

Unique spatiotemporal activation pattern of Cdc42 by Gef1 and Scd1 promotes different events during cytokinesis

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ABSTRACT The Rho-family GTPase Cdc42 regulates cell polarity and localizes to the cell division site. Cdc42 is activated by guanine nucleotide exchange factors (GEFs). We report that Cdc42 promotes cytokinesis via a unique spatiotemporal activation pattern due to the distinct action of its GEFs, Gef1 and Scd1, in fission yeast. Before cytokinetic ring constriction, Cdc42 activation, is Gef1 dependent, and after ring constriction, it is Scd1 dependent. Gef1 localizes to the actomyosin ring immediately after ring assembly and promotes timely onset of ring constriction. Gef1 is required for proper actin organization during cytokinesis, distribution of type V myosin Myo52 to the division site, and timely recruitment of septum protein Bgs1. In contrast, Scd1 localizes to the broader region of ingressing membrane during cytokinetic furrowing. Scd1 promotes normal septum formation, and *scd1*Δ cells display aberrant septa with reduced Bgs1 localization. Thus we define unique roles of the GEFs Gef1 and Scd1 in the regulation of distinct events during cytokinesis. Gef1 localizes first to the cytokinetic ring and promotes timely constriction, whereas Scd1 localizes later to the ingressing membrane and promotes septum formation. Our findings are consistent with reports that complexity in GTPase signaling patterns enables exquisite precision over the control of cellular processes.

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INTRODUCTION

Cytokinesis is the final step in cell division in which the cell separates into two through the formation of an actomyosin-based cytokinetic ring that subsequently constricts, concurrent with membrane ingression (Goyal *et al.*, 2011). Seminal work over the years has given us a good understanding of actomyosin ring assembly (Pollard, 2010; Lee *et al.*, 2012). However, we do not understand what enables concurrent ring constriction and septum formation or how the multiple steps in cytokinesis are precisely coordinated. A recent model indi-

cates that septum ingression, rather than actomyosin ring constriction, provides the force required to overcome internal turgor pressure for membrane furrowing (Proctor *et al.*, 2012). Septum ingression requires the polarized delivery of septum-synthesizing enzymes, the β -glucan synthases, at the division site. Thus polarized delivery of proteins and membrane for proper partitioning of two daughter cells is a critical step in cytokinesis (Wang *et al.*, 2002; Albertson *et al.*, 2005; Boucrot and Kirchhausen, 2007; McCusker and Kellogg, 2012). It is not clear how polarized delivery is regulated at the cell division site and coordinated with actomyosin contractility.

In the fission yeast *Schizosaccharomyces pombe*, a major regulator of polarized delivery and actin organization in cell growth is the conserved GTPase Cdc42 (Martin *et al.*, 2007; Bendezu *et al.*, 2012). Cdc42 displays remarkable oscillatory behavior that promotes cell morphology and polarity (Das *et al.*, 2012). However, it is not clear whether Cdc42 promotes cytokinesis or what its role may be next to Rho and other well-studied GTPases in this process (Jordan and Canman, 2012; Chircop, 2014). In budding yeast, Cdc42 activity is down-regulated during septum formation and abscission (Atkins *et al.*, 2013; Onishi *et al.*, 2013), which suggests that Cdc42 has a

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Abbreviations used: BAR, Bin/Amphiphysin/Rvs167; CRIB, Cdc42/Rac interactive binding peptide; DMSO, dimethyl sulfoxide; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; RFP, red fluorescent protein; SPB, spindle pole body.

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negative role in cytokinesis. Constitutively activated Cdc42 leads to cytokinesis failure in HeLa cells and *Drosophila* embryos (Dutartre *et al.*, 1996; Crawford *et al.*, 1998). In mouse oocytes, Cdc42 is required for polar body protrusion and asymmetric cytokinesis (Ma *et al.*, 2006; Bielak-Zmijewska *et al.*, 2008; Zhang *et al.*, 2008a; Leblanc *et al.*, 2011; Liu, 2012; Maddox *et al.*, 2012; Dehapiot *et al.*, 2013). In *Xenopus* embryos, both constitutively active and dominant-negative forms of Cdc42 lead to cytokinetic failure (Drechsel *et al.*, 1997). These reports suggest that Cdc42 needs to be tightly regulated during cytokinesis, similar to what is observed of Cdc42 in polarity establishment.

Cdc42 is active when it is GTP bound and inactive when it is GDP bound (Bos *et al.*, 2007). Cdc42 is activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (Bos *et al.*, 2007). Fission yeast has only two Cdc42 GEFs, Scd1 and Gef1 (Chang *et al.*, 1994; Coll *et al.*, 2003), making it a simple system with which to understand Cdc42 activation. During polarization, Gef1 promotes growth at the second (new) end in fission yeast (Coll *et al.*, 2003; Das *et al.*, 2012), and Scd1 is required for establishing polarity (Kelly and Nurse, 2011). Although *gef1* and *scd1* mutants show distinct cellular phenotypes, suggesting distinct roles for each in polarity, a *gef1Δscd1Δ* double mutant is not viable, suggesting that they are also partially redundant (Coll *et al.*, 2003). During polarization, Scd1 and Gef1 undergo unique regulatory pathways to maintain cell shape and form (Coll *et al.*, 2003; Das *et al.*, 2009, 2015). Scd1 and Gef1 localize to the cell division site, suggesting a role for these GEFs in cytokinesis (Hirota *et al.*, 2003). Gef1 interacts with the Bin/Amphiphysin/Rvs167 (N-BAR) domain-containing protein Hob3, which promotes cytokinesis (Rincon *et al.*, 2007). The roles of Gef1 and Scd1 in cytokinesis are unclear, however, and the relative contributions of each GEF during cytokinesis have not been investigated.

Here we report that, after cytokinetic ring assembly, Cdc42 is activated in a unique spatiotemporal manner at the division site through the distinct functions of Gef1 and Scd1. In addition, the distinct localization pattern of the two GEFs corresponds with their role in cytokinesis. Loss of Gef1-mediated Cdc42 activation leads to a delay in the onset of ring constriction, whereas Scd1 is required for septum formation. Therefore Cdc42 is spatiotemporally activated by two distinct GEFs to promote different events during cytokinesis. These results suggest additional layers of complexity in the regulation of a single GTPase during a complex cellular process. This is likely a general operating principle inherent to this family of regulatory proteins.

RESULTS

Cdc42 is activated at the division site after actomyosin ring assembly

Cdc42 localizes to the cell division site in fission yeast (Merla and Johnson, 2000; Rincon *et al.*, 2007), and previous reports showed that Cdc42 is activated at the division site (Tatebe *et al.*, 2008). To analyze the dynamics of Cdc42 activation at the division site, we studied the localization of a green fluorescent protein (GFP)-tagged Cdc42/Rac interactive-binding peptide ([CRIB] from *S. cerevisiae* Gic2 protein)-domain bioprobe that is used to detect activated Cdc42 (Tatebe *et al.*, 2008). The CRIB domain specifically binds to GTP-bound (active) Cdc42. CRIB-3xGFP localization at the cell division site was compared with that of spindle pole body (SPB) marker Sad1-mCherry and the cytokinetic ring marker type II myosin light chain Rlc1-Tomato. We found that CRIB-3xGFP localized to the cell division site 12 min after initial SPB separation, when the Rlc1-Tomato ring is assembled, and persisted to the end of cytokinesis

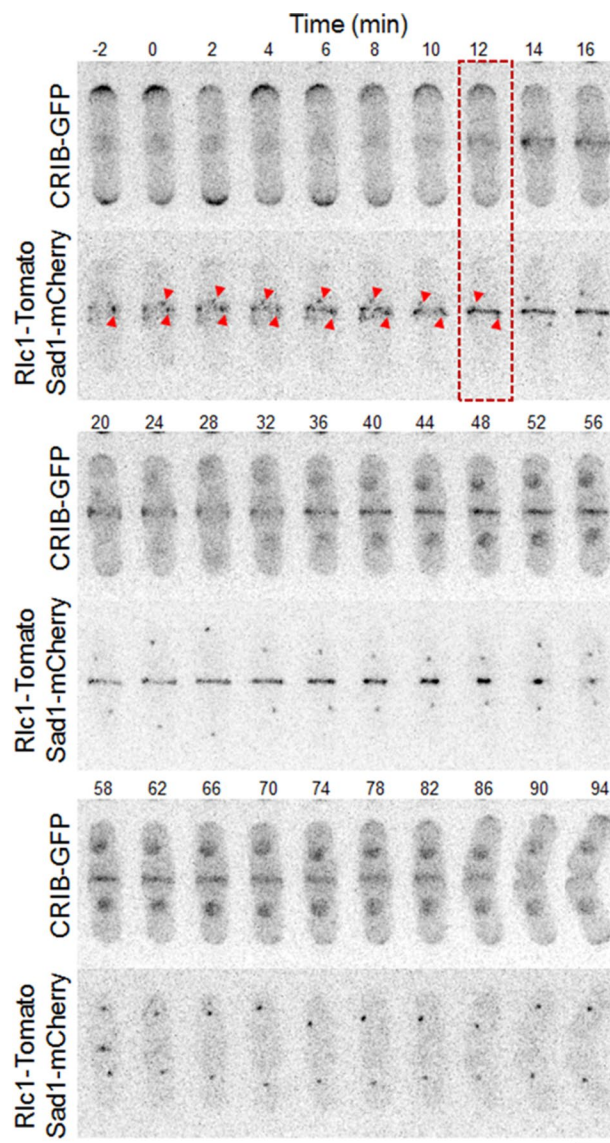


FIGURE 1: Cdc42 is activated at the site of cell division during cytokinesis. Time-series images showing the appearance and duration of the CRIB-3xGFP signal at the site of cell division. SPB separation by Sad1-mCherry and the cytokinetic ring protein by Rlc1-Tomato (bottom). Red arrowheads show initial stages of SPB marker position. Red box depicts onset of Cdc42 activation. Bar, 5 μ m. Time is in minutes.

