Assembly and architecture of precursor nodes during fission yeast cytokinesis

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The contractile ring is essential for cytokinesis in most fungal and animal cells. In fission yeast, cytokinesis nodes are precursors of the contractile ring and mark the future cleavage site. However, their assembly and architecture have not been well described. We found that nodes are assembled stoichiometrically in a hierarchical order with two modules linked by the positional marker anillin Mid1. Mid1 first recruits Cdc4 and IQGAP Rng2 to form module I. Rng2 subsequently recruits the myosin-II subunits Myo2 and Rlc1. Mid1 then independently recruits the F-BAR protein Cdc15 to form module II. Mid1, Rng2, Cdc4, and Cdc15 are stable node components that accumulate close to the plasma membrane. Both modules recruit the formin Cdc12 to nucleate actin filaments. Myo2 heads point into the cell interior, where they efficiently capture actin filaments to condense nodes into the contractile ring. Collectively, our work characterizing the assembly and architecture of precursor nodes defines important steps and molecular players for contractile ring assembly.

Introduction

Cytokinesis requires coordination of cleavage site selection, assembly and constriction of a contractile ring, and targeted membrane fusion to partition a mother cell into two daughter cells (Balasubramanian et al., 2004; Barr and Gruneberg, 2007; Pollard and Wu, 2010). In animal cells, the spindle midzone, astral microtubules, or both specify where to assemble the contractile ring (Bringmann and Hyman, 2005; Barr and Gruneberg, 2007). In Saccharomyces cerevisiae, the division site is determined by the bud site selection machinery (Balasubramanian et al., 2004). In Schizosaccharomyces pombe, the anillin-like protein Mid1 provides a positional cue for cleavage site selection (Sohrmann et al., 1996). Subsequently, the contractile ring is assembled at the specified site. Contractile rings are essential for force production and for guiding membrane fusion at the cleavage site in most fungi, amoebas, and animal cells (Hales et al., 1999; Pollard and Wu, 2010). However, molecular mechanisms of the coordination of cytokinesis stages remain elusive.

The fission yeast S. pombe is a favorite model for the study of cleavage site selection and contractile ring assembly (Mishra and Oliferenko, 2008; Roberts-Galbraith and Gould, 2008; Bathe and Chang, 2010). In S. pombe, the current model of cytokinesis proposes cytokinesis nodes as precursors of the contractile ring (Wu et al., 2006; Vavylonis et al., 2008). Although interphase nodes are essential for coordination of cell size control and the cell cycle (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009), deletion of the cdr2 kinase, the organizer of interphase nodes, does not obviously affect the cytokinesis nodes and contractile ring formation, suggesting that these nodes can assemble independently (Almonacid et al., 2009; Moseley et al., 2009).

Cytokinesis nodes contain at least seven conserved proteins that assemble independently of actin filaments: Mid1, myosin-II heavy chain Myo2, essential light chain Cdc4, regulatory light chain Rlc1, formin Cdc12, IQGAP Rng2, and F-BAR protein Cdc15 (Pollard and Wu, 2010). Except for Mid1 and Rlc1, these proteins are essential for contractile ring assembly and/or maturation. Mid1 was reported to recruit Myo2 to nodes by interacting with the Myo2 tail (Motegi et al., 2004). Myosin-II is a hexamer composed of Myo2, Cdc4, and Rlc1, which produces a force to condense nodes into a contractile ring as well as to constrict the ring (Lord and Pollard, 2004; Vavylonis et al., 2008).
Myo2 directly interacts with Cdc4 and Rlc1 through its two IQ domains at the neck region (Motegi et al., 2000; Naqvi et al., 2000), which connects an N-terminal ATPase motor domain and an α-helical tail. Formin Cdc12 nucleates linear actin filaments for the contractile ring (Kovar et al., 2003; Coffman et al., 2009). IQGAP Rng2 is essential for bundling and arranging actin filaments into the contractile ring (Takai et al., 2009). Rng2 also interacts with Cdc4 as revealed by immunoprecipitation (IP; D’souza et al., 2001), most likely via its multiple IQ domains. F-BAR protein Cdc15 interacts with formin Cdc12 (Carnahan and Gould, 2003) and is essential for ring maturation and the Mid1-independent ring assembly (Wu et al., 2003; Wachtler et al., 2006; Hachet and Simanis, 2008).

Mid1, the first protein to appear in cytokinesis nodes (Paoletti and Chang, 2000; Wu et al., 2003), is essential for division site specification, as loss of Mid1 results in the loss of cytokinesis nodes and, thus, randomly placed contractile rings (Sohrmann et al., 1996; Wu et al., 2006). However, it was poorly understood how Mid1 recruits other proteins to form functional cytokinesis nodes and a contractile ring. The relative simplicity of cytokinesis nodes makes them an ideal structure to determine interactions and architecture of these proteins, which will provide insights into the assembly and function of the more complex contractile ring.

Here, we study the Mid1-dependent node assembly during fission yeast cytokinesis. We systematically determine localization dependencies of node proteins, their molecular stoichiometry, protein dynamics in vivo, and their physical interactions. In addition, we have determined the node architecture at a nanometer resolution using single-molecule high resolution colocalization (SHREC). We find that cytokinesis nodes are assembled stoichiometrically in a hierarchical order through two Mid1-dependent modules. Both modules recruit formin Cdc12 to nucleate actin filaments. Moreover, the defined node architecture provides strong support for the proposed mechanism of contractile ring assembly.

**Results**

In this study, we define the separation of spindle pole bodies (SPBs) labeled with Sad1-CFP, which marks the initiation of mitosis, as time 0. We define cytokinesis nodes as discrete protein clusters (20–65) close to the equatorial plasma membrane during the G2/M transition.

**Cdc4 and IQGAP Rng2 localize to cytokinesis nodes independently of Myo2**

To investigate the assembly of cytokinesis nodes, we first tested whether the myosin-II heavy chain Myo2 recruits its light chains Rlc1 and Cdc4 and IQGAP Rng2 to the nodes through its two IQ domains. Deletion of the IQ domains prevents the interactions between Myo2 and its light chains (Naqvi et al., 2000; D’souza et al., 2001). In wild-type (wt) cells at 25°C, a broad band of Rlc1, Cdc4, and Rng2 nodes appeared at the cell equator at similar times (Fig. 1 A, left). In myo2-IQ1ΔIQ2Δ cells with both IQ domains deleted, Cdc4 and Rng2 nodes still appeared with normal timing (Fig. 1 A, right). The contractile ring also formed normally as previously reported (Naqvi et al., 2000). In contrast, no Rlc1 nodes were observed, and Rlc1 localization to the ring was significantly delayed. In this background, Rlc1 ring localization depends on Myp2 (Naqvi et al., 2000; Wu et al., 2003). Thus, Rlc1, but not Cdc4 or Rng2, depends on the IQ domains of Myo2 for localization to cytokinesis nodes.

To find whether other domains of Myo2 are required for Cdc4 and Rng2 localization to cytokinesis nodes, we examined protein localization in myo2Δ cells (see Materials and methods). The maternal contribution of Myo2 was negligible because we observed no monomeric YFP (mYFP)-Myo2 signal in myo2Δ cells at any cell cycle stage, and Rlc1 localized to a clump instead of to nodes or a contractile ring (Fig. S1, A and B). In contrast, Cdc4 and Rng2 nodes were observed in myo2Δ cells (Fig. 1 B and Table I). However, ~10 min after their appearance, the nodes began to clump. These results were confirmed using an N-degron-myo2 strain (Fig. S1, C and D). In summary, Myo2 is critical for node condensation into a contractile ring and for Rlc1 node localization. However, it is not required for Cdc4 and Rng2 localization to cytokinesis nodes.

