdc42p in the vicinity of the landmarks (Fig. 1A). As $Cdc42p^{Q61L}$ binds GTP immediately following its synthesis and then retains the GTP, it would never cycle through a GDP-bound form that could interact with Rsr1p or the GEF, so the Rsr1p-GEF-mediated mechanism for generating a localized pool of GTP-bound Cdc42p would be unavailable to $Cdc42p^{Q61L}$.

Cells lacking landmark proteins or Rsr1p fail to polarize to the "correct" sites at the poles of the cell, but they nevertheless polarize effectively towards a single random site upon receipt of the cell cycle cue.[11,16] This "symmetry-breaking" polarization does not require polymerized actin or tubulin, but does require the scaffold protein Bem1p.[5] A model consistent with these data is that a core set of scaffold proteins and effectors of Cdc42p "co-polymerize" together with Cdc42p to form a multiply interacting patch that constitutes the polarization site.[5,17] The key mystery in this process is how cells ensure that one and only one patch is formed upon receipt of the cell cycle cue, even when no pre-existing asymmetry is apparent.

Mathematical models to explain symmetry-breaking behavior usually invoke a short-range amplification mechanism that can take a small spatial inequality arising due to stochastic fluctuations in starting component concentrations, and turn it into a dramatically larger asymmetry.[18] Based on biochemical and genetic data (see legend, Fig. 1), we suggested that the amplification mechanism in yeast involves the scaffold protein Bem1p and the PAK-family kinase Cla4p.[5] Prior to initiating polarization, Bem1p and Cla4p are thought to be autoinhibited and evenly distributed throughout the cell cortex. However, when the intramolecular interactions are relieved (presumably stimulated by GTP-Cdc42p), Bem1p can form a complex with Cdc24p, GTP-Cdc42p, and Cla4p in which Cla4p phosphorylates the associated GEF.[19] We suggest that this activates Cdc24p to enhance local GTP-loading of Cdc42p, promoting further assembly of additional interacting complexes in the vicinity (Fig. 1B). As with the landmark-Rsr1p pathway discussed above, a central feature of this model is that localized GTP-loading of Cdc42p mediated by the GEF serves to localize Cdc42p itself. Thus, neither the landmark nor the symmetry-breaking pathway would be accessible to $Cdc42p^{Q61L}$.

The above analysis suggests that one reason that GTP hydrolysis by Cdc42p is essential for polarization is simply that such hydrolysis is necessary to convert Cdc42p to a GDP-bound form that can productively interact with the GEF, which serves to localize the Cdc42p. Recent studies suggested that artificial activation of Cdc24p throughout the cell cortex, presumably yielding abundant GTP-Cdc42p, did not promote polarization, and indeed inhibited polarization,[6] providing strong support for this view. However, not all of the data are easily explained by this "localized GTP-loading" model. In particular, mutations in Cdc42p that mildly retard GTP hydrolysis or elimination of several Cdc42p-directed GAPs did not seem to affect polarity establishment, suggesting that the process is moderately insensitive to the exact rate of GTP hydrolysis.[20-22] Moreover, expression of $Cdc42p^{Q61L}$ or $Cdc42p^{G60D}$ did not eliminate polarization triggered by less-abundant endogenous Cdc42p, suggesting that the amplification process can function even with a high threshold of unlocalized GTP-Cdc42p.[4,5] The unexpected robustness of Cdc42p polarization to perturbations of GTP hydrolysis rate or global GTP-Cdc42p threshold raises the possibility that other pathways that do not involve GTP-GDP cycling may also contribute to polarization. Although such mechanisms are not sufficient to polarize endogenous Cdc42p, they may explain why overexpressed $Cdc42p^{Q61L}$ or $Cdc42p^{G60D}$ can induce some polarity.

Cdc42p POLARIZATION IN MAMMALIAN CELLS

Most studies on Cdc42p in mammalian cells have focused on identification of effector pathways by which Cdc42p can influence cytoskeletal organization and vesicle traffic.[1,23] The yeast studies outlined above highlight the importance of properly localizing the activity of Cdc42p, and Cdc42p is known to concentrate at the front of several mammalian polarized cells. Elegant experiments in which $Cdc42p^{Q61L}$ was tethered to a trans-membrane anchor demonstrated that downstream effects of Cdc42p on the actin cytoskeleton were only triggered when the cortical Cdc42p was clustered using antibodies to the extracellular domain of the anchor.[24] More recently, similar results were obtained upon antibody-mediated clustering of a scaffold protein, and in that case Cdc42p was no longer required for the cytoskeletal regulation.[25] Thus, the mechanism whereby endogenous Cdc42p becomes clustered in response to a polarizing stimulus is likely to be key for polarization of scaffolds and cytoskeletal elements.

