Cooperation between Rho-GEF Gef2 and its binding partner Nod1 in the regulation of fission yeast cytokinesis

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ABSTRACT Cytokinesis is the last step of the cell-division cycle, which requires precise spatial and temporal regulation to ensure genetic stability. Rho guanine nucleotide exchange factors (Rho GEFs) and Rho GTPases are among the key regulators of cytokinesis. We previously found that putative Rho-GEF Gef2 coordinates with Polo kinase Plo1 to control the medial cortical localization of anillin-like protein Mid1 in fission yeast. Here we show that an adaptor protein, Nod1, colocalizes with Gef2 in the contractile ring and its precursor cortical nodes. Like gef2Δ, nod1Δ has strong genetic interactions with various cytokinesis mutants involved in division-site positioning, suggesting a role of Nod1 in early cytokinesis. We find that Nod1 and Gef2 interact through the C-termini, which is important for their localization. The contractile-ring localization of Nod1 and Gef2 also depends on the interaction between Nod1 and the F-BAR protein Cdc15, where the Nod1/Gef2 complex plays a role in contractile-ring maintenance and affects the septation initiation network. Moreover, Gef2 binds to purified GTPases Rho1, Rho4, and Rho5 in vitro. Taken together, our data indicate that Nod1 and Gef2 function cooperatively in a protein complex to regulate fission yeast cytokinesis.

INTRODUCTION Cytokinesis is the last step of the cell cycle and is essential for cell proliferation and differentiation. Most proteins and key events in cytokinesis are evolutionarily conserved from fungal to human cells (Pollard and Wu, 2010; Green et al., 2012; Lee et al., 2012; Woka and Bi, 2012). In the fission yeast Schizosaccharomyces pombe, anillin-related protein Mid1 plays a crucial role in early stages of cytokinesis (Chang et al., 1996; Sohmann et al., 1996; Bähler et al., 1998a; Paoletti and Chang, 2000; Lee and Wu, 2012; Saha and Pollard, 2012a). Mid1 resides in the nucleus and in protein complexes called nodes at the medial cortex during interphase (Bähler et al., 1998a; Paoletti and Chang, 2000; Almonacid et al., 2011). Together with the DYRK kinase Pom1, these medial nodes control cell size and mitotic entry (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009; Hachet et al., 2011). During G2/M transition, more Mid1 is released from the nucleus to the cortical nodes by Polo kinase Plo1 via phosphorylation of Mid1 (Bähler et al., 1998a; Almonacid et al., 2011). These Mid1 nodes mature into cytokinesis nodes by recruiting other proteins, such as IQGAP Rng2, myosin-II, F-BAR protein Cdc15, and formin Cdc12 (Wu et al., 2003, 2006; Motegi et al., 2004; Almonacid et al., 2011; Laporte et al., 2011; Padmanabhan et al., 2011). Then the nodes and actin filaments condense into a compact ring through a search, capture, pull, and release mechanism (Vavylonis et al., 2008; Chen and Pollard, 2011; Ojkic et al., 2011; Laporte et al., 2012). The compact ring matures and constricts, guiding the formation of a division septum (Pollard and Wu, 2010; Proctor et al., 2012). The cell is then divided into two daughter cells with the degradation of primary septum.
The F-BAR protein Cdc15 is essential for cytokinesis (Fankhauser et al., 1995; Carnahan and Gould, 2003; Roberts-Galbraith et al., 2009, 2010; Arasada and Pollard, 2011). In early cytokinesis, Mid1 recruits Cdc15 to cytokinesis nodes, which in turn recruits the formin Cdc12 to nucleate actin filaments (Carnahan and Gould, 2003; Kovar et al., 2003; Laporte et al., 2011). Cdc15 is also essential for contractile-ring maturation and assembly regulated by the septation initiation network (SIN) pathway (Wachter et al., 2006; Hachet and Simanis, 2008; Laporte et al., 2012). During late cytokinesis, Cdc15 and another F-BAR protein, Imp2, recruit C2-domain protein Fic1 and paxlillin Pxl1 to ensure the maintenance and integrity of the contractile ring (Pinar et al., 2008; Roberts-Galbraith et al., 2009).

The contractile ring and septation/septum formation are regulated by the SIN pathway, which is composed of a GTPase and a kinase cascade (Wachter et al., 2006; Hachet and Simanis, 2008; Krapp and Simanis, 2008; Johnson et al., 2012). The SIN proteins localize at the spindle pole body (SPB) via scaffold proteins Cdc11 and Sid4 (Chang and Gould, 2000; Krapp et al., 2001; Tomlin et al., 2002; Morrell et al., 2004). SIN pathway signaling is controlled by the activation of the GTPase Spg1 by Polo kinase, and the inactivation by the two component GTPase-activating proteins Cdc16 and Byr4 (Schmidt et al., 1997; Furge et al., 1998, 1999; Jwa and Song, 1998; Tanaka et al., 2001; Krapp et al., 2008). The GTP-bound Spg1 interacts with kinase Cdc7 and causes its redistribution to the new SPB (Fankhauser and Simanis, 1994; Cerutti and Simanis, 1999; Mehta et al., 2008). The downstream kinases and their binding partners, including Sid1-Cdc14 and Sid2-Mob1, are then activated and recruited onto the SPB (Fankhauser and Simanis, 1993; Balasubramanian et al., 1998; Sparks et al., 1999, 2003; Guertin et al., 2008; Hou et al., 2000; Salimova et al., 2000). Activated Sid2-Mob1 is then relocated to the contractile ring to promote contractile-ring constriction and septum formation (Jin et al., 2006; Chen et al., 2008).

Besides the equivalents of the SIN pathway, MEN and Hippo pathways, Rho GTPase Rho1/RhoA and its activators, the Rho guanine nucleotide exchange factor (GEF; Ect2, Pebble, etc.) are involved in division-site specification and contractile-ring formation by activating myosin-II and actin assembly in budding yeast and animal cells (Lehner, 1992; Imanura et al., 1997; O’Keefe et al., 2001; Tolliday et al., 2002; Bement et al., 2005; Yuce et al., 2005; Nishimura and Yonemura, 2006; Yoshida et al., 2006; Watanabe et al., 2010; Su et al., 2011). In contrast, Rho GTPases in S. pombe regulate only later stages of cytokinesis and cell polarity (Garica et al., 2006b; Perez and Rincón, 2010). Fission yeast has six Rho GTPases (Cdc42 and Rho1-5) and seven Rho GEFs (Gef1-3, Rgf1-3, and Scd1). Cdc42, regulated by Gef1 and Scd1, is essential for cell polarity and morphogenesis (Coll et al., 2003; Hirota et al., 2003; Rincón et al., 2007). Rho-GEFs Rgf1-3 activate Rho1, which is essential for cell-wall synthesis, septum formation, and cell polarization (Tajadura et al., 2004; Morrell-Falvey et al., 2005; Mutoh et al., 2005; Garcia et al., 2006a, 2009; Wu et al., 2010). Rho2 is involved in cell morphology and septum formation by regulating cell wall α-glucan biosynthesis (Calonge et al., 2000). Rho3 regulates exocytosis (Nakano et al., 2002; Wang et al., 2003; Kita et al., 2011). Rho4 controls the secretion of lytic enzymes for septum degradation (Nakano et al., 2003; Santos et al., 2003, 2005). Rho5 is a parologue of Rho1 and shares similar functions (Nakano et al., 2003; Rincón et al., 2006). GEFs that regulate Rho2-5 GTPases are unknown, except that Rgf1 and Rgf2 might weakly interact with Rho5 (Mutoh et al., 2005).

Recently we and others found that the putative Rho-GEF Gef2 localizes to cortical nodes and coordinates with Polo kinase Plo1 to regulate division-site selection (Moseley et al., 2009; Ye et al., 2012; Guzman-Vendrell et al., 2013). In gef2A plo1 double mutants, Mid1 localization to the cortical nodes and the contractile ring is severely affected and the division site is misplaced. In addition, these studies showed that Gef2 interacts with Mid1 N-terminus (Ye et al., 2012; Guzman-Vendrell et al., 2013), which is essential for Mid1 function (Almonacid et al., 2009, 2011; Lee and Wu, 2012). The substrate GTPases for Gef2 and the regulation of Gef2, however, are largely unknown.

Here we show that Nod1 forms a complex with Gef2 to regulate cytokinesis. Nod1 and Gef2 are interdependent for their localization to cortical nodes and the contractile ring. Their localization at the contractile ring also depends on the physical interaction between Nod1 and the F-BAR protein Cdc15. Like gef2A, nod1Δ suppresses SIN mutants by reducing cell lysis. In addition, the GEF domain of Gef2 interacts with GTPases Rho1, Rho4, and Rho5 in vitro. Thus it is possible that the Gef2/Nod1 complex may activate and function through Rho GTPases during cytokinesis.

**RESULTS**

**Nod1 is a Gef2-related protein that localizes to cortical nodes and the contractile ring**

We previously found that the putative Rho-GEF Gef2 plays a role in division-site positioning in cooperation with Polo kinase, Plo1 (Ye et al., 2012). Concurrently, we identified a novel protein, Nod1 (SPAC12B10.10; Jourdain et al., 2013), in the S. pombe protein database with sequence similarity to Gef2. Nod1 is annotated as a sequence orphan with 419 amino acids (aa; www.pombase.org/spombe/result/SPAC12B10.10). Although it has no GEF domain, Nod1 shares 18% identity and 34% similarity with Gef2 C-terminal aa 636–1101 (Figure 1A). The structure prediction program suggested that Nod1 is a helix-rich protein with no predicted domain (Jones, 1999; Wood et al., 2012).

To determine Nod1’s functions, we first tagged Nod1 with monomeric enhanced green fluorescent protein (GFP) at its C-terminus and examined its localization. Of interest, Nod1 colocalized with Gef2 throughout the cell cycle at interphase nodes, cytokinesis nodes, and the contractile ring (Figure 1B). We next counted Nod1 molecule numbers in cells by measuring its local and global fluorescence intensity (Wu and Pollard, 2005; Laporte et al., 2011). In our previous study, we used strain kanMX6-Pgel2-mECitrine-gef2 (JW3825) to measure the intensity of Gef2 (Ye et al., 2012). We found that the kanMX6 cassette in the strain affected Gef2 expression level, similar to N-terminal tagged F-BAR protein Cdc15 (Wu and Pollard, 2005). We therefore used the kanMX6 looped-out mECitrine-gef2 strain (JW4912) to quantitatively Gef2 molecules globally and locally. The global Gef2 level was one-third in the kan-sensitive strain (JW4912), whereas the local Gef2 concentrations at the contractile ring and cortical nodes were similar to the original data (Ye et al., 2012). Compared to Gef2 (1440 ± 660 molecules/cell, 570 ± 90 molecules at the contractile ring, and 16 ± 5 molecules/interphase node), Nod1 had 1520 ± 700 molecules/cell, 770 ± 150 molecules at the contractile ring, and 15 ± 5 molecules/interphase node (Figure 1C). Thus the ratio of Nod1 to Gef2 in interphase nodes and contractile ring is ∼1:1 and 1.35:1, respectively.

