Abstract

In budding yeast, the major regulator of the mitotic exit network (MEN) is Tem1, a GTPase, which is inhibited by the GTPase-activating protein (GAP), Bfa1/Bub2. Asymmetric Bfa1 localization to the bud-directed spindle pole body (SPB) during metaphase also controls mitotic exit, but the molecular mechanism and function of this localization are not well understood, particularly in unperturbed cells. We identified four novel Cdc5 target residues within the Bfa1 C-terminus: 452S, 453S, 454S, and 559S. A Bfa1 mutant in which all of these residues had been changed to alanine (Bfa14A) persisted on both SPBs at anaphase and was hypo-phosphorylated, despite retaining its GAP activity for Tem1. A Bfa1 phospho-mimetic mutant in which all of these residues were switched to aspartate (Bfa14D) always localized asymmetrically to the SPB. These observations demonstrate that asymmetric localization of Bfa1 is tightly linked to its Cdc5-dependent phosphorylation, but not to its GAP activity. Consistent with this, in kinase-defective cdc5-2 cells Bfa1 was not phosphorylated and localized to both SPBs, whereas Bfa14D was asymmetrically localized. BFA14D cells progressed through anaphase normally but displayed delayed mitotic exit in unperturbed cell cycles, while BFA14D cells underwent mitotic exit with the same kinetics as wild-type cells. We suggest that Cdc5 induces the asymmetric distribution of Bfa1 to the bud-directed SPB independently of Bfa1 GAP activity at anaphase and that Bfa1 asymmetry fine-tunes the timing of MEN activation in unperturbed cell cycles.

Introduction

In eukaryotes, mitotic entry is driven by a rise in cyclin-dependent kinase (Cdk) activity, which is required for the formation of a bipolar spindle and chromosome segregation (For a review, see [1]). For cells to subsequently undergo cytokinesis and enter the G1 phase of the next cell cycle, Cdk-mediated phosphorylation events are reversed and Cdk activity declines (For reviews, see [2,3]). In budding yeast, this transition, called mitotic exit, is triggered by a signaling cascade known as the mitotic exit network (MEN). The MEN activates and releases the Cdc14 phosphatase from the nucleolus, and this phosphatase reverses the phosphorylation of Cdk substrates and inactivates the mitotic Cdns (For a review, see [4]).

The MEN must be tightly regulated for each daughter cell to receive a complete set of chromosomes. When the MEN is prematurely activated in cells undergoing mitosis, genomic instability results [5]. Therefore, the MEN is a crucial target of various checkpoints that keep mitotic Cdk activity high until the daughter chromosomes have segregated properly. The MEN coordinates spindle position and mitotic progression in asymmetrically dividing cells such as budding yeast, where the division plane is predetermined. A pathway called the spindle position checkpoint (SPOC) ensures that the MEN is activated only if the extended mitotic spindle is correctly positioned. When spindles misalign relative to the division plane, mitotic exit is delayed by preventing the MEN activation [6].

The Tem1 GTPase functions to activate the MEN [7]. The MEN signaling cascade is triggered when the two-component GTPase-activating protein (GAP) for Tem1, composed of Bfa1 and Bub2, becomes inactivated. The polo kinase Cdc5 also contributes to MEN activation by directly phosphorylating and inhibiting the GAP activity of Bfa1/Bub2 and/or disrupting its interaction with Tem1 [8,9]. Impaired Bfa1/Bub2 GAP activity allows mitotic exit in cells that have either mitotic exit defects or activated checkpoints [10]. Consistent with this, Bfa1 remains unphosphorylated when the SPOC prevents mitotic exit [8]. Lte1, which was once suggested to promote mitotic exit as a putative guanine nucleotide exchange factor (GEF) for Tem1, has been reported to contribute to mitotic exit by controlling asymmetric Bfa1 localization and cell polarization [11,12]. A recent study demonstrated that loading of Tem1 onto the spindle pole bodies (SPBs) is required for activation of the MEN [13]. Thus a misaligned spindle markedly delays mitotic exit in cells with low GAP activity for Tem1 [10]. These recent studies have suggested more complex ways by which MEN is regulated, including localization of MEN components to the SPB, together with the GTPase-switch model for Tem1.
Author Summary

