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13. ABSTRACT (Maximum 200 Words)

Cdc42p and other proteins polarize to a cap at the presumptive bud site and the tip of the bud. The observation that Cdc42p polarizes in the complete absence of F-actin has been confirmed repeatedly. These studies used LatA as a method to completely depolymerize actin, and suggested that Cdc42p polarization is actin-independent. Polarized secretion and endocytic uptake require F-actin cables and patches, respectively. When LatA is applied to yeast cells neither actin patches nor cables are detectable, disrupting all F-actin-dependent processes. Thus, Cdc42p polarization occurs by a non-secretory pathway.

Is the polarized cap static? Current models of Cdc42p function propose that the cap is an organizing center for polarity. Since it is associated with the plasma membrane, the cap may be static, acting as a polymerization site for actin cables by formins, assembly of septins, signal transduction, etc. Our data indicate that the cap is, in reality, dynamic. We show that the loss of actin cables, but not actin patches, leads to Cdc42p delocalization. We propose that the initial polarization of Cdc42p is an actin-independent process; continued polarization is the result of two competing processes: endocytic removal of the plasma membrane, and a deposition process, dependent on polarized secretion.

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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 tpm2*Δ 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 *tpm2*Δ 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 tpm2*Δ 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 *tpm2*Δ 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 *rdi1*Δ cells with Lat-B. After 2 hr of incubation, the *rdi1*Δ 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.**

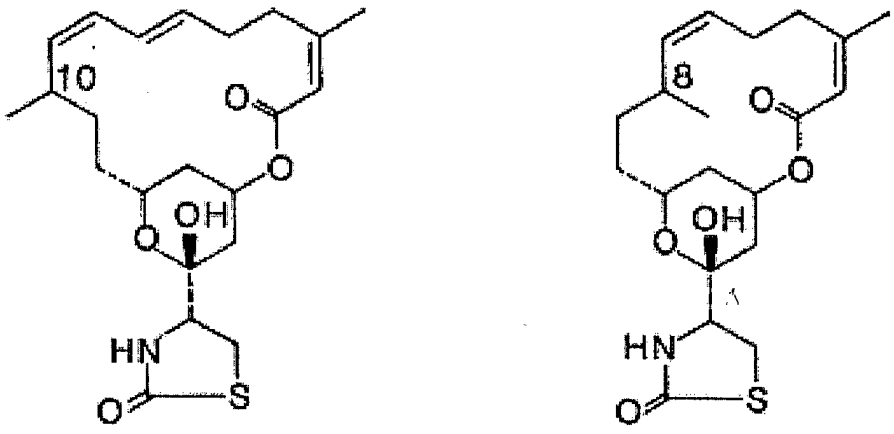


Figure1.1 **Structure of the latrunculins.**

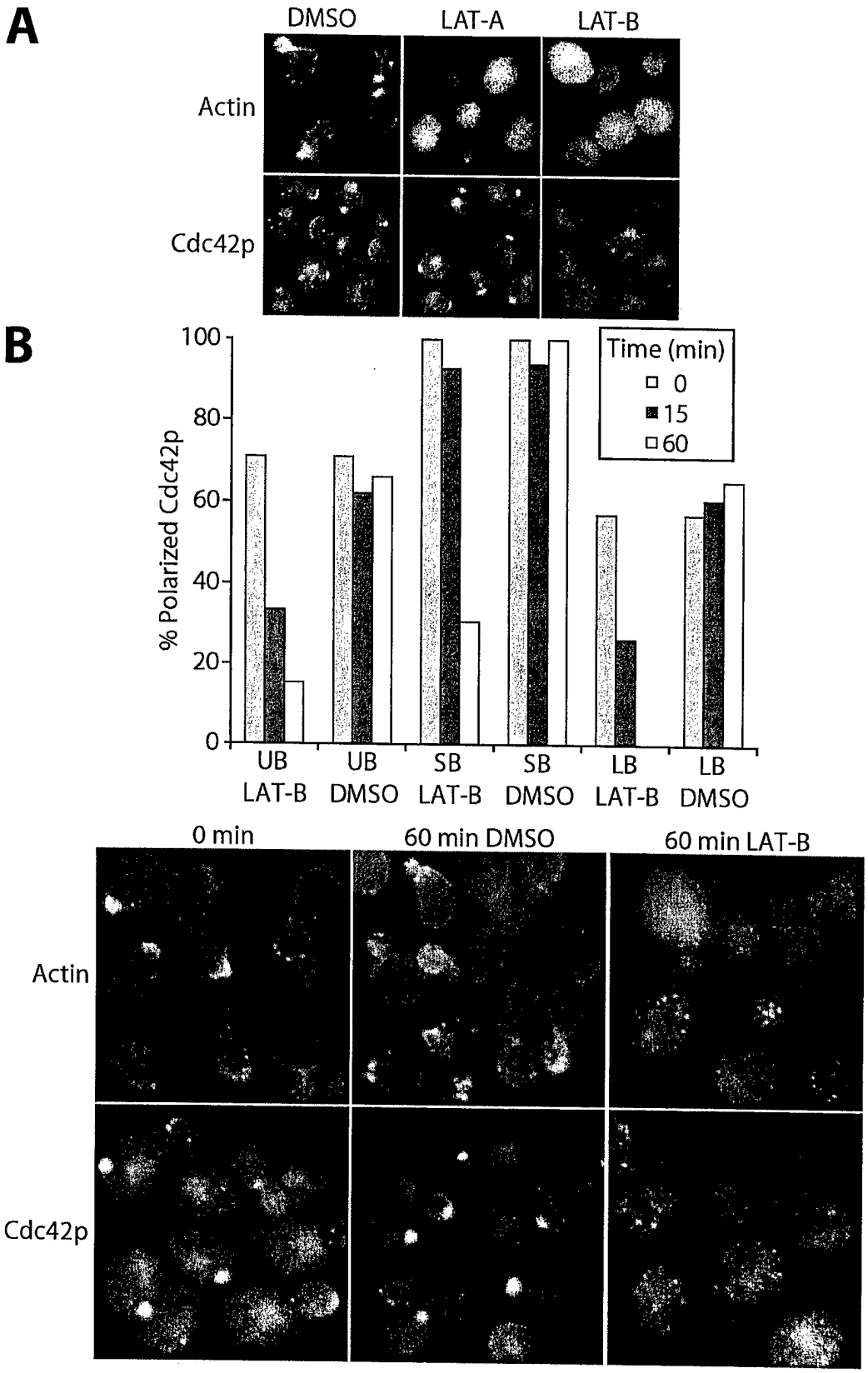
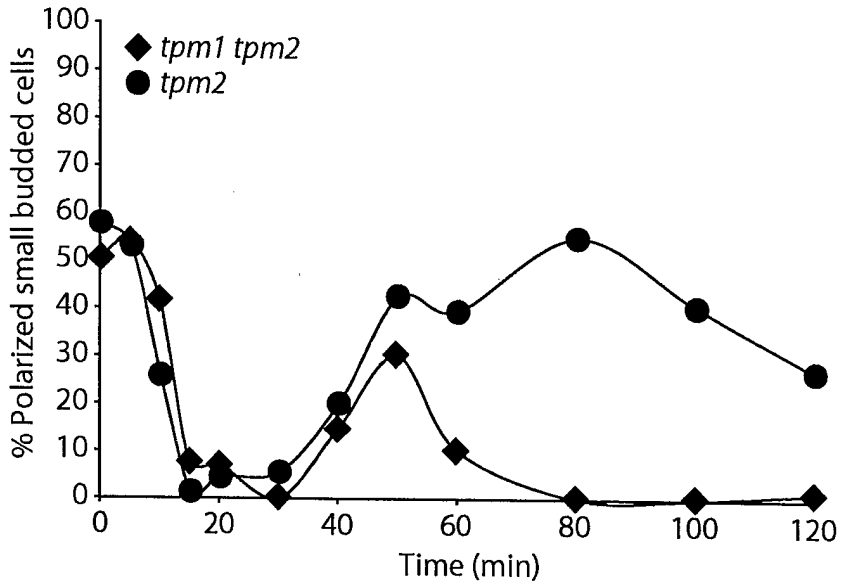


Figure1.2 **Latrunculin-B treatment causes Cdc42p to depolarize. A.** Wild type (DLY5) cells treated with 100 M Latrunculin -A, 200 M Latrunculin B or DMSO as control for 2 hr. **B.** Timecourse of loss of Cdc42p polarization. Wild type (DLY5) cells were treated with Latrunculin-B for 15 and 60 min. 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



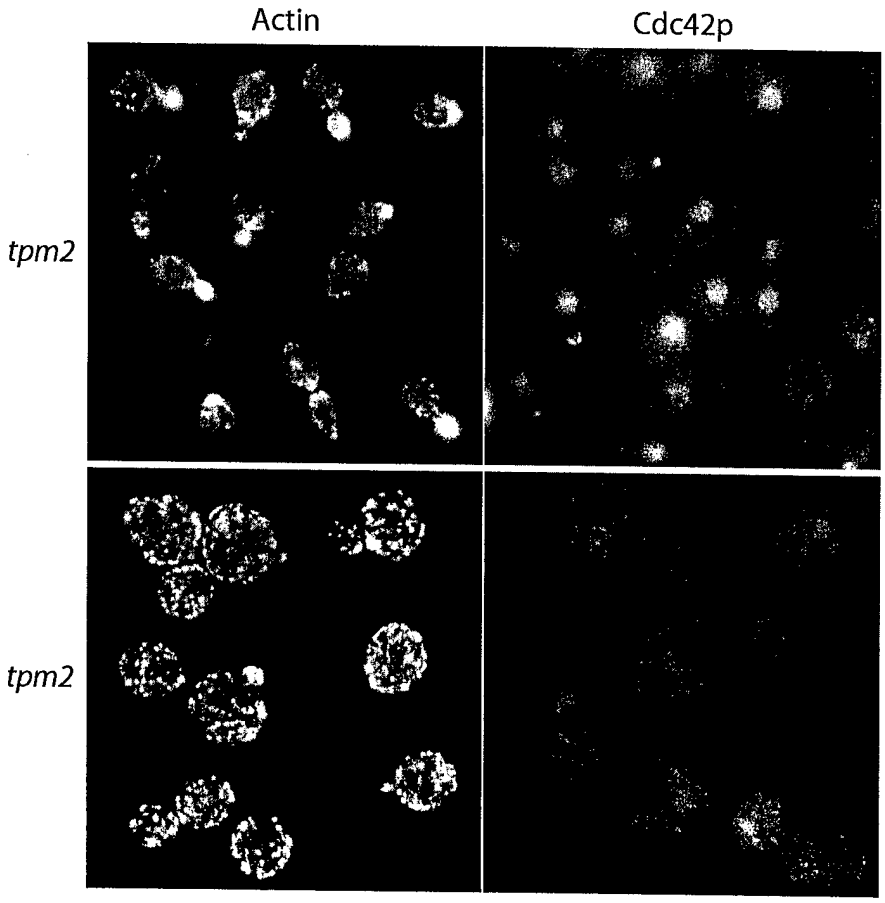


Figure1.3 **Loss of cables results in Cdc42p depolarization.** *tpm1-2 tpm2* cells and *tpm2* cells as control were grown at 24 °C until mid-log phase, then shifted to 34.5°C for the times indicated. Graph, percent small -budded cells with polarized Cdc42p vs. time of incubation at 34.5°C. In both cases, 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.