**Myo2 node localization depends on both Cdc4 and Rng2**

We next investigated whether Myo2 depends on Rlc1, Cdc4, or Rng2 for localization to cytokinesis nodes. Rlc1 deletion did not affect node localization of Myo2 or other node proteins (Fig. S1 E). In contrast, Cdc4 and Rng2 were required for Myo2 localization to nodes. In wt cells at 36°C, Myo2 appeared in nodes at ~6.1 ± 1.9 min (n = 30 cells; Fig. 1 C). Although the protein level of Myo2 was not obviously affected by the cdc4-8 or rng2-D5 mutation (see Fig. 5 B and Fig. S2 A, middle), no Myo2 accumulated in nodes in temperature-sensitive cdc4-8, rng2-D5, or rng2-346 mutants (n > 35 cells for each) at 36°C nor in cdc4Δ- and rng2Δ-null mutants at 25°C (Fig. 1, C and D; and not depicted). In cdc4-8 cells at 5–7 min, Myo2 appeared as faint structures (Fig. 1 C) that either aggregated into clumps or disappeared. We observed no Myo2 structures after latrunculin A (Lat-A) treatment (Fig. S2 B), indicating that these structures are actin dependent, unlike nodes (Wu et al., 2006). In rng2 mutants, all cells formed a clump at 6–7 min (Fig. 1, C and D). The same genetic dependency was found for Rlc1 (Fig. S2 D and not depicted). Together, these results indicate that Myo2 and Rlc1 node localization depends on both Rng2 and Cdc4.

**Cdc4 and Rng2 are interdependent for node localization, and both affect Mid1 levels in cytokinesis nodes**

To better understand how Myo2 is recruited to cytokinesis nodes, we investigated whether the interaction between Cdc4 and Myo2 is required for Myo2 node localization. Cells expressing mYFP-Myo2-IQ1ΔIQ2Δ formed nodes at ~4.1 ± 2.1 min (n = 63 cells) and normal contractile rings at 36°C, similar to wt mYFP-Myo2 (~3.9 ± 1.7 min; n = 29; Fig. 2 A). Because Myo2-IQ1ΔIQ2Δ cannot interact with Cdc4 (D’souza et al., 2001), this result suggests that Myo2 can localize to cytokinesis nodes independently of Cdc4, which seems to conflict with our
It has been suggested that Mid1 recruits Myo2 to nodes (Motegi et al., 2004). One explanation for our data, therefore, could be that cdc4 and rng2 mutations affect Mid1 localization in nodes so that Myo2 fails to localize properly. In cells findings that Myo2 failed to localize to nodes in cdc4-8 and cdc4Δ mutants (Fig. 1, C and D). Thus, we hypothesized that Cdc4 recruits a protein that in turn helps recruit Myo2 to cytokinesis nodes.

Figure 1. Myo2 localization to cytokinesis nodes depends on both Cdc4 and Rng2. (A) Rlc1, but not Cdc4 and Rng2, depends on IQ domains of Myo2 for node localization. Time courses of Rlc1-mYFP, mYFP-Cdc4, and mYFP-Rng2 localization in asynchronous wild type (WT) and the myo2-IQ1ΔIQ2Δ mutant at 25°C (n > 20 cells for each). (B) Localization of Cdc4 (n = 11 cells) and Rng2 (n = 16) in myo2Δ after growing for 24 h at 25°C. (C) Time courses of the localization of Myo2 in temperature-sensitive cdc4 and rng2 mutants (n > 35 cells for each) at the restrictive temperature 36°C (see Materials and methods). (D) Time courses of the localization of Myo2 in cdc4Δ and rng2Δ (n > 10 cells for each) after growing for 12 h at 25°C. The cell boundary is marked with dashed lines; elapsed times on micrographs are in minutes; arrows indicate node appearance; the SPB separation (Sad1-CFP, red) is defined as time 0; the time 0 on micrographs without Sad1-CFP is relative. Bars, 2 µm.
from germinated wt, cdc4Δ, and rng2Δ spores, Mid1 localized in the nucleus during interphase and then exited from the nucleus and formed nodes (Fig. 2 B). Mid1 stayed in nodes for ~30 min in the deletion strains before moving back to the daughter nuclei without forming a contractile ring or clumps. Mid1 localized in nodes with ~25% lower fluorescence intensity in cdc4-8 cdr2Δ than in cdr2Δ, although the Mid1 concentration in cells was the same (Fig. 2, C and D). Similar reduction was observed in rng2-D5, cdc4Δ, and rng2Δ cells. Thus, our data suggest that Cdc4 and Rng2 affect Mid1 accumulation to nodes. However, the partial reduction in Mid1 level alone cannot explain the complete lack of Myo2 nodes in cdc4Δ and rng2Δ mutants.

Rng2 is required for Myo2 node localization (Fig. 1, C and D) and interacts with Cdc4 through its multiple IQ domains (D’souza et al., 2001). To ask whether Myo2 could depend on Rng2 in cdc4Δ mutants, we investigated Rng2 localization in cdc4-8 (Fig. 2 E) and in cdc4Δ cells. At 36°C, no Rng2 nodes, clumps, or rings were observed in dividing cells (n = 20 cells), although Rng2 protein level was not affected by the cdc4-8 mutation (Fig. S2 A, left). The same results were obtained in cdc4Δ cells at 25°C (unpublished data). Next, we investigated the dependency of Cdc4 localization on Rng2. Similar to Myo2 and Rlc1, Cdc4 formed no nodes or rings but formed a clump in rng2-D5, rng2-346, or rng2Δ cells (Fig. 2 F and not depicted), whereas Cdc4 protein level was not obviously affected in rng2-D5 (Fig. S2 A, right). Collectively, these results suggest that Cdc4 and Rng2 are interdependent for node localization and help clarify how Myo2 accumulation depends on the presence of Cdc4.

Table I. Genetic dependencies of protein localization to cytokinesis nodes

<table>
<thead>
<tr>
<th>Node protein</th>
<th>wt)a</th>
<th>No F-actin)b,c</th>
<th>mid1-d</th>
<th>rng2</th>
<th>cdc4-e</th>
<th>myo2-f</th>
<th>rlc1-g</th>
<th>cdc15-h</th>
<th>cdc12-i</th>
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<tr>
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<td>+ +</td>
<td>+ -</td>
<td>+ -</td>
<td>-</td>
<td>+ -</td>
<td>ND</td>
<td>ND</td>
<td>+ -</td>
<td>+ -</td>
</tr>
<tr>
<td>Rng2</td>
<td>+ +</td>
<td>+ -</td>
<td>- -</td>
<td>-</td>
<td>- +</td>
<td>+ -</td>
<td>ND</td>
<td>+ -</td>
<td>+ -</td>
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<tr>
<td>Cdc4</td>
<td>+ +</td>
<td>+ -</td>
<td>- +</td>
<td>+</td>
<td>+ -</td>
<td>ND</td>
<td>ND</td>
<td>+ -</td>
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<tr>
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<td>+ +</td>
<td>+ -</td>
<td>- +</td>
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<td>- +</td>
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<tr>
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<td>- +</td>
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<td>- +</td>
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<td>ND</td>
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<tr>
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<td>+ +</td>
<td>ND</td>
<td>ND</td>
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</table>

Localization under test conditions. Data is given as node, ring, and clump, respectively. Except for rlc1, all null and temperature-sensitive mutants were tested, and the same results were obtained. Italic +/- indicates the genetic dependencies tested in this study; non-italic +/- indicates the published genetic results not tested in this study. Because our experiments focus on the node assembly, the published genetic dependencies on ring and clump formation were not cited here.

aWu et al., 2006.
bWu et al., 2003.
cLahA treatment.
dIn the absence of Mid1, cytokinesis nodes do not form. After a delay, cells usually assemble a contractile ring that is generally mislocalized and constricts slowly (Wu et al., 2003; Hachet and Simanis, 2008; Huang et al., 2008).
eSahrmann et al., 1996; Böhler et al., 1998a.
gMotegi et al., 2000: no Myo2 signal was detected in the cdc4 temperature-sensitive strain. But in our study, we show that Myo2 localizes as a faint disorganized structure.
hMulvihill et al., 2001.
iLe Goff et al., 2000.
jHuang et al., 2008.
kNaqvi et al., 2000.
lHachet and Simanis, 2008.
mCoffman et al., 2009.