One well-studied model system for polarization is the chemotactic migration of neutrophils, in which Cdc42p is required for unidirectional polarization.[26] Whereas migration is usually directed by a gradient of chemoattractant concentration, neutrophils also initiate randomly directed migration when exposed to a uniform concentration of chemoattractant. Thus, they exhibit both cue-driven and symmetry-breaking forms of polarization, as described above for yeast. Recent studies showed that directional sensing and polarization by neutrophils required a complex with remarkably similar architecture to the yeast Bem1p-scaffolded complex (see Fig. 1C).[27] It is, as yet, unclear exactly how this complex participates in polarization, but we suggest that such complexes provide a localized amplification mechanism to stimulate clustering of Cdc42p and associated polarity factors, creating a self-reinforcing cortical zone that gives a cell its sense of direction.

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Polarity establishment in yeast

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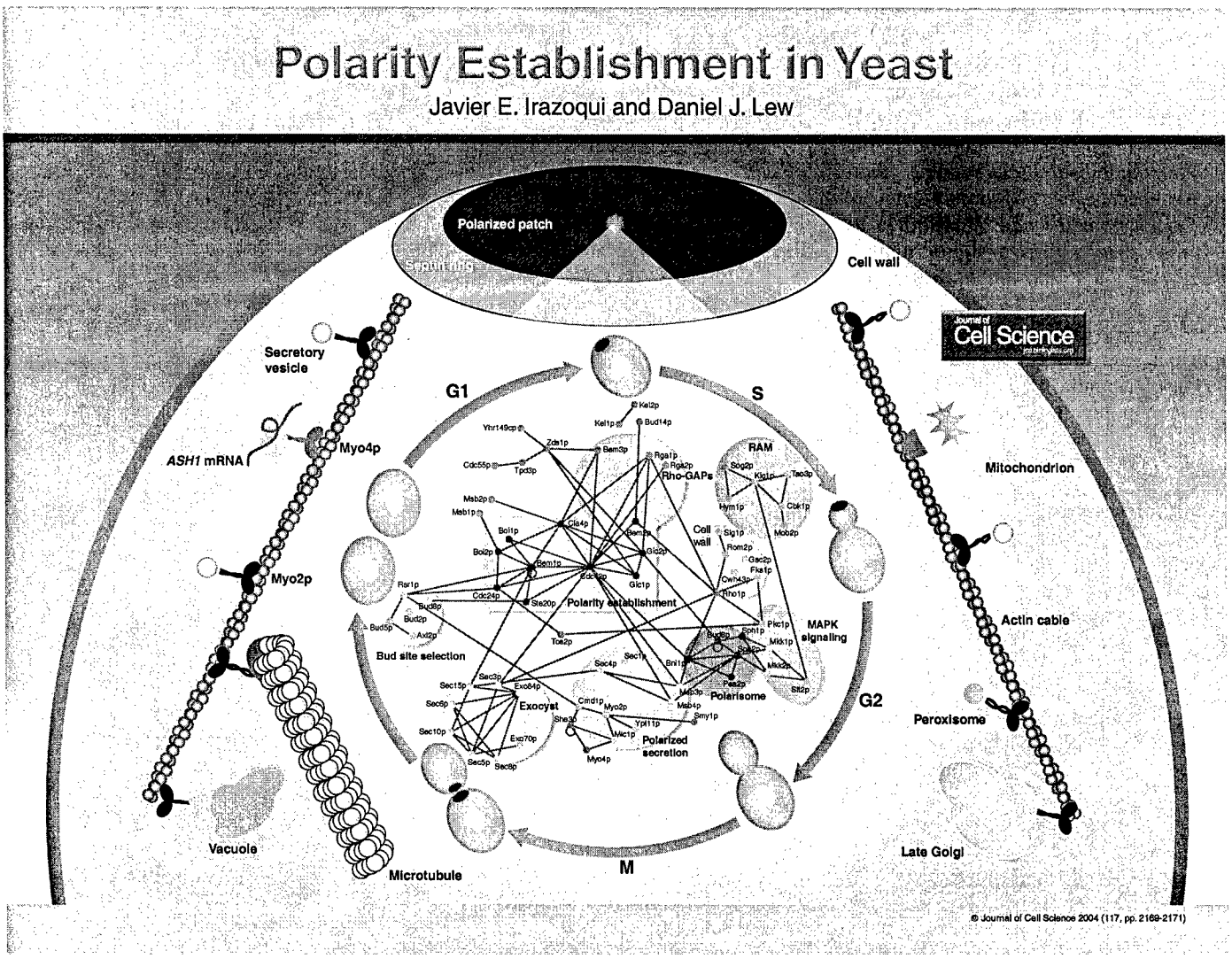
The unicellular yeast *Saccharomyces cerevisiae* is a model system for the establishment of cell polarity. Yeast cells proliferate by budding, which involves specialization of a small patch of the mother cell cortex and polarization of many cell constituents towards that patch, promoting growth of the bud. Genetic approaches have uncovered a large number of genes involved in these processes, yielding a working model for polarity establishment.

