

# The Rho-GEF Gef3 interacts with the septin complex and activates the GTPase Rho4 during fission yeast cytokinesis

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**ABSTRACT** Rho GTPases, activated by Rho guanine nucleotide exchange factors (GEFs), are conserved molecular switches for signal transductions that regulate diverse cellular processes, including cell polarization and cytokinesis. The fission yeast *Schizosaccharomyces pombe* has six Rho GTPases (Cdc42 and Rho1–Rho5) and seven Rho GEFs (Scd1, Rgf1–Rgf3, and Gef1–Gef3). The GEFs for Rho2–Rho5 have not been unequivocally assigned. In particular, Gef3, the smallest Rho GEF, was barely studied. Here we show that Gef3 colocalizes with septins at the cell equator. Gef3 physically interacts with septins and anillin Mid2 and depends on them to localize. Gef3 coprecipitates with GDP-bound Rho4 in vitro and accelerates nucleotide exchange of Rho4, suggesting that Gef3 is a GEF for Rho4. Consistently, Gef3 and Rho4 are in the same genetic pathways to regulate septum formation and/or cell separation. In *gef3Δ* cells, the localizations of two potential Rho4 effectors—glucanases Eng1 and Agn1—are abnormal, and active Rho4 level is reduced, indicating that Gef3 is involved in Rho4 activation in vivo. Moreover, overexpression of active Rho4 or Eng1 rescues the septation defects of mutants containing *gef3Δ*. Together our data support that Gef3 interacts with the septin complex and activates Rho4 GTPase as a Rho GEF for septation in fission yeast.

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## INTRODUCTION

Cytokinesis is the last step of the cell cycle. It results in a mother cell splitting into two daughter cells. The basic mechanism for cytokinesis is conserved from yeast to humans. Cytokinesis requires the coordination of several key events: cleavage-site selection, assembly of the actomyosin contractile ring, constriction and disassembly of

the contractile ring, plasma membrane deposition, septum formation or extracellular matrix remodeling, and daughter cell separation/midbody abscission (Balasubramanian *et al.*, 2004; Barr and Gruneberg, 2007; Pollard and Wu, 2010; Green *et al.*, 2012). Most of the events are controlled by Rho GTPases and their regulators (Hall, 1998, 2012; Garcia *et al.*, 2006b; Jordan and Canman, 2012). Rho GTPases are highly conserved molecular switches that control signal transduction pathways. Rho guanine nucleotide exchange factors (GEFs) catalyze the exchange from GDP to GTP and thus activate Rho GTPases (Jaffe and Hall, 2005). Rho GTPase-activating proteins (GAPs) turn off Rho GTPases by catalyzing their intrinsic hydrolase activity (Jaffe and Hall, 2005).

The fission yeast *Schizosaccharomyces pombe* is an excellent model system for studying cytokinesis (Gould and Simanis, 1997; Roberts-Galbraith and Gould, 2008; Lee *et al.*, 2012). The anillin-related protein Mid1 plays a crucial role in division-site selection together with the Polo kinase Plo1 and DYRK kinase Pom1 (Sohrmann *et al.*, 1996; Bähler and Pringle, 1998; Bähler *et al.*, 1998a; Paoletti and Chang, 2000; Celton-Morizur *et al.*, 2006; Almonacid *et al.*, 2011; Ye *et al.*, 2012). Mid1 is essential for the assembly of cytokinesis nodes, the precursors of the contractile ring

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Abbreviations used: aa, amino acid; DH, DBL homology; DIC, differential interference contrast; EMM5S, Edinburgh minimal medium plus five supplements; FL, full length; FRAP, fluorescence recovery after photobleaching; GEF, guanine nucleotide exchange factor; IP, immunoprecipitation; mant-GDP, *N*-methylanthraniloyl-GDP; mCFP, monomeric cyan fluorescent protein; mECitrine, monomeric enhanced Citrine; mEGFP, monomeric enhanced green fluorescent protein; PH, pleckstrin homology; RBD, Rho-binding domain; ROI, region of interest; SPB, spindle pole body; ts, temperature sensitive; wt, wild type.

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(Wu *et al.*, 2006; Hachet and Simanis, 2008; Coffman *et al.*, 2009; Almonacid *et al.*, 2011; Laporte *et al.*, 2011; Padmanabhan *et al.*, 2011; Lee and Wu, 2012). The cytokinesis nodes condense into the contractile ring through a search, capture, pull, and release mechanism (Vavylonis *et al.*, 2008; Laporte *et al.*, 2010, 2012; Ojkic *et al.*, 2011). The ring matures during anaphase B by recruiting more proteins under the regulation of the septation initiation network pathway (Wu *et al.*, 2003; Hachet and Simanis, 2008; Huang *et al.*, 2008). Then the ring constricts, and the division septum is formed (Wu *et al.*, 2003; Proctor *et al.*, 2012). The septum contains a primary septum flanked by two secondary septa (Johnson *et al.*, 1973). During cell separation, the primary septum and the surrounding cell wall on cell sides are degraded by hydrolytic enzymes, including  $\beta$ -glucanase Eng1 and  $\alpha$ -glucanase Agn1, and the secondary septa remodel to become the cell wall at the new ends of the daughter cells (Martin-Cuadrado *et al.*, 2003, 2008; Dekker *et al.*, 2004; Sipiczki, 2007).

Both budding yeast and fission yeast have six homologous Rho GTPases, named Rho1–Rho5 and Cdc42 (Garcia *et al.*, 2006b; Park and Bi, 2007; Perez and Rincon, 2010). Cdc42 is essential for cell-polarity establishment and maintenance, actin organization, and septation (septum formation and/or cell separation) during cytokinesis (Adams *et al.*, 1990; Johnson and Pringle, 1990; Caviston *et al.*, 2003; Rincon *et al.*, 2007; Howell *et al.*, 2012; Atkins *et al.*, 2013; Onishi *et al.*, 2013; Slaughter *et al.*, 2013). Rho1 is essential for cell-wall formation and integrity, actomyosin-ring assembly, and septation (Arellano *et al.*, 1997; Nakano *et al.*, 1997; Tolliday *et al.*, 2002; Yoshida *et al.*, 2006; Levin, 2011). Rho5 is suggested to be a functional homologue of Rho1 in *S. pombe* (Nakano *et al.*, 2005; Rincon *et al.*, 2006), whereas in *Saccharomyces cerevisiae* it is involved in mediating stress response (Schmitz *et al.*, 2002; Singh *et al.*, 2008). Rho2 is nonessential and also involved in cell-wall synthesis by regulating  $\alpha$ -glucan synthase (Madaule *et al.*, 1987; Calonge *et al.*, 2000). In budding yeast, Rho3 is crucial for polarized cell growth by regulating exocytosis and formin activation, and its roles are often shared with Rho4 (Matsui and Toh, 1992; Imai *et al.*, 1996; Adamo *et al.*, 1999; Doignon *et al.*, 1999; Robinson *et al.*, 1999; Wu *et al.*, 2010a). Similarly, in fission yeast, Rho3 functions in polarized cell growth and modulates the exocyst complex for cell separation (Nakano *et al.*, 2002; Wang *et al.*, 2003). It has been suggested that *S. pombe* Rho4 participates in the delivery of certain secretory vesicles and regulates the localization of glucanases Eng1 and Agn1 during cell separation (Nakano *et al.*, 2003; Santos *et al.*, 2003, 2005). However, the relationship between Rho3 and Rho4 is unknown in fission yeast. The exocyst is an octameric protein complex (containing Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) that tethers secretory vesicles to the plasma membrane during polarized cell growth and cytokinesis (TerBush *et al.*, 1996; Guo *et al.*, 1999; Hsu *et al.*, 2004; He *et al.*, 2007a,b; Mukherjee *et al.*, 2014). The exocyst complex is involved in transporting and secreting the hydrolytic enzymes Eng1 and Agn1 for daughter cell separation in fission yeast cytokinesis (Wang *et al.*, 2002; Martin-Cuadrado *et al.*, 2005).

Seven Rho GEFs—Scd1, Gef1–Gef3, and Rgf1–Rgf3—have been identified in *S. pombe* (Iwaki *et al.*, 2003; Garcia *et al.*, 2006b; Wu *et al.*, 2010b). Among these, Scd1 and Gef1 are GEFs for Cdc42 (Coll *et al.*, 2003; Hirota *et al.*, 2003), and Rgf1–Rgf3 function as GEFs for Rho1 and possibly Rho5 (Tajadura *et al.*, 2004; Morrell-Falvey *et al.*, 2005; Mutoh *et al.*, 2005; Garcia *et al.*, 2006a, 2009). Recently we and others found that Gef2 localizes to cortical nodes and the contractile ring and plays roles in division-site positioning and septation (Moseley *et al.*, 2009; Ye *et al.*, 2012; Guzman-Vendrell *et al.*, 2013; Jourdain *et al.*, 2013; Zhu *et al.*, 2013; Akamatsu

*et al.*, 2014). Gef2 binds to Rho4, Rho1, and Rho5 in vitro and regulates Rho4 localization in vivo (Zhu *et al.*, 2013). However, the functions and substrate GTPase(s) of Gef3 were still unknown.

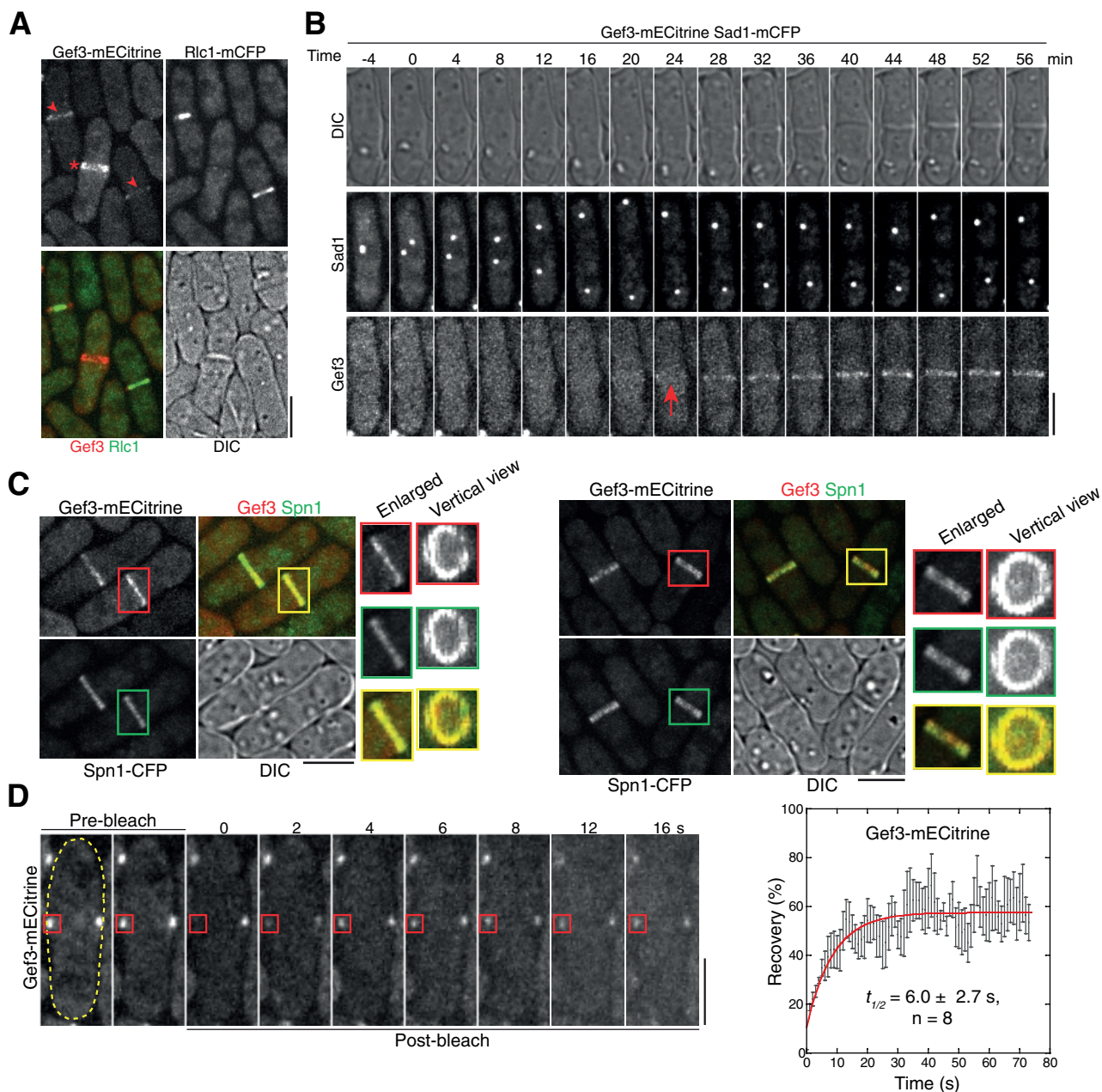
Besides Rho GTPases and their GEFs, septins are also essential for cytokinesis in many cell types (Longtine *et al.*, 1996; Gladfelter *et al.*, 2001; Caudron and Barral, 2009; Oh and Bi, 2011). Septins are GTP-binding proteins that usually associate with the plasma membrane and function as scaffolds and diffusion barriers (Hall *et al.*, 2008; Oh and Bi, 2011). In budding yeast, septins localize to the mother-bud neck as filaments and are required for the localizations of ~100 proteins (Byers and Goetsch, 1976; Gladfelter *et al.*, 2001; McMurray and Thorner, 2009; Oh and Bi, 2011). *S. pombe* has seven septins, and four of them (Spn1–Spn4) are expressed in vegetative cells and localize to the division site during cytokinesis (Longtine *et al.*, 1996; Berlin *et al.*, 2003; Tasto *et al.*, 2003; Wu *et al.*, 2003, 2010b; An *et al.*, 2004; Petit *et al.*, 2005; Onishi *et al.*, 2010). Spn1 and Spn4 are the core members for septin-complex assembly (An *et al.*, 2004; McMurray and Thorner, 2008). Mid2, an anillin homologue related to *S. cerevisiae* Bud4 and *Candida albicans* Int1, colocalizes with septins and stabilizes septin rings during cytokinesis in fission yeast (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Deletion mutants of septins or *mid2* are viable but display a delay in the separation of daughter cells (Berlin *et al.*, 2003; Tasto *et al.*, 2003). One function of the septin ring is to regulate the localization of glucanases Eng1 and Agn1, delivered by exocytosis during cell separation (Martin-Cuadrado *et al.*, 2005). However, unlike budding yeast, very few fission yeast proteins have been reported to localize to the division site in a septin-dependent manner, and thus our knowledge on septin functions in fission yeast cytokinesis is quite limited. Of note, a Rho GEF (SA-RhoGEF) was identified to be a binding partner of a mammalian septin Sept9b and act as a link between septins and Rho signaling (Nagata and Inagaki, 2005). Hence the relationship between septins and Rho GEFs is of great interest.

