Actin cables and the exocyst form two independent morphogenesis pathways in the fission yeast

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ABSTRACT **Cell morphogenesis depends on polarized exocytosis. One widely held model posits that long-range transport and exocyst-dependent tethering of exocytic vesicles at the plasma membrane sequentially drive this process. Here, we describe that disruption of either actin-based long-range transport and microtubules or the exocyst did not abolish polarized growth in rod-shaped fission yeast cells. However, disruption of both actin cables and exocyst led to isotropic growth. Exocytic vesicles localized to cell tips in single mutants but were dispersed in double mutants. In contrast, a marker for active Cdc42, a major polarity landmark, localized to discreet cortical sites even in double mutants. Localization and photobleaching studies show that the exocyst subunits Sec6 and Sec8 localize to cell tips largely independently of the actin cytoskeleton, but in a *cdc42* and phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2)–dependent manner. Thus in fission yeast long-range cytoskeletal transport and PIP_2-dependent exocyst represent parallel morphogenetic modules downstream of Cdc42, raising the possibility of similar mechanisms in other cell types.**

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INTRODUCTION

Polarized exocytosis is a fundamental cell biological process in which secretory vesicles are transported by motor proteins along a polarized cytoskeleton and tethered, docked, and fused at a landmark-defined surface region. Fusion of vesicles with the plasma membrane relies on the formation of a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Sudhof and Rothman, 2009), whose assembly is facilitated by the exocyst, an eight-subunit tethering complex initially identified in the budding yeast (TerBush *et al.*, 1996; He and Guo, 2009).

The budding yeast model system has been instrumental in defining a simple linear sequence of events for polarized exocytosis (Pruyne *et al.*, 2004). Activated Cdc42 forms the initial cortical polarizing cue, which activates actin nucleators of the formin family to build polarized actin cables (Evangelista *et al.*, 1997, 2002; Imamura *et al.*, 1997; Sagot *et al.*, 2002; Dong *et al.*, 2003). These cables form tracks for the vectorial transport of myosin V–driven exocytic vesicles toward the site of polarization (Pruyne *et al.*, 1998). At the plasma membrane, vesicles are tethered by the exocyst complex and undergo SNARE-mediated exocytosis for cell morphogenesis. Localization of exocyst components to the growth site relies on two distinct actin cable–dependent and independent mechanisms. Two exocyst subunits, Sec3 and Exo70, directly bind to Cdc42 and the phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP_2; Finger *et al.*, 1998; Boyd *et al.*, 2004; He *et al.*, 2007; Zhang *et al.*, 2008). All other exocyst subunits are transported toward the site of polarization with exocytic vesicles along actin cables, where they join the other two subunits to form a functional tethering complex (Ayscough *et al.*, 1997; Pruyne *et al.*, 1998; Roumanie *et al.*, 2005; Zajac *et al.*, 2005; Zhang *et al.*, 2005). Thus polarized exocytosis is directly dependent on cytoskeletal organization. Accordingly, disruption of myosin V or exocyst components in budding yeast leads to a complete loss of polarized growth (Novick *et al.*, 1980; Johnston *et al.*, 1991).

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Abbreviations used: CRIB, Cdc42/Rac-interactive binding; DMSO, dimethyl sulfoxide; EMM, Edinburgh minimal media; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; LatA, latrunculin A; MBC, methyl benzimidazole-2-yl-carbamate; PIP_2, phosphatidylinositol 4,5-biphosphate; RFP, red fluorescent protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TRITC, tetramethylrhodamine B isothiocyanate.

To probe the conservation of cell morphogenesis mechanisms, we use the fission yeast *Schizosaccharomyces pombe*, which is phylogenetically distant from the budding yeast and displays distinct rodlike morphology. Here again, Cdc42 is likely to form the most upstream polarizing cue and activates the formin For3 for assembly of polarized actin cables from cell ends (Feierbach and Chang, 2001; Nakano *et al.*, 2002; Martin *et al.*, 2007; Rincon *et al.*, 2009). Along these cables, type V myosin, Myo52, transports vesicular cargoes containing the glucan synthases Bgs1 and Bgs4 necessary for cell wall remodelling and polarized growth at cell tips (Feierbach and Chang, 2001; Motegi *et al.*, 2001; Win *et al.*, 2001; Cortes *et al.*, 2005; Mulvihill *et al.*, 2006). Surprisingly, deletion mutants of *for3* or *myo52* are viable and, despite a partial loss of rod shape, cells remain competent for polarized cell growth. Fission yeast encodes two additional formins, Fus1 and Cdc12, and one other type V myosin, Myo51, but none of these have reported roles in polarized growth (Petersen *et al.*, 1995; Chang *et al.*, 1997; Motegi *et al.*, 2001; Win *et al.*, 2001). Cells with gene disruption in essential components required for actin cable formation, such as profilin and tropomyosin, are likewise able to polarize growth (Balasubramanian *et al.*, 1992, 1994). Microtubules are also not required for polarized growth, although they provide spatial information in part by recruiting the formin For3 to cell tips (Martin *et al.*, 2005). Core components of the exocytic machinery, such as the v-SNARE Syb1, are essential for cell morphogenesis (Edamatsu and Toyoshima, 2003), but the exocyst, while essential for cell separation and viability, is dispensable for polarized cell growth (Wang *et al.*, 2002). These observations suggest there may be significant variations in the process of cell morphogenesis in diverse cell types.

Here we show that polarized cell growth can occur independently of cytoskeletal transport in fission yeast. We show that transport of vesicles along actin cables and their tethering at cell tips by the exocyst form two parallel morphogenesis modules, individually dispensable, but together essential, for polarized cell growth. Localization of these modules to cell tips is under control of Cdc42 but independent of each other. Specifically, localization of the exocyst is largely independent of the actin cytoskeleton but depends on PIP_2, indicating that membranes can polarize a cell in the absence of linear cytoskeletal elements.