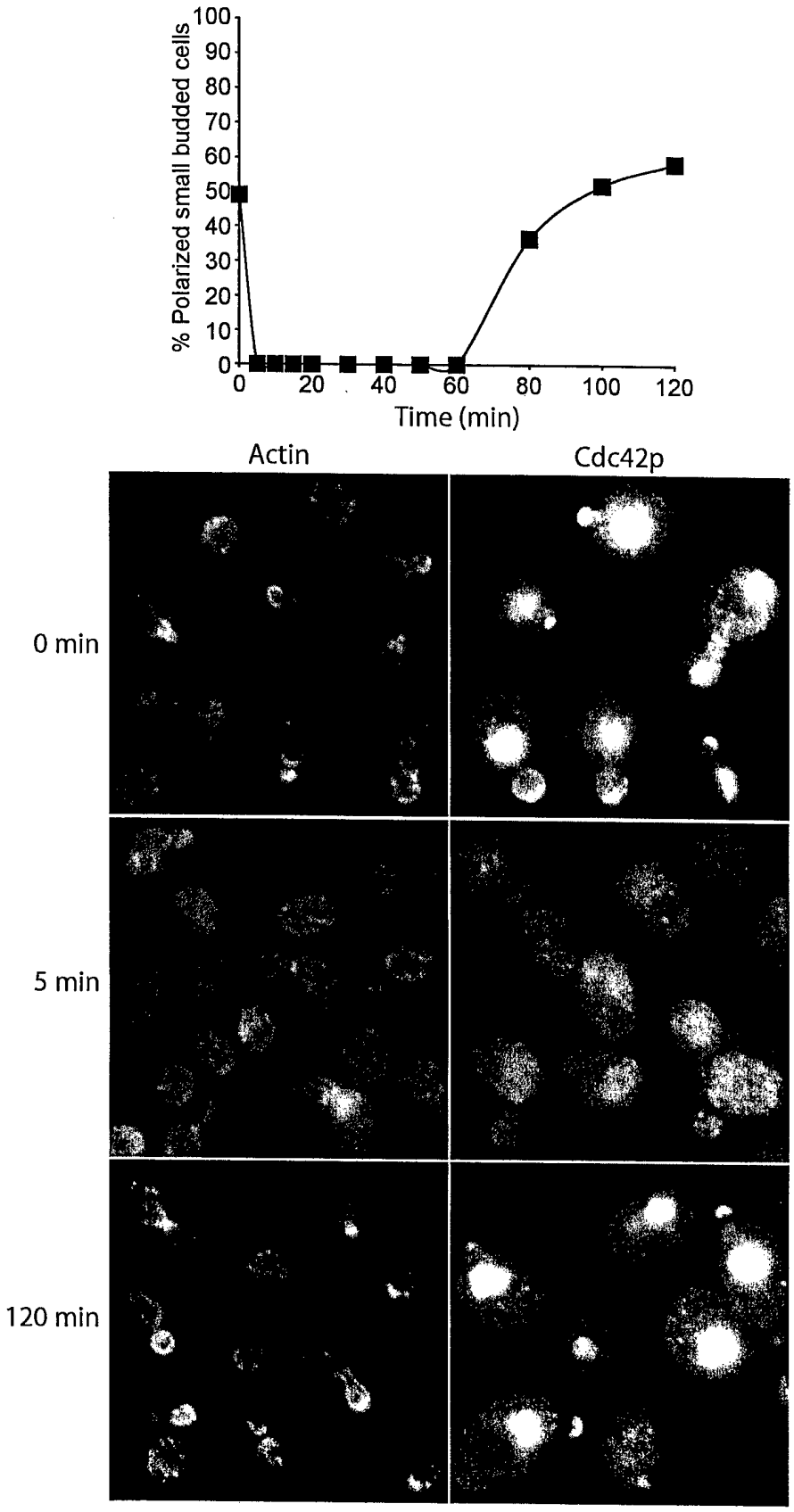


Figure1.4 **Osmotic shock results in Cdc42p depolarization.** Wild type cells (DLY5) were grown to mid-log phase previous to the addition of 0.4 N NaCl. Graph, percentage of small-budded cells that have polarized Cdc42p vs. time of incubation in 0.4 N NaCl. Below, micrographs of cells after 0, 5, and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

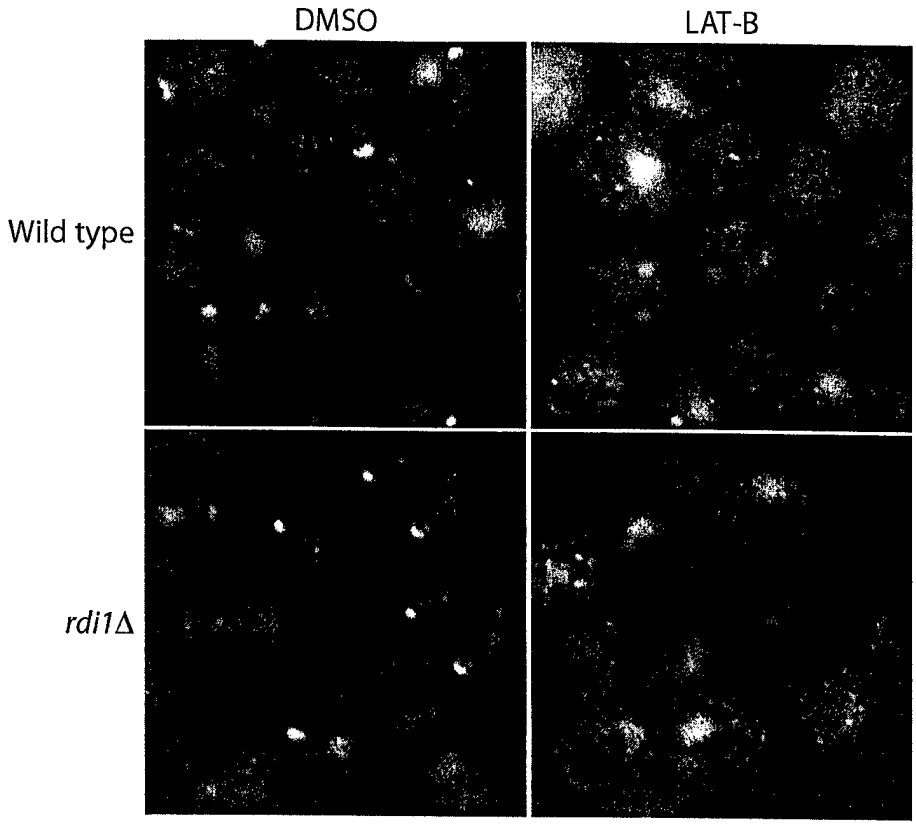


Figure1.5 **Rdi1p is not required for the depolarization of Cdc42p caused by Latrunculin-B.** Wild type cells (DLY5) or *rdi1* cells (MOSY20) were incubate d in 200 M Latrunculin B, or DMSO as control, for 2 hr. Fluorescence microscopy as in Figure1 .1.

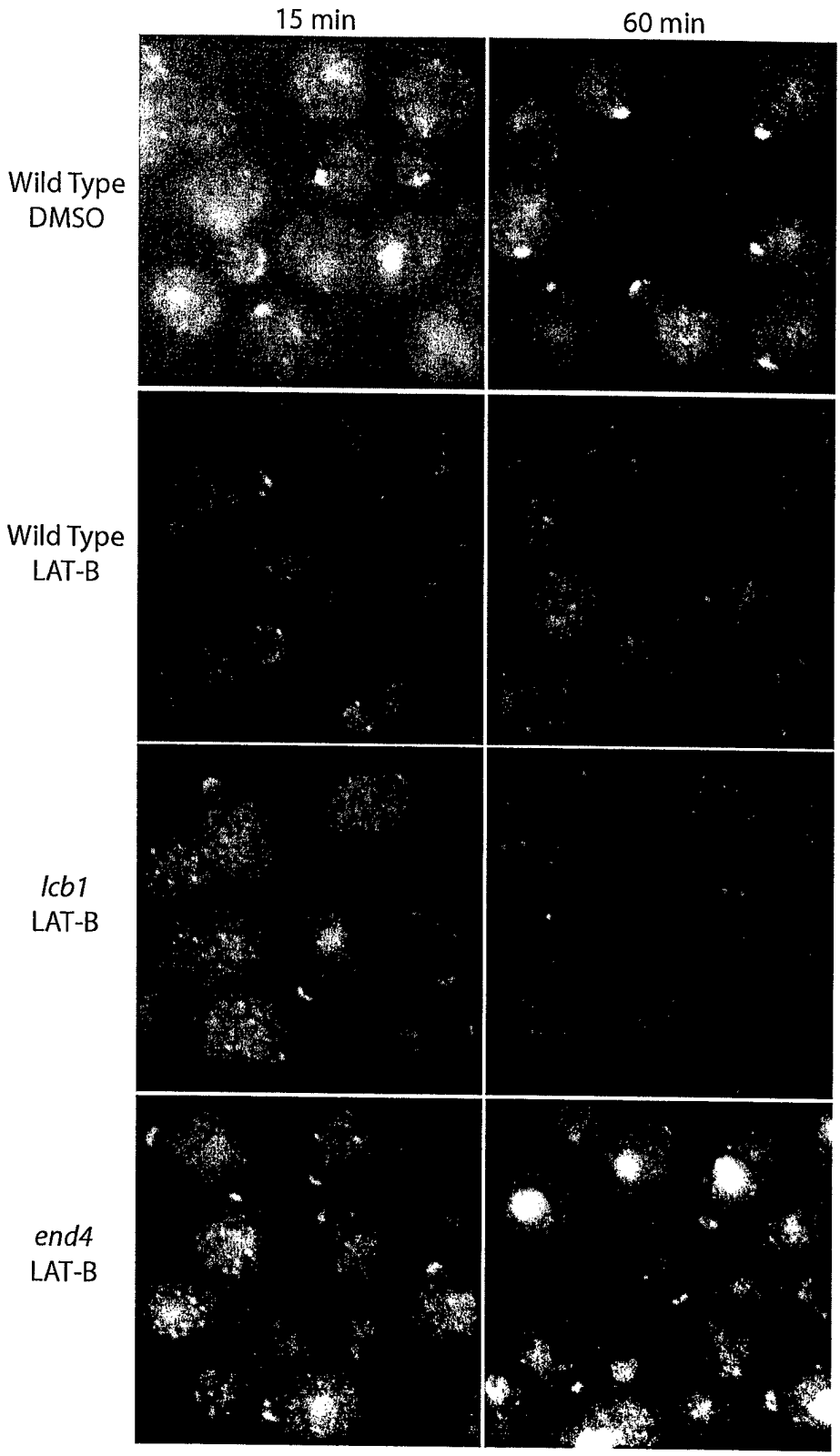


Figure1.6 **Endocytosis mutants do not depolarize Cdc42p in response to Latrunculin B treatment.** Wild type (RH1800), *lcb1-100* (RH3809) and *end4-1* (RVS152) cells were grown to mid-log phase at 24°C, shifted to 37°C for 1 hr and then treated with 200 M Latrunculin B or DMSO for 15 min and 60 min. *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.

Scaffold-mediated symmetry breaking by Cdc42p

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Running Title: Cdc42p symmetry breaking

Key words: *CDC42, BEM1*, Polarity, Cytoskeleton, Actin, *RSR1*

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Abstract

Cell polarization is usually directed towards specific spatial cues, but cells lacking those cues are capable of developing polarity by symmetry breaking. The conserved Rho-family GTPase Cdc42p becomes concentrated at a cortical site during polarization and is essential for cytoskeletal polarization. We found that without spatial cues, yeast Cdc42p was still polarized to a random cortical site even in the absence of polymerized actin or tubulin. This symmetry breaking behavior required the scaffold protein Bem1p. 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.

Introduction

Cell polarization, involving the asymmetric organization of cytoskeletal fibers, organelles, and other cell components, is crucial for a multitude of cell functions.[1] Polarization generally occurs along a single well-defined axis that is frequently determined by environmental cues such as chemoattractant gradients or cell-cell contacts. However, polarization can also occur spontaneously in the apparent absence of such cues, through a process called symmetry breaking.[2,3] Such polarization is as asymmetric and unidirectional as that which occurs in the presence of the normal spatial cue, suggesting that spatial signals serve primarily to bias a cell-intrinsic symmetry-breaking process in the desired direction.[3-6] The molecular basis for cellular symmetry-breaking is therefore of considerable interest.

The Rho-family GTPase Cdc42p is required for polarity establishment in many cell types[1,7]. In both yeast and animal cells, Cdc42p itself is concentrated beneath the plasma membrane at the site(s) of polarization[8-11], and clustering of GTP-loaded Cdc42p at a cortical site is sufficient to direct cytoskeletal polarization towards that site[12]. Previous studies have shown that Cdc42p acts at an early step in polarization towards a spatial cue, indicating that it may serve to bias the symmetry-breaking process to the specified site or that it may be a key element of the symmetry-breaking process itself.[7,10,11,13-15] Consistent with the latter possibility, overexpression of Cdc42p in yeast cells that normally polarize towards specific spatial cues causes them to polarize in a random direction[16].

