INTRODUCTION
To ensure proper segregation of genetic material to daughter cells during cell division, the onset of cytokinesis must be coordinated with the completion of mitosis. In the yeast Schizosaccharomyces pombe, a GTPase-driven signaling pathway termed the septation initiation network (SIN) participates in this control (reviewed in Krapp et al., 2004b; Wolfe and Gould, 2005). The SIN controls the final stages of cell division, including actomyosin ring contraction and formation of the division septum. Loss-of-function mutations in SIN components, Sid2, with its binding partner Mob1, and regulates the SIN scaffold, Cdc11

ABSTRACT The Schizosaccharomyces pombe septation initiation network (SIN) is an Spg1-GTPase–mediated protein kinase cascade that triggers actomyosin ring constriction, septation, and cell division. The SIN is assembled at the spindle pole body (SPB) on the scaffold proteins Cdc11 and Sid4, with Cdc11 binding directly to SIN signaling components. Proficient SIN activity requires the asymmetric distribution of its signaling components to one of the two SPBs during anaphase, and Cdc11 hyperphosphorylation correlates with proficient SIN activity. In this paper, we show that the last protein kinase in the signaling cascade, Sid2, feeds back to phosphorylate Cdc11 during mitosis. The characterization of Cdc11 phosphorylation provides evidence that Sid2-mediated Cdc11 phosphorylation promotes the association of the SIN kinase, Cdc7, with the SPB and maximum SIN signaling during anaphase. We also show that Sid2 is crucial for the establishment of SIN asymmetry, indicating a positive-feedback loop is an important element of the SIN.
localizes to the medial ring when Sid1 binds the SPBs; Sid2 medial localization depends on all other upstream SIN components (Sparks et al., 1999; Hou et al., 2000; Salimova et al., 2000). It is therefore speculated that Sid2-mediated phosphorylation events provide the final output of the SIN, but substrates other than the Cdc14 family phosphatase, Clp1 (Chen et al., 2008), have yet to be identified.

SIN inactivation at the end of division involves removal of a positive regulator, Etd1, from the daughter cell that inherited active Spg1-Cdc7 (Garcia-Cortes and McCollum, 2009) and increases in Spg1 GAP activity at both SPBs (Li et al., 2000). Bry4 and Cdc16 comprise the Spg1 GAP (Furge et al., 1998). These components localize to the SPB during interphase to prevent Spg1 activation, are transiently lost from SPBs during early mitosis when Cdc7 localizes to both SPBs, and then localize to the old SPB during anaphase (Li et al., 2000).

Assembly of SIN signaling components and their regulators occurs at the SPB on a platform comprised of the SIN scaffolding components Cdc11 and Sid4 (Chang and Gould, 2000; Krapp et al., 2001, 2004a; Tomlin et al., 2002; Morrell et al., 2004). Cdc11 binds directly to Sid2, Cdc16, and Spg1 through its N-terminal domain, while its C-terminal, leucine-rich repeats tether it to Sid4 (Krapp et al., 2001; Morrell et al., 2004). The Cdc11 N-terminus also interacts with Cdk1-Cdc13/cyclin B, and multiple sites of Cdk1 phosphorylation are predicted (Morrell et al., 2004). Indeed, Cdc11 is hyperphosphorylated during mitosis, although this modification has been correlated with SIN activation and depends upon both Cdc7 and its upstream regulator, the Plo1 kinase (Krapp et al., 2003, 2004a). However, the specific kinase involved in Cdc11 phosphorylation and the functional consequence of Cdc11 phosphorylation remained undefined.

In this study, we investigated the regulation of Cdc11 phosphorylation and its involvement in SIN function. In addition to our previous finding that Cdc11 phosphorylates Cdc11 during mitosis (Morrell et al., 2004), we found that Sid2 also phosphorylates Cdc11 on multiple sites. Sid2 phosphorylation of Cdc11 affects the dynamics of Cdc7 and Sid2 and promotes the robustness of SIN signaling. Unexpectedly, our study also revealed that Sid2 is required for the development of Cdc7 asymmetry, uncovering a positive-feedback autoamplification loop in the SIN.