Here we show that Gef3 localizes to the division site in a septin-dependent manner in fission yeast. Gef3 and the Rho GTPase Rho4 bind to each other and are in the same genetic pathways to regulate septation. Gef3 possesses GEF activity toward Rho4 in vitro and activates Rho4 in vivo. Rho4 overexpression can rescue cell-separation defects in mutants containing *gef3* $\Delta$ . Thus we conclude that the Gef3 is a GEF for Rho4 and is involved in the final stages of cytokinesis.

## RESULTS

### Gef3 colocalizes with septins at the cell-division site

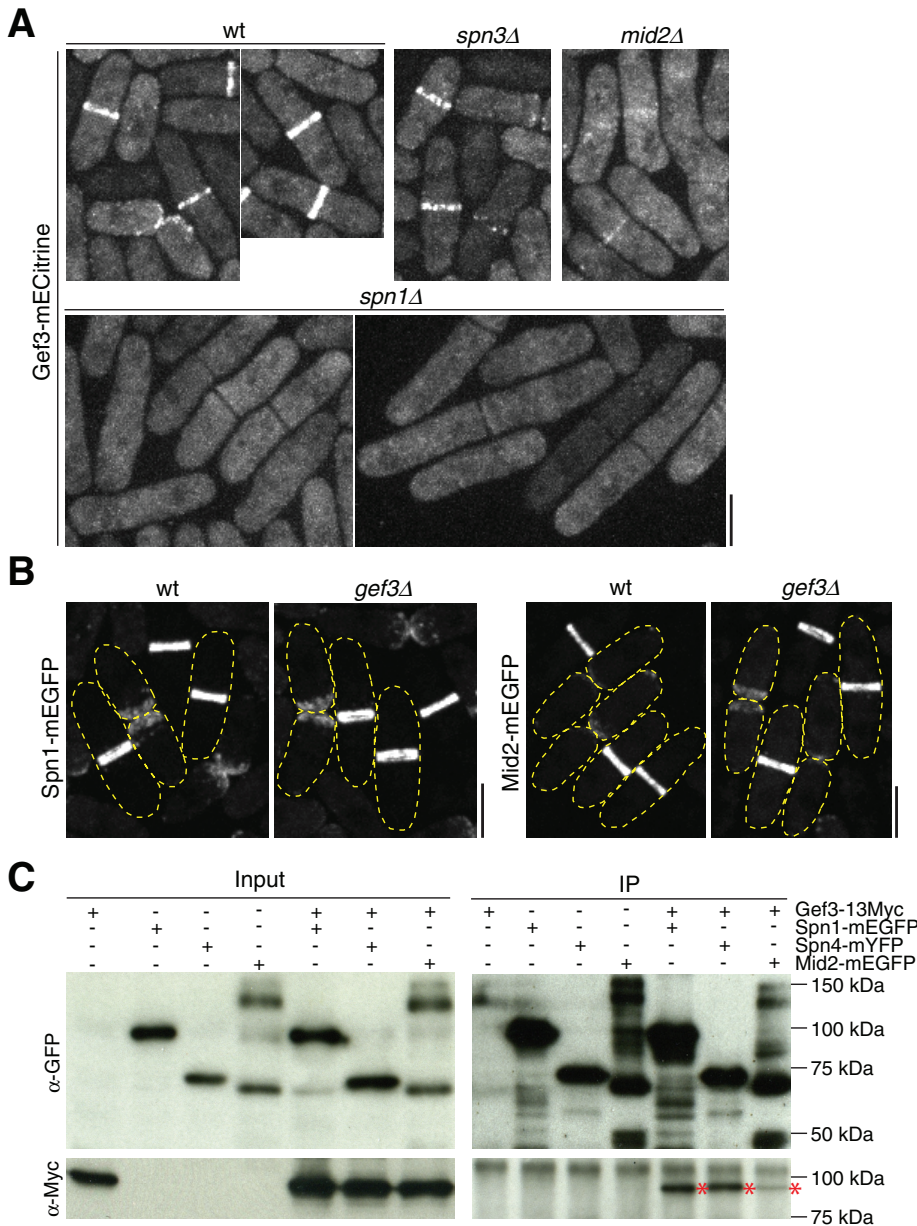
The putative Rho GEF Gef3 was the smallest and least-studied Rho GEFs in *S. pombe* (Iwaki *et al.*, 2003; Garcia *et al.*, 2006b; Perez and Rincon, 2010). To explore the functions of Gef3, we first tagged it with the yellow fluorescent protein (YFP) variant monomeric enhanced Citrine (mECitrine; Griesbeck *et al.*, 2001; Ye *et al.*, 2012) at its C-terminus and examined its localization (Figure 1). Gef3-mECitrine concentrated at the cell-division site with varied intensity during cytokinesis (Figure 1A). Using monomeric cyan fluorescent protein (mCFP)-tagged myosin regulatory light chain Rlc1 and SUN-domain protein Sad1 as the contractile-ring and spindle-pole body (SPB) marker (Wu *et al.*, 2003), respectively, we found that Gef3 appeared at the cell equator as a nonconstricting ring at the end of anaphase B (Figure 1, A and B, and Supplemental Figure S1, A and B). Gef3 intensity at the division site seemed to increase during septum formation (Figure 1, A and B, and Supplemental Figure S1, A and B). However, its diameter remained the same until cell separation, when it spread to new cell ends (Supplemental Figure S1B, 57 min).



**FIGURE 1:** Gef3 colocalizes with septins at the cell-division site. (A) Gef3-mECitrine localizes to the cell-division site but does not constrict with Rlc1-mCFP-labeled contractile ring. DIC, differential interference contrast. Arrowheads mark cells with constricting contractile rings. The asterisk marks a cell with a complete septum after contractile-ring disassembly. (B) Time course (in minutes) of Gef3 localization at the division site using the SPB protein Sad1 as a cell-cycle marker. Time 0 marks SPB separation. The arrow marks the appearance of Gef3 at the division site. (C) Colocalization of Gef3 and the septin Spn1 in septin single (left) and double rings (right). Enlarged and vertical views of the ring structures within the boxed regions are shown to the right. (D) FRAP analysis of Gef3 in cells with complete septa. Left, representative cell before (prebleach) and after (postbleach) photobleaching. The boxed region was bleached at time 0. The cell boundary is marked with broken lines. Right, mean recovery of eight cells. Mean  $\pm$  SEM is plotted, and the curve fit is in red. Bars, 5  $\mu$ m.

The timing and pattern of Gef3 localization resemble those of septins (Berlin *et al.*, 2003; Tasto *et al.*, 2003; Wu *et al.*, 2003). Thus we examined whether Gef3 colocalizes with Spn1, a core component of the septin complex (An *et al.*, 2004). Indeed, Gef3 colocalized with Spn1 as either single (Figure 1C, left) or double rings (Figure 1C, right) from its appearance to just before cell separation (Supplemental Figure S1B). To determine Gef3 dynamics, we performed fluorescence recovery after photobleach-

ing (FRAP) assays in cells with complete septa to avoid interference from protein recruiting to the division site. Different from septins, which form stable rings at the division site (Berlin *et al.*, 2003), Gef3 was quite dynamic, with a recovery half-time of  $6.0 \pm 2.7$  s after photobleaching (Figure 1D). Taken together, the results show that Gef3 colocalizes with septins at the division site during septum formation and cell separation, but Gef3 is more dynamic than septins.



**FIGURE 2:** Gef3 depends on septins to localize and physically interacts with the septin complex. (A) Gef3 localization in wt, *spn1Δ*, *spn3Δ*, and *mid2Δ* cells. (B) Spn1 (left) and Mid2 (right) localization in wt and *gef3Δ* cells. The cell boundary of most cells is marked with broken lines. (C) Gef3-13Myc co-IP with Spn1, Spn4, and Mid2. Asterisks mark the Gef3-13Myc bands after pull down. Bars, 5  $\mu$ m.

### Gef3 localization depends on septins

Septins are scaffolding proteins and essential for localization of ~100 proteins to the bud neck in budding yeast (Versele and Thorner, 2005; Caudron and Barral, 2009; Oh and Bi, 2011; Spiliotis and Gladfelter, 2012; Wloka and Bi, 2012). It is still a mystery why septins are not essential and very few proteins depend on septins to localize in fission yeast (Berlin *et al.*, 2003; Tasto *et al.*, 2003; An *et al.*, 2004; Martin-Cuadrado *et al.*, 2005; Wu *et al.*, 2010b). Given the colocalization of Gef3 and septins, we tested their interdependence for localization to the division site. Of interest, Gef3 localization at the division site was abolished in *spn1Δ* cells (Figure 2A), in which no other septins can localize (An *et al.*, 2004). Gef3 intensity was also reduced in *spn3Δ* cells (Figure 2A), a deletion in which the septin

structure is slightly compromised (An *et al.*, 2004). The anillin-like protein Mid2 regulates septin dynamics and localization in *S. pombe* (Berlin *et al.*, 2003; Tasto *et al.*, 2003; Wu *et al.*, 2010b). In *mid2Δ* cells, the septin level at the division site is reduced, and septins spread to the septum instead of the double rings (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Consistently, Gef3 intensity at the division site was dramatically reduced and spread to the septum as septins in *mid2Δ* cells (Figure 2A and unpublished data). In contrast, the localizations of Spn1 and Mid2 were not affected in *gef3Δ* cells (Figure 2B). Thus septins are essential for Gef3 localization.

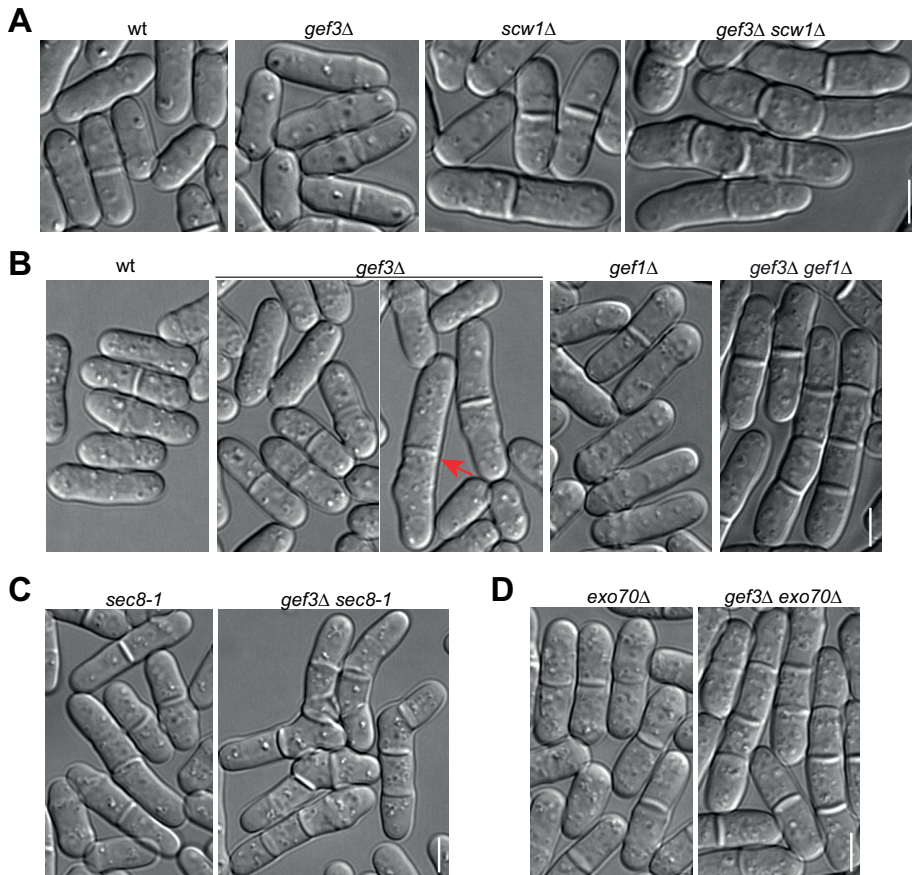
The localization dependence suggests that Gef3 and septins may form protein complexes. Indeed, Gef3-13Myc was pulled down by the septins Spn1-monomeric enhanced green fluorescent protein (mEGFP) and Spn4-mYFP and Mid2-mEGFP in coimmunoprecipitation (co-IP) assays (Figure 2C). Together these data suggest that Gef3 is recruited to the division site through physical interactions with septins and/or anillin Mid2, although the nature of the interactions needs further study.