Yeast cells are born with positional landmarks that mark the poles of the cell and guide subsequent polarization and bud emergence to those sites.[17] The landmarks communicate with Cdc42p through the Ras-family GTPase Rsr1p[16,18,19], which in its GTP-bound form interacts

directly with the Cdc42p exchange factor (GEF) Cdc24p[20,21]. In the absence of Rsr1p, yeast cells no longer recognize the appropriate site, but they do polarize Cdc42p to a random location, form buds, and proliferate at a wild-type rate[16,19]. Thus, like other cells, yeast appear to have the capacity for symmetry-breaking polarization.

Recent studies have highlighted the role of the cytoskeleton in symmetry-breaking behavior.[6,22] However, we now show that yeast cells lacking Rsr1p are able to polarize Cdc42p even in the absence of microtubules or F-actin, indicating that the symmetry breaking capacity of yeast cells is independent of these cytoskeletal elements. Polarization of Cdc42p in cells lacking Rsr1p required Bem1p, a scaffold protein that interacts directly with Cdc24p and GTP-Cdc42p as well as effectors of Cdc42p and other scaffold proteins. Moreover, we show that polarization in yeast is dependent upon GTP hydrolysis by Cdc42p. In aggregate, our findings suggest that symmetry breaking in yeast occurs through the assembly of a cortical patch of polarity proteins organized by GTP-cycling Cdc42p and Bem1p, which can occur independent of cytoskeletal components.

Results

Landmark-independent Cdc42p polarization does not require microtubules or F-actin

To assess the cytoskeletal requirements for symmetry breaking, we examined Cdc42p polarization in an *rsr1*Δ *bud8*Δ strain. 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[23]. This residual ability was eliminated upon loss of the distal pole landmark Bud8p, so that *rsr1*Δ *bud8*Δ daughter cells polarized and formed buds randomly with regard to the poles (Fig. 1A). We isolated unbudded wild-type and *rsr1*Δ *bud8*Δ daughter cells by centrifugal

elutriation and incubated them 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). We found that polarization of Cdc42p in *rsr1*Δ *bud8*Δ cells did not require microtubules or F-actin (Fig. 1B,C). Thus, yeast cells possess a landmark-independent and cytoskeleton-independent ability to polarize Cdc42p.

Landmark-independent Cdc42p polarization requires the scaffold protein Bem1p

The finding that Cdc42p was able to polarize in the simultaneous absence of effective landmarks, microtubules, and microfilaments suggested that Cdc42p and associated polarity factors possess an intrinsic capacity for symmetry-breaking behavior. We reasoned that proteins important for symmetry-breaking would be essential for cell viability in strains lacking effective landmarks, but might be dispensable in strains that employed landmarks to guide polarization. To search for such proteins, we tested whether non-lethal mutations affecting proteins that colocalize with Cdc42p and interact with Cdc42p (physically or genetically) would become lethal in strains lacking Rsr1p. We found that *rsr1*Δ *bem1*Δ double mutants were inviable (Fig. 2A), whereas the other double mutants tested were not (Table I). The inviability of *rsr1*Δ *bem1*Δ double mutants was confirmed by crosses in three distinct strain backgrounds (Table I), and was not specific to spore germination because *rsr1*Δ *bem1*Δ mutants kept alive by a *BEM1* plasmid were unable to proliferate on medium selecting for plasmid loss (Fig. 2B). Synthetic lethality was also observed for *bem1*Δ strains lacking the Rsr1p GEF Bud5p or GAP Bud2p (which are also incapable of recognizing spatial landmarks [19,24,25]) (Fig. 2A), but not for strains lacking individual spatial landmarks (where the remaining landmarks are able to trigger polarization through Rsr1p[7,17]) (Table I). *BEM1* was isolated in genetic screens designed to identify components involved in bud emergence or in mating projection formation[25-27], and *bem1*Δ

mutants display significant defects in cytoskeletal polarity and fail to maintain Cdc24p at the polarization site [26,28], indicating that Bem1p is important for polarity even in cells that contain Rsr1p. These results indicate that Bem1p becomes essential in cells that cannot utilize the spatial cues that normally guide polarization.

To evaluate the basis for the observed synthetic lethality, we used error-prone PCR mutagenesis to generate a temperature-sensitive *bem1* allele, *bem1-8*. As shown in Fig. 2B, *bem1-8 rsr1*Δ mutants were viable at 30°C but inviable at 39°C. Unbudded *bem1-8 rsr1*Δ daughter cells were isolated by centrifugal elutriation and incubated at 39°C to assess how they lost viability. Like control *rsr1*Δ cells, the double mutants entered the cell cycle and formed spindles as detected by anti-tubulin staining (Fig. 2C). However, unlike the *BEM1 rsr1*Δ control, the *bem1-8 rsr1*Δ cells did not polarize Cdc42p or form buds at 39°C (Fig. 2C). Thus, cells require either Bem1p or Rsr1p function in order to polarize Cdc42p.

Requirements for Bem1p localization and function

Bem1p is a scaffold protein that contains several domains mediating interactions with other polarity establishment proteins[20,21,28-34] and phosphoinositides[35-37] (see Fig. 3A and legend). In addition, the *Schizosaccharomyces pombe* homolog of Bem1p is capable of assuming a "closed" conformation through an intramolecular interaction (Fig. 3A).[38] To assess the roles of the various domains of Bem1p in symmetry-breaking, we generated a panel of point mutations impairing interactions mediated by the individual domains (Fig. 3A). Plasmids expressing wild-type or mutant Bem1p at expression levels comparable to wild-type (Fig. 3B) were tested for their ability to rescue the viability of *bem1*Δ *rsr1*Δ mutants (Fig. 3C). Bem1p^{P208L}, Bem1p^{R369A}, and Bem1p^{K482A} were inactive in this assay (Fig. 3C), suggesting that interactions mediated by the SH3-2 domain, the PX domain, and the PB1 domain are all essential for Bem1p function in

symmetry breaking. In contrast, Bem1p^{P355A} appeared to be even more potent at rescuing *bem1*Δ *rsr1*Δ cells than wild-type Bem1p (Fig. 3C), consistent with the autoinhibitory role proposed for the intramolecular interaction in *S. pombe*.[38]

Bem1p, like Cdc42p, is localized at the presumptive bud site and the tips of small- and medium-sized buds.[39,40] 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. 4A). These results suggest that interactions mediated by the SH3-2 domain and the PB1 domain are important for Bem1p localization, whereas the 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. 4B), indicating that the wild-type Bem1p could promote the localization of this mutant.

Role of GTP hydrolysis in Cdc42p polarization

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, whose binding to many effectors disrupts autoinhibitory intramolecular interactions to unleash effector activity.[1] However, some Cdc42p actions, including septin ring assembly in yeast and oncogenic transformation in mammalian cells, appear to require GTP hydrolysis by Cdc42p, suggesting that GTP cycling of Cdc42p (rather than just GTP-loading) is important.[41,42] The finding that Cdc42p can undergo symmetry-breaking polarization independent of the cytoskeleton prompted us to ask whether establishment of a polarization site is carried out by GTP-Cdc42p or whether it requires cycling of Cdc42p between GDP- and GTP-bound forms.

The question of whether a GTP-locked Cdc42p (i.e. 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.[43] To circumvent this problem, we generated vectors expressing lower levels of Cdc42p^{Q61L} from a weak galactose-inducible promoter (*EG43*).[41] On galactose medium, cells containing *EG43-CDC42*^{Q61L} expressed levels of Cdc42p^{Q61L} about 50% lower than those of total endogenous Cdc42p in wild-type cells (Fig. 5A), which is still likely to be higher than endogenous GTP-Cdc42p levels. At these levels, wild-type Cdc42p was fully functional and Cdc42p^{Q61L} no longer caused cell death (Fig. 5B,D, and Fig. 6C), although it did cause defects in septin organization as previously reported.[41]

To ask whether non-lethal levels of Cdc42p^{Q61L} could substitute for wild-type Cdc42p, we introduced *EG43-CDC42*^{Q61L} and *EG43-CDC42* into *cdc42-17* cells (*cdc42-17* is a very tight temperature-sensitive allele). On galactose medium, wild-type Cdc42p rescued the temperature sensitivity of *cdc42-17* but Cdc42p^{Q61L} did not (Fig. 5B). Following a shift to restrictive temperature, no polarization of Cdc42p^{Q61L} (or Cdc42-17p) was observed (Fig. 5C). Thus, Cdc42p function requires its ability to hydrolyse GTP, and GTP-locked Cdc42p cannot polarize in the absence of functional endogenous Cdc42p.

The requirement for GTP hydrolysis could reflect a need to generate a *localized* pool of GTP-Cdc42p, perhaps involving GTP hydrolysis by Cdc42p away from the polarization site. In that case, delocalized Cdc42p^{Q61L} would be unable to polarize or to cause polarization of other factors. Moreover, Cdc42p^{Q61L} would interfere with the effective polarization of endogenous Cdc42p by raising the GTP-Cdc42p concentration all over the cell cortex. Alternatively, GTP hydrolysis by Cdc42p might be a required step in the assembly of a polarization site. For

instance, it could be that binding of GTP-Cdc42p to polarization effectors drives them to adopt an "open" conformation, but that subsequent GTP hydrolysis is required for the incorporation of the effectors into functional polarization complexes.

To begin to distinguish between these possibilities, we expressed *EG43-CDC42*^{Q61L} in a *cdc42-1* mutant. The *cdc42-1* strain contains mutations in the promoter and coding region of *CDC42* that result in greatly reduced expression of Cdc42-1p even at permissive temperature[44](Fig. 5A). Nevertheless, *cdc42-1 EG43-CDC42*^{Q61L} cells proliferated on galactose medium at permissive temperature (Fig. 5D), indicating that the small amount of Cdc42-1p was able to function in the presence of larger amounts of Cdc42p^{Q61L}. As shown previously[44,45], anti-Cdc42p staining was not sufficiently sensitive to detect Cdc42-1p, but we did detect clear Cdc42p polarization in *cdc42-1 EG43-CDC42*^{Q61L} cells grown at permissive temperature on galactose medium (Fig. 5E), implying that Cdc42p^{Q61L} was able to polarize in these cells. Thus, Cdc42p^{Q61L} is able to polarize to a site established by Cdc42-1p, but is unable to establish a site of polarization on its own.

Polarization of overexpressed GTP-locked Cdc42p

The findings described above indicate that GTP hydrolysis by Cdc42p is essential for its ability to establish a polarization site. In apparent contradiction to this conclusion, recent studies reported that overexpression of Cdc42p^{Q61L} from the *GAL1* promoter induced "spontaneous" polarization in G1-arrested cells, which lack the cell cycle signal that normally triggers polarization.[22,40]In agreement with those findings, we also observed polarization of overexpressed Cdc42p^{Q61L} (Fig. 6A) and F-actin (data not shown) in G1-arrested cells. In similar experiments, we observed polarization of overexpressed Cdc42p^{Q61L} in *cdc24-4* cells in which the GEF Cdc24p was inactivated (Fig. 6B). As described previously[22,40,46], polarization

sometimes occurred towards more than one site (Fig. 6B) and was accompanied by cell lysis (data not shown). Thus, overexpressed Cdc42p^{Q61L} is able to cause 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. 6C) and led to accumulation of Cdc42p^{Q61L} to levels much higher than those of the total endogenous Cdc42p (Fig. 6D), raising 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. 6D) in G1-arrested cells (Fig. 6A) or *cdc24-4* cells (Fig. 6B), 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.

Discussion

Perhaps the most obvious manifestation of polarized cell organization is the oriented arrangement of cytoskeletal fibers. Moreover, elegant recent studies in cell-free systems have shown that the properties of cytoskeletal polymers are well-suited to promote symmetry-breaking polarization.[6] Together, these features have encouraged the view that cytoskeletal organization is intrinsically biased towards a polarized morphology, imparting cells with the capacity for symmetry-breaking.[2] Our findings suggest that cells contain another network, centered around the GTPase Cdc42p, that is capable of symmetry-breaking behavior in the absence of polymerized actin or tubulin.