RESULTS
Sid2 phosphorylates Cdc11
We had previously shown that Cdc11 binds Sid2 directly (Krapp et al., 2004a; Morrell et al., 2004), and we also found that Cdc11 copurified in a Sid2 tandem affinity purification (TAP; Supplemental Figure S1). Therefore, we investigated whether Sid2 phosphorylated Cdc11. While neither maltose binding protein (MBP) nor the C-terminal half of Cdc11 was phosphorylated by Sid2-Myc13 immunoprecipitates prepared from cdc16-116 cells (in which Sid2 is active [Sparks et al., 1999]), the N-terminal 660 amino acids of Cdc11 were phosphorylated (Figure 1A). Phosphorylation of Cdc11 occurred exclusively on serine residues (Figure 1B). Cdc11 phosphorylation was due to Sid2-Myc13 and not a coprecipitating kinase, because Sid2-Myc13 from mob1-R4 mutant cells phosphorylated MBP-Cdc11-(1-660) very inefficiently (Figure 1C). Mob1 is an essential subunit of the Sid2 kinase (Hou et al., 2000; Salimova et al., 2000). Furthermore, Sid2-Myc13 from cdc16-116 mutant cells phosphorylated MBP-Cdc11-(1-660) better (Figure 1C).

To narrow down the region of Cdc11 that was phosphorylated, we tested additional Cdc11 fragments fused to MBP as Sid2-Myc13 substrates. MBP-Cdc11-(488-660) was not phosphorylated by Sid2-Myc13 (Figure S2A), but Cdc11 residues 1–282, 110–160, and 301–493 were (Figure S2B and unpublished data), indicating that multiple Sid2-Myc13 phosphorylation sites reside in the first 488 amino acids of Cdc11. Accordingly, phosphotryptic peptide mapping of MBP-Cdc11-(1-660) generated five major (1–5), one minor (6), and a few very minor phosphopeptides (Figures 2B, left panel, and S2B, top panel). All of these were accounted for by phosphopeptides derived from various N-terminal Cdc11 fragments (Figure S2B and unpublished data).

Sid2 substrate specificity was defined previously as RXXS (Chen et al., 2008). Cdc11 contains nine RXXS motifs within its first 488 amino acids. To determine which of these was phosphorylated in vivo, we purified Cdc11 using both C-terminal and N-terminal TAP fusions and subjected it to two-dimensional liquid chromatography tandem mass spectrometry (2D-LC MS/MS) analysis. Phosphorylation of residues 121, 122, 264, 345, and 418, in addition to eight Cdk1 consensus (S/T)P sites, which we previously showed were not essential to Cdc11 function (Morrell et al., 2004), were detected multiple times (Table 1 and Supplemental Table S1). Mutating serines 121, 122, 264, 345, and 418 to alanines (S5A) greatly diminished the ability of Sid2-Mob1 to phosphorylate MBP-Cdc11-(1-660) in vitro (Figure 2A), and tryptic phosphopeptides 1–5 were eliminated (Figures 2B, middle panel, and S3A). From these data, we conclude that residues 121, 122, 264, 345, and 418 are the major Sid2 targets within Cdc11. Like Sid2-mediated phosphorylation of the Clp1 phosphatase (Chen et al., 2008), phosphorylation of Cdc11 by Sid2 created recognition sites for 14-3-3 protein binding in vitro (Figure S3B).

We next tested whether phosphorylation of Cdc11 by Sid2 affected its function. Plasmids expressing cdc11-S5A or the corresponding aspartic acid mutations (cdc11-SSD) rescued cdc11-123, cdc11-136, and cdc11::ura4 cells (Figure S4, A and B, and unpublished data), indicating Sid2 phosphorylation is not essential for Cdc11 function. Each mutant was then integrated into the genome at the cdc11 locus by replacing cdc11::ura4. In the following experiments, we used untagged and epitope-tagged cdc11 alleles so the phosphorylation state, localization, and function of the phosphomutants could be evaluated.

Cdc11 phosphorylation was detected previously as gel shifts that increased during mitosis (Krapp et al., 2003). The SDS–PAGE mobilities of Cdc11-GFP, Cdc11-SSA-GFP, and Cdc11-SSD-GFP were therefore examined during a synchronous cell cycle generated by arrest-release of the cdc25-22 mutant. As described previously (Krapp et al., 2003), Cdc11 migrated in at least two bands during interphase and became extensively modified during mitosis (Figure 2C). This is in accord with the > 13 phosphorylation sites on mitotic Cdc11 identified by 2D-LC MS/MS (Tables 1 and S1). In contrast, with wild-type, Cdc11-SSA migrated throughout the cell cycle as a more discrete band in SDS–PAGE (Figure 2C) that comigrated with the faster-migrating bands of Cdc11 (Figure 2D), indicating that phosphorylation of Sid2 sites generates a significant fraction of modified Cdc11 during mitosis. Cdc11-SSD also did not display significant gel shifts compared with wild-type Cdc11 (Figure 2, C and D). Phosphatase treatment of Cdc11 immunoprecipitates confirmed that the slowly migrating forms of Cdc11 are due to phosphorylation (Figure 2E). Phosphatase treatment of Cdc11-SSA immunoprecipitates showed that phosphorylation still occurred on this mutant protein (Figure 2E), consistent with the presence of Cdk1 consensus phosphorylation sites identified by 2D-LC MS/MS (Tables 1 and S1).