RESULTS

Actin cable– and microtubule-independent polarized cell growth

We created a triple formin mutant with the nonessential *for3*Δ and *fus1*Δ deletions and the temperature-sensitive allele *cdc12-112*. Upon inactivation of *cdc12-112* at the restrictive temperature of 36°C for 5 h, the cells became elongated due to a block in cytokinesis but grew in a polarized manner as shown by fluorescent lectin staining (Figure 1, A and B). Actin cables were absent in these mutants, although short, wavy weakly-stained actin filaments could occasionally be detected (Supplemental Figure S1), as previously noted in *for3*Δ single mutants (Nakano *et al.*, 2002). Thus disruption of all formins does not prevent polarized cell growth. This finding is in agreement with the fact that loss-of-function alleles or deletion mutants of tropomyosin (*cdc8*) or profilin (*cdc3*), essential for actin cable stability and formation, respectively, still permit polarized growth (Balasubramanian *et al.*, 1992, 1994). In addition, we confirmed that complete deletion of all myosin V motors (in a *myo51*Δ *myo52*Δ double mutant) does not abrogate polarized cell growth (Motegi *et al.*, 2001; Win *et al.*, 2001; see Figure 1D). Addition of the microtubule-destabilizing drug methyl benzimidazole-2-yl-carbamate (MBC) led to defects in the placement of growth sites but did not abrogate polarized cell growth in either triple formin mutant, tropomyosin or profilin mutant, or double myosin V deletion mutant (Figure 1, C and E, and unpublished data). Thus fission yeast can polarize growth, albeit less efficiently, in the absence of both actin cable– and microtubule-based transport, suggesting that transport of vesicles along linear cytoskeletal filaments is not essential for this process.

To substantiate this finding, we examined the localization of exocytic vesicles by monitoring green fluorescent protein (GFP)–tagged cargoes (the glucan synthases Bgs1 and Bgs4) and v-SNARE (Syb1; Figure 1F and unpublished data). In wild-type cells, these fluorescent markers localize to sites of growth, labeling the cell end cortex, and mark internal sites that probably represent Golgi or recycling compartments (Cortes *et al.*, 2002, 2005; Edamatsu and Toyoshima, 2003). Consistent with the ability of actin cable-less cells to polarize growth, we found that exocytic vesicle markers still localized to cell ends and to internal sites in *for3*Δ, *myo51*Δ *myo52*Δ, and *cdc3-313* cells grown at restrictive temperature for 90 min, although cell end accumulation was in some cases less prominent (Figures 1F and 4B). In this and all subsequent experiments, we used the *for3*Δ single mutant, rather than the triple formin mutant, because *fus1* and *cdc12* did not appear to play any role in cell elongation or actin cable formation. In addition, fluorescence recovery after photobleaching (FRAP) experiments in which we photobleached GFP-Bgs1 signal from the cell tip either in the presence or absence of MBC showed no significant difference in the rate of recovery between wild-type and *myo51*Δ *myo52*Δ cells (Figure 1, G and H). Finally, we found that *for3*Δ or *myo51*Δ *myo52*Δ cells secreted acid phosphatase as efficiently as wild-type cells, suggesting no significant defect in general exocytosis (Figure 1I). Thus cells devoid of cytoskeletal long-range transport are able to polarize vesicles to cell ends, suggesting the existence of an independent pathway for polarized exocytosis.

Actin cables are dispensable for the localization of the exocyst to cell tips

In search of this second pathway, we investigated the localization of the exocyst. In wild-type cells, the exocyst localizes to septum and cell tips (Wang *et al.*, 2002). In *for3*Δ or *myo52*Δ cells, the exocyst subunit Sec6-GFP localized to cell ends and septum correctly (Figure 2A). We also imaged Sec8-GFP but discovered that *for3*Δ *sec8-GFP* or *myo52*Δ *sec8-GFP* cells displayed highly aberrant morphologies, suggesting that the Sec8-GFP fusion is not fully functional. However, even in these aberrantly shaped cells, Sec8-GFP localized correctly to identifiable tips and septa (Figure 2A). Further disruption of microtubules with MBC had no effect on either exocyst subunit localization (Supplemental Figure S2). These results suggest that exocyst localization to cell tips is independent of long-range cytoskeletal transport.

To test whether the exocyst was able to actively localize to cell tips in the absence of F-actin, we used *for3*Δ cells or wild-type cells treated with latrunculin A (LatA) to disrupt actin cables or all actin structures, respectively, and performed FRAP experiments. In control dimethyl sulfoxide (DMSO)–treated cells and *for3*Δ cells, Sec6-GFP fluorescence recovered rapidly at cell tips, with a half-time of about 5 s, and efficiently to about 65% of the initial signal (Figure 2, B, D, and E). The amount of signal bleached corresponded to about 25% of the total cellular signal, with a maximum possible recovery of 75%, suggesting a nonexistent or very small immobile fraction. Five minutes after