Polarization of Cdc42p involves its concentration at a small patch of the cell cortex. A large number of proteins co-localize together with Cdc42p at the time of polarization, including

scaffold and effector proteins that bind to GTP-Cdc42p and display additional interactions amongst themselves.[47,48] Given our findings that Cdc42p polarization occurred efficiently in cells lacking cytoskeletal elements and effective landmarks, it seems likely that polarity establishment in yeast consists of the assembly of a patch of multiply-interacting polarity components at a cortical site. A speculative model for how such a patch might be assembled in the absence of effective landmarks is presented in Fig. 7. We suggest that a local increase in GTP-Cdc42p concentration, caused by stochastic fluctuations, results in the "opening" of local autoinhibited scaffold and effector proteins, including (importantly) Bem1p. GTP hydrolysis by Cdc42p then promotes efficient assembly of these proteins into scaffolded complexes, perhaps by accelerating release of Cdc42p to allow other interactions and/or by triggering further conformational changes in the effectors/scaffolds. 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. 7).

This model accommodates our finding that GTP hydrolysis by Cdc42p is required for establishing a polarization site. However, the requirement for GTP hydrolysis was not limited to symmetry-breaking, as GTP-locked Cdc42p was not functional even in cells containing landmarks and Rsr1p. In principle, the requirement for GTP hydrolysis could reflect a need to reduce the GTP-Cdc42p concentration at sites other than the polarization site, in order to generate or maintain a sufficiently steep spatial gradient of GTP-Cdc42p. However, this hypothesis seems unlikely to account for our observations, because the GTP-locked Cdc42p was itself polarized in cells containing functional endogenous Cdc42p, and because GTP-locked

Cdc42p did not disrupt polarization triggered by the much less abundant endogenous Cdc42-1p at permissive temperature. Instead, we favor the hypothesis that GTP hydrolysis by Cdc42p constitutes an obligatory step in the assembly of a polarization site. Precedent for the action of a GTPase in this manner comes from the role of translation elongation factors in protein synthesis, where GTP hydrolysis by the elongation factor is an obligate step in the assembly of a polypeptide chain.[49] Once a polarization site is assembled, the high concentration of GTP-Cdc42p binding sites within the polarization patch would readily explain the observed concentration of GTP-locked Cdc42p at that site.

We found that in the absence of effective landmarks, the scaffold protein Bem1p was essential for Cdc42p polarization. Bem1p was essential in cells lacking Rsr1p, or the Rsr1p-GEF Bud5p, or the Rsr1p-GAP Bud2p, consistent with previous work indicating that Rsr1p function requires the ability to cycle between GTP- and GDP-bound states.[18] It is also worth noting that the same screen for conditional polarity mutants that first identified *CDC42*[50] also yielded a strain containing two mutations required in combination for the *cdc42*-like phenotype; mutations that were later found to reside in *BEM1* and *BUD5*.[25] What is the role of Bem1p in symmetry breaking polarization? Symmetry-breaking behavior is thought to require short-range positive feedback mechanisms whereby a small cluster of one or more key polarity factors (arising at a random location through stochastic fluctuations in component concentrations) can promote the recruitment of more such factors to the polarization site.[2] 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 that Bem1p can bring together the upstream GEF Cdc24p, GTP-Cdc42p, and downstream effectors of Cdc42p, and that

assembly of such complexes promotes feedback phosphorylation of Cdc24p by effector kinases.[33,40]

Among animals and fungi, Cdc42p is one of the most highly conserved of the Ras-superfamily GTPases (yeast and human Cdc42p are 80% identical, as compared with 66% identity between yeast and human Rho and 34% identity between yeast and human Ras).[48,51,52] Moreover, human Cdc42p can functionally replace yeast Cdc42p, indicating a high degree of evolutionary conservation.[52] As in yeast, Cdc42p has been implicated in polarity establishment in a variety of animal cells.[1,13,53] However, both the spatial cues for polarization and the cytoskeletal elements that respond to those cues vary from cell type to cell type (e.g. Cdc42p directs actin polarization in fibroblasts but microtubule polarization in astrocytes). Consistent with these system-specific features, different sets of Cdc42p effectors appear to be important for cytoskeletal polarization in different cell types.[10,11,15] What, then, is the conserved essence of Cdc42p function? We speculate that the symmetry breaking ability of Cdc42p is the key feature that has maintained the involvement of Cdc42p in cell polarization across more than a billion years of evolution.

Methods

Yeast strains, plasmids and PCR manipulations.

Standard media and methods were used for plasmid manipulations [54] and yeast genetic manipulations [55]. The yeast strains used are listed in Table II, plasmids are listed in Table III and oligonucleotides are listed in Table IV.

The crippled *GAL* promoter was described previously [41].

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 the synthetic lethality analysis, *bem1::KAN* was amplified by PCR using genomic DNA from DLY5631 and primers BEM1-480 and SWE1TERM and incorporated by transformation into the homozygous deletion strains as indicated in Table I. Alternatively, *bem1::URA3* was generated as described by Chenevert et al[27]. 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 amplifying by PCR the *rsr1::KAN* locus, using genomic DNA from DLY5631 as a substrate and oligos RSR1-163 and RSR1+1443 as primers, and transformation of DLY5 or DLY5077. Correct intergration 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 harboring the mutations described in Table I, 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 available upon request

For the 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[33], DLY4000 harbors a genomic insertion at the *BEM1* locus that expresses wild type *BEM1* with a C-terminal 12X *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 12X*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 the generation of temperature-sensitive alleles of *BEM1*, DLY5023 was transformed with the PCR product obtained from amplification of the *bem1::KAN* 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 rsr1::HIS* YEpLac181-*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 [56], using pDLB2226 as template and primers BEM1-480 and SWE1TERM. The PCR product was co-transformed into DLY5452 together with pDLB2226 previously digested with *Hin*DIII and *Sal*I and gel-purified to isolate the ~8 kb vector backbone. This linear backbone contains *BEM1p* and the 5' 300 bp of *BEM1* separated from the 12X*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 unto

dextrose medium containing 5-FOA to force the loss of pDLB1974 and select against null alleles of *BEM1*. Colonies were then replica-plated unto 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, harboring *bem1-8*, was isolated by plasmid rescue and re-transformed into DLY5452 to verify the phenotype.

The R316A, P355A and R369A alleles of *BEM1* were generated by site-directed mutagenesis using pDLB2226, the primers listed in Table IV and the QuickChange PCR kit (Stratagene, La Jolla, CA), according to 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.

The wild type *BEM1* and the point mutant alleles were transferred to YEpLac181 (high-copy *LEU2)* excising the *BEM1p-BEM1*-12X*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.[41]

The generation of the *cdc42::GAL1p-CDC42::LEU2* and *cdc42::GAL1p-CDC42*^{Q61L}*::LEU2* alleles was described previously.[57]

Fluorescence staining and Microscopy.

Cells were stained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich Corp., St. Louis, MO) to visualize DNA (Pringle, 1991), with 10 mg/ml Calcofluor (Sigma-Aldrich Corp.) to visualize chitin[58], or with rhodamine-phalloidin (Molecular Probes, Eugene, OR) to visualize F-actin [59]. To localize Cdc42p, cells were fixed for a total of 3 h in 3.6% formaldehyde as described [60]. Fixed cells were incubated with 0.5% SDS and processed for immunofluorescence as described [61]. Anti-Cdc42p antibody (used at 1:200 dilution) was generously provided by Patrick Brennwald (UNC-Chapel Hill). To visualize myc-tagged Bem1p, cells were fixed and treated as for Cdc42p immunofluorescence. Monoclonal anti-myc antibody (9E10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:200 dilution. Cy3-conjugated goat anti-mouse IgG and Cy3-conjugated donkey anti-goat IgG (purchased from Jackson ImmunoResearch, West Grove, PA) at 1:200 dilutions, were added sequentially after 1 hr incubations. Cells were examined using a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY). 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).

Preparation of protein extracts and western blotting.

Procedures for harvesting and lysis of yeast cells, SDS-PAGE and immunoblotting were as described [33]. Anti-myc monoclonal antibody (9E10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:1000 dilution. Anti-Cdc42p polyclonal antibody was used at a 1:500

dilution. Anti-Cdc11p polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) 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 hr intervals. Cell number was determined using a hemacytometer, and the samples were diluted to a final cell count of $2x10^4$ cells/ml. 5 µl (~100 cells) were then spotted unto 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 $2x10^6$ cells/ml. Subsequent 10-fold serial dilutions were performed, and 5 µl (~10^4, 10^3, 10^2 and 10 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.

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Figure legends

Figure 1. Landmark-independent Cdc42p polarization does not require polymerized actin or tubulin. A. Diploid wild type (DLY5077) and *rsr1*Δ *bud8*Δ (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 based on the position of the birth scar, indicated with *). 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 (DLY5077) and *rsr1*Δ *bud8*Δ (DLY5026) early G1 daughter cells were isolated by centrifugal elutriation and incubated in rich medium with Latrunculin-A (100 μM) and nocodazole (15 μg / mL) or with DMSO (vehicle control), as indicated. After 2 h at 30°C the localization of Cdc42p (left), F-actin (center), and tubulin (right) was visualized by fluorescence microscopy. The anti-Cdc42p antibody sometimes stains the nuclear region as well as the polarization site, but this staining was not reproducible and it is not clear whether it represents a pool of Cdc42p or a cross-reacting antigen. However, the occasional nuclear staining did not affect the identification of Cdc42p polarized to presumptive bud sites, bud tips and mother-bud necks. The bright dots stained by anti-tubulin in nocodazole-treated cells are the spindle pole bodies, which retain some tubulin following nocodazole treatment. **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 with Latrunculin A and nocodazole (2 h). At least 200 cells were scored in each case.

Figure 2. Landmark-independent Cdc42p polarization requires Bem1p. A. Tetrad dissection of asci derived from the indicated heterozygous diploid strains (DLY5029, DLY5030 and DLY5031). The 2:2 segregation for viability indicates the inability of the *bem1*Δ *rsr1*Δ, *bem1*Δ *bud2*Δ, and *bem1*Δ *bud5*Δ spores to form colonies. **B.** Left, *bem1*Δ *rsr1*Δ cells carrying a *URA3*-marked *BEM1* plasmid (DLY5452) as well as *LEU2*-marked plasmids containing vector alone (YEpLac181), wild-type *BEM1* (pDLB2226), or *RSR1* (pDLB671) were plated onto dextrose medium and medium containing 5-fuoro-orotic acid (FOA) at 30°C as indicated. On this medium, cells that still contained the *URA3*-marked plasmid are killed, and only cells that have lost the plasmid are able to grow.[62] Cells with empty vector were inviable on 5-FOA medium (which forces the simultaneous lack of Rsr1p and Bem1p) whereas cells with the *BEM1* or the *RSR1* plasmid grew well on both media. Right, *RSR1 bem1*Δ cells (DLY5694) and *bem1*Δ *rsr1*Δ cells carrying *LEU2*-marked plasmids containing wild-type *BEM1* (DLY6146), or *bem1-8* (DLY6147) were plated onto rich medium at 30°C and 39°C as indicated. Cells with Rsr1p but not Bem1p, or with Bem1p but not Rsr1p, were viable at both temperatures. The *bem1-8* plasmid allowed growth at 30°C but not 39°C. **C.** Early G1 daughter cells were isolated by centrifugal elutriation from *bem1*Δ *rsr1*Δ 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. At 1 h intervals, cells were fixed and stained to visualize Cdc42p and tubulin localization. Representative cells from the 39°C 3 h sample are illustrated (left), and the proportion of unbudded cells displaying polarized Cdc42p staining is plotted in the graph (right). At least 200 cells were scored in each time point.