Polarized structures

The poster depicts a yeast mother cell shortly before the emergence of a bud. A large number of proteins become concentrated in a polarized patch about 0.5 μm diameter. A ring of cytoskeletal filaments called septins surrounds the patch, and actin cables course through the cytoplasm, terminating near the patch. The organization of septin filaments within the ring is unknown. Although the actin cables are depicted as single filaments for simplicity, in fact each cable is thought to consist of many shorter filaments linked into parallel bundles, with each filament oriented so that the barbed (plus) end points towards the polarized patch. Myosin motors (Myo2p and Myo4p) travel along the cables towards the patch, transporting many types of cargo, including secretory vesicles, various organelles, the plus ends of cytoplasmic microtubules and RNA-protein complexes. Mitochondria are also transported along cables, though perhaps without need for myosin motors. Polarized secretion of vesicles carrying cell wall remodeling enzymes and new cell wall constituents promotes local cell wall deformation and bud emergence. Trafficking of other cargos serves to segregate organelles into the growing bud and to orient the microtubule spindle along the mother-bud axis. Not depicted are cortical actin patches, mobile short-lived structures linked to endocytosis, which are 'born' near the polarized patch.



(See poster insert)

Polarization during the cell cycle

The cell cycle of budding yeast is depicted in the cell interior. Cell cycle commitment in late G1 phase triggers the assembly of the polarized patch (red) and septin ring (green), as well as the polarization of actin cables and cortical actin patches (not shown). Polarized secretion leads to bud emergence (at about the time of the G1/S transition) and then bud growth. The septin ring spreads to form an hourglass-shaped collar in the cell cortex at the neck, remaining there throughout bud growth and acting as a diffusion barrier that prevents the mixing of integral and peripheral membrane proteins between the mother cortex and the bud cortex. Early on during bud growth the polarized patch remains focused at the bud tip, but then becomes broader and dissipates as the bud enlarges. Following mitosis most of the proteins of the polarized patch reassemble at the mother-bud neck and the septin hourglass splits into two rings during cytokinesis.

Components of the polarized patch

All of the proteins known to localize within the polarized patch are depicted in the network at the center of the figure, derived using Osprey software from data curated at the *Saccharomyces* genome database (http://genome-www.stanford.edu/Saccharomyces/) and edited by the authors. Each protein is marked by a circle, and reported protein-protein interactions among this set are shown as connecting lines between the circles (these include reports of two-hybrid and co-immunoprecipitation data as well as direct interactions among recombinant proteins). Interacting proteins not known to localize to the patch were excluded. The proteins are divided into color-coded functional groupings.

Bud site selection

The location of the polarized patch is not random within the cell. Newborn cells carry spatial landmarks (including Bud8p at the distal tip) that influence the location of the polarized patch in the subsequent cell cycle. The Rsr1p GTPase and its guanine nucleotide exchange factor (GEF) Bud5p and GTPase-activating protein (GAP) Bud2p are required for proper localization of the polarized patch to the site specified by the spatial landmarks, and in their absence the polarized patch forms at a random location.