We performed fluorescence recovery after photobleaching (FRAP) assays on interphase nodes to determine Nod1 dynamics at the cell cortex. Nod1 fluorescence recovered with a half-time ($t_{1/2}$) of 170 ± 77 s, and the mobile fraction was ∼40%, similar to Gef2 ($t_{1/2}$ = 180 ± 85 s, 37% mobile fraction; Figure 1D). This indicates that both Nod1 and Gef2 are relatively stable on the plasma membrane compared with some other cytokinesis proteins (Laporte et al., 2011). Together these data suggested that Nod1 might play a role in cytokinesis together with the putative Rho-GEF Gef2.
Regulation of division-site positioning by Nod1 and Gef2

Nod1 shares similarity with Gef2 C-terminus. Top, schematics of Gef2 and Nod1 domains or their colocalization (Figure 1, A and B), with identical and similar (D/E, I/L/V, K/R, N/Q, and S/T) aa are shaded in black and gray, respectively. (B–F) Cells were grown and imaged at 25°C. (B) Colocalization of Nod1 with Gef2 in cortical nodes and the contractile ring (strain JW4457). Top, maximum intensity projection. Bottom, single slice at cell bottom. (C) Molecule numbers of mECitrine-Gef2 (JW4912) and Nod1-mECitrine (JW4008) globally in whole cells and locally in the contractile ring and interphase nodes. (D) FRAP analysis of Nod1-mECitrine (JW4008) and Gef2 (JW3825). Cells were bleached at time zero. Mean ± SEM. (E, F) Nod1 and Gef2 have similar function in division-site positioning. (E) Differential interference contrast (DIC) images and (F) quantification of the division-site positioning. The abnormal septa are defined as septa not placed within the central 20% of the cell or not within 80–100° angle to the long axis of the cell. Strains used: wt (JW81), nod1Δ (JW3773), gef2Δ (JW1826), nod1Δ gef2Δ (JW3814), plo1-ts18 (IH1600), nod1Δ plo1-ts18 (JW3815), gef2Δ plo1-ts18 (JW3078), and nod1Δ gef2Δ plo1-ts18 (JW3873). Bars, 5 μm.

**FIGURE 1:** Nod1 colocalizes with Gef2 in cortical nodes and the contractile ring and shares similar function with Gef2 in division-site selection. (A) Nod1 shares similarity with Gef2 C-terminus. Top, schematics of Gef2 and Nod1 domains or regions. The similar regions between Nod1 and Gef2 are marked with the same pattern. Bottom, sequence alignment between Gef2 aa 601–1101 (top row) and FL Nod1 (bottom row) using Vector NTI program. Identical and similar (D/E, I/L/V, K/R, N/Q, and S/T) aa are shaded in black and gray, respectively. (B–F) Cells were grown and imaged at 25°C. (B) Colocalization of Nod1 with Gef2 in cortical nodes and the contractile ring (strain JW4457). Top, maximum intensity projection. Bottom, single slice at cell bottom. (C) Molecule numbers of mECitrine-Gef2 (JW4912) and Nod1-mECitrine (JW4008) globally in whole cells and locally in the contractile ring and interphase nodes. (D) FRAP analysis of Nod1 (JW4008) and Gef2 (JW3825). Cells were bleached at time zero. Mean ± SEM. (E, F) Nod1 and Gef2 have similar function in division-site positioning. (E) Differential interference contrast (DIC) images and (F) quantification of the division-site positioning. The abnormal septa are defined as septa not placed within the central 20% of the cell or not within 80–100° angle to the long axis of the cell. Strains used: wt (JW81), nod1Δ (JW3773), gef2Δ (JW1826), nod1Δ gef2Δ (JW3814), plo1-ts18 (IH1600), nod1Δ plo1-ts18 (JW3815), gef2Δ plo1-ts18 (JW3078), and nod1Δ gef2Δ plo1-ts18 (JW3873). Bars, 5 μm.

**Nod1 regulates division-site positioning cooperatively with Polo kinase Plo1**

Interphase nodes are important for cell-size control and mitotic entry in fission yeast (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009; Hachet et al., 2011; Deng and Moseley, 2013). As reported (Jourdain et al., 2013), we found that similar to the length of dividing gef2Δ cells (Ye et al., 2012), dividing nod1Δ cells were 16.2 ± 1.0 μm long (n = 148 septating cells), slightly but significantly longer than 14.4 ± 0.9 μm of wild-type (wt) cells (n = 117, p < 0.001). Thus Nod1 and Gef2 play a role in cell-size control.

Gef2 coordinates with Polo kinase, Plo1, to recruit anillin-related protein Mid1 to the cortical nodes for division-site specification (Ye et al., 2012). Because of the sequence similarity between Nod1 and the C-terminus of Gef2 and their colocalization (Figure 1, A and B), we hypothesized that Nod1 has a function similar to Gef2 at early cytokinesis. To test this hypothesis, we crossed nod1Δ to the
Nod1 and Gef2 are in the same genetic pathway.

Nod1 and Gef2 are interdependent on their C-termini for localization to cortical nodes

Because Gef2 and Nod1 are in the same genetic pathway, we tested whether they affect each other’s localization. In wt cells, Gef2 localized to cortical nodes and the contractile ring (Figure 2A). Node localization was abolished, however, and contractile ring localization was greatly reduced in nod1Δ (Figure 2A). Gef2 was detected at the contractile ring with 115 ± 50 molecules, at ~20% of wt levels, in nod1Δ cells (p < 0.001). Nod1 also failed to localize to cortical nodes in gef2Δ, and the localization to the contractile ring was reduced to ~60% of wt level, with 460 ± 130 molecules (p < 0.001; Figure 2A). The loss of localizations was not due to global protein concentration, since Nod1 and Gef2 protein levels were not significantly affected in the absence of one another (Figure 2B). Thus Gef2 and Nod1 are interdependent for localization to cortical nodes (Jourdain et al., 2013) and partially interdependent for localization to the contractile ring.

Gef2 C-terminal aa 957–1101 are necessary and sufficient for its cellular localization (Ye et al., 2012). To test which region of Nod1 is important for its localization, we truncated Nod1 at its native chromosomal locus under the control of nod1Δ promoter based on the sequence alignment between Gef2(601–1101) and Nod1 (Figures 1A and 2E). N-terminal truncations of Nod1 still localized to the cortical nodes and contractile ring (Figure 2C, top). When the last 91 aa of Nod1 from the C-terminus were truncated, however, Nod1 failed to localize to cortical nodes, but it still localized to the contractile ring with lower intensity (Figure 2C, bottom). We conclude that Nod1 C-terminal aa 329–419 are both essential and sufficient for Nod1 node localization.

Next we studied how the Nod1 and Gef2 truncations affect each other’s localization (Figure 2, D and E). Gef2 localized to both cortical nodes and the contractile ring in nod1Δ (329-419) but only localized to the contractile ring weakly when the last 91 aa of Nod1 were truncated in nod1Δ(329-419) (Figure 2D), which is similar to Gef2 localization in nod1Δ (Figure 2A). Similarly, Nod1 localized normally in gef2Δ(1-956) but failed to localize to cortical nodes when Gef2 C-terminal aa 957–1101 were truncated (Figure 2D). Together Nod1 and Gef2 are interdependent on their C-termini for cortical node localization and partially interdependent on their C-termini for localization to the contractile ring (Figure 2E).

Nod1 physically interacts with Gef2 through their C-termini

Based on the interdependence between Nod1 and Gef2 for localization, we hypothesized that the two proteins interact with each other at their C-termini.

To examine whether Nod1 and Gef2 function in the same or parallel genetic pathways, we tested the genetic interactions among nod1Δ, gef2Δ, and plo1-ts18 (Ye et al., 2012). 95% of plo1-ts18 cells had abnormal septa at 25°C (Figure 1, E and F). Moreover, nod1Δ and gef2Δ also had the same strong synthetic interactions with mutations known to affect early cytokinesis, such as mid1, rng2, and cdc4-8, but not with mutations in cell-size control such as cdc2Δ and bfl1Δ (see Table 1 later in the article). Thus Nod1 shares a similar function with Gef2 in division-site specification and contractile-ring assembly (Ye et al., 2012; Jourdain et al., 2013).

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As expected, Nod1 interacted with Gef2(957–1101) (Figure 3A) and Gef2 with Nod1(329–419) (Figure 3B) in co-IP assays. These data suggested that Nod1 and Gef2 interact with each other in vivo through their C-termini.

We tested whether the interaction might be direct between Nod1 and Gef2 through yeast two-hybrid assays (Figure 3C). Full length (FL) Nod1 displayed positive interaction with Gef2 and Gef2(957–1101) but not with Gef2(1–956), whereas FL Gef2 bound to Nod1 and Nod1(210–419) but not to Nod1(1–209). Moreover, Nod1(210–419) interacted with Gef2(957–1101). In summary, Nod1 and Gef2 physically interact with each other through their C-termini, and the interaction is critical for their localization.

The F-BAR protein Cdc15 recruits Nod1 and Gef2 to the contractile ring through its interaction with the Nod1 N-terminus

Gef2 localizes to cytokinesis nodes and the contractile ring in btf1Δ, although interphase-node localization is abolished (Ye et al., 2012). The timings of appearance at cytokinesis nodes for Gef2 in btf1Δ and the F-BAR protein Cdc15 in wt cells are similar (Laporte et al., 2011; Ye et al., 2012). Thus we observed Gef2 and Nod1 localization in the temperature-sensitive mutant cdc15-140 at the restrictive temperature (Figure 4, A and B). After 2 h at 36°C, both Gef2 and Nod1 formed some aggregates, and signals were weaker in cdc15Δ than at 25°C (Figures 2A and 4, A and B). Gef2 and Nod1 still localized to cortical nodes with low intensity, but their contractile-ring localizations were greatly reduced in cdc15-140 cells (Figure 4, A and B). Unlike in gef2Δ cells, the contractile-ring localization of Nod1 was completely abolished in gef2Δ cdc15-140 cells (Figure 4B). Together our data indicate that the contractile-ring localizations of Nod1 and Gef2 depend on each other and on the F-BAR protein Cdc15.