Sid2-mediated Cdc11 phosphorylation affects Cdc7 localization and SIN signaling
To assay whether Cdc11 phosphorylation at Sid2 sites affects cytokinesis, we first took a genetic approach. Although we did not detect synthetic genetic interactions with mutations in sid4, spg1, sid1,
or sid2 (unpublished data), cdc11-S5A and cdc11-S5A-GFP were synthetically lethal with cdc7-24 and synthetically sick with cdc7-A20, whereas the cdc11-SSD mutation had no effect on the growth of these strains (Figures 3A and S4C, and unpublished data). These results indicate that loss of Cdc11 phosphorylation at Sid2 sites compromises Cdc11 function, and this is particularly deleterious if Cdc7 function is also reduced.

To examine how cytokinesis was altered in cdc11-S5A cells, we used time-lapse microscopy to measure the period from the onset of contractile ring (CR) assembly to the completion of CR constriction. CRs form within 1 min of SPB separation (Wu et al., 2003). Therefore we tracked SPBs with the Sid4–red fluorescent protein (RFP) marker (Chang and Gould, 2000), together with the CR component, Cdc15–green fluorescent protein (GFP) (Fankhauser et al., 1995). Whereas wild-type and cdc11-SSD cells took 48 and 50 min respectively from SPB separation and CR formation to clearance of Cdc15-GFP from the division site, cdc11-SSA cells took significantly longer, 57 min (Figure 3B). Furthermore, every step from CR formation through constriction was slower in each cell (unpublished data).

To explain the delayed and compromised cytokinesis, we examined whether the cdc11-SSA or cdc11-SSD mutations affected the subcellular distribution of SIN components. The Cdc11 phosphosite mutants themselves localized normally to the SPB throughout the cell cycle (Figure S4D). Similarly, there were no changes to the constitutive SPB localization of Sid4 or Spg1 (unpublished data). However, time-lapse imaging of Cdc7-GFP revealed a reproducible and statistically significant change in its distribution in cdc11-SSA cells. Whereas Cdc7-GFP remained at two SPBs for 22.0 ± 1.7 min in wild-type cells, it became asymmetric more rapidly in cdc11-SSA cells (14.4 ± 0.7 min; Figure 3C). Following initial symmetry breaking, Cdc7 became undetectable at one SPB when the spindle reached a length of 8 μm in wild-type cells (tracked using Sid4-RFP), whereas this occurred at a spindle length of 6 μm in cdc11-SSA cells (Figure 3D). These results indicated that Cdc11 phosphorylation influenced the retention of Cdc7 at the old SPB. Time-lapse imaging of Sid2-GFP also revealed a difference in the persistence of Sid2-GFP at the division site in the cdc11-SSA mutant. In wild-type cells, Sid2-GFP remained an average of 28.75 ± 1.1 min, whereas Sid2-GFP was detected at the division site for only 22.75 ± 0.6 min in the cdc11-SSA mutant.


<table>
<thead>
<tr>
<th>Consensus kinase</th>
<th>Residue(s)</th>
<th>Number of phosphopeptides</th>
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<tbody>
<tr>
<td>1. Cdk1: (S/T)P</td>
<td>S98</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>S103</td>
<td>3</td>
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<tr>
<td></td>
<td>S136</td>
<td>5</td>
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<td></td>
<td>S393</td>
<td>3</td>
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<tr>
<td></td>
<td>S558</td>
<td>61</td>
</tr>
<tr>
<td>2. Sid2: RXXS</td>
<td>S121,122</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>S264</td>
<td>8</td>
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<td></td>
<td>S301</td>
<td>3</td>
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<tr>
<td></td>
<td>S345</td>
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<td></td>
<td>S418</td>
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The results are collated from six separate experiments. The phosphorylated residue(s) number is given, followed by the number of phosphopeptides identified containing the phosphorylated residue.

**TABLE 1:** Phosphosites identified by MS analysis of Cdc11 purified from cells.

(Figure 3E). In the cdc11-550 mutant, Sid2-GFP remained an average of 30.50 ± 0.9 min. Thus the phosphostatus of Cdc11 at the SPBs likely affects propagation of the SIN signal to the cell division site.