Polarity establishment

The Rho-family GTPase Cdc42p and its GEF Cdc24p are essential for assembly of the polarized patch and septin ring, and for the polarization of actin cables and cortical actin patches. As such, they are considered the master regulators of polarity establishment in yeast. Genetic studies indicate that the Cdc42p effectors Cla4p, Ste20p, Gic1p and Gic2p, as well as the scaffold proteins Bem1p, Boi1p and Boi2p act together with Cdc42p and Cdc24p to establish polarity.

Rho-GAPs

At least four GAPs can stimulate GTP hydrolysis by Cdc42p. Phenotypic analysis suggests that Bem2p is important for polarity establishment, although it also has links to Rho1p and the control of cell wall integrity and MAPK signaling. By contrast, Rga1p, Rga2p and Bem3p have been implicated in promoting the assembly of the septin ring around the polarized patch.

Polarisome

The polarisome is a protein complex thought to form a link between polarity establishment factors and actin cables. The formin Bni1p promotes nucleation and growth of actin cables, and Spa2p and Bud6p are important for Bni1p localization and function. Although actin polarization is essential for bud growth, the polarisome components are not essential, perhaps because another formin, Bnr1p, is recruited to the septin ring and can nucleate polarized actin cables from there.

Polarized secretion

The type V myosin Myo2p, with associated light chains Mlc1p and Cmd1p (calmodulin), transports secretory vesicles containing the Rab-family GTPase Sec4p to the polarized patch. The SNARE-binding protein Sec1p is also polarized and essential for vesicle fusion with the plasma membrane. Myo2p also transports organelles and microtubules along actin cables, while the related Myo4p transports mRNA-protein complexes, generating mother-bud differences in protein translation.

Exocyst

The exocyst is a multiprotein complex that tethers secretory vesicles to the plasma membrane prior to fusion.

Cell wall

Glucan polymers make up a large portion of the cell wall, and the glucan synthases Fks1p and Gcs2p extrude the polymers across the plasma membrane at sites of cell growth. A cell wall protein (Cwh43p) and a putative sensor of cell wall stress (Slg1p) are also polarized, as is the Rho1p GEF Rom2p. Rho1p is a multifunctional GTPase that activates glucan synthase as well as the protein kinase C Pkc1p.

MAPK signaling

Cell wall stress activates the 'cell integrity' MAPK signaling cascade, several members of which are found in the polarized patch (Rho1p, Pkc1p, Mkk1p, Mkk2p and the MAPK Slt2p). This pathway activates transcription of cell-wall-related genes and contributes to halting of the cell cycle under conditions of stress, through the morphogenesis checkpoint.

RAM

A recently identified signaling pathway termed the RAM (regulation of Ace2p activity and cellular morphogenesis) contains interacting components required for optimal polarization as well as asymmetric mother/daughter gene expression. During bud growth these proteins are localized to the polarized patch, but some components relocate to the daughter cell nucleus in the bud following mitosis.

A hierarchical model for cell polarization

The transition of a yeast cell from an unpolarized state (where only the bud

site selection proteins are spatially restricted) to the polarized state depicted in the poster involves the near-simultaneous polarization of all of the structures discussed above. Examination of whether specific proteins or structures can become polarized in the absence of others has led to a hierarchical model for cell polarization. In response to a cell cycle cue, Cdc42p together with a subset of polarized patch proteins clusters into a patch at a location usually designated by the bud site selection landmarks. These proteins promote the independent assembly of the septin ring and the actin cables (and possibly also the actin patches). The septins then recruit a host of proteins to the ring, and the cables deliver more cargo, such as proteins and organelles, to the patch. Although this model accounts for most known aspects of polarization, there is probably a reinforcing cross-talk among these structures once they are polarized. For instance, the recruitment of the formin Bnr1p to the septin ring might reinforce polarized actin cable assembly, and polarized patch factors including Rho1p, Bud6p, and even Cdc42p itself might be delivered to the patch on secretory vesicles traveling along actin cables.

Recommended reading

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Go ahead, break my symmetry!

Kendall J. Blumer and John A. Cooper

Work in this issue advances our understanding of how the small G protein Cdc42 functions to polarize budding yeast. Remarkably, Cdc42 can polarize in the absence of upstream cues or positive feedback from the cytoskeleton. Polarization requires the scaffold protein Bem1 and cycling of Cdc42 between its GTP- and GDP-bound states.