### Gef3 regulates septation in later stages of cytokinesis

Septins are involved in septation by regulating the localizations of  $\beta$ -glucanase Eng1 and  $\alpha$ -glucanase Agn1 (Martin-Cuadrado *et al.*, 2005). Gef3 localization and its dependence on septins suggest that it has a function in later stages of cytokinesis. Indeed, *gef3Δ* cells had mild septation defects at 36°C (Figure 3, A and B; for quantification, see later discussion of Figure 6, B and D): higher percentage of septating cells, some long, septating cells (Figure 3B, arrow), and occasionally a few multiseptated cells (unpublished data). The phenotype indicates that Gef3 functions redundantly in late cytokinesis. To identify the proteins that play an overlapping role with Gef3, we tested genetic interactions between *gef3Δ* and other cytokinetic mutations (Table 1). Of

interest, *gef3Δ* had strong synthetic genetic interactions with *scw1Δ*, *gef1Δ*, and exocyst mutants (Figure 3 and Table 1), as described later.

Scw1 is an RNA-binding protein, and *scw1Δ* has strong synthetic genetic interaction with *spn1Δ* (Wu *et al.*, 2010b). *gef3Δ scw1Δ* double mutant displayed very strong additive defects in septation (see Table 1 for classification of genetic interactions), with more septating and multiseptated cells than single mutants (Figure 3A; see Figure 6B for quantification). Similarly, *gef3Δ* also had very strong synthetic interaction with *gef1Δ*, a deletion mutant of the Cdc42 GEF *gef1* (Coll *et al.*, 2003; Hirota *et al.*, 2003). Most *gef3Δ gef1Δ* cells contained one or more septa (Figure 3B; see Figure 6D for quantification). Intriguingly, *gef3Δ* had strong synthetic interactions



**FIGURE 3:** Synthetic genetic interactions of *gef3Δ* with other cytokinesis mutations. (A–D) DIC images showing synthetic interactions between *gef3Δ* and *scw1Δ* (A), *gef1Δ* (B), and exocyst mutants *sec8-1* (C) and *exo70Δ* (D) at 25°C (A, C) or after shifting to 36°C for 6 h (B, D). The arrow marks a long *gef3Δ* cell likely with delay in cell separation. Bars, 5 μm.

with formin *for3Δ* mutant and *cdc42-1625*, a temperature-sensitive (ts) mutant of *cdc42*, in cell polarization (Table 1 and Supplemental Figure S2A). In addition, *gef3Δ* had strong synthetic interaction in cell separation and cell polarization with *myo52Δ* (Supplemental Figure S2B), a myosin V deletion defective in exocytic vesicle transport to the sites of polarized growth (Win *et al.*, 2001; Bendezú and Martin, 2011). In contrast, *gef3Δ* had no or mild genetic interactions with mutations in arrestin *art1* (which has strong synthetic interaction with *spn1Δ*; Wu *et al.*, 2010b), in septins *spn1* and *spn4*, in Polo kinase *plo1* (which has strong synthetic interaction with *gef2Δ*; Ye *et al.*, 2012), in β-glucan synthase *bgs1*, in other Rho GEFs *gef2*, *rgf1-3*, and *scd1*, and in Rho GTPases *rho1* to *rho5* (Table 1). Together these data suggest that Gef3 may regulate late cytokinesis together with Scw1 and Gef1.

The exocyst complex is involved in daughter cell separation by targeting the hydrolytic enzymes Eng1 and Agn1 to the septum (Martin-Cuadrado *et al.*, 2005). We then tested whether *gef3Δ* had genetic interactions with exocyst mutations. The ts mutant *sec8-1* was defective in cell separation even at the permissive temperature, but multiseptated *sec8-1* cells are rare (Wang *et al.*, 2002, 2003; Martin-Cuadrado *et al.*, 2005). However, most *gef3Δ sec8-1* cells contained multiple septa (Figure 3C). In addition, *gef3Δ* had very strong synthetic interaction with another exocyst mutant, *exo70Δ* (Figure 3D). Because the GTPase Rho3 modulates exocyst activity during cell separation and *rho3Δ* is synthetic lethal with exocyst mutants in fission yeast (Wang *et al.*, 2003), the genetic interactions

between *gef3Δ* and exocyst mutants suggested that Rho3 could be a potential substrate for Gef3. However, unlike Rho3, which partially depends on the exocyst complex for localization (Wang *et al.*, 2003), no obvious defect in Gef3 localization was observed in exocyst mutants at 25 (unpublished data) or 36°C (Supplemental Figure S2C). Collectively these genetic interactions indicate that Gef3 is involved in later stages of cytokinesis to regulate septum formation and/or cell separation.

### Gef3 preferentially interacts with Rho4 GTPase in vitro

To identify the potential Rho GTPase(s) that Gef3 regulates, we tested the interactions between purified full-length (FL) Gef3 and all six Rho GTPases in vitro. The hexahistidine (6His)-tagged Gef3 was purified from *Escherichia coli* (Supplemental Figure S3A, asterisk) and then was pulled down by purified recombinant glutathione S-transferase (GST)-tagged Rho proteins under the nucleotide-depletion condition (Supplemental Figure S3B). Gef3 interacted with Rho1, Rho4, and Rho5 but not with Rho3 or Cdc42 (Supplemental Figure S3, B and C). Apparently, the co-IP between Rho4 and Gef3 was the most efficient. Compared to the GST control, GST-Rho4 pulled down ~35 times more 6His-Gef3 (Supplemental Figure S3C).

Because Rho GEF functions are often regulated by posttranslational modifications such as phosphorylation (Cherfilis and Zeghouf, 2013), we also purified 3FLAG-Gef3 from *S. pombe* (Figure 4A, asterisk) and repeated the Rho GTPase pull-down assay (Figure 4, B and C). Consistently, Gef3 still preferentially bound to Rho4 (Figure 4C), although it was also pulled down by other Rho GTPases less efficiently. Together these data suggest that Gef3 has the strongest interaction with Rho4 GTPase.

### Gef3 is a Rho4 GEF in vitro

To determine whether Gef3 might act as a Rho GEF, we first tested whether Gef3 prefers GDP-bound Rho GTPase, a prerequisite for being a GEF. GST-Rho1, 3, 4, and 5 preloaded with GTPγS (a non-hydrolyzable analogue of GTP) or GDP or nucleotide depleted were incubated with yeast extract from cells expressing 3FLAG-Gef3. For Rho1 and Rho3, GTP or GDP had no obvious effect on the amount of Gef3 that was pulled down (Figure 5A). Gef3 coprecipitated with GTPγS-Rho5 more efficiently than GDP- or nucleotide-free Rho5 (Figure 5A). In contrast, Gef3 coprecipitated with GDP-bound and nucleotide-free Rho4 much more efficiently than GTPγS-Rho4 (Figure 5A), which suggests that Gef3 associates preferentially with inactive Rho4. Consistently, Gef3 pulled down more GDP-locked constitutive inactive Rho4-T28N compared with wt Rho4 or GTP-locked constitutive active Rho4-G23V (Figure 5B). Together the results indicate that Gef3 may function as a GEF for Rho4, but may have other GEF-independent functions with other Rho GTPases.

Next we tested directly whether Gef3 stimulates the release of GDP from Rho4 in vitro. Purified GST-Rho4 (Figure 5C) was

Mutation 1	Mutation 2	Genetic interaction at 25°C <sup>a</sup>	Mutation 1	Mutation 2	Genetic interaction at 25°C <sup>a</sup>
<i>gef3Δ</i>	<i>scw1Δ</i>	+++	<i>gef3(70-525)</i>	<i>gef1Δ</i>	+++ <sup>b</sup>
<i>gef3Δ</i>	<i>gef1Δ</i>	+++ <sup>b</sup>	<i>gef3(ΔDH)</i>	<i>gef1Δ</i>	+++ <sup>b</sup>
<i>gef3Δ</i>	<i>exo70Δ</i>	+++ <sup>b</sup>	<i>gef3(ΔDH)</i>	<i>scw1Δ</i>	+++
<i>gef3Δ</i>	<i>sec8-1</i>	+++	<i>gef3(ΔDH)</i>	<i>exo70Δ</i>	+++ <sup>b</sup>
<i>gef3Δ</i>	<i>for3Δ</i>	+++ <sup>c</sup>	<i>gef3(ΔDH)</i>	<i>sec8-1</i>	+++
<i>gef3Δ</i>	<i>cdc42-1625(A158V)</i>	++ <sup>c</sup>	<i>rho4Δ</i>	<i>scw1Δ</i>	+++
<i>gef3Δ</i>	<i>myo52Δ</i>	++	<i>rho4Δ</i>	<i>gef1Δ</i>	+++ <sup>b</sup>
<i>gef3Δ</i>	<i>art1Δ</i>	*	<i>rho4Δ</i>	<i>exo70Δ</i>	+++ <sup>b</sup>
<i>gef3Δ</i>	<i>spn1Δ</i>	+ <sup>b</sup>	<i>rho4Δ</i>	<i>sec8-1</i>	+++
<i>gef3Δ</i>	<i>spn4Δ</i>	+ <sup>b</sup>	<i>rho4Δ</i>	<i>for3Δ</i>	+++ <sup>c</sup>
<i>gef3Δ</i>	<i>plo1-ts18</i>	-	<i>rho4Δ</i>	<i>myo52Δ</i>	++
<i>gef3Δ</i>	<i>bgs1-191</i>	-	<i>rho4Δ</i>	<i>art1Δ</i>	*
<i>gef3Δ</i>	<i>gef2Δ</i>	+ <sup>b</sup>	<i>rho4Δ</i>	<i>plo1-ts18</i>	-
<i>gef3Δ</i>	<i>rgf1Δ</i>	-	<i>rho4Δ</i>	<i>bgs1-191</i>	-
<i>gef3Δ</i>	<i>rgf2Δ</i>	-	<i>rho4Δ</i>	<i>rgf1Δ</i>	-
<i>gef3Δ</i>	<i>rgf3-s44</i>	*	<i>rho4Δ</i>	<i>rgf2Δ</i>	-
<i>gef3Δ</i>	<i>scd1Δ</i>	-	<i>rho4Δ</i>	<i>rgf3-s44</i>	*
<i>gef3Δ</i>	<i>rho1-596</i>	-	<i>rho4Δ</i>	<i>scd1Δ</i>	-
<i>gef3Δ</i>	<i>rho2Δ</i>	-	<i>rho4Δ</i>	<i>rho1-596</i>	-
<i>gef3Δ</i>	<i>rho3Δ</i>	+ <sup>b</sup>	<i>rho4Δ</i>	<i>rho2Δ</i>	-
<i>gef3Δ</i>	<i>rho4Δ</i>	+ <sup>b</sup>	<i>rho4Δ</i>	<i>rho5Δ</i>	-
<i>gef3Δ</i>	<i>rho5Δ</i>	-	<i>rho4Δ</i>	<i>agn1Δ</i>	+++ <sup>b</sup>
<i>gef3Δ</i>	<i>agn1Δ</i>	+++ <sup>b</sup>	<i>rho4Δ</i>	<i>agn1Δ</i>	+++ <sup>b</sup>
<i>gef3(1-270)</i>	<i>gef1Δ</i>	+++ <sup>b</sup>	<i>rho3Δ</i>	<i>scw1Δ</i>	+++ <sup>d</sup>
<i>gef3(1-69)</i>	<i>gef1Δ</i>	+++ <sup>b</sup>	<i>rho3Δ</i>	<i>gef1Δ</i>	-

<sup>a</sup>Cells were grown exponentially in YE5S liquid medium before checking the morphology under DIC. The severity of defects compared with the parental mutants (in terms of abnormal septation) was classified as follows: +++, very strong synthetic interaction and very severe septation defects; ++, strong synthetic interaction and prominent septation defects; +, mild synthetic interaction and moderate septation defects; -, no additive septation defects; \*, no additive septation defects and mild suppression of cell lysis in mutation 2.