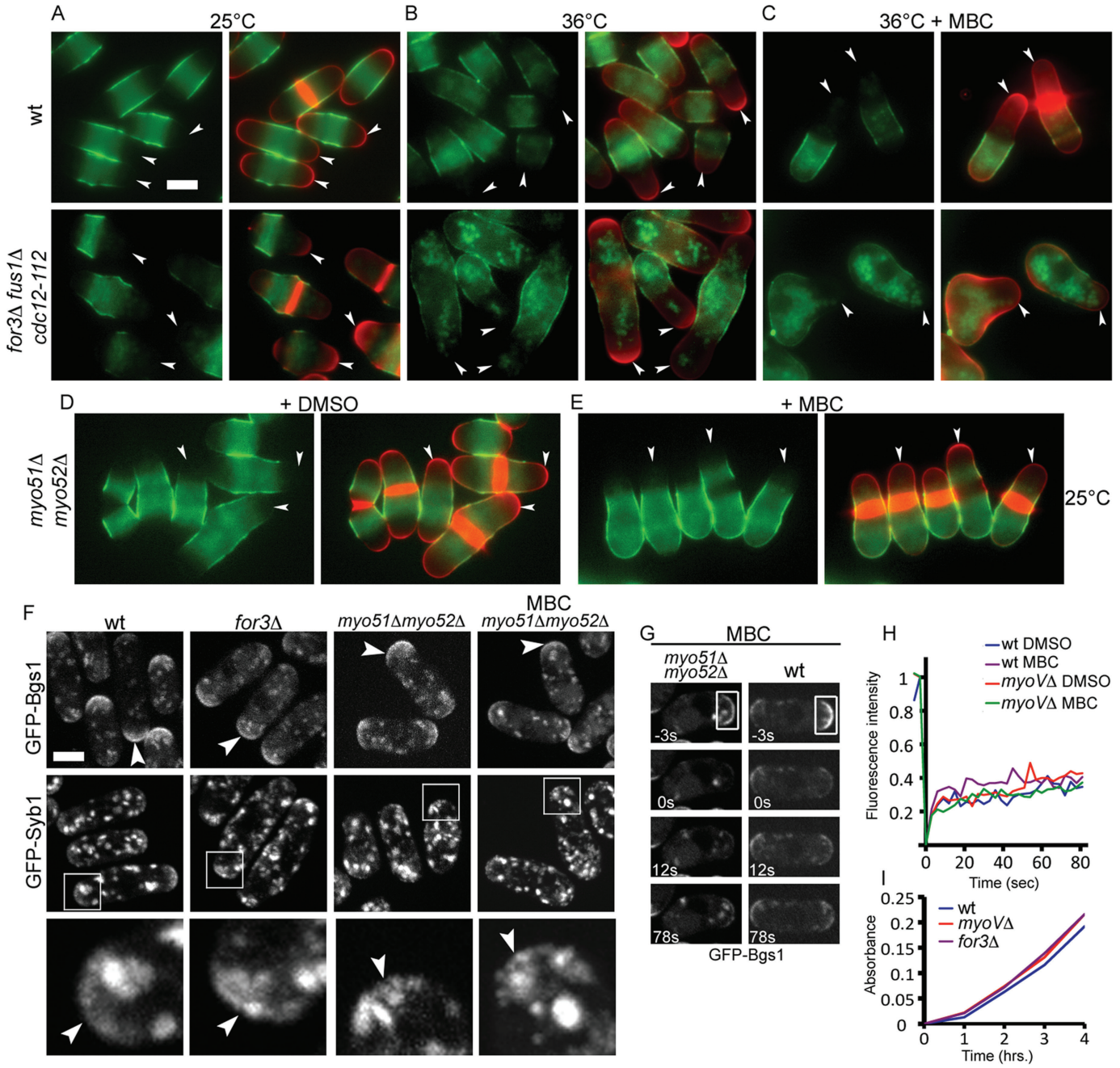

FIGURE 1: Polarized actin cables and microtubules are not required for polarized cell growth. (A–E) Wild-type, *for3Δ fus1Δ cdc12-112* and *myo51Δ myo52Δ* mutant cells stained with TRITC-lectin (green; all panels) and calcofluor (red; right panels). Cells were grown at 25°C (A, D, E) or 36°C (B, C) or were treated with MBC for 5 h as indicated (C and E). Clearing of fluorescent lectin at cell ends shows regions of growth (arrowheads). (F) GFP-Bgs1 and GFP-Syb1 signals in wild-type, *for3Δ*, and *myo51Δ myo52Δ* grown with or without MBC for 30 min. Arrowheads indicate enrichment of fusion proteins. Boxed regions are shown enlarged in the bottom row. (G) Recovery of GFP-Bgs1 to bleached tips in wild-type and *myo51Δ myo52Δ* in the presence of MBC. (H) Average recovery of GFP-Bgs1 to bleached tips (n = 15–20). GFP-Bgs1 shows a similar half-time of recovery of <5 s and immobile fraction of 60–66% in all strains. (I) Secretion of acid phosphatase in wild-type, *myo51Δ myo52Δ*, and *for3Δ* cells. Bars, 5 μm.

LatA addition, Sec6-GFP was still localized at or in the vicinity of cell tips in most cells. After 30 min, Sec6 was still cortical in all cells, and its localization was either polar or displaced from the cell tip toward cell sides (Figure 2C). Upon photobleaching, Sec6-GFP fluorescence recovered with a half-time of about 10 sec, even when it was displaced from cell tips, but only to about 40% of the initial signal (Figure 2, B–D). Thus complete actin depolymerization does not prevent the localization of Sec6 to cell tips but causes a larger immobile fraction. Because complete actin depolymerization disrupts actin patches in addition to cables, and thus endocytosis, one possibility is that the changes observed in Sec6 behavior may be due to endocytic recycling defects. Similarly, Exo70–red fluorescent protein (RFP) localized to cell tips independently of the actin

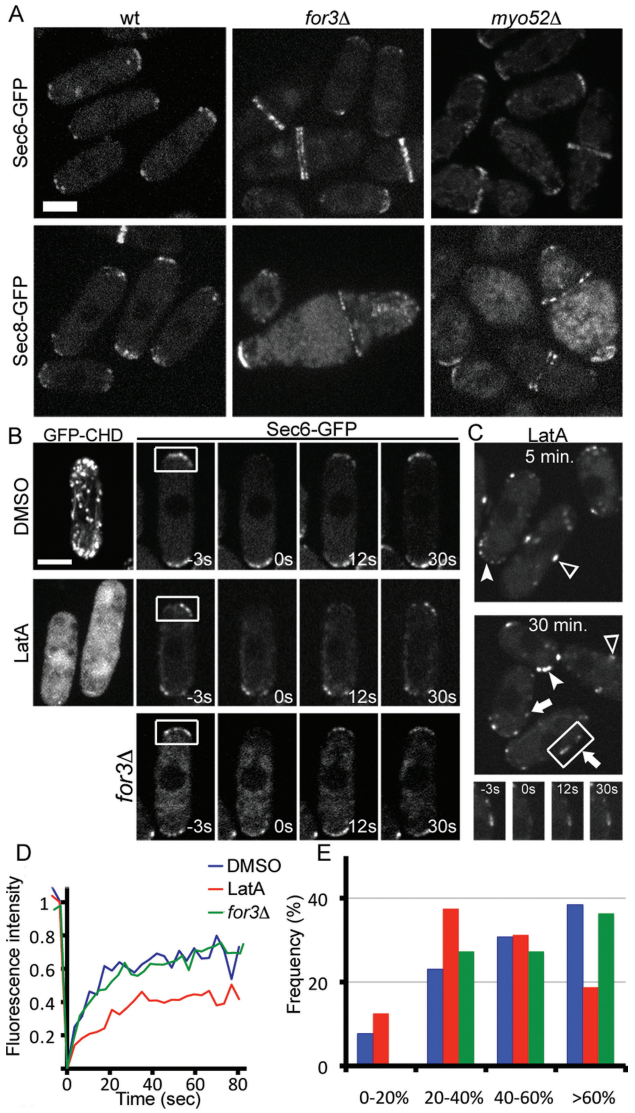

FIGURE 2: The exocyst localizes to cell tips in absence of actin cables. (A) Sec6-GFP and Sec8-GFP signals in wild-type, *for3*Δ, and *myo52*Δ cells. (B) Recovery of Sec6-GFP to bleached tips in the presence of LatA or DMSO. Actin depolymerization was confirmed by coimaging of cells expressing the F-actin binding protein GFP-CHD (left panels). (C) Sec6-GFP in wild-type treated with LatA for 5 and 30 min. Bottom panels show recovery of side wall staining bleached in inset. Arrowheads, arrows, and open triangles indicate tip, cell side, and division site localization, respectively. Bars, 5 μm. (D) Average recovery of Sec6-GFP to bleached tips (n = 15). Control, *for3*Δ, and LatA-treated cells show 5-, 5-, and 10-s half-time of recovery and 35%, 35%, and 60% immobile fractions, respectively. (E) Frequency of excellent (>60%), good (40–60%), medium (20–40%), or poor (0–20%) recovery to bleached tips by averaging recovery from 40 to 80 s.

cytoskeleton (Supplemental Figure S3; Sharifmoghadam *et al.*, 2010). Taken together, our experiments demonstrate that the exocyst can localize to cell tips largely independently of the actin cytoskeleton.