Figure 3. Domains of Bem1p required for symmetry-breaking. A. Domain structure of Bem1p and mutation sites. Top: proteins and lipids known to bind individual domains of Bem1p directly are indicated.[20,29,30,33,36,37] Bottom, sequence alignment (generated by MacVector, Accelrys, San Diego) of short stretches in each domain from Bem1p and homologues from *Saccharomyces cerevisiae* (S.c.), *Saccharomyces bayanus* (S.b.), *Candida albicans* (C.a.), *Schizzosaccharomyces pombe* (S.p.) and *Yarrowia lipolytica* (Y.l.) . Residues mutated in this study are indicated in bold and marked by arrowheads, using the *S. cerevisiae* numbering. P208L disrupts SH3-2 function.[32] P355A mutates the second proline residue in the Class I PxxP motif 348-KRIMPYIP-355[63], disrupting interaction with SH3-2 [38]. R369 lies in the lipid-binding pocket of the PX domain and is critical for lipid binding[64,65]. K482A disrupts binding of the PB1 domain to Cdc24p.[34] **B.** Analysis of Bem1p expression by immunoblotting with anti-myc antibody. Low-copy CEN plasmids (Lo) or high-copy 2 μm plasmids (Hi) expressing wild-type or mutant forms of Bem1p tagged at the C-terminus with 12 c-myc epitopes and expressed from the *BEM1* promoter were transformed into *bem1*Δ cells. A strain containing a single copy of *BEM1-12myc* at the genomic *BEM1* locus was used as positive control (BM) and a strain transformed with empty vector (YEpLac181) was used as negative control (V). To control for gel loading and membrane transfer, the same membrane was probed with anti-Cdc11p. Although some mutants were underexpressed compared to wild-type Bem1p (particularly the P208L mutant), expression levels from the high-copy plasmids were comparable (within a factor of 2) to the wild-type. **C.** Functional analysis of *bem1* mutants. *rsr1*Δ *bem1*Δ cells carrying a *URA3*-marked *BEM1* plasmid as well as high-copy *LEU2*-marked plasmids containing vector alone or the indicated alleles of *BEM1* were grown in medium containing uracil but lacking leucine to mid-log phase. Approximately 10^4, 10^3, 10^2 and 10 cells were spotted onto medium lacking

leucine to examine viability of cells containing the 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 the wild-type and P355A mutant Bem1p were able to rescue the viability of *rsr1*Δ *bem1*Δ cells.

Figure 4. Subcellular localization of Bem1p variants. High-copy plasmids expressing myc-tagged wild-type or mutant forms of Bem1p as indicated were transformed into *bem1*Δ (**A**, DLY5694) and *BEM1* (**B**, DLY5) cells. Bem1p localization was assessed by anti-myc immunofluorescence analysis of log-phase cells. Differing brightness of staining probably results from different copy numbers of the 2 μm plasmids. Vector, YEpLac181.

Figure 5. Cdc42p function requires GTP hydrolysis. A. Cdc42p levels of expression were determined by immunoblotting using anti-Cdc42p antibody in *cdc42-1* (DLY680), *cdc42-1 EG43p-CDC42*^{Q61L} (DLY5296), *CDC42* (DLY1) and *cdc42-1 EG43p-CDC42* (DLY5293) cells grown in galactose medium to mid-log phase. To control for gel loading and membrane transfer, the same membrane was probed with anti-Cdc11p. **B.** *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. **C.** *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. **D.** *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. **E.** *cdc42-1 EG43p-CDC42*^{Q61L} cells (DLY5296) were grown to mid-log phase in dextrose (left) or galactose (right) medium at 24°C, and Cdc42p localization was visualized by immunofluorescence microscopy.

Figure 6. Spontaneous Cdc42p^{Q61L} polarization only occurs upon 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, 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** above. **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/spot were plated onto dextrose (repressing) medium (bottom left). Quantitations of the number of colony-forming cells from three independent repeats of this experiment were plotted as mean viability (±

standard deviation) vs. 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 after the indicated time Cdc42p levels of expression were determined by immunoblotting using anti-Cdc42p antibody. To control for gel loading and membrane transfer, the same membrane was probed with anti-Cdc11p.

Figure 7. Cdc42p function in symmetry-breaking and polarity establishment. Components involved in polarity establishment in yeast become highly concentrated in a "polarization patch" (from a diffuse starting distribution) following commitment to cell cycle progression. We suggest that assembly of this patch occurs through a locally self-promoting feedback loop: GTP loading of Cdc42p is catalyzed by the GEF Cdc24p (step 1), generating GTP-Cdc42p which interacts with autoinhibited ("closed") effector and scaffold proteins and switches them to the active ("open") conformation (step 2). For scaffold proteins, this allows new interactions to take place, leading to 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 take place 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, and 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.[66] 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.

Table I. Synthetic lethal analysis with *rsr1Δ* and *bem1Δ*

Mutant combination	Viability
rsr1Δ cdc24-4	+
rsr1Δ cdc42-1	+
rsr1Δ bem1Δ	-*
rsr1Δ bem2Δ	+
rsr1Δ bem3Δ	+
rsr1Δ spa2Δ	+
rsr1Δ boi1Δ	+
rsr1Δ boi2Δ	+
rsr1Δ bni1Δ	+
rsr1Δ msb1Δ	+
rsr1Δ cla4Δ	+
rsr1Δ cdc12-6	+
bem1Δ rsr1Δ	-*
bem1Δ bud2Δ	-*
bem1Δ bud5Δ	-*
bem1Δ bud3Δ	+
bem1Δ bud4Δ	+
bem1Δ bud7Δ	+
bem1Δ bud8Δ	+
bem1Δ bud9Δ	+
bem1Δ bud8Δ bud9Δ	+
bem1Δ bud10Δ	+

* Synthetic lethality was confirmed in the YEF473, S288C, and BF264-15Du backgrounds.

Table II. Yeast strains used in this study[a]

Strain	*Relevant genotype*	*Source*
DLY1	a	67
DLY5	a/α	67
DLY664	a/α *cdc24-4/cdc24-4*	68
DLY680	α *cdc42-1*	68
DLY2601	a/α *cdc28-13/cdc28-13* pGAL-*CDC42*	This study
DLY2603	a/α *cdc28-13/cdc28-13* pGAL-*CDC42*^{Q61L}	This study
DLY2605	a/α *cdc24-4/cdc24-4* pGAL-*CDC42*	This study
DLY2607	a/α *cdc24-4/cdc24-4* pGAL-*CDC42*^{Q61L}	This study
DLY2611	a *spa2::URA3*	69
DLY2623	a *msb1::URA3*	69
DLY3002	a *cdc42::GAL1p-CDC42::LEU2*	This study
DLY3003	a *cdc4 ::GAL1p-CDC42*^{Q61L}*::LEU2*	This study
DLY3005	a/α *cdc42::GAL1p-CDC42::LEU2*	This study
DLY3006	a/α *cdc42::GAL1p-CDC42*^{Q61L}*::LEU2*	This study
DLY3032	a *cdc42-1*	68
DLY3340	α *bem3::LEU2*	41
DLY3368	a *rsr1::URA3*	69
DLY3462[2]	α *bni1::HIS3*	E. Bi
DLY3947	α *bem2::TRP1*	69
DLY4000	a *BEM1-12Xmyc::HIS3*	33
DLY4780	a/α *cla4::TRP1/ cla4::TRP1*	This study
DLY4864	α *cdc12-6::LEU2*	70
DLY5023[2]	a/α *rsr1::HIS3/rsr1::HIS3*	J. Pringle
DLY5024[2]	a/α *bud2::HIS3/bud2::HIS3*	J. Pringle
DLY5025[2]	a/α *bud5::HIS3/bud5::HIS3*	J. Pringle
DLY5026[2]	a/α *rsr1::HIS3/rsr1::HIS3 bud8::HIS3/bud8::HIS3*	J. Pringle
DLY5029[2]	a/α *rsr1::HIS3/rsr1::HIS3 bem1::URA3/BEM1*	This study
DLY5030[2]	a/α *bud2::HIS3/bud2::HIS3 bem1::URA3/BEM1*	This study
DLY5031[2]	a/α *bud5::HIS3/bud5::HIS3 bem1::URA3/BEM1*	This study
DLY5068[2]	a *rsr1::TRP1*	J. Pringle
DLY5069[2]	α *rsr1::HIS3*	J. Pringle
DLY5075[2]	a/α *bud8::TRP1/bud8::TRP1 bud9::HIS3/bud9::HIS3*	J. Pringle
DLY5077[2]	a/α	J. Pringle
DLY5078	α *cdc24-4 GAL1p-SWE1::URA3*	41
DLY5240	a *CDC42 his2::EG43p-CDC42*^{Q61L}*::HIS2*	41
DLY5293	α *cdc42-1 his2::EG43p-CDC42::HIS2*	This study
DLY5296	α *cdc42-1 his2::EG43p-CDC42*^{Q61L}*::HIS2*	This study
DLY5299	a *cdc42-17 his2::EG43p-CDC42::HIS2/his2*	This study
DLY5301	a *cdc42-17 his2::EG43p-CDC42*^{Q61L}*::HIS2/his2*	This study
DLY5452[2]	a *rsr1::HIS3 bem1::KAN* YEpLac195-*BEM1*	This study
DLY5489	a *rsr1::KAN*	This study
DLY5555	a/α *cdc24-4/cdc24-4 CDC42/CDC42 EG43p-CDC42::HIS2/his2*	This study
DLY5557	a/α *cdc28-13/cdc28-13 CDC42/CDC42 EG43p-CDC42::HIS2/his2*	This study
DLY5560	a/α *cdc24-4/cdc24-4 CDC42/CDC42 EG43p-CDC42*^{Q61L}*::HIS2/his2*	This study
DLY5562	a/α *cdc28-13/cdc28-13 CDC42/CDC42 EG43p-CDC42*^{Q61L}*::HIS2/his2*	This study
DLY5570	a/α *cla4::TRP1/cla4::TRP1 rsr1::KAN/RSR1*	This study
DLY5631[1]	a *bem1::KAN*	Research Genetics
DLY5681	α *rsr1::KAN*	This study
DLY5694[2]	a *bem1::KAN*	This study
DLY6146	a *rsr1::HIS3 bem1::KAN* pRS315-*BEM1-12XMYC*	This study
DLY6147	a *rsr1::HIS3 bem1::KAN* pRS315-*bem1-8-12XMYC*	This study

MOSY122	a *cdc42-17*	This study
MOSY176	a *boi1::HIS2*	This study
MOSY181	a *boi2 ::HIS2*	This study
[1]	a/α *rsr1::KAN/rsr1::KAN*	Research Genetics
[1]	a/α *bud2::KAN/bud2::KAN*	Research Genetics
[1]	a/α *bud3::KAN/bud3::KAN*	Research Genetics
[1]	a/α *bud4::KAN/bud4::KAN*	Research Genetics
[1]	a/α *bud7::KAN/bud7::KAN*	Research Genetics
[1]	a/α *bud8::KAN/bud8::KAN*	Research Genetics
[1]	a/α *bud9::KAN/bud9::KAN*	Research Genetics
[1]	a/α *bud10::KAN/bud10::KAN*	Research Genetics

[a]All strains except [1,2] are in the BF264-15Du (Richardson *et al.*, 1989) background (*ade1 his2 leu2-3,112 trp1-1 ura3Δns*).
[1]Are in the in the BY4743 (S288C) background (*his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0*).
[2]Are in the YEF473 background (*his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*).

