Exo-endocytic trafficking and the septin-based diffusion barrier are required for the maintenance of Cdc42p polarization during budding yeast asymmetric growth

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ABSTRACT Cdc42p plays a central role in asymmetric cell growth in yeast by controlling actin organization and vesicular trafficking. However, how Cdc42p is maintained specifically at the daughter cell plasma membrane during asymmetric cell growth is unclear. We have analyzed Cdc42p localization in yeast mutants defective in various stages of membrane trafficking by fluorescence microscopy and biochemical fractionation. We found that two separate exocytic pathways mediate Cdc42p delivery to the daughter cell. Defects in one of these pathways result in Cdc42p being rerouted through the other. In particular, the pathway involving trafficking through endosomes may couple Cdc42p endocytosis from, and subsequent redelivery to, the plasma membrane to maintain Cdc42p polarization at the daughter cell. Although the endo-exocytotic coupling is necessary for Cdc42p polarization, it is not sufficient to prevent the lateral diffusion of Cdc42p along the cell cortex. A barrier function conferred by septins is required to counteract the dispersal of Cdc42p and maintain its localization in the daughter cell but has no effect on the initial polarization of Cdc42p at the presumptive budding site before symmetry breaking. Collectively, membrane trafficking and septins function synergistically to maintain the dynamic polarization of Cdc42p during asymmetric growth in yeast.

INTRODUCTION

Cell polarity is a universal theme in eukaryotes and is important for a wide range of biological processes, including cell division, directional migration, tissue organization, and organogenesis. Polarity is established through the asymmetric distribution of proteins and lipids at the plasma membrane (see McCaffrey and Macara, 2009; Nelson, 2009; Orlando and Guo, 2009). Many of the key components that control cell polarization are evolutionarily conserved. The budding yeast *Saccharomyces cerevisiae* reproduces by the asymmetrical growth of its daughter cell ("budding") and provides an excellent model system for the study of the molecular mechanisms of cell polarity.

Cdc42 is a member of the Rho family of small GTPases that plays a central role in cell polarity in most eukaryotic cells (Johnson, 1999; Etienne-Manneville, 2004; Park and Bi, 2007). In budding yeast, Cdc42p is found at both the plasma membrane and internal vacuolar compartments (Ziman *et al.*, 1993; Eitzen *et al.*, 2001; Muller *et al.*, 2001; Richman *et al.*, 2002). The localization of Cdc42p at specific domains of the plasma membrane is important for coordinating the polarized organization of actin cytoskeleton and membrane trafficking during polarized cell growth (for a review, see Pruyne *et al.*, 2004; Park and Bi, 2007). The early establishment of Cdc42p polarization (sometimes referred to as "symmetry breaking") has been intensively studied over these years, and two mechanisms are found to be important for the localization of Cdc42p at the presumptive sites of bud emergence. One involves an adaptor-based signaling system, including Bem1p, Cdc24p (the guanine nucleotide exchange factor for Cdc42p), and the Cdc42p

downstream effectors that provide a positive-feedback loop, which amplifies an initial stochastic cluster of Cdc42p signal to break symmetry (Butty et al., 2002; Irazoqui et al., 2003; Kozubowski et al., 2008; Howell et al., 2009). The other involves membrane trafficking along actin cables for the delivery of Cdc42p to the daughter cell (Wedlich-Soldner et al., 2003; Pruyne et al., 2004; Wedlich-Soldner et al., 2004; Zajac et al., 2005; Kozminski et al., 2006; Gao and Bretscher, 2009). These two interconnected systems account for the polarized localization of Cdc42p for symmetry breaking (Slaughter et al., 2009a).

At the early bud establishment stage, Cdc42p appears as a "cap" at the presumptive site of bud emergence. Once the bud appears, Cdc42p is localized at the tip of the bud, where it controls actin organization and secretory machinery for continued cell surface expanding. How Cdc42p is maintained at the daughter cell is an open question. It is possible that, like the early stages of budding, the directional transport of vesicles to the daughter cell continuously deliver Cdc42p to the bud. But the exocytic pathways that transport Cdc42p are uncharacterized. Also, the continuous delivery of Cdc42p by exocytosis may cause its diffusion along the cell cortex away from the bud. Removal of the laterally diffused Cdc42p by endocytosis may counteract this diffusion. However, whether endocytosis is sufficient to counteract the lateral dispersal is unknown.

In addition to endo-exocytic trafficking, another major factor that needs to be considered for the *maintenance* of Cdc42p localization is the septin barrier. After the initial establishment of Cdc42p polarity, the septins are recruited to the bud by Cdc42p and subsequently assemble into a ring between the mother and daughter cells (Barral et al., 2000; Iwase et al., 2006). It will be interesting to examine whether the septin barrier serves to prevent Cdc42p diffusion.

In this paper, we characterized the different trafficking routes that mediate the transport of Cdc42p to the daughter cell. Furthermore, We found that endo-exocytic trafficking and septins synergistically control the maintenance of Cdc42p polarization. The study of Cdc42p in the simple organism budding yeast may reveal intersecting factors and general principles underlying eukaryotic cell polarity.

RESULTS

Cdc42p remain polarized at the daughter cell plasma membrane when the exocytic routes are separately blocked

In the budding yeast, at least two distinct populations of secretory vesicles are responsible for transporting cargoes to the plasma membrane (Harsay and Bretscher, 1995; Harsay and Schekman, 2002). Named for one of the cell wall endoglucanases it carries, the first population is known as "Bgl2p vesicles." The second population was named "invertase vesicles," which contain cargoes such as the periplasmic enzymes invertase and acid phosphatase (Harsay and Bretscher, 1995). These two types of vesicles are delivered to the plasma membrane via different routes: the cargo of invertase vesicles is first sorted through endosomes, whereas Bgl2p vesicles are mostly originated from the *trans*-Golgi network (Harsay and Bretscher, 1995; Chuang and Schekman, 1996; Yuan et al., 1997; Ziman et al., 1998; Harsay and Schekman, 2002). To understand the nature of Cdc42p trafficking and ultimately the dynamics of its polarization, it is important to determine the role of these pathways on Cdc42p transport to the daughter cells.