We next investigated whether Cdc15 physically interacts with Gef2 and Nod1. Monomeric yellow fluorescent protein (mYFP–Cdc15) in wt cells were similar (Laporte et al., 2011; Ye et al., 2012). The fact that Cdc15 recruits the Nod1/Gef2 complex to the contractile ring indicated that Nod1 and Gef2 might have additional functions during late cytokinesis besides their role in division-site positioning. Indeed, we found that nod1Δ and gef2Δ had synthetic genetic interactions with cdc15-140. The double mutants nod1Δ cdc15-140 and gef2Δ cdc15-140 failed to form colonies, whereas cdc15-140 mutant still grew at 30°C (Figure 5A; see Table 1 later in the paper). At 25°C, both cdc15-140 single mutant and the double mutants resembled wt (Figure 5B, top). After 6 h at 30°C, cells proliferated with a mean cell length of 11.9 μm for wt and 17.3 μm for cdc15-140 cells. In contrast, most nod1Δ cdc15-140 and gef2Δ cdc15-140 cells were significantly longer, with mean cell length of 26.5 and 28.3 μm, respectively (Figure 5, B and C).
Approximately 30% cdc15-140 cells were defective in contractile-ring assembly and stability, however, and the ring eventually collapsed into aggregates (Figure 5, F and G). Consequently, Bgs1 dispersed around the cell cortex, and the cells became elongated and swollen. These defects were more pronounced in nod1Δ cdc15-140 and gef2Δ cdc15-140 cells, for which Rlc1-tdTomato levels at the division site were significantly reduced to ∼30% of those in cdc15-140 single mutant (Figure 5, E and G). Approximately 52% of nod1Δ cdc15-140 and gef2Δ cdc15-140 cells failed to maintain the contractile ring (Figure 5, E and F, Supplemental Figure S1, and Supplemental Videos S1–S3). Thus our data suggest that Nod1 and Gef2 help to stabilize the contractile ring.

Nod1 and Gef2 suppress mutants in the SIN pathway and affect Sid2 kinase localization

The SIN pathway regulates contractile-ring maturation, stability, and septum formation (Krapp and Simanis, 2008; Roberts-Galbraith and Gould, 2008). We reported that gef2Δ suppresses cdc11-136 and sid2-250 mutants in the SIN pathway, but the mechanism is unknown (Ye et al., 2012). We tested whether nod1Δ suppressed SIN mutants, using gef2Δ as a control (see Table 1 later in the paper).
FIGURE 5: Nod1 and Gef2 affect contractile-ring stability. (A) nod1Δ and gef2Δ display synthetic interaction with cdc15-140. Serial dilutions (3×) of indicated strains (JW81, JW1743, JW4259, JW4016, JW2854, and JW2937) on YES plates at 25, 30, and 36°C, respectively. (B–D) nod1Δ cdc15-140 and gef2Δ cdc15-140 cells display typical cytokinesis defects with elongated and multinucleated cells. Relevant strains used in A were cultured in YES liquid at 25°C (top) or 30°C (bottom) for 6 h before imaging. (B) Before imaging at 30°C, cells were stained with Hoechst for 10 min at 30°C to visualize DNA (green). DIC in gray. (C) Cell length and (D) number of nuclei in cells grown at 30°C for 6 h. (E–G) Nod1 and Gef2 affect contractile-ring stability during cytokinesis at 30°C. Rlc1 and Bgs1 were used to monitor the contractile ring and septum formation. Cells were grown at 30°C for 6 h before imaging at 30°C. Strains used: JW5357, JW5329, and JW5330. (E) Time courses of selected images from a movie with 1-min delay. The entire series can be viewed in Supplemental Videos S1 and S2. (F) Quantification of cells that fail to maintain the contractile ring (CR) after ring assembly. (G) Mean intensity of Rlc1-tTomato at CR. Rlc1 intensity is significantly reduced in nod1Δ cdc15-140 (p < 0.001) and gef2Δ cdc15-140 (p < 0.001) cells vs. cdc15-140 cells. Bars, 5 μm.

Both gef2Δ and nod1Δ partially restored cell growth of cdc7-24 at 30°C (Figure 6A, top) and of cdc11-136 at both 30 and 36°C (Figure 6A, middle). Surprisingly, unlike gef2Δ, nod1Δ did not suppress sid2-250 (Figure 6A, bottom; see Discussion). To explore the mechanism of the suppression of SIN mutants by gef2Δ and nod1Δ, we examined cell morphology of SIN single mutants and SIN gef2Δ or SIN nod1Δ double-mutant cells. cdc7-24 and sid2-250 displayed cell lysis (Figure 6B and Supplemental Figure S2). Except for nod1Δ sid2-250, all double mutants partially restored cell viability by reducing cell lysis. Approximately 60% gef2Δ sid2-250 cells survived at a semipermissive temperature of 30°C, whereas only ~20% sid2-250 and nod1Δ sid2-250 were viable (Figure 6C). On the other hand, cells overexpressing Gef2 from 3nmt1 or 41nmt1 promoter under inducing conditions were synthetic lethal with sid2-250 at 30°C and synthetic sick with sid2-1 from 30 to 36°C (Figure 6D). Taken together, our data suggest that both Nod1 and Gef2 negatively affect the SIN pathway or the process regulated by the pathway.

We next tested whether Sid2 localization is affected in gef2Δ and nod1Δ. Sid2 localizes to the SPB, the contractile ring, and the septum during cytokinesis (Sparks et al., 1999). Sid2 appeared at the contractile ring at the beginning of anaphase B, and the level gradually increased until the contractile ring started to constrict (Figure 6, E, top row, and F; as reported (Sparks et al., 1999; Tebbs and Pollard, 2013). In gef2Δ and nod1Δ, Sid2 appeared at the contractile ring at a similar timing as in wt. Recruitment of Sid2 to the division site, however, was defective. By the end of anaphase B, Sid2 intensity at the division site in gef2Δ and nod1Δ was only ~20% of that in wt (Figure 6, E, middle and lower rows, and F; p < 0.001 for both gef2Δ and nod1Δ vs. wt). Moreover, the peak level of Sid2 at the division site in gef2Δ and nod1Δ was reduced to 57 and 46% that of wt (Figure 6F; p < 0.005 for both gef2Δ and nod1Δ vs. wt). Both wt and mutant cells expressing Sid2-GFP spent more time in mitosis. Because Sid2 regulates proper spindle elongation during anaphase (Mana-Capelli et al., 2012), it seems that Sid2-GFP may not be fully functional. Together these data suggest that Gef2 and Nod1 play a role in recruiting Sid2 to the contractile ring.

Gef2 interacts with Rho GTPases in vitro and is involved in Rho4 localization
Rho GTPases regulate contractile-ring formation, septum formation, and degradation during cytokinesis (Arelano et al., 1997; Nakano et al., 1997, 2003, 2005; Tolliday et al., 2002; Santos et al., 2003; Tajadura et al., 2004; Mutoh et al., 2005; Yoshida et al., 2006). To further dissect the role of Gef2, we tested the interactions...
FIGURE 6: nod1Δ and gef2Δ suppress SIN mutants by reducing cell lysis. (A) Serial dilutions (3×) of indicated strains on YE5S or YE5S + phloxin B (red dye accumulated in dead cells) plates at 25, 30, and 36°C. Strains used: wt (JW81), cdc7-24 (TP34), nod1Δ (JW4259), nod1Δ cdc7-24 (JW4304), gef2Δ (JW2854), gef2Δ cdc7-24 (JW3021), cdc11-136 (TP47), nod1Δ cdc11-136 (JW4306), gef2Δ cdc11-136 (JW2972), sid2-250 (YDM429), nod1Δ sid2-250 (JW4294), and gef2Δ sid2-250 (JW3009). (B, C) gef2Δ but not nod1Δ partially rescued cell lysis in sid2-250. Cells were grown in liquid culture at 25°C and then shifted 30°C for 6 h. (B) DIC images of sid2 mutant strains used in A. (C) Percentage of viable cells. Dead or lysed cells are stained red. (D) Strains grown in liquid culture at 25°C were challenged with yeast extract peptone dextrose (YPD) at 30°C, and glucose was added at 32°C, and then cell viability was measured. (E) Strains grown in liquid culture at 25°C were challenged with 0.1 M NaCl at 30°C, and cell viability was measured. (F) Fluorescence intensity at the division site was quantified for Sld2::GFP.
FIGURE 7: Gef2 GEF domain binds to GTPases Rho1, Rho4, and Rho5 in vitro. (A, B) Purified GST-Rho GTPases and GST control were bound to the beads and then incubated with purified His-Gef2 domain (aa 211–600) of Gef2. The amount of pulled down Gef2 was detected by Western blotting (A) and quantified (B). The intensities of His-Gef2(GEF) bands were measured, background subtracted, corrected for Rho GTPase amount, and normalized by setting the intensity of His-Gef2(GEF) in GST control as 1. The experiment was repeated, and mean ± SD is shown in B. (C–E) rho4Δ suppresses SIN mutants. Strains used: JW81, JW3041, YDM429, JW5505, TP34, JW5503, TP47, and JW5504. (C) Serial dilutions (3×) of indicated strains on YE5S or YE5S + phloxin B plates at 25, 30, and 36°C for 3 d. (D) DIC images of cells grown in liquid culture at 25°C or after 6 h at 30°C (Figure 7D). At 25°C, both rho4Δ and rho4Δ sid2-250 resembled wt, whereas sid2-250 displayed slight cell lysis. At 30°C, only ~20% sid2-250 cells were viable, whereas ~85% cells survived in rho4Δ sid2-250 double mutant (Figure 7E). Thus rho4Δ resembled gef2Δ (Figure 6, A–C) in the suppression of the SIN mutants. Together these data suggest that Gef2 functions through Rho4 GTPase to regulate late cytokinesis.

We next determined whether Gef2 or Nod1 affect Rho4 localization. GFP-Rho4 localized to the cell–division site, as well as to the cell periphery, in wt cells (Nakanuma et al., 2003; Santos et al., 2003). Although its localization was not abolished, Rho4 intensity at the division site was reduced to 82 and 75% of wt level in gef2Δ and nod1Δ, respectively (Figure 7F and G; p < 0.005 for both gef2Δ and nod1Δ vs. wt). Thus Gef2 and Nod1 are involved in concentrating Rho4 GTPase at the division site during cytokinesis.