(Figure 1 and Supplemental Movie S1). In fission yeast, after actomyosin ring assembly, the ring enters a maturation/dwell phase in which the diameter of the ring stays constant (Laporte *et al.*, 2010). At the end of the maturation/dwell phase, the ring initiates constriction (Laporte *et al.*, 2010). We found that Cdc42 is activated after cytokinetic ring assembly at the onset of the maturation/dwell phase during cytokinesis (Figure 1 and Supplemental Movie S1).

Next we tested to see whether Gef1 and Scd1 are required for activation of Cdc42 at the cell division site. In *gef1Δ* cells, Cdc42 activation at the cell division site is significantly delayed (Figure 2, A and B, and Supplemental Movie S2). In *gef1Δ* cells, CRIB-3xGFP appeared at the cell division site -40 ± 4.8 min after SPB marker separation, compared with 13 ± 2.4 min in *gef1+* cells ($n > 17$, $p = 1.8E-16$; Figure 2, A and B, and Supplemental Movie S2). In *scd1Δ* cells, CRIB-3xGFP localization was not delayed at the cell division

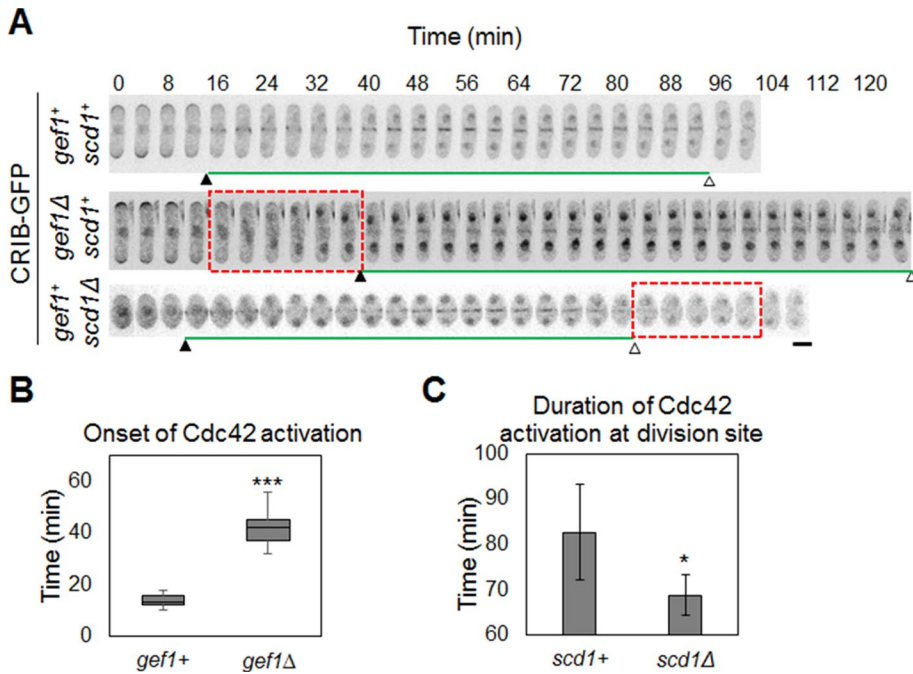


FIGURE 2: Cdc42 activation pattern during cytokinesis. (A) Time-series images of CRIB-3xGFP signals during cytokinesis in the indicated cells. Black arrowheads indicate the appearance of CRIB-3xGFP at the site of cell division, and white arrowheads indicate the end of the CRIB-3xGFP signal at the site of cell division. Red box, absence of Cdc42 activation. $n > 10$. Bar, 5 μm . (B) Quantification of onset of Cdc42 activation cells as indicated ($***p < 0.001$). (C) Quantification of duration of Cdc42 activation in cells as indicated ($*p < 0.05$). Error bar, SD. Time is in minutes.

site but disappeared early (Figure 2, A and C, and Supplemental Movie S3). Duration of Cdc42 activation at the cell division site was 82 ± 10.6 min in control cells and 68 ± 4.5 min in *scd1Δ* cells ($n > 8$, $p = 0.003$; Figure 2, A and C, and Supplemental Movie S3). Note that *scd1Δ* cells display cell polarity defects and are mainly round (Chang *et al.*, 1994). Gef1- and Scd1-mediated Cdc42 activation overlapped at the 40- to 80-min time points (Figure 2A). These results suggest that Gef1 and Scd1 activate Cdc42 sequentially, with significant temporal overlap during cytokinesis.

Cdc42 GEFs Gef1 and Scd1 display a distinct spatiotemporal localization pattern during cytokinesis

Our findings suggest that after ring assembly, Cdc42 activation at the onset of maturation/dwell phase is likely Gef1 dependent, whereas Scd1 activates Cdc42 in the later stages of cytokinesis. This indicates a distinct temporal pattern for the Cdc42 GEFs at the cell division site. To test this, we studied the localization of Gef1 and Scd1 throughout cytokinesis using Gef1-3x yellow fluorescent protein (3xYFP) and Scd1-3xGFP as markers. Both Gef1 and Scd1 are low-abundance proteins with weak signals and thus are not suitable for time-lapse images (Das *et al.*, 2012). We performed live-cell microscopy in Gef1-3xYFP- or Scd1-3xGFP-expressing cells, with Cdc15-Tomato as a cytokinetic ring marker and bright-field images for septum detection. During ring assembly (Figure 3A, stage I), Gef1-3xYFP was absent from the cell division site. After the formation of the cytokinetic ring (Figure 3A, stage II), we detected Gef1-3xYFP at the cell division site (93% cells, $n > 15$). In cells containing a constricting Cdc15-Tomato ring (Figure 3A, stage III), Gef1-3xYFP also appeared to undergo constriction. At the end of ring constriction (Figure 3A, stage IV), Gef1-3xYFP was absent from the cell division site. In contrast, we detected Scd1-3xGFP in only a small num-

ber of cells in stage II (Figure 3B, 28% cells, $n > 15$). In stage III, Scd1-3xGFP overlapped with the constricting ring but also extended beyond the Cdc15-Tomato ring and was still visible at the end of constriction in stage IV (Figure 3B). Scd1-3xGFP was not detected at the cell division site during cell separation (Figure 3B, stage V). This indicates that Gef1 localizes to the cell division site immediately after ring formation and is lost as the ring constricts (Figure 3A), whereas Scd1 localizes just before ring constriction and follows the constricting ring (Figure 3B).

To analyze localization of Gef1 at the cell division site in greater detail, we compared Gef1-3xYFP with Cdc15-Tomato. Three-dimensional (3D) reconstructed Cdc15-Tomato rings colocalized with Gef1-3xYFP both before and during constriction, suggesting that Gef1 localizes to the cytokinetic ring (Figure 3C). Gef1-3xYFP could not be detected after ring constriction. Further, Gef1-3xYFP did not localize to the site of cell division when cells were treated with latrunculin A (Supplemental Figure S1B). This suggests that Gef1 localization is dependent on the presence of the actomyosin ring. In contrast, Scd1-3xGFP did not colocalize with the Cdc15-Tomato ring before ring constriction (Figure 3D). Scd1-3xGFP appeared to localize to the constricting Cdc15-Tomato ring and to its outer periphery (Figure 3D). Scd1-3xGFP was still detected at the periphery of the constricted ring, suggesting that Scd1 localized to the ingressing membrane that followed the cytokinetic ring. Indeed, after ring constriction, Scd1-3xGFP colocalized with CellMask Orange-stained membrane barrier at the cell division site (Supplemental Figure S2A), suggesting that Scd1 localized to the furrow membrane.