During mitosis the replicated chromosomes are distributed equally to the daughter cells. Once the chromosomes have segregated properly, a pathway called the mitotic exit network (MEN) becomes activated to complete mitosis. How MEN activation is coordinated with segregation of the chromosomes is currently a focus of interest. In budding yeast, Tem1 initiates MEN activation and Bfa1 negatively regulates Tem1 with Bub2. The polo kinase Cdc5 also activates MEN by directly phosphorylating and inhibiting Bfa1. The spindle pole body (SPB), which corresponds to the mammalian centrosome, acts as a platform for these MEN components. The Bfa1/Bub2 complex localizes to SPBs and regulates the association of Tem1 with the SPBs. When the spindle aligns correctly along the mother-bud axis, Bfa1/Bub2 is restricted to the bud-oriented SPB. Conversely, when the spindle is misaligned, Bfa1/Bub2 is present on both SPBs and mitotic exit is delayed, suggesting that the spatial distribution of Bfa1/Bub2 controls the timing of mitotic exit. In this study, we identified new Cdc5 target phosphorylation residues in Bfa1 that function in its asymmetric distribution on SPBs and showed that the asymmetric Bfa1 distribution was required for timely mitotic exit during unperturbed cell cycle of the budding yeast.

The SPB acts as a scaffold for many MEN components (For a review, see [4]). The association of Tem1 with SPBs depends on Bfa1 and Bub2, which are mutually required for the other’s localization to the SPB [14]. The Bfa1/Bub2 complex localizes to SPBs in an asymmetric manner: as the spindle aligns along the mother-bud axis and the dividing nucleus migrates to the bud neck, the complex is exclusively found on the bud-oriented SPB [14,15]. Conversely, on misoriented spindles that lead to delayed mitotic exit, Bfa1/Bub2 is present on both SPBs. This suggests that the spatial distribution of Bfa1/Bub2 is directly connected to the control of mitotic exit [15,16]. Consistent with this hypothesis, a Bub2 variant that localizes to both SPBs throughout the cell cycle prevented mitotic exit in certain MEN-impaired mutants [17]. Also a recent quantitative analysis showed that Bfa1 dynamics at the SPBs establishes asymmetry in MEN signaling and regulates MEN activity. Bfa1 associates with both SPBs in a transient fashion, but its binding to the daughter SPB (SPBd) is stabilized by cell polarity determinants and their interactions with microtubules [18]. As a consequence, Bfa1 accumulates on the SPBd during metaphase, whereas it disappears from the mother SPB (SPBm), thereby establishing Bfa1 asymmetry [18]. When the spindles are improperly positioned, Bfa1 association becomes highly dynamic on both SPBs, which is required for proper SPOC function [19].

Despite the role in the fidelity of mitosis, the molecular details governing the asymmetry of Bfa1/Bub2 positioning have yet to be fully elucidated. Furthermore, the importance of the asymmetry in the unperturbed cell cycle remains unclear. Bfa1 asymmetry is required for recruiting MEN components exclusively to the SPBd during metaphase [18]. Bfa1 reaches its maximum phosphorylation state when it associates preferentially with the SPBd, whereas Bfa1 is unphosphorylated and localizes to both SPBs in SPOC-activated cells [8,16]. We have identified new phosphorylation sites on Bfa1 that function in directing its asymmetric distribution to SPBs. We present evidence that the phosphorylation of these sites by Cdc5 does not inhibit Bfa1 GAP activity, but induces Bfa1 asymmetry and achieves timely MEN activation during unperturbed mitotic progression.

Results

Bfa1 localizes to both SPBs in the kinase-defective cdc5-2 mutant

Bfa1 is a cell cycle-regulated phosphoprotein [8] that forms a complex with Bub2 and negatively controls the activation of Tem1, a key upstream regulator of mitotic exit [9,9]. Cdc5 polo kinase phosphorylates Bfa1 during mitosis to down-regulate Bfa1/Bub2, and thus activates mitotic exit [9,9]. In cdc15-2 cells, Bfa1 becomes phosphorylated by Cdc5 and Tem1 is activated, but mitotic exit is not permitted since Cdc15 acts downstream of Tem1 in the MEN [7]. Thus, we used the cdc15-2 mutant, which contains wild-type Cdc5, as a control for Bfa1 phosphorylation and localization in Figure 1. In this experiment, we compared wild-type Cdc5 in the cdc15-2 strain to the cdc3-1 and cdc5-2 mutants to characterize the effects of these mutations on late mitosis at the restrictive temperature [20,21].

When α-factor-synchronized cdc15-2 cells were released at restrictive temperature (35°C), Bfa1 was detected as a sharp band in G1 phase; the band accumulated as slower-migrating forms during mitosis, and finally attained its maximal phosphorylation states (Figure 1A left). When treated with phosphatase, the slower-migrating forms collapsed into a sharp band (Figure 1A right). The two cdc5 mutations, cdc5-1 and cdc5-2, affected Bfa1 differently. Bfa1 was phosphorylated as usual in the cdc5-1 mutant, whereas the cdc5-2 mutant was severely defective in phosphorylating Bfa1 at the restrictive temperature (Figure 1A; [8]).