<sup>b</sup>The synthetic interaction is (even) more obvious at 36°C.

<sup>c</sup>The synthetic interaction is reflected by loss of polarity instead of septation defects.

<sup>d</sup>The synthetic interaction is reflected by increased cell lysis instead of septation defects.

**TABLE 1: Genetic interactions of double mutants described in this study.**

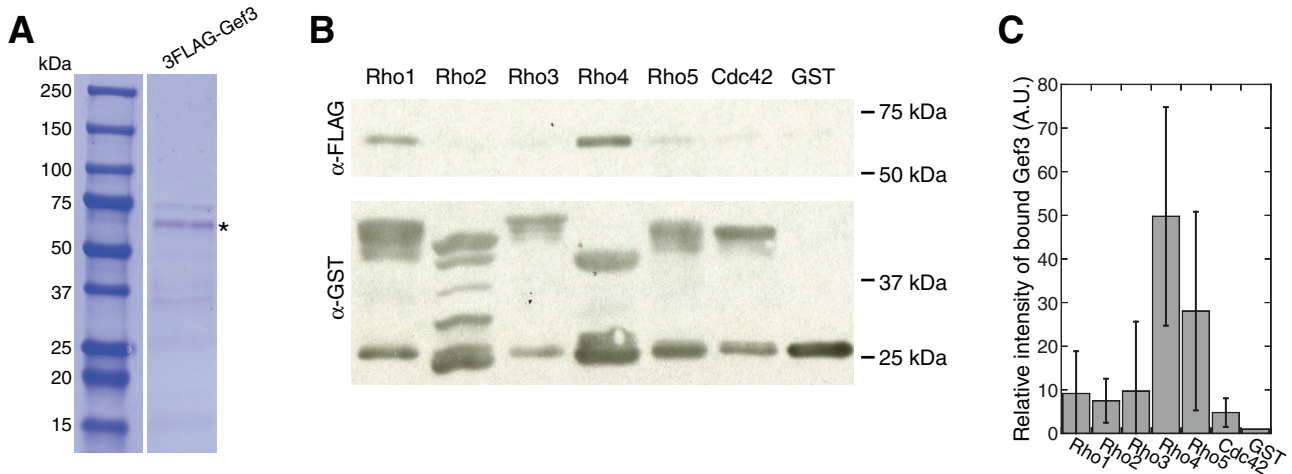
preloaded with *N*-methylanthraniloyl-GDP (mant-GDP), a fluorescent GDP analogue, before addition of Gef3. Both 6His-Gef3 purified from *E. coli* and 3FLAG-Gef3 purified from *S. pombe* accelerated mant-GDP release from Rho4 in a Gef3 concentration-dependent manner (Figure 5, D and E, and Supplemental Figure S3D), indicating that Gef3 has GEF activity toward Rho4 in vitro.

### Gef3 and Rho4 are in the same genetic pathways to regulate cytokinesis

To elucidate the relationship between Gef3 and Rho4 in vivo, we test whether Gef3 and Rho4 function in the same genetic pathway (Figure 6 and Table 1). We expected *gef3Δ* and *rho4Δ* to have similar genetic interactions with other mutations if Gef3 activates Rho4 in vivo. Indeed, *rho4Δ* had very strong synthetic interactions with *scw1Δ*, *gef1Δ*, and exocyst mutants. The double mutants displayed severe defects in septum formation and/or cell separation (Figure 6, Table 1, and Supplemental Figure S2, D and E). The septation indices of *rho4Δ scw1Δ*, *rho4Δ gef1Δ*, *rho4Δ sec8-1*, and *rho4Δ exo70Δ*

double mutants were almost identical to those of *gef3Δ scw1Δ*, *gef3Δ gef1Δ*, *gef3Δ sec8-1*, and *gef3Δ exo70Δ*, respectively (Figure 6, B, D, and F, and Supplemental Figure S2E). The synthetic interaction of *gef3Δ* and *rho4Δ* with *myo52Δ*, *for3Δ*, and exocyst mutants suggests that Gef3 and Rho4 may play roles in actin cable- and exocyst-dependent delivery of vesicles for cell separation and polarity establishment (Figures 3, C and D, and 6, E and F, Table 1, and Supplemental Figure S2, B, D, and E). On the other hand, like *gef3Δ*, *rho4Δ* had no or mild genetic interactions with mutations in arrestin *art1*, polo kinase *plo1*, β-glucan synthase *bgs1*, other Rho GEFs *rgf1-3*, *scd1*, and Rho GTPases *rho1*, *rho2*, and *rho5* (Table 1).

Because *rho3Δ* is synthetic lethal with exocyst mutant *sec8-1* and plays roles in late cytokinesis (Nakano *et al.*, 2002; Wang *et al.*, 2003; Kita *et al.*, 2011), we also tested the genetic interactions between *rho3Δ* and *scw1Δ* or *gef1Δ* (Figure 6, A–D, and Table 1). *rho3Δ* did not show similar genetic interactions with these two mutations as *gef3Δ* and *rho4Δ*. *rho3Δ scw1Δ* cells were shorter and rounder and had more cell lysis than single mutants (Figure 6A), whereas *gef3Δ*



**FIGURE 4:** Gef3 binds to the Rho GTPase Rho4 in vitro. (A) Coomassie blue staining of SDS-PAGE showing purified 3FLAG-Gef3 from *S. pombe*. The asterisk marks the 3FLAG-Gef3 band with the expected size. (B, C) Gef3 binds to Rho4 in pull-down assays. Purified GST-Rho GTPases and GST control were bound to beads, depleted for nucleotides, and then incubated with purified 3FLAG-Gef3. The amount of pulled-down Gef3 was detected by Western blotting (B; representative of four independent assays) and quantified (C; mean  $\pm$  1 SD). The intensities of 3FLAG-Gef3 bands were normalized by the intensities of pulled-down Rho GTPases. The intensity of 3FLAG-Gef3 band in GST control was set as 1.

*scw1Δ* and *rho4Δ scw1Δ* had higher septation indices and more multiseptated cells (Figure 6, A and B). In addition, *rho3Δ* had no synthetic genetic interaction with *gef1Δ* (Figure 6, C and D). Taken together, the results indicate that Rho4 but not Rho3 is in the same genetic pathways as Gef3 in regulating septum formation and/or cell separation.

#### Recruitment and maintenance of Rho4 at the division site are affected in *gef3Δ* and *spn1Δ* cells

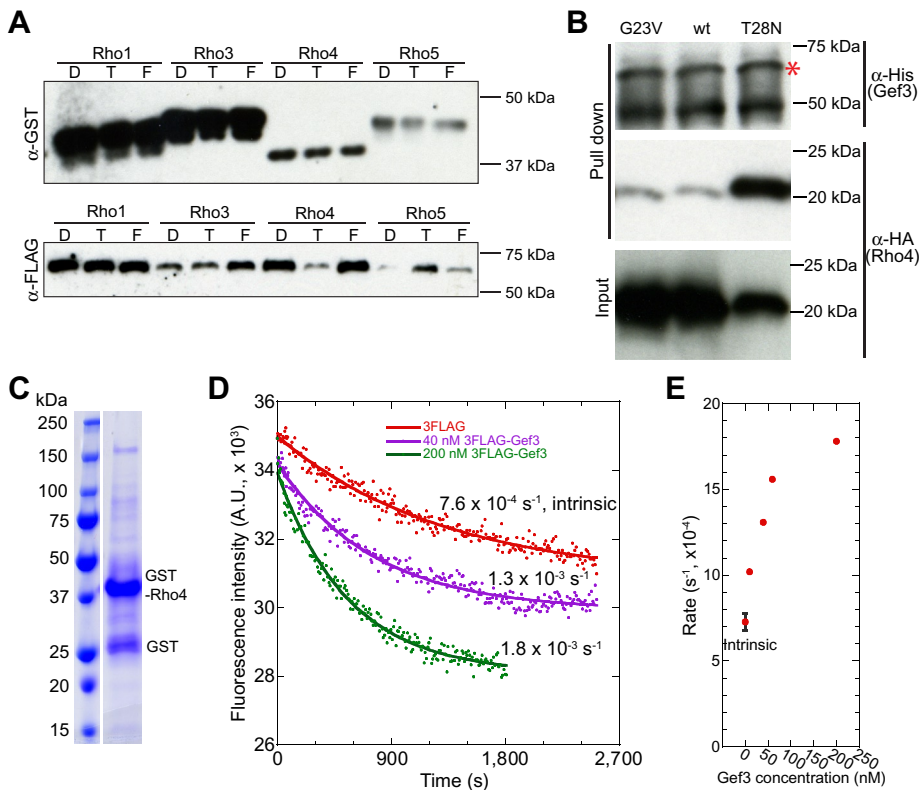
Besides activating Rho GTPases, GEFs are often involved in recruiting Rho GTPases to the functioning site in vivo (Garcia *et al.*, 2006b; Yoshida *et al.*, 2009; Zhu *et al.*, 2013), so we tested whether Gef3 affects Rho4 localization to the division site. Because Rho3 modulates exocytosis and regulates cell separation (Wang *et al.*, 2003; Kita *et al.*, 2011), we tested its localization in *gef3Δ*, although our in vitro binding and genetic data do not support that Rho3 is Gef3 substrate. Rho4 localizes to the cell-division site during septum formation and cell separation (Figure 7A; Nakano *et al.*, 2003; Santos *et al.*, 2003). Rho3 localizes to the division site, Golgi, endosomes, and plasma membrane (Supplemental Figure S4A; Nakano *et al.*, 2002; Wang *et al.*, 2003; Kita *et al.*, 2011). Like Gef3, both Rho4 and Rho3 localized to ring structures when they first appeared at the division site (Figure 7A and Supplemental Figure S4A, red boxes). As the septum formed, Rho3 and Rho4 formed disk-like structures (Figure 7A and Supplemental Figure S4A, blue boxes), which is different from Gef3, as it stayed as the hollow rings even in cells with a complete septum (Figure 7A, blue box). The difference in localization between Rho4 and Gef3 implies that Rho4 activation by Gef3 may be spatially restricted.

Although we detected no obvious changes in Rho4 localization and intensity in either the ring (arrows) or the disk (arrowheads) in unsynchronized *gef3Δ* cells (Figure 7, B and C), *spn1* deletion did partially affect Rho4 localization. Whereas Rho4 localization and intensity at the ring were not obviously affected in *spn1Δ* cells (Figure 7, B and C, arrow), Rho4 intensity at the division site in cells with a complete septum (the disk stage) was significantly reduced

(Figure 7, B and C, arrowheads). To determine whether this effect is caused by the delayed cell separation in *spn1Δ* cells, we followed the localization and intensity of Rho4 with time-lapse microscopy (Figure 7D and Supplemental Figure S4B). In wt cells, Rho4 intensity reached the peak ~24 min after its appearance at the division site (Figure 7D, red line). Then the intensity dropped to ~50% of the peak value when the daughter cells separated (Figure 7D, red line). In *spn1Δ* cells, Rho4 recruitment was similar to wt cells, whereas Rho4 intensity decreased faster and further (Figure 7D, green line). Of interest, in *gef3Δ* cells, Rho4 levels at the division site peaked at ~32 min, but the intensity dropped at the similar rate as in wt cells (Figure 7D, purple line). On the other hand, the localization and intensity of Rho3 in neither the ring (arrows) nor the disk (arrowheads) were affected by *gef3Δ* or *spn1Δ* (Supplemental Figure S4, C and D). Moreover, Gef3 localization and intensity at the division site were not affected by *rho4Δ* or *rho3Δ* (Figure 7E and Supplemental Figure S4E). Together these data suggest that septins regulate the localization of both Rho4 and Gef3 during cytokinesis and that Gef3 only plays a minor role in Rho4 recruitment.