To analyze how this distinct spatiotemporal pattern of Gef1 and Scd1 localization influenced Cdc42 activation at the cell division site, we observed CRIB-3xGFP localization in 3D reconstructed cells. In control cells, CRIB-3xGFP appeared as a ring before cytokinetic ring constriction (Figure 3E). The CRIB-3xGFP ring appeared to localize the outer edge of the Rlc1-Tomato ring. Previous reports indicated that the cytokinetic ring interacts with the membrane (Wachtler *et al.*, 2003; Takeda *et al.*, 2004) and that Cdc42 is a membrane protein (Hall, 2005). It is possible that the GEF at the ring activates Cdc42 at the ring-membrane interface. In agreement with this, CRIB-3xGFP also appeared in the ingressing membrane and at the membrane barrier after constriction (Figure 3E). In *gef1Δ* cells, CRIB-3xGFP was absent before cytokinetic ring constriction but was visible in the ingressing membrane during constriction and at the membrane barrier after constriction (Figure 3E). In *scd1Δ* cells, CRIB-3xGFP appeared at the edge of the ring before and during cytokinetic ring constriction but was absent after constriction (Figure 3E). These observations indicate that Cdc42 is activated by its GEFs in a distinct spatiotemporal manner. Gef1 localizes to the cytokinetic ring and activates Cdc42 immediately after cytokinetic ring assembly, whereas Scd1 localizes to the cell division site during cytokinetic ring constriction and activates Cdc42 along the ingressing membrane.

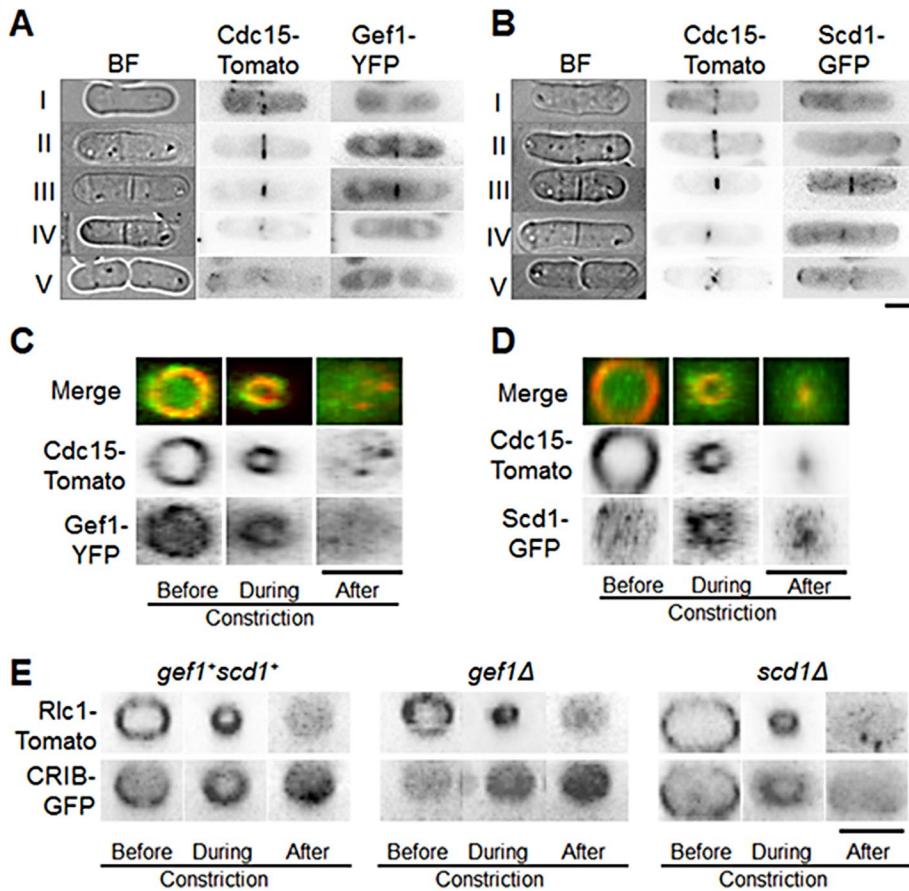


FIGURE 3: Localization of Cdc42 GEFs Gef1 and Scd1 during cytokinesis. (A, B) Cells expressing Cdc15-Tomato with either Gef1-3xYFP or Scd1-3xGFP in the following cytokinetic stages: I, cytokinetic ring assembly; II, after cytokinetic ring assembly; III, cytokinetic ring constriction; IV, end of constriction and septum formation; V, cell separation. (C) Gef1-3xYFP colocalization with Cdc15-Tomato at the cytokinetic ring before, during, and after constriction. (D) Localization of Scd1-3xYFP with Cdc15-Tomato at the cytokinetic ring before, during, and after constriction. (E) CRIB-3xGFP localization in the indicated cells at the site of cell division before, during, and after cytokinetic ring constriction. The images are 3D reconstructed cells rotated to an angle of 90°. Bars, 5 μ m.

Of interest, in several *scd1* Δ cells, CRIB-3xGFP was also visible well after ring constriction (Figure 2A) and in the ingressing membrane during constriction (Figure 3E). To address this, we studied Gef1-3xYFP localization in *scd1* Δ cells. We found that in *scd1* Δ cells, Gef1-3xYFP shows random localization at the cell membrane and division site (Supplemental Figure S2B). Random Gef1 localization may contribute to Cdc42 activation at the membrane furrow after ring constriction in *scd1* Δ cells.

Gef1 promotes onset of actomyosin ring constriction

We find that Cdc42 is activated at the division site at the onset of ring maturation during cytokinesis. What is the significance of the maturation phase during cytokinesis in fission yeast? Why does the actomyosin ring not start constriction as soon as it is assembled? One possible explanation is that during maturation, the actomyosin ring and/or the division site prepare to successfully form the membrane furrow (Laporte *et al.*, 2010). Because Cdc42 is activated at the onset of maturation, we posited that it might promote events during maturation. To understand the role of Cdc42 activation during maturation, we studied cytokinetic events in *gef1* Δ mutants, since these mutants fail to activate Cdc42 at the onset of maturation. We did not find any change in the localization of ring assembly proteins in *gef1* $^{+}$ and

gef1 Δ cells (Supplemental Figure S1A). Further, analysis of different cytokinetic events did not show any delay in ring assembly in *gef1* Δ mutants (Figure 4C and Supplemental Movie S4). This is in agreement with our observation that Cdc42 activation and Gef1 recruitment at the division site occurred after the actomyosin ring was assembled. Of interest, in the *gef1* Δ mutants, the duration of the maturation phase was prolonged compared with *gef1* $^{+}$ cells, leading to a delay in the onset of ring constriction (Figure 4, A and B, Supplemental Figure S3, and Supplemental Movie S4). We used Cdc15-GFP as a cytokinetic ring marker and Sad1-mCherry as a marker for the SPB. Under normal conditions, the cytokinetic ring forms in \sim 13 min after SPB separation (Figure 4, A and C). Ring constriction initiates 33 min after SPB separation, with a 30-min duration (Figure 4, A, B, and D). The time line for cytokinetic events observed in this study is comparable to that reported earlier (Wu *et al.*, 2003). In *gef1* Δ cells, ring constriction initiated 38.4 ± 3.2 min after SPB separation, compared with 32.9 ± 3.4 min in *gef1* $^{+}$ cells ($n > 11$, $p = 0.0006$; Figure 4, A and B, and Supplemental Movie S4). We did not observe any change in the duration of actomyosin ring constriction in *gef1* Δ cells, suggesting that once initiated, ring constriction progressed normally in these cells (Figure 4D and Supplemental Movie S4). Similar results were observed with other cytokinetic ring markers, such as Rlc1-GFP (Supplemental Figure 3).

Because Gef1 is a known activator for Cdc42 (Coll *et al.*, 2003), we tested whether the cytokinetic defect observed in *gef1* Δ mutants was indeed due to loss of Cdc42 activity. We expressed constitutively active Cdc42, *cdc42G12V*, in *gef1* Δ cells. We studied the timing of cytokinetic events in *gef1* $^{+}$ and *gef1* Δ strains expressing *cdc42* $^{+}$ or *cdc42G12V*, using Cdc15-GFP and Sad1-mCherry. Because the cells were grown in minimal media, the cytokinetic events lasted longer than in cells grown in rich media. The onset of cytokinetic ring constriction in *cdc42* $^{+}$ *gef1* Δ cells was delayed (44.9 ± 7.2 min) compared with that in *cdc42* $^{+}$ *gef1* $^{+}$ cells (35.8 ± 3.3 min; Supplemental Figure S4, A and B). However, in *gef1* Δ cells expressing Cdc42G12V, the onset of cytokinetic ring constriction was similar (34.5 ± 7.3 min) to that of *cdc42* $^{+}$ *gef1* $^{+}$ and *cdc42G12V gef1* $^{+}$ (34.8 ± 3.6 min) cells ($n > 16$, $p = 0.88$; Supplemental Figure S4, A and B). Thus the cytokinetic defect observed in *gef1* Δ cells can be alleviated via the expression of constitutively active Cdc42. This, together with our findings on Cdc42 activation (Figure 2, A and B), indicates that Gef1 promotes cytokinesis through the activation of Cdc42. Of interest, cells expressing constitutively activated Cdc42 display cytokinetic defects after ring constriction. Cells overexpressing constitutively active Cdc42G12V showed a high septation index (0.71 ± 0.1) compared with control cells (0.18 ± 0.08), indicating a delay in cell separation ($n > 450$, $p = 9.6E-17$; Supplemental Figure S4). Thus, whereas constitutively active Cdc42 can promote timely onset of ring constriction, the later stages of cytokinesis are disrupted in these mutants.