**DISCUSSION**

In this study we found that Nod1, a new player in cytokinesis, regulates division-site positioning and contractile ring stability together with the putative Rho-GEF Gef2 (Figure 8). In addition, we identified the potential Rho GTPase substrates for Gef2,
suggesting the possible involvement of Gef2 GEF activity and Rho
GTPases in the regulation of cytokinesis.

The roles of Rho GTPases during cytokinesis
Among the seven Rho GEFs in S. pombe, Gef2 and Gef3 have no
identified Rho substrates. We find that Gef2 interacts with Rho1,
Rho4, and Rho5 in vitro (Figure 7, A and B). It is unclear whether
Gef2 interacts with and activates these Rho GTPases in vivo, but
these data provide insight into Gef2’s functions as a potential Rho
GEF. In previous study, we reported that deletion of Gef2 DH do-
main causes defects in division-site positioning in ∼50% plo1-ts18
mutant cells at 25°C (Ye et al., 2012). Therefore it is possible that
the GEF activity of Gef2 is involved in division-site placement. Rho1
regulates cell integrity and septum formation during late cytokinesis
in fission yeast (Nakano et al., 1997; Mutoh et al., 2005). Its ho-
mologs RhoA or Rho1 in animal cells and budding yeast, however,
are active in early cytokinesis for division-site selection and contrac-
tile-ring assembly (Imamura et al., 1997; Tolliday et al., 2002; Bement
et al., 2005; Piekny et al., 2005; Yoshida et al., 2006; Watanabe
et al., 2010). The presence and function of Gef2 in the cortical
nids might suggest a role of Rho1 during early cytokinesis if Gef2 indeed
activates Rho1 in vivo. One difficulty in studying RhoA or Rho1,
however, is that its native concentration is low, and therefore it
is difficult to detect Rho1 at the division site during early cytokinesis
by fluorescence microscopy. Whether Rho1 participates in division-
site positioning in fission yeast remains to be tested, and we cannot
rule out the possibility that other Rho candidates are also involved.

Of the six Rho GTPases in fission yeast, Rho1 and Cdc42 are rela-
tively well studied, whereas our knowledge on Rho2-5 is limited. For
example, no Rho GEFs have been assigned to Rho2, Rho3, and
Rho4. Rho4 affects the localization and activity of β-glucanase Eng1
and α-glucanase Agn1, which results in cell separation defects (Na-
kano et al., 2003; Santos et al., 2003, 2005). Rho5 is a Rho1 paral-
logue that shares similar functions (Nakano et al., 2005). How Rho4
and Rho5 are regulated and localized is unknown. Our data suggest
that Gef2 might be a GEF for Rho4 or Rho5 and help recruit Rho4 to
the division site. Rho4 localization, however, is only partially depen-
dent on Gef2 (Figure 7, F and G). More efforts are needed to inves-
tigate whether and how Gef2 works with these Rho GTPases.

Localization of Node and Gef2 during the cell cycle
We and others found that Gef2 coordinates with Polo kinase, Plo1, to recruit anillin-like
protein Mid1 to the cortical nodes during G2/M transition (Ye et al., 2012; Guzman-
Ventrell et al., 2013; Jourdain et al., 2013). During the course of that study, we identi-
nied Node1 as a Gef2-related protein and binding partner. We found that Gef2 and
Node1 form a complex, which is important for their cortical node localization and functions.
These results are consistent with a recent re-
port on Node1 (Jourdain et al., 2013). Gef2 and
Node1 are stable in interphase nodes, as revealed by FRAP assays. Besides a GEF
(DH-PH) domain, Gef2 has no other known structures or motifs (Figure 1A; Iwaki et al.,
2003). Blt1 was reported to recruit Gef2 to the interphase nodes (Ye et al., 2012; Guz-
man-Ventrell et al., 2013; Jourdain et al., 2013). It is likely that Blt1 interacts with Node1
and Gef2 through their C-termini (Figure 8).

Both Node1 and Gef2 have enriched α-helix structures at C-termini
(Jones, 1999). Gef2 still localizes to cytokinesis nodes in blt1Δ, so
Gef2 must have other binding partners during early mitosis. We pre-
viously showed that Gef2 interacts with Mid1(300–350) in vivo (Ye et al., 2012). Although we found that Mid1(1–580), which includes the Gef2-binding region, depended on Gef2 C-terminus for node
localization, no positive interactions were observed between Mid1(300–350) and several regions of Gef2 or Node1 in yeast two-
hybrid assays (unpublished data). Thus the interactions between
Gef2 and Mid1 may be indirect.

Although the majority of Gef2 is recruited to the contractile ring
through the cortical nodes, our localization independence data reveal
that both Node1 and Gef2 are capable of localizing to the contractile
ring without each other. We find that F-BAR protein Cdc15 physi-
cally interacts with Node1 and recruits Node1 to the contractile ring
(Figures 4 and 8). Cdc15 appears at cytokinesis nodes ∼5 min be-
fore SPB separation and is continuously recruited to the contractile
ring during mitosis (Wu and Pollard, 2005; Laporte et al., 2011).
Consistently, the contractile ring contains ∼40% more molecules of
Node1 than Gef2 (Figure 1C). Node1 intensity at the contractile ring
in gef2Δ also increases during ring maturation at late mitosis. With-
out Node1, Gef2 can still localize to the division site during later stages of cytokinesis (Figure 2A), although Gef2 does not interact with Cdc15 in yeast two-hybrid assays. It is possible that Gef2 de-
pends on alternative mechanisms to localize. One attractive candi-
date is a Rho GTPase. We found that Gef2 can interact with Rho1,
Rho4, and Rho5, and all of them localize to the division site at late
cytokinesis (Nakano et al., 2003, 2005; Santos et al., 2003; Mutoh
et al., 2005). In budding yeast, activated Cdc42 recruits the Rho-
GEF Cdc24 and scaffold protein Bem1 to activate more Cdc42 and
establish cell polarity (Butty et al., 2002; Slaughter et al., 2009; Bi
and Park, 2012). It is possible that Gef2 and its Rho substrates are
involved in a similar positive feedback loop to regulate cytokinesis.

Node1 and Gef2 coordinate with F-BAR protein Cdc15 to
maintain contractile-ring stability
Cdc15 has multiple functions during cytokinesis. During early cy-
tokinesis, Cdc15 recruits the formin Cdc12 to promote contractile-
ring assembly (Carnahan and Gould, 2003; Kovar et al., 2003;

FIGURE 8: Model of Node1 and Gef2 localization and interactions with other proteins on the
cytosolic side of the plasma membrane during the cell cycle. i) During interphase, Node1 and
Gef2 localize to interphase nodes via Blt1 or other interphase-node proteins, ii) where they help
to recruit and stabilize anillin-related protein Mid1. iii) The nodes mature into cytokinesis nodes
and coalesce into the contractile ring as more Mid1 and other cytokinesis proteins like F-BAR
protein Cdc15 arrive at the division site. iv) Cdc15 continuously recruits or stabilize the Node1/
Gef2 complex during ring maturation, which helps to maintain the contractile-ring integrity and
stability. v) Mid1 disappears from the ring at the onset of its constriction. For clarity, the
potential interactions between Gef2 and Rho GTPases are not shown.

3196 | Y.-H. Zhu et al.

Molecular Biology of the Cell
be related to the scaffolding protein Mid1. Mid1 is anchored to the equatorial cortex through the cooperation of its own lipid-binding domains and other cytokinesis proteins, including Cdr2, Gef2, and Blt1 (Almonacid et al., 2009; Lee and Wu, 2012; Ye et al., 2012; Guzman-Vendrell et al., 2013). Mid1 is more dynamic and mobile at the division site without Gef2 (Ye et al., 2012). As a result, the recruitment and maintenance of the contractile-ring components might be less effective during late mitosis, which aggravates the cdc15-mutant phenotype. It is also possible that Rho1 and/or Rho5 GTPases are also involved in contractile-ring stability and their activities are compromised in nod1Δ and gef2Δ cells. Further experiments are needed to distinguish these possibilities.

**Node1 and Gef2 suppress the SIN pathway**

The SIN pathway includes a small GTPase and several protein kinases and their adaptors, which form a kinase cascade on the SPB (Fankhauser and Simanis, 1993, 1994; Furge et al., 1998, 1999; Sparks et al., 1999; Chang and Gould, 2000; Guertin et al., 2000; Hou et al., 2000; Salimova et al., 2000; Tomlin et al., 2002). The activation of SIN pathway leads to contractile-ring constriction and septum formation (Wachtler et al., 2006; Hachet and Simanis, 2008; Krapp and Simanis, 2008; Johnson et al., 2012). This is executed by translocation of kinase Sid2 and its adaptor Mob1 from the SPB to the contractile ring (Sparks et al., 1999; Hou et al., 2000; Chen et al., 2008). Discoveries of suppressors of SIN pathway mutants, especially those of sid2Δ, have helped us understand how SIN pathway regulates cytokinesis (Jiang and Hallberg, 2001; Jin and McCollum, 2003; Jin et al., 2006; Goyal and Simanis, 2012). Here we found that nod1Δ and gef2Δ suppress the SIN mutants by improving cell survival at the semipermissive temperature, whereas single-SIN-mutant cells lyse when trying to separate with defective septa (Figure 6, A–C, and Table 1). We also observed that Sid2 accumulation at the division site is delayed and compromised in nod1Δ and gef2Δ cells (Figure 6, E and F). Similar results were observed in IQGAP mg2Δ without the IQ motifs (Tebbs and Pollard, 2013), suggesting a requirement of intact contractile ring for Sid2 stable localization.

Therefore the contractile ring components, including Gef2 and Nod1, may regulate the SIN pathway through direct or indirect influence on contractile-ring localization of Sid2. It is still possible, however, that the defects caused by nod1Δ and gef2Δ affect the rates of contractile-ring maturation and constriction, allowing more time for septum synthesis. Consistently, increasing the amount and activity of β-glucan synthase Bgs1 by overexpressing Rho1 GTPase or its GEF Rgf3 can rescue sid2Δ mutants (Jin et al., 2006).

Rho4 GTPase, however, might be also involved in the suppression of sid2-250 by gef2Δ. We found that Gef2 binds to Rho4 in vitro. Of interest, deletion of rho4Δ or its effector eng1Δ or agn1 partially suppresses sid2-250 (Jin et al., 2006), which is consistent with our results (Figure 7C–E). Thus it is likely that suppression of SIN mutants by gef2Δ is due to a reduced function of Rho4 and its effectors. Consistently, we found that Rho4 localization to the division site was slightly but significantly reduced in both nod1Δ and gef2Δ cells (Figure 7, F and G). This suggests that Gef2 and Nod1 contribute to Rho4 localization besides the undefined role of Rho4 activation. The cell-separation defect of rho4Δ is mild even at 36°C (Santos et al., 2003), suggesting that other mechanisms and pathways are involved in septum degradation. Further studies are needed to identify the redundant pathways.