Here we examined the maintenance of Cdc42p polarization in secretory mutants defective in both exocytic routes or specifically in one of them. The exocyst is an octameric protein complex containing Sec3p, Sec5p, Sec6p, Sec8p, Sec10, Sec15p, Exo70p, and Exo84p. It mediates the tethering of secretory vesicles to the plasma
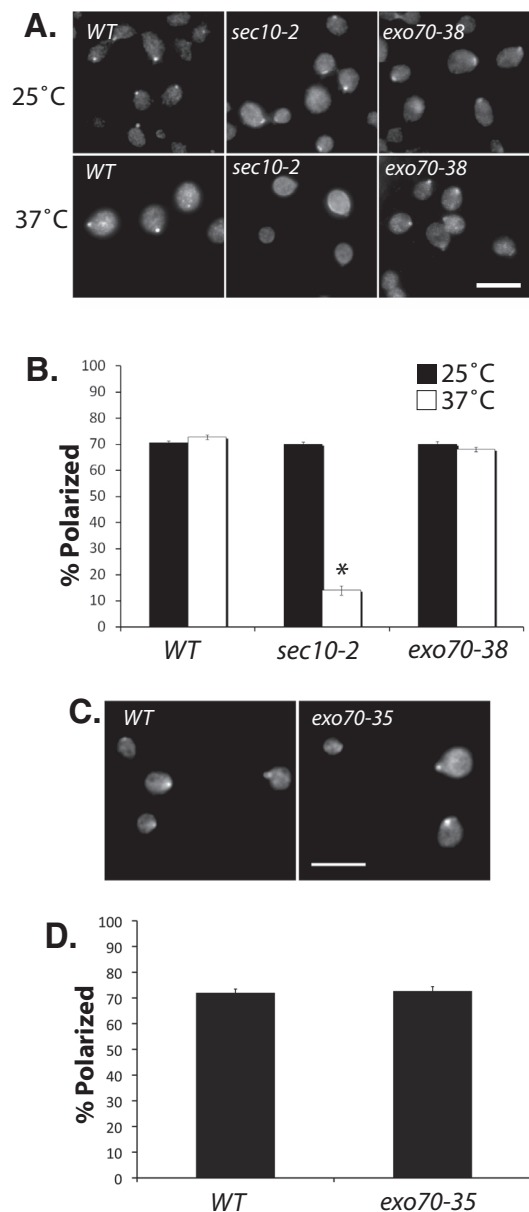

FIGURE 1: Cdc42p is depolarized in the *sec10* mutant but remains polarized in the *exo70* mutant cells. (A) Immunofluorescence staining of Cdc42p in wild-type (*WT*), *sec10-2*, and *exo70-38* cells. Cells were grown to log phase at 25°C and shifted to 37°C for 120 min, fixed, and immunostained for Cdc42p. Bar = 10 μm. (B) Quantification of Cdc42p polarization in *WT*, *sec10-2*, and *exo70-38* cells. Fifty small-budded cells were counted for each group (n = 3). Error bars represent standard error. *, p < 0.05. (C) *WT* and *exo70-35* cells were grown to log phase at 25°C, fixed, and immunostained for Cdc42p. Bar = 10 μm. (D) Quantification of Cdc42p polarization in the *WT* and *exo70-35* cells. Fifty cells were counted for each group (n = 3).

membrane prior to fusion (for a recent review, see He and Guo, 2009). Mutations in most of the exocyst subunits, such as Sec5p and Sec10p, lead to a block of both classes of secretory vesicles. Mutations in the Exo70p component, however, primarily affect Bgl2p secretion (He et al., 2007). Using affinity-purified anti-Cdc42p antibodies, we performed immunofluorescence to examine the localization of endogenous Cdc42p in these mutant cells. In the *sec10-2* mutant, Cdc42p became depolarized in most of the cells at the restrictive temperature (Figure 1, A and B). Similarly, Cdc42p is

depolarized in the *sec5-24* mutant soon after shifting to the restrictive temperature of 34°C (Supplemental Figure 1). For the *exo70* mutants, we use two different alleles. One is defective in secretion at 37°C (*exo70-38*) and another at 25°C (*exo70-35*), both of which are blocked in Bgl2p secretion, but not invertase secretion (He *et al.*, 2007). The *exo70-38* mutant has comparable growth defect to the *sec10-2* mutant at 37°C (He *et al.*, 2007). Surprisingly, Cdc42p is well polarized at the daughter cell in these *exo70* mutants (Figure 1, A and C, results quantified in Figure 1, B and D, respectively). The polarized localization of Cdc42p in the *exo70* mutants, but not the *sec10* and *sec5* mutants, suggests two possibilities. First, Cdc42p may be delivered primarily via the invertase vesicles rather than the Bgl2p vesicle to the bud. Second, Cdc42p may be transported through both pathways but rerouted to the invertase pathway when the Bgl2p pathway is blocked.
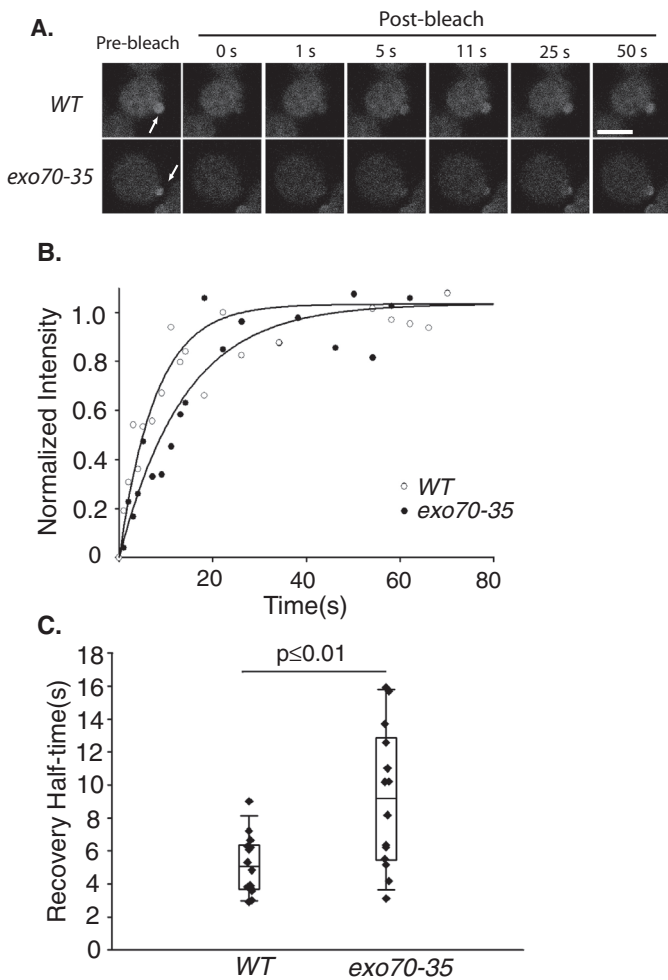
In the *exo70* mutants, although Cdc42p appears polarized, the delivery of Cdc42p to the bud may be delayed because only one of the exocytic routes is functional. We therefore compared green fluorescent protein (GFP)-Cdc42p targeting to the bud tip in *exo70-35* and wild-type cells using fluorescence recovery after photobleaching (FRAP). We photobleached the GFP-Cdc42p in the bud tip of small daughter cells and then measured the fluorescence recovery at various time points after the bleach (Figure 2A; representative quantification in Figure 2B). In the wild-type cells, the average half-time for GFP-Cdc42p fluorescence recovery was 5.2 s. However, the average half-time for GFP-Cdc42p fluorescence recovery was 9.1 s in the *exo70-35* mutant (Figure 2C). The longer half-life for GFP-Cdc42p polarization in the *exo70* mutant is consistent with its defect in one of the delivery routes.