F-BAR protein Cdc15 depends on Mid1 for localization to cytokinesis nodes

We next addressed which node protein is required for Cdc15 localization to cytokinesis nodes. In wt cells at 36°C, Cdc15 localized to nodes at 0.9 ± 1.3 min (n = 21 cells). Surprisingly, all cdc4-8, rng2-35, and rng2-346 dividing cells displayed Cdc15 nodes. The nodes appeared later than in wt (3.7 ± 2 min; n = ~30 cells each) and aggregated into clumps instead of forming a contractile ring (Fig. S2 C and not depicted). Furthermore, Cdc15 still localized to cytokinesis nodes in cdc4Δ and rng2Δ cells (Fig. 3 A).

The delayed appearance of Cdc15 in nodes in cdc4Δ and rng2Δ mutants may result from the reduced Mid1 levels in nodes (Fig. 2, B–D). Indeed, we detected no Cdc15 nodes in mid1Δ cells, although Cdc15 still concentrated at endocytic patches and a mislocalized and tilted ring (Fig. 3 A). Conversely, the Mid1 level in nodes was not affected in cdc15 mutants. Collectively, these results suggest that Cdc15 localization to nodes depends only on Mid1.

Cdc4 and Rng2 node localization are independent of Cdc15

Cdc15 localization to cytokinesis nodes is independent of Cdc4 and Rng2 (Fig. 3 A). We explored whether Cdc15 plays a role in the accumulation or maintenance of Cdc4 and Rng2 to nodes using cdc15Δ mutants. We detected no mYFP-Cdc15 signals in cdc15Δ cells (Fig. S3 A), suggesting a negligible maternal contribution of Cdc15. Cdc4 and Rng2 nodes were observed in cdc15Δ cells (Fig. S3, B and C; and Table I). These nodes condensed slowly into an unstable ring that collapsed before its disappearance. It took
Formin Cdc12 is targeted to cytokinesis nodes through overlapping pathways

We next tested dependencies for Cdc12 localization to cytokinesis nodes. We found Cdc12 nodes in 
\[ \text{rng}2^{-} \], \[ \text{cdc}4^{-} \], and \[ \text{cdc}15^{-} \] cells (Fig. 3 B). However, the mean fluorescence intensity
\[ \sim 15 \text{ min} \] for Cdc4 node condensation into a ring in \[ \text{cdc}15^{-} \] cells 

\[ (n = 11) \] compared with \[ \sim 10 \text{ min} \] in wt cells \[ (n = 18) \]. In addition, Myo2 also localized to nodes normally in \[ \text{cdc}15^{-} \text{I40} \] cells at \[ 36^\circ \text{C} \] (Fig. S3 D). Thus, Cdc15 is not required for the accumulation or maintenance of Cdc4, Rng2, or Myo2 in cytokinesis nodes.
Furthermore, Cdc12 nodes were not detected in rng2-D5 cdc15-140 cells at the semipermissive temperature of 30°C, whereas they were in the single mutants (n = 23 cells; Fig. 3 D). These data suggest that Cdc4–Rng2 and Cdc15 have an overlapping function in recruiting formin Cdc12 to cytokinesis nodes.

Figure 3. Genetic dependencies for node localization of Cdc15 and Cdc12 and node assembly pathways. (A) Cdc15 node localization depends on Mid1 but not on Cdc4 or Rng2. Time courses of Cdc15 localization in wt, cdc4Δ (n = 28 cells), mid1Δ, and rng2Δ (n = 17 cells) after 12 h at 25°C. (B) Cdc12 localizes to nodes in rng2Δ (n = 17 cells), cdc4Δ (n = 10), and cdc15Δ (n = 16) after growing for 12 h at 25°C. (C) Cdc12 partially colocalizes with Cdc15 in nodes. The boxed regions are enlarged (bottom), and blue arrows indicate Cdc12 nodes containing no Cdc15. (D) Time courses of Cdc12 localization in cdc15-140, rng2-D5, and rng2-D5 cdc15-140 mutants after 1 h at 30°C. (E) The localization hierarchy for cytokinesis node assembly. The complete and partial dependencies of node localization on a specific protein are depicted by solid and dashed lines, respectively. The two modules are colored differently. The cell boundary is marked with dashed white lines; elapsed times on micrographs are in minutes; arrows indicate node appearance; the SPB separation (Sad1-CFP, red) is defined as time 0; the time 0 on micrographs without Sad1-CFP is relative. ELC, essential light chain. RLC, regulatory light chain. Bars, 2 µm.

of Cdc12-3YFP nodes was ~40% lower in each mutant than in wt cells (P < 10−4 each compared with wt). In addition, only 37% of Cdc12 nodes (or clusters of nodes) contained Cdc15 in wt cells (n = 350 Cdc12 nodes; Fig. 3 C), which indicates that Cdc12 can localize or be maintained in nodes without Cdc15.

Furthermore, Cdc12 nodes were not detected in rng2-D5 cdc15-140 cells at the semipermissive temperature of 30°C, whereas they were in the single mutants (n = 23 cells; Fig. 3 D). These data suggest that Cdc4–Rng2 and Cdc15 have an overlapping function in recruiting formin Cdc12 to cytokinesis nodes.
On the other hand, Rng2, Myo2, Cdc15, and Mid1 localized to nodes, but not clumps, in cdc12Δ cells (Fig. S3, E–H; and Table I), suggesting that Cdc12 is downstream of other node proteins in the localization hierarchy and is required for clump formation.

We hypothesized that the clumping observed in rng2-D5 mutants may represent failed ring formation as predicted by the search, capture, pull, and release (SCPR) model (Vaylonis et al., 2008). Consistent with this hypothesis, Cdc15, Rlc1, and Cdc12 colocalized with Myo2 in clumps in rng2-D5 cells (Fig. S2, C–E). Furthermore, clump formation and stability depended on actin filaments, as revealed by Lat-A treatment (Table I).

In summary of the localization dependencies, we proposed two modules through which cytokinesis nodes are assembled hierarchically (Fig. 3 E). Module I consists of Mid1 recruiting Cdc4 and Rng2, which subsequently recruits Myo2 and Rlc1. Cdc4 and Rng2 provide a positive feedback on Mid1 recruitment. Module II assembles independently and consists of Mid1 recruiting Cdc15. Both modules are involved in recruiting formin Cdc12.

**Temporal pathway and stoichiometry of node assembly**

We hypothesized that proteins upstream in our localization hierarchy (Fig. 3 E) should appear earlier in the nodes. Thus, we quantified the timing of node appearance and absolute protein levels of the seven node proteins (see Materials and methods) with high resolution (Fig. 4 A and Table II). Consistent with its scaffolding role, interphase nodes each accumulated ~17 Mid1 molecules well before cytokinesis nodes appeared as indicated by the first plateau (from greater than −15 to −12.5 min; Fig. 4 A). At −10 min, Mid1 levels reached a second plateau of 28 molecules per cytokinesis node.

Supporting the localization hierarchy, we observed that Cdc4 and Rng2 appeared in nodes earlier than Myo2 and Rlc1. The accumulation of Cdc4 and Rng2 was biphasic, and that of Myo2–Rlc1 was monophasic. Cdc4 and Rng2 nodes appeared with similar timing at −11.9 ± 1.4 min and −12.5 ± 1.1 min, respectively (P = 0.9; Fig. 4 A, arrows). The numbers of molecules in each node (134 for Cdc4 and 9 for Rng2) remained constant until −5 min, defining the first plateau. The molecule numbers then increased to 286 and 28, respectively, reaching the second plateau at SPB separation (time 0; Fig. 4 A).