Table III. Plasmids used in this study

Plasmid	Vector	Insert	Source
pDLB659	YIpEG43-*HIS2*	*CDC42*^{Q61L}	[41]
pDLB664	YIpEG43-*HIS2*	*CDC42*	[41]
pDLB671	2μm *LEU2*	*RSR1*	A. Bender
pDLB678	pPB321	genomic clone including *BEM1* locus	A. Bender
pDLB1822	YIpUBEM1-P208L	*bem1*^{P208L}	[32]
pDLB1974	YEpLac195	*BEM1* genomic locus	This study
pDLB2226	pRS315	*BEM1p-BEM1-12XMYC-SWE1t*	This study
pDLB2261	pRS315	*BEM1p-bem1-8-12XMYC-SWE1t*	This study
pDLB2278	YIpUBEM1-K482A	*bem1*^{K482A}	[34]
pDLB2282	pRS315	*BEM1p-bem1*^{P208L}*-12XMYC-SWE1t*	This study
pDLB2284	pRS315	*BEM1p-bem1*^{R316A}*-12XMYC-SWE1t*	This study
pDLB2285	pRS315	*BEM1p-bem1*^{P355A}*-12XMYC-SWE1t*	This study
pDLB2288	pRS315	*BEM1p-bem1*^{R369A}*-12XMYC-SWE1t*	This study
pDLB2336	pRS315	*BEM1p-bem1*^{K482A}*-12XMYC-SWE1t*	This study
pDLB2374	YEpLac181	*BEM1p-BEM1-12XMYC-SWE1t*	This study
pDLB2375	YEpLac181	*BEM1p-bem1*^{P208L}*-12XMYC-SWE1t*	This study
pDLB2376	YEpLac181	*BEM1p-bem1*^{R316A}*-12XMYC-SWE1t*	This study
pDLB2377	YEpLac181	*BEM1p-bem1*^{P355A}*-12XMYC-SWE1t*	This study
pDLB2378	YEpLac181	*BEM1p-bem1*^{R369A}*-12XMYC-SWE1t*	This study
pDLB2379	YEpLac181	*BEM1p-bem1*^{K482A}*-12XMYC-SWE1t*	This study
pMOSB164	pCRII	*BOI2::HIS2*	This study
pMOSB178	pCRII	*BOI1::HIS2*	This study
pRS315	*CEN LEU2*		[71]
YEpLac181	2μm *LEU2*		[72]
YEpLac195	2μm *URA3*		[72]

Table IV. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')
MOSKOW1.2	GATCCATATGCTGAAAAACTTCAAAC
DOWNSTREAM	GCGCGTCGACAATATCGTGAACGGAAATTTTCAG
MOSKOW1.4	GAATGGTTCATTGCTAAGCC
R316A-1	AGGCAACTGAAAGCTTACTATCAAGATTTCTACGATCTGCAGG
R316A-2	CCTGCAGATCGTAGAAATCTTGATAGTAAGCTTTCAGTTGCCT
P355A-1	TATAATGCCCTATATCGCCGGCCCCGTTCC
P355A-2	GGAACGGGGCCGGCGATATAGGGCATTATA
R369A-1	CAAAAAAAGCAAAGGAGGACTTGAATATATATGTGGCAGACC
R369A-2	GGTCTGCCACATATATATTCAAGTCCTCCTTTGCTTTTTTTG
BEM1-480	TAAAGAGCTCAACGGCATCACATCTGGGG
SWE1TERM	TTAAAACTCGAGGGCCCATAAGCACGTGTGGG
BEM1-744	TATAGGTTATCACCATACTCCG
MACBEM1-2	TCTTCTGAATTTTACGTCAGC
RSR1-163	ATTGCGTTCGTTCTTAACTACGCC
RSR1+1443	CTCAGAAAAAAGGTTAGAGCAAGGC

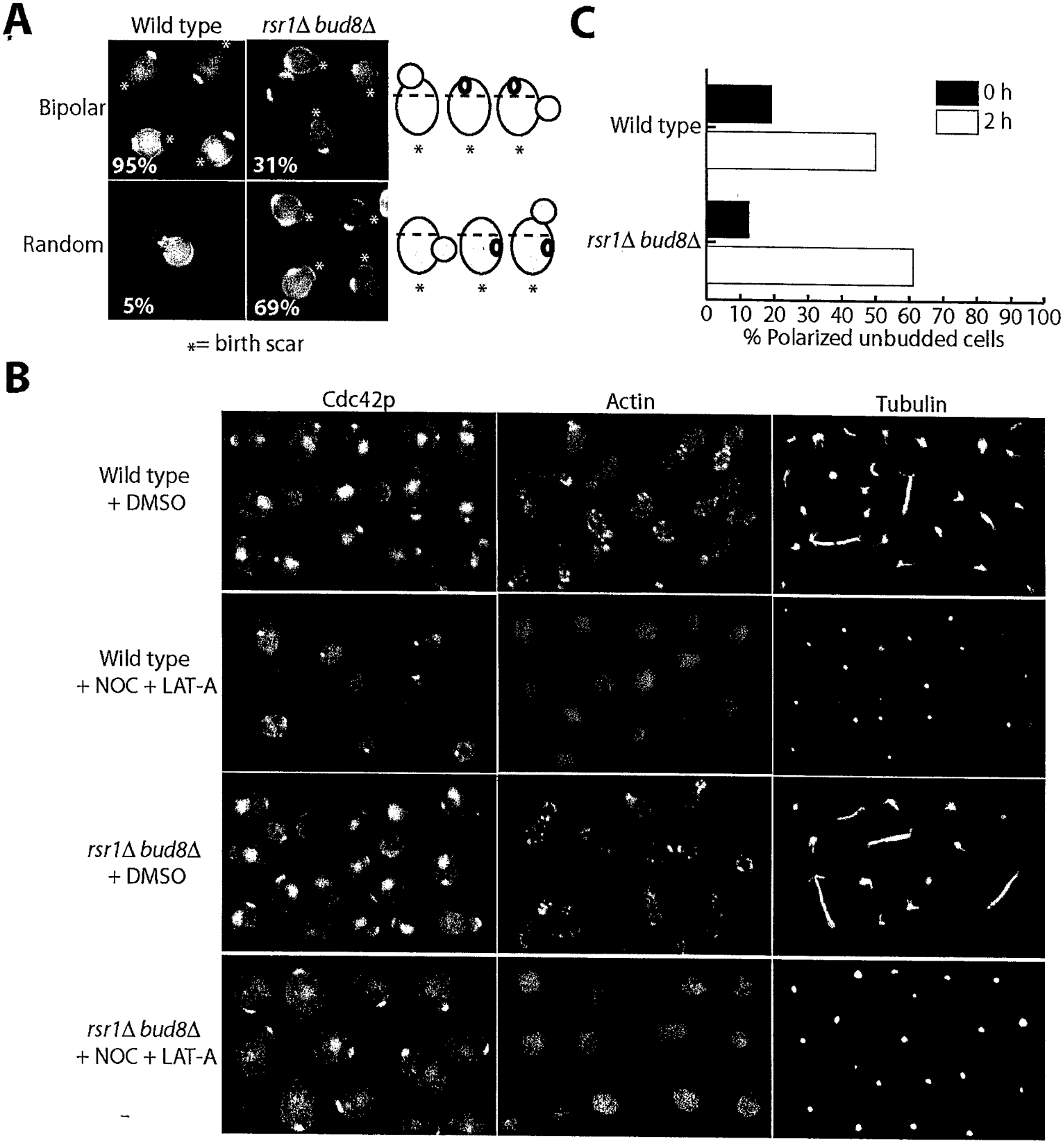

A
Wild type
rsr1Δ bud8Δ
Bipolar
Random
95%
31%
5%
69%
*= birth scar
C
0 h
2 h
Wild type
rsr1Δ bud8Δ
0 10 20 30 40 50 60 70 80 90 100
% Polarized unbudded cells
B
Cdc42p
Actin
Tubulin
Wild type
+ DMSO
Wild type
+ NOC + LAT-A
rsr1Δ bud8Δ
+ DMSO
rsr1Δ bud8Δ
+ NOC + LAT-A

Figure 1, Lew

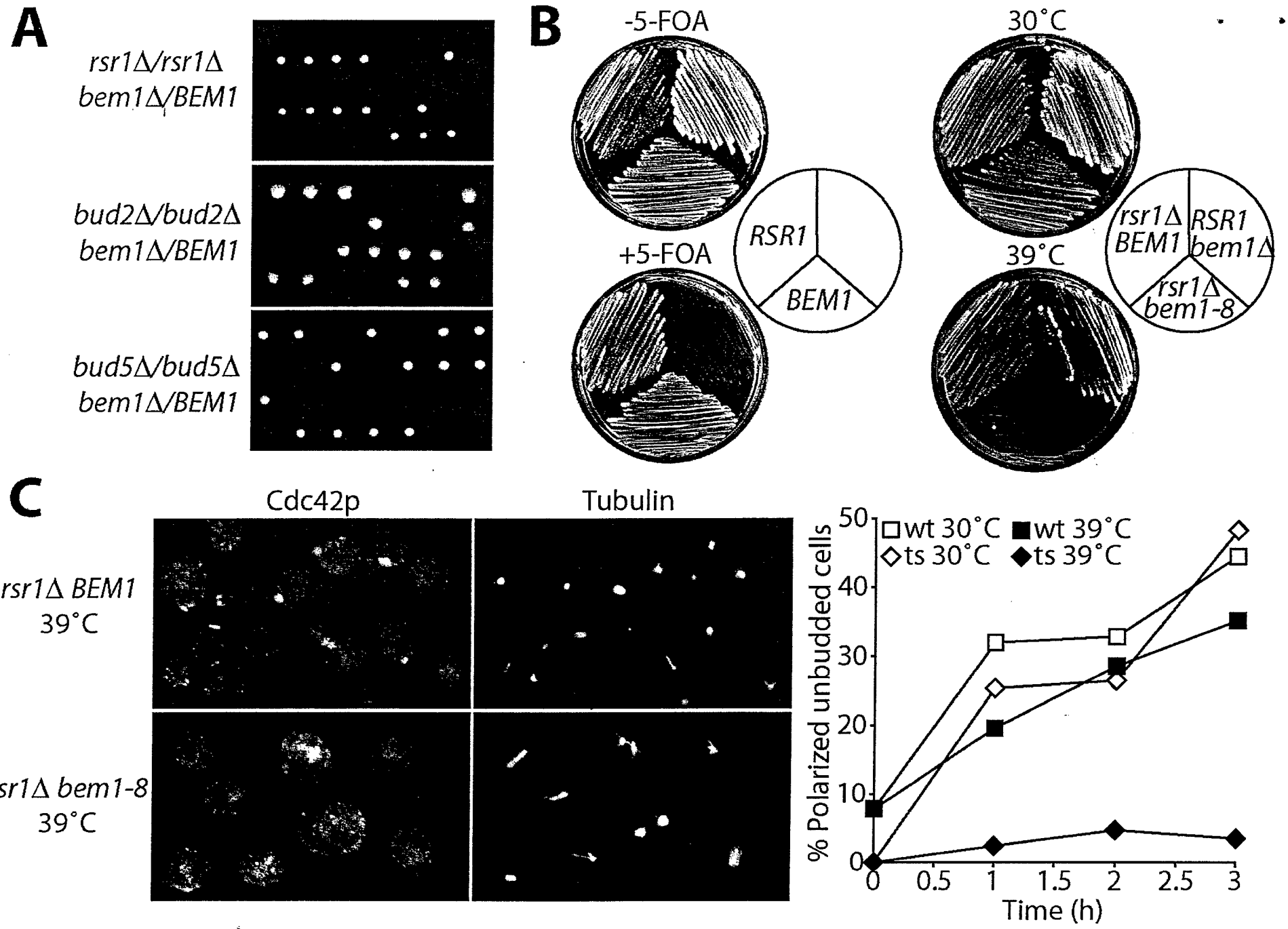

A
rsr1Δ/rsr1Δ
bem1Δ/BEM1
bud2Δ/bud2Δ
bem1Δ/BEM1
bud5Δ/bud5Δ
bem1Δ/BEM1
B
-5-FOA
+5-FOA
RSR1
BEM1
30°C
39°C
rsr1Δ BEM1
RSR1 bem1Δ
rsr1Δ bem1-8
C
Cdc42p
Tubulin
rsr1Δ BEM1
39°C
rsr1Δ bem1-8
39°C
% Polarized unbudded cells
wt 30°C
wt 39°C
ts 30°C
ts 39°C
Time (h)
0
10
20
30
40
50
0.5
1
1.5
2
2.5
3