We hypothesized that phosphorylation of Bfa1 by Cdc5 might influence its subcellular location. Before examining this possibility we investigated cell cycle progression and the arrest points in cdc3-1 and cdc5-2 cells. We shifted G1-synchronized cells to 35°C, and counted every 10 min the number of large budded cells with elongated nuclei stretching along the neck or two segregated nuclei (Figure 1B). Compared to the cdc15-2 mutant, both cdc5 mutants exhibited a delay during nuclear elongation (more markedly in cdc5-2 than in cdc3-1), but eventually arrested as large budded cells with separated nuclei, a phenotype similar to that of the cdc15-2 mutant (Figure 1B). Quantitative analysis of pole-to-pole distances in cells with segregated nuclei revealed that the spindle length relative to cell length was shorter in cdc3-1 and cdc5-2 cells than in cdc15-2 cells; mean spindle length was approximately 83% of that of cdc15-2 for cdc3-1, and 53% for the cdc5-2 mutant (Figure 1C). These results demonstrate that the cdc3-1 and cdc5-2 mutants have defects in nuclear spindle elongation but eventually undergo nuclear division with spindles that are not fully elongated; thereafter they arrest as large budded cells with separate nuclei, as does the cdc15-2 mutant. Hereafter, for simplicity, we refer to the arrest point of cdc5-1, cdc5-2, and cdc15-2 as late anaphase.

We then examined the localization of Bfa1 in cdc3-1, cdc5-2 and cdc15-2 cells, where Bfa1 and Spec42 were fused to GFP and RFP, respectively. Bfa1-GFP was found on both SPBs immediately after their separation and before the nucleus moved to the bud neck [14,18]. During metaphase of the cdc15-2 cells, when the spindle was oriented along the division axis and the nucleus was positioned at the bud neck, Bfa1-GFP was predominantly localized to the SPB closest to the bud neck (Figure 1D; [18]). Bfa1 continued to be selectively localized in cdc15-2 cells, with elongated dividing nuclei or segregated nuclei (Figure 1D). The cdc3-1 mutant displayed a Bfa1-GFP localization pattern similar to the cdc15-2 mutant, whereas Bfa1-GFP remained associated with both SPBs in the cdc5-2 mutant, even after nuclear segregation (Figure 1D). In anaphase-arrested cells, Bfa1-GFP was present on both SPBs in 90.9±3.0% of cdc5-2 cells, whereas it was asymmetrically localized...
on the SPB^d in 88.0±1.2% ofcdc5-1 and 92.3±1.5% ofcdc5-2 cells (Figure 1E).

We also observed that the fluorescence intensity ofBfa1-GFP on the SPBs in cdc5-2 cells was only about 25–28% of that in cdc5-1 cells (Figure 1F). Recently, Monje-Casas and Amon [18] showed that the intensity ofBfa1-GFP fluorescence is a good measure of the affinity ofBfa1 for the SPB, and its dynamics. We therefore suggest that the phosphorylation ofBfa1 byCdc5 regulates the dynamics of its behavior, and leads to its asymmetric distribution on the two SPBs.

A decrease inBfa1/Bub2 GAP activity is not required forBfa1 asymmetry

SinceCdc5 inhibitsBfa1/Bub2 GAP activity towardTem1 by phosphorylatingBfa1 [9], the presence ofBfa1 on both SPBs in cdc5-2 cells (Figure 1) could be due to uninhibitedBfa1/Bub2 GAP activity or to the absence ofBfa1 phosphorylation. To distinguish between these possibilities, we examined the localization of GAP activity-defective variants ofBfa1. In in vitroTem1 GTPase assays withBub2, GAP activity was almost completely absent inBfa1^W422A, markedly decreased in bothBfa1^M413I andBfa1^D416A, and slightly decreased inBfa1^G411E [10]. The cdc5-2 mutant was used to analyze the localization of eachBfa1 variant at anaphase. We integratedGFP-fusedBFA1, BFA1^G411E, BFA1^M413I, BFA1^D416A, andBFA1^W422A into cdc5-2SPC42-RFP::Bfa1 cells, and released these cells fromG1 arrest at 35°C. Southern and Western blots verified that theBfa1 mutants were integrated as single copies and were expressed at similar levels to wild-typeBfa1 (Figure S1). We reasoned that ifBfa1 asymmetry was promoted by inhibition of its GAP activity, the mutant forms ofBfa1 would establishBfa1 asymmetry prematurely and localize to only one SPB throughout the cell cycle. In fact, however, like wild-typeBfa1, they associated with both SPBs immediately after SPB separation and before nuclear migration to the bud neck (data not shown). In addition, most of theBfa1^M413I, Bfa1^D416A, andBfa1^W422A forms were present on both SPBs even after chromosome segregation, despite their low GAP activities (Figure 2A and 2B). Bfa1^M413I, Bfa1^D416A, andBfa1^W422A bound to both SPBs in 78.4±1.7, 68.7±0.6, and 70.5±10.1% of anaphase-arrested cells, respectively, while most wild-typeBfa1 (88.0±1.2%) andBfa1^G411E (83.2±4.5%) was asymmetrically localized to the SPB^d. To exclude the possibility that residual GAP activity ofBfa1^M413I, Bfa1^D416A, andBfa1^W422A was responsible for their association with both SPBs at anaphase, we constructed another mutant,Bfa1^DDR2 (Deletion of Direct Repeat 2; Bfa1^G411E M413I D416A W422A), which was predicted to have no GAP activity; indeed, in vitro assays revealed that theBfa1^DDR2/Bub2 complex completely failed to stimulateTem1 GTPase activity (Figure 2C). Consistent with this, Bfa1^DDR2 was utterly unable to prevent mitotic exit in vivo (Figure S2). After confirming single copy integration and normal expression levels (Figure S1), we observed thatBfa1^DDR2 also persisted at both SPBs in anaphase (76.0±5.3%); Figure 2A and 2B), clearly demonstrating that inhibition of GAP activity does not induceBfa1 asymmetry. These results suggest that the persistence ofBfa1 on both SPBs in the cdc5-2 mutant is not due to failure to inhibit GAP activity.