In conclusion, we find that the Nod1/Gef2 complex functions in division-site positioning, contractile-ring maintenance, and septation besides its role in cell-size control. We also discover the potential Rho GTPase substrates for Gef2. It will be very informative to

**TABLE 1:** Genetic interactions of nod1Δ with other mutations affecting cytokinesis and cell-size control.

Laporte et al., 2011). During ring maturation at anaphase, Cdc15, together with the SIN pathway and the F-BAR protein Imp2, is believed to be important for maintaining contractile-ring stability and integrity (Wachtler et al., 2006; Hachet and Simanis, 2008; Huang et al., 2008; Roberts-Galbraith et al., 2009). The exact mechanism remains elusive.

Here we add another layer of complexity to the function of Cdc15 during late cytokinesis. In nod1Δ cdc15-140 and gef2Δ cdc15-140, most cells form a fragile contractile ring and become elongated and multinucleated (Figure 5). The severely reduced level of the myosin regulatory light chain Rlc1 suggests loss of proteins from the contractile ring (Figure 5, E–G, Supplemental Figure S1, and Supplemental Videos S1–S3). One possible explanation could

| Strain      | Temperature (°C)a | 25 | 30 | 32 | 36 | gef2Δb |
|-------------|-------------------|----|----|----|----|--------|
| plo1-ts18   |                   | +++| ++ | ++ | +  | Same   |
| plo1-ts18 nod1Δ |               | +  | +  | +  | +/-| Same   |
| mid1-6     |                   | +++| +++| +++| +  | Same   |
| mid1-6 nod1Δ |                  | +++| ++ | ++ | ++ | Same   |
| mid1-366   |                   | +++| +++| +++| +  | Same   |
| mid1-366 nod1Δ |                | +  | +  | +  | +  | Same   |
| mg2-5      |                   | +++| ++ | ++ | -  | Same   |
| mg2-5 nod1Δ |                   | ++ | +  | +  | -  | Same   |
| mg2-346    |                   | +++| ++ | ++ | -  | Same   |
| mg2-346 nod1Δ |                | ++ | +  | +  | -  | Same   |
| cdc4-8     |                   | +++| +++| +++| +  | Same   |
| cdc4-8 nod1Δ |                  | +++| +  | +  | -  | Same   |
| cdc15-140  |                   | +++| +  | +  | -  | Same   |
| cdc15-140 nod1Δ |                 | +++| -  | -  | -  | Same   |
| nod1Δ      |                   | +++| +++| +++| ++| Same   |
| cdr2Δ      |                   | +++| +++| +++| +++| Same   |
| cdr2Δ nod1Δ |                  | +++| +++| +++| +++| Same   |
| blt1Δ      |                   | +++| +++| +++| +++| Same   |
| blt1Δ nod1Δ |                  | +++| +++| +++| +++| Same   |
| klp8Δ      |                   | +++| +++| +++| +++| Same   |
| klp8Δ nod1Δ |                  | +++| +++| +++| +++| Same   |
| cdc7-24    |                   | +++| +  | -  | -  | Same   |
| cdc7-24 nod1Δ |                 | +++| +  | -  | -  | Same   |
| cdc11-136  |                   | +++| +++| +++| +/-| Same   |
| cdc11-136 nod1Δ |                | +++| +++| +++| +/-| Same   |
| sid2-250   |                   | ++ | -  | -  | -  | Different |
| sid2-250 nod1Δ |                | ++ | -  | -  | -  | Different |
| sid2-250 gef2Δ |               | ++ | +/-| -  | -  | Different |

aGrowth and color of colonies on YE5S + phloxin B plates at various temperatures. +++, similar to wt; ++, mild defects or cell lysis; +, cell lysis with reduced growth rate; +/-, severe cell lysis and slow growth; -, inviable.

bThe genetic interactions of nod1Δ were compared with those of gef2Δ with corresponding mutants.
investigate whether Gef2 has GEF activity toward the Rho GTPase candidates and whether Nod1 affects Gef2 activity in addition to its localization.

**MATERIALS AND METHODS**

**Strains and genetic, molecular, and cellular methods**

Table 2 lists the strains used in this study. We used PCR-based gene targeting and standard yeast genetics to construct strains (Moreno et al., 1991; Bähler et al., 1998b). All tagged and truncation strains are regulated under endogenous promoters or 5′ untranslated region (UTR) and integrated into native chromosomal loci, except for the overexpression strains that are integrated at native loci under the control of 3nmt1 or 41nmt1 promoter, which is repressed by thiamine (Maundrell, 1990).

Nod1 C-terminal truncations and Nod1 overexpression were constructed as previously described (Bähler et al., 1998). For N-terminal truncations, nod1 5′ UTR −300 to +3 base pairs was cloned into pFA6a-kanMX6-P3ntm1-mECitrine at BglII and PacI sites to replace the 3nmt1 promoter. The resulting plasmid (JQW560) was then used as the template for PCR amplification and gene targeting. Primers were designed according to desired truncation sites, and the PCR products were transformed into wt cells. The resulting strains were sequenced. Some kanMX6 marker at 5′ end of nod1 or gef2 gene was looped out by crossing the strains to wt cells.

To test the functionalities of tagged FL Nod1, both N- and C-terminally tagged Nod1 strains were crossed to plo1-ts18. Double mutants had <10% abnormal septa at 25°C, which is similar to plo1-ts18 single mutant but different from the ~95% abnormal septa in plo1-ts18 nod1Δ. Thus both N- and C-terminally tagged Nod1 are functional.

For DNA staining, cells were incubated with 10 μg/ml Hoechst 33258 for 10 min in the dark before imaging in the 4',6-diamidino-2-phenylindole (DAPI) channel as described (Wu et al., 2011).

**Microscopy and data analysis**

Strains were restreaked from −80°C stock and grown 1–2 d on yeast extract plus five supplements (YE5S) plates at 25°C. Cells were then inoculated and kept in exponential phase for ~48 h at 25°C except where noted. Before microscopy, cells were washed in Edinburgh minimal medium plus five supplements (EMM5S) twice to reduce autofluorescence and imaged on EMM5S with 20% gelatin pad with 5 μM n-propyl-gallate as described (Laporte et al., 2011; Ye et al., 2012).

| Strain | Genotype | Source/reference |
|--------|----------|------------------|
| JW81   | h+ ade6-210 ura4-D18 leu1-32 | Wu et al. (2003) |
| JW1063 | h+ mYFP-cdc15 ade6-M216 leu1-32 ura4-D18 | Wu and Pollard (2005) |
| JW1636 | h+ mid1-6 ade6-M210 leu1-32 ura4-D18 | Coffman et al. (2013) |
| JW1743 | cdc15-140 ade6-M210 leu1-32 ura4-D18 | Coffman et al. (2013) |
| JW1824 | h+ klp8Δ::kanMX4 ade6 leu1-32 ura4-D18 | Kim et al. (2010) |
| JW1825 | h+ blt1Δ::kanMX4 ade6-M216 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW1826 | h+ gef2Δ::kanMX4 ade6 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW2249 | mg2-346 ade6-M210 leu1-32 ura4-D18 | This study |
| JW2255 | h+ mid1-366 ade6-M210 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW2854 | h+ gef2Δ::hphMX6 ade6 leu1-32 ura4-D18 | This study |
| JW2937 | cdc15-140 gef2Δ::kanMX4 ade6 leu1-32 ura4-D18 | This study |
| JW2972 | h+ cdc11-136 gef2Δ::hphMX6 ade6 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW3009 | gef2Δ::hphMX6 sid2-250 ade6 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW3021 | gef2Δ::hphMX6 cdc7-24 ade6 leu1-32 ura4-D18 | This study |
| JW3041 | h+ rho4Δ::kanMX4 ade6 leu1-32 ura4-D18 | Kim et al. (2010) |
| JW3078 | h+ gef2Δ::hphMX6 plo1-ts18::ura4+ ade6 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW3078 | h+ gef2Δ::hphMX6 plo1-ts18::ura4+ ade6 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW3204 | h+ gef2-13Myc-hphMX6 ade6-M210 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW3235 | gef2-13Myc-hphMX6 mYFP-cdc15 ade6-M210 leu1-32 ura4-D18 | This study |
| JW3561 | h+ kanMX6-3nmt1-gef2 ade6-M216 leu1-32 ura4-D18 | This study |
| JW3562 | h+ kanMX6-41nmt1-gef2 ade6-M216 leu1-32 ura4-D18 | This study |
| JW3622 | h+ gef2-13Myc-hphMX6 ade6-M210 leu1-32 ura4-D18 | This study |
| JW3773 | h+ nod1Δ::kanMX6 ade6-M210 leu1-32 ura4-D18 | This study |
| JW3814 | h+ nod1Δ::kanMX6 gef2Δ::kanMX4 ade6 leu1-32 ura4-D18 | This study |
| JW3815 | nod1Δ::kanMX6 plo1-ts18::ura4+ ade6-M210 ura4-D18 leu1-32 | Ye et al. (2012) |
| JW3825 | h+ kanMX6-Pgef2-mECitrine-4Gly-gef2 ade6-M216 leu1-32 ura4-D18 | Ye et al. (2012) |
| JW3826 | h+ kanMX6-Pgef2-mECitrine-4Gly-gef2-(957-1101) ade6-M216 leu1-32 ura4-D18 | Ye et al. (2012) |