#### Gef3 modulates the localization of glucanases Eng1 and Agn1 at the division site

Next we tested whether Gef3 affects the localizations of potential Rho4 downstream effectors, which may reflect the localization of active Rho4. Because Rho4 regulates the secretion of  $\beta$ -glucanase Eng1 and  $\alpha$ -glucanase Agn1 during cell separation (Santos *et al.*, 2005), we checked the localizations of Eng1 and Agn1 in *gef3Δ*. In wt cells with complete septa, Eng1 localized to nonconstricting rings in ~50% of cells (Figure 8A, arrowhead), whereas the rest of the cells also had a weak Eng1 dot signal in the center of the septa besides the ring signal (ring plus dot; Figure 8, A and B; wt, red box). However, Eng1 ring signal was lost at the division site in 10% of *gef3Δ* cells with complete septa (Figure 8, A and B, arrows). In the majority of *gef3Δ* cells (90%), Eng1 formed a nonuniform disk at the septum, with brighter signals at the ring and the center (Figure 8, A and B, red box). Consistent with a previous report (Santos *et al.*, 2003,



**FIGURE 5:** Gef3 is a Rho GEF of Rho4 *in vitro*. (A) Gef3 coprecipitates more efficiently with GDP-Rho4 than GTP $\gamma$ S-Rho4. Purified GST-Rho1, 3, 4, and 5 were bound to beads, preloaded with either GDP (D) or GTP $\gamma$ S (T), or depleted for nucleotides (F) and incubated with yeast extract from cells expressing 3FLAG-Gef3. Experiments were performed three times for Rho4 and twice for Rho1, Rho3, and Rho5, and a representative Western blotting is shown. (B) Gef3 pulls down Rho4-T28N more efficiently than wt and Rho4-G23V. Purified 6His-Gef3 was bound to beads and incubated with yeast extracts from cells expressing 3HA-tagged Rho4-G23V, Rho4 (wt), or Rho4-T28N (Santos *et al.*, 2003). Asterisk marks the expected 6His-Gef3 band. (C) Coomassie blue staining of SDS-PAGE showing purified GST-Rho4 from *E. coli*. (D, E) Gef3 accelerates nucleotide exchange of mant-GDP bound GST-Rho4 in GEF activity assays. Fluorescence intensity was monitored to reflect the disassociation of mant-GDP from Rho4. (D) Nucleotide exchanges in the presence of 3FLAG peptide as a control (intrinsic) or two concentrations of 3FLAG-Gef3 shown with the calculated rates after curve fit (see *Materials and Methods*). (E) Fluorescence decrease rate vs. Gef3 concentration. The intrinsic rate is the mean  $\pm$  1 SD from three independent assays.

2005), *rho4* $\Delta$  cells showed the same mislocalization pattern for Eng1 (Figure 8, A and B), which is also similar to reported Eng1 localization in exocyst mutants (Martin-Cuadrado *et al.*, 2005). In addition, as in *rho4* $\Delta$  cells (Santos *et al.*, 2005), Agn1 localization at the division site was undetectable or significantly reduced in *gef3* $\Delta$  cells (Supplemental Figure S5A). Moreover, both *gef3* $\Delta$  and *rho4* $\Delta$  were synthetic sick with *agn1* $\Delta$  in terms of cell separation (Supplemental Figure S5B), which was expected, given that *agn1* $\Delta$  *eng1* $\Delta$  displayed additive defects in cell separation (Dekker *et al.*, 2004). Thus Gef3 modulates the normal localizations of glucanases Eng1 and Agn1, likely by regulating Rho4 activity.

### Rho GEF Gef3 activates Rho4 GTPase *in vivo*

The identical effects of *gef3* $\Delta$  and *rho4* $\Delta$  on Eng1 and Agn1 localization suggest that Gef3 activates Rho4 for cell separation *in vivo*. To test this idea, we performed pull-down experiments using cell extracts to compare the amount of active Rho4 using Rho-binding domain (RBD) of human Rhotekin protein. Because RBD preferentially binds to GTP-RhoA in mammalian cells (Reid *et al.*, 1996; Ren *et al.*, 1999), we first tested whether GST-RBD prefers

active Rho4 in *S. pombe*. GST-RBD pulled down active GTP-locked Rho4-G23V more efficiently than wt Rho4 and inactive Rho4-T28N (Figure 8C). Next we compared active Rho4 in wt and *gef3* $\Delta$  cells. *gef3* $\Delta$  cells had about half the amount of GTP-Rho4 as wt cells (Figure 8, D and E), suggesting that Gef3 activates Rho4 *in vivo*.

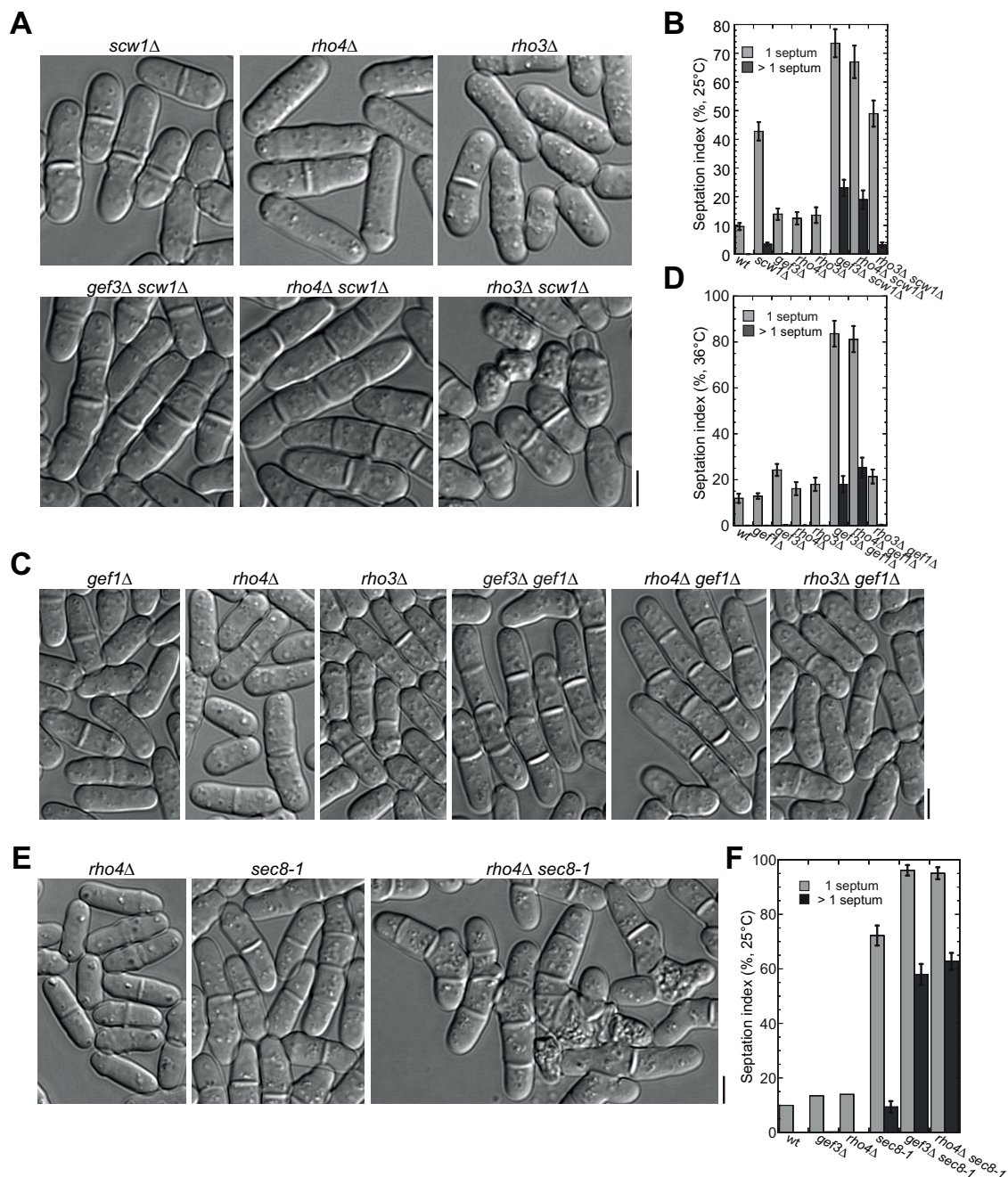
Our genetic data provide further support that Gef3 activates Rho4 *in vivo*. Overexpression of constitutively active Rho4-G23V reduced the septation index of *gef3* $\Delta$  *scw1* $\Delta$  cells from ~54% (with empty vector) to ~24% after 24 h of induction. The percentage of multiseptated cells was also reduced from ~14 to ~4% (Figure 8, F and G). Overexpression of wt and the constitutively inactive Rho4-T28N also partially rescued *gef3* $\Delta$  *scw1* $\Delta$  mutant (Figure 8, F and G), probably due to the basal or residual Rho GTPase activity. Moreover, overexpression of Eng1 (Figure 8, F and G) and Agn1 (unpublished data) also partially rescued the septation defects in *gef3* $\Delta$  *scw1* $\Delta$ . In contrast, overexpression of wt Rho4, Rho4-G23V, and Rho4-T28N did not rescue *scw1* $\Delta$  cells (Supplemental Figure S5, C and D), indicating that the rescue of *gef3* $\Delta$  *scw1* $\Delta$  is specific to the Gef3-Rho4 pathway. Thus high levels of active Rho4 and its potential effectors Eng1 and Agn1 can compensate the loss of Gef3. Taken together, these data suggest that Gef3 activates Rho4 GTPase as a GEF *in vivo*.

### N-terminus of Gef3 is important for its localization, and DBL homology domain and C-terminus of Gef3 are critical for its function

To understand how Gef3 localizes and functions, we performed domain analyses. Most GEFs of Rho GTPases contain both DBL homology (DH) and pleckstrin homology (PH) domains (Cherfils and Zeghouf, 2013), whereas Gef3 only contains the DH domain (amino acids [aa] 76–267, Figure 9A; Garcia *et al.*, 2006b). Of interest, both Gef3 C-terminal truncations, Gef3(1–270) and Gef3(1–69), together with the truncation without the DH domain (Gef3 $\Delta$ DH), still localized to the rings at the division site, although the signals were weaker than that of FL Gef3 (Figure 9, A and B). In contrast, neither of the N-terminal truncations Gef3(70–525) and Gef3(267–525) localized to the division site (Figure 9, A and B), although all the truncations were expressed and detected by Western blotting (Figure 9C).

Consistent with the idea that Gef3 regulates cytokinesis as a Rho4 GEF, Gef3 $\Delta$ DH was not sufficient for Gef3 function, although it still localized to the division site. *gef3* $\Delta$ DH had synthetic interactions with *gef1* $\Delta$ , *scw1* $\Delta$ , and exocyst mutant *exo70* $\Delta$  and *sec8-1* mutants (Figure 9, D and E, and Table 1), and the phenotypes of the double mutants resembled those of *gef1* $\Delta$  *gef3* $\Delta$  (Figure 9, D and E), *scw1* $\Delta$  *gef3* $\Delta$ , and *sec8-1* *gef3* $\Delta$ . Of interest, the two C-terminal truncation mutants *gef3*(1–270) and *gef3*(1–69) also had synthetic interactions with *gef1* $\Delta$ , *scw1* $\Delta$ , and *sec8-1* mutants (Figure 9, D and E, and Table 1). Thus aa 271–525 and the DH domain are both important for Gef3





**FIGURE 6:** Gef3 and Rho4 are in the same genetic pathways. (A–F) *rho4Δ* shows similar genetic interactions as *gef3Δ*. DIC images (A, C) and septation indices (B, D) showing that *gef3Δ* and *rho4Δ*, but not *rho3Δ*, have similar synthetic genetic interactions with *scw1Δ* at 25°C (A, B) or with *gef1Δ* at 36°C (C, D; grown at 36°C for 6 h before imaging). (E, F) DIC images (E) and quantification (F) of synthetic interactions of exocyst mutant *sec8-1* with *gef3Δ* and *rho4Δ* at 25°C. Mean  $\pm$  1 SD from three independent experiments is plotted for *sec8-1* and double mutants, and only one set of representative data is shown for wt, *gef3Δ*, and *rho4Δ*, as they are repetitive to B. For each strain,  $n > 600$  cells in each of the three experiments in B, D, and F. Bars, 5  $\mu$ m.

function. Given that Gef3 lacks the conventional PH domain, it is possible that the C-terminus may become necessary to facilitate Gef3's function as a GEF. Together these data indicate that the N-terminal aa 1–69 are required for Gef3 localization, and the C-terminus including the DH domain is required for Gef3 function during septation.