**Cdc7 binds Cdc11 directly**

Cdc7 is recruited to SPBs primarily by activated GTP-bound Spg1 (Sohrmann et al., 1998). However, the observation that cdc7 overexpression rescued an spg1 null mutation suggested that Cdc7 might also interact with another SIN component at the SPB (Schmidt et al., 1997). We reasoned that if it did so, Cdc7 might display localization dynamics different than those of other SIN signaling components in fluorescence recovery after photobleaching (FRAP) experiments. Our previous FRAP studies had indicated that Spg1 and Sid2 had half-lives of recovery of less than 1 min, but the half-lives had not been measured precisely nor had we measured Cdc7-GFP dynamics at all (Morrell et al., 2004). We found that Cdc7-GFP had a significantly longer half-life of recovery during anaphase (67 ± 4.7 s) than either SPB-localized Sid2 (12.6 ± 0.7 s) or Spg1 (12.2 ± 0.9 s) (Figure 4, A and B). Cdc7 also had a significantly larger immobile fraction, a result that is consistent with the idea that Cdc7 interacts with a second SPB component. Given the above data showing that Cdc11 phosphostatus influenced Cdc7 dynamics, we wondered whether Cdc11 might be that component. Consistent with this possibility, we found that Cdc11-GFP is stably anchored at the SPB, because it does not recover appreciably after photobleaching (Figure 4C), as we had previously estimated (Morrell et al., 2004). Thus Cdc11 could represent a second SPB-interacting protein for Cdc7. Accordingly, directed two-hybrid analysis detected an association of the C-terminus of Cdc7 with the N-terminus of Cdc11 (Figure 4D). The interaction is likely sensitive to the phosphostatus of Cdc11, because the Cdc11-550 mutant interacted more robustly with Cdc7 than did the Cdc11-55A mutant (Figure 4E, top panel), although these bait proteins were produced at the same levels in cells (Figure 4E, bottom panel). MBP-Cdc11-(1-660) could also pull down Cdc7 produced in vitro (Figure 4F), suggesting that this association occurs directly. We therefore surmise that Cdc7-Cdc11 interaction contributes to the stabilization of Cdc7 SPB association and that this interaction is likely enhanced by Cdc11 phosphorylation.

**Sid2 contributes to SIN symmetry breaking**

Given that Sid2-mediated phosphorylation of Cdc11 affected the behavior of Cdc7 at the SPB, we predicted that Cdc7 dynamics would be influenced by the loss of Sid2 activity. Thus Cdc7-GFP was imaged by time-lapse microscopy in sid2-250 mutant cells that had been synchronized in G2 prior to temperature shift and imaging. Unexpectedly, we found that Cdc7 SPB localization never became asymmetric as cells proceeded through anaphase, as it did in wild-type cells (Figure 5A, compare top and bottom panels). This was surprising, because previous studies reported that Cdc7-HA localizes asymmetrically in the sid1-239 and sid2-250 mutants (Sparks et al., 1999; Guertin et al., 2000). While we cannot explain the apparent discrepancy, it is possible that fixation led to some loss or masking of the HA epitope in previous studies. Cdc7 also did not become asymmetric in cdc11-55A (n = 4) or cdc11-55D (n = 11) mutants containing the sid2-250 mutation (unpublished data). Furthermore, symmetric Cdc7 localization was detected during the second anaphase in the sid2-250 mutant (Figure 5B). Because Sid2 activity depends upon Sid1 (Sparks et al., 1999), we tested whether Cdc7 became asymmetric in a sid1 mutant strain. As in the case with sid2-250 mutants, we found that Cdc7 remained symmetrically localized during anaphase in fixed sid1-239 cells arrested at the restrictive temperature (Figure 5C). Time-lapse imaging further confirmed that Cdc7 was either symmetric all through mitosis or the generation of Cdc7 asymmetry was delayed until mitotic spindle breakdown in sid1-239 cells (Figure 5, D and E). These results indicate that Sid1 and Sid2 are required for generating SIN asymmetry.