**TABLE 2:** *S. pombe* strains used in this study.

Continues
| Strain     | Genotype                                      | Source/reference          |
|------------|-----------------------------------------------|---------------------------|
| JW3861     | h\(^+\) nod1\(\Delta\)::kanMX6 mid1-6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW3873     | nod1\(\Delta\)::kanMX6 gef2\(\Delta\)::kanMX4 plo1.ts18::ura4^+ ade6 leu1-32 ura4-D18 | This study                |
| JW3875     | h\(^-\) nod1\(\Delta\)::kanMX6 mid1-366 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4008     | h\(^-\) nod1-mECitrine-kanMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4010     | h\(^-\) nod1-tdTomato-hphMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4013     | h\(^-\) nod1-13Myc-hphMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4014     | nod1\(\Delta\)::kanMX6 kanMX6-Pgef2-mECitrine-4Gly-gef2 ade6 leu1-32 ura4-D18 | This study                |
| JW4015     | h\(^-\) nod1::kanMX6 cdc4-8 ade6 leu1-32 ura4-D18 | This study                |
| JW4016     | h\(^-\) nod1::kanMX6 cdc15-140 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4038     | nod1-mECitrine-kanMX6 gef2\(\Delta\)::hphMX6 ade6 leu1-32 ura4-D18 | This study                |
| JW4042     | h\(^+\) nod1::kanMX6 mg2-D5 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4043     | h\(^-\) nod1::kanMX6 mg2-346 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4098     | nod1\(\Delta\)::kanMX6 cdr2\(\Delta\)::kanMX6 ade6 leu1-32 ura4-D18 | This study                |
| JW4099     | h\(^-\) nod1::kanMX6 blt1\(\Delta\)::kanMX4 ade6 leu1-32 ura4-D18 | This study                |
| JW4226     | h\(^-\) kanMX6-Pgef2-tdTomato-4Gly-gef2 ade6-M210 leu1-32 ura4-D18 | Ye et al. (2012)          |
| JW4256     | nod1-tdTomato-hphMX6 kanMX6-Pgef2-mECitrine-4Gly-gef2-(957-1101) ade6 leu1-32 ura4-D18 | This study                |
| JW4259     | h\(^+\) nod1::hphMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4294     | nod1\(\Delta\)::hphMX6 sid2-250 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4295     | klp8\(\Delta\)::kanMX4 nod1\(\Delta\)::hphMX6 ade6 leu1-32 ura4-D18 | This study                |
| JW4304     | nod1\(\Delta\)::hphMX6 cdc7-24 ade6 leu1-32 ura4-D18 his2 or his7 | This study                |
| JW4306     | nod1\(\Delta\)::hphMX6 cdc11-136 ade6-M210 leu1-32 ura4-D18 his2 or his7 | This study                |
| JW4325     | h\(^-\) nod1(1-209)-mECitrine-kanMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4326     | h\(^-\) nod1(1-328)-mECitrine-kanMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4330     | nod1-13Myc-hphMX6 kanMX6-Pgef2-mECitrine-4Gly-gef2 ade6 leu1-32 ura4-D18 | This study                |
| JW4331     | nod1-13Myc-hphMX6 kanMX6-Pgef2-mECitrine-4Gly-gef2-(957-1101) ade6 leu1-32 ura4-D18 | This study                |
| JW4355     | nod1-tdTomato-hphMX6 kanMX6-Pgef2-mECitrine-4Gly-gef2-(1-956)-TADH1-hphMX6 ade6 leu1-32 ura4-D18 | This study                |
| JW4359     | h\(^-\) nod1(1-328)-mECitrine-kanMX6 kanMX6-Pgef2-tdTomato-4Gly-gef2 ade6 leu1-32 ura4-D18 | This study                |
| JW4453     | h\(^+\) kanMX6-Pnod1-mECitrine-nod1 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4455     | h\(^-\) kanMX6-Pnod1-mECitrine-nod1(329-419) ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4457     | nod1-mEGFP-hphMX6 kanMX6-Pgef2-tdTomato-4Gly-gef2 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4750     | Pnod1-mECitrine-nod1 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4856     | h\(^-\) Pnod1-mECitrine-nod1(329-419) ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4909     | rho4A::kanMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4910     | h\(^-\) rho4A::kanMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW4912     | Pgef2-mECitrine-4Gly-gef2 ade6 leu1-32 ura4-D18 | This study                |
| JW5027     | cdc15-140 nod1-mECitrine-kanMX6 gef2\(\Delta\)::hphMX6 ade6 leu1-32 ura4-D18 | This study                |
| JW5028     | cdc15-140 nod1-mECitrine-kanMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW5065     | h\(^+\) Pnod1-mECitrine-nod1(210-419) ade6-M210 leu1-32 ura4-D18 | This study                |
| JW5093     | kanMX6-Pnod1-mECitrine-nod1 gef2-13Myc-hphMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW5095     | kanMX6-Pnod1-mECitrine-nod1(329-419) gef2-13Myc-hphMX6 ade6-M210 leu1-32 ura4-D18 | This study                |
| JW5107     | kanMX6-Pgef2-tdTomato-4Gly-gef2 kanMX6-Pnod1-mECitrine-nod1(329-419) ade6-M210 leu1-32 ura4-D18 | This study                |
| JW5120     | nod1-13Myc-hphMX6 mYFP-cdc15 ade6 leu1-32 ura4-D18 | This study                |

**TABLE 2:** *S. pombe* strains used in this study. Continued

Volume 24 October 15, 2013 Rho-GEF Gef2 and Nod1 in cytokinesis | 3199
2012). For long movies, cells were washed in YE55 and resuspended in YE55 with 5 μM n-propyl-gallate. Then 2-μL concentrated cells were spotted onto a coverglass-bottom dish (Delta TPG Dish; Bio- techs, Butler, PA) and covered with a layer of YE55 agar before imaging at 23.5°C or in a preheated climate chamber (stage top incubator INUB-PPZ12-F1 equipped with UNIV2-D35 dish holder; Tokai Hit, Shizuoka-ken, Japan) for imaging at the restrictive temperatures for certain mutants.

Microscopy was performed at 23.5–25°C except where noted. To visualize cell morphology, DNA, and septum, Hoechst-stained cells were imaged with a 100×/1.4 numeral aperture (NA) Plan-Apo objective lens on a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) equipped with a Nikon cooled digital camera DS-Qi1 (Nikon) or a spinning disk confocal microscope (UltraView VERS; PerkinElmer Life and Analytical Sciences, Waltham, MA) with 440- and 568-nm solid state lasers and a back-thinned, electron-multiplying charge-coupled device camera (Hamamatsu C9100-13) without binning.

Images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD), UltraVIEW, or Volocity (PerkinElmer) software. Fluorescence images shown in figures and movies are maximum projections of images stacks at 0.4- to 0.6-μm spacing except where noted. Nod1 and Gef2 molecules in cells were counted globally or locally by measuring fluorescence intensity as described (Laporte et al., 2011). Briefly, tagged Nod1 or Gef2 cells were mixed with wt cells and imaged with 11 z-sections with 0.4-μm spacing on the UltraView ERS confocal system. The offset was subtracted from images that were then corrected for uneven illumination. Mean intensity in whole cells was measured in sum intensity projections and images that were then corrected for uneven illumination. Mean intensity in whole cells was measured in sum intensity projections and images that were then corrected for uneven illumination.

| Strain    | Genotype                                                                 | Source/reference                  |
|-----------|---------------------------------------------------------------------------|-----------------------------------|
| JW5329    | h\(^+\) gef2Δ::kanMX4 cdc15-140 GFP-bgs1-1 leu1\(^+\) bgs1Δ::ura4\(^+\) rlc1-tdTomato-natMX6 adel6 leu1-32 ura4-D18 | This study                        |
| JW5330    | nod1Δ::kanMX6 cdc15-140 GFP-bgs1-1 leu1\(^+\) bgs1Δ::ura4\(^+\) rlc1-tdTomato-natMX6 adel6-M210 leu1-32 ura4-D18 | This study                        |
| JW5357    | h\(^+\) cdc15-140 GFP-bgs1-1 leu1\(^+\) bgs1Δ::ura4\(^+\) rlc1-tdTomato-natMX6 adel6-M210 leu1-32 ura4-D18 | This study                        |
| JW5360    | sid2-250 kanMX6-3nmt1-gef2 adel6 leu1-32 ura4-D18                         | This study                        |
| JW5361    | sid2-250 kanMX6-41nmt1-gef2 adel6 leu1-32 ura4-D18                         | This study                        |
| JW5405    | sid2-1 kanMX6-3nmt1-gef2 adel6 leu1-32 ura4-D18                           | This study                        |
| JW5406    | sid2-1 kanMX6-41nmt1-gef2 adel6 leu1-32 ura4-D18                           | This study                        |
| JW5503    | rho4Δ::kanMX4 cdc7-24 adel6 leu1-32 ura4-D18 his-366                      | This study                        |
| JW5504    | rho4Δ::kanMX4 cdc11-136 adel6 leu1-32 ura4-D18                            | This study                        |
| JW5505    | rho4Δ::kanMX4 sid2-250 adel6 leu1-32 ura4-D18                             | This study                        |
| JW5580    | gef2Δ::kanMX4 sid2-GFP-ura4 ade6-M210 leu1-32 ura4-D18                    | This study                        |
| JW5581    | nod1Δ::kanMX6 sid2-GFP-ura4 ade6-M210 leu1-32 ura4-D18                    | This study                        |
| JW5582    | Pgef2-mECitrine-4Gly-gef2 Pmyo2-mCFP-myo2 adel6 leu1-32 ura4-D18           | This study                        |
| JW5583    | cdc15-140 Pgef2-mECitrine-4Gly-gef2 Pmyo2-mCFP-myo2 adel6 leu1-32 ura4-D18 | This study                        |
| IH1600    | h\(^+\) plo1.ts18::ura4 ura4-D18 leu1-32 adel6-M210 his2                  | Maciver et al. (2003)             |
| JM578     | h\(^+\) cdc7-24 his7-366 leu1-32 adel6-M216 ura4-D18                      | Thomas Pollard (Yale University, New Haven, CT) |
| PPG1580   | rho4Δ::kanMX6 leu1::GFP-rho4 leu1-32 ura4-D18                             | Thomas Pollard (Yale University, New Haven, CT) |
| TP7       | h\(^+\) cdc4-8 his7-366 leu1-32 ura4-D18 adel6-M216                       | Thomas Pollard (Yale University, New Haven, CT) |
| TP34      | h\(^+\) cdc7-24 his7-366 leu1-32 adel6-M216 ura4-D18                      | Thomas Pollard (Yale University, New Haven, CT) |
| TP47      | h\(^+\) cdc11-136 ura4-D18 leu1-32 his7-366                               | Bezanilla et al. (1997)           |
| VS2367    | h\(^+\) sid2-1 adel6-M210 leu1-32 ura4-D18                               | Salimova et al. (2000)            |
| YMD26     | h\(^+\) mg2-D5 adel6-210 ura4-D18 leu1-32                                 | Eng et al. (1998)                 |
| YMD415    | h\(^+\) sid2-GFP-ura4 adel6-M210 leu1-32 ura4-D18                        | Sparks et al. (1999)              |
| YMD429    | h\(^+\) sid2-250 adel6-M210 leu1-32 ura4-D18                             | Sparks et al. (1999)              |

**TABLE 2:** *S. pombe* strains used in this study. Continued
avoid overlapping with other nodes. The global and local intensities of Nod1 and Gef2 were then normalized to molecule numbers using previous Gef2 data as a reference (Wu and Pollard, 2005; Wu et al., 2008; Ye et al., 2012).