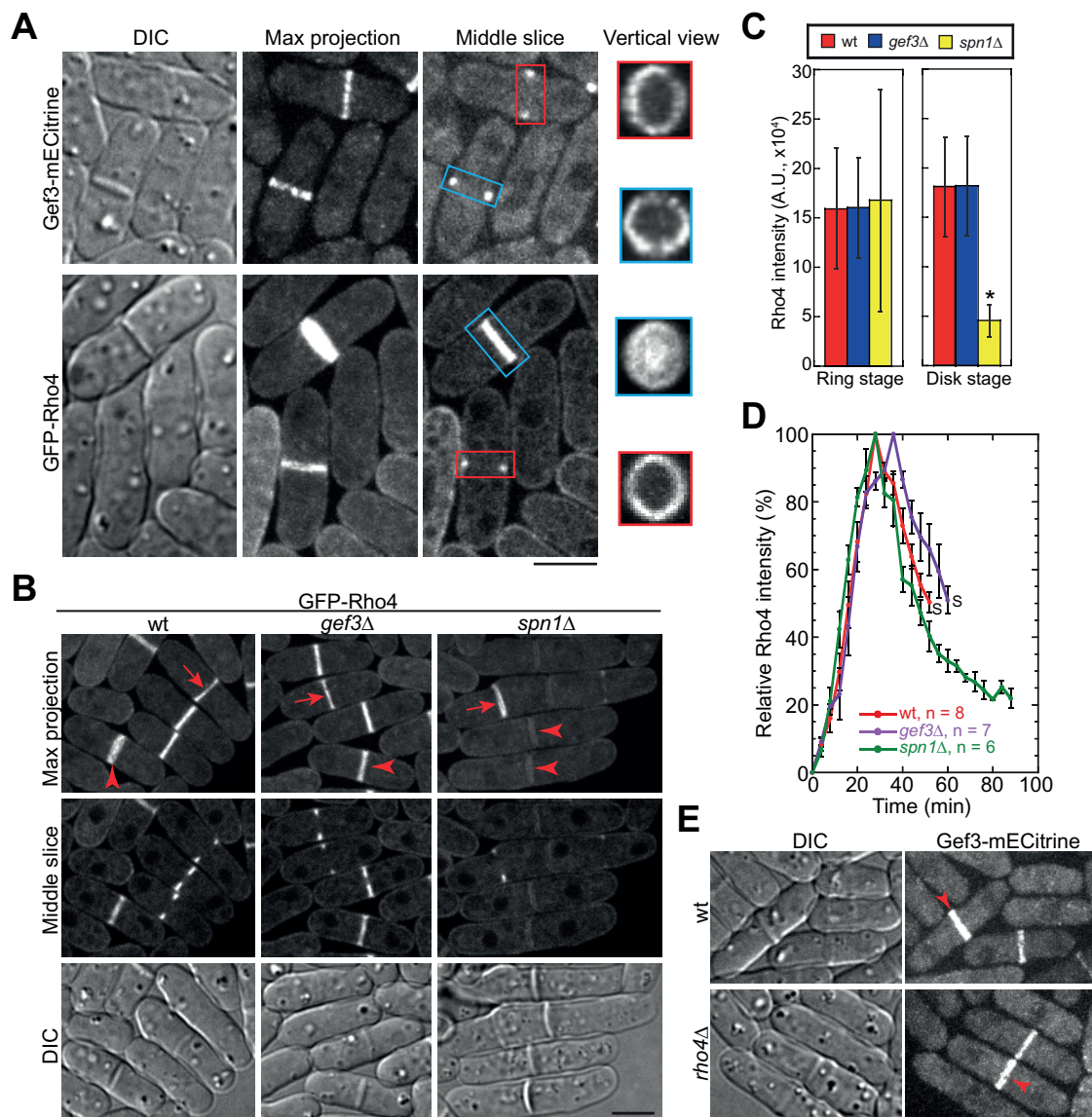
## DISCUSSION

In this study, we find that Gef3, unlike the other six predicted (or proven) Rho GEFs, depends on septins for localization to the division

site. Gef3 plays roles in the regulation of septation during fission yeast cytokinesis. With combined genetic and biochemical experiments, we identify GTPase Rho4 as a substrate for the Rho GEF Gef3.

### Gef3 is a Rho GEF for the GTPase Rho4

The putative Rho GEF Gef3 was the least characterized among the predicted Rho GEFs in *S. pombe*. In this study, several lines of evidence support that Gef3 has GEF activity toward the GTPase Rho4: 1) purified Gef3 coprecipitates most efficiently with purified

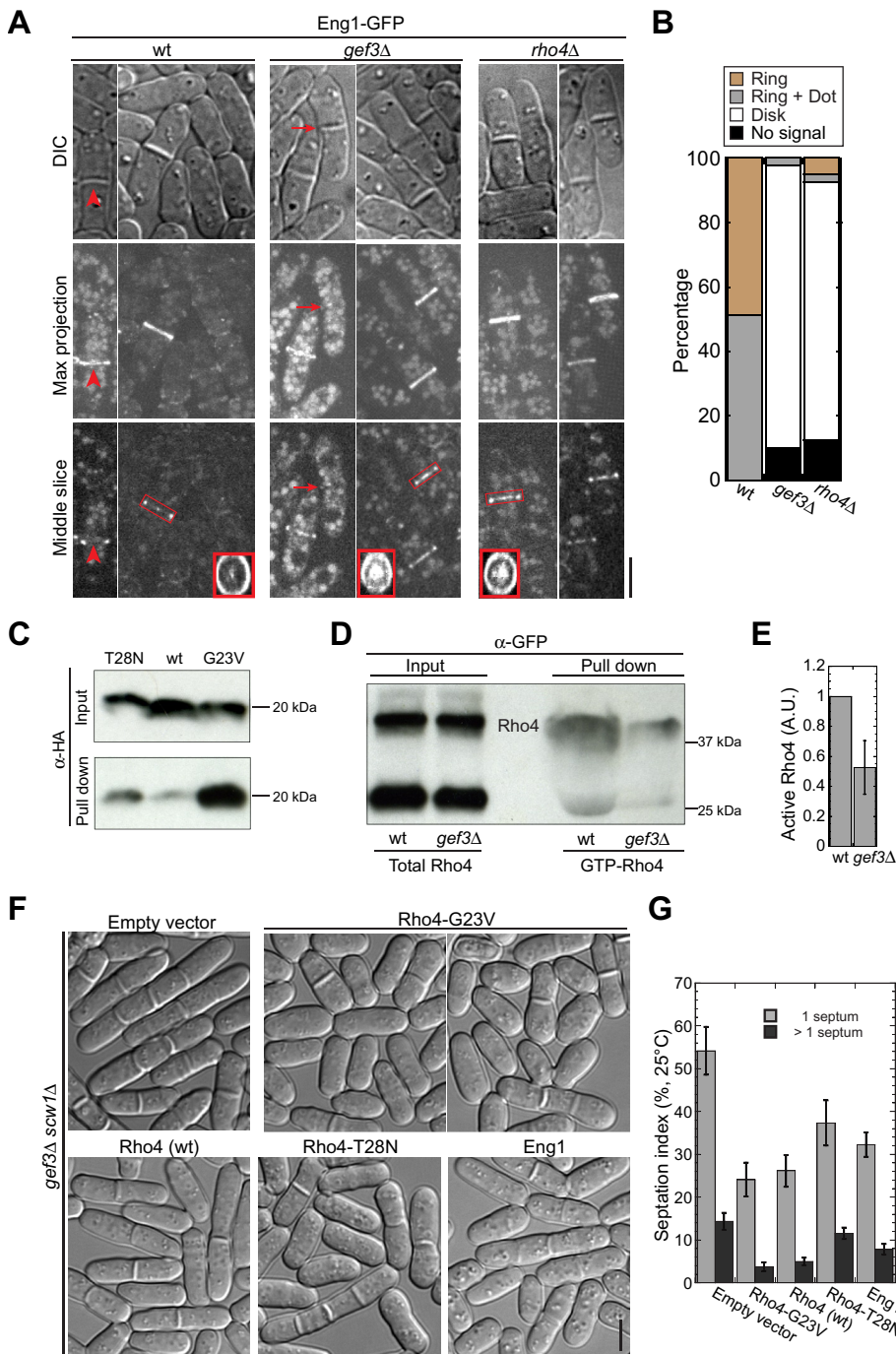


**FIGURE 7:** Recruitment and maintenance of Rho4 at the division site are affected in *gef3Δ* and *spn1Δ* cells. (A) Localization of Gef3 and Rho4 in maximum-intensity projection, the middle slice of z-stacks, or vertical view of color-boxed regions. Red boxes, cells at the start of septum formation; blue boxes, cells with complete septa. (B, C) Localization (B) and intensity quantification (C) of Rho4 at the division site in wt, *gef3Δ*, and *spn1Δ* cells. (B) The maximum projection and middle slice of z-stacks are shown. Arrows, cells at the start of septum formation (ring stage); arrowheads, cells with complete septa (disk stage). (C) Fluorescence intensities (mean  $\pm$  1 SD) at the ring and disk stage. \* $p < 0.001$  compared with wt from t test. For each strain,  $n > 40$  cells at each stage. (D) Time-lapse analysis of GFP-Rho4 intensity at the division site during cytokinesis. The peak intensity of Rho4 for each measured cell is set as 100%. Time 0 marks the time point just before Rho4 appears at the division site (see Supplemental Figure S4B for example). Mean  $\pm$  SEM for each time point. S, cell separation. Note that most *spn1Δ* cells did not separate at the last time point shown in the graph (88 min). (E) Gef3 localization is not affected by *rho4Δ*. Arrowheads mark cells with complete septa. Bars, 5  $\mu$ m.

Rho4 in vitro when nucleotides are depleted (Figure 4 and Supplemental Figure S3); 2) Gef3 prefers to bind GDP-bound Rho4 (Figure 5, A and B); 3) Gef3 accelerates nucleotide exchange of Rho4 in vitro (Figure 5, D and E); 4) Gef3 and Rho4 are in the same genetic pathways (Figure 6 and Table 1); 5) *gef3Δ* and *rho4Δ* have similar effects on the localization of Eng1 (Figure 8, A and B), a potential effector of active Rho4; 6) *gef3Δ* cells have less active Rho4 than wt cells (Figure 8, C–E); 7) overexpression of constitutively active and wt Rho4 rescues septation defects in *gef3Δ* *scw1Δ* but not *scw1Δ* cells (Figure 8, F and G, and Supplemental Figure S5, C and D); and 8) the DH domain of Gef3 is important

for its function (Figure 9). Together our data indicate that Gef3 is a GEF of Rho4 GTPase.

We previously found that putative Rho GEF Gef2 regulates Rho4 localization in vivo and binds to Rho4 in vitro, suggesting that Gef2 could be another GEF of Rho4 (Zhu *et al.*, 2013). Here we find that *gef2Δ* is slightly synthetic sick with *gef3Δ* (Table 1) in septation. This suggests that both Gef2 and Gef3 are involved in regulating Rho4 during septation. However, they may use different mechanisms, given that *gef2Δ* reduces Rho4 localization at the division site (Zhu *et al.*, 2013) but *gef3Δ* only mildly delays Rho4 recruitment (Figure 7D). Moreover, Gef2 localizes to the contractile ring and



**FIGURE 8:** Gef3 regulates the localization of glucanases and Rho4 activity in vivo. (A, B) Eng1 localization is affected in *gef3Δ* cells. Micrographs (A) and quantification (B) showing that Eng1 localization at division site is affected in *gef3Δ* and *rho4Δ* cells. The arrowhead indicates a wt cell with Eng1 in the nonconstricting ring. Vertical views of the regions marked with red boxes are shown at the bottom corners. Arrows mark a *gef3Δ* cell without an intact Eng1 ring at the division site. (B) Only cells with complete septa were counted. For each strain,  $n > 40$  cells. (C) RBD pulls down constitutively active Rho4-G23V more efficiently than wt and constitutively inactive Rho4-T28N. Yeast extracts from cells expressing 3HA tagged wt or mutant Rho4 were incubated with 200  $\mu$ g of GST-RBD. The amount of Rho4 was detected by Western blotting. (D, E) Western blotting (D) and quantification (E) showing that *gef3Δ* cells have less active Rho4 than wt cells. (E) Mean  $\pm$  1 SD from three independent experiments. The level in wt is normalized to 1. (F, G) Overexpression of Rho4 or Eng1 rescues the septation defects in *gef3Δ scw1Δ* cells with plasmids grown under inducing conditions in liquid EMM5S – leucine for 24 h. (G) Mean  $\pm$  1 SD from three independent experiments, and  $n > 600$  for each strain in each experiment. Bars, 5  $\mu$ m.

