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Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth *via* Rga GTPase-activating proteins

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Running title: Lipid flipping regulates polarized morphogenesis

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Summary

An important problem in polarized morphogenesis is how polarized transport of membrane vesicles is spatiotemporally regulated. Here, we report that a local change in the transbilayer phospholipid distribution of the plasma membrane regulates the axis of polarized growth. Type 4 P-type ATPases Lem3p-Dnf1p and -Dnf2p are putative heteromeric phospholipid flippases in budding yeast that are localized to polarized sites on the plasma membrane. The *lem3Δ* mutant exhibits prolonged apical growth due to a defect in the switch to isotropic bud growth. In *lem3Δ* cells, the small GTPase Cdc42p remains polarized at the bud tip where phosphatidylethanolamine remains exposed on the outer leaflet. Intriguingly, phosphatidylethanolamine and phosphatidylserine stimulate GTPase-activating protein (GAP) activity of Rga1p and Rga2p toward Cdc42p, whereas PI(4,5)P₂ inhibits it. We propose that a redistribution of phospholipids to the inner leaflet of the plasma membrane triggers the dispersal of Cdc42p from the apical growth site, through activation of GAPs.

Introduction

Polarized membrane growth, which occurs by membrane transport and insertion to a specific area of the cell surface, plays an essential role in cell morphogenesis, but it is largely unknown how it is spatio-temporally regulated (Lecuit and Pilot, 2003). The budding yeast *Saccharomyces cerevisiae* undergoes changes in polarized membrane growth that are dependent on rearrangements of the actin cytoskeleton (Pruyne and Bretscher, 2000a). During the early phase of budding, the bud grows apically, and later switches to an isotropic phase, resulting in an ellipsoidal bud shape. Cells that are delayed in the switching step form an elongated bud (Lew and Reed, 1993). During cytokinesis, cells repolarize the growth site to the mother-daughter neck of the cell division site (bud neck). The small GTPase Cdc42p plays a key role in directing these events by polarizing regulators of the actin cytoskeleton to growth sites (Johnson, 1999). The timing of the growth switch is regulated in a cell cycle-dependent manner: activation of the cyclin-dependent kinase Cdc28p by Clb cyclins at an early G2 phase triggers the rearrangement of the actin cytoskeleton for the apical-isotropic growth switch and entry into mitosis; inactivation of Clb/Cdc28p at the end of mitosis leads to the repolarization of the actin cytoskeleton to the bud neck (Lew and Reed, 1995). However, it is not known how the reorganization of the actin cytoskeleton is spatially regulated downstream of Clb/Cdc28p.

Various cell types display an asymmetric distribution of phospholipids across the plasma membrane (Devaux, 1991). This lipid asymmetry is generated and maintained by ATP-driven lipid transporters or translocases that vary in their lipid

specificity (Devaux, 1991). A subfamily (classified as “flippase”) of the type 4 P-type ATPases has been implicated in the translocation of phospholipids from the external to the cytosolic leaflet. Dnf1p and Dnf2p, members of this subfamily in the budding yeast, are hypothesized to translocate phospholipids from the outer to the inner leaflet of the plasma membrane (Pomorski et al., 2003). Lem3p, a member of the Cdc50p family, is a potential non-catalytic subunit of both Dnf1p and Dnf2p, and is essential for the exit of Dnf1p and Dnf2p from the endoplasmic reticulum (Kato et al., 2002; Saito et al., 2004; Furuta et al., 2007). These proteins are primarily localized to the plasma membrane of polarized growth sites, such as emerging buds, small buds, and the bud neck of dividing cells (Hua et al., 2002; Pomorski et al., 2003; Saito et al., 2004; Furuta et al., 2007), but their functions remain to be elucidated. In this study, we propose that the transbilayer phospholipid redistribution by Lem3p-Dnf1p and -Dnf2p (Lem3p-Dnf1/2p) at the apical growth site changes the mode of membrane growth by triggering downregulation of Cdc42p.

Results

The *lem3Δ* Mutant Exhibits Defects in the Apical-isotropic Growth Switch

The *lem3Δ* mutant as well as the *dnf1Δ dnf2Δ* mutant displayed hyperpolarized growth at lower temperatures (18°C), resulting in an elongated cell shape (**Figure 1A** and data not shown) (Hua et al., 2002). In this study, we analyzed phenotypes of the *lem3Δ* mutant at 18°C, because the mutant displayed more prominent hyperpolarized

growth at this temperature. However, hyperpolarization of the cortical actin cytoskeleton and polarity regulators (see below) to the bud tip was also observed at 30°C (data not shown).

We first examined distributions of the actin cytoskeleton. Cortical actin patches (small foci of actin filaments) and actin cables (bundles of actin filaments) within buds are randomly distributed beneath the cell surface in large-budded cells of the wild type, presumably representing the isotropic growth phase (**Figure 1B**) (Pruyne and Bretscher, 2000a). Strikingly, cortical actin patches remained polarized to the bud tip and actin cables also appeared to extend along the mother-daughter axis in the *lem3Δ* mutant (**Figure 1B**). These results suggest that the loss of Lem3p-Dnf1/2p causes defects in the apical-isotropic growth switch, resulting in prolonged apical growth. This prolonged apical growth in the *lem3Δ* mutant was not due to a mitotic delay. In addition, the repolarization of the actin cytoskeleton to the bud neck at a post-mitotic phase was largely normal in the *lem3Δ* mutant (Supplemental Results and **Figure S1A**). We next examined whether the prolonged apical growth is due to the retention of Cdc42p and other regulators of polarized growth at the apical bud tip. To clearly compare the growth pattern of a bud between the wild type and the *lem3Δ* mutant, we examined large-budded cells with divided nuclei in an asynchronous culture at 18°C (**Figure 1C, S1B, and S1C**). In these analyses, the bud-neck localization pattern was excluded, because it did not vary between the wild type and the *lem3Δ* mutant, as for the actin cytoskeleton in **Figure S1A** (data not shown). Thus, cells in a late mitotic phase, but not in cytokinesis, were analyzed. Cdc42p was localized to the bud tip in