To investigate the role of invertase vesicles in Cdc42p trafficking, we used the *vps1* and *pep12* deletion mutants. Vps1p is a dynamin-like protein in yeast that mediates the generation of vesicles from the Golgi for subsequent delivery of vesicles to prevacuolar compartments (PVC; equivalent to late endosomes) or vacuole (Nothwehr *et al.*, 1995; Bryant and Stevens, 1998). Pep12p (a.k.a. Vps6p) is a t-SNARE protein involved in the fusion of Golgi or early endosomal vesicles to PVCs (Becherer *et al.*, 1996; Bryant and Stevens, 1998, Gerrard *et al.*, 2000). It was demonstrated that deletion of either *VPS1* or *PEP12* blocks the biogenesis of invertase vesicles, and invertase is rerouted to the Bgl2p pathway for exocytosis (Gurunathan *et al.* 2002; Harsay and Schekman, 2002). We found that Cdc42p polarization was slightly disturbed in *vps1Δ* cells and normal in *pep12Δ* cells (Figure 3, A and C; quantified in Figure 3, B and D). We have also examined the GFP-tagged Cdc42p and internal FM 4-64 labeling of vacuoles in the same cells. In the *pep12Δ* cells, whereas the vacuoles had abnormal morphologies (often appearing

FIGURE 2: Targeting of Cdc42p to the bud is delayed in the *exo70-35* cells. GFP-Cdc42p fluorescence at the daughter cell was bleached, and the recovery of the fluorescence signal was monitored over time. (A) Montages of FRAP of the wild-type and *exo70-35* cells. The GFP-Cdc42p fluorescence recovers slower in the *exo70-35* mutant cell. Arrows point to the regions of photobleaching. Scale bar represents 2 μm. (B) Fitting curves of one set of FRAP experiments on the wild-type and *exo70-35* cells. The normalized fluorescence intensity is plotted over time. The curve fitting was performed using SigmaPlot as described in *Materials and Methods*. (C) Recovery half-times for the wild-type and *exo70-35* mutant cells were plotted. Bottom and top of the box are the lower and upper quartiles, respectively. The band near the middle of the box is the median. Whiskers represent standard errors. n = 14, p ≤ 0.01.

FIGURE 3: Polarized localization of Cdc42p in *vps1Δ* and *pep12Δ* cells. (A) The *WT* and *vps1Δ* cells were grown to log phase, fixed, and immunostained for Cdc42p. (B) Quantification of Cdc42p polarization in *WT* and *vps1Δ* cells. Fifty small-budded cells were counted for each group (n = 3). Error bars represent standard errors. *, p < 0.05. (C) The *WT* and *pep12Δ* cells were immunostained for Cdc42p. (D) Quantification of Cdc42p polarization in *WT* and *pep12Δ* cells. Fifty small-budded cells were counted for each group (n = 3). (E) Percentage of invertase secretion in *WT* and *vps1Δ* cells (n = 3). (F) Percentage of invertase secretion in *WT* and *pep12Δ* cells (n = 3). Error bars represent standard errors.

as a single, enlarged vacuole) consistent with its previous categorization as a class D *vps* mutant (Conibear and Stevens, 1998), GFP-Cdc42p remains polarized at the plasma membrane similar to the wild-type cells (Supplemental Figure 2).

Because cargoes of invertase vesicles can be rerouted to the Bgl2p pathway (Gurunathan *et al.* 2002; Harsay and Schekman, 2002), Cdc42p may still be able to reach the plasma membrane. We examined the secretion of invertase in these cells and found that it was also slightly affected in *vps1Δ* cells and normal in *pep12Δ* cells (Figure 3, E and F). The invertase secretion data are consistent with the rerouting model, in which it was demonstrated previously by density gradients that the invertase peak fractions were shifted to the lighter density vesicles carrying Bgl2p in *vps* mutants (Gurunathan *et al.* 2002; Harsay and Schekman, 2002).

Together, the analyses of the *exo70* mutants versus the *vps1Δ* and *pep12Δ* mutants suggest that Cdc42p can be rerouted between each of the exocytic routes. It would be ideal to test the localization of Cdc42p in *exo70 vps1Δ* or *exo70 pep12Δ* double mutant cells, in which both pathways are inhibited. However, *exo70* mutants are synthetic lethal with *vps1Δ* or *pep12Δ* (He *et al.*, 2007). On the other hand, the synthetic lethality suggests the vital role of both pathways in exocytosis and polarized cell growth.
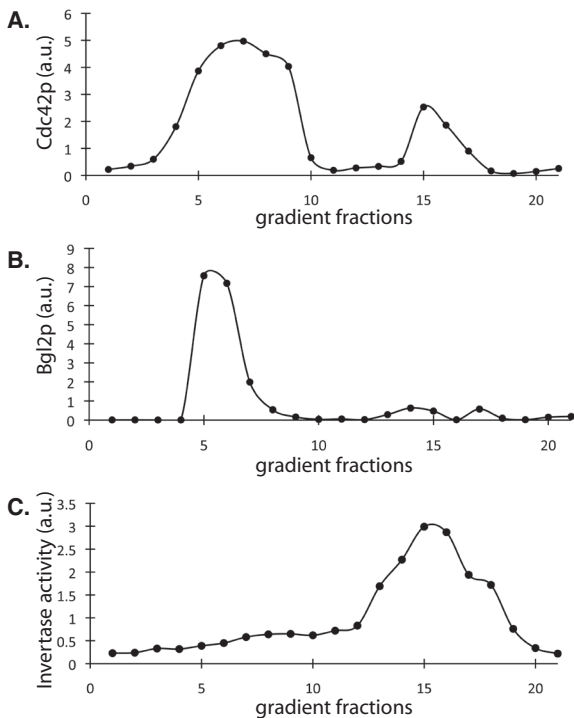

FIGURE 4: Analysis of Cdc42p association with different classes of secretory vesicles by Percoll density gradients. The *sec10-2* mutant cells, which have block in both Bgl2p and invertase secretion, were used to analyze Cdc42p association with vesicles. A *sec10-2* mutant strain with a copy of HA-Cdc42p integrated at its *URA3* locus on the chromosome was used for membrane preparation as described in *Materials and Methods*. The secretory vesicle preparation was then subjected to Percoll density gradients. The collected fractions were analyzed by Western blot to measure the distributions of HA-Cdc42p (A) and Bgl2p (B) or tested for invertase activity (C). Two major peaks in the HA-Cdc42p blot were observed: one around fractions 5–9, which corresponds to the lighter Bgl2p vesicles, and the other around fractions 14–17, which corresponds to denser invertase vesicles.