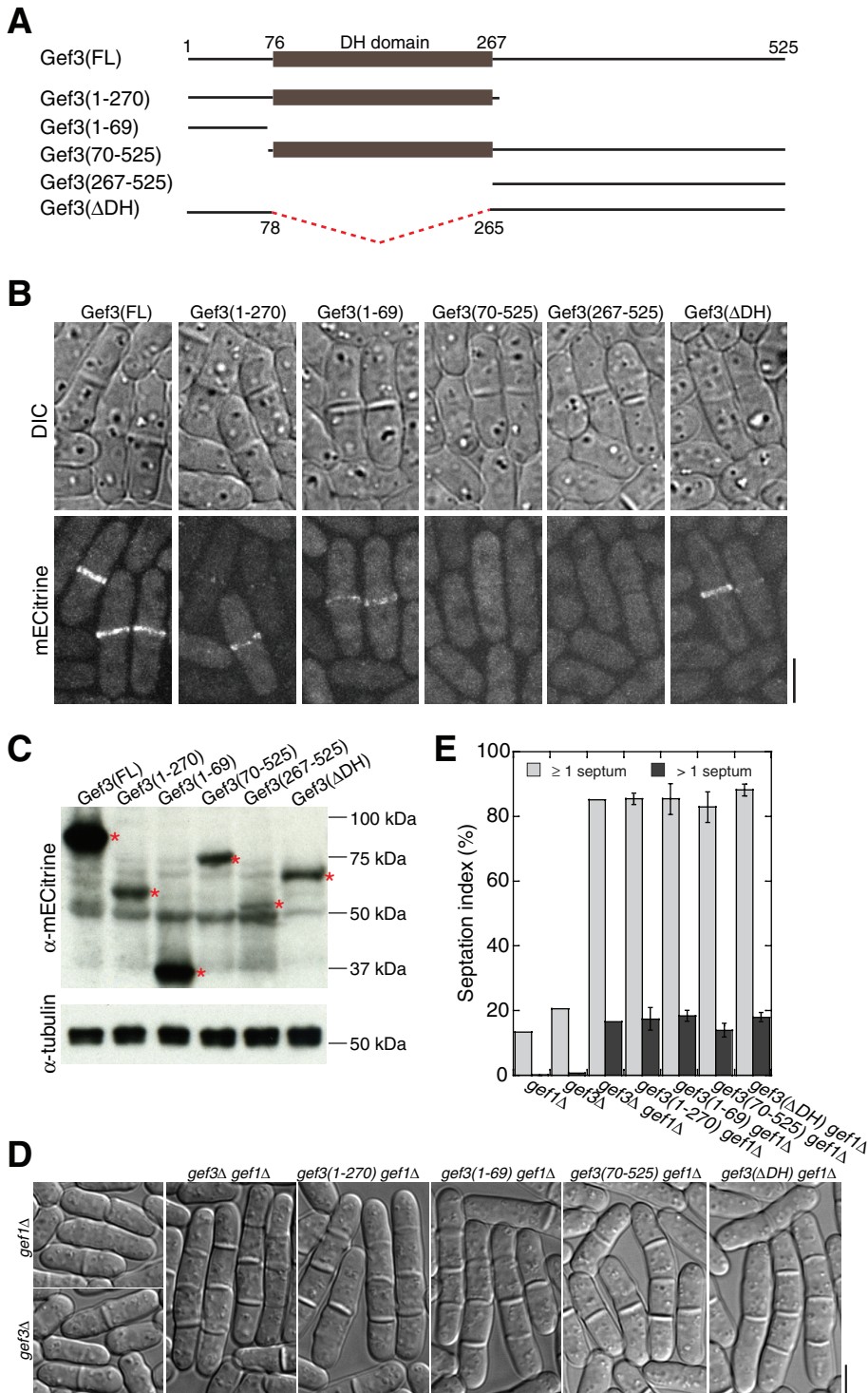
Gef3 to the septin ring, and thus their regulations of Rho4 may be spatially and temporally separated.

Cell separation in *S. pombe* is achieved by the concerted actions of  $\beta$ -glucanase Eng1 and  $\alpha$ -glucanase Agn1 to digest the primary septum and the surrounding cell wall on cell sides (Martin-Cuadrado *et al.*, 2003; Dekker *et al.*, 2004; Sipiczki, 2007). The proper localizations of Eng1 and Agn1 require the functions of the exocyst complex, septins, and active Rho4 (Martin-Cuadrado *et al.*, 2005; Santos *et al.*, 2005). Rho4 also regulates septum formation, given that *rho4Δ* cells have thicker secondary septa with abnormal structure by electron microscopy (Nakano *et al.*, 2003) and *rho4Δ* has very strong synthetic interaction with *gef1Δ* (Figure 6, C and D), a deletion mutant of the Cdc42 GEF involved in septum formation (Hirota *et al.*, 2003). Further studies are needed to distinguish whether the defects in *gef3Δ* and *gef3Δ*-containing double mutants are caused by cell separation, septum formation, or both.

### Gef3 may have Rho4-independent functions

The mild synthetic septation defects in *gef3Δ rho4Δ* double mutant (Table 1) and partial colocalization of Gef3 and Rho4 (Figure 7A) suggest that Gef3 may have Rho4-independent functions, which may or may not depend on its GEF activity. Besides Rho4, Gef3 also interacts with Rho1 and Rho5 in *in vitro* binding assays (Figure 4 and Supplemental Figure S3). Both Rho1 and Rho5 are activated by several Rho GEFs (Tajadura *et al.*, 2004; Mutoh *et al.*, 2005; Nakano *et al.*, 2005; Garcia *et al.*, 2006a, 2009; Rincon *et al.*, 2006) and have multiple downstream effectors (Arellano *et al.*, 1996, 1999; Nakano *et al.*, 2005; Rincon *et al.*, 2006; Prosser *et al.*, 2011). However, the fact that the GDP-bound Rho1 and Rho5 are not preferred in the binding assay (Figure 5A) does not support that Gef3 is a GEF for Rho1 and Rho5. Consistently, the lack of synthetic genetic interactions between *gef3Δ* and mutations in *rho1* and *rho5*, in their known Rho GEFs (*rgf1*, *rgf2*, and *rgf3*), and in their effectors or regulators (*bgs1* and *art1*) further indicates that Gef3 is not a major GEF for Rho1 or Rho5 (Table 1).

During the final preparation of our manuscript, Muñoz *et al.* (2014) reported that Gef3 physically interacts with active, GTP-bound Rho3 when they were overexpressed, but the level of active Rho3 *in vivo* was not affected by Gef3 levels, indicating that Gef3 acts as a scaffold but not a GEF to stabilize GTP-Rho3. Our *in vitro*



**FIGURE 9:** Domain analyses for Gef3. (A) Schematics of Gef3 domains and truncations constructed. (B, C) Localizations (B) and protein levels (C) of FL and truncated Gef3. Asterisks on Western blotting mark the bands with the expected sizes for FL and truncated Gef3. Tubulin was blotted as a loading control. (D, E) DIC images (D) and septation index (E) showing synthetic interactions between *gef3* truncations and *gef1 $\Delta$* . Cells were grown at 36°C for 6 h before imaging. (E) Mean  $\pm$  1 SD from three independent experiments, and  $n > 600$  cells for each strain in each experiment. Bars, 5  $\mu$ m.

binding assays agree with their finding that Gef3 is not a GEF of Rho3. However, our genetic data do not support strong functional connection between Gef3 and Rho3 in vivo. Further studies on

GeF–Cdc42 modules are essential for division-site selection and cell polarization (Chant *et al.*, 1995; Gladfelter *et al.*, 2002; Iwase *et al.*, 2006; Bi and Park, 2012; Kang *et al.*, 2013; Sadian *et al.*, 2013).

Gef3–Rho3 physical interaction would be of interest.

Gef3 has the least efficient physical interaction with nucleotide-free Cdc42 (Figure 4 and Supplemental Figure S3); thus it is unlikely that Gef3 is a GEF for Cdc42. Gef1 and Scd1 are known GEFs for Cdc42 (Coll *et al.*, 2003; Hirota *et al.*, 2003). The very strong synthetic interaction between *gef3 $\Delta$*  and *gef1 $\Delta$*  in septation indicates that Gef3 and Gef1/Cdc42 may play an overlapping function in septum formation. The polarity defects in *gef3 $\Delta$  cdc42-1625* and *gef3 $\Delta$  for3 $\Delta$*  (Table 1 and Supplemental Figure S2A) suggest that Gef3 also has a function in cell polarity. Although Gef3 only localizes to the division site and transiently to the new cell ends, its role in cell polarization is consistent with growth-polarity defects reported in mutants with defects in final stages of cytokinesis (Bohnert and Gould, 2012). Alternatively, Gef3 and Rho4 may indirectly regulate cell polarity by affecting exocyst-dependent delivery of vesicles to polarized growth sites, especially given that *gef3 $\Delta$*  and *rho4 $\Delta$*  are both synthetic sick with *myo52 $\Delta$* , a mutant defective in long-distance vesicle transport along actin cables (Win *et al.*, 2001; Bendezú and Martin, 2011).

#### Conserved septins-anillin-Rho GEF module at cell-division site

Septins are usually associated with the plasma membrane and function as a scaffold or diffusion barrier to regulate cellular processes, including cytokinesis and cell polarization (Kinoshita, 2003; Bertin *et al.*, 2008; Oh and Bi, 2011). In budding yeast, septins are essential and critical for localization of many proteins to the mother-bud neck/division site (Oh and Bi, 2011). In fission yeast, very few proteins have been identified to be septin dependent at the division site, which agrees with the mild phenotype of septin deletion in this yeast (Longtine *et al.*, 1996; Berlin *et al.*, 2003; Tasto *et al.*, 2003; An *et al.*, 2004). Consistent with a recent report (Muñoz *et al.*, 2014), we found that Gef3 localization is completely abolished in *spn1 $\Delta$*  and severely compromised without anillin Mid2 (Figure 2A). The *spn1 $\Delta$*  mutant also affects Rho4 maintenance at the septum (Figure 7, B–D). Moreover, our co-IP data are consistent with the idea that Gef3, septins, and anillin Mid2 form a protein complex (Figure 2C).

It is well known that septins–Bud3–Bud4 (anillin-like) and septins–Cdc24 (Cdc42 GEF)–Cdc42 modules are essential for division-site selection and cell polarization (Chant *et al.*, 1995; Gladfelter *et al.*, 2002; Iwase *et al.*, 2006; Bi and Park, 2012; Kang *et al.*, 2013; Sadian *et al.*, 2013).

Recently Kang *et al.* (2014) reported that Bud3 is a GEF of Cdc42 during polarity establishment. Although the functions of budding yeast Bud3 and fission yeast Gef3 are apparently different and Gef3 is only one-third as long as Bud3, both of them contain only a DH domain instead of typical DH-PH domain as in other Rho GEFs. Considering the high divergence between budding yeast and fission yeast, the septins-anillin-Rho GEF complex might present one of the most conserved modules for septin functions. Consistently, Bud3 homologues in other fungi have been suggested to function as a Rho4 GEF for contractile-ring and septum formation (Justa-Schuch *et al.*, 2010; Si *et al.*, 2010). In animal cells, interactions between septins, anillin, and the Rho GEFs Ect2 and Pebble during cytokinesis have also been reported (Oegema *et al.*, 2000; Hickson and O'Farrell, 2008; Silverman-Gavrila *et al.*, 2008; Frenette *et al.*, 2012). Moreover, a mammalian septin Sept9b was found to bind to a Rho GEF (SA-RhoGEF) and regulate its localization and GEF activity (Nagata and Inagaki, 2005). Thus the septins-anillin-Rho signaling is a highly conserved module in cell polarization and cytokinesis.

In conclusion, we revealed the role of Gef3 in septation during cytokinesis and found that Gef3 physically interacts with the septins-anillin complex and its localization depends on septins. We found that Rho4 GTPase is the substrate of Gef3. It will be interesting to test whether Gef3 also regulates other Rho GTPases, although not necessarily acting as a GEF. Finally, we propose that septins-anillin-Rho GEFs is a highly conserved module functioning at the cell-division site.

## MATERIALS AND METHODS

### Strains and genetic, molecular, and cellular methods

Strains used in this study are listed in Supplemental Table S1. PCR-based gene targeting and standard yeast genetics were used to construct strains (Moreno *et al.*, 1991; Bähler *et al.*, 1998b). All tagged and truncated strains for *gef3* and *rho3* are regulated under their endogenous promoters and integrated into native chromosomal loci, except the overexpression strain JW6030 for purifying 3FLAG-Gef3 from *S. pombe*, which is integrated at *gef3* native locus under the control of inducible *3nmt1* promoter (Maundrell, 1990). GFP-Rho4 strain contains *Prho4-GFP-rho4* integrated at *leu1* locus with the endogenous *rho4* deleted (a gift from Beatriz Santos, Universidad de Salamanca, Spain; Santos *et al.*, 2003).

The N-terminal tagging and truncations of *gef3* and *rho3* were made by first cloning the promoter regions of *gef3* (−439 to +6) and *rho3* (−193 to +6) into the pFA6a-kanMX6-P3nmt1-mECitrine plasmid at *Bgl*II and *Pac*I sites to replace the *3nmt1* promoter. The resulting plasmids JQW755 and JQW756 were sequenced and used as templates to amplify kanMX6-Pgef3-mECitrine or kanMX6-Prho3-mECitrine fragments flanked with homologous sequences from the designated positions at *gef3* or *rho3* locus, which were then transformed into wt strain as described (Bähler *et al.*, 1998b). We used PCR to confirm the positive transformants. The functionalities of Gef3 tagged at N- or C-terminus were tested by crossing the strains to *gef1Δ*. The growth and morphology of the double mutants were similar to those of *gef1Δ* single mutant at different temperatures, indicating that the tagged proteins are functional.

The Gef3(ΔDH) truncation was made as follows: Pgef3-gef3-mECitrine-kanMX6 was cloned into Topo vector (JQW769). Then the sequence encoding aa 79–264 of Gef3 was removed by PCR as described (Lee and Wu, 2012) to obtain the plasmid JQW770. This resulting plasmid was sequenced and then used as the template to amplify Pgef3-gef3(ΔDH)-mECitrine-kanMX6 fragment. The purified PCR product was transformed into *gef3Δ* strain (JW5492) to obtain

the *gef3*(ΔDH) truncation mutant with the mECitrine tag at its C-terminus.

Cells were grown in exponential phase in yeast extract medium with five supplements (YE5S) or Edinburgh minimal medium plus five supplements (EMM5S) at 25°C for 36–48 h before microscopy or other experiments as previously described (Wu *et al.*, 2006; Coffman *et al.*, 2013), except where noted. Plasmids for overexpression of Eng1 (Santos *et al.*, 2005) and wt, constitutively active, and constitutively inactive Rho4 (gifts from Beatriz Santos; Santos *et al.*, 2003) were under the control of *3nmt1* promoter. The plasmids were transformed into yeast as described (Bähler *et al.*, 1998b) and EMM5S – leucine was used to induce the expression of *3nmt1* promoter.