48% of the *lem3Δ* cells, while no specific localization was observed in the wild-type cells (**Figure 1C**). GFP-tagged polarity regulators (Pruyne and Bretscher, 2000a, 2000b), including Bni1p, a Cdc42p effector that promotes actin cable assembly, Myo2p, a type V myosin that transports secretory vesicles along actin cables, Spa2p, a polarisome component required for the proper localization of Bni1p, and Exo70p and Sec15p, exocyst components that play a role in vesicle targeting and docking to the plasma membrane, were also localized to the bud tip in the majority (60 ~ 85%) of *lem3Δ* cells, while they were largely dispersed to the bud cortex in the wild type (**Figure S1B** and **S1C**).

Taken together, these results show that the *lem3Δ* mutant is defective in the apical-isotropic growth switch independent of the cell cycle control due to the persistent localization of Cdc42p and polarity regulators to the apically growing bud tip. Interestingly, the prolonged bud-tip localization of Cdc42p in the *lem3Δ* mutant was independent of efficient vesicle transport along actin cables toward the bud tip (Supplemental Results and **Figure S2**). This result suggests the existence of a stable membrane domain at the bud tip to which Cdc42p and its effectors are localized during the apical growth.

Cdc42p Is Polarized to an Apical Membrane Domain Where Phosphatidylethanolamine Is Exposed on the Outer Leaflet

It has been shown that phosphatidylethanolamine (PE), which is generally enriched in the inner leaflet of the plasma membrane (Devaux, 1991), is specifically exposed on the

outer leaflet at polarized sites during the early stage of budding (Iwamoto et al., 2004). This PE exposure is enhanced in the *lem3Δ* mutant, probably due to the loss of the inward translocation of PE (Iwamoto et al., 2004). PE exposure is also observed at the cytokinesis site in mammalian cells (Emoto et al., 1996). PE in the outer leaflet of the plasma membrane can be probed in living cells with biotinylated Ro09-0198 (Bio-Ro), a peptide that specifically binds to PE (Emoto et al., 1996; Iwamoto et al., 2004). We examined the distribution of PE in the outer leaflet during bud growth in the wild type and the *lem3Δ* mutant and compared it with that of Cdc42p. In wild-type cells, PE was exposed at polarized sites during the early stages of budding, at the presumptive bud site and the bud tip of tiny- to small-budded cells where Cdc42p was localized (**Figure 2A, (i) and (ii)**); the PE exposure was observed at the bud tip in 71% (n >100) of tiny- to small-budded cells. Cdc42p was then redistributed from the bud tip to the bud cortex in the small- to medium-budded cell phase, which represents the cell population undergoing the apical-isotropic growth switch (**Figure 2A, (iii)**) (Pruyne and Bretscher, 2000a). At this phase, cells with PE exposed on the outer leaflet decreased to 11% (n > 100). In medium- and large-budded cell phases, neither the PE exposure nor the Cdc42p polarization could be detected (**Figure 2A, (iv) and (v)**). These results suggest the existence of a membrane domain at the apical growth site where PE is exposed on the outer leaflet. Consistent with this, PE exposure, as well as Cdc42p polarization with prolonged apical growth, was observed at the bud tip in G2-arrested cells induced by depletion of Clb1p in *clb2Δ clb3Δ clb4Δ* cells (**Figure 2C**) (Lew and Reed, 1993). In contrast to wild-type cells, the PE exposure and Cdc42p remained

polarized to the bud tip throughout the budding processes in the *lem3Δ* mutant (**Figure 2B, (i) - (v)**); the PE exposure was still observed at the bud tip in 80% ($n > 100$) of the large-budded cells (**Figure 2B, (v)**). These results suggest that the translocation of phospholipids such as PE to the inner leaflet by Dnf1/2p leads to the dispersal of Cdc42p from the polarized plasma membrane site, resulting in the apical-isotropic growth switch.

Cdc42p-GTP Seems to Be Maintained at the Apical Membrane Domain by Slowed Hydrolysis of GTP Rather Than by GDP/GTP Exchange

Rga1p, Rga2p, and Bem3p are GTPase-activating proteins (GAPs) for Cdc42p (Smith et al., 2002). Overexpression of N-terminally truncated-Bem3p containing the GAP domain (Kadota et al., 2004) or full-length Rga1p suppressed the hyperpolarized growth as well as the prolonged bud-tip localization of Cdc42p in the *lem3Δ* mutant (**Figure 3A and 3B**). Overexpression of Cdc42p GAPs affected neither the growth rate nor the localization of Cdc42p to the incipient bud site and the bud tip during the early stage of budding in the *lem3Δ* mutant (**Figure 3C** and data not shown). These results suggest that the hyperpolarized growth in the *lem3Δ* mutant is caused by the prolonged bud-tip localization of Cdc42p-GTP. Surprisingly, Cdc24p, the sole guanine-nucleotide exchange factor for Cdc42p, was relocalized from the bud tip to the bud cortex after the small-budded phase in the *lem3Δ* mutant as in the wild type (Supplemental Results and **Figure S3**).