**Sid2 activity peaks with SIN asymmetry**

Although it has been established that Sid2 activity requires Cdc7 function (Sparks et al., 1999), it has not previously been tested how Sid2-Mob1 activity relates to the development of Cdc7 asymmetry, which is generally considered a reflection of SIN activation. Thus we monitored Sid2 kinase activity in synchronized cdc7-GFP cells. Sid2-Mob1 kinase activity was very low in interphase and spindle checkpoint–arrested cells (Figure 6, A and B), consistent with the previous observation that Cdc11 phosphorylation does not occur at these times (Krapp et al., 2003). Sid2 activity peaked with the development of asymmetric Cdc7 SPB localization as expected (Krapp et al., 2004b; Wolfe and Gould, 2005), but it was detected earlier (Figure 6A). This result is consistent with the finding that the SIN participates in contractile ring assembly prior to anaphase (Hachet and Simanis, 2008) and the possibility that Cdc11 begins to be phosphorylated by Sid2 before anaphase B ensues.

**DISCUSSION**

Biological signaling pathways typically contain positive- and negative-feedback loops (Brandman and Meyer, 2008). In this paper, we have presented evidence that the ultimate protein kinase of the SIN pathway, Sid2, feeds back to control the establishment of Cdc7 asymmetry, which correlates with effective SIN signaling and timely SIN silencing (Sohrmann et al., 1998; Garcia-Cortes and McCollum, 2009; Singh et al., 2011).

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Cdc7 is necessary for Sid2 activity (Sparks et al., 1999), and this dependency might explain why changes in Cdc11 SDS-gel mobility were observed previously in cdc7 mutants (Krapp et al., 2003). However, we cannot exclude the possibility that Cdc7 participates directly in Cdc11 phosphorylation, although this was not detected previously (Krapp et al., 2003). Our evidence that Sid2 is responsible for a significant fraction of mitotic Cdc11 phosphorylation is consistent with the previous observation that SIN-dependent Cdc11 phosphorylation does not occur in spindle checkpoint–arrested cells (Krapp et al., 2003), a condition in which we have shown that Sid2 is inactive. Also, Cdc11 phosphorylation requires Sid4 function, evidence that it occurs at SPBs (Krapp et al., 2003), and we have shown here and previously that Sid2 forms a physical complex with Cdc11 (Morrell et al., 2004), which is constitutively SPB-localized (Krapp et al., 2001; Tomlin et al., 2002). We conclude that Sid2-Mob1 is active at the SPBs to phosphorylate Cdc11, as well as at the site of division.

On the basis of our characterization of SIN dynamics in cdc11 phosphomutants, we propose the following model for the role of Cdc11 phosphorylation in SIN signaling (Figure 6C). Spg1 and Cdc7 become activated at both SPBs during metaphase. This leads to Sid2 activity on both SPBs and phosphorylation of Cdc11. However, phosphatases, such as the recently described SIN-inhibitory complex (SIP) that promotes Cdc11 dephosphorylation and is present at SPBs at this time (Singh et al., 2011), limit the extent of Cdc11 phosphorylation. Upon anaphase initiation in wild-type cells, the limited amount of Cdc11 phosphorylation diminishes at the old SPB, due to increased phosphatase activity at that pole (which is enriched in SIP; Singh et al., 2011), and this promotes the recruitment of the Cdc16-By4 GAP complex for Spg1 (Furge et al., 1998) that binds preferentially to hypophosphorylated Cdc11 (Krapp et al., 2003). This then contributes to loss of Cdc7 from the old SPB. As more active Cdc7 accumulates at the new SPB, so do active Sid1 and Sid2, leading to maximal levels of SIN signaling. Increased Cdc11 phosphorylation resulting from increased Sid2 activity at the new SPB would promote the progressive accumulation of Cdc7 at the new SPB (Garcia-Cortes and McCollum, 2009). We propose that cdc11-5A cells break Cdc7 symmetry more rapidly than wild-type cells because Cdc11 phosphorylation is not present at all to help retain Cdc7 at the old SPB. The new SPB also cannot develop a robust positive-feedback loop in the absence of Cdc11 phosphorylation. As a result, cytokinesis takes longer in cdc11-5A cells and is sensitive to perturbation. Indeed, if Cdc7 function is further compromised by mutation, cytokinesis fails completely in cdc11-5A cells. It may be that the SIN uses a "feedback-first" strategy to ensure switch-like activation and achieve a bistable state, as do other cell cycle signaling networks (reviewed in Ferrell, 2011). In contrast with cdc11-5A, the cdc11-S5D mutant behaved similarly to wild-type cdc11, which suggests that it does not represent either the fully phosphorylated or dephosphorylated form of the protein. Indeed, it is worth noting that aspartates cannot provide docking sites for 14-3-3 proteins (Chen et al., 2008;