Bfa1 asymmetry is tightly linked to its phosphorylation byCdc5

We then asked if phosphorylation byCdc5 is required for the asymmetric distribution ofBfa1 on SPBs. We examined phosphorylation of theBfa1 mutants shown in Figure 2 using SDS-PAGE mobility shift assays in the cdc5-2 background. Whenα-factor- synchronized cells were released at 35°C, Bfa1^G411E became phosphorylated with similar kinetics to wild-typeBfa1 (Figure 3A). In contrast, slower-migrating forms ofBfa1^M413I, Bfa1^D416A, Bfa1^W422A, andBfa1^DDR2 were not detected after the release fromG1 arrest (Figure 3A). We did not observe any mobility shift of theseBfa1 mutants even whenCdc5 was overexpressed (Figure S3). Using yeast two-hybrid assays we showed that theseBfa1 mutants interacted withCdc5 like wild-typeBfa1, demonstrating that the lack ofBfa1 phosphorylation inCdc5 in these mutants is not due to reduced interaction withCdc5 (Figure S6B).

To examine the extents of phosphorylation of theBfa1 mutants, we purified GST-Cdc5 and GST-Cdc5KD (a kinase-dead control) fromS. cerevisiaeand incubated them withMBP-Bfa1 proteins (MBP-Bfa1^M413I, -Bfa1^D416A, -Bfa1^W422A, and -Bfa1^DDR2) expressed inE. colietd and purified. As the amount ofGST-Cdc5 was increased, wild-typeBfa1 began to appear as multiple, slower migrating forms and eventually appeared as the slowest migrating form, while theBfa1 mutants remained as multiple, less slowly-migrating forms (Figure 3B). The results of these in vitro kinase assays differed slightly from the in vivo results in which slower migrating forms of theBfa1 mutants were rarely seen (Figure 3A; Figure S3). This difference is probably due to either non-specific phosphorylation by the excessiveCdc5 activity used, or the presence ofCdc5 sites that are not easily phosphorylated in vivo. In either case, theseBfa1 mutants were obviously resistant to phosphorylation byCdc5. Note thatBfa1^G411E localized asymmetrically toSPB^d and was phosphorylated byCdc5 like wild-typeBfa1, whereas the otherBfa1 variants (Bfa1^M413I, Bfa1^D416A, Bfa1^W422A, andBfa1^DDR2) were distributed to both SPBs and were

Image
not phosphorylated as efficiently as wild-type Bfa1 by Cdc5. We thus conclude that the asymmetric distribution of Bfa1 is probably linked to its phosphorylation by Cdc5.

**Novel Cdc5 phosphosites are involved in the asymmetric distribution of Bfa1**

Eleven Cdc5 phosphosites have been mapped previously, and substituted with Ala in the Bfa1-11A mutant [8]. To confirm the relationship between Bfa1 localization and phosphorylation, we examined the distribution of Bfa1-11A on SPBs. Since Bfa1-11A mobility is not greatly retarded under conditions that normally produce hyperphosphorylated wild-type Bfa1 (Figure 4A), we expected this mutant to be present on both SPBs in anaphase cells. However, most of the Bfa1-11A (90.1 ± 3.4%) was only associated with SPBd, like wild-type Bfa1 (88.0 ± 1.25%) (Figure 4B). We therefore examined whether Bfa1-11A was further phosphorylated by Cdc5. When MBP-fused Bfa1-11A was incubated with GST-Cdc5 and γ[32P] ATP, 32P incorporation was observed, whereas no 32P incorporation was detected in reactions with an equivalent amount of GST-Cdc5KD (Figure 4C). These observations are consistent with a previous report that mutation of these 11 residues reduces in vitro phosphorylation of Bfa1 by only 75% [8], and demonstrate that not all Cdc5 phosphorylation sites are mutated in Bfa1-11A.