The above results suggest that Cdc42p-GTP is maintained by slowed hydrolysis of GTP rather than by GDP/GTP exchange. Interestingly, the *rga1Δ rga2Δ* mutant exhibited an elongated cell shape at 18°C (**Figure 3D**), as previously reported (Smith et al., 2002). We examined the localization of Myo2p-GFP and Gic1p-GFP, a Cdc42p effector (Pruyne and Bretscher, 2000a), in large-budded *rga1Δ rga2Δ* cells with divided nuclei, as in **Figure 1C**. Myo2p-GFP remained localized to the bud tip, albeit with a lower frequency (58%) compared to the *lem3Δ* mutant (80%) (**Figure 3E**). Similarly, late mitotic cells with polarized Gic1p increased from 5% in the wild type to 19% and 52% in *rga1Δ rga2Δ* and *lem3Δ* mutants, respectively (data not shown). We did not analyze a mutant in combination with the *bem3Δ* mutation, because it caused abnormal cell morphology, possibly due to defective assembly of septins (Smith et al., 2002; Caviston et al., 2003). These results suggest that the *rga1Δ rga2Δ* mutant exhibited hyperpolarized growth independent of cell cycle control to a lesser extent than that of the *lem3Δ* mutant. Thus, Rga1p and Rga2p are also required for the switch from the apical to isotropic growth phase. Suppression of the hyperpolarized growth of the *lem3Δ* mutant by overexpression of Cdc42p GAPs suggest that they function downstream of Lem3p-Dnf1/2p. Consistently, the *rga1Δ rga2Δ* mutant exhibited PE exposure only during early stages of budding, as the wild-type cells did (data not shown) (see **Figure 2A**), suggesting that Dnf1/2p translocated PE in the *rga1Δ rga2Δ* mutant with normal timing in the cell cycle.

**The GAP Activities of Rga1p and Rga2p Are Stimulated by
Phosphatidylethanolamine and Phosphatidylserine, but Inhibited by
Phosphatidylinositol-4,5-bisphosphate**

The results described above, taken together with previous observations that Cdc42p GAPs are localized to polarized sites including the bud tip (Caviston et al., 2003; our unpublished results), raise a possibility that Rga1p and Rga2p are maintained in an inactive state at the apical growth site and become activated when Lem3p-Dnf1/2p translocates phospholipids to the cytosolic leaflet. If inwardly translocated phospholipids including PE directly regulate Rga1p and Rga2p, stimulation of GAP activity by these phospholipids might be expected. To examine effects of various phospholipids on the GAP activity, the lipid-modified Cdc42p and the full-length Cdc42p GAPs were expressed and affinity-purified from Sf9 insect cells and yeast cells, respectively, as a fusion protein with glutathione S-transferase (GST).

We first examined whether the intrinsic GTPase activity of Cdc42p was affected by various phospholipids, but it was not affected by phosphatidylcholine (PC), PE, phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) (**Figure S4**). We then examined the GAP activity of Rga1p and Rga2p in the presence of these phospholipids. We chose a dose of GAP, which gave a 40~50% stimulation of GTP hydrolysis further from intrinsic GTP hydrolysis. Consistent with our idea, PE significantly stimulated GAP activity of both Rga1p and Rga2p (**Figure 4**). PS also stimulated the activity of Rga GAPs to an extent similar to that by PE. It was reported that inward translocation of

fluorescence (NBD)-labeled PS was minimally affected in the *lem3Δ* and *dnf1Δ dnf2Δ* mutants (Kato et al., 2002; Pomorski et al., 2003; Hanson et al., 2003). However, a recent report suggests that PS is also exposed on the outer leaflet of the plasma membrane in the *dnf1Δ dnf2Δ* mutant; the *dnf1Δ dnf2Δ* mutant exhibited hypersensitive growth to a PS-binding drug papuamide B (Parsons et al., 2006). Thus, Dnf1/2p seems to inwardly translocate PS as well as PE. In contrast, although PC was suggested to be flipped by Lem3p-Dnf1/2p (Kato et al., 2002; Pomorski et al., 2003; Hanson et al., 2003), it did not exhibit any effects. PI was also potent for activation of GAP-stimulated GTP hydrolysis. Asymmetric distribution of PI across the plasma membrane, in which PI is enriched in the cytoplasmic leaflet like PE and PS, was reported in many cell types including yeast (Boon and Smith, 2002). However, it is unknown whether Lem3p-Dnf1/2p inwardly translocates PI. Further studies are needed to clarify the physiological significance of this effect in the apical-isotropic switch. PA exhibited a weak stimulation of GAP activity. Because the uptake of NBD-PA was unaffected by *dnf1Δ dnf2Δ* mutations (Pomorski et al., 2003), PA may be involved in another cell function *via* Rga GAPs rather than in the apical-isotropic switch. We confirmed that the observed stimulation of GTP hydrolysis was not due to enhanced release of GTP from Cdc42p by using [α - 32 P]GTP instead of [γ - 32 P]GTP (data not shown).

Surprisingly, PI(4,5)P₂ completely inhibited the GAP-stimulated GTP hydrolysis (**Figure 4**), suggesting that Rga1p and Rga2p may be maintained in an inactive state by PI(4,5)P₂ at the apical membrane domain. We examined whether

PI(4,5)P₂ was concentrated at the bud tip using GFP-fused PI(4,5)P₂-specific PH domain from rat phospholipase C (PLC)- δ 1 (Fujii et al., 1999), but the GFP-PH was uniformly localized to the plasma membrane in both wild-type and *lem3* Δ mutant cells (data not shown). However, it could be envisaged that the apical membrane domain was not accessible to exogenously expressed GFP-PH due to occupancy by endogenous polarity regulators. Boi1p, a PH domain-containing polarity regulator implicated in PI(4,5)P₂ binding (Hallett et al., 2002), remained localized to the bud tip in the *lem3* Δ mutant (Supplemental Results and **Figure S5**). In addition to the role for localization of polarity regulators, PI(4,5)P₂ in the apical membrane domain may keep Rga1p and Rga2p in an inactive state.

We also examined effects of phospholipids on GAP activity of Bem3p. The GAP activity was weakly stimulated by PS and PI, and was not stimulated by PE, whereas it was completely inhibited by PI(4,5)P₂ (**Figure S6**). Although the involvement of Bem3p in the apical-isotropic switch needs to be clarified, negative regulation by PI(4,5)P₂ seems to be a common mechanism to Cdc42p GAPs in budding yeast.