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**FIGURE 2:** Identification of Sid2-mediated Cdc11 phosphorylation sites. (A) Sid2-Myc13 was immunoprecipitated from cdc16-116 cells that had been shifted to 36ºC for 2.5 h and incubated with MBP-Cdc11-(1-660) (wild-type) or MBP-Cdc11-S5A. (B) Phosphotyrosine peptides from the indicated Cdc11 proteins were separated in two dimensions with the anode on the left. The positions of major phosphopeptides are numbered. The asterisks indicate minor phosphopeptides. Equal counts of wild-type MBP-Cdc11-(1-660) or the S5A mutant were analyzed individually (left two panels) or mixed together (right panel) to prove the correct assignment of phosphorylation sites. (C) Sid2 phosphorylation sites contribute to Cdc11 mitotic phosphorylation. cdc11-GFP, cdc11-S5A-GFP, and cdc11-S5D-GFP strains were synchronized in G2 phase using temperature shift of cdc25-22 to 36ºC for 4 h, and samples were taken every 15 min after return to 25ºC. Cdc11 proteins were isolated from cell pellets by immunoprecipitation and detected by immunoblotting with anti-GFP antibody. Cell cycle progression was monitored by formation of septa. (D) Samples from the 60-min time point in (C) were run on the same gel to demonstrate comigration of the most rapidly migrating Cdc11 band. (E) An anti-GFP immunoprecipitate of wild-type Cdc11 or Cdc11-S5A isolated from a similar cdc25 block-and-release experiment at the indicated seption indices was treated (+) or not (−) with λ-phosphatase before immunoblotting.
at the cell division site was analyzed as in C. p value and SE are included.

cdc11
measured for the indicated number of cells for each strain, and analyzed by unpaired two-tailed
SE are included. (D) The average length of the spindle when Cdc7 broke symmetry was
Cdc7-GFP proteins detected at 2 SPBs was analyzed by unpaired two-tailed
test. p value and SE are included. (E) Still images from representative movies of Sid2-GFP in
Sid2 activity, which we hypothesize begins
phosphorylation state influences Cdc7 dy
phosphates from phosphatase action and
mechanism we propose here for
And Amon, 2011). One factor is the activa
Cdc11 protein–protein interactions and SIN
Cdc7-GFP in cdc11, cdc11-S5A, and cdc11-S5D cells.
Cdc7 directly associates with Cdc11. 
Spg1, Cdc7 directly associates with Cdc11. 
Spg1-binding domain (Schmidt et al., 2004a). Thus, it is likely
how initial Sid2 phosphorylation affects its function? It might be that phosphoryl
Cdc11 adopts a different confor
nerve the recruitment of S. cerevisiae Cdc15, the orthologue of S. pombe Cdc7, to SPBs (Rock and Amon, 2011). One factor is the activation of Tem1, the homologue of Spg1, and the other factor is the protein kinase activity of Cdc5, the homologue of Plo1. Interestingly, it was speculated that Nud1, the S. cerevisiae homologue of Cdc11, might be the direct or indirect target of Cdc5’s activity in promoting Cdc15 SPB association (Rock and Amon, 2011), as was reported previously for Cdc11 (Schmidt et al., 1997; Krapp et al., 2003, 2004a). Thus, it is likely that the mechanism we propose here for Cdc11 phosphorylation is conserved.

How could phosphorylation of Cdc11 affect its function? It might be that phosphorylated Cdc11 adopts a different conformation than the dephosphorylated form. In this case, 14-3-3 protein binding would protect phosphates from phosphatase action and stabilize the phosphorylated conformation, which might interact better with other SIN components such as Cdc7. Structural studies will be required to test this possibility. Alternatively, 14-3-3 protein binding might be involved more directly in stabilizing Cdc11–protein interactions and SIN signaling.