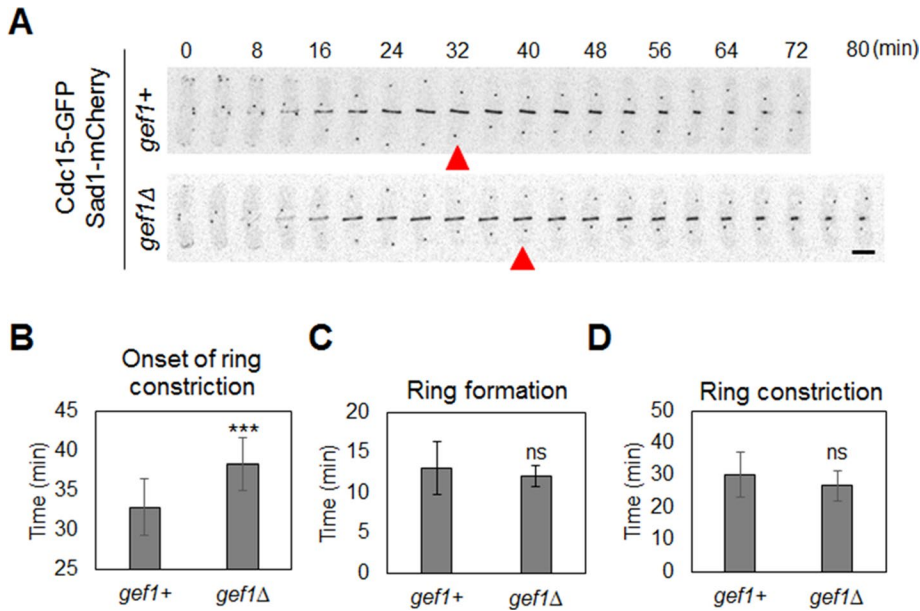


FIGURE 4: Gef1 promotes the timely onset of ring constriction. (A) Time-series images of cytokinetic ring represented by Cdc15-GFP in indicated cells during cytokinesis. Sad1-mCherry represents SPB. Red arrowhead indicates the onset of ring constriction. (B) Quantification of timing of onset of ring cytokinetic ring constriction in indicated cells ($***p < 0.001$). (C) Quantification of timing of cytokinetic ring formation in indicated cells. (D) Quantification of duration of cytokinetic ring constriction in indicated cells; 11 cells. Bar, 5 μm . ns, not significant. Error bars, SD. Time is in minutes.

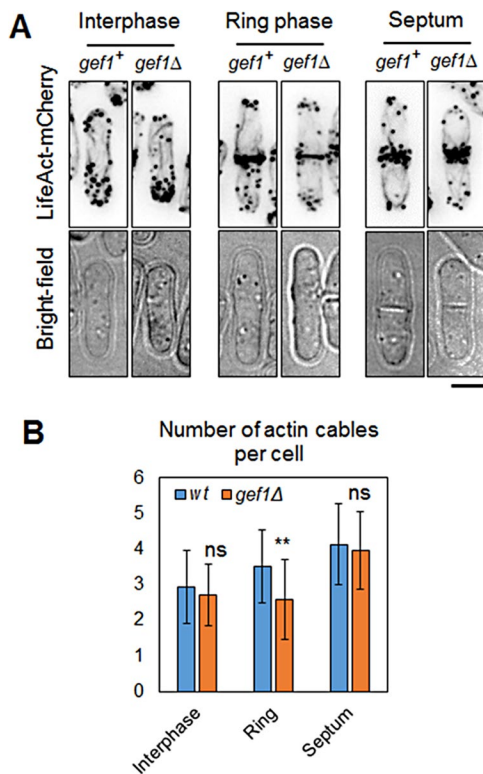


FIGURE 5: Gef1 is required for nonmedial actin cables during cytokinesis. (A) Actin probe LifeAct-mCherry-expressing *gef1+* and *gef1Δ* cells were analyzed during interphase, the ring phase of cytokinesis, and septation. Cells were visualized under bright field to determine septation. Bar, 5 μm (B) Quantification of nonmedial actin cables as determined in the cells described. $n > 23$, $**p = 0.0039$. Error bars, SD. ns, not significant.

The delay in onset of ring constriction in *gef1Δ* cells could be due to mitotic delays. We analyzed mitotic events in *gef1+* and *gef1Δ* cells by measuring the distance between the SPB markers Sad1-mCherry over time. Onset of anaphase is marked by the separation of the SPB (Nabeshima *et al.*, 1998). No change was detected in the timing of anaphase A and anaphase B in *gef1+* and *gef1Δ* cells (Supplemental Figure S5). Thus delay in the onset of cytokinetic ring constriction in *gef1Δ* mutants is independent of mitotic events. Therefore these findings suggest that Gef1-dependent Cdc42 activation during maturation is required to promote timely onset of ring constriction.

Gef1 promotes nonmedial actin cables during cytokinesis

To understand the molecular details of the role of Gef1 in cytokinesis, we studied known downstream effectors of Cdc42 during cytokinesis. In budding yeast, Cdc42 has been shown to promote exocyst-mediated delivery (Zhang *et al.*, 2008b; Wu *et al.*, 2010) and recruitment of the septin proteins (Gladfelter *et al.*, 2002, 2005; Caviston *et al.*, 2003). In fission yeast, the exocyst proteins Sec3 and Exo70 require functional

Cdc42 for proper localization (Bendezu and Martin, 2011; Estravis *et al.*, 2011; Bendezu *et al.*, 2012). However, we did not see any change in the localization of exocyst proteins or septin proteins in either *gef1Δ* or *scd1Δ* mutants, as compared with control cells (Supplemental Figure S6). This suggests that Gef1 and Scd1 function independently of the exocyst complex and septin ring during cytokinesis.

Cdc42 has also been shown to promote actin organization in fission yeast during polarization. We did not observe any change in the actin ring during cytokinesis in *gef1Δ*, compared with *gef1+* cells. However, *gef1Δ* cells appeared to have fewer actin cables along the long axis of the cell during cytokinesis (Figure 5A). It was reported that during cytokinesis, nonmedial actin cables incorporate into the actomyosin ring (Huang *et al.*, 2012). Although studies have shown that these nonmedial actin cables are not sufficient to form stable actin rings, it is not clear what precise role these cables perform during cytokinesis (Huang *et al.*, 2012; Coffman *et al.*, 2013). We found that in *gef1Δ* cells, the number of nonmedial actin cables was reduced compared with *gef1+* cells. We counted nonmedial actin cables in *gef1+* and *gef1Δ* cells expressing the actin probe LifeAct-mCherry (Huang *et al.*, 2012). Cells in interphase did not show any change in the number of actin cables (Figure 5, A and B). We next compared cells in the ring phase of cytokinesis. For this, we considered only cells with a distinct actin ring but without a septum (as determined by bright-field imaging). We found an average of 3.5 ± 1 nonmedial actin cables in *gef1+* cells compared with 2.6 ± 1.1 cables in *gef1Δ* cells ($n > 23$, $p = 0.0039$; Figure 5B). Septated cells, however, did not show any significant difference in the number of nonmedial actin cables (Figure 5B). Because we observed only a moderate (26%) decrease in the number of nonmedial actin cables in *gef1Δ* cells, and to eliminate any artifactual errors due the LifeAct-mCherry probe, we confirmed our findings by Alexa Fluor-phalloidin staining of *gef1+* and *gef1Δ* cells. Similar to LifeAct-mCherry cells,