Conversely, we also tested the localization of For3 and Myo52 in exocyst mutants: Both proteins localized normally to cell tips in *nmt-sec8* depletion strains (Figure S4). In addition, actin cables formed normally in *sec8-1* mutants. Thus actin cables are functional for transport in the absence of a functional exocyst.

Loss of both exocyst and actin cables results in failure to polarize growth

The localization of the exocyst at cell tips suggests that it may be involved in polarized exocytosis for polar growth. However, previous genetic examination of exocyst function revealed an essential role for cell division but not polarized growth (Wang *et al.*, 2002): Temperature-sensitive inactivation (*sec8-1*) or depletion (*nmt81-sec8*) of Sec8 in haploid cells and sporulation of *sec8* deletion from diploids led to arrest of multiseptated, yet elongated, cells. We confirmed this finding by sporulating a double heterozygous mutant *sec6*Δ/*sec6+ sec8*Δ/*sec8+*: Single- or double-deletion mutant spores exhibited the same terminal phenotype of elongated, often septated, cells (Figure 3A). *sec8*Δ *exo70*Δ and *sec8-1 exo70*Δ double-mutant cells showed similar phenotype. Thus in an otherwise wild-type background, the exocyst is not essential for polarized cell growth.

In contrast, when we combined exocyst and actin cable mutants, double-mutant cells were spherical or misshapen, suggesting a failure to properly polarize growth (Figure 3, B and C, and Supplemental Figure S5). This phenotype was observed at 25°C in combinations of *for3*Δ or *myo52*Δ with *sec8-1*, *exo70*Δ, or *sec8* depletion (*nmt81-sec8*). Double mutants with *sec8-1* or *exo70*Δ also exhibited synthetic lethality or sickness at 25°C, respectively, although these cells could be maintained alive by growth in 1 M sorbitol, which alleviates the temperature-sensitive lethality of *sec8-1* single mutants (Martin-Cuadrado *et al.*, 2005). As mentioned above, *for3*Δ *sec8-GFP* and *myo52*Δ *sec8-GFP* were also sick and formed round and aberrantly shaped cells, suggesting that *sec8-GFP* represents a mild loss-of-function allele of *sec8*, which is deleterious for polarized growth in absence of functional actin cables. Finally, a double mutant of profilin *cdc3-313* allele and *sec8-1* also led to isotropic growth at restrictive temperature, as shown by fluorescent lectin staining (Figure 3D). Thus even mild disruption of the exocyst (*sec8-GFP* or *exo70*Δ) in cells devoid of actin cable transport leads to isotropic growth, while more complete disruption of the exocyst in cells with intact cables (*sec8*Δ *sec6*Δ, *sec8*Δ *exo70*Δ, or *sec8-1 exo70*Δ) still permits polarized growth.

Exocytic cargoes fail to polarize in the absence of cables and exocyst

We also examined the localization of exocytic vesicles upon Sec8 depletion in wild-type and *for3*Δ cells using the markers described above. In the *nmt-sec8* strain, all three markers localized to cell ends, in agreement with the fact that this strain still polarizes growth (Figure 3, E and F, and unpublished data). However, the markers assumed a distinct appearance from wild-type or *for3*Δ cells: First, they localized predominantly in the vicinity of cell tips and showed a reduced signal at the cell tip cortex, especially GFP-Syb1. Second, other cellular regions were largely devoid of signal. Similar observations were made in the *sec8-1* strain, with the cortical signal decreasing over time at restrictive temperature (Figure 4B and unpublished data). This suggests that in the absence of a functional exocyst, exocytic vesicles accumulate at cell tips, where they are able to fuse, albeit less efficiently than in wild-type cells. This may create a bottleneck in membrane trafficking, leading to gradual accumulation of all vesicles near cell tips.

By contrast, tip accumulation of exocytic vesicles was undetectable when Sec8 was depleted in the *for3*Δ background (Figure 3, G and H). Instead, large numbers of internal sites were labeled. We also detected weak uniform cortical staining of both Bgs1 and Bgs4 cargoes, but not Syb1, suggesting that vesicles may still inefficiently fuse with the plasma membrane but without spatial constraints. Similarly, *cdc3-313 sec8-1* double mutants that had not yet undergone