Several factors might explain how initial Sid2 activity, which we hypothesize begins equally at both SPBs during metaphase (Figure 6C), could become asymmetric. As mentioned above, Cdc11 dephosphorylation at the old SPB is likely to be a factor and result from the asymmetric action of one or more PP2A phosphatase complexes that have an impact on Cdc11 phosphostatus and SIN signaling (Le Goff et al., 2001; Krapp et al., 2003; Lahoz et al., 2010; Singh et al.,
Growth on activation, which would then promote a strong positive-amplification loop on that SPB (Figure 6C). This possibility is consistent with previous studies showing that Cdc7 asymmetry and maximum SIN activation depend upon Cdk1 inhibition (Guertin et al., 2000; Chang et al., 2001; Dischinger et al., 2008). We have shown that Cdc7 asymmetry requires Sid2 function and that Cdc11 is a Sid2 target. However, Cdc7 becomes asymmetrically localized to SPBs in cdc11 phosphomutants, indicating that additional Sid2 targets must exist that contribute to the positive-feedback loop. Candidates for additional Sid2 substrates include Cdc7 itself; the Spg1 GAP component Byr4, which is a heavily phosphorylated protein (Song et al., 1996; Krapp et al., 2008; Johnson and Gould, 2011); the Polo kinase Plo1, which is essential for SIN signaling (Ohkura et al., 1995; Bahler et al., 1998a; Mulvihill et al., 1999); and one or more phosphatase complexes that influence the establishment of SIN asymmetry and SIN signaling (Le Goff et al., 2001; Krapp et al., 2003; Lahoz et al., 2010; Singh et al., 2011). Further study will be required to define the complete repertoire of Sid2 substrates influencing the establishment of asymmetric SIN signaling.

**MATERIALS AND METHODS**

**Strains and media**

*S. pombe* strains (Table S2) were grown in yeast extract (YE) medium or minimal medium with appropriate supplements (Moreno et al., 1991). Transformations were performed by the lithium acetate method or electroporation (Keeney and Boeke, 1994; Gietz et al., 1995). Epitope-tagged strains were constructed as described previously (Wach et al., 1994; Bahler et al., 1998b) so that open reading frames were tagged at the 3’ end of endogenous loci with the GFP-Kan<sup>R</sup> or a TAP-Kan<sup>R</sup> cassette. Appropriate tagging was confirmed by PCR and immunoblotting. Strain construction and tetrad analysis were accomplished through standard methods.

**TAP and MS analysis**

Proteins were purified by TAP as previously described (Tasto et al., 2001) and subjected to MS analysis as previously detailed (McDonald et al., 2002; Roberts-Galbraith et al., 2009).

In vitro kinase and phosphatase assays

The Sid2-Mob1 kinase complex was purified by immunoprecipitation with anti-Myc antibodies from sid2-Myc<sub>13</sub> cdc16-116 cells that had been shifted to 36°C for 2.5–4 h. Kinase assays were performed as described (Sparks et al., 1999). Relative kinase activity was measured by determining the incorporation of <sup>32</sup>P into MBP-Cdc11-(1-660) with a scintillation counter, dividing by the amount

Additional Cdk1 activity may be involved. In *S. cerevisiae*, Cdk1 inhibits *S. cerevisiae* Cdc15 (Cdc15 is analogous to *S. pombe* Cdc7) (Jaspersen and Morgan, 2000) and shows asymmetric localization to SPBs during anaphase (Konig et al., 2010). If the analogous mechanism operates in *S. pombe*, relief of Cdk1-mediated Cdc7 inhibition at the new SPB may contribute to initial asymmetric Sid2 activation, which would then promote a strong positive-amplification loop on that SPB (Figure 6C). This possibility is consistent with previous studies showing that Cdc7 asymmetry and maximum SIN activation depend upon Cdk1 inhibition (Guertin et al., 2000; Chang et al., 2001; Dischinger et al., 2008). We have shown that Cdc7 asymmetry requires Sid2 function and that Cdc11 is a Sid2 target. However, Cdc7 becomes asymmetrically localized to SPBs in cdc11 phosphomutants, indicating that additional Sid2 targets must exist that contribute to the positive-feedback loop. Candidates for additional Sid2 substrates include Cdc7 itself; the Spg1 GAP component Byr4, which is a heavily phosphorylated protein (Song et al., 1996; Krapp et al., 2008; Johnson and Gould, 2011); the Polo kinase Plo1, which is essential for SIN signaling (Ohkura et al., 1995; Bahler et al., 1998a; Mulvihill et al., 1999); and one or more phosphatase complexes that influence the establishment of SIN asymmetry and SIN signaling (Le Goff et al., 2001; Krapp et al., 2003; Lahoz et al., 2010; Singh et al., 2011). Further study will be required to define the complete repertoire of Sid2 substrates influencing the establishment of asymmetric SIN signaling.
Sid2 kinase dictates SIN asymmetry