Figure 2, Lew

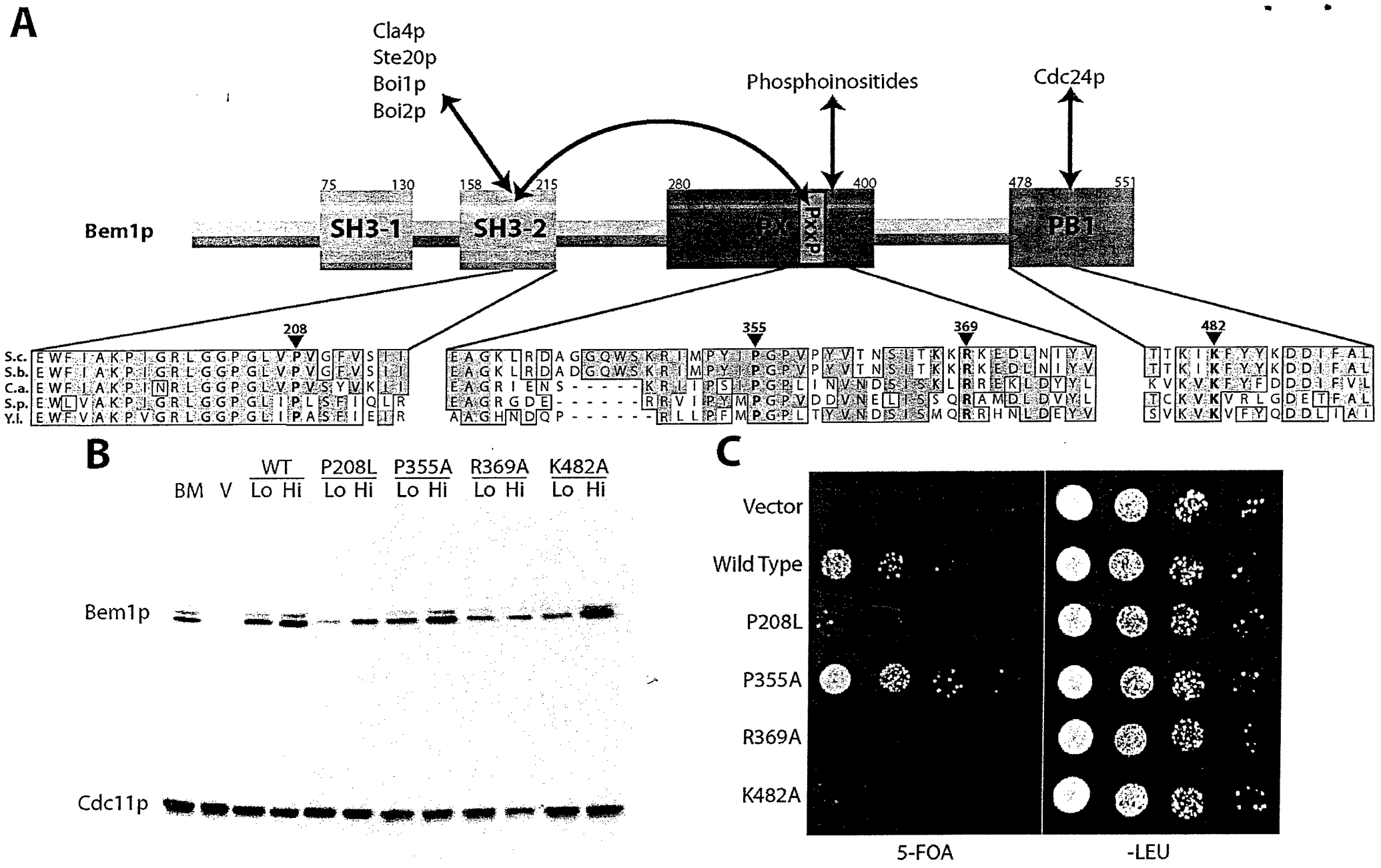

A
Cla4p
Ste20p
Boi1p
Boi2p
Phosphoinositides
Cdc24p
Bem1p
SH3-1
SH3-2
PX
PXXP
PB1
75
130
158
215
280
400
478
551
208
355
369
482
S.c.
S.b.
C.a.
S.p.
Y.l.
B
WT
P208L
P355A
R369A
K482A
BM
V
Lo Hi
Bem1p
Cdc11p
C
Vector
Wild Type
P208L
P355A
R369A
K482A
5-FOA
-LEU

Figure 3, Lew

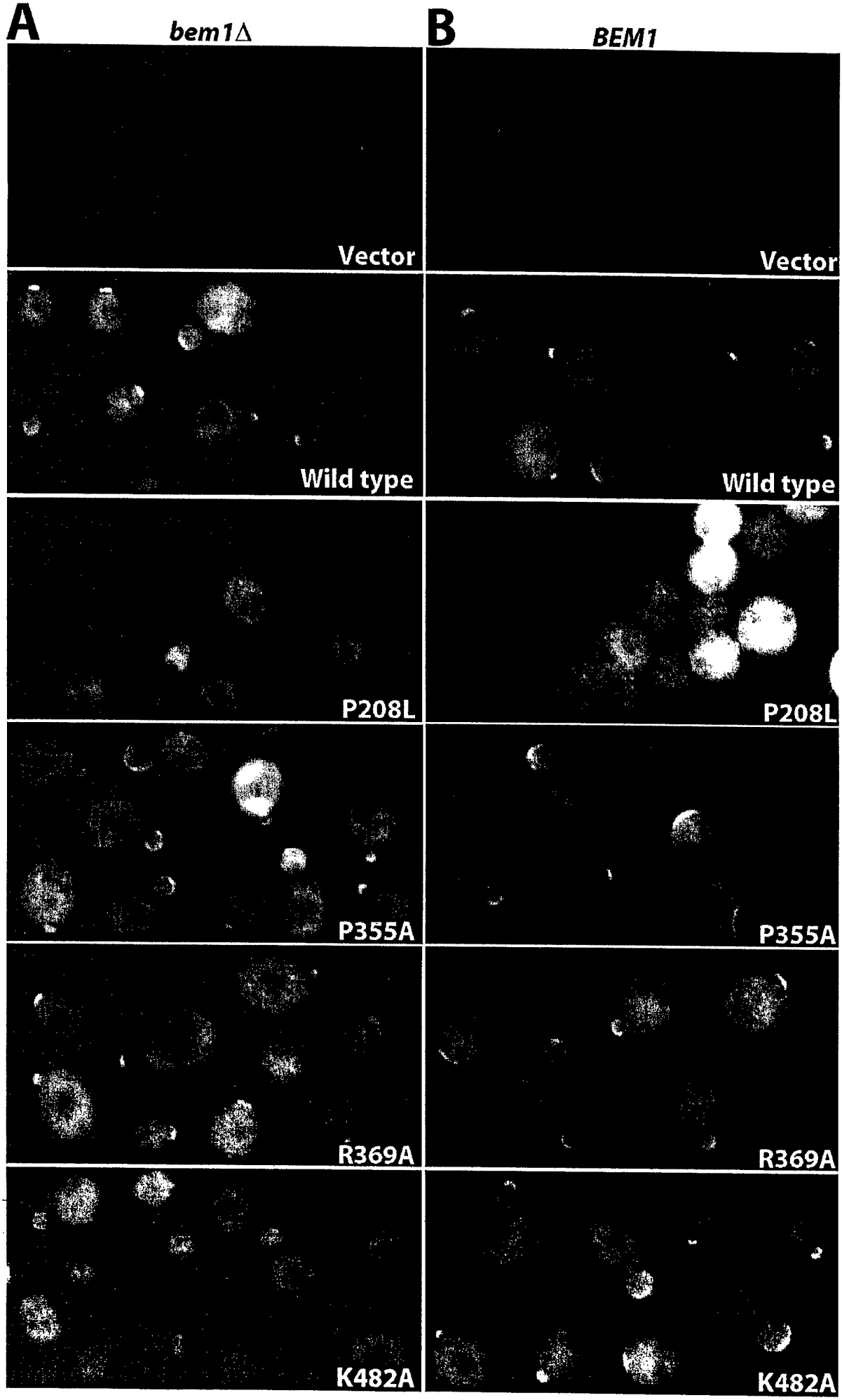

A
bem1Δ
B
BEM1
Vector
Vector
Wild type
Wild type
P208L
P208L
P355A
P355A
R369A
R369A
K482A
K482A