Discussion

We herein propose a model in which the apical-isotropic switch is triggered by inward translocation of phospholipids such as PE across the plasma membrane by Lem3p-Dnf1/2p. Because the activation of Clb/Cdc28p is a prerequisite for the apical-isotropic growth switch (Lew and Reed, 1995), Clb/Cdc28p may act upstream of

Lem3p-Dnf1/2p. Interestingly, Dnf2p was identified in a proteomic library as one of proteins that were directly phosphorylated by Clb2p/Cdc28p (Ubersax et al., 2003).

We hypothesized that the Dnf1/2p-mediated phospholipid translocation dissolves the apical membrane domain by stimulating GTP hydrolysis of Cdc42p through GAPs, and this was supported by biochemical data. Consistently, yeast cell lysates from *CLB2*-overexpressing cells (post-apical-isotropic switch) contained more Cdc42p GAP activities than those from *SWE1*-overexpressing cells (pre-apical-isotropic switch), whereas GDP/GTP exchange activity was unchanged (D. Lew, personal communication). Stimulation of Rga GAP activities was not due to ionic nature of phospholipids; Rga1p and Rga2p were activated by neutral (PE) as well as anionic (PS and PI) phospholipids. PE and PS may interact with distinct domains of Rga proteins for activation, but other plausible mechanisms are: (1) that phospholipids affect the structure of Cdc42p-GTP to enhance the interaction with a GAP and (2) that phospholipids enhance the interaction between Cdc42p and a GAP by interacting with both proteins. Because our preparation of GST-Cdc42p is a lipid-modified form, it is able to associate with phospholipid vesicles (our unpublished results). It should be noted that effects of phospholipids on the GAP activities of the mammalian p190 Rho/Rac GAP also required C-terminal prenylation of Rho and Rac (Ligeti et al., 2004).

Although expression of a PI(4,5)P₂-specific PH domain did not reveal concentration of PI(4,5)P₂ at the bud tip, Boi1p, which had been implicated in PI(4,5)P₂ binding, was localized to the bud tip in the *lem3Δ* mutant. In mammalian cells, the same GFP-fused PH domain from rat PLC- δ 1 was localized to the cytokinesis site

where PE was exposed on the outer leaflet (Emoto et al., 2005). A subtle increase of PI(4,5)P₂ in the bud tip membrane or a normal level of PI(4,5)P₂ as in other regions of the plasma membrane may be sufficient for inhibition of Rga GAPs. Because no known lipid-binding motif has been found in Rga 1/2p (Smith et al., 2002), a novel domain that interacts with PI(4,5)P₂ may be present, or PI(4,5)P₂ may prevent the prenylated Cdc42p from interacting with GAPs.

Taken together, phospholipid flipping by Dnf1/2p would result in activation of Rga GAPs *via* two different mechanisms: (1) stimulation of GAP activity by PE and PS; (2) release from inhibition by dispersal of PI(4,5)P₂ from the bud tip. Although PC did not affect GAP activity of Rga 1/2p, inward translocation of PC may contribute to the second mechanism. In mammalian systems, physiological significance for modulation of GAP activity by phospholipids remains obscure. Because there are multiple type 4 P-type ATPases (thus putative flippases) in mammals (Paulusma and Oude Elferink, 2005), the reported effects of phospholipids (Ligeti et al., 2004; Ahmed et al., 1995) may reflect regulation by flippase-mediated transbilayer changes in phospholipid composition.

The PE content in the outer leaflet of the apical membrane needs to be examined in future studies to substantiate that a sufficient amount of PE for GAP activation would be translocated to the inner leaflet by Dnf1/2p. The results of chemical labeling experiments suggested that only 1% of the total plasma membrane PE was exposed on the outer leaflet in a wild-type asynchronous culture (Pomorski et al., 2003). However, it is possible that PE would not be exposed on the outer leaflet once

it has been cleared from the outer leaflet by Dnf1/2p during the apical-isotropic switch.

In our estimation, the surface area of the Bio-Ro-stained region represents approximately 0.2% of the total plasma membrane surface area of the cells in a wild-type asynchronous culture (number of examined cells > 100; our unpublished results). If one combines this estimation with the untested assumption that the concentrations of PE within the outer leaflet are vanishingly small everywhere but this apical membrane region, the PE content in the outer leaflet at the bud tip would correspond roughly to 80% of the total bud tip PE.

How does PE become exposed on the outer leaflet of the plasma membrane during the bud site assembly? One possibility is that the proteins that transport phospholipids from the inner to outer leaflet (e.g. ABC transporters) (Pohl et al., 2005) are specifically activated at the incipient bud site. Alternatively, this outward PE transport mechanism could operate at late Golgi membranes to form bud site-directed vesicles with PE enriched in the non-cytosolic leaflet. It should be noted that flippase activities of Dnf1/2p seem to be suppressed when they are localized on post-Golgi secretory vesicles (Alder-Baerens et al., 2006). It is an interesting question whether this outward PE transport is coupled with the assembly of Cdc42p at the cytosolic leaflet. To maintain the PE-exposed membrane domain, continuous polarized flow of those vesicles or sustained outward movement of phospholipids may counteract the diffusion of PE from the bud tip. However, another interesting possibility is that the domain might be composed of a lipid microdomain such as “lipid rafts”. A specific membrane domain that requires sphingolipids for its formation has been suggested to

exist at the tip of mating projection (Proszynski et al., 2006). This domain may share a similarity in lipid composition with that at the bud tip, as PE exposure was also observed in the tip of the mating projection (Iwamoto et al., 2004).

The lipid composition in the cytoplasmic leaflet of the apical membrane domain also needs to be investigated, especially in the light of how Cdc42p is preferentially localized to this domain. Electrostatic interaction between the carboxy-terminal polybasic region (K¹⁸³KSKK¹⁸⁷) of Cdc42p and PI(4,5)P₂ might play an important role (Supplemental Results and Discussion and **Figure S7**).