FRAP analysis
FRAP assays were performed using the photokinesis unit on the UltraVIEW Vox confocal system, similar to the assays described before (Coffman et al., 2009; Laporte et al., 2011). The best focal plane for bleaching was chosen from z-stacks. Selected ROIs were bleached to <50% of the original fluorescence intensity after five prebleach images were collected. One hundred postbleach images with 10-s delay were collected. The images were then corrected for background and photobleaching during image acquisition at non-bleached sites. We normalized prebleach intensity of the ROI to 100%, the intensity just after bleaching to 0%, and the end of the bleaching time as time 0. Intensity of every three consecutive post-bleaching time points was averaged to reduce noise. The data were then plotted and fitted using the exponential equation $y = m_1 + m_2 \exp(-t/m_3)$, where $m_3$ is the off-rate (KaleidaGraph; Synergy Software, Reading, PA). The half-time of recovery was calculated as $t_{1/2} = (\ln 2)/m_3$. The $p$ values in this study were calculated using two-tailed Student's t-test.

IP and Western blotting
IP assay and Western blotting were carried out as previously described (Laporte et al., 2011; Lee and Wu, 2012). Briefly, mECitrine-tagged proteins were pulled down from fission yeast cell extract by protein G covalently coupled magnetic Dynabeads (100.04D; Invitrogen, Carlsbad, CA) with polyclonal anti-GFP antibodies (NB600-308; Novus Biologicals, Littleton, CO). The bead samples were then boiled in sample buffer after washing three times. The protein samples were then separated in SDS–PAGE, and Western blotting was performed using monoclonal anti-GFP antibody (11814460001, 1:2000 dilution; Roche, Mannheim, Germany) or monoclonal anti-His antibody (9E10, 1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The anti-tubulin monoclonal TAT1 antibody was used at 1:20,000 dilution (Woods et al., 1989). Anti-mouse secondary antibody was used at 1:5000 dilution.

Yeast two-hybrid assays
β-Galactosidase activity assays were performed to semiquantitatively detect protein interactions in yeast two-hybrid assays (Laporte et al., 2011). DNAs or cDNAs of interest were constructed into vectors with either VP16 activation domain or GBT9 DNA-binding domains. Yeast two-hybrid assays were performed in the yeast cytokinesis mutants. Genetics 149, 1265–1275.

Protein purification and the interaction between Gef2 and Rho GTPases
Pull-down assays between recombinant 6His-Gef2 (GEF) and GST-Rho proteins were adapted from a previous study (Iwaki et al., 2003). Expression of 6His-tagged GEF domain of Gef2 (aa 211–600) was induced when ArcticExpress RIL cells (230193; Agilent Technologies, Santa Clara, CA) carrying the plasmid were grown at 10°C for 18 h after adding 1 mM isopropyl-β-D-thiogalactoside (IPTG; Saha and Pollard, 2012b). After sonication (output 9, 50% duty cycle, 4x 20 pulses) and ultracentrifugation (25,000 rpm for 15 min, then 38,000 rpm for 30 min), 6His-Gef2 (GEF) was purified on Talon Metal Affinity Resin (635501; Clontech, Mountain View, CA) followed by gel filtration with a HiLoad 16×60 Superdex 200 (17-5175-01; GE Healthcare, Buckinghamshire, United Kingdom) in phosphate buffer (50 mM sodium phosphate, pH 6.2, 0.3 M NaCl, 1 mM dithiothreitol [DTT]). The purified His-Gef2 (GEF) was then dialyzed into the final binding buffer (25 mM 3-N-morpholinosopropanesulfonic acid, pH 7.2, 60 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphonate, 1 mM DTT, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor tablets). GST and GST-Rho1 to Rho5 and Cdc42 were purified from BL21(DE3)pLysS cells (69451; Novagen, EMD Chemicals, Darmstadt, Germany; induced with 0.5 mM IPTG at 15°C for 6 h) using glutathione–Sepharose beads (17-5132-01; GE Healthcare). The beads with Rho proteins were then incubated at 30°C for 10 min with buffer containing 50 mM Tris (pH 7.5), 1 mM DTT, and 5 mM EDTA to deplete nucleotides. Then 500 μl of 0.25 μM 6His-Gef2 (GEF) in binding buffer was added to 30 μl 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 detect by Western blotting. Rho GTPases were detected by monoclonal anti-GST antibody (3G10/1B3, 1:5000 dilution; NB600-446, Novus Biologicals), and bound 6His-Gef2 (GEF) was detected by anti-His antibody (631212, 1:10,000 dilution; Clontech). Secondary anti-mouse antibody was used at 1:5000 dilution.

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REFERENCES
Almonacid M, Celton-Morizur S, Jakubowski JL, Dingli F, Loew D, Mayeux A, Chen JS, Gould KL, Clifford DM, Paoletti A (2011). Temporal control of contractile ring assembly by Plo1 regulation of myosin II recruitment by Mid1/anillin. Curr Biol 21, 473–479.

Almonacid M, Moseley JB, Janvore J, Mayeux A, Fraiser V, Nurse P, Paoletti A (2009). Spatial control of cytokinesis by Cdc2 kinase and Mid1/anillin nuclear export. Curr Biol 19, 961–966.

Arasada R, Pollard TD (2011). Distinct roles for F-BAR proteins Cdc15p and Cdc42p in cortical constriction of the cell envelope. Curr Biol 21, 1450–1459.

Avellan-M, Duran A, Perez P (1997). Localization of the Schizosaccharomyces pombe rho1p GTPase and its involvement in the organisation of the actin cytoskeleton. J Cell Sci 110, 2547–2555.

Bähler J, Steever AB, Wheatley S, Wang Y-L, Pringle JR, Gould KL, McCollum D (1998a). Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. J Cell Biol 143, 1603–1616.

Bähler J, Wu J-Q, Longtine MS, Shah NG, McKenzie A III, Steever AB, Wach A, Philippsen P, Pringle JR (1998b). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast 14, 943–951.

Balasubramanian MK, McCollum D, Chang L, Wong KC, Naqvi NI, He X, Sazer S, Gould KL, McCollum D (2011b). Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. J Cell Biol 143, 1603–1616.
Bezanilla M, Forsburg SL, Pollard TD (1997). Identification of a second myosin-II in Schizosaccharomyces pombe: Myp2p is conditionally required for cytokinesis. Mol Biol Cell 8, 2693–2705.

Bi E, Park H-O (2012). Cell polarization and cytokinesis in budding yeast. Genetics 191, 347–387.

Butty AC, Perrijaquet N, Pettit A, Jaquenoud M, Segall JE, Hofmann K, Zwahlen C, Peter M (2002). A positive feedback loop stabilizes the guanine-nucleotide exchange factor Cdc24 at sites of polarization. EMBO J 21, 1556–1576.

Calonge TM, Nakano K, Arrallano M, Ari A, Katayama S, Toda T, Mabuchi I, Pérez P (2000). Schizosaccharomyces pombe Rho2p GTPase regulates cell wall α-glucan biosynthesis through the protein kinase Pck1p. Mol Biol Cell 11, 4393–4401.

Cavander RH, Gould KL (2003). The PCH family protein, Cdc15p, recruits two F-actin nucleation pathways to coordinate cytokinetic actin ring formation in Schizosaccharomyces pombe. J Cell Biol 162, 851–862.

Cerutti L, Simanis V (1999). Asymmetry of the spindle pole bodies and spg1p GAP segregation during mitosis in fission yeast. J Cell Sci 112, 2313–2321.

Chang F, Pollard A, Nurse P (1996). Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. J Cell Sci 109, 131–142.

Chang L, Gould KL (2000). Sid4p is required to localize components of the septation initiation pathway to the spindle pole body in fission yeast. Proc Natl Acad Sci USA 97, 5249–5254.

Chen C-T, Feoktistova A, Chen JS, Shim YS, Clifford DM, Gould KL (2000). Sid2 is a guanine-nucleotide exchange factor Cdc14p at sites of polarization. Curr Biol 10, 1594–1599.

Chen Q, Pollard TD (2011). Actin filament severing by coflin is more important for assembly than constriction of the cytokinetic contractile ring. J Cell Biol 195, 485–498.

Coffman VC, Nile AH, Lee I-J, Liu HY, Wu J-Q (2009). Roles of formin nodes for cytokinesis in Schizosaccharomyces pombe. Mol Biol Cell (in press).

Collin PM, Trillo Y, Ametazaurra A, Pérez P (2003). Gef1p, a new guanine nucleotide exchange factor for Cdc42p, regulates polarity in Schizosaccharomyces pombe. Mol Biol Cell 14, 313–323.

Deng L, Moseley JB (2013). Compartmentalized nodes control mitotic entry signaling in fission yeast. Mol Biol Cell 24, 1872–1881.

Eng K, Naqvi N, Wong KC, Balasubramanian MK (1998). Rng2p, a protein required for cytokinesis in fission yeast, is an activator of the actomyosin ring and the spindle pole body. Curr Biol 8, 611–621.

Fankhauser C, Raymond A, Cerutti L, Utzig S, Hofmann K, Simanis V (1995). The Pom1p kinase and Cdc14p in regulating the onset of cytokinesis in fission yeast. EMBO J 14, 1803–1815.

Guzman-Vendrell M, Baldissard S, Almonacid M, Mayeux A, Paololeti A, Moseley JB (2013). Blt1 and Mid1 provide overlapping membrane anchors to position the division plane in fission yeast. Mol Cell Biol 33, 418–428.

Hachet O, Berthelot-Grosjean M, Kokkoris K, Vincenzetti V, Moosbrugger J, Martin SG (2011). A phosphorylation cycle shape gradients of the DYRK family kinase Pom1 at the plasma membrane. Cell 145, 1116–1128.

Hachet O, Simanis V (2008). Mid1p/anillin and the septation initiation network orchestrate contractile ring assembly for cytokinesis. Genes Dev 22, 3205–3216.

Hirot a K, Tanaka K, Ohta K, Yamamoto M (2003). Gef1p and Scd1p, the Two GDP-GTP exchange factors for Cdc42p, form a ring structure that shrinks during cytokinesis in Schizosaccharomyces pombe. Mol Biol Cell 14, 3617–3627.

Hou M-C, Salek J, McCollum D (2000). Mob1p interacts with the Sid2p kinase and is required for cytokinesis in fission yeast. Curr Biol 10, 619–622.

Huang Y, Yan H, Balasubramanian MK (2008). Assembly of normal actomyosin rings in the absence of Mid1p and cortical nodes in fission yeast. J Cell Biol 183, 979–988.