Myo2 and Rlc1 displayed almost constant molecular levels (55 for Myo2 and 41 for Rlc1) after the last node formed (Fig. 4 A). These two proteins appeared in nodes later than Cdc4–Rng2, at −10.2 ± 2.5 min and −10.2 ± 1.8 min, respectively (Fig. 4 A, arrow). These times are statistically different from those measured for Cdc4 and Rng2 (P < 0.05, comparing the timing of Cdc4 or Rng2 to Myo2 or Rlc1). Consistent with the timing, >50% of Cdc4 nodes had no Rlc1 during node appearance (Fig. 4, B and C). In contrast, Myo2 and Rlc1 always colocalized in nodes regardless of the number of nodes at the cell equator (Fig. 4, B and C). These results suggest that Rng2 and Cdc4 are the first proteins localized to cytokinesis nodes after Mid1. Cdc15 appeared in nodes later than module I at −4.9 min with 36 molecules at the plateau, and Cdc12 appeared last in nodes at 1 min with approximately six to eight molecules in each node (Fig. 4 A), which is consistent with the localization hierarchy (Fig. 3 E).

**FRAP analysis indicates that node proteins display different dynamics**

Scaffolding proteins are usually more stable components of protein complexes. Many proteins are known to be very dynamic in contractile rings (Pelham and Chang, 2002; Clifford et al., 2008; Roberts-Galbraith et al., 2009), but little is known about their stabilities within cytokinesis nodes. We used FRAP to test the proposed pathways for node assembly (Fig. 3 E). Mid1 recovered slowly in nodes after photobleaching with a half-time of 3.03 min (Fig. 4 D and Table II), which is consistent with its scaffolding function.

The recovery of Cdc4 in nodes after photobleaching was biphasic, with a fast (t1/2 = 0.20 min) and a slow (1.45 min) recovery phase (Fig. 4 E). The fast phase depended on Cdc4’s interaction with Myo2, as it was lost in the myo2-IQ1IQ2Δ strain (t1/2 = 1.39 min; Fig. 4, E and G). In addition, Rng2 was not very dynamic in nodes, with a recovery half-time of 1.77 min in wt and 1.39 min in the myo2-IQ1IQ2Δ mutant; the latter is identical to the slow phase of Cdc4 (Fig. 4, F and G). In contrast, Myo2 and Rlc1 were highly dynamic in the nodes with a similar recovery half-time (0.51 min) and extent of recovery (Fig. 4 H and Table II).

Recovery of Cdc15 in nodes after photobleaching was biphasic, with a fast (t1/2 = 0.07 min) and a slow (t1/2 = 1.10 min) phase (Fig. 4 I). Previously, we have shown that Cdc12 is dynamic in nodes (t1/2 = 0.5 min; Coffman et al., 2009). In summary, the FRAP experiments suggest that Mid1, Cdc4, Rng2, and Cdc15 are scaffolding proteins that recruit more dynamic components Myo2, Rlc1, and Cdc12 to the nodes, which is consistent with the localization hierarchy (Fig. 3 E).

**Physical interactions among cytokinesis node proteins**

To further test the node-assembly pathway (Fig. 3 E), we investigated some of the key physical interactions among the node proteins. Mid1 coimmunoprecipitated with both Cdc4 and Rng2 (Fig. 5 A) from lysates of asynchronous cultures. Reciprocal co-IPs confirmed the interactions (unpublished data). In contrast, co-IP between Myo2 and Mid1 was not as efficient, even though more Myo2 than Rng2 was present (Fig. 5 A). As a negative control, Mid1 did not coimmunoprecipitate with Rlc1 under the same conditions (unpublished data). Interestingly, Mid1 did not coimmunoprecipitate with Myo2 in extracts from rng2-D5 cells grown at 36°C (Fig. 5 B). This suggests that the physical interaction between Myo2 and Mid1 depends on Rng2 in nodes and/or in the contractile ring. Indeed, Myo2 coimmunoprecipitated with both Rng2 and Cdc4 (Fig. 5 C). Actin filaments were not involved in the Myo2–Mid1 and Myo2–Rng2 physical interactions we observed because we detected no actin after IP (Fig. S2 F and not depicted).

Previous data (D’Souza et al., 2001) and ours (Figs. 2, E and F; and 4, A and E–G) suggest that Cdc4 and Rng2 may directly interact with each other. Indeed, they interacted in yeast two-hybrid assays, and the interaction was lost in budding yeast cells expressing Cdc4-8 at restrictive temperatures (Fig. 5 D).
Based on the localization hierarchy (Fig. 3 E), we proposed that Mid1 directly recruits Cdc15 to cytokinesis nodes. Consistent with this hypothesis, Cdc15 interacted with itself and with Mid1 in yeast two-hybrid assays (Fig. 5 E). Moreover, Mid1 coimmunoprecipitated with Cdc15 (Fig. 5 F). Together, the aforementioned data suggest that Mid1 may directly recruit Cdc4–Rng2 and Cdc15 to cytokinesis nodes, and Rng2 recruits Myo2 or stabilizes the Myo2–Mid1 interaction in nodes.
Architecture of cytokinesis nodes determined by SHREC

Our experiments of node architecture provide further support for the node-assembly pathways (Fig. 3 E). Cytokinesis nodes are separated by 558 ± 138 nm before node condensation (n = 40 distances measured between centroids of different Rlc1 nodes). To further investigate node assembly, we used SHREC (Churchman et al., 2005; Joglekar et al., 2009) to determine the relative positions and distances of the seven node proteins within the same nodes. The imperfect alignment of two laser channels introduces an offset (Fig. S4 A), but our method has an accuracy of 10 nm after offset correction (Fig. S4, B and C). Because of the difficulties in measuring small distances using the maximum likelihood method, the longer distances we measured might be more accurate than the shorter ones (see Materials and methods). We measured two distances at right angles to the cell’s long axis, the axis of Rlc1-Mid1 is at 71° to the plasma membrane (Celton-Morizur et al., 2004). If we assume the plasma membrane is parallel to the SPB separation, which is defined as time 0. The timing for Mid1 represents the timing that the Mid1 level reaches the cytokinesis node plateau. NA, not applicable.