It remains unknown how Cdc42p-GDP is dispersed from the bud tip after the apical-isotropic switch, but endocytosis is one plausible route: Irazoqui et al. (2005) have shown that dispersal of Cdc42p occurs by endocytosis during bud site assembly, and that once a bud has formed, Cdc42p becomes more resistant to dispersal.

Although the *dnf1Δ dnf2Δ* mutant is only slightly defective in endocytosis as assayed by the uptake of FM4-64 at 15°C (Pomorski et al., 2003), phospholipid flipping by Dnf1/2p may indirectly regulate endocytosis of Cdc42p; the apical membrane domain may prevent Cdc42p from being endocytosed, or Cdc42p-GTP itself might be resistant to endocytosis.

This study presents what is, to our knowledge, the first direct evidence for the involvement of phospholipid translocation at the plasma membrane in the directional regulation of polarized membrane growth. It has also been suggested that PE redistribution to the inner leaflet of the plasma membrane at the cleavage furrow is a crucial step for cytokinesis in mammalian cells (Emoto et al., 1996). Local changes of

lipid distribution in the inner leaflet of the plasma membrane may be an important aspect for the spatial regulation of cortical cytoskeletons and their regulators during formation of cell polarity or cell morphogenesis.

Experimental Procedures

Yeast methods

Details on yeast media, genetic techniques, strains, and plasmids are given in Supplemental Experimental Procedures.

Microscopy

Cells were usually grown at 30°C to logarithmic phase and then cultured at 18°C for 3 h before observation. Longer cultivation (12 h) at 18°C essentially gave the same phenotypes in the *lem3* mutants (data not shown). To observe GFP-tagged Cdc24p in living cells, collected cells were resuspended in synthetic medium, and observed immediately. To simultaneously observe GFP-tagged proteins and DNA, cells were fixed for 10 min at 18°C with formaldehyde (Wako Pure Chemicals, Osaka, Japan) to a final concentration of 3.7 or 5% in the medium, washed three times with phosphate buffered saline (PBS), and then stained with a DNA-specific dye, 4',6-diamidino-2-phenylindole (DAPI, Sigma Chemical, St. Louis, MO) as described previously (Kawasaki et al., 2003). To observe filamentous actin, formaldehyde-fixed cells were stained with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin

(Sigma Chemical) as described previously (Saito et al., 2002), and then with DAPI. Immunofluorescence staining of Cdc42p was performed essentially as described using rabbit anti-Cdc42p polyclonal antibodies diluted at 1:500 (Kozminski et al., 2000). Bound antibodies were visualized with Cy3-conjugated donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted at 1:500. After ten washes in PBS, cells were stained with DAPI. Staining of PE in the outer leaflet of the plasma membrane was performed with a biotinylated Ro09-0198 peptide (Bio-Ro) as described (Iwamoto et al., 2004) with the modifications (see Supplemental Experimental Procedures). Cells were observed under a Nikon ECLIPSE E800 microscope (Nikon Instec, Tokyo, Japan) and images were acquired using a cooled digital charge-coupled device camera (C4742-95-12NR; Hamamatsu Photonics, Hamamatsu, Japan) as described (Saito et al., 2004). Observations are based on the characterization of >100 cells.

GAP assay

Details on protein and liposome preparations are given in Supplemental Experimental Procedures. To load Cdc42p with GTP, GST-Cdc42p (2.7 pmol) was incubated for 20 min at 20°C with 7,000-10,000 cpm/pmol [γ -³²P]GTP or [α -³²P]GTP (PerkinElmer Life and Analytical Sciences, Boston, MA) in 10 μ l of reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol [DTT], 1 mg/ml BSA, 0.1% Na cholate, 1 μ M GTP). After incubation, GTP-bound Cdc42p was diluted twofold into stop buffer (50 mM Tris-HCl, pH 7.5, 19 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 0.1%

Na cholate), and maintained on ice. GST-Rga1p (0.27 pmol), GST-Rga2p (0.64 pmol), GST-Bem3p (1.51 pmol) or control GST (18.5 pmol) was preincubated for 10 min at 20°C in 30 µl of the liposome suspension. To this mixture, GTP-bound Cdc42p was added (50 µl total volume), and incubated for the indicated time at 20°C. The reaction was terminated by adding 1 ml of cold wash buffer (50 mM Tris-HCl, pH 7.5, 25 mM MgCl₂), followed by rapid filtration through 0.45-µm nitrocellulose membranes (PerkinElmer Life and Analytical Sciences). The membranes were thoroughly washed with 14 ml of cold wash buffer. Protein-bound [³²P]GTP remaining on the filter was measured by Cerenkov counting using an LSC-5100 liquid scintillation counter (Aloka Co, Tokyo, Japan). All three GAPs stimulated release of ³²P from [γ -³²P]GTP-loaded Cdc42p in a dose- and time-dependent manner (data not shown). The ³²P release was not observed when [α -³²P]GTP was used instead of [γ -³²P]GTP, indicating that the observed ³²P release was due to hydrolysis of GTP, but not due to nucleotide dissociation (data not shown).

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Figure Legends

Figure 1. Hyperpolarized growth in the *lem3Δ* mutant. Wild-type (YKT39) and

lem3Δ mutant (YKT496) cells were grown at 30°C to logarithmic phase and then cultured at 18°C for 3 h. **(A)** Cell morphology in an asynchronous culture. **(B)** Organization of the actin cytoskeleton in late mitotic cells. After fixation, cells were stained with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin and 4',6-diamidino-2-phenylindole (DAPI) for filamentous actin and nuclei, respectively. **(C)** Localization of Cdc42p in late mitotic cells. Cdc42p was visualized by immunofluorescence with the anti-Cdc42p antibody. Large-budded cells with divided nuclei, recognized by DAPI staining as in **(B)**, were categorized as having Cdc42p polarized to the bud tip (black), localized to the bud cortex (dark gray), and delocalized (light gray) ($n > 100$). The bud-neck localization pattern was excluded from the categorization (see text). Representative images are shown in the left panels. Bars, 5 μm .