Imamura H, Tanaka K, Hara T, Umikawa M, Kamei T, Takahashi K, Sasaki T, Taki Y (1997). Bni1p and Bnr1p: downstream target of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in Saccharomyces cerevisiae. EMBO J 16, 2745–2755.

Iwaki N, Karatsu K, Miyamoto M (2003). Role of guanine nucleotide exchange factors for Rho family GTPases in the regulation of cell morphology and actin cytoskeleton in fission yeast. Biochem Biophys Res Commun 312, 414–420.

Jiang W, Hallberg RL (2001). Correct regulation of the septation initiation network in Schizosaccharomyces pombe requires the activities of par1 and par2. Genetics 158, 1413–1429.

Jin Q-W, McCollum D (2003). Scw1p antagonizes the septation initiation network to regulate septum formation and cell separation in the fission yeast Schizosaccharomyces pombe. Eukaryot Cell 2, 510–520.

Jin Q-W, Zhou M, Bimbo A, Balasubramanian MK, McCollum D (2006). A role for the septation initiation network in septum assembly revealed by genetic analysis of sid2–250 suppressors. Genetics 172, 2101–2112.

Johnson AE, McCollum D, Gould KL (2012). Polar opposites: fine-tuning cytokinesis through SIN asymmetry. Cytoskeleton (Hoboken) 69, 586–599.

Jones DT (1999). Protein secondary structure prediction based on position-specific scoring matrices. J Mol Biol 292, 195–202.

Jourdain I, Brzezinska EA, Toda T (2013). Fission yeast Nod1 is a component of cortical nodes involved in cell size control and division site placement. PLoS One 8, e54142.

Jwa M, Song K (1998). Byr4, a dosage-dependent regulator of cytokinesis in S. pombe, interacts with a possible small GTPase pathway including Spg1 and Cdc16. Mol Cells 8, 240–245.

Kim DU et al. (2010). Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. Nat Biotechnol 28, 617–623.

Kita A, Li C, Yu Y, Umeda N, Doi A, Yasuda M, Ishiwata S, Taga A, Horiuchi Y, Sugira R (2011). Role of the small GTPase Rh3 in Golgi/endosome trafficking through functional interaction with adaptin in fission yeast. PLoS One 6, e16842.

Kovar DR, Kuhn JR, Tichy AL, Pollard TD (2003). The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. J Cell Biol 161, 875–887.

Krapp A, Collin P, Cano Del Rosario E, Simanis V (2008). Homoeostasis between the GTPase Spg1p and its GAP in the regulation of cytokinesis in S. pombe. J Cell Sci 121, 601–606.

Krapp A, Schmidt S, Cano E, Simanis V (2001). S. pombe cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole body. Curr Biol 11, 1559–1568.

Krapp A, Simanis V (2008). An overview of the fission yeast septation initiation network (SIN). Biochem Soc Trans 36, 411–415.

Laporte D, Coffman VC, Lee I-J, Wu J-Q (2011). Assembly and architecture of precursor nodes during fission yeast cytokinesis. J Cell Biol 192, 1005–1021.
Laporte D, Ojci D, Vaylonis D, Wu J-Q (2012). α-Actinin and fimbrin cooperate with myosin II to organize actomyosin bundles during contractile-ring assembly. Mol Biol Cell 23, 3094–3110.

Lee I-J, Coffman VC, Wu J-Q (2012). Contractile-ring assembly in fission yeast cytokinesis: Recent advances and new perspectives. Cytoskeleton (Hoboken) 69, 751–763.

Lee I-J, Wu J-Q (2012). Characteristics of Mid1 domains for targeting and scaffolding in fission yeast cytokinesis. J Cell Sci 125, 2973–2985.

Lehner CF (1992). The pebble gene is required for cytokinesis in Drosophila. J Cell Sci 103, 1021–1030.

MacLver FH, Tanaka K, Robertson AM, Hagan IM (2003). Physical and functional interactions between polo kinase and the spindle pole component Cull12 regulate mitotic commitment in S. pombe. Genes Dev 17, 1507–1523.

Mana-Capelli S, McLean JR, Chen C-T, Gould KL, McCollum D (2012). The kinesin-14 Klp2p is negatively regulated by the SIN for proper spindle elongation and telophase nuclear positioning. Mol Biol Cell 23, 4952–4960.

Martin SG, Berthelot-Grosjean M (2009). Polar gradients of the DYRK-family kinase Pom1 couple cell length with the cell cycle. Nature 459, 852–856.

Maundrell K (1990). Chara, a highly transcribed gene completely repressed by thiamine. J Biol Chem 265, 10857–10864.

Mehta S, Gould KL (2006). Identification of functional domains within the septation initiation network kinase, Cdc7. J Biol Chem 281, 9935–9941.

Moreno S, Klar A, Nurse P (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol 194, 795–823.

Morrell-Falvey JL, Ren L, Feoktistova A, Haese GD, Gould KL (2005). Cell wall remodeling at the fission yeast cell division site requires the RhoGEF Rgf3p. J Cell Sci 118, 5563–5573.

Morrell JL et al. (2004). Sid4p-Cdc11p assembles the septation initiation network and its regulators at the S. pombe SPB. Curr Biol 14, 579–584.

Moseley JB, Mayeux A, Paoletti A, Nurse P (2009). A spatial gradient coordinates cell size and mitotic entry in fission yeast. Nature 459, 857–860.

Motej F, Mishra M, Balasubramanian MK, Mabuchi I (2004). Myosin-II reorganization during mitosis is controlled temporally by its dephosphorylation and spatially by Mid1 in fission yeast. J Cell Biol 165, 685–695.

Mutoh T, Nakano K, Mabuchi I (2005). Rho1-GEFs Rgfl and Rgl2 are involved in formation of cell wall and septum, while Rgfl is involved in cytokinesis in fission yeast. Genes Cells 10, 1189–1202.

Nakano K, Arii R, Mabuchi I (1997). The small GTP-binding protein Rho1 is a multifunctional protein that regulates actin localization, cell polarity, and septum formation in the fission yeast Schizosaccharomyces pombe. Genes Cells 2, 679–694.

Nakano K, Arii R, Mabuchi I (2005). Small GTPase Rho5 is a functional homologue of Rho1, which controls cell shape and septation in fission yeast. FEBS Lett 579, 5181–5186.

Nakano K, Imai J, Arii R, Toh EA, Matsui Y, Mabuchi I (2002). The small GTPase Rho3 and the diaphanous/formin For3 function in polarized cell growth in fission yeast. J Cell Sci 115, 4629–4639.

Nakano K, Mabuchi I (2003). The small GTPase Rho4 is involved in formation of cell wall and septum, while Rgf3 is involved in cytokinesis in fission yeast. Genes Cells 10, 1189–1202.

Nishimura Y, Yonemura S (2006). Centralspinindole regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. J Cell Sci 119, 104–114.

O’Keefe L, Somers WG, Harley A, Saint R (2001). The role of Pom1 kinase in mitotic commitment and septation in Schizosaccharomyces pombe. Curr Biol 11, 5181–5186.

Padmanabhan A, Bakka K, Sevugan M, Naqvi NI, D’Souza V, Tang X, Mishra M, Balasubramanian MK (2011). IQGAP-related Rng2p organizes cortical nodes and ensures position of cell division in fission yeast. Curr Biol 21, 467–472.

Paoletti A, Chang F (2000). Analysis of mid1p, a protein required for placement of the cell division site, reveals a link between the nucleus and the cell surface in fission yeast. Mol Biol Cell 11, 2757–2773.

Pérez P, Rincón SA (2010). Rho GTPases: regulation of cell polarity and growth in yeasts. Biochem J 426, 243–253.

Pieńko A, Werner M, Glotzer M (2005). Cytokinesis: welcome to the Rho zone. Trends Cell Biol 15, 651–658.

Pinar M, Coll PM, Rincón SA, Pérez P (2008). Schizosaccharomyces pombe Pa fileId is a pafiles homologue that modulates Rho1 activity and participates in cytokinesis. Mol Biol Cell 19, 1727–1738.
Watanabe S, Okawa K, Miki T, Sakamoto S, Morinaga T, Segawa K, Arakawa T, Kinoshita M, Ishizaki T, Narumiya S (2010). Rho and anillin-dependent control of mDia2 localization and function in cytokinesis. Mol Biol Cell 21, 3193–3204.

Wloka C, Bi E (2012). Mechanisms of cytokinesis in budding yeast. Cytoskeleton (Hoboken) 69, 710–726.

Wood V et al. (2012). PomBase: a comprehensive online resource for fission yeast. Nucleic Acids Res 40, D695–D699.

Woods A, Sherwin T, Sasse R, MacRae TH, Baines AJ, Gull K (1989). Definition of individual components within the cytoskeleton of Trypanosoma brucei by a library of monoclonal antibodies. J Cell Sci 93, 491–500.

Wu J-Q, Kuhn JR, Kovar DR, Pollard TD (2003). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. Dev Cell 5, 723–734.

Wu J-Q, McCormick C, Pollard TD (2008). Counting proteins in living cells by quantitative fluorescence microscopy with internal standards. Methods Cell Biol 89, 253–273.

Wu J-Q, Pollard TD (2005). Counting cytokinesis proteins globally and locally in fission yeast. Science 310, 310–314.

Wu J-Q, Sirotnik V, Kovar DR, Lord M, Beltzner CC, Kuhn JR, Pollard TD (2006). Assembly of the cytokinetic contractile ring from a broad band of nodes in fission yeast. J Cell Biol 174, 391–402.

Wu J-Q, Ye Y, Wang N, Pollard TD, Pringle JR (2010). Cooperation between the septins and the actomyosin ring and role of a cell-integrity pathway during cell division in fission yeast. Genetics 186, 897–915.

Wu P, Zhao R, Ye Y, Wu J-Q (2011). Roles of the DYRK kinase Pom2 in cytokinesis, mitochondrial morphology, and sporulation in fission yeast. PLoS One 6, e28000.

Ye Y, Lee I-J, Runge KW, Wu J-Q (2012). Roles of putative Rho-GEF Gef2 in division-site positioning and contractile-ring function in fission yeast cytokinesis. Mol Biol Cell 23, 1181–1195.

Yoshida S, Kono K, Lowery DM, Bartolini S, Yaffe MB, Ohya Y, Pellman D (2006). Polo like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. Science 313, 108–111.

Yuce O, Piekny A, Glotzer M (2005). An ECT2-centralspindlin complex regulates the localization and function of RhoA. J Cell Biol 170, 571–582.