Table II. Node protein dynamics revealed by FRAP and numbers of molecules in nodes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Half-time, fast/slow phase (number of cells)</th>
<th>Recovery, fast/slow</th>
<th>Molecules (polypeptides) per node, first/second plateau (number of nodes)</th>
<th>Timing of node appearance (number of nodes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>%</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>mid1-mYFP</td>
<td>ND</td>
<td>ND</td>
<td>17.1 ± 7.4 (4)/27.7 ± 10 (23)</td>
<td>−11.2 ± 1.1 (9)</td>
</tr>
<tr>
<td>mid1-mECitrine</td>
<td>3.03 ± 1.32 (10)</td>
<td>68.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>mid1-mECitrine cdr2Δ</td>
<td>1.97 ± 0.77 (10)</td>
<td>57.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>mYFP-rng2</td>
<td>1.77 ± 1.07 (14)</td>
<td>75.4</td>
<td>9.4 ± 5.6 (25)/28.0 ± 16.9 (25)</td>
<td>−12.5 ± 1.1 (8)</td>
</tr>
<tr>
<td>mYFP-rng2 myo2-IQ1Δ/Q2Δ</td>
<td>1.39 ± 0.82 (10)</td>
<td>56.7</td>
<td>10.3 ± 3.4 (16)/30.3 ± 9.4 (16)a</td>
<td>−12.8 ± 1.0 (16)a</td>
</tr>
<tr>
<td>mYFP-rng2 cdr2Δ</td>
<td>1.29 ± 0.69 (14)</td>
<td>57.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>mYFP-cdc4</td>
<td>0.20 ± 0.14 (11)</td>
<td>21.1/22.3</td>
<td>134.1 ± 93.9 (29)/286.3 ± 130.4 (29)</td>
<td>−11.9 ± 1.4 (11)</td>
</tr>
<tr>
<td>mYFP-cdc4 myo2-IQ1Δ/Q2Δ</td>
<td>NA/1.39 ± 0.89 (24)</td>
<td>NA/53.4</td>
<td>107.5 ± 59.7 (16)/265.0 ± 90.1 (16)a</td>
<td>−11.4 ± 1.5 (16)a</td>
</tr>
<tr>
<td>mYFP-myo2</td>
<td>0.51 ± 0.22 (14)</td>
<td>59.5</td>
<td>55.3 ± 32.8 (31)</td>
<td>−10.2 ± 2.5 (9)</td>
</tr>
<tr>
<td>mYFP-myo2-IQ1Δ/Q2Δ</td>
<td>0.48 ± 0.31 (18)</td>
<td>71.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>mYFP-myo2 cdr2Δ</td>
<td>0.52 ± 0.22 (11)</td>
<td>79.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rlc1-mYFP</td>
<td>0.51 ± 0.22 (18)</td>
<td>61.9</td>
<td>41.3 ± 23 (31)</td>
<td>−10.2 ± 1.8 (14)</td>
</tr>
<tr>
<td>mYFP-cdc15</td>
<td>0.07 ± 0.02/1.10 ± 0.67 (15)</td>
<td>57.3/27.3</td>
<td>36.3 ± 16.9 (21)/−4.9 ± 0.6 (12)</td>
<td></td>
</tr>
<tr>
<td>mYFP-cdc15 cdr2Δ</td>
<td>0.08 ± 0.04/0.83 ± 0.62 (15)</td>
<td>50.9/49.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>cdc12-3YFP</td>
<td>0.50 ± 0.17 (11)</td>
<td>69.3</td>
<td>6.9 ± 2.9 (16)</td>
<td>1.0 ± 0.6 (16)</td>
</tr>
</tbody>
</table>

Timing of node appearance is relative to the SPB separation, which is defined as time 0. The timing for Mid1 represents the timing that the Mid1 level reaches the cytokinesis node plateau. NA, not applicable.

Table II (continued)

*Not statistically different from wt.

Coffman et al., 2009.

Table II. Node protein dynamics revealed by FRAP and numbers of molecules in nodes

Cytokinesis node assembly and architecture • Laporte et al. 1013

Published March 21, 2011
Cytokinesis nodes are proposed to be precursors of the contractile ring in fission yeast (Wu et al., 2006; Vavylonis et al., 2008). They are essential for efficient ring assembly, which involves actin filaments and >30 proteins, at the correct cleavage plane (Coffman et al., 2009; Pollard and Wu, 2010). However, little was known about the assembly and architecture of nodes. In animal cells, anillin and myosin-II are enriched in node-like foci (Noguchi et al., 2001; Straight et al., 2003; Werner et al., 2007; Zhou and Wang, 2008). In addition, cytokinesis nodes also resemble the contractile unit of the contractile ring in Caenorhabditis elegans (Carvalho et al., 2009). Thus, studying the assembly and architecture of the relatively simple cytokinesis nodes will help us to decipher the much more complex contractile ring during cytokinesis.

Here, we find that cytokinesis nodes are assembled hierarchically, with two modules linked by the anillin-like protein Mid1 in fission yeast (Fig. 3 E). Module I consists of Mid1 recruiting myosin-II essential light chain Cdc4 and IQGAP Rng2, which subsequently binds myosin-II heavy chain Myo2 and regulatory light chain Rlc1. Module II assembles independently at a later stage and consists of Mid1 recruiting F-BAR protein Cdc15. The two modules allow assembly of a compact contractile ring by recruiting actin-nucleating formin Cdc12. (compare Fig. 6, E and F), which is consistent with the distances measured for other myosin-IIs (Shih et al., 2000; Xiao et al., 2003).

Next, we determined that the 2D distance between Myo2-GFP and Rlc1-tdTomato is 42 nm, with the Myo2 C terminus closer to the plasma membrane (Fig. 6 G). The 2D distance between Rng2 and Rlc1 is 47 nm (Fig. 6 H), which suggests that Rng2 is close to the Myo2 tail. The 2D distance between Cdc4 and Rlc1 is 53 nm (Fig. S4 G), which places Cdc4 close to Rng2. Given that Rng2 has ≤11 IQ domains (Wang et al., 2004) and a vast excess of Cdc4 in nodes (Table II), we assume that our measurements mainly reflect the Cdc4 fraction that binds to Rng2. Lastly, the 2D distance from Rlc1 to Cdc12 is 63 nm, which places Mid1, Cdc15, and Cdc12 very close to each other in nodes (Figs. 6 J and S4 H). Together, the node architecture is consistent with the protein interactions we discovered and further validates the node assembly pathways (Fig. 3 E).

Lastly, we determined the location of actin filaments relative to the orientation of Myo2 heads by measuring the angles of actin filaments from Cdc12 nodes at the cell sides. Actin filaments/bundles anchored by Cdc12 nodes display a small angle of 8.2 ± 8.0° (n = 44 filaments) to the cell’s long axis away from the plasma membrane (Fig. 6 I). Given the mean distance between nodes, this small angle suggests that actin filaments could be readily captured by Myo2 heads (Fig. 6 J and see Discussion).
Figure 6. **Node architecture revealed by the distances between node proteins.** (A) Diagrams of cell sides and cell top and bottom used to measure distances between two proteins tagged with GFP (green dot) and tdTomato (red dot). Distances measured at cell sides are depicted as D and at cell top and bottom as d. After fitting these distances with the non-Gaussian equation, the predicted distances D and d between the two fluorophores were used to calculate the 2D distance using the Pythagorean theorem. (B–H) Non-Gaussian probability distribution fits for protein distance measurements in nodes. Except for GFP-Psy1/Rlc1-tdTomato, each panel contains two graphs representing the histograms of measurements at the cell sides and at cell top and bottom, colored in orange and blue, respectively. The curves are the maximum likelihood fit. Dashed lines represent the distances predicted by maximum likelihood estimation. For each measurement, numbers of analyzed nodes are indicated. The orientation of the protein pair toward the plasma membrane is depicted at the bottom of the graphs. The distances predicted by maximum likelihood estimation (D and d) and the calculated 2D distance are shown along with the orientation. (I) Angles of the GFP–calponin homology domain-marked actin filaments anchored by Cdc12-tdTomato nodes to the long axis of the cell. Only filaments at cell sides were measured. n = 44. (J) Model for cytokinesis node assembly and architecture. Protein distances, stoichiometries (except Cdc12), and timings of appearance are depicted.
The anillin Mid1-Cdc4-IQGAP Rng2 module for cytokinesis node assembly

In module I, Mid1, Cdc4, and Rng2 are the stable components to initiate node assembly. Mid1 molecules bind to the plasma membrane as oligomers to form a platform for other proteins to bind (Celton-Morizur et al., 2004). Cdc4 and Rng2 display almost identical recovery rates after photobleaching and identical recruitment kinetics (Fig. 4), which suggest that Cdc4 and Rng2 form a subcomplex and may associate/disassociate from Mid1 together. Once assembled, the Mid1–Cdc4–Rng2 complex recruits dynamic proteins Myo2-Rlc1 and formin Cdc12 to nodes (Fig. 3 E). The dynamic nature of Myo2 and Cdc12 could contribute to contractile ring assembly, as transient interactions between nodes and actin filaments are required during node condensation into a contractile ring (Vavylonis et al., 2008). Rng2, Cdc4, and Cdc15 are more stable in nodes (t1/2 = 1.2–1.8 min) than in the contractile ring (t1/2 = 0.3–0.5 min; Pelham and Chang, 2002; Clifford et al., 2008; Roberts-Galbraith et al., 2009), which suggests that actin filaments and changes in binding partners in the contractile ring may affect their dynamics. In addition, departure of interphase node proteins from the division site may also make cytokinesis proteins more dynamic, as revealed by our FRAP results in cdr2Δ cells (Table II).