Figure 2. Phosphatidylethanolamine (PE) is exposed on the plasma membrane at the apical growth site. To visualize PE in the outer leaflet, wild-type (YKT39) and *lem3Δ* mutant (YKT496) cells were treated with 100 μM biotinylated Ro-peptide (Bio-Ro) for 3.5 h and with 30 μM Bio-Ro for 15 min, respectively, on ice. After fixation, cells were spheroplasted and labeled with FITC-conjugated streptavidin. Cdc42p was separately stained by immunofluorescence. **(A, B)** Localization of PE and Cdc42p during bud growth in the wild type **(A)** and the *lem3Δ* mutant **(B)** grown at 18°C. Cells were visually assigned as unbudded **(i)**, tiny- to small-budded ($\sim 1/3$ mother cell length) **(ii)**, small- to medium-budded ($1/3 \sim 1/2$ mother cell length) **(iii)**,

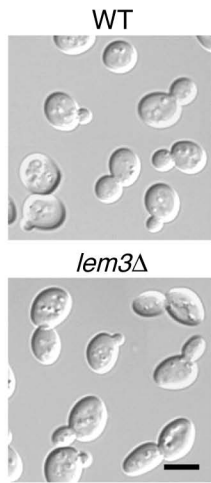
medium-budded ($1/2 \sim 2/3$ mother cell length) (**iv**), and large-budded ($2/3 \sim 1$ mother cell length) (**v**). (**C**) The PE exposure and the Cdc42p polarization at the bud tip during the prolonged apical growth in G2-arrested cells. DLY384 (*GAL1p-CLB1 clb1Δ clb2Δ clb3Δ clb4Δ*) cells were grown to logarithmic phase in a rich medium containing galactose (YPGA) at 30°C and then incubated in YPDA for 2 h at 30°C. Bars, 5 μm.

Figure 3. Hyperpolarized growth is caused by Cdc42p-GTP. (**A - C**) Suppression of hyperpolarized growth in the *lem3Δ* mutant at 18°C by the overexpression of Cdc42p GTPase-activating proteins, N-terminally truncated-Bem3p containing the GAP domain and full-length Rga1p. The *lem3Δ* mutant cells (YKT496) carrying a plasmid YEplac195 (vector), YEp24-*BEM3ΔN* (pKT1364), or YEplac195-*RGA1* (pDLB1537) were cultured in synthetic medium without uracil. (**A**) Morphology of transformed cells. (**B**) Polarized localization of Cdc42p. Late mitotic cells ($n > 100$) were categorized as described in **Figure 1C**. (**C**) Localization of Cdc42p in an unbudded- to tiny-budded cell phase. (**D and E**) Hyperpolarized growth in the *rga1Δ rga2Δ* mutant. (**D**) Morphology of the *rga1Δ rga2Δ* mutant (YKT1340) at 18°C. (**E**) Polarized localization of Myo2p-GFP in late mitotic cells of the wild type (YKT512), the *lem3Δ* mutant (YKT1324), and the *rga1Δ rga2Δ* mutant (YKT1341) at 18°C. Late mitotic cells ($n > 100$) were categorized as described in **Figure 1C**. Images in the upper panel are the bud-tip localization patterns of Myo2p-GFP in the *rga1Δ rga2Δ* mutant. Bars, 5 μm.

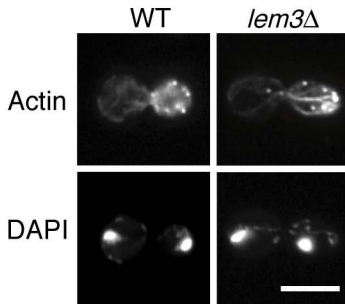
Figure 4. Effects of various phospholipids on GAP activities of Rga1p and Rga2p. [γ - ^{32}P]GTP-bound Cdc42p was incubated with GST-Rga1p for 15 min or GST-Rga2p for 20 min at 20°C in the presence or absence (-) of phospholipids at a final concentration of 210-290 μM . After incubation, GTP hydrolysis was assayed by measuring the radioactivity of [γ - ^{32}P]GTP bound to Cdc42p using a nitrocellulose-filtration method. Results are expressed as a percentage of the relative radioactivity to the intrinsic GTP hydrolysis during incubation, which was measured in the presence of a control GST protein instead of an Rga protein. The data are the mean $\pm$ SD of at least three independent experiments. * $P < 0.01$ in comparison to the no phospholipid condition as determined by Student's *t* test. Lipids used were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂).

Figure 1

A



B



C

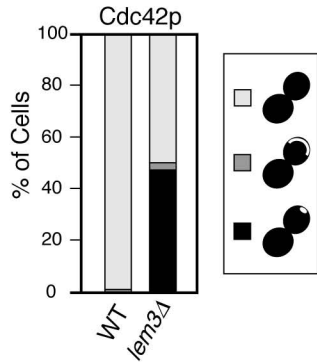
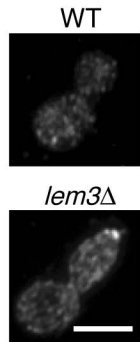
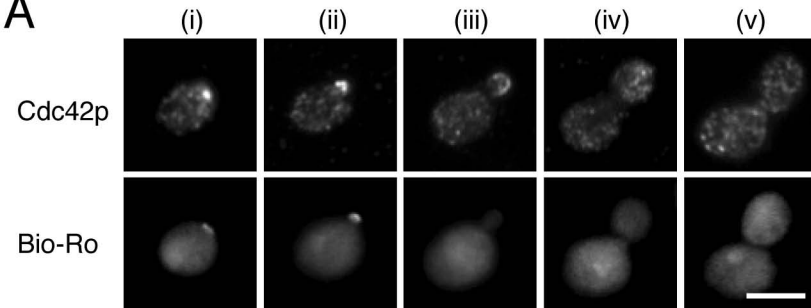
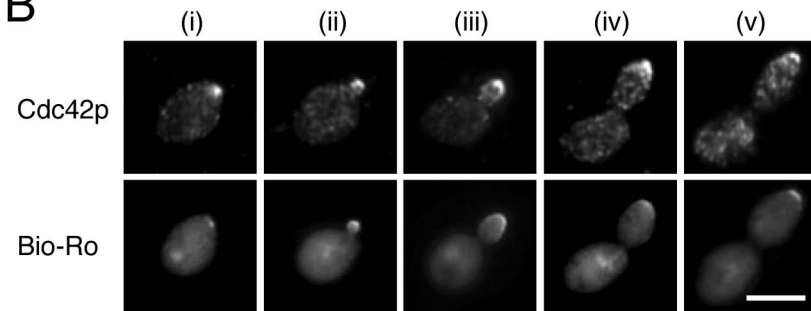