Based on these results, we reasoned that there are unidentified Cdc5 phosphosites and that these could be responsible for the asymmetric distribution of Bfa1-11A. We further hypothesized that these novel sites are not efficiently phosphorylated on Bfa1M413I, Bfa1D416A, and Bfa1W422A, thereby causing these Bfa1 variants to persist at both SPBs in anaphase. If that were the case, the introduction of the M413I, D416A, or W422A mutation into Bfa1-11A should impair Bfa1-11A asymmetry and reduce its phosphorylation. Indeed, 32P incorporation into Bfa1M413I-11A, Bfa1D416A-11A, and Bfa1W422A-11A was less efficient than into Bfa1-11A (Figure 4C), and Bfa1M413I-11A, Bfa1D416A-11A, and Bfa1W422A-11A were each symmetrically distributed to both SPBs (Figure 4D). Consistent with this, when Cdc5 was overexpressed to force phosphorylation of Bfa1 by Cdc5, we detected a mobility shift in Bfa1-11A but not in Bfa1M413I-11A, Bfa1D416A-11A, or Bfa1W422A-11A (Figure S4A). However, in the anaphase-arrested cdc15-2 background, the mobilities of Bfa1M413I-11A, Bfa1D416A-11A, and Bfa1W422A-11A were nearly the same as that of Bfa1-11A (Figure 4A), suggesting that phosphorylation of the residue(s) responsible for asymmetry is not efficient in cells expressing endogenous Cdc5 levels. Together, these results support the presence of unidentified Cdc5 target residues required for establishing Bfa1 asymmetry.

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**Figure 2. Localization of GAP activity-defective Bfa1 mutants.** (A) The anaphase localization of Bfa1 mutants. cdc15-2SPC42-RFP cells expressing the indicated BFA1-GFP mutants (YSK2545, 2547, 2549, 2551, 2553, and 2557) were synchronized with α-factor at 25°C, released at 35°C for 3 h, and processed for confocal microscopy. (B) The quantification of (A). The average of two independent counts is plotted with standard deviations (n = 200). Arrows indicate Bfa1 localized to the SPBm. Bar, 5 μm. (C) In vitro GTPase assays of wild-type MBP-Bfa1 and MBP-Bfa1DDR2 (5 μg), as described in Materials and Methods. doi:10.1371/journal.pgen.1002450.g002
Mutation of 452S, 453S, 454S, and 559S disrupts Bfa1 asymmetry

We previously showed that the C-terminal 184 residues of Bfa1 (Bfa1-D8391–574) sufficiently inhibit the MEN and localize predominantly to the SPBd (Figure 5A and 5B; [22]). As expected, GFP-fused Bfa1-D8M413I, Bfa1-D8D416A, and Bfa1-D8W422A were found at both SPBs (Figure 5B). In addition, the Bfa1-D8 mutants were less efficiently phosphorylated by Cdc5; Bfa1-D8 was more intensely labeled by 32P and detected in a slower-migrating form than Bfa1-D8M413I, Bfa1-D8D416A, and Bfa1-D8W422A (Figure 5C). This indicated that the putative Cdc5 target site(s) responsible for Bfa1 asymmetry was probably located within the C-terminal 184 residues of Bfa1.

We next searched for possible kinase targets within the C-terminal 184 residues of Bfa1. Cdc5 is a Ser/Thr protein kinase, and Bfa1-D8 contains 23 Ser and 12 Thr residues (Figure 5A). We first systematically mutated 21 of the 23 Ser residues to Ala in Bfa1-D8 and constructed 16 different Ser mutants as GFP fusion proteins: Bfa1S395A, Bfa1S404A, Bfa1S426A, Bfa1SSS452AAA, Bfa1SKS459AAA, Bfa1S469A, Bfa1S478A, Bfa1S490A, Bfa1SS504AA, Bfa1SS510AA, Bfa1S530A, Bfa1S541A, Bfa1S551A, Bfa1S555A, Bfa1S559A, and Bfa1S571A (Figure 5A, Table 1). Both 424S and 447S were excluded because they were included in Bfa1-11A. We integrated each of the Ser mutants into cdc15-2D BFA1 cells, and examined their localization at anaphase. The results are summarized in Table 1. Most of the GFP-fused Ser mutants exhibited the localization pattern of wild-type Bfa1-GFP (Table 1; and data not shown). However, the percentage of anaphase-arrested cells with Bfa1-GFP on both SPBs was significantly increased in cdc15-2BFA1SSS452AAA cells (Figure 5D and 5E, Table 1). Cells expressing the substitutions of each Ser in Bfa14A, GFP-tagged Bfa1S452A, Bfa1S453A, or Bfa1S454A, or Bfa1S559A, also had larger numbers of anaphase cells with Bfa1-GFP on both SPBs (Table 1), demonstrating that all three Ser residues contribute to Bfa1 asymmetry. In contrast, mutation of some of the Thr residues to Ala around the newly identified 452S, 453S, 454S, and 559S sites (465T, 497TT, 500T, 537T, 546T, 552T, and 572T; Figure 5A) had little effect on Bfa1 asymmetry (Table 1; data not shown).