Detection of Cdc42p in both classes of exocytic vesicles

Invertase vesicles and Bgl2p vesicles have different densities (Harsay and Bretscher, 1995). Thus we examined the distribution of Cdc42p in these vesicles using density gradients. Secretory vesicle preparations from *sec10-2*, a mutant with defects in both Bgl2p and invertase secretion (He *et al.*, 2007), were subjected to Percoll density gradients. The collected fractions were tested for invertase enzymatic activity and analyzed by Western blot for the distributions of Bgl2p and Cdc42p. Two major peaks in the HA-Cdc42p blot were observed (Figure 4A), one around fractions 5-9, which comigrate with the lighter Bgl2 vesicles (Figure 4B), and the other around fractions 14-17, which correspond to invertase activity (Figure 4C). The amount of Cdc42p in the lighter fractions was greater than that in the denser fractions. It is known that the majority of the exocytic vesicles are the Bgl2p-containing light-density vesicles, and the invertase vesicles constitute a small percentage of the total vesicles (Harsay and Bretscher, 1995). Therefore, we cannot infer from these results that the majority of Cdc42p are carried via the Bgl2p vesicles. The cofractionation of Cdc42p with both invertase activity and Bgl2p indicates that both vesicle populations are involved in the transport of Cdc42p to the plasma membrane. This result is also consistent with the fluorescence microscopy data above (Figures 1–3).

The coupling of endocytosis and exocytosis during Cdc42p polarization

The association of Cdc42p with the invertase vesicles indicates that endosomal compartments are involved in Cdc42p trafficking. As such, it is likely that the Cdc42p on the plasma membrane is endocytosed as cargo into these endosomal compartments before being recycled back to the bud through another round of exocytosis. Indeed, previous studies using the F-actin disrupting drug latrunculin and Arp2/3 mutants have implicated endocytosis in Cdc42p polarization (Irazoqui *et al.*, 2005; Marco *et al.*, 2007). Also, for protein cargoes such as the v-SNARE protein Snc1p and the cell wall stress sensor Wsc1p, endocytosis was shown to be important for counteracting the lateral diffusion along the plasma membrane to maintain their polarized localization (Valdez-Taubas and Pelham, 2003; Piao *et al.*, 2007). Here we directly examined the localization of Cdc42p in endocytosis mutants. Rvs161p is a yeast amphiphysin homolog implicated in the internalization step of endocytosis (Lombardi and Riezman, 2001). End4p (a.k.a. Sla2p) is a transmembrane protein that links actin to clathrin coats for endocytosis (Raths *et al.*, 1993; Henry *et al.*, 2002; Kaksonen *et al.*, 2003). Surprisingly, we found that neither *end4-1* nor *rvs161Δ* had an effect on Cdc42p polarization (Figure 5A, results quantified in Figure 5B). This is different from the observation on the transmembrane cargo Snc1p, in which endocytosis is sufficient to counteract its lateral dispersal (Valdez-Taubas and Pelham, 2003). The difference between Cdc42p and Snc1p is likely caused by their different diffusion rates, which is explicated later in the *Discussion*.

Although the endocytosis mutants showed no difference in static Cdc42p localization in small-budded cells, we suspected that a block of Cdc42p recycling from the plasma membrane might affect the kinetics of Cdc42p polarization. Therefore, we performed FRAP experiments to compare GFP-Cdc42p localization in wild-type and *rvs161Δ* cells. We detected a longer half-life for GFP-Cdc42p fluorescence recovery at the bud tip in the *rvs161Δ* cells (6.9 s) versus the wild-type cells (5.1 s) (Figure 5, C and D). The data suggest that defects in endocytosis affect Cdc42p dynamics, likely by impinging on its recycling from the plasma membrane.
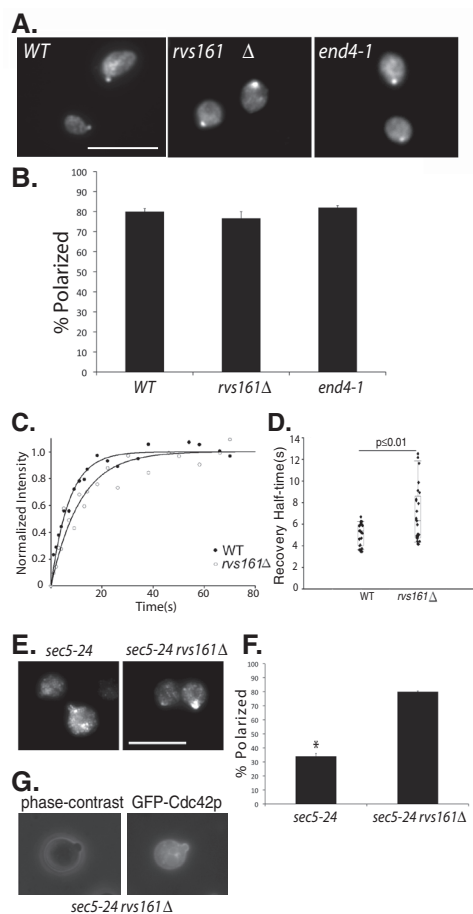
FIGURE 5: Cdc42p polarization in endocytosis mutants. (A) The wild-type, *rvs161Δ*, and *end4-1* cells were grown to log phase, incubated at 37°C for 90 min, fixed, and immunostained for Cdc42p. Cdc42p is polarized in the endocytosis mutants. Scale bar = 10 μm. (B) Quantification of Cdc42p polarization in wild-type, *rvs161Δ*, and *end4-1* cells. Fifty cells were counted for each group (n = 3). Error bars represent standard error. (C) FRAP curves of GFP-Cdc42p in wild-type and *rvs161Δ* cells. The normalized fluorescence intensity is plotted over time using SigmaPlot. (D) Recovery half-times for GFP-Cdc42p in wild-type and *rvs161Δ* cells. Bottom and top of the box are the lower and upper quartiles, respectively. The band near the middle of the box is the median. Whiskers represent standard errors. n = 25, p ≤ 0.01. (E) The *sec5-24* and *sec5-24 rvs161Δ* cells were grown to early log phase, shifted to 35°C for 90 min, fixed, and immunostained for Cdc42p. Although mostly depolarized in the *sec5-24* mutant, Cdc42p is well polarized in the *sec5-24 rvs161Δ* double mutant cells. (F) Quantification of Cdc42p polarization in the *sec5-24* and *sec5-24 rvs161Δ* double mutant cells. Fifty cells were counted for each group (n = 3). Error bars represent standard error. *, p ≤ 0.01. (G) The *sec5-24 rvs161Δ* cells were transformed with GFP-Cdc42, grown to early log phase, and shifted to 35°C for 90 min. GFP-Cdc42p was then observed using fluorescence microscopy. GFP-Cdc42p is enriched at daughter cell plasma membrane.