Figure 2

A



B



C

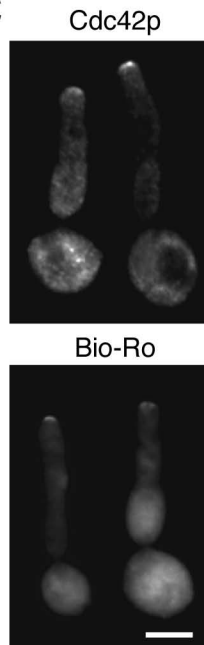


Figure 3

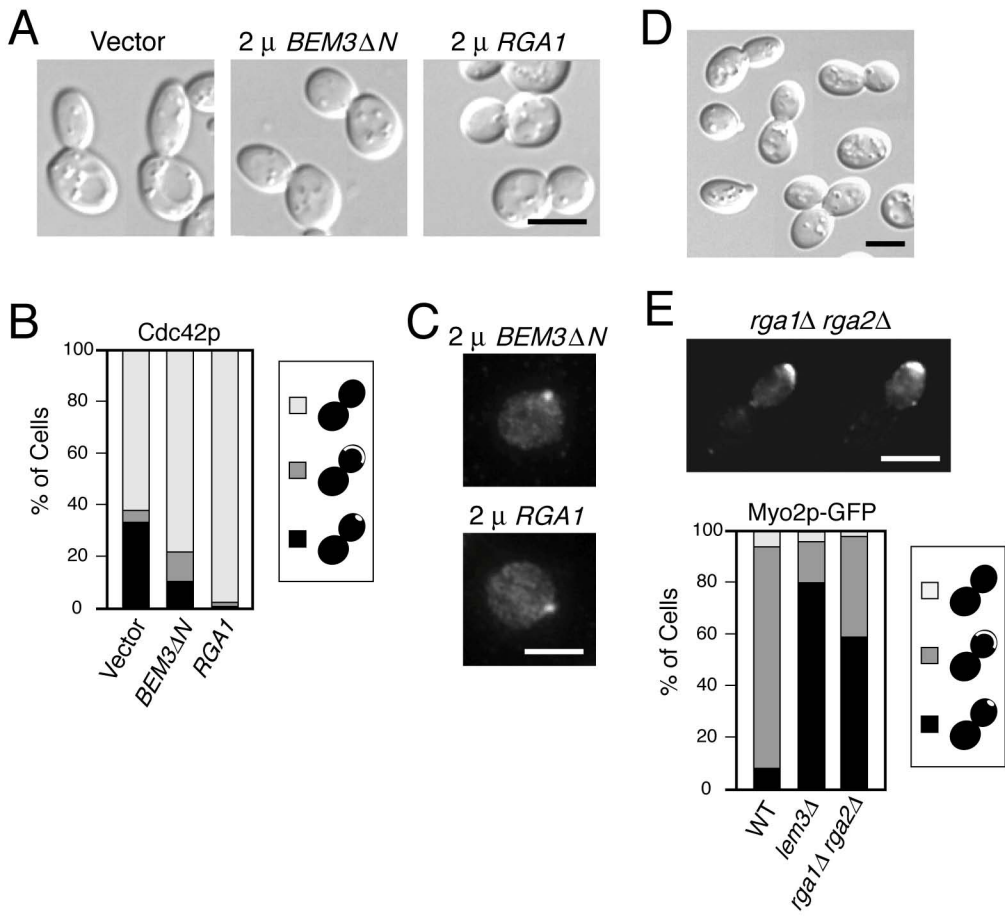


Figure 4

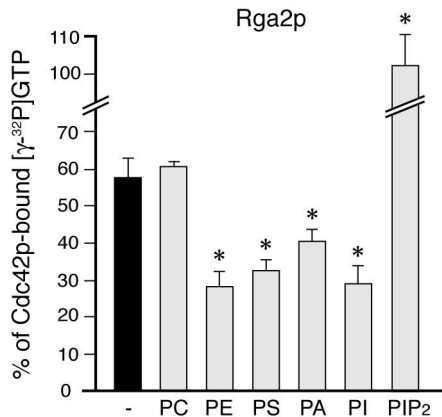
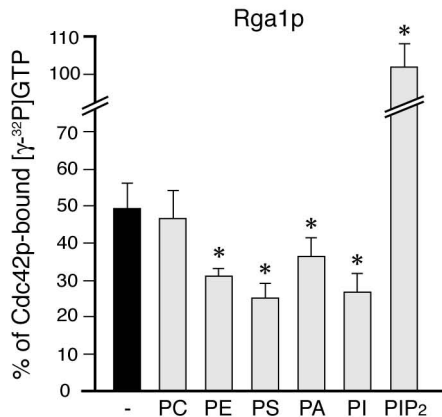
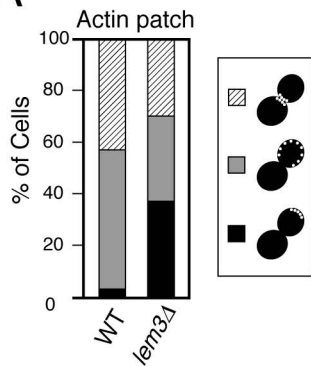
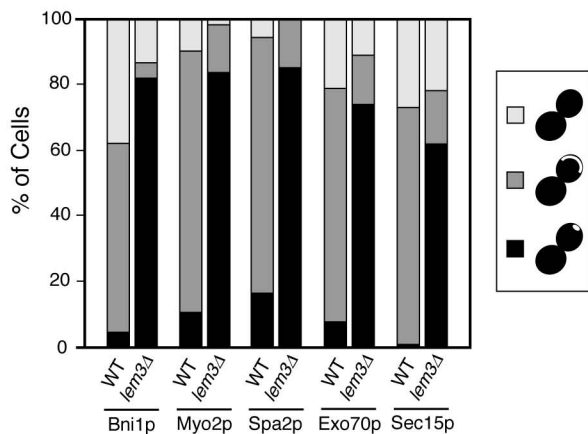


Figure S1

A



B



C

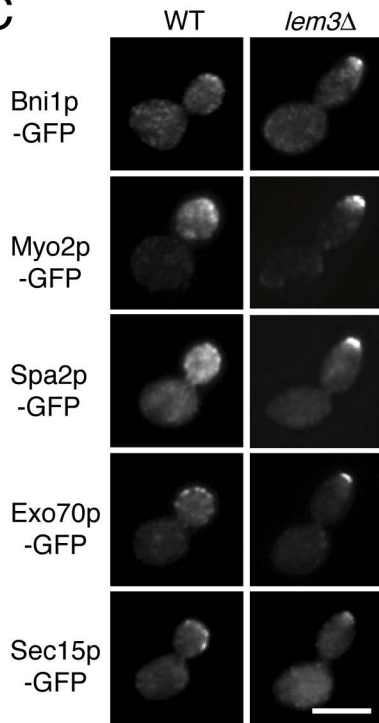


Figure S2

lem3Δ bni1Δ

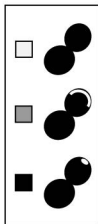
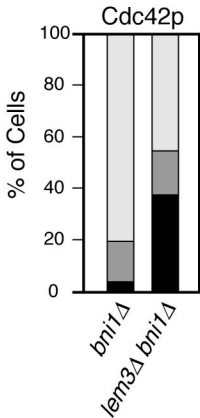
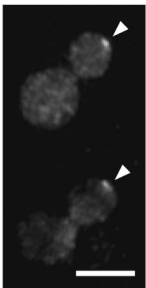