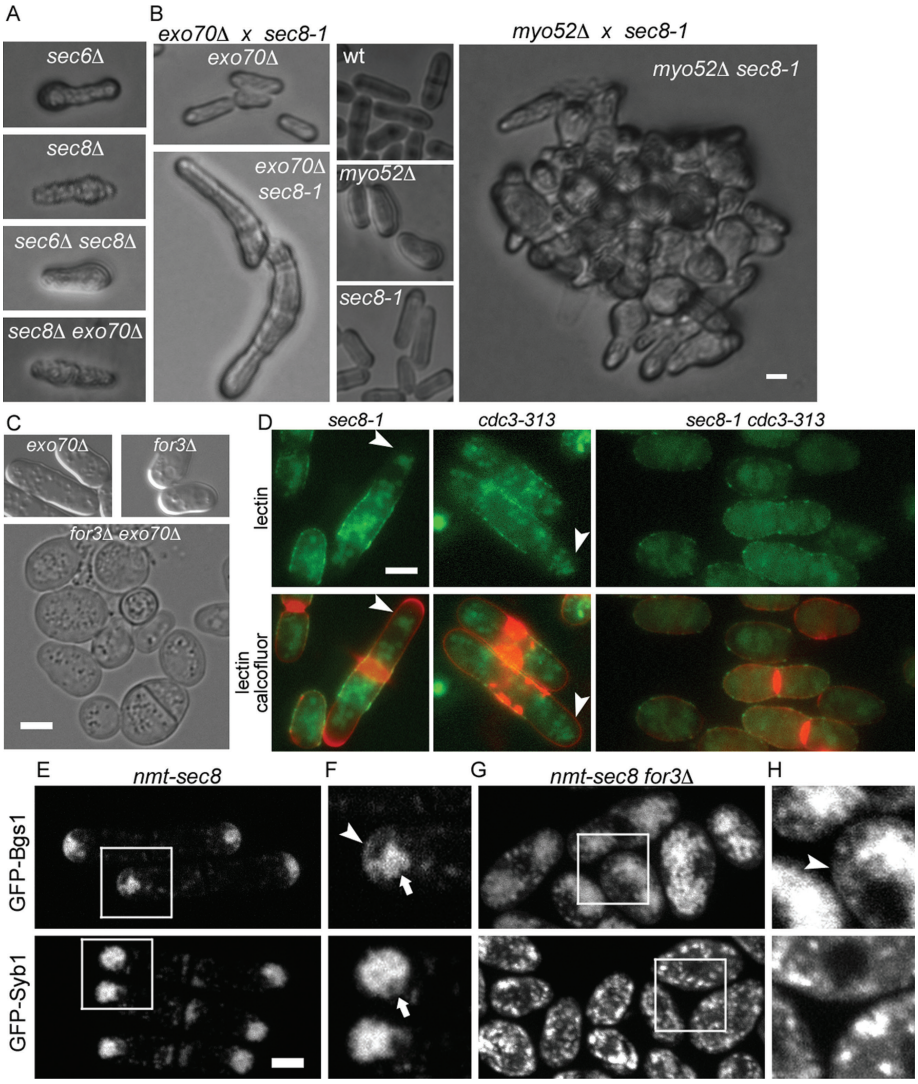

FIGURE 3: Loss of both actin cable machinery and the exocyst results in failure to polarize growth. (A) Germinated *sec6*Δ, *sec8*Δ, *sec6*Δ *sec8*Δ, and *sec8*Δ *exo70*Δ spores on YE5S at 25°C. (B) Cells with indicated genotype from cross of *exo70*Δ × *sec8-1* or *myo52*Δ × *sec8-1*. (C) *exo70*Δ, *for3*Δ, and *for3*Δ *exo70*Δ cells. (D) *sec8-1*, *cdc3-313*, and *sec8-1 cdc3-313* cells stained with TRITC-lectin (green; all panels) and calcofluor (red; bottom panels). Note clearing of lectin staining at cell ends of single mutants (arrowheads) but absence of clearing and patchy appearance in double mutant cells. (E–H) GFP-Bgs1 and GFP-Syb1 signals in *nmt-sec8* and *for3*Δ *nmt-sec8* cells depleted for Sec8. (F, H) Enlargements of insets marked in (E, G). Arrowheads indicate cortical and arrows indicate subcortical polar accumulation of fusion proteins. Bars, 5 μm.

visible shape change after 90 min of growth at 36°C mostly lacked tip enrichment of RFP-Bgs4 (see Figure 4B). Residual tip localization was observed in only 21% of cells (n = 103), whereas it was retained in over 74% in wild type and each single mutant. In summary, exocytic cargoes can concentrate to cell tips in absence of either actin cables or exocyst but not in absence of both. Thus actin cables and exocyst provide distinct but partly redundant input for polarized exocytosis.

Actin cable and exocyst pathways lie downstream of Cdc42

Because disruption of both actin cables and exocyst leads to isotropic growth, we investigated whether the polarity establishment machinery was compromised in double mutants. To label regions of Cdc42 activity, we used a Cdc42/Rac-interactive binding (CRIB)-GFP fusion, which localizes to cell tips in wild-type cells (and artifactually to the nucleus; Tatebe *et al.*, 2008). CRIB–GFP localization was not altered in single mutants. In aberrantly shaped *nmt-sec8 for3*Δ double mutants, it also labeled distinct cortical regions, indicating that localization and activation of the Cdc42 landmark is intact even in these misshapen cells (Figure 4A). Similarly, in *cdc3-313 sec8-1* double mutants grown at restrictive temperature for 90 min, CRIB-GFP localized correctly to at least one cell end in 86% of cells (as compared to over 76% in either single mutants or wild-type cells), although 78% of these cells lacked all cell tip Bgs4 cargo (Figure 4B). These results indicate that the cell polarity establishment machinery is largely intact in cells lacking morphogenesis information.

The above results suggest that both actin cables and exocyst may function downstream of Cdc42. We previously showed that For3 localization and activity are controlled by Cdc42 (Martin *et al.*, 2007; Rincon *et al.*, 2009). In *cdc42-1625* cells, in which Cdc42 localizes correctly to cell tips (Martin *et al.*, 2007) but displays severely compromised activity, both Sec6-GFP and Sec8-GFP failed to efficiently localize to cell tips (Figure 4C). Similar observations were made in a second *cdc42* mutant allele, *cdc42-879* (Rincon *et al.*, 2009). Thus both formin and exocyst are under control of Cdc42.

We investigated the localization of active Cdc42 upon actin disruption. Five minutes after LatA addition, CRIB-GFP was still localized at or in the vicinity of cell tips in most cells. After 30 min, CRIB-GFP was still cortical, but its localization was either polar or displaced from the cell tip toward cell sides (Figure 4D). This behavior upon LatA treatment is similar to that of Sec6-GFP. Examination of For3-3GFP localization after 5 and 30 min of LatA treatment also showed initial cell tip localization followed by cortical displacement (Figure 4D; Martin and Chang, 2006). This suggests that active Cdc42 may recruit formin and the exocyst to these ectopic sites.

Exocyst pathway is dependent on PIP_2

Because all cytoskeletal transport is dispensable for the localization of the exocyst to cell tips, we tested whether it depends on the lipid composition of the membrane. In *myo1*Δ, a deletion in a type I myosin essential for ergosterol domain organization (Takeda and Chang, 2005), Sec8-GFP was localized correctly to cell tips, suggesting that ergosterol domains do not significantly contribute to the localization of the exocyst (unpublished data). In *its3-1* cells, which bear a mutation in the major phosphatidylinositol 4-phosphate 5-kinase and show vastly reduced levels of PIP_2 (Zhang *et al.*, 2000), both Sec6-GFP and Sec8-GFP were delocalized from cell tips after 90 min at restrictive temperature (Figure 5A). Careful examination of their localization in single focal sections, however, revealed that they were still cortical but not enriched at tips (unpublished data). In contrast, CRIB-GFP and Myo52-GFP localized correctly to