To better study the coupling of endocytosis and exocytosis in Cdc42p polarization, we constructed a *sec5-24 rvs161Δ* double mutant strain. We chose the *sec5-24* allele because it is partially defective in exocytosis at 34°C or 35°C. Rather than a complete blockage, a small amount of Cdc42p can still be delivered to the bud. In the *sec5-24 rvs161Δ* double mutant growing at 25°C, endocytosis is already inhibited due to the *rvs161* deletion. A partial block in exocytosis can then be introduced by a temperature shift

to 35°C. As shown in Figure 5, E and F, whereas Cdc42p was partially depolarized in the *sec5-24* mutant, it became well polarized in the *sec5-24 rvs161Δ* double mutant cells. This polarized localization pattern of Cdc42p was also observed using GFP-Cdc42p expressed in the *sec5-24 rvs161Δ* cells; GFP-Cdc42p is enriched at the daughter cell plasma membrane (Figure 5G). This result suggests that, in a system where the delivery of Cdc42p is partially affected, inhibition of endocytosis helps to preserve Cdc42p polarization, likely by preventing its continuous removal. The data further demonstrate a coupling of endocytosis and exocytosis during Cdc42p polarization.
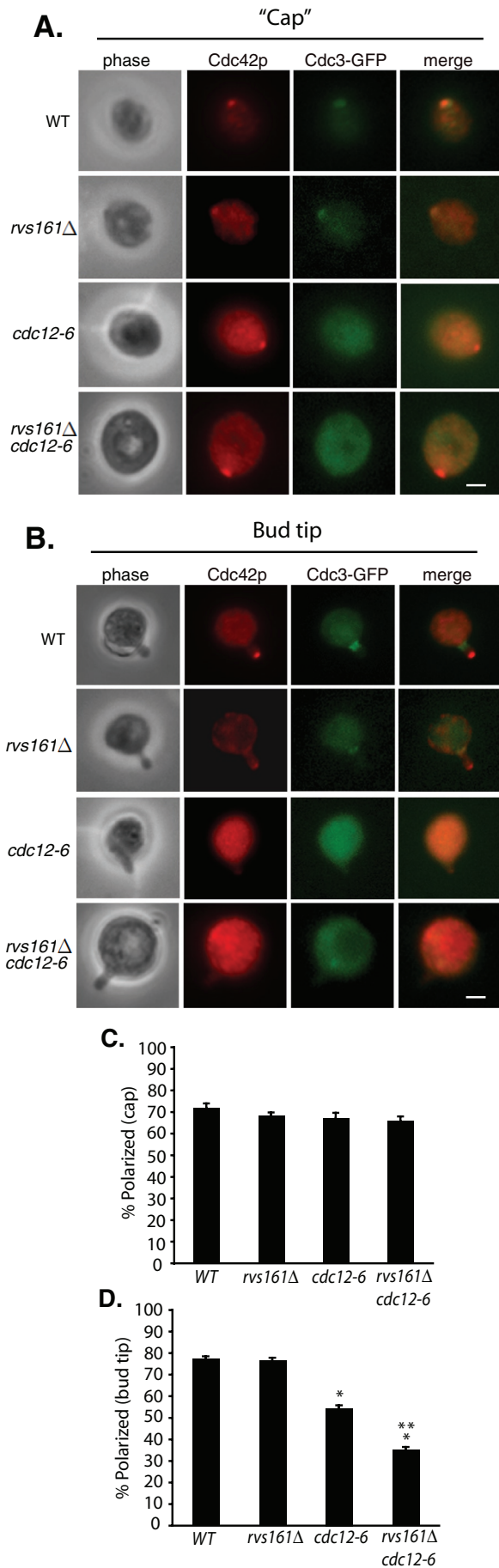
The septin ring is required for Cdc42p polarization
The observation that Cdc42p remains polarized in *rvs161* and *end4* mutants suggests that endocytosis is not sufficient to restrict Cdc42p from diffusion. What maintains Cdc42p at the bud in the endocytosis mutants? Septins are filamentous GTPases that form a diffusion barrier around the bud neck (Barral *et al.*, 2000). It is possible that the apparent Cdc42p polarization in endocytosis mutants results from the diffusion barrier imposed by the septins. Therefore, we examined Cdc42p localization in the *cdc12-6* mutant defective in Cdc12p, a component of septins. Cells were grown to early log phase at 25°C and shifted to 37°C for 90 min. In the *cdc12-6* and *cdc12-6 rvs161Δ* mutants, we did not detect significant changes in the polarized localization of Cdc42p at the presumptive budding site (the Cdc42p "caps") (Figure 6, A and C). However, in cells that had already generated small buds, Cdc42p became depolarized from the bud tips in some of the *cdc12-6* mutant cells. In the *cdc12-6 rvs161Δ* double mutants, most of the cells lost Cdc42p from the bud tip (Figure 6, B and D). These results suggest that the septin diffusion barrier is important for the maintenance of Cdc42p polarization in the daughter cells after symmetry breaking but does not affect the early Cdc42p clustering during the establishment of cell polarity. We have also examined the Cdc42p localization in cells released from G₀ phase synchronization. After temperature shifted to 37°C for 90 min, most of the cells generated small buds. We observed a similar decrease in Cdc42p polarization in *cdc12-6* and the *rvs161Δ cdc12-6* double mutants as we saw in small-budded cells of asynchronous culture (Supplemental Figure 3).