Sid2 is required for SIN asymmetry. (A) Still images captured every 2 min from a movie of a representative cdc7-GFP sid2-250 cell (total cells examined = 10) held at 36°C, or wild-type cells at 36°C (total cells examined = 5). (B) sid2-250 cdc7-GFP mCherry-atb2 cells were shifted to 36°C for 5 h and fixed with methanol. At least 50 anaphase cells in three separate experiments were examined to determine the indicated percentage of SPB pairs with Cdc7 at both SPBs. (C) sid1-239 cdc7-GFP mCherry-atb2 cells were shifted to 36°C for 5 h and fixed with methanol. At least 50 anaphase cells in three separate experiments were examined to determine the indicated percentage of SPB pairs with Cdc7 at both SPBs. (D) Time-lapse imaging of Cdc7 and mCherry-α-tubulin in sid1-239 cells. sid1-239 cdc7-GFP mCherry-atb2 cells were shifted to 36°C for 3 h and then still images were captured every 1.5 min at 36°C using a spinning-disk confocal microscope and the percentage of cells with asymmetric Cdc7 localization was quantified.

FIGURE 5: Sid2 is required for SIN asymmetry. (A) Still images captured every 2 min from a movie of a representative cdc7-GFP sid2-250 cell (total cells examined = 10) held at 36°C, or wild-type cells at 36°C (total cells examined = 5). (B) sid2-250 cdc7-GFP mCherry-atb2 cells were shifted to 36°C for 5 h and fixed with methanol. At least 50 anaphase cells in three separate experiments were examined to determine the indicated percentage of SPB pairs with Cdc7 at both SPBs. (C) sid1-239 cdc7-GFP mCherry-atb2 cells were shifted to 36°C for 5 h and fixed with methanol. At least 50 anaphase cells in three separate experiments were examined to determine the indicated percentage of SPB pairs with Cdc7 at both SPBs. (D) Time-lapse imaging of Cdc7 and mCherry-α-tubulin in sid1-239 cells. sid1-239 cdc7-GFP mCherry-atb2 cells were shifted to 36°C for 3 h and then still images were captured every 1.5 min at 36°C using a spinning-disk confocal microscope and the percentage of cells with asymmetric Cdc7 localization was quantified.
Phosphoamino acid analysis and tryptic peptide mapping

[32P]labeled proteins were subjected to partial acid hydrolysis and tryptic digestion while bound to polyvinylidene fluoride membrane and analyzed as described in McCollum et al. (1999) and references therein.

Expression of labeled and recombinant fusion proteins

MBP, MBP-fusion proteins, GST, and GST-fusion proteins were produced in BL21 bacterial cells and purified from bacterial lysates using amylase beads (New England Biolabs, Ipswich, MA) or GST bind resin (Novagen, Billerica, MA), as specified by the manufacturers. Proteins were also produced in vitro in the presence of [35S]methionine (Trans-label; ICN Pharmaceuticals, Irvine, CA) via the TNT Coupled Reticulocyte lysate system (Promega, Madison, WI). Purified MBP fusion proteins bound to amylace resin were mixed with [35S]labeled proteins in binding buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM EDTA, 0.1% bovine serum albumin, and 0.1% NP-40) and incubated at 4°C for 1 h. The beads were washed once with 100 μl binding buffer before being resolved by SDS–PAGE.

Yeast two-hybrid analyses

Yeast two-hybrid assays were done as described using S. cerevisiae strain PJ69-4A and the pGBT9 and pGAD vectors (James et al., 1996). β-Galactosidase reporter enzyme activity in the two-hybrid strains was measured with the Galacto-Star chemiluminescent reporter assay system according to the manufacturer’s instructions (Tropix, Bedford, MA), with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate. Results were normalized by subtracting the activity associated with the empty prey vector.

Molecular biology techniques

All plasmid constructions were performed by standard molecular biology techniques. All DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). All sequences were PCR-amplified with TaqPlus Precision (Stratagene, Santa Clara, CA) according to the manufacturer’s protocol. Site-directed mutagenesis was carried out using Quickchange (Stratagene) according to the manufacturer’s protocols.

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Immunoprecipitations and immunoblotting

Cell pellets were frozen in a dry ice/ethanol bath and lysed by bead disruption in HEN buffer with inhibitors (Krapp et al., 2001). Proteins were immunoprecipitated from various amounts of protein lysates using anti-GFP (Roche, Indianapolis, IN) or anti-Myc (9E10) followed by Protein G Sepharose beads (GE Healthcare, Waukesha, WI).

For immunoblotting, proteins were resolved by 8% or 10% SDS–PAGE, transferred by electroblotting to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA) and incubated with the set of primary antibodies indicated at 1 μg/ml. Primary antibodies were detected with secondary antibodies coupled to Alexa Fluor 680 (Invitrogen, Carlsbad, CA) or IRDye800 (LI-COR) and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).
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