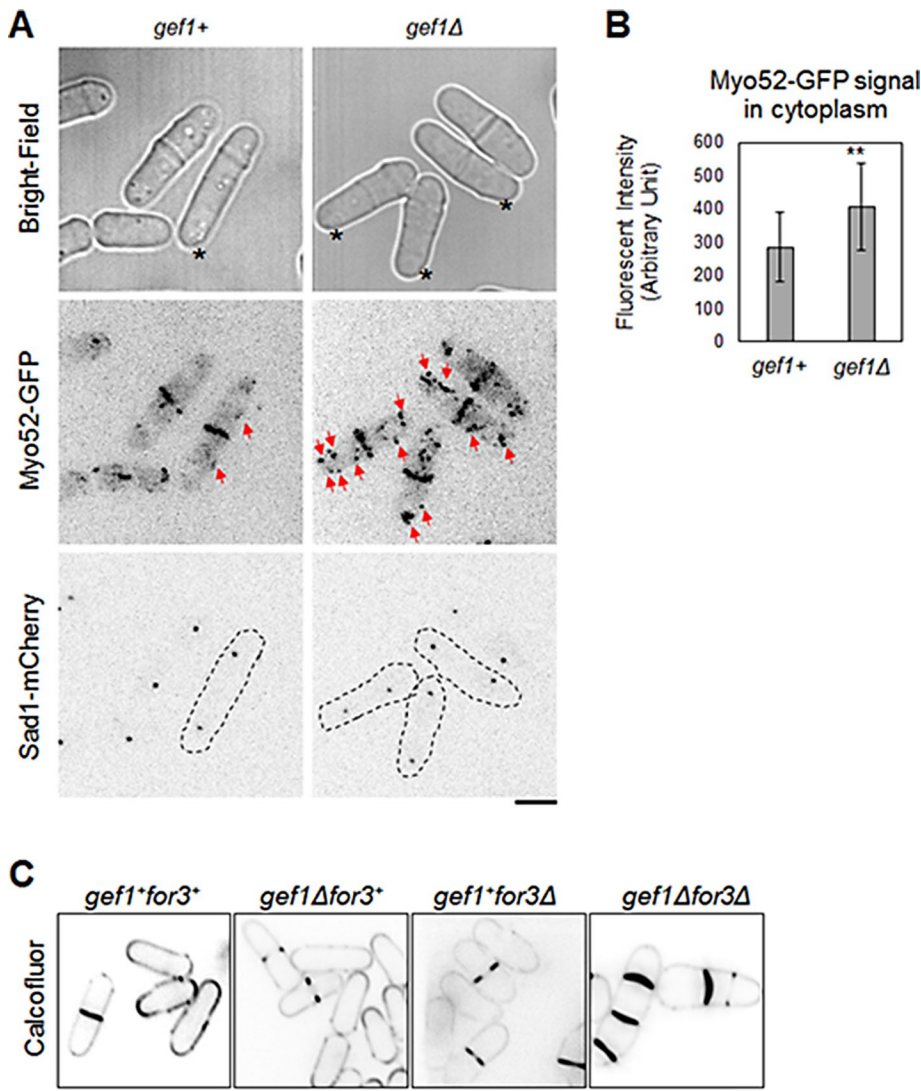


FIGURE 6: Gef1 promotes type V myosin Myo52 localization to the cell division site. (A) Type V myosin Myo52-GFP distribution in *gef1+* and *gef1Δ* cells. Cells with comparable distance of SPB marker Sad1-mCherry were selected. Bright-field images ensured nonseptating cells were selected. Red arrows mark cytoplasmic Myo52-GFP patches. Asterisks mark cells in the ring phase of cytokinesis. (B) Quantification of Myo52-GFP signal in the cytoplasm in *gef1+* and *gef1Δ* cells as described; 22 cells, $**p = 0.0015$. Error bars, SD. (C) Calcofluor staining of *gef1+for3+*, *gef1Δ*, *for3Δ*, and *gef1Δfor3Δ* cells grown at 35°C. Bars, 5 μ m.

phalloidin staining also displayed reduced nonmedial actin cables in *gef1Δ* cells (Supplemental Figure S7).

In addition to fewer nonmedial actin cables, *gef1Δ* cells also displayed more disorganized distribution of the type V myosin motor protein Myo52. The Myo52 motor protein walks along actin cables and carries cargo to the site of delivery (Win *et al.*, 2001). Myo52 promotes delivery of proteins to site of cell division and is involved in the later stages of cytokinesis (Mulvihill *et al.*, 2006). Under normal conditions, Myo52-GFP is localized to the site of cell division during cytokinesis, with very little localization in the cytoplasm (Figure 6A; Win *et al.*, 2001; Mulvihill *et al.*, 2006). In *gef1Δ* cells, Myo52-GFP is localized to the cell division site, similar to *gef1+* cells, but also shows increased patches of Myo52-GFP distribution throughout the cytoplasm (Figure 6A, red arrows). Quantification of Myo52-GFP intensity in the cytoplasm indicates a 42% increase in signal in *gef1Δ* cells as compared with *gef1+* cells ($p = 0.0015$, $n = 22$).

The nonmedial actin cables are polymerized mainly by the formin Cdc12 (Huang *et al.*, 2012). The formin For3 also likely polymerizes actin cables during cytokinesis but to a much smaller extent (Huang *et al.*, 2012). The formins undergo autoinhibition and are activated by Rho GTPases to promote actin assembly (Kovar, 2006; Martin *et al.*, 2007). However, previous reports indicate that Cdc12 is not regulated by this mechanism (Yonetani *et al.*, 2008). The formin For3 has been shown to be activated by Cdc42 to promote actin organization during polarization (Martin *et al.*, 2007). We asked whether Gef1-mediated Cdc42 activation promotes For3-dependent actin organization during cytokinesis. Although For3 is involved in cytokinesis, its functional role in cytokinesis is not clearly understood (Coffman *et al.*, 2013). We studied the genetic relationship between *gef1* and *for3*. If Gef1 functions upstream of For3, we expect to see an epistatic relationship between *gef1* and *for3*. Instead, we find that *gef1Δ* mutants are sensitive to loss of *for3*. Both *gef1Δ* and *for3Δ* mutants grow normally at 25 and 36°C. We found that, compared with the single mutants, *gef1Δfor3Δ* double mutants display multiple septa defects at 36°C (Figure 6C). This suggests that For3 and Gef1 function in parallel pathways to promote cytokinesis.

Gef1 is required for recruitment of the septum-synthesizing enzyme Bgs1 to the division site

Previous reports showed that Bgs1 is essential for ring constriction, and cells expressing *bgs1* temperature-sensitive mutants form a proper actomyosin ring but do not initiate constriction (Liu *et al.*, 1999; Proctor *et al.*, 2012; Cortes *et al.*, 2015). The growing septum provides the force to overcome internal turgor pressure, thereby promoting membrane furrowing and actomyosin ring constriction (Proctor *et al.*, 2012). Bgs1 is recruited to the cell division site in a

Myo52-dependent manner (Mulvihill *et al.*, 2006). Because *gef1Δ* cells displayed a delay in the onset of ring constriction and disorganized Myo52 localization at the division site, we postulated that Gef1 promotes Bgs1 recruitment to the cell division site to promote timely onset of ring constriction. To test this, we studied the recruitment of Bgs1-GFP in *gef1+* and *gef1Δ* cells at the cell division site. We analyzed cells expressing Bgs1-GFP, Rlc1-Tomato, and Sad1-mCherry over time. Similar to previous reports, we find that in *gef1+* cells, Bgs1-GFP is recruited to the cell division site 16.5 ± 4.8 min after SPB separation and 5.8 ± 3.1 min after actomyosin ring assembly (Cortes *et al.*, 2015). Thus Bgs1 is recruited to the division site during the maturation phase of cytokinesis before onset of ring constriction. In *gef1Δ* cells, Bgs1-GFP was recruited to the cell division site 22.7 ± 4.9 min after SPB separation and 13 ± 4 min after actomyosin ring formation ($n > 14$, $p = 2.7E-5$; Figure 7, A and C, and Supplemental Movie S5). The delay (~ 6.2 min) in Bgs1-GFP recruitment is comparable to