To confirm these results, we constructed Bfa14A, in which all four Ser residues were substituted with Ala (SSS452AAA S559A referred to as 4A). Bfa14A-GFP was detected on both SPBs in 79.2 ± 4.8% of anaphase cells, compared with 67.3 ± 6.5% for cdc15-2BFA13A and 29 ± 2.6% for cdc15-2BFA1S559A cells (Figure 5D and 5E). Quantification of the GFP signal in anaphase-arrested cells showed that the fluorescence intensity of Bfa14A-GFP was nearly the same at both SPBs, weaker than that of wild-type Bfa1-GFP at the SPBd (Figure 1). In order to verify that the symmetric localization of Bfa14A was not caused by defective interaction with Cdc5, we compared the physical interactions of Bfa14A and wild-type Bfa1 with Cdc5 using yeast two-hybrid assays. As shown in Figure S6A, Bfa14A interacted as strongly with Cdc5 as wild-type Bfa1. The asymmetric localization of Tem1 to the daughter SPB in anaphase depends on Bfa1 and Bub2 [14]. Therefore we asked whether the presence of the Bfa14A mutant on both SPBs affected the asymmetric localization of Tem1. To answer this question we integrated GFP-fused BFA14A or BFA1 into cdc15-2TEM1-RFPΔbfa1 cells, arrested these cells in G1, and released them at 35°C. As expected, Tem1-RFP followed the localization pattern of Bfa1-GFP in anaphase: it was present on both SPBs in 67.3 ± 6.5% of cdc15-2BFA14A cells, and 29 ± 2.6% of cdc15-2BFA1SSS452AAA cells (Figure 5D and 5E, Table 1). Cells expressing the substitutions of each Ser in Bfa14A, GFP-tagged Bfa1S452A, Bfa1S453A, or Bfa1S454A, or Bfa1S559A, also had larger numbers of anaphase cells with Bfa1-GFP on both SPBs (Table 1), demonstrating that all three Ser residues contribute to Bfa1 asymmetry. In contrast, mutation of some of the Thr residues to Ala around the newly identified 452S, 453S, 454S, and 559S sites (465T, 497TT, 500T, 537T, 546T, 552T, and 572T; Figure 5A) had little effect on Bfa1 asymmetry (Table 1; data not shown).
background and distributed asymmetrically to the SPBd in the wild-type background (Figure 5G).