Mid1 localization is highly regulated to restrict the division site to the cell equator. Initially, Mid1 is mainly localized in the nucleus. Polo kinase is indicated to phosphorylate and release Mid1 from the nucleus to initiate cytokinesis node formation (Bähler et al., 1998a). Mid1 is maintained as a band of nodes at the cell equator by a balance of positive and negative signals (Celton-Morizur et al., 2006; Padte et al., 2006; Huang et al., 2007; Zhang et al., 2010). Here, we find a novel positive feedback regulation for Mid1 by Cdc4 and Rng2 during cytokinesis node formation (Figs. 2 and 3 E). Consistently, Cdc4 and Rng2 are recruited to nodes when Mid1 level increases to reach the second plateau (Fig. 4 A). Thus, both Mid1 phosphorylation by Polo kinase and binding with Cdc4–Rng2 are involved in doubling the levels of Mid1 in cytokinesis nodes (Fig. 4 A).

In mammalian cells, a positive feedback loop also reinforces the localization of anillin at the cleavage site (D’Avino, 2009).

Our finding that Myo2 node localization depends on Rng2 is surprising because it has been proposed that Mid1 recruits Myo2 by binding to the last 133 amino acids of the Myo2 tail, but the tail fragment was overexpressed in the study (Motegi et al., 2004). We did observe a faint band in Myo2–Mid1 IPs when both proteins are expressed at their native levels, which suggests a weak interaction. However, the interaction is not sufficient for Myo2 localization to nodes without Rng2 (Figs. 1, 2, and 5). The localization dependencies and physical interaction between Rng2 and Myo2 suggest that Rng2 recruits Myo2 to nodes or stabilizes the interaction between Myo2 and Mid1.

We find that Rng2, Cdc4, Myo2 tail, and Cdc12 are all close to Mid1 and the plasma membrane, which is consistent with their physical interactions (Figs. 5, 6, and S4). The Myo2 head is 80 nm from Mid1. Interestingly, anillin and myosin-II also do not colocalize perfectly but display a periodic pattern in HeLa cells, suggesting that this architecture may be conserved in animal cells (Straight et al., 2005). Recombinant Myo2 tails are 87 nm long, and sequence analyses suggest that the tail is folded back (Bezanilla and Pollard, 2000; Motegi et al., 2004), which is consistent with our measurements.

Analysis of node motion and Monte Carlo simulations supports a SCPR model for contractile ring assembly from cytokinesis nodes (Vavylonis et al., 2008). The SCPR model assumed that myosin motors in one node capture and pull actin filaments growing from other nodes. Direct observations and simulations support this assumption. Our data show that actin filaments nucleated by Cdc12 are mostly near the plasma membrane with a small tilt of ≤8° away from the membrane (Fig. 6 I). Cortical endoplasmic reticulum tubules restrict node movement (Zhang et al., 2010), so they might also confine actin filaments close to the plasma membrane. The distances between nodes are ~560 nm. The barbed ends of actin filaments are ~80 nm from the Myo2 head in the same node, and the Myo2 tail is oriented at ~70° to the plasma membrane. This configuration enables Myo2 heads to capture the pointed ends of actin filaments from neighboring nodes very efficiently (Fig. 6 J). Thus, node architecture is consistent with the SCPR model (Vavylonis et al., 2008). In addition, the distances between Myo2 and other node proteins suggest that the two-headed myosin-II might not be assembled into bipolar filaments (Fig. 6 J). Further studies are needed to determine whether Myo2 assembles and/or aligns into minifilaments during ring maturation and constriction in fission yeast as myosin-II in other systems (Sinard et al., 1989; Mahajan and Pardee, 1996).

The anillin Mid1 and F-BAR protein Cdc15 module for cytokinesis node assembly

Cdc15 appears in nodes later than the proteins in module I but before node condensation. In FRAP assays, Cdc15 exhibits biphasic recovery, with a slow phase (t1/2 = 1.1 min), supporting its role as a scaffolding protein in nodes to recruit formin Cdc12 by physical interaction (Carnahan and Gould, 2003). The N terminus of Cdc15 lies very close to Mid1, consistent with the physical interactions between Cdc15 and Mid1 (Fig. 5, E and F) as well as Cdc15 and the plasma membrane (Takeda et al., 2004). Although Cdc15 is not required for node and contractile ring assembly, node condensation into a ring is slower, and the ring collapses before constriction without Cdc15 (Wachtler et al., 2006; Hachet and Simanis, 2008). Our data confirms that module I is sufficient to assemble a compact contractile ring.

When module I functions normally, Cdc15 is only required for the maturation of the compact ring during anaphase B (Pollard and Wu, 2010).

The interactions between modules I and II and their downstream proteins

The two modules for node assembly can cooperate with each other for successful assembly of the contractile ring in at least four ways: (1) Cdc4 and Rng2 provide a positive feedback for Mid1 recruitment, and Mid1 in turn is essential for the assembly of both modules; (2) both modules are involved in recruiting Cdc12 to nodes; (3) actin filaments nucleated by Cdc12 can recruit and/or bind to Rng2 (Takai et al., 2009), Myo2, and other actin-binding proteins; (4) Cdc15 physically interacts with...
Rng2 (Roberts-Galbraith et al., 2010) and, thus, links the two modules together in the contractile ring.

The interactions between the two modules and their downstream proteins can explain the two overlapping pathways for contractile ring assembly in fission yeast: the Mid1–nodes pathway (Wu et al., 2006; Vavylonis et al., 2008) and the septation initiation network (SIN)–Cdc15 pathway (Wachtler et al., 2006; Hachet and Simanis, 2008). Without Mid1, cells cannot form nodes, and a randomly placed contractile ring is sometimes formed using the SIN–Cdc15 pathway. It takes much longer to form and constrict the misplaced ring (Huang et al., 2008). In mid1Δ cells, Cdc15, Cdc4, Rng2, Myo2, Cdc12, and the SIN pathway are all essential for formation of the misplaced contractile ring. Because Cdc15 can bind to the plasma membrane (Takeda et al., 2004), it might anchor or recruit other proteins to the plasma membrane in mid1 mutants. Then the myosin-II motor and formin Cdc12 could work together to form contractile filaments/bundles and/or a misplaced ring. In the absence of Mid1, Rng2 might be recruited to the plasma membrane by binding to Cdc15 (Roberts-Galbraith et al., 2010) or to the actin filaments nucleated by Cdc12 (Takaine et al., 2009). These multiplex protein interactions among the node proteins may explain the plasticity of contractile ring formation.

Contractile ring assembly in other model systems

Most proteins involved in contractile ring assembly, including the seven node proteins, are conserved during evolution. Stepwise recruitment for ring assembly was described in the seven node proteins, are conserved during evolution. Stepwise recruitment for ring assembly was described in model systems

Contractile ring assembly in other model systems

Most proteins involved in contractile ring assembly, including the seven node proteins, are conserved during evolution. Stepwise recruitment for ring assembly was described in model systems

**Materials and methods**

### Strains, growing conditions, and genetic and cellular methods

Table S1 lists the S. pombe strains used in this study. All tagged genes are under the control of endogenous promoters and integrated at their native chromosomal loci. Functionalities of newly tagged strains were tested by examining the growth and morphology at different temperatures and by crossing tagged strains with mutations [Table S1] known to have synthetic interactions with mutations in the tagged genes. Cells were grown in G1 exponential phase for 36–48 h before microscopy as previously described [Wu et al., 2006]. Most temperature-sensitive strains for microscopy were synchronized by growing exponential cultures with 20 mM hydroxyurea (Sigma-Aldrich) for 4 h at 25°C and then 1 h at 36°C. Cells were then washed twice with prewarmed 36°C YE5S medium, and the cell cycle was resumed at 36°C in YE5S for 1 h before imaging at 36°C.