DISCUSSION
A major step in morphogenesis is the asymmetric distribution of a specific set of proteins at the cell cortex. Cdc42p is such a protein and is concomitantly a determinant of cell polarity. Studying the establishment and maintenance of Cdc42p localization is important to our understanding of the mechanisms of cell polarization. Previous studies using yeast as a model system have provided important insights into the early establishment of cell polarity (sometimes referred as "symmetry breaking"). Here biochemical and microscopic analyses of yeast mutants have allowed us to study factors that cooperatively maintain Cdc42p polarization after symmetry breaking.

We found that Cdc42p is transported along at least two exocytic routes in yeast (the "Bgl2p" and "invertase" pathways) (see model in Figure 7). Blocking either one of them does not inhibit Cdc42p transport, but rather results in the rerouting of Cdc42p to the other pathway. The invertase pathway involves trafficking through endosomal compartments, which may also mediate the recycling of proteins, such as the general amino acid permease (Gap1p) (Roberg *et al.*, 1997) and iron transporters (Strochlic *et al.*, 2007), to the cell surface in response to a changing environment. Our finding that some Cdc42p is delivered through endosomes is consistent with the

A. "Cap"

	phase	Cdc42p	Cdc3-GFP	merge
WT				
rvs161Δ				
cdc12-6				
rvs161Δ cdc12-6				

B. Bud tip

	phase	Cdc42p	Cdc3-GFP	merge
WT				
rvs161Δ				
cdc12-6				
rvs161Δ cdc12-6				

C. % Polarized (cap) — WT, rvs161Δ, cdc12-6, rvs161Δ cdc12-6

D. % Polarized (bud tip) — WT, rvs161Δ, cdc12-6 (*), rvs161Δ cdc12-6 (**)

FIGURE 6: Septins regulate Cdc42p polarization in small-budded cells but not during bud emergence. Wild-type, rvs161Δ, cdc12-6, and rvs161Δ cdc12-6 cells expressing Cdc3-GFP were grown to early log phase, shifted to 37°C for 90 min, and then immunostained for Cdc42p. (A) For cells during early bud emergence, Cdc42p remained polarized at the presumptive budding sites in both the wild-type and mutant cells. Scale bar represents 2 μm. (B) For cells that had generated small buds, Cdc42p was depolarized in some of the cdc12-6 mutant, and diffused in most of the rvs161Δ cdc12-6 double mutant cells. Scale bar represents 2 μm. Cdc42p polarization was quantified in cells during early bud emergence (C) and small-budded stage (D). Groups of 50 cells were counted (n = 3) for the wild-type and each mutant. Error bars represent standard error. *, p < 0.01 vs. WT cells. **, p < 0.01 between cdc12-6 and cdc12-6 rvs161Δ cells.

previous observation that a portion of Cdc42p was localized in vacuoles, the endosomal compartments of yeast (Ziman *et al.*, 1993; Eitzen *et al.*, 2001; Muller *et al.*, 2001; Richman *et al.*, 2002). In mammalian cells, intermediate compartments such as recycling endosomes have been well characterized not only for their recycling of transmembrane receptors (e.g., growth factors receptors) and metal transporters (e.g., transferrin receptors), but also for proteins that are critical to the generation of epithelial asymmetry such as E-cadherin (Desclozeaux *et al.*, 2008; Orlando and Guo, 2009) and integrins important for directional cell migration (Bretscher, 1996; Li *et al.*, 2005; Caswell and Norman, 2006; Jovi *et al.*, 2007; Balasubramanian *et al.*, 2010). Here endosomal trafficking serves to couple endocytosis of Cdc42p from, and subsequent exocytosis back to, the plasma membrane. Thus this recycling mechanism seems to be an evolutionarily conserved strategy in eukaryotic cell polarization.

Because many cargoes associated with invertase vesicles may originate from the plasma membrane through internalization, we examined the role of endocytosis at the plasma membrane in Cdc42p polarization. Our FRAP analyses of Cdc42p polarization in endocytosis mutants, and the use of the *sec5-24 rvs161Δ* double mutant, demonstrate that endocytosis is indeed tightly coupled to exocytosis in regulating the dynamics of Cdc42p polarization, which further underscores the importance of endosomal trafficking in Cdc42p recycling. Surprisingly, however, our microscopy study clearly shows that Cdc42p remains well polarized at the bud tip in yeast mutants that are defective in different steps of endocytosis (*end4-1* and *rvs161Δ*). This is in contrast to the scenario of transmembrane cargoes such as Snc1p and Wsc1p, in which blocking endocytosis is sufficient to cause depolarization (Valdez-Taubas and Pelham, 2003; Piao *et al.*, 2007). Therefore, although Cdc42p and these transmembrane proteins are all initially delivered in a polarized manner to the bud, their diffusion from the bud membrane is different. Based on previous studies, Cdc42p has a diffusion constant of 0.036 μm²/s, whereas Snc1p has a diffusion constant of 0.0025 μm²/s (Valdez-Taubas and Pelham, 2003; Marco *et al.*, 2007). The more than 10-fold faster diffusion rate of Cdc42p clearly calls for mechanisms that counteract lateral dispersion in addition to endocytosis. Cdc42p differs from Snc1p and Wsc1p in its mode of membrane association. Instead of being incorporated into the lipid bilayers via a transmembrane domain, Cdc42p is prenylated at a C-terminal cysteine residue for its membrane attachment (Johnson, 1999). It was recently clearly demonstrated that Rdi1p, which extracts Cdc42p from the membrane, mediates the fast recycling of Cdc42p during early bud emergence (Slaughter *et al.*, 2009b). However, Rdi1p alone is probably not sufficient for the maintenance of Cdc42p at the daughter cell because Cdc42p
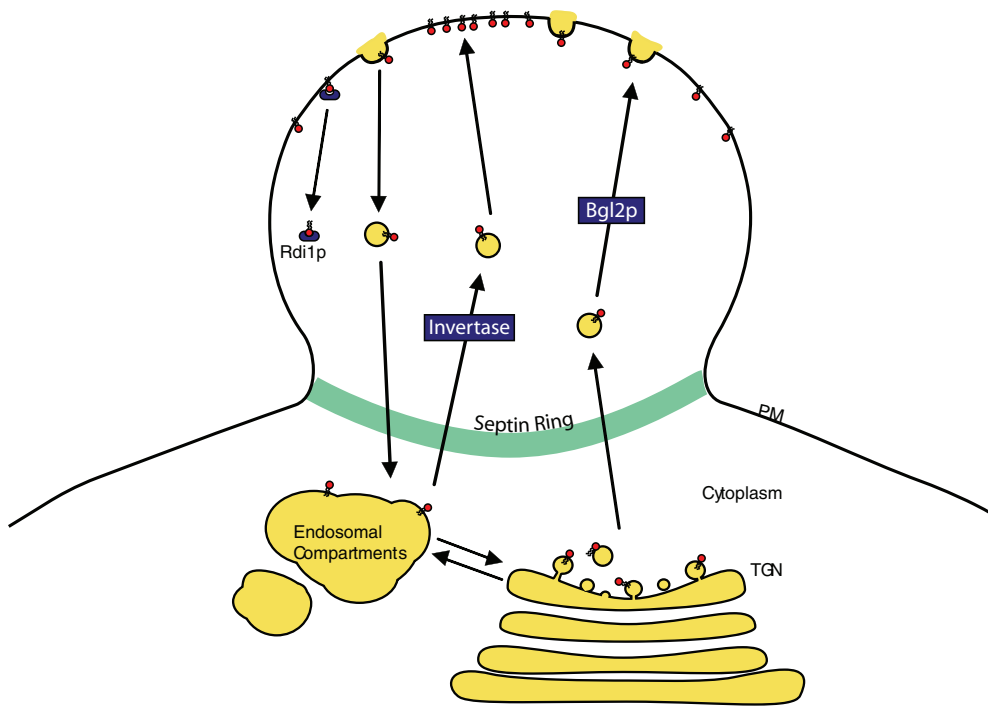
FIGURE 7: Schematic representation of the factors that regulate Cdc42p polarization in the daughter cell plasma membrane. Cdc42p is trafficked to the tip of the daughter cell through both the Bgl2p and invertase pathways. Once at the bud tip, Cdc42p may diffuse laterally along the plasma membrane. Endocytosis internalizes Cdc42p from the plasma membrane. The internalized Cdc42p can be carried as cargo back to the daughter cell through the endosomal compartments along the invertase pathway. Cdc42p can also be recycled from the daughter cell membrane by Rdi1p. The septin ring acts as a diffusion barrier to prevent Cdc42p from diffusing out of the daughter cell. PM, plasma membrane; TGN, *trans*-Golgi network. Cdc42p is indicated as small red circles with lipid tails.