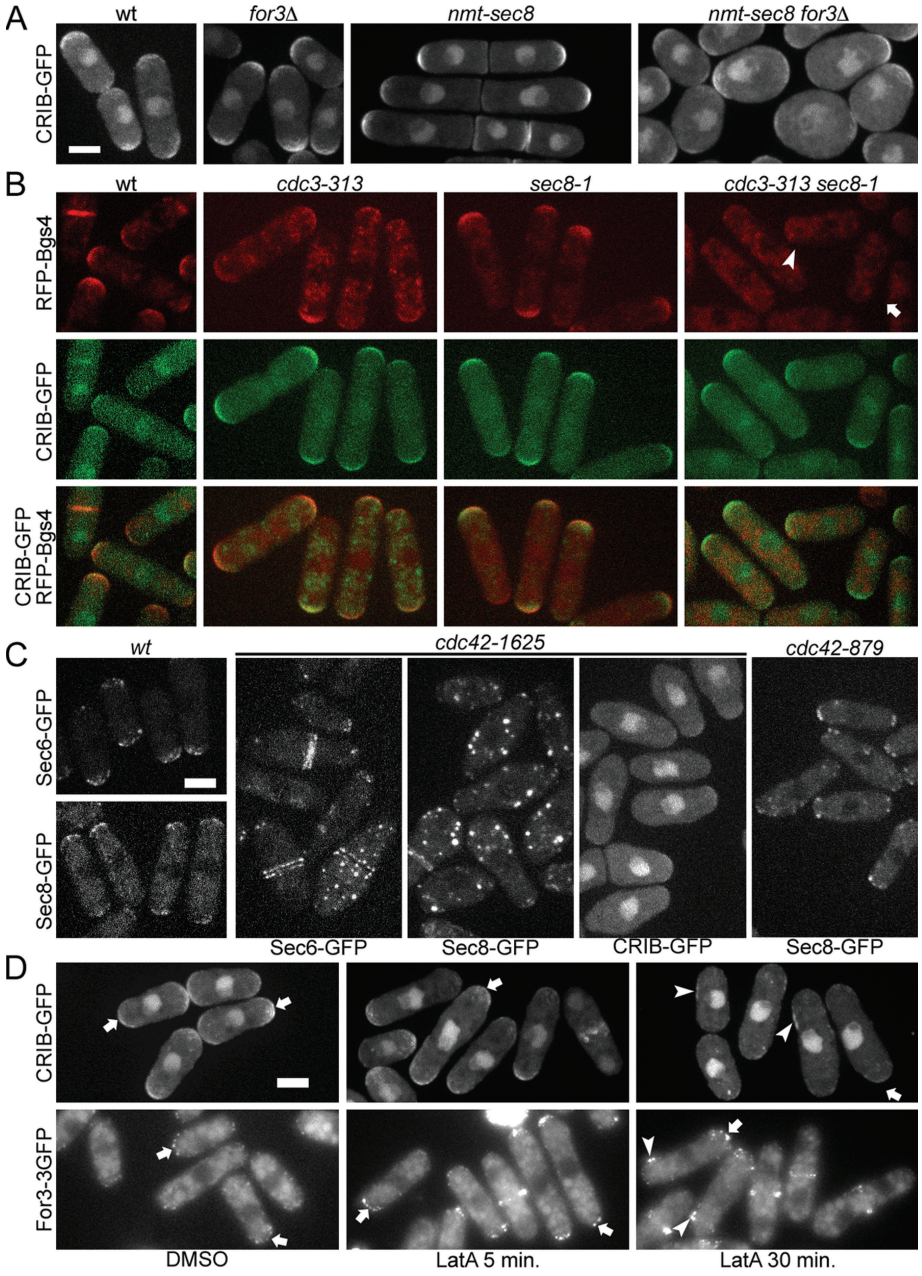

FIGURE 4: Cdc42 activity is independent of actin cable and exocyst and is necessary for exocyst localization. (A) CRIB-GFP in wild-type, *for3Δ*, *nmt-sec8*, and *for3Δ nmt-sec8* cells depleted for Sec8. (B) RFP-Bgs4 (red) and CRIB-GFP (green) in wild-type, *cdc3-313*, *sec8-1*, and *cdc3-313 sec8-1* cells grown for 90 min at 36°C. (C) Sec6-GFP and Sec8-GFP in wild-type (left panels), *cdc42-1625* (middle panels), and *cdc42-879* mutant cells. CRIB-GFP in *cdc42-1625* mutant is also shown as labeled. (D) Localization of CRIB-GFP and For3-3GFP in wild-type cells treated with LatA for 5 and 30 min. Arrows indicate polar and arrowheads lateral localization of fusion proteins. Bars, 5 μm.

cell tips, indicating that the polarity establishment machinery and actin cables are functional for polarized transport. As *its3-1* mutants display phenotypes similar to *sec8-1*, with accumulation of multi-septated cells at restrictive temperature (Zhang *et al.*, 2000), we generated double mutants between *its3-1* and *for3Δ* and between *its3-1* and *sec8-1*. While both combinations were synthetic lethal at 25°C, *its3-1 for3Δ* double mutants produced rounded and misshapen cells, whereas *its3-1 sec8-1* arrested as elongated, often septated, cells (Figure 5B). Thus PIP_2 levels contribute to exocyst localization to cell tips.

DISCUSSION

Polarized exocytosis is fundamental for many cellular processes, including cell morphogenesis. We report here that exocytic vesicle transport along actin cables and tethering by the exocyst—two processes often thought to function sequentially—can sustain cell morphogenesis in the fission yeast independently of each other.

We present multiple lines of evidence to support the conclusion that the actin cable machinery and the exocyst form two autonomous "morphogenetic modules." First, actin cables polarize independently of the exocyst. Second, the exocyst localizes independently of the cytoskeleton and instead relies on PIP_2. Third, the two modules are genetically redundant for cell shape. Finally, the localization of both modules is under control of the small GTPase Cdc42. In contrast, Cdc42 localization and activity are not affected by the disruption of either or both morphogenetic modules, a finding that raises important questions regarding the mechanisms of Cdc42 localization. Together these results define a clear hierarchical organization where Cdc42 establishes a polar landmark, which activates parallel cytoskeleton-dependent and membrane-dependent morphogenetic modules that translate this polar localization in actual polar growth (Figure 6).

Cell morphogenesis in the absence of cytoskeleton-dependent vesicle transport

Cytoskeletal transport, along either microtubules or linear actin filaments, underlies the targeted delivery of cargoes. As such it is thought to play fundamental roles for cell morphogenesis in many cell types. For instance, actin cables are essential to sustain polarized growth through the cell cycle in the budding yeast. Similarly, class II formins and thus the actin structures they assemble are essential for cell morphogenesis in the moss *Physcomitrella patens* (Vidali *et al.*, 2009). The flagellated protozoan parasite *Giardia intestinalis* lacks formins altogether (Chalkia *et al.*, 2008) but completely depends on microtubules for cell morphogenesis (Mariante *et al.*, 2005). In contrast, we show that simultaneous disruption of both actin cables and microtubules in fission yeast still permits significant polarized cell growth throughout the cell cycle, indicating the existence of alternative cytoskeleton-independent morphogenesis pathways.