Polarity is a fundamental feature of almost all cells, and how cells break symmetry to achieve polarity is an important and topical question. The budding yeast *Saccharomyces cerevisiae* is a good experimental system in this regard because bud formation represents a simple case of polarity. Indeed, much progress has been made. First, we know that the cell specifies the location of the bud formation site, and some genes/proteins that direct site selection have been defined. These bud-site selection proteins appear at the place on the membrane where the bud will form, directed by polarized structures from the previous cell division. Second, a signalling network centred around the Rho-type GTPase Cdc42 polarizes to this site. Finally, the cytoskeleton (including actin and septins) assembles at the site. We know that Cdc42 directs polarized assembly of the cytoskeleton and that Bud proteins direct the positioning of Cdc42 (refs 1, 2). Now, work from Lew and colleagues on page 1062 furthers our understanding of how polarization of Cdc42 occurs[3].

One interesting question concerns to what extent positive feedback loops or cooperative protein interactions contribute to the polarization of Cdc42; for example, does the polarized actin cytoskeleton provide positive feedback to reinforce polarization of Cdc42? In particular, actin cables deliver secretory vesicles to the site of bud formation and growth, causing polarized secretion and growth. Perhaps Cdc42 is delivered to the site by these vesicles, a hypothesis supported by the fact that Cdc42 is directly associated with membranes through a lipid modification.

In terms of cooperativity, can Cdc42 alone self-assemble into a structure at the bud site? Indeed, yeast cells lacking Bud proteins still form a bud. Furthermore, only one bud forms, and that bud is both associated with and dependent on polarization of Cdc42 at that location. So, one might imagine that Cdc42 has a strong intrinsic tendency to self-assemble and that Bud proteins merely need to nudge the process at the desired location.

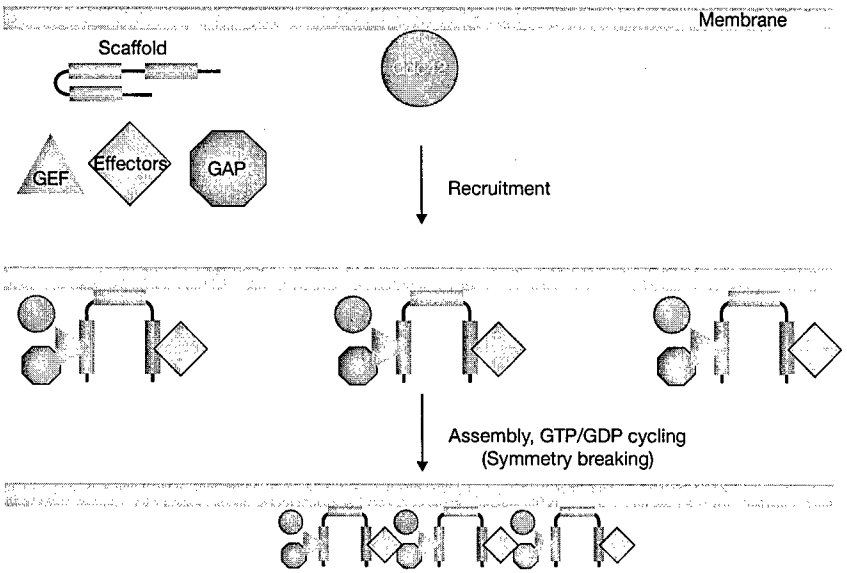

Figure 1 Symmetry breaking by Cdc42. In the absence of upstream polarity cues, Cdc42 associates with assorted proteins, including a scaffold, a GEF, a GTPase-activating protein (GAP) and an effector, to form a multisubunit complex. Next, the complex assembles into a polymer, a process driven by GTP/GDP cycling of Cdc42. One polymer forms, and its location establishes the new bud site.

Self-assembly is well understood and accepted for the cytoskeleton, where protein subunits such as actin spontaneously form filaments that possess polarity. Actin-binding proteins associate with the filaments and with each other to create larger polarized structures. In addition, spontaneous fluctuations of position can be sufficient to break symmetry in a model system containing actin filaments[4].