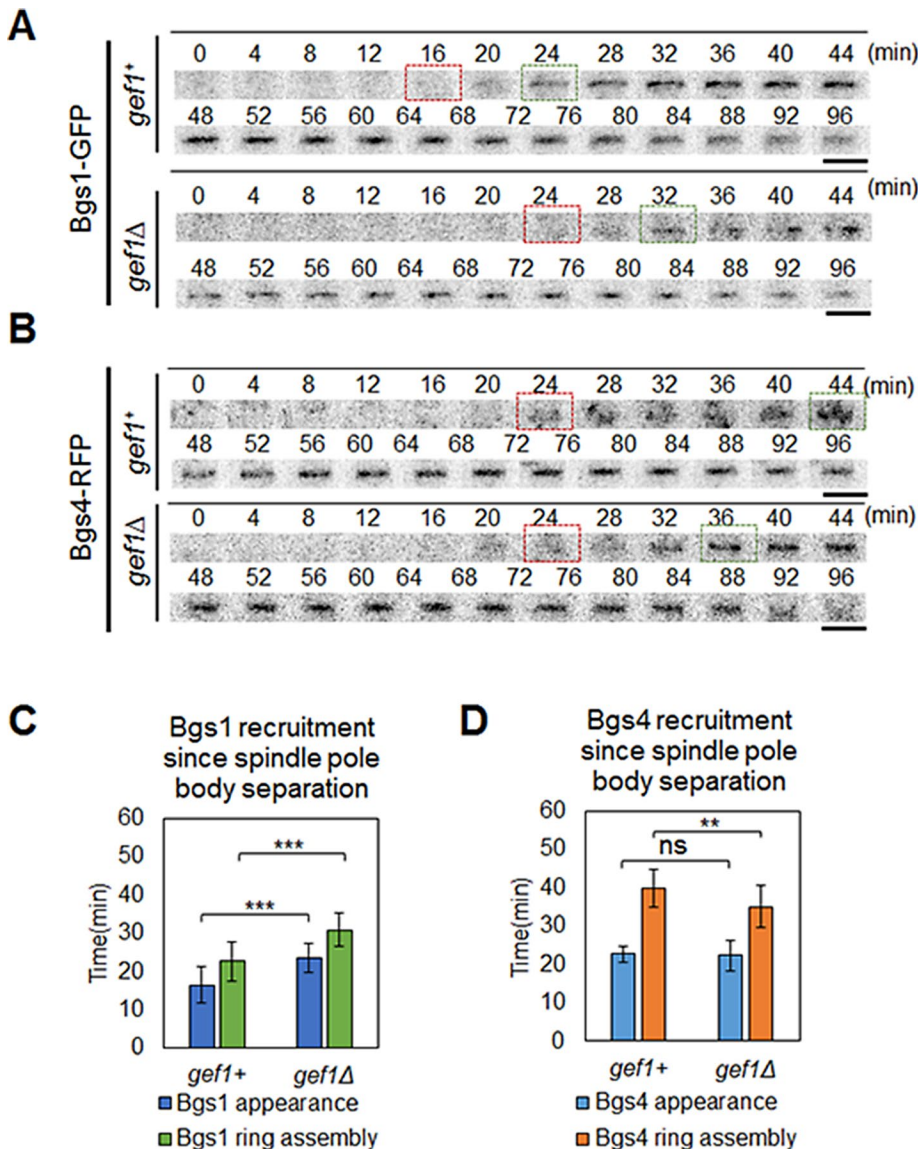


FIGURE 7: Gef1 promotes recruitment of Bgs1 but not Bgs4 to the site of cell division. Localization of septum protein (A) Bgs1-GFP and (B) Bgs4-RFP at the site of cell division. Time 0 represents SPB separation. Red box, onset of Bgs1-GFP or Bgs4-RFP recruitment to the site of cell division. Green box, appearance of Bgs1-GFP or Bgs4-RFP as a ring at the division site. Bars, 5 μ m. (C) Quantification of time of recruitment of Bgs1-GFP with reference to cytokinetic ring formation as depicted by Rlc1-Tomato; >14 cells. Time in minutes. *** $p < 0.001$. Error bars, SD. (D) Quantification of time of recruitment of Bgs4-RFP with reference to SPB separation as depicted by Cdc11-GFP; 21 cells. Time in minutes. ns, not significant; ** $p < 0.01$. Error bars, SD.

the delay in the onset of ring constriction (~5.5 min) in *gef1* Δ cells as compared with *gef1*⁺ cells. Because Bgs1 is eventually recruited in *gef1* Δ cells, it is possible that Bgs1 is also recruited by alternate pathways that may include other Rho GTPases or even Scd1.

It is possible that the delay in Bgs1 delivery at the division site was due to an overall delay in the onset of ring constriction and elongation of the maturation phase. In such a scenario, other proteins recruited during the maturation phase would also show a delay in recruitment at the division site. The ring protein Cdc15 is known to increase in level during the maturation phase (Wu and Pollard, 2005). We studied the fold increase in Cdc15-GFP level in *gef1*⁺ and *gef1* Δ cells throughout the ring maturation phase. We did not see a change in either the level or the timing of Cdc15-GFP recruitment in these cells (Supplemental Figure S8). This suggests that the delayed

recruitment of Bgs1 in *gef1* Δ cells is not due to an overall delay in the maturation phase. In addition, we found that the timing of the delivery of Bgs4 to the cell division site was not affected in *gef1* Δ cells as compared with *gef1*⁺ cells with reference to SPB separation (Figure 7, B and D). In *gef1*⁺ cells, Bgs4–red fluorescent protein (RFP) was recruited 22.7 \pm 2 min, and in *gef1* Δ cells, 22.3 \pm 4 min, after SPB separation ($n > 20$, $p = 0.67$; Figure 7, B and D). This suggests that Bgs1 recruitment is specifically affected by Gef1 at the division site.

Scd1 promotes normal septum formation

Next we studied cytokinetic events in *scd1* Δ mutants to study the role of Scd1 in cytokinesis. Using Rlc1-Tomato as a ring marker and Sad1-mCherry as a SPB marker, we compared the timing for actomyosin ring assembly, onset of ring constriction, and duration of constriction in *scd1*⁺ and *scd1* Δ cells. There was no change in ring assembly or onset of constriction in *scd1*⁺ and *scd1* Δ cells (Supplemental Figure S9, A–C, and Supplemental Movie S6). However, the duration of ring constriction was longer in *scd1* Δ cells (44.9 \pm 5.8 min) than in *scd1*⁺ cells (31.4 \pm 3.3 min; $n = 14$, $p = 6.2E-7$; Supplemental Figure S9, A and D, and Supplemental Movie S6). One explanation for longer duration of ring constriction could be the wider diameter of *scd1* Δ cells. Loss of *scd1* leads to increased cell width (Kelly and Nurse, 2011), and as a result, the circumference of the actomyosin ring is significantly larger in these cells than in *scd1*⁺ cells. To test this, we calculated the rate of ring constriction in *scd1*⁺ and *scd1* Δ cells. Rates of ring constriction were 0.371 \pm 0.039 and 0.395 \pm 0.035 μ m/min in *scd1*⁺ and *scd1* Δ cells, respectively, suggesting that the ring constriction rate is comparable in these cells.

Because Scd1 activated Cdc42 along the membrane during ingression, we hypothesized that Scd1 plays a role in septum formation. Cdc42 is required for Bgs1 delivery to

the cell tips (Estravis *et al.*, 2012). During cytokinesis, Bgs1 synthesizes linear $\beta(1,3)$ -glucans to form the primary septum (Cortes *et al.*, 2002, 2007). We hypothesized that Scd1 promotes Bgs1 recruitment at the site of cell division. We found that Bgs1-GFP signal in *scd1* Δ cells ($n = 21$) showed a 48% decrease ($p = 0.001$) at the septum compared with *scd1*⁺ ($n = 13$; Figure 8A and Supplemental Figure S10A). This suggests that loss of *scd1* leads to reduced recruitment of Bgs1 at the septum. Bgs1-GFP is distributed evenly at the cell division site in *scd1*⁺ cells (Figure 8A). However, in *scd1* Δ cells, Bgs1-GFP is localized mainly at the periphery of the ring, with very little signal at the ingressing membrane (Figure 8A). We also compared Bgs1-GFP localization in *gef1* Δ and *scd1* Δ cells. Bgs1-GFP is evenly distributed at the cell division site in *gef1* Δ cells, unlike in *scd1* Δ cells (Figure 8A). This indicates that Gef1 is required for recruitment of Bgs1 at the