of Cdc42p from the daughter cell. Future quantitative studies in live cells may reveal the dynamic interplays between the septin-based diffusion restriction and endocytosis-based recycling of Cdc42p during asymmetric cell growth.

In summary, we demonstrate that endo-exocytic coupling and septins synergistically contribute to the maintenance of Cdc42p at the daughter cell membrane (Figure 7). First, the delivery of Cdc42p involves at least two routes of exocytic trafficking to the cell surface. The route involving endosomal trafficking serves as a mechanism that couples the removal of Cdc42p from the cell surface with its subsequent redelivery to the daughter cell membrane. As the endo-exocytotic pathways are not sufficient for maintaining Cdc42p at the daughter cell, a diffusion barrier imposed by septins helps to further restrict the dispersal of Cdc42p along the plasma membrane. These factors may work with Rdi1p (Slaughter *et al.*, 2009b) to synergistically maintain Cdc42p polarization during asymmetrical growth of the yeast cell. Collectively, the multiple inputs to Cdc42p may fine-tune the spatial and temporal distribution of this master regulator of cell morphogenesis for polarized cell growth and possibly effective adaptation to the changing environments.

These regulatory mechanisms on small GTPases may be evolutionarily conserved in mammalian cells (Palamidessi *et al.*, 2008; Misaki *et al.*, 2010). Studies using budding yeast will continue to present important conceptual frameworks for future studies of cell morphogenesis in higher eukaryotic systems.

MATERIALS AND METHODS

Strains and growth conditions

The genotypes of the yeast strains used in this project are listed in Table 1. Standard methods were used for yeast media, growth, and genetic manipulations (Guthrie and Fink, 1991). For the generation of deletion mutants, primers targeting the promoter and terminator regions of *RVS161*, *VPS1*, and *PEP12* were used to amplify the DNA fragments containing *KanMX* as well as the flanking sequences of *RVS161*, *VPS1*, and *PEP12* from deletion strains generated in the *S. cerevisiae* Genome Deletion Project. The obtained PCR products were transformed into the wild-type, *sec5-24*, or *cdc12-6* yeast cells to disrupt the *RVS161*, *VPS1*, or *PEP12* genes from the chromosome, and cells were selected by growing on plates containing 100 μg/ml G418. Expression of GFP-tagged Cdc42p in yeast was performed by integrating GFP-Cdc42 into the *ura3-52* locus (*YIp211-GFP-CDC42*, *URA3*, cut with *Stu*I for integration). To observe septins by fluorescence microscopy, a plasmid containing the GFP-tagged septin subunit *CDC3* (*CEN*, *URA3*) was transformed into yeast cells. The culturing and G$_0$ synchronization of yeast cells by saturating growth were performed as previously described (Zhang *et al.*, 2008).

is still polarized to the bud in *rdi1Δ* cells (Irazoqui *et al.*, 2005; Guo lab, unpublished data). Thus additional factors may exist that work together with endocytosis and Rdi1p to maintain Cdc42p polarization after bud formation.

A cortical barrier can serve to maintain polarity, following the premise that a protein is initially positioned within the barrier by delivery, clustering, and/or local amplification. A major event after bud emergence is the assembly of septin rings between the mother and nascent daughter cells. We speculate that the septins may carry out this barrier function after the establishment of Cdc42p polarity. A previous time-lapse microscopy study demonstrated that septins first appear at the presumptive budding site, and ring assembly takes place minutes after; the polarized recruitment of septins depends on Cdc42p, but not Rsr1p, a ras family small GTPase involved in bud site-selection (Iwase *et al.*, 2006). Here we found that septins are important for the maintenance of Cdc42p in small daughter cells but not in cells undergoing early bud emergence. These data support a role of the septins in the *maintenance* of Cdc42p localization. In the *cdc12-6* mutant, some cells lost Cdc42p polarization at the bud tip. Further disruption of *RVS161* in the septin mutant led to diffusion of Cdc42p. This result suggests that both recycling and the cortical barrier work together to maintain Cdc42p polarization. It is possible that the speed of Cdc42p diffusion along the plasma membrane is faster than the actions of endocytosis; as such, a septin-based barrier becomes necessary for the maintenance of the polarity. The involvement of a barrier function also suggests the existence of a positive feedback loop between Cdc42p and septins: although Cdc42p is needed for the polarized recruitment of septin, the formation of the septin ring in turn helps to restrict the diffusion