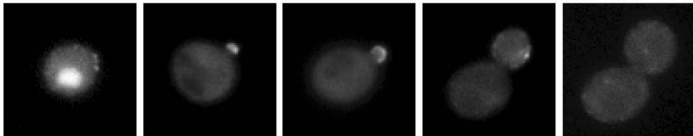


Figure S3

WT



lem3Δ



Figure S4

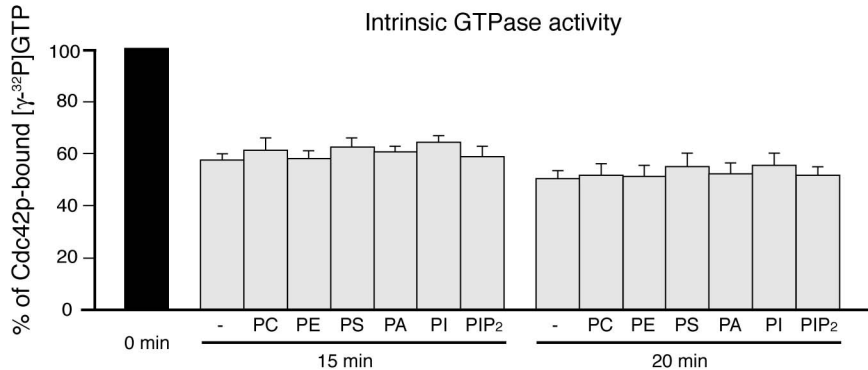


Figure S5



lem3Δ

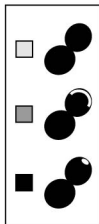
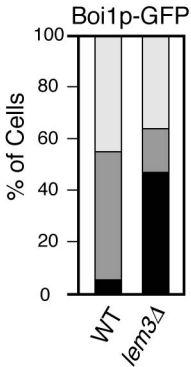


Figure S6

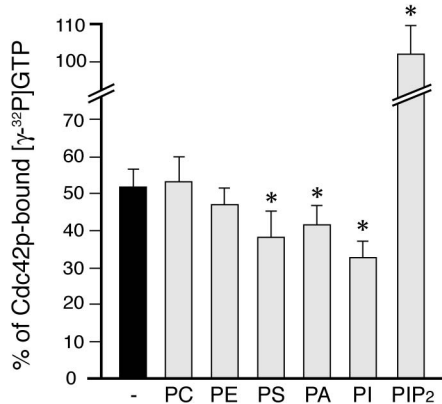
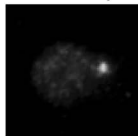


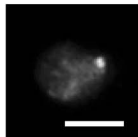
Figure S7

A

Cdc42p

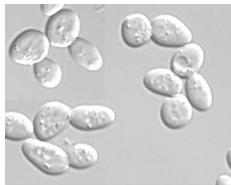


Cdc42^{K183-187Q}p

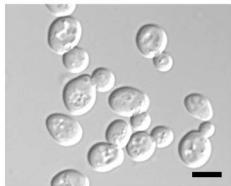


B

CDC42



cdc42^{K183-187Q}



C