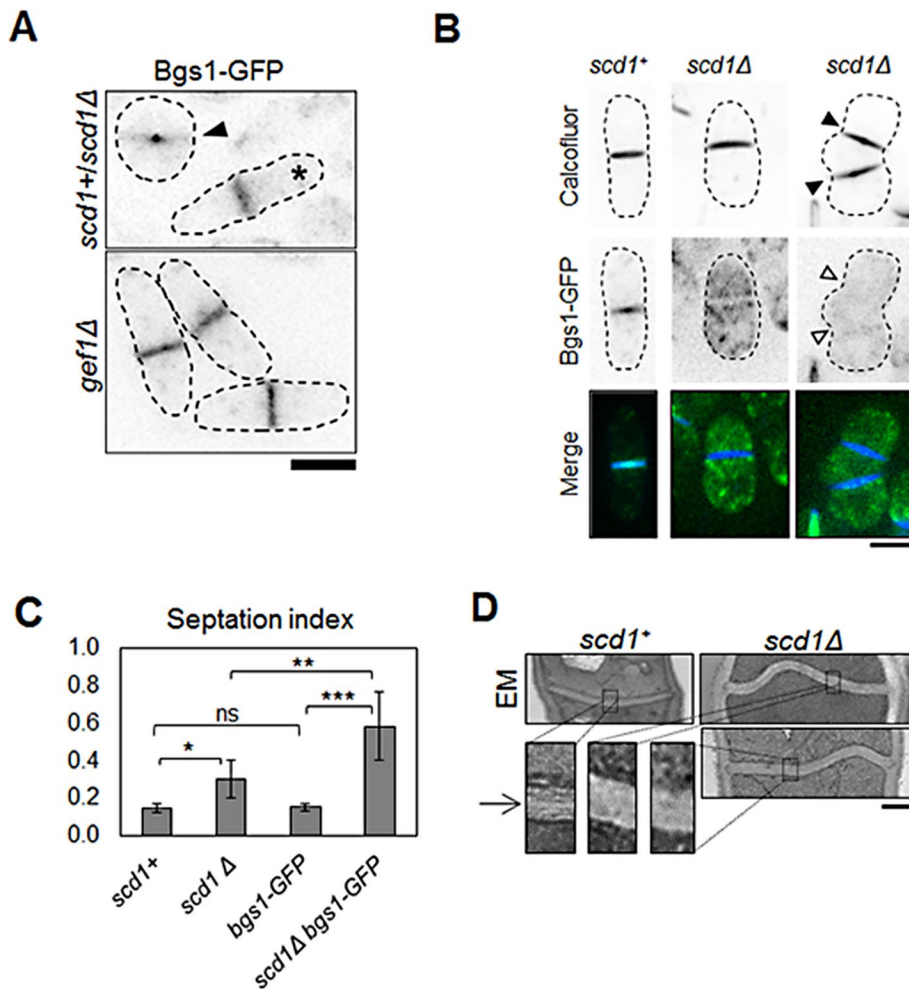


FIGURE 8: Scd1 is required for normal septum formation. (A) Cells expressing Bgs1-GFP in *scd1⁺* and *scd1Δ* cells analyzed in the same field. *scd1⁺* cells are depicted by asterisks, and *scd1Δ* cells are depicted by arrowheads. Bottom, Bgs1-GFP-expressing *gef1Δ* cells shown for comparison. Bar, 5 μ m. (B) Calcofluor staining of *scd1⁺* and *scd1Δ* cells expressing Bgs1-GFP. *scd1Δ* cells with multiple septa (arrowheads). Bar, 5 μ m. (C) Quantification of septation index in cells as indicated; >223 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant. Error bars, SD. (D) Transmission electron microscopy of septum in *scd1⁺* and *scd1Δ* cells. Sections of septum (black box) are zoomed at 4 \times to show primary septum defects. Bar, 500 nm.

periphery of the ring, whereas Scd1 is required for recruitment to the ring periphery and the ingressing membrane.

We also found that the septation index in *scd1⁺* cells was $\sim 14 \pm 2\%$, whereas in *scd1Δ* cells, it was $30 \pm 10\%$, which suggested a defect in cytokinesis. The increased septation index in *scd1Δ* cells could be due to prolonged ring constriction in these cells. However, the septation index in *scd1Δ* cells expressing Bgs1-GFP was higher ($58 \pm 19\%$) than in either *scd1⁺* or *scd1Δ* cells (Figure 8C). The septation index in *scd1⁺* cells expressing Bgs1-GFP ($15 \pm 2\%$) was similar to that in *scd1⁺* cells alone (Figure 8C). Furthermore, in *scd1Δ bgs1-GFP* mutants, $45 \pm 12\%$ of the septated cells showed multiple septa ($n > 286$, $p = 1.0E-4$; Figure 8B and Supplemental Figure S10B). We did not see any multiple septa in *scd1⁺* cells expressing Bgs1-GFP (Figure 8A). Fluorescent tagging of proteins has been reported to cause diminished protein activity in some cases (Cortes et al., 2015). It is possible that fluorescently tagged Bgs1 could have reduced function, rendering these cells more sensitive to loss of *scd1*. These results suggest that *scd1Δ* cells are sensitive to the levels of functional Bgs1 and that even minor perturbation of its function can lead to cytokinetic defects.

Because our data indicated that loss of *scd1* resulted in septum defects, we studied septum morphology in *scd1⁺* and *scd1Δ* cells. We performed transmission electron microscopy in *scd1⁺* and *scd1Δ* cells. *scd1⁺* cells showed fairly straight and even septum, whereas in *scd1Δ* cells, the septum was buckled and uneven (Figure 8D). Moreover, the septum in *scd1Δ* cells appeared thicker (360 ± 171 nm) than in *scd1⁺* cells (170 ± 50 nm; $n > 16$, $p < 0.001$). In fission yeast, septation involves that formation of the primary septum be followed by the formation of the secondary septum (Cortes et al., 2007). Using electron microscopy, it is possible to observe the primary and secondary septum in normal cells (Cortes et al., 2007). The secondary septum appears darker and flanks the primary septum (Figure 8D). The distinction between primary and secondary septum was not clear in *scd1Δ* cells compared with *scd1⁺* cells (Figure 8D). Electron microscopy did not show any primary septum defects in *gef1Δ* cells (Supplemental Figure S11). However, the septum in *gef1Δ* (369 ± 71 nm, $n > 11$) cells also appeared thicker, similar to *scd1Δ* cells.

We find that in *scd1Δ* cells the ingressing septum has reduced Bgs1 levels but the septum appears thicker than in *scd1⁺* cells. This could be due to increased secondary septum formation in these mutants. The secondary septum is dependent on the β -glucan synthase, Bgs4. We find a small increase in Bgs4 levels in *scd1Δ* cells. Bgs4-RFP levels increased by 18% in *scd1Δ* cells compared with *scd1⁺* cells ($n > 20$, $p = 0.04$; Supplemental Figure S10, C and D). This supports the idea that *scd1Δ* cells contain larger amounts of secondary septum. Further studies will determine whether Scd1 indeed promotes primary septum and restricts secondary septum formation.

DISCUSSION

Whereas Cdc42 is a major regulator of cell growth and polarity, its role in cytokinesis is not clear. Here we report that loss of Cdc42 activation leads to cytokinetic defects in fission yeast. During cytokinesis, Cdc42 is activated immediately after actomyosin ring assembly and persists until cell separation. Cdc42 activation is Gef1 dependent just after ring assembly and Scd1 dependent after constriction. Gef1 and Scd1 display unique localization patterns during cytokinesis, resulting in a spatiotemporal activation pattern of Cdc42. Corresponding to their localization patterns, the GEFs promote distinct cytokinetic events. Gef1 localizes to the cytokinetic ring immediately after ring assembly and constricts with the ring. Gef1 promotes timely onset of ring constriction, nonmedial actin cables, type V myosin organization, and timely recruitment of Bgs1. Scd1 localizes to the ingressing membrane and promotes septum formation. Scd1 promotes Bgs1 localization to the septum and is required for normal septum morphology. Loss of *scd1* results in primary septum defects, with septum buckling and overall increase in thickness. Cells lacking *scd1* did not show any delay in the onset of ring constriction, whereas

gef1 mutants did not show aberrant primary septum. Taken together, these findings suggest that the GEFs have unique roles in cytokinesis, as determined by their spatiotemporal localization patterns.

What is the molecular mechanism by which Gef1 and Scd1 regulate cytokinetic events? We find that cells lacking *gef1* show a moderate decrease in nonmedial actin cables during the ring phase of cytokinesis. We also find a synthetic genetic interaction between *gef1* and *for3*, suggesting that they function in parallel pathways to promote actin organization. It is possible that Gef1 promotes actin cable stability rather than synthesis during cytokinesis. Of interest, in actin-interacting protein *aip1* mutants, actin cables are stable and prominent during cytokinesis and display earlier onset of actomyosin ring constriction (Chen *et al.*, 2015). Further studies will address the role of Gef1 in actin organization during cytokinesis. Reduced actin cables and disrupted distribution of the type V myosin Myo52 in *gef1Δ* cells could lead to delays in Bgs1 delivery. Indeed, Myo52 promotes Bgs1 recruitment to the division site during cytokinesis (Mulvihill *et al.*, 2006). We also observed reduced Bgs1 levels at the septum in *scd1Δ* cells. This agrees with previous reports that Cdc42 specifically promotes Bgs1 delivery to the cell tips (Estravis *et al.*, 2012).