Strain	Genotype
NY179	*Mat a ura3-52 leu2-3, 112*
NY776	*Mat α ura3-52 leu2-3, 112 sec5-24*
NY784	*Mat α ura3-52 leu2-3, 112 sec10-2*
GY1132	*Mat a trp1, leu2, his3, ura3, lys2*
GY1201	*Mat α ura3-52 leu2-3 112 his3Δ200 trp1 exo70::HIS3 exo70-38,TRP1 CEN*
GY2836	*Mat a ura3-52 leu2-3 112 trp1 his3Δ200 exo70::HIS3 exo70-35, TRP1 CEN*
GY2955	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 vps1:KanMX*
GY2956	*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pep12:KanMX*
GY3016	*Mat a ura3-52 leu2-3, 112 rvs161::KanMX*
GY3017	*Mat α ura3-52 leu2-3, 112 sec5-24 rvs161::KanMX*
GY3500	*Mat α ura3-52, leu2, his3, trp1, end4-1*
GY3503	*Mat a leu2, ura3, cdc12-6*
GY3586	*Mat a leu2, ura3, cdc12-6 rvs161::KanMX*
GY3601	*Mat α ura3-52 leu2-3 112 his3Δ200 trp1 pep12::LEU2*
GY3605	*Mat α ura3-52 leu2-3, 112 sec5-24, GFP-Cdc42 URA3*
GY3606	*Mat α ura3-52 leu2-3, 112 sec5-24 rvs161::KanMX, GFP-Cdc42 URA3*
GY3607	*Mat α leu2-3112 ura3-52 trp1 his3Δ200 pep12::LEU2, GFP-Cdc42 URA3*
GY3608	*Mat a ura3-52 leu2-3, 112, Cdc3-GFP CEN URA3*
GY3609	*Mat a ura3-52 leu2-3, 112 rvs161::KanMX , Cdc3-GFP CEN URA3*
GY3610	*Mat a leu2, ura3, cdc12-6, Cdc3-GFP CEN URA3*
GY3611	*Mat a leu2, ura3, cdc12-6 rvs161::KanMX , Cdc3-GFP CEN URA3*

TABLE 1: Yeast strains and genotypes.

Immunofluorescence microscopy

Yeast strains were grown overnight at 25°C in yeast-extract peptone dextrose (YPD). The final cell density (A$_{600}$) was between 0.5 and 1.0 U. The cells were then shifted to their restrictive temperatures for various times as indicated. After the shift, the cells were fixed with 4.4% formaldehyde. The cells were then transferred to isotonic media and the cell walls removed by zymolyase and β-mercapthoethanol. After attachment to 8-well microslides treated with poly-lysine (Carlson Scientific, Peotone, IL), the spheroplasts were treated with 0.5% SDS in phosphate-buffered saline (PBS) buffer containing 1 mg/ml bovine serum albumin (PBS/bovine serum albumin [BSA]) for 5 min. The cells were then washed 10 times with 50 µl per well of PBS/BSA, and nonspecific binding was blocked for 30 min in the same buffer. The incubation with polyclonal antibody against Cdc42p (diluted 1:500, kind gift from Keith Kozminski) was performed overnight at 4°C. After 10 washes with PBS/BSA, the cells were incubated for 1 h at room temperature with AlexaFluor594-conjugated goat anti–rabbit immunoglobulin (Ig)G antibody (diluted 1:1000; Molecular Probes, Eugene, OR). The cells were washed 10 times with PBS/BSA, and the slides were then mounted with Fluoro-Gel mounting media (Electron Microscopy Sciences,

Hatfield, PA). The digital images were captured by a fluorescence microscope (DM IRB; Leica, Bannockburn, IL) using a 100× objective and a high-resolution charge-coupled device camera (model ORCA-ER; Hamamatsu Photonics, Bridgewater, NJ).

FRAP experiments

The dynamic localization of GFP-tagged Cdc42p in small-budded cells was analyzed by FRAP. Cells were grown to log phase in synthetic complete media (SC) at 25°C. One or two milliliters of culture was pelleted and resuspended in 20 µl fresh SC media. Slides containing agar pads were prepared with 2% agar dissolved in SC medium containing appropriate amino acids. Next, 2 µl of the suspension was dropped onto the agar pads; Corning number 1½ coverslips were placed on top of the agar pads; and a mixture of Vaseline, lanolin, and candlewax (modified form of VALAP) was used to seal in the cells and prevent drying. Bleaching and imaging was performed using a Leica TCS SL laser scanning confocal microscope using an oil-immersion 63× objective. Small buds were photobleached using an Argon laser at 488 nm, and pre- and post-bleach images were collected over time. GFP-Cdc42p intensity was normalized to the intensity at final equilibrium, which is calculated as the average intensity from 60 s to 80 s. For each time point, the background intensity was subtracted and the data were normalized by dividing each data point by the average prebleached value. The fluorescence recovery over time was analyzed by SigmaPlot. Curve is fit by the equation $I = a(1 - e^{-bt})$, where I is the normalized intensity. The recovery half-times were calculated from the graph by measuring the time when the cells reach half of the final intensity.

Separation of vesicles by Percoll density gradients

Vesicle fractionation was performed as previously described (Harsay and Bretscher, 1995; He *et al.*, 2007). Briefly, the *sec10-2* mutant cells carrying a copy of HA-Cdc42p at the *URA3* locus on the chromosome were grown overnight in YPD media. Yeast cells (100-200 A$_{600}$ U) were washed with YPD media and shifted to 37°C for 90 min in YPD media containing 0.1% glucose to induce invertase production and post-Golgi secretory accumulation. After washing in cold PBS containing 10 mM sodium azide, cells were resuspended in spheroplast medium (1.4 M sorbitol, 50 mM KPi [pH 7.5], 10 mM azide, 40 mM β-mercapthoethanol) with 0.15 mg/ml of zymolyase-100T and converted to spheroplasts during a 45-min incubation at 37°C. The spheroplasts were resuspended in 1.5 ml of lysis buffer (0.8 M sorbitol in 10 mM triethanolamine, 1 mM EDTA [pH 7.2]) containing protease inhibitors, homogenized by 20 strokes with a 2-ml Wheaton tissue grinder, and centrifuged at 500 × g for 3 min. The supernatant was spun at 10,000 × g in an Eppendorf 5415R bench top centrifuge for 10 min at 4°C, and then further centrifuged at 100,000 × g in a TLA100 rotor (Beckman Coulter, Brea, CA) for 1 h at 4°C to give the final supernatant and pellet. These pellets were fractionated on 20–55% Percoll step gradients (4 ml in TLA100.3 tubes), and 160-µl fractions were collected for density measurement, invertase quantification and Western blot analysis with anti-Sec4p and anti-Bgl2p polyclonal antibodies. Invertase assays were performed as described previously (Novick *et al.*, 1980).

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