452S, 453S, 454S, and 559S can be phosphorylated by Cdc5 in vivo and in vitro

We then asked if 452S, 453S, 454S, and 559S of Bfa1 are phosphorylated by Cdc5. Measuring phosphorylation of these four Ser without phosphorylation of the 11 known targets was not feasible. Therefore, since the N-terminus of Bfa1 contains nine of the Cdc5 targets of Bfa1-D8, we used the C-terminal 184 residues of Bfa1 in in vitro kinase assays. MBP-tagged Bfa1-D8, Bfa1-D8-11A, Bfa1-D8K-A, and Bfa1-D8A-11A were incubated with GST-Cdc5 or GST-Cdc5KD. The extents of phosphorylation were determined from the resulting mobility shifts and phospho-Bfa1-D8 bands by Phos-tag SDS-PAGE (Figure 6A). The band representing Bfa1-D8K-11A was tighter in mobility shifts and the phospho-Bfa1-D8K-A-11A forms were not detected in Phos-tag SDS-PAGE (Figure 6A). The extents of phosphorylation were further confirmed by measuring γ-[32P] incorporation and Bfa1-D8K-A-11A less intensely labeled with 32P than that of Bfa1-D8-11A.
(Figure 6B), demonstrating that the 4A mutations reduce Bfa1 phosphorylation by Cdc5. In addition, we speculated that the slower-migrating forms of Bfa1-11A observed in CDC5-overexpressing cells (Figure S4A) resulted, at least in part, from the phosphorylation of residues, such as 452S, 453S, 454S, and S559, required for Bfa1 asymmetry. Indeed, the 4A mutations abolished the slower migrating forms of Bfa1-11A in cells overexpressing CDC5 (Figure S4A), consistent with the results in Figure 6A and 6B. Thus, we suggest that Cdc5 phosphorylates 452S, 453S, 454S, and S559, as shown in blue. Ser residues that were mutated to Ala in this study are shown in red, and Thr are shown in green. (B,D) The localization of Bfa1-D8 substrates was observed from reactions with GST-Cdc5KD. (E) The quantification of (D). Anaphase cells with the indicated phenotype were counted observed by fluorescence microscopy. Arrows indicate Bfa1-GFP and Tem1-RFP localized to the SPBm. Bar, 5 μm. D, GFP signal at the SPBd. M, GFP signal at the SPBm. (G) The anaphase localization of Tem1-RFP in CDC5 rules Bfa1 asymmetry for timely mitotic exit. To confirm this we used mass spectrometry (MS) to map Bfa1 phosphorylation sites (Figure 6C). Recombinant MBP-Bfa1 was phosphorylated in vitro with GST-Cdc5 or with GST-Cdc5KD as a negative control (Figure 6C). Subsequently, MBP-Bfa1 was purified by SDS-PAGE, excised, digested with trypsin, and analyzed by phosphopeptide-selective precursor ion-scanning liquid chromatography (LC) MS. The success of this approach was assessed by seeing if we could detect the 11 previously identified Cdc5 phosphorylation sites. Although we were unable to identify eight of the 11 phosphorylation residues of Bfa1, likely due to their presence on extremely small (457S-460K; three amino acids) or large (474F-477K; 48 amino acids) trypsin peptides, we did detect phosphorylated forms of 457S, 461T, and 441S with high frequency. The same tandem LC-MS/MS analysis identified two phosphopeptide species, 455S pSpFLR (data not shown), containing 455S and 457S, and 452S pSpFLR, containing 452S and 455S, two of the four Bfa1 phosphorylation residues responsible for asymmetry identified above. Phosphorylation of 452S and 455S was not detected by this approach.

To ask if the GAP activity of Bfa1A affects its association with both SPBs at anaphase, we directly measured the GAP activity of Bfa1A/Bub2. When MBP-Bfa1A was added to the reaction

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**Table 1. Summary of Bfa1 localization on SPBs in various Bfa1 mutants.**

| Strains | Mutation sites | Localization to both SPBs | Localization to both SPBs |
|---------|----------------|---------------------------|---------------------------|
| **Bfa1** | Wild type      | 12 ± 1.2%                 | S395A                     | As wild type               |
| **Bfa1 D8** |              |                           | S404A                     | As wild type               |
| **Bfa1G411E** |              | 16.8 ± 4.5%               | S426A                     | As wild type               |
| **Bfa1M413I** | M413I         | 78.4 ± 1.7%               | S459A, S460A, S461A       | As wild type               |
| **Bfa1D8M413I** |              |                           | S469A                     | As wild type               |
| **Bfa1D8D416A** | D416A         | 68.7 ± 0.6%               | S478A                     | As wild type               |
| **Bfa1D8G411E** |              |                           | S490A                     | As wild type               |
| **Bfa1W422A** | W422A         | 70.5 ± 10.1%              | S504A, S505A, S506A       | As wild type               |
| **Bfa1D8W422A** |              |                           | S510A, S511A              | As wild type               |
| **Bfa1DD2** | G411E M413I D416A W422A | 76 ± 5.3% | S530A | As wild type               |
| **Bfa111A M413I** |              | 6.3 ± 6.5%               | S551A                     | As wild type               |
| **Bfa111A D416A** | D416A         | 63 ± 4.9%                | S555A                     | As wild type               |
| **Bfa111A W422A** | W422A         | 40.9 ± 7.1%              | S559A                     | As wild type               |
| **Bfa1S452A** | S452A         | 22.8 ± 3.5%              | S559A                     | As wild type               |
| **Bfa1S453A** | S453A         | 29.2 ± 2.6%              | S559A                     | As wild type               |
| **Bfa1S454A** | S454A         | 79.2 ± 4.8%              | S559A                     | As wild type               |
| **Bfa1S452A S453A S454A S559A** |              | 0.7 ± 1.5%               | S559A                     | As wild type               |
| **Bfa1T7A S17A T24A S30A T35A T47A S54A T62A S69A S424A S447A** |              | 9.9 ± 3.4%               | S559A                     | As wild type               |

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together with Tem1 and GST-Bub2, as in the experiment of Figure 2C, γ-P increased rapidly with kinetics similar to those obtained with wild-type Bfa1, indicating that the 4A substitutions had no effect on Bfa1 GAP activity (Figure 6D). We also examined the control of mitotic exit by Bfa14A in vivo. BFA14A cells arrested as large budded cells, as did wild-type BFA1 cells, in the presence of various checkpoint-activating signals (Figure S5A–S5C). Together, these results show that the Bfa14A has functional GAP activity and demonstrate that the presence of Bfa14A at both SPBs is independent of GAP activity.