Diploid strains with both copies of the gene for localization analysis tagged with YFP were constructed by standard genetic methods (Moreno et al., 1991). One copy of the essential genes from the diploid strains was deleted using the KSura4+ marker as previously described (Bähler et al., 1998b). Resulting heterozygous diploid strains were grown for 2 d on SPA5S plates to induce sporulation, and then tetrads were inoculated in 2% glusulase for 12 h at 25°C to kill the remaining diploid cells. After five washes in 1.7 g/liter of the rinsing medium (Yeast Nitrogen Base; BD), spores were germinated and grown in YE5S-ura liquid medium at 25°C for 12 or 24 h before imaging. Extra spores were kept at 4°C for repeating experiments. Cells from germinated wt spores were used as controls. The null mutant cells were identified by the failure in contractile ring formation and cytokinesis in time-lapse videos. Because fusion yeast diploid cells are not stable for long-term storage, only the parental strains are listed in Table S1.

We constructed the N-degron-myo2 strain as previously described (Rajagopalan et al., 2004) under the control of the myo2 promoter. The N-degron-HA fragment was cloned into the pFA6a plasmid (IQW246). The fragment kanMX6-Pmyo2-N-degron-HA flanked with ~70-bp homology to myo2 was integrated at the myo2 locus. Positive clones were confirmed by PCR over both junctions.

### Microscopy and data analysis

Cells for microscopy were collected from liquid cultures, centrifuged at 5,000 rpm, and then washed into EM5S for imaging. Live-cell microscopy was performed using a thin layer of EMM5 liquid medium with 20% gelatin (Sigma-Aldrich) and 0.1 mM n-propyl-gallate and observed at 23–25°C as previously described [Wu et al., 2006; Coffman et al., 2009]. To observe cells at 36°C, cells were spun down for 30 s at 7,000 rpm and placed on EM5S + 2% agar pads prewarmed for 10 min at 36°C. All slides, coverslips, and cultures were kept at 36°C during preparations of samples to maintain cells at 36°C. An Objective Heater system (Bioptechs) was used to maintain the temperature at 36°C or other temperatures for microscopy of temperature-sensitive mutants. In general, fluorescence intensity was lower at 36°C than at 25°C, making it more difficult to observe nodes in some strains.

For imaging, we used a 100×/1.4 NA objective lens (Nikon) on a spinning-disk confocal microscope [UltraView ERS, PerkinElmer] with a 440-nm solid-state laser, 488-, 514-, and 568-nm argon ion lasers, and a cooled charge-coupled device camera (ORCA-AG; Hamamatsu) with a 2 × 2 binning or no binning [for distance measurement]. Maximum intensity projections of color images, grayscale montages, and other image analyses were performed using ImageJ [National Institutes of Health]. Images in figures are maximum intensity projections of 2 sections spaced at 0.2–0.8 μm except where noted. For genetic dependencies, the time intervals in time-lapse movies were as follows: 1 min for mYFP-Cdc4 and Rlc1-mYFP; 1.5–2 min for mYFP-Cdc15; and 2 min for Cdc12-3YFP, mYFP-Rng2, YFP-Myo2, and Mid1-monomeric enhanced Citrine [mCitrine].

### FRAP analysis

We used the Photokinesis unit on the confocal system [UltraView ERS] for all FRAP experiments [Coffman et al., 2009]. The FRAP data were gathered by first taking z sections to determine the optimum cell and plane for bleaching, which contained several nodes whose intensity appeared to have reached the plateau stage and had not started condensation. Then we collected five prebleach images, bleached several nodes in a single plane, and acquired 50 or 100 postbleach images appropriate to the timing of recovery. A region of interest [ROI] was selected at each site that was bleached >50% of the original signal. After subtracting the background and correcting for photobleaching during image acquisition at nonbleached sites [Vavylonis et al., 2008], intensity values at each ROI were

Cytokinesis node assembly and architecture • Laporte et al.

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normalized against the mean prebleach value, which was set to 100%. We averaged intensity values of postbleaching from three consecutive images for each ROI to reduce noise (Vayyolinos et al., 2008). The averaged value at each time point across all ROIs was plotted as the percentage of recovery with the bleaching time defined as time 0 and the fluorescence intensity after bleaching set to 0%.

Fit curves were obtained from the mean of all ROIs and from individual ROIs separately to obtain SDs. The single exponential curve equation used in Kaleidagraph software (Synergy Software) is \( y = m_0 + m_1 \times e^{-(x - x_0)/t_1} \), in which \( m_0 \) is the offset rate. The offset rate was used to calculate the half-time of the recovery by the equation \( t_{1/2} = \ln(2)/t_1 \). The variable \( m_1 \) is the plateau of the recovery. Some curves were determined to have two phases of recovery by plotting the recovery rates from a single exponential fit, \( k_{off} \times t = -\ln(y/x - m_3)/m_3 \), in which \( y_0 \) is the y value at time 0, and \( x \) is the x value at time x. The resulting curve can be fit with either one or two linear fits, indicating one or two offset rates as in Fig. 4 (E and F), in which the slope of each line is \( k_{off} \). Biphasic recovery curves were then fit with the double exponential equation \( y = m_0 + m_1 \times e^{-x/m_3} + x_0 \times e^{-x/m_5} \), in which \( m_0 \) and \( m_3 \) are the off rates for the slow and fast phases, respectively. The contribution of the fast phase to the total recovery is calculated by \( m_2/(y_0 - m_1) \), \( m_3 \) is the difference between the intensity right after bleach and the plateau, such that in a single exponential fit, \( y_0 - m_1 = m_2 \), whereas in a double exponential fit, \( y_0 - m_1 = m_2 + m_4 \). Note that \( m_3 \) and \( m_4 \) are both negative for FRAP fits. To obtain a plateau for each recovery curve, images were collected with different time intervals: a 1-s (Cdc15 in mid-cell, Cdc4 in myo2\(^{+}\), Cdc15 in cdc2\(^{-}\), and Rlc1\(^{+}\)), 10-s (all Reg2, Cdc4 in myo2\(^{+}\)IQ1J0232A, and mid1 in cdc2\(^{-}\)), and 20-s delay (mid1 interphase nodes).

Counting numbers of molecules during node assembly

We counted molecules in nodes based on fluorescence intensity (Wu and Pollard, 2005) with some modifications. We define one polypeptide as one molecule throughout the paper, except there are three tandem YFP molecules for every Cdc12 in the Cdc12-3YFP fusion polypeptide. For intensity comparison, images were collected using the same laser power and imaging settings. The same strain expressing Rlc1-YFP was imaged at the beginning and end of the experiments to ensure that the laser intensity remained constant.