### Microscopy and data analysis

Microscopy was performed as previously described (Coffman *et al.*, 2009, 2013; Wang *et al.*, 2014). Briefly, cells in exponential phase in liquid cultures were collected by centrifugation at 5000 rpm for 30 s. For fluorescence microscopy, cells were washed twice with EMM5S to reduce autofluorescence. Live-cell microscopy was performed using a thin layer of liquid EMM5S with 20% gelatin (Sigma-Aldrich, St. Louis, MO) and 5 μM *n*-propyl-gallate and observed at 23–25°C except where noted.

Three microscopy systems were used to acquire images with Nikon 100×/1.4 numerical aperture Plan-Apo objective lenses (Nikon, Melville, NY). For fluorescence images and time-lapse movies, a spinning disk confocal system (UltraVIEW Vox CSUX1 system; PerkinElmer, Waltham, MA) with 440-, 488-, 515-, and 561-nm solid-state lasers and a back-thinned electron-multiplying charge-coupled device (CCD) camera (C9100-13; Hamamatsu, Bridgewater, NJ) on a Nikon Ti-E microscope without binning was used. For most differential interference contrast (DIC) images used for the quantifications of septation defects, a Nikon Eclipse Ti inverted microscope equipped with a Nikon cooled digital camera DS-Q11 was used. Owing to the high cytoplasmic signals (and/or autofluorescence), we imaged cells expressing Eng1-GFP or Agn1-GFP using the UltraVIEW ERS system, which has lower sensitivity but better spatial resolution. The spinning disk confocal system (PerkinElmer) is equipped with 488-nm argon ion laser and a cooled CCD camera (ORCA-AG; Hamamatsu) on a Nikon Eclipse TE2000-U microscope.

Image analyses were performed using Volocity (PerkinElmer) and ImageJ (National Institutes of Health, Bethesda, MD). Images in figures are maximum-intensity projections of z-sections spaced at 0.3–0.5 μm except where noted. For Figure 8A, the z-spacing is 0.1 μm. To quantify the fluorescence intensity of Rho3 and Rho4 at the division site, we summed the intensity from 13 z-sections spaced at 0.4 μm (Coffman *et al.*, 2011). Then a rectangular region of interest (ROI) that is big enough to include >90% of ring or septum signal was used to measure the intensity. The intensity of the cytoplasmic signal in a concentric rectangular ROI (approximately twice as big as the ROI that elongated along the cell long axis) was used for background subtraction (Wu and Pollard, 2005; Wu *et al.*, 2008; Coffman and Wu, 2012). The *p* values in this study were calculated using two-tailed Student's *t* tests.

### FRAP analysis

FRAP assays were performed using the photokinesis unit on UltraVIEW Vox confocal system, similar to the assays described previously (Coffman *et al.*, 2011; Zhu *et al.*, 2013; Wang *et al.*, 2014). Briefly, the middle focal plane of cells with complete septa was chosen to bleach the fluorescence signal at the division site. Selected ROIs were bleached to <50% of the original fluorescence intensity

after four prebleach images were collected. One hundred images with 1-s delay were collected after photobleaching. The images were then corrected for background and photobleaching during image acquisition. The prebleach intensity of the ROI was normalized to 100% and the intensity just after bleaching to 0%. The end time of the bleach was set as time 0. Intensities of every three consecutive postbleaching time points were averaged to reduce noise. Then the intensity data were plotted and fitted using the exponential equation  $y = m_1 + m_2 \exp(-m_3x)$ , where  $m_3$  is the off-rate (Kaleidagraph; Synergy Software, PA). The half-time of recovery was calculated using the equation  $t_{1/2} = (\ln 2)/m_3$ .

### IP and Western blotting

IP assays and Western blottings were carried out as previously described (Laporte *et al.*, 2011; Lee and Wu, 2012; Wang *et al.*, 2014) with the following modifications: 1) 200 mg (Figure 2C) and 40 mg (Figure 9C) of lyophilized cells for each strain were used for IPs and Western blottings, respectively, due to the low abundance of Gef3; 2) The monoclonal anti-Myc antibody (1:1000 dilution; 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect Gef3-13Myc. Rho GTPases were detected by monoclonal anti-GST antibody (3G10/1B3, 1:10,000 dilution; NB600-446; Novus Biologicals, Littleton, CO). A monoclonal antibody against FLAG (1:2000 dilution; F1804; Sigma-Aldrich) was used to detect 3FLAG-Gef3, and 6His-Gef3 was detected by anti-His antibody (1:10,000 dilution; 631212; Clontech, Mountain View, CA). Secondary anti-mouse antibody was used at 1:10,000 dilution for all the primary antibodies mentioned. In addition, triple-hemagglutinin (HA)-tagged wt and mutant Rho4 were detected by anti-HA antibody (1:2000 dilution; 3F10; Roche, Mannheim, Germany), and a secondary anti-rat antibody (A5795; Sigma-Aldrich) was used at 1:4000 dilution.

### Protein purification

Purification of 6His-tagged protein from *E. coli* was carried out as previously described (Zhu *et al.*, 2013) with the following modifications: BL21(DE3)pLysS cells (69451; Novagen, EMD Chemicals, Darmstadt, Germany) carrying 6His-Gef3 expression plasmid (JQW734) were grown at 37°C for 6 h after adding 1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG; Saha and Pollard, 2012).

Purification of 3FLAG-Gef3 from *S. pombe* was performed similarly to previously described (Wang *et al.*, 2014). Briefly, 3FLAG-Gef3 was overexpressed in *spn1 $\Delta$*  background, since Gef3 was released to cytoplasm (Figure 2A). The *3nmt1-3FLAG-Gef3 spn1 $\Delta$*  cells (JW6030) were induced in EMM5S at 30°C for 48 h and collected for lyophilization. Approximately 3 g of lyophilized cells were broken by grinding, and then the cell powder was mixed with 40 ml of cold HK extraction buffer (25 mM Tris, pH 7.5, 1% NP40, 300 mM NaCl, 5 mM EDTA, 15 mM ethylene glycol tetraacetic acid [EGTA], 60 mM  $\beta$ -glycerophosphate, 500  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitors [Roche]). The cell extract was cleared by two rounds of centrifugation at 4°C (21,000 rpm for 10 min; 38,000 rpm for 30 min). Then cell extract (~35 ml) was incubated with 600  $\mu$ l of beads covalently coated with anti-FLAG antibody (F2426; Sigma-Aldrich) at 4°C for 1.5 h. The beads were collected by centrifugation at 4000 rpm for 2 min and washed once with equal volume (35 ml) of HK extraction buffer, four times with an equal volume (35 ml) of washing buffer (25 mM Tris, pH 7.5, 300 mM NaCl, 5 mM EDTA, 500  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM PMSF, and 1 mM DTT), and twice with 1 ml of washing buffer. To elute 3FLAG-Gef3, we incubated beads with 1 ml of 3FLAG peptide (F4799; Sigma-Aldrich) at a final concentration of 200  $\mu$ g/ml in the washing buffer at 4°C for 30 min.

GST and GST-tagged Rho1–Rho5 and Cdc42 were expressed and purified from BL21(DE3)pLysS cells (induced with 0.5 mM IPTG at 15°C for 6 h) using glutathione–Sephacel beads (17-5132-01; GE Healthcare, Chalfont St Giles, United Kingdom) as described previously (Zhu *et al.*, 2013). Bead-bound GST or GST-Rhos were treated to deplete nucleotides or load GDP/GTP $\gamma$ S for the following pull-down assays as described next. The GST-Rho4 used in the GEF assays was eluted by 100 mM reduced glutathione.

### In vitro pull-down assays for Gef3 and Rho GTPases

For pull-down assays of nucleotide-depleted Rho GTPases, the purified 6His-Gef3 or 3FLAG-Gef3 was dialyzed into the final binding buffer (25 mM 3-(N-morpholino)propanesulfonic acid [MOPS], pH 7.2, 60 mM  $\beta$ -glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, and protease inhibitor tablets [Roche]) as previously described (Iwaki *et al.*, 2003; Zhu *et al.*, 2013). The beads with GST control and GST-Rho proteins were incubated with buffer containing 50 mM Tris, pH 7.5, 1 mM DTT, and 5 mM EDTA to deplete nucleotides at 30°C for 10 min. Then 500  $\mu$ l of ~0.25  $\mu$ M 3FLAG-Gef3 or 6His-Gef3 in binding buffer was added to 30  $\mu$ l of beads with each nucleotide-depleted Rho protein and incubated at 4°C for 1 h. After incubation, glutathione beads were washed with 1 ml of binding buffer three times, and the bound proteins were detected by Western blotting.

The pull-down assays for GDP- or GTP $\gamma$ S-preloaded Rho1, Rho3, Rho4, and Rho5 were performed as described (Kozminski *et al.*, 2003; Singh *et al.*, 2008) with minor modification. Briefly, 30  $\mu$ l of GST or GST-Rho bound beads were first incubated in Buffer I (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM EDTA, 10% glycerol, 0.1% Triton X-100, and protease inhibitors [Roche]) for 1 h at room temperature to deplete nucleotide. Then beads were washed with Buffer I containing 5 mM MgCl<sub>2</sub> and then incubated with Buffer I containing 5 mM MgCl<sub>2</sub> plus 0.5 mM GTP $\gamma$ S or 0.5 mM GDP (Roche) for 30 min at room temperature to load the desired nucleotide. To stabilize the nucleotide-bound Rho GTPases, the beads were resuspended in Buffer I without EDTA but containing 10 mM MgCl<sub>2</sub> plus 0.5 mM GTP $\gamma$ S or 0.5 mM GDP and incubated 20 min at room temperature. To pull down Gef3, the beads were incubated with yeast extracts prepared from 50 mg of lyophilized cells expressing 3FLAG-Gef3 (JW6030) at 4°C for 1.5 h. After incubation, beads were washed with 1 ml of Buffer I without EDTA but containing 10 mM MgCl<sub>2</sub> three times, and the bound proteins were detected by Western blotting.

To test the preference of Gef3 in binding with Rho4 (Figure 5B), we induced cells transformed with 3HA-Rho4 plasmids (wt, G23V, and T28N) in EMM5S – leucine for 20 h before collection for cell extracts. We incubated 30  $\mu$ l of Talon metal affinity resin (635501; Clontech) carrying 6His-Gef3 with yeast extracts from 30 mg of lyophilized cells at 4°C for 1.5 h.

### GEF activity assay

In vitro nucleotide exchange assay was performed as described (Kang *et al.*, 2014) with modifications. Briefly, purified GST-Rho4 was incubated with 50-fold molar excess of mant-GDP (Invitrogen, Carlsbad, CA) in a nucleotide-loading buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, and 2 mM DTT) for 25 min at 25°C. After termination of nucleotide loading with 20 mM MgCl<sub>2</sub>, excess mant-GDP was removed by running through NAP™-5 gravity column (17-0853-01; GE Healthcare), and mant-GDP-loaded GST-Rho4 was switched into the GEF assay buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1% glycerol, and 1 mM DTT). A GEF assay was performed by mixing 40  $\mu$ l of ~2  $\mu$ M mant-GDP-loaded Rho4 with

5  $\mu$ l of GEF assay buffer containing 3FLAG-Gef3 (with different concentrations) or 3FLAG in the presence of 400  $\mu$ M GDP. Fluorescence intensity was monitored at  $\sim$ 20°C every 10 s using the Infinite M1000Pro plate reader (Tecan Group, Männedorf, Switzerland), with excitation at 360 nm and emission at 440 nm. Data were fitted with a monophasic exponential curves using  $y = m_1 - m_2 \exp(-m_3x)$ , where  $m_3$  is the fluorescence decrease rate plotted in Figure 5, D and E (KaleidaGraph; Synergy Software, PA).

### Active Rho4 pull-down assay

We carried out active Rho pull-down assays using commercial GST-RBD kit (16116; Thermo Scientific, Waltham, MA). Briefly, 50 mg of lyophilized cells were lysed with 600  $\mu$ l of the lysis buffer (25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP40, 5% glycerol, and protease inhibitors). The cell extracts were cleared by two rounds of centrifugation at 4°C (5000 rpm for 5 min; 15,000 rpm for 30 min). Protein concentration was measured using the Bradford method. Then 200  $\mu$ g of GST-RBD protein coupled to glutathione-Sepharose beads was incubated with 3 mg of proteins from the cell extracts at 4°C for 1.5 h to immunoprecipitate active Rho GTPases. The beads were then washed three times with lysis buffer, and the bound proteins were detected by Western blotting. To test whether GST-RBD prefers active Rho4 (Figure 8C), we incubated bead-bound 200  $\mu$ g of GST-RBD at 4°C for 1.5 h with yeast extracts from 30 mg of lyophilized cells expressing 3HA-Rho4 plasmids (wt, G23V, and T28N). Cells transformed with the Rho4 plasmids were induced in EMM5S – leucine for 20 h before collection.

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