Cytokinesis is a highly complex process involving several temporally organized events (Pollard, 2010). In fission yeast, once the actomyosin ring assembles, it does not immediately start constriction but undergoes a maturation phase (Lee *et al.*, 2012). Ring constriction initiates at the end of maturation phase and accompanies membrane furrow ingression and septum formation (Lee *et al.*, 2012). Although the significance of the maturation phase is not clear, proteins required for the later stages of cytokinesis are recruited in this phase (Lee *et al.*, 2012). We find that Cdc42 is not required for actomyosin ring assembly but is required for timely onset of ring constriction. We show that Gef1 at the assembled ring during maturation ensures timely Bgs1 recruitment. We posit that the primary septum built by Bgs1, recruited during maturation, provides the force to overcome the internal turgor pressure to enable ring constriction and membrane furrowing. This supports the idea that the maturation phase prepares the ring/division site for membrane furrowing. Although our data suggest that Gef1 promotes recruitment of Bgs1 to the division site, it is possible that Gef1 also promotes recruitment of other cytokinetic proteins to ensure timely onset of ring constriction. Further studies will address the role of Gef1 in cytokinesis in more details. In most animal cells, actomyosin ring constriction initiates immediately after assembly without a maturation phase. However, in *Drosophila* embryos, during cellularization, actomyosin ring constriction is biphasic, with a significantly slow initial constriction rate (Royou *et al.*, 2004). This is probably due to membrane expansion events required for furrowing at this stage in the embryo (Figard *et al.*, 2013). Thus it is possible that events similar to ring maturation may be present even in animal cells that require special conditions for successful cytokinesis.

Although Scd1 is required for proper primary septum formation, our data also suggest that Cdc42 activation at the septum restricts overall septum formation, as indicated by thicker and buckling septa in *scd1Δ* cells. Whereas Cdc42 activation is required for proper ring constriction and septation, excessive activation is detrimental to cytokinesis, as constitutively active Cdc42 shows severe cell separation defects. This is in agreement with reports on budding yeast, in which Cdc42 inhibits abscission during the final stage of cytokinesis (Atkins *et al.*, 2013; Onishi *et al.*, 2013). Taken together, our findings suggest that Cdc42 may act to ensure proper organization of different temporal events during cytokinesis.

Gef1- and Scd1-mediated activation of Cdc42 at the cell division site shows significant temporal overlap (Figure 1B), and *gef1Δscd1Δ*

mutants are inviable (Coll *et al.*, 2003; Hirota *et al.*, 2003). Thus it is possible that Cdc42 activation is essential for cytokinesis and the two GEFs are partially redundant. Given the conserved nature of Cdc42, it is conceivable that Cdc42 is also required for cytokinesis in other eukaryotes. In *Caenorhabditis elegans*, the PAR proteins, including Cdc42, are required for robust cytokinesis, likely through the regulation of actin organization (Jordan *et al.*, 2016). How are the GEFs spatiotemporally organized, and what is the significance of this in cytokinesis? Why does the cell require different GEFs to activate Cdc42 during the same cellular process? The two GEFs differ structurally, in that Scd1 contains a pleckstrin homology domain required for localization (Endo *et al.*, 2003), whereas Gef1 contains an N-BAR domain (Das *et al.*, 2015). The structural differences in the two GEFs could contribute to distinct localization patterns and/or functions through protein interactions. In addition, the different GEFs may have different kinetics of Cdc42 activation or different feedback mechanisms that could contribute to the spatiotemporal pattern. Indeed, Cdc42 shows an oscillatory activation pattern, which is maintained through feedback mechanisms for the establishment of cell polarity in yeasts (Das *et al.*, 2012; Howell *et al.*, 2012). Distinct patterns of GTPase activation have also been reported to be critical for their function in other eukaryotes (Burkel *et al.*, 2012; Bement and von Dassow, 2014; Vaughan *et al.*, 2014). Our findings indicate a unique GEF-dependent spatiotemporal activation pattern for Cdc42 that corresponds to its function. Pattern organization established by the different regulators may well emerge to be critical for GTPase function.

Understanding the molecular details of GTPase pattern establishment and cellular function in eukaryotes is complicated by the presence of multiple GEFs and GAPs (Miyamoto and Yamauchi, 2010; Shi, 2013; Gadea and Blangy, 2014). The significance of multiple GTPase regulators in promoting different cellular processes is not clear. It is possible that Rho-family GTPases coordinate multiple cellular events through its different regulators. In fission yeast, Cdc42 has only two GEFs, mutants of which show distinct phenotypes during cytokinesis, as reported here. Thus fission yeast provides an advantage for the study of Cdc42 in cytokinesis. Future work is required to understand how Cdc42, activated by different GEFs, mediates distinct functions during cytokinesis and how Cdc42 regulators collaborate to fine-tune its spatiotemporal activation.

MATERIALS AND METHODS

Strains and cell culture

The *S. pombe* strains used in this study are listed in Supplemental Table S1. All strains were isogenic to the original strain 972. Cells were cultured in yeast extract (YE) medium or Edinburgh minimal medium plus required supplements. Standard techniques were used for genetic manipulation and analysis (Moreno *et al.*, 1991). Unless specified, cells were grown exponentially at 25°C.

Microscopy

All images were acquired at room temperature (23–25°C) with a VT-Hawk two-dimensional array laser scanning confocal microscopy system (Visitech International, Sunderland, UK) with an Olympus IX-83 inverted microscope with a 100×/numerical aperture 1.49 UAPO lens (Olympus, Tokyo, Japan) and electron-multiplying charge-coupled device digital camera (Hamamatsu, Hamamatsu City, Japan).

For still, z-series, and time-lapse images (<5 min), the cells were mounted directly on glass slides with a #1.5 coverslip (Fisher Scientific, Waltham, MA) and imaged immediately. For z-series, images were acquired with a depth interval of 0.4 μm. For time-lapse images >5 min, the cells were placed in 3.5-mm glass-bottom culture

dishes (MatTek, Ashland, MA) and overlaid with YE medium plus 1% agar. Ascorbic acid (100 μ M) as an antioxidant was added to the culture to minimize fluorescence toxicity to the cell, as reported previously (Frigault *et al.*, 2009). Images were acquired by MetaMorph (Molecular Devices, Sunnyvale, CA) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD). Statistically significant difference between two groups of cells was determined by *p* value from Student's *t* test.

Electron microscopy

Transmission electron microscopy was performed as described previously (Chappell and Warren, 1989). Cells were washed three times in sterile water, fixed for 1 h in 2% potassium permanganate at room temperature, and then harvested by centrifugation, washed three times in sterile water, resuspended in 70% ethanol, and incubated overnight at 4°C. Samples were then dehydrated by sequential washes in 90% ethanol (twice for 15 min) and washed in 100% ethanol (three times for 20 min). The pellet was resuspended in propylene oxide for 10 min, incubated in a 1:1 mixture of propylene oxide and Spurr's medium for 1 h, and incubated in neat Spurr's medium for 1 h. This was followed by another change of medium and incubation at 65°C for 1 h. Finally, samples were embedded in Spurr's medium in a capsule, and resin in the medium was allowed to polymerize at 60°C overnight. Blocks were sectioned with a diamond knife and stained with uranyl acetate and lead citrate. The cells were then examined in a Zeiss Libra 200MC electron microscope (Oberkochen, Germany) at the University of Tennessee Imaging Core facility.

Expression of constitutively active Cdc42

DNA fragment of *cdc42G12V* was cloned into the vector *pjk148* under the thiamine-repressible *nmt41* promoter and integrated into the *leu1-32* loci in *gef1⁺* and *gef1 Δ* cells. Cells were grown in the absence of thiamine to promote expression of Cdc42G12V. Empty vector *pjk148* in *gef1⁺* cells and *gef1 Δ* cells was used as control. Cells expressing Cdc42G12V displayed mixed morphological defects, from round to almost normal, rod-shaped cells. Round cells were not analyzed further to avoid pleiotropic effects due to high *cdc42G12V* levels. We assumed that polarized cells expressed low or moderate levels of Cdc42G12V and used them for further analysis.

Cell staining

To stain the septum and cell wall, cells were stained in YE liquid with 50 μ g/ml Calcofluor White M2R (Sigma-Aldrich, St. Louis, MO) at room temperature. Cells were washed with fresh YE liquid once before imaging. For CellMask Orange staining, cells were stained in YE liquid with 5 μ g/ml CellMask Orange in dimethyl sulfoxide (Thermo Fisher Scientific) for 5 min at room temperature in the dark. Cells were washed with fresh YE liquid before imaging.

Latrunculin A treatment

Cells were treated with 100 μ M latrunculin A in dimethyl sulfoxide (DMSO) in YE medium for 30 min before imaging. Control cells were treated with only 0.1% DMSO in YE medium.

Analysis of Bgs1-GFP and Bgs4-RFP levels at the septum

Cultures of *scd1⁺* and *scd1 Δ* cells expressing Bgs1-GFP or Bgs4-RFP were grown to OD 0.5 at 32°C and then mixed in equal volumes before imaging at 488 nm. Quantification of Bgs1-GFP and Bgs4-RFP in the septa of dividing cells was performed in ImageJ by measuring the fluorescence intensity restricted to fully formed septa (confirmed by bright field). Images were normalized for background fluorescence by subtracting the intensity of a cell-free region within the same image.

The mean normalized intensity was calculated for cells of each genotype within the same image. Significance was determined through comparison of each genotype's mean normalized intensities using a Student's two-tailed *t* test assuming unequal variance.

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