We used the full width at half-maximum to determine the sampling interval along the z axis (Hirschberg et al., 1998; Wu and Pollard, 2005). The full width at half-maximum for our confocal system was 0.44 ± 0.07 nm in the z direction. Thus, z sections spaced at 0.4 μm of strains expressing monomeric EGFP (mEGFP)-tagged Mid1 were collected for comparison to an EGFP-tagged MotB motor in Escherichia coli, which is known to contain a mean of 22 molecules (Leake et al., 2006). The E. coli strain expressing EGFP-MotB (JPA750) was grown in tryptone broth with shaking overnight at 30°C and then washed and suspended in motility buffer (10 mM potassium phosphate and 0.1 mM EDTA, pH 7.0) for imaging as previously reported (Leake et al., 2006). The strain was imaged on bare slides and washed three times with 1 ml of cold 1% NP-40 buffer and split into two 30 mg lyophilized cells (\(1.2 \times 10^9 \) cells) per sample resuspended in 500 μl IP buffer [1% NP-40 buffer, 1 mM PMSF, and protease inhibitor (Roche)]. Under these conditions, ~50% of the cells were lysed (Liu et al., 2010). After low speed centrifugation at 3,000 rpm for 30 s at 4°C, 200 μl supernatant was centrifuged again at 13,000 rpm for 10 min at 4°C. 130 μl supernatant was transferred to use as a cell lysate. 120 μl lysate was added to the antibody-coupled beads and incubated for 90 min at 4°C. Beads were washed five times with 1 ml of cold 1% NP-40 buffer. In some cases, a more stringent buffer (1% NP-40 buffer with 200 mM NaCl) was used in washing steps. Then, 50 μl sample buffer was added to the beads and boiled for 5 min to elute the proteins. Reciprocal co-IPs were performed using the monoclonal antibody against myc (sc-40; Santa Cruz Biotechnology) as a control.

IP and immunoblotting

We immunoprecipitated S. pombe cell lysates using the polyclonal antibody against YFP (NB600-308; Novus Biologicals) from strains expressing tagged proteins under the control of endogenous promoters and integrated at their native chromosomal loci. 30 μl protein G covalently coupled magnetic Dynabeads (Invitrogen) was washed three times with 1 ml of cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4) and 2 mM EDTA. The antibody-coupled magnetic beads were then washed three times with 3 ml PBS and once with 1 ml 1% NP-40 buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 20 mM glycerophosphate, and 0.1 mM Na3VO4).

We immunoprecipitated S. pombe cell lysates using the polyclonal antibody against YFP (NB600-308; Novus Biologicals) from strains expressing tagged proteins under the control of endogenous promoters and integrated at their native chromosomal loci. 30 μl protein G covalently coupled magnetic Dynabeads (Invitrogen) was washed three times with 1 ml of cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4) and 2 mM EDTA. The antibody-coupled magnetic beads were then washed three times with 3 ml PBS and once with 1 ml 1% NP-40 buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 20 mM glycerophosphate, and 0.1 mM Na3VO4).

The slight difference in intensity of GFP variants was corrected using strains expressing integrated Rlc1-GFP under the control of the native promoter. Comparison between cellular concentrations of Rlc1-mEGFP and Rlc1-EGFP was performed as previously described (Wu and Pollard, 2005). In brief, offset pixel intensity was subtracted from all images. Then, the images were corrected for uneven illumination. Cell size-corrected pixel intensity in sum images was measured for each strain and for a wt strain with no fluorescent tag (n = 60 cells for each strain). Then, the wt auto-occupancy was subtracted from both Rlc1 strains. We found that mEGFP is 1.14× brighter than EGFP.

We first used EGFP-MoAb as a standard to quantify the mean numbers of molecules in Mid1-mEGFP nodes in a single stack. Offset intensity value from the system was subtracted from each image, and images of purified GFP were used to correct for uneven illumination in the field as previously described (Wu and Pollard, 2005). Intensities of individual nodes in a single stack were measured using a circular ROI in ImageJ, and background corrections were made using five equal-sized ROIs from nearby cytoplasmic areas without nodes. The size of each ROI was optimized using Gaussian fits of the signal intensity in the x-y axis to measure >90% of the signal. Node intensities were obtained from the sum of two to three consecutive z sections. Mid1 nodes were measured after the signal from the nucleus had disappeared to ensure the measurement of only cytokinesis nodes. Node intensities were divided by 1.14 to correct for the slight difference in intensity between mEGFP and EGFP and then compared with the intensity of EGFP-MoB motors that contain 22 molecules (Leake et al., 2006) to obtain the mean number of Mid1 molecules in individual nodes.

We next determined the number of all protein molecules in nodes over time. Three z sections spaced at 0.2 μm were collected every 20 s for 20 min using strains expressing mYFP or 3FYP-tagged node proteins. mYFP is 1.1× brighter than YFP, and Cdc12-3YFP is 3× brighter than Cdc12-YFP (Wu and Pollard, 2005). Photobleaching during image acquisition was corrected. Individual node intensities over time were measured, and background fluorescence from the mean of five nearby ROIs of the same size was subtracted. The slice with a maximum intensity that could be tracked over time was used for measurements. The fraction of signal present in the best focal plane was determined to be 45–55% of the total in each node. The mean intensity from the maximum intensity plane was divided by this fraction to obtain the total intensity in nodes. The time of node appearance, except that of Mid1, was defined as an intensity ≥25% higher than the cytoplasmic background. The timing of the transition for Mid1 from interphase nodes to cytokinesis nodes was defined as the time that the Mid1 node signal increased to ≥25% higher than that of interphase nodes. The number of molecules in nodes over time was determined by setting the second plateau of Mid1-mYFP nodes to 27.7, the value obtained by comparison to MoAb. The ratios of plateaus of cytokinesis nodes are similar to the numbers counted previously (Wu and Pollard, 2005), validating the method. Before condensation, nodes move very little by diffusion (Vayyolinos et al., 2008). Some nodes could be tracked for several minutes even after the beginning of condensation but before the appearance with offset point of the beginning of node condensation is used to align all the nodes temporally. Each data point in the plateaus in Fig. 4 A is the averaged value at each time point after all the nodes appeared.

The time of the beginning of node condensation relative to the SPB separation was measured in the strain expressing GFP-Mo2s 1a-mEGFP at 25°C, resulting in 2.3 min on average. Thus, the time point at the beginning of node condensation for each cell in the videos for counting molecules was set to 2.3 min so that time 0 represents SPB separation.
nonadherent tubes. One tube was incubated with 100 µM Lat-A, and the other was incubated with equal volume DMSO at room temperature for 5–15 min with shaking. Beads were then collected by the magnetic rack. After removing the supernatant, beads were washed once with 1 ml of cold 1% NP-40 buffer. 50 µl sample buffer was added to the beads and boiled for 5 min to elute the proteins.

Yeast two-hybrid assays
For two-hybrid assays, cdc4, cdc4-8, cdc15, mid1, and rog2 open reading frames were fused with the VP16 activation domain and GAL4 DNA-binding domain in JGQ270 (TRP1, 2µm) and JGQ271 (LEU2, 2µm) plasmids, respectively (gifts from J. Hopper, The Ohio State University, Columbus, OH). The S. cerevisiae strain Mo203 was used for the assays ([1]281–011; Invitrogen). β-Galactosidase assays were performed as described in the α-nitrophenyl β-galactopyranoside assay (Sigma-Aldrich), and β-galactosidase units were defined as [A420 \times 10,000] / Ascoh/min/ml.

Measurements of molecular distances in nodes
We measured protein distances in nodes using the SHREC method (Churchman et al., 2005; Joglekaj et al., 2009) with some modifications. Measurements were made in strains simultaneously expressing two node proteins tagged with GFP (except mECitrine for Mid1) and tdTomato under the control of their native promoters. The distance between the centroids of GFP and tdTomato reflects the mean distance separating two labeled protein clusters within the same node.

To determine the distance between Rlc1-tdTomato and the plasma membrane, we used GFP-Psy1, a t-SNARE protein, as a membrane marker. For two-hybrid assays, cdc4, cdc4-8, cdc15, mid1, and rog2 open reading frames were fused with the VP16 activation domain and GAL4 DNA-binding domain in JGQ270 (TRP1, 2µm) and JGQ271 (LEU2, 2µm) plasmids, respectively (gifts from J. Hopper, The Ohio State University, Columbus, OH). The S. cerevisiae strain Mo203 was used for the assays ([1]281–011; Invitrogen). β-Galactosidase assays were performed as described in the α-nitrophenyl β-galactopyranoside assay (Sigma-Aldrich), and β-galactosidase units were defined as [A420 \times 10,000] / Ascoh/min/ml.

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