How prevalent are these alternative pathways? Although, to our knowledge, fission yeast is the only organism shown to date to sustain polarized cell growth and survive in absence of cytoskeletal vesicular transport, two recent studies have also suggested the existence of additional actin cable–independent morphogenesis mechanisms in the budding yeast. First, cells exiting from prolonged

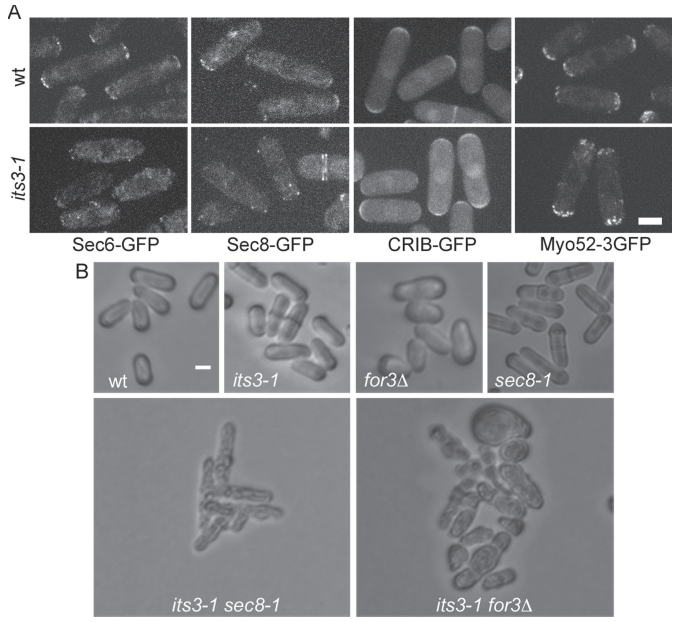

FIGURE 5: The localization of the exocyst, but not of active Cdc42 or Myo52, is dependent on PIP_2. (A) Sec6-GFP, Sec8-GFP, CRIB-GFP, and Myo52-3GFP signals in wild-type and *its3-1* mutant cells grown at 36°C in EMM for 90 min. (B) *its3-1 sec8-1* and *its3-1 for3*Δ double-mutant and control cells. Bar, 5 μm.

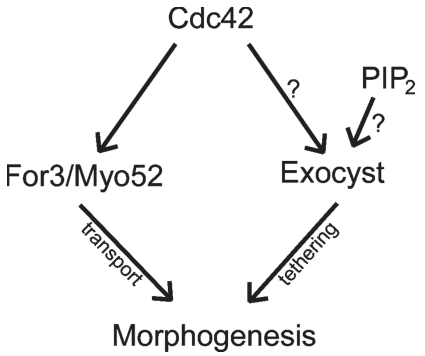

FIGURE 6: Model for parallel morphogenesis pathways under the control of Cdc42. Cdc42 sets up cell polarity and regulates two parallel morphogenetic modules for polarized cell growth, the formin-dependent actin cable module and the exocyst module, contributing to transport and tethering of exocytic vesicles, respectively. The exocyst is also controlled by PIP_2 levels at the plasma membrane. Direct links between Cdc42, PIP_2, and the exocyst are as yet unknown.

(7 d) quiescence can form small buds in the absence of all F-actin (Sahin *et al.*, 2008). This requires a functional exocyst but not endocytosis. Second, small bud formation also occurs during vegetative growth in the absence of functional formins or tropomyosins but not in the absence of functional myosin V (Yamamoto *et al.*, 2010). In contrast to the first example, this small bud growth requires components of the endocytic machinery. Although in neither situation was polarized growth sustained beyond formation of a small bud, this nevertheless suggests that alternative cytoskeletal transport–independent pathways contribute to shape cells.

Spatial determinants of exocyst localization

We have shown that in fission yeast, one alternative morphogenetic pathway requires the exocyst, as this complex is essential for polarized growth in the absence of actin cables. This complex thus provides key spatial information to identify the tips as zones of growth. How is the exocyst localized to cell tips? Our live microscopy and FRAP experiments demonstrate that the cytoskeleton is largely dispensable while both the phosphoinositide PIP_2 and the small GTPase Cdc42 are required for exocyst accumulation to cell tips. Interestingly, actin cables are not required for tip localization, but complete disruption of F-actin caused a larger fraction of Sec6 to remain at the cortex and, over time, spread laterally. This displacement is also observed for CRIB-GFP, labeling active Cdc42, and For3. One interpretation is that actin patches, and thus endocytosis, participate in the recycling of active Cdc42 off the membrane and may be important for maintaining tip localization. Displaced Cdc42 may remain competent at recruiting formin and the exocyst, suggesting that this small GTPase is a key spatial determinant. The role of the phosphoinositide PIP_2 is less clear as it is not apparently polarized in fission yeast (Zhang *et al.*, 2000). However, both Cdc42 and PIP_2 may function to link the exocyst with the plasma membrane at cell tips.

Interactions between the exocyst and both PIP_2 and Cdc42 or other small GTPases have been shown to mediate cortical attachment in *S. cerevisiae* and metazoan systems (Zhang *et al.*, 2001, 2008; Inoue *et al.*, 2003; He *et al.*, 2007; Liu *et al.*, 2007; Wu *et al.*, 2010). These may thus represent conserved mechanisms to anchor the exocyst at the plasma membrane. In *S. cerevisiae*, only two exocyst subunits—Sec3 and Exo70—localize to the bud tip independently of actin cable–mediated transport by directly binding Cdc42 and PIP_2 (Zhang *et al.*, 2001, 2008; Boyd *et al.*, 2004; He *et al.*, 2007; Wu *et al.*, 2010). Intriguingly, it is unclear whether the fission yeast genome encodes any Sec3 homologue (Wang *et al.*, 2002) and Exo70 is nonessential (Wang *et al.*, 2003) and thus unlikely to serve as a unique spatial landmark. It is therefore unknown whether the specific interaction domains of the exocyst with PIP_2 or Cdc42 are conserved.

Exocyst-independent polarized exocytosis

That fission yeast cells can polarize growth until the next cell division in the absence of a functional exocyst raises the question of the mechanisms for exocyst-independent polarized exocytosis. The accumulation of vesicular markers beneath the cell tips in exocyst mutants suggests that polarized exocytosis is much less efficient in this situation. We show that this depends on actin cables and type V myosin. We can envisage two nonexclusive hypotheses. First, exocytosis may be possible in the absence of any tethering complex: Polarized vesicle transport may lead to a high local concentration of fusion-competent vesicles in the vicinity of the cell tip, sufficient for the formation of SNARE complexes independently of proper tethering. Alternatively, a second tethering factor may exist. Instances of exocyst-independent exocytic events have been documented in metazoan systems, such as neurotransmitter release in neuronal cells (Murthy *et al.*, 2003). One attractive idea is that the actin cable module may provide its own tethering function. Direct roles for actin and myosin in vesicle docking near the plasma membrane have been proposed in mammalian cells (Malacombe *et al.*, 2006; Desnos *et al.*, 2007), suggesting that these cytoskeletal elements may fulfill similar functions in the fission yeast. The recent observation that budding yeast cells devoid of actin cables, but not of type V myosin, are able to form small buds also suggests the possibility that type V myosin may play an additional transport-independent function in this organism (Yamamoto *et al.*, 2010). Thus the fission yeast model may prove particularly useful in dissecting these exocyst-independent tethering events.