Figure 4, Lew

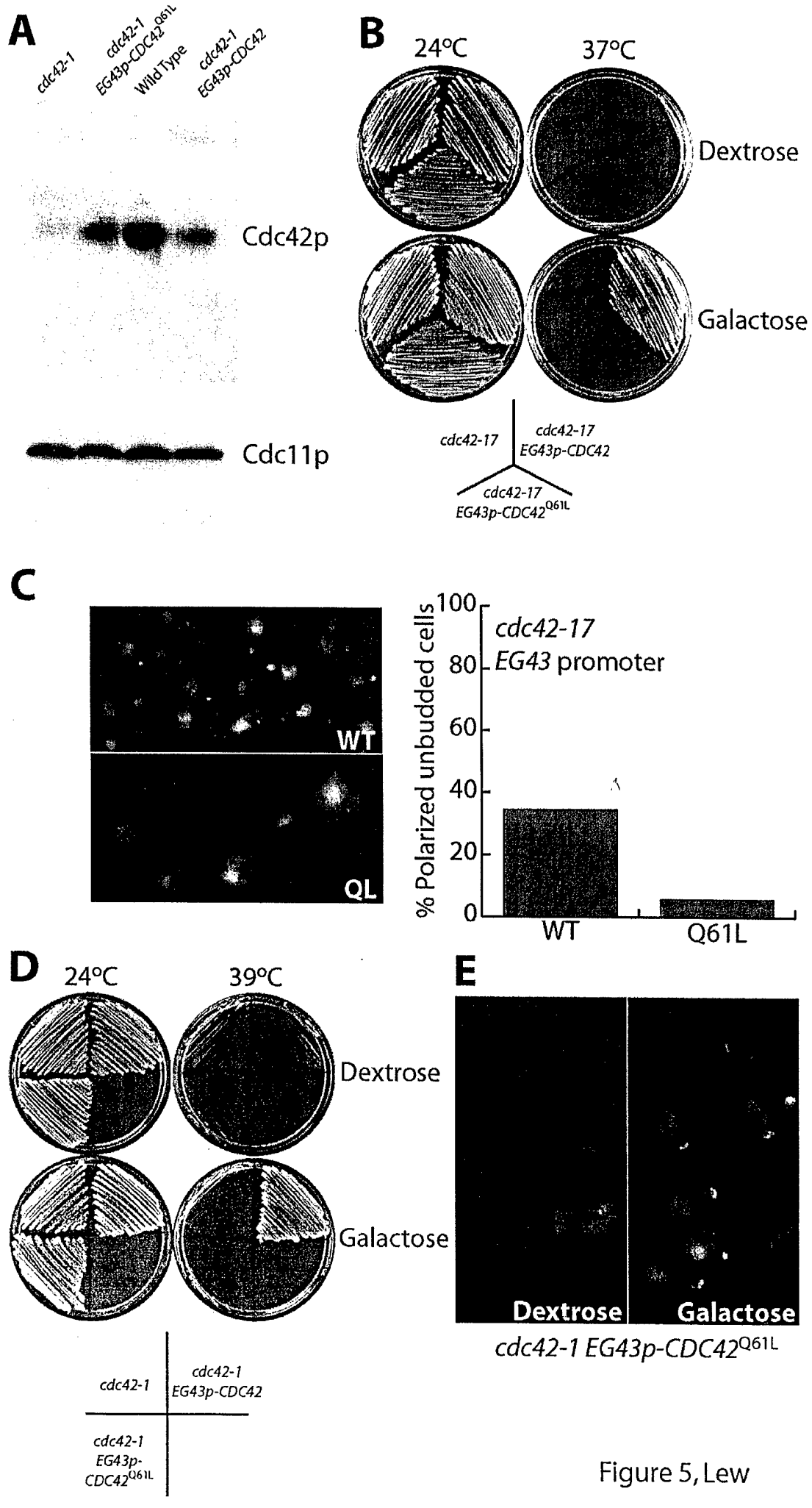
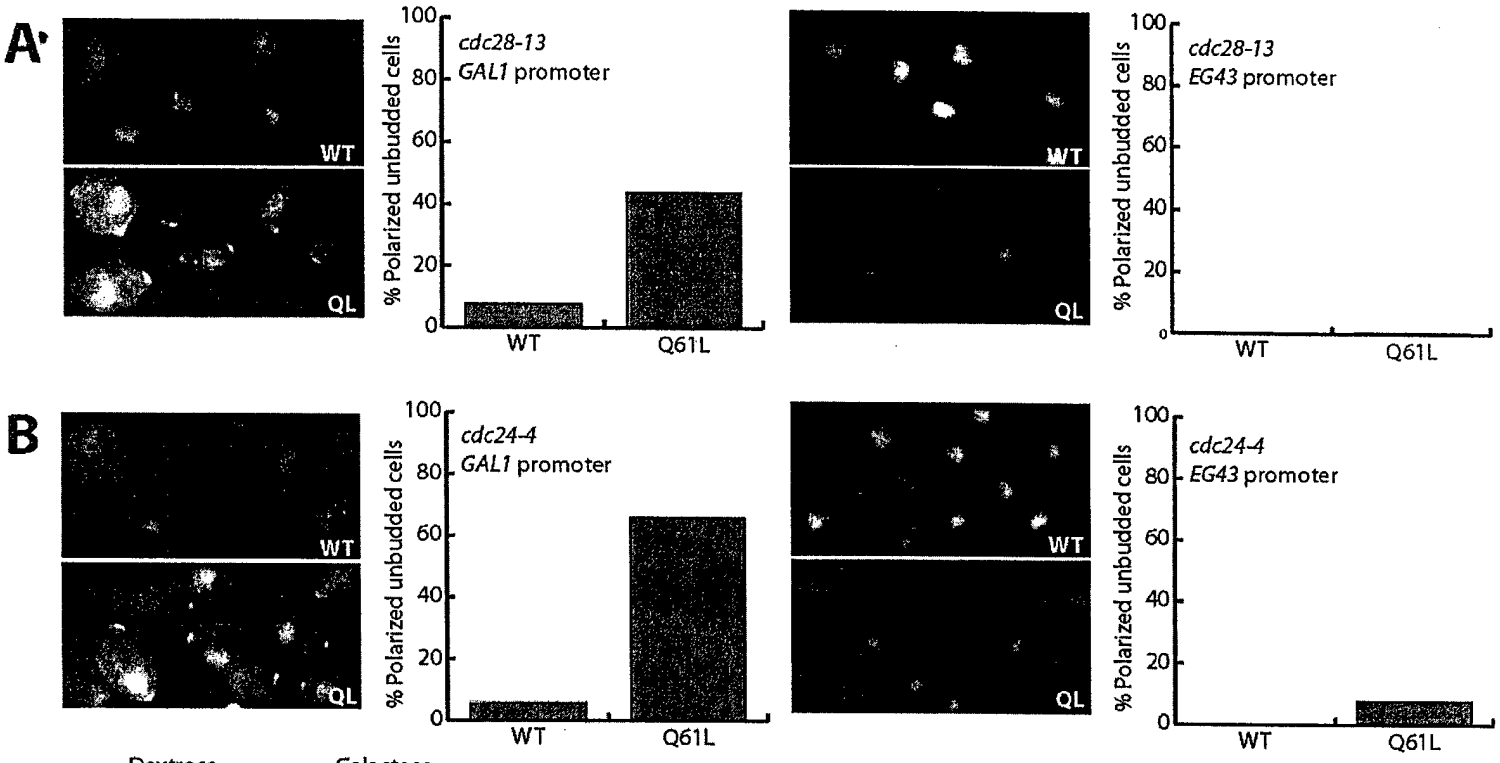
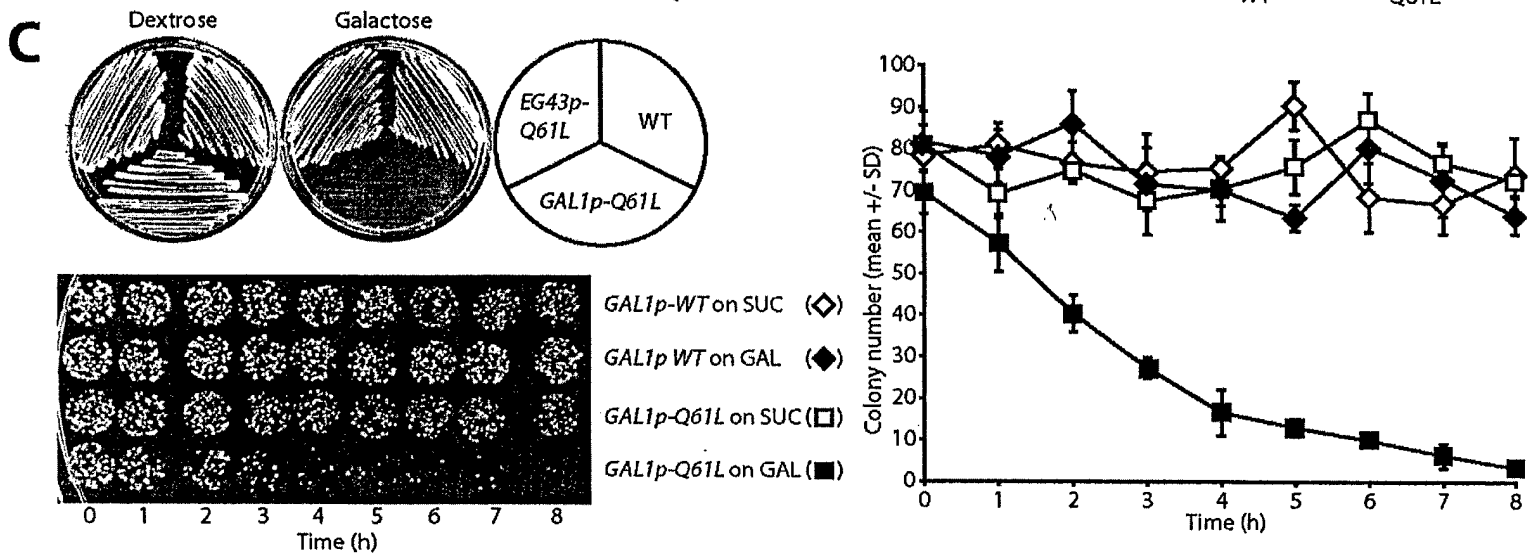

A
cdc42-1
cdc42-1 EG43p-CDC42Q61L
Wild Type
cdc42-1 EG43p-CDC42
Cdc42p
Cdc11p
B
24°C
37°C
Dextrose
Galactose
cdc42-17
cdc42-17 EG43p-CDC42
cdc42-17 EG43p-CDC42Q61L
C
WT
QL
% Polarized unbudded cells
100
80
60
40
20
0
cdc42-17
EG43 promoter
WT
Q61L
D
24°C
39°C
Dextrose
Galactose
cdc42-1
cdc42-1 EG43p-CDC42
cdc42-1 EG43p-CDC42Q61L
E
Dextrose
Galactose
cdc42-1 EG43p-CDC42Q61L

Figure 5, Lew


A
WT
QL
% Polarized unbudded cells
cdc28-13
GAL1 promoter
WT
Q61L
cdc28-13
EG43 promoter
B
cdc24-4
GAL1 promoter
cdc24-4
EG43 promoter


C
Dextrose
Galactose
EG43p-Q61L
WT
GAL1p-Q61L
GAL1p-WT on SUC
GAL1p WT on GAL
GAL1p-Q61L on SUC
GAL1p-Q61L on GAL
Time (h)
Colony number (mean +/- SD)

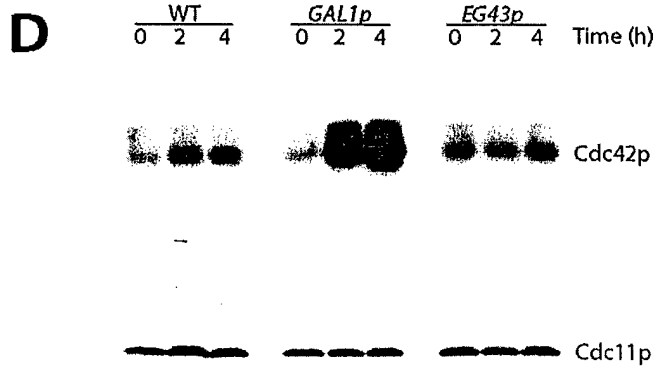

D
WT
GAL1p
EG43p
Time (h)
Cdc42p
Cdc11p

Figure 6, Lew

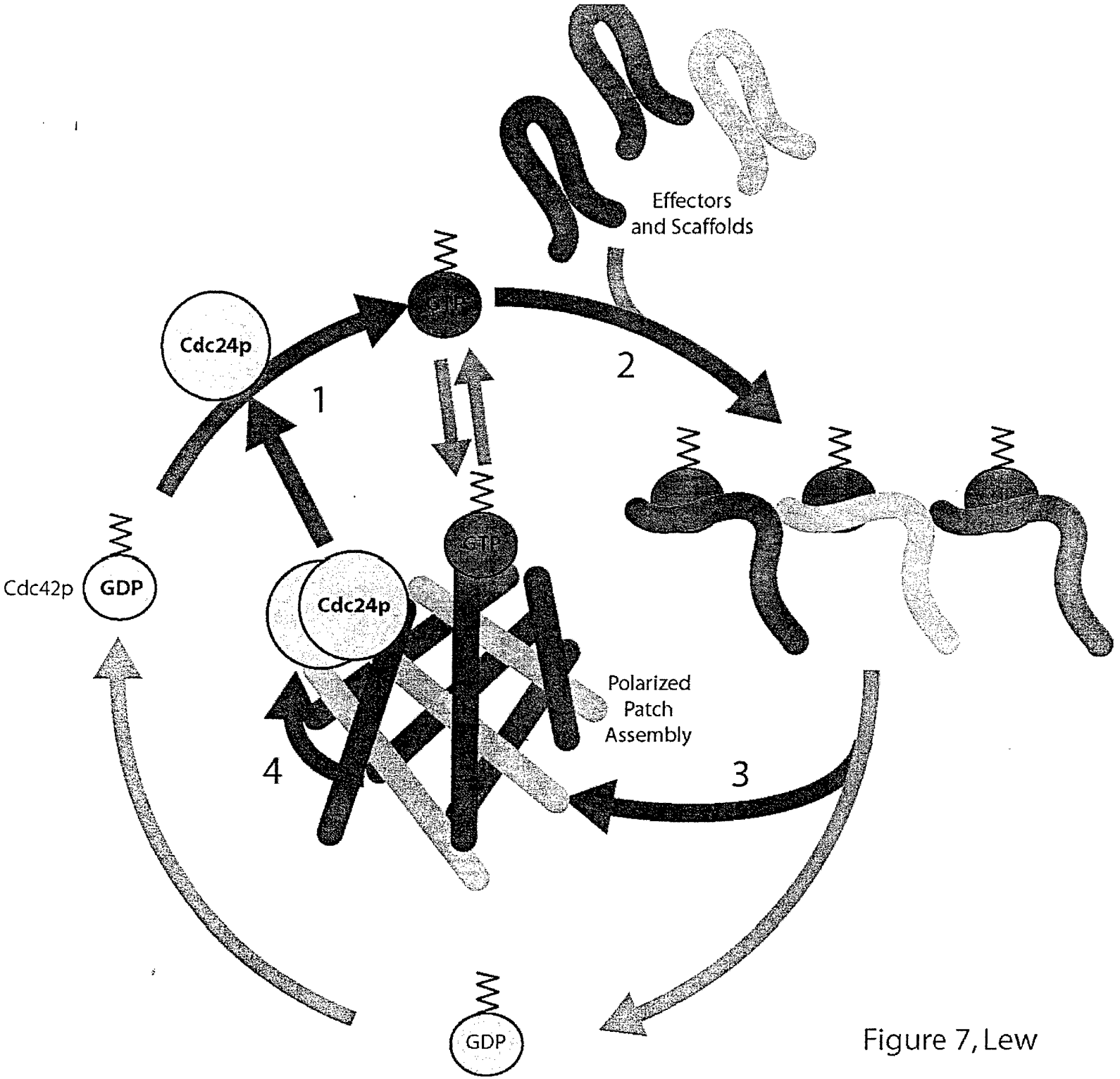

Effectors
and Scaffolds
GTP
Cdc24p
1
2
GTP
Cdc42p
GDP
Cdc24p
Polarized
Patch
Assembly
4
3
GDP

Figure 7, Lew