In signalling, assembly is based largely on the concept of scaffolding, where one protein binds to several other proteins. If the scaffold or any of its binding proteins can interact with itself or with other proteins then one can readily envision the formation of a polymer of multicomponent protein complexes. Polymerization may be cooperative and self-propagating, so that only one polymer forms at a given space and time. So, does such a phenomenon exist for Cdc42 in yeast? The answer seems to be yes. Lew and colleagues asked whether Cdc42 could polarize to a single location at the plasma membrane in the absence of upstream cues (Bud genes/proteins) and in the absence of potential positive feedback from the actin cytoskeleton[3]. First, they deleted two genes involved in bud-site selection, producing a cell with no evidence of an upstream cue. Next, they treated the cells with an actin toxin under conditions that remove all evidence of structures based on filamentous actin (notably, the cables that mediate polarized secretion) and then asked if Cdc42 was polarized to one site on the membrane: it was.

At this point, one might imagine that Cdc42 polarizes entirely on its own through cooperative self-assembly. That seems to be a stretch: the scaffold notion is more likely. Indeed, Cdc42 is known to interact with many other proteins. On the other hand, almost all of the Cdc42 interactors are considered to be either upstream cues, such as Bud proteins, or downstream effectors that induce actin and septin assembly. Lew and colleagues found that the Bem1 protein seems to function as a

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scaffold: first, Bem1 was found to be necessary for self-polarization of Cdc42; second, mutations changing single amino acids in any one of several domains of Bem1 caused loss of Cdc42 polarization, similarly to the complete absence of Bem1.

G proteins seem to function in two ways — switching or cycling. In the switch model, the protein has some activity when in a particular nucleotide state (usually when bound to GTP). The strength of activity is proportional to the amount of GTP-containing protein; oncogenic Ras seems to function in this way. In the cycling model, activity is produced only through transitioning between states. The G protein EF-Tu regulates translation elongation by a cycling mechanism. Cycling is also used by cytoskeletal motors, which hydrolyze ATP to produce a conformational change that is converted into molecular motion. In mammalian cells, cycling of Cdc42 has been implicated in oncogenic transformation[5].

Lew and colleagues asked whether the switch or the cycling model accounts for the observed polarization of Cdc42 by expressing a GTP-locked Cdc42 mutant. In addition, they used different promoters to control expression levels. When expressed at levels comparable with that of the endogenous protein, polarization of Cdc42 was not observed. High expression levels did result in polarization. This is in agreement with another recent study showing that polarization of Cdc42 is dependent on the actin cytoskeleton[6], suggesting that positive feedback through the actin cytoskeleton and secretion may promote Cdc42 polarization along the lines discussed above. Bi and colleagues recently proposed such a model to account for poor polarization of Cdc42 in mutants defective for secretion[7]. Together, these studies question the extent to which Cdc42 polarization is caused by Bud proteins, filamentous actin, polarized secretion and scaffolding proteins such as Bem1 in wild-type cells. Elucidating this complex issue will most probably require quantitative methods to follow Cdc42 polarization in cells in response to small changes in the activity of each proposed cause of polarization.

The results from Lew and colleagues support a cycling, rather than switch, mode of action for Cdc42 during self-polarization. Other recent studies also indicate that Cdc42 cycles between the two nucleotide-bound states to induce bud formation[8] and septin assembly[9,10]. How might this be explained? Perhaps Cdc42 uses the energy of GTP hydrolysis to execute a conformational change in itself and associated proteins, allowing new interactions that are necessary for polymerization of the Cdc42 structure (the 'Cdc42-some'). Lew and colleagues suggest another interesting possibility. Cells contain more kinds of guanine nucleotide-exchange factors (GEFs) than they do G proteins. A common explanation for this difference is that individual GEFs are localized or activated by different upstream signals, thus placing the G protein into its active GTP state at different times and places. Instead, one might now imagine that a specific GEF mediates the assembly of Cdc42-GDP with other proteins into a macromolecular complex. In this model, GTP exchanges for GDP and the complex cooperatively self-assembles into a polymer (Fig. 1).

In summary, the data of Lew and colleagues, in combination with other recent studies, makes important advances in our understanding of how small G proteins function in budding yeast. Novel ideas for the molecular action of Cdc42 are provided by the findings that it can polarize without positional cues or actin filaments, that scaffold proteins are involved, and that GTPase cycling is required. G proteins are essential for mediating signals that direct cell polarization and macromolecular assembly in all eukaryotes, so the lessons learned in yeast may help illuminate the path of research in other systems. □

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