Parallel or linear organization of morphogenesis modules

Our results indicate that actin cable–mediated transport and the exocyst form two largely independent morphogenesis modules in fission yeast. This contrasts with the budding yeast situation, where, except for the initial formation of small buds mentioned above, both modules are essential for morphogenesis. The composition of both modules appears to be conserved in the two systems, with the possible exception of Sec3. But the mechanism of exocyst localization is distinct: While most exocyst subunits are transported along actin cables in budding yeast (Boyd *et al.*, 2004), exocyst localization has been uncoupled from actin cable transport in fission yeast. This suggests that the two modules are in a parallel configuration in the fission yeast but in a linear relationship in the budding yeast. Thus whereas the modules are likely conserved, the wiring between them is different between the two species.

Are there evolutionary advantages for these distinct wiring designs? These may represent adaptations that underlie diverse cellular morphologies. One can speculate that a linear link between the two modules promotes the transport of exocyst and vesicles through a narrow bud neck and ensures accuracy in the site of vesicle delivery. In contrast, the fission yeast morphology may tolerate a broader zone of vesicle fusion, and a parallel configuration would provide robustness to the system. Given the vast array of cell morphologies in eukaryotes, it will be interesting to test whether distinct wiring of largely identical modules also underlies shape and functional diversity in other cell types.

MATERIALS AND METHODS

Strains, growth conditions, and pharmacological inhibitor

S. pombe strains used in this study are listed in Supplemental Table S1. Standard genetic methods and growth conditions were used. Unless otherwise stated, cells were grown in Edinburgh minimal media (EMM) supplemented with appropriate amino acids (ALU) or YE5S at 25°C. Tetrads were germinated on YE5S at 25°C, and synthetic lethal strains were imaged directly on this medium. MBC (Sigma, St. Louis, MO) was used at a final concentration of 25 μg/ml from a stock of 2.5 mg/ml in DMSO. A 100× stock solution of Latrunculin A in DMSO (Phillip Crews, UC Santa Cruz) was used at a final concentration of 200 μM. Control experiments with DMSO alone had no effect on polarized growth or GFP fusion protein localization.

For construction of *sec6::nat* and *exo70::nat* mutants, a PCR-based approach was used with plasmid pFA6a-NatMX6 and oligos with homology to 78 nt directly upstream of the start codon and 78 nt downstream the stop codon. Strains were confirmed by diagnostic PCR for both sides of the gene replacements.

For depletion of Sec8 from the *nmt-sec8* strain, precultures were grown in EMM lacking thiamine and diluted for 20 h in either YE5S, which naturally contains thiamine, or EMM supplemented with increasing amounts of thiamine. We experimentally determined the best concentration of thiamine to use (Supplemental Figure S4). Strong depletion of Sec8 with 5 μg/ml thiamine led to the rapid appearance of the multiseptated phenotype, which probably masked the role of Sec8 in polarized cell growth in the *for3*Δ background. In contrast, milder depletion of Sec8 with lower concentration of thiamine permitted cell division to proceed for a longer time and led to formation of round cells in the *for3*Δ background. We thus routinely used 0.25 μl/ml thiamine grown for 20 h for Sec8 depletion.

Microscopy

Microscopy was performed with either a spinning disk confocal microscope or a wide-field fluorescence microscope, essentially as described (Martin and Berthelot-Grosjean, 2009). Stacks of Z-series confocal sections were acquired at 0.3-μm intervals with either UltraVIEW or Volocity (PerkinElmer, Waltham, MA) software. All images of GFP, RFP, and phalloidin fluorescence are two-dimensional maximum-intensity projections of Z-series slices. For3-3GFP images in Figure 4D were acquired as described previously (Martin and Chang, 2006). All differential interference contrast, lectin, and calcofluor imaging was done by wide-field microscopy acquired with the Leica AS AF software (Leica MicroSystems, Bannockburn, IL). Figures were prepared with ImageJ 1.41 and Adobe Photoshop Elements 6.

FRAP experiments were performed with the Photokinesis module attachment for the spinning disk confocal microscope (PerkinElmer). Photobleaching was accomplished by applying 488-nm laser to cell tips or side wall. Images were acquired before photobleaching, immediately after, and subsequently at regular intervals. For FRAP analysis of Sec6-GFP, cells were overlaid on a 2% agarose EMM-ALU pad made with DMSO or LatA, sealed with VALAP, and imaged within 5 min. For GFP-Bgs1 FRAP, cells were incubated with DMSO or MBC for 30 min before imaging on glass slides. Analysis of FRAP was done as described previously (Martin and Chang, 2006).

Imaging of cells at 36°C (Figures 4B and 5A) was performed with an objective heater and Delta T specimen warming dish from Bioptech (Butler, PA). Cells were adhered to Delta T dishes with equal volume of 100 μg/ml lectin (*Bandeiraea simplicifolia*; Sigma) washed with and grown in EMM. All other imaging was done at room temperature (23°C).

Fluorescent lectin and actin staining

Cells were grown overnight in EMM at 25°C to log phase and stained with 5 μg/ml tetramethylrhodamine B isothiocyanate (TRITC)–lectin (stock of 5 mg/ml in dH_2O, Sigma) for 10 min. Cells were then washed twice and resuspended in EMM. Cells were then diluted to OD_{600} = 0.2 in EMM and grown for 5 h before being stained with calcofluor white from 200× stock (2 mg/ml in 100 mM Tris-HCl, pH 9.0). Actin staining was performed as described (Martin *et al.*, 2005).

Measurement of acid phosphatase secretion

Acid phosphatase was assayed as previously described with minor modifications (Wang *et al.*, 2002). Cells were grown overnight to OD_{600} = 0.6 to 0.8 in EMM at 25°C and washed twice with EMM. Cells were then diluted to OD_{600} = 0.3, and 500 μl was removed and designated 0 h. The 500-μl aliquot was centrifuged, and 350 μl of the supernatant was added to 350 μl of 2 mM *p*-nitrophenyl phosphate, 0.1 M sodium acetate, pH 4.1, prewarmed to 30°C. Reactions were incubated at 30°C for 10 min and then stopped by the addition of 350 μl 1 M NaOH. This was repeated every hour for each strain. Absorbance was read at 405 nm, and values were normalized by subtracting the value at 0 h.

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