Asymmetric localization of Bfa1 fine-tunes the timing of MEN activation

Since Bfa1 is found on both SPBs in cells with misaligned spindles [16], it has been proposed that symmetrically localized Bfa1, and in particular, the Bfa1 associated with the SPB in mother cells, contributes to the arrest of mitotic exit [17]. However, the BFA14A/BUB2/BFA14A/BUB2 cells grew well and did not show any apparent cell cycle delay in unperturbed conditions, despite having Bfa1 on both SPBs throughout the cell cycle. To better understand the function of
Bfa1 asymmetry in mitotic exit, we examined cell cycle progression in these asymmetry-defective BFA1 mutants. Following G1 synchronization and release at room temperature, we monitored large budded cells with two divided nuclei (for simplicity, anaphase cells). In both wild-type and mutant BFA1 cultures, anaphase cells began to accumulate approximately 70 min after release (Figure 7A). In BFA1 cells, numbers of anaphase cells began to decrease about 110 min after release. Interestingly, in the BFA1M413I, BFA1D414A, BFA1V423A, and BFA1A44 cultures, the decrease in anaphase cells was delayed by about 10 min (Figure 7A).

To further examine the delay in cell cycle progression in the Bfa1 mutant cells, we measured Pds1 and Sic1 levels. Pds1 is an anaphase inhibitor that is degraded upon sister chromatid separation [23] and Sic1 is a negative regulator of mitotic CDKs that accumulates following activation of the MEN (For a review, see [24]). Consistent with Figure 7A, the wild-type and all the BFA1 mutants exhibited a drop in Pds1 levels approximately 60 min after release (Figure 7B and 7C), indicating that Bfa1 asymmetry and its persistent association with the SPB does not alter the timing of anaphase onset. Importantly, Sic1 accumulation in the wild-type began about 80 min after release, whereas it began about 90 min after release in BFA1M413I, BFA1D414A, BFA1V423A, and BFA1A44 cells (Figure 7B and 7C). BFA1G411E cells, with a normal Bfa1 distribution, displayed the Pds1 and Sic1 kinetics of the wild-type (data not shown). These results show that asymmetry-defective BFA1 cells activate the MEN approximately 10 min later than cells with normal Bfa1 localization.

We confirmed the 10 min delay of mitotic exit in BFA1A44 mutant cells by examining the dynamics of Cdc14 release in BFA1 and BFA1A44 mutant cells by live cell analysis using time-lapse confocal microscopy. Mitotic exit requires full activation of Cdc14 by releasing it from the nucleolus in late anaphase [25]. As shown in the captured images of Figure 7D, Cdc14 was fully released out of the nucleolus 95 and 105 min after BFA1A44 mutant cells were released from G1 arrest, while wild-type BFA1 cells released Cdc14 approximately 10 min earlier. When we examined time lapse images of several BFA1A44 and wild-type cells, the average time of Cdc14 release was 95.8 ± 9.1 min after release from G1 arrest in the BFA1A44 mutant and 86.7 ± 13.5 min in BFA1A44 wild-type (Figure 7D). These observations clearly demonstrate that in the asymmetry-defective BFA1A44 cells the activation of MEN is delayed by approximately 10 min compared with wild-type BFA1 cells. Moreover, we also tested the asymmetry of BFA1A44 cells upon cell division by confocal microscopy. As shown in Figure S6C and S6D, BFA1A44 cells divided asymmetrically (S6.3% of the cells, 3.0% in the wild-type) when they were grown at restrictive temperatures [8]. While the cells expressed wild-type BFA1, the localization of Cdc14 was symmetric, whereas it was asymmetric in the cells expressing BFA1A44. These results show a strong dependence on Bfa1 asymmetry for timely mitotic exit.

Phospho-mimicking of 452S, 453S, 454S, and 559S results in Bfa1 asymmetry and timely mitotic exit

In order to confirm that Cdc5-dependent phosphorylation of 452S, 453S, 454S, and 559S in Bfa1 controls the asymmetric localization of Bfa1 for timely mitotic exit, we constructed BFA1D45 (SS452DDD S559D referred to as 4D) that mimics the negative charges due to phosphorylation. GFP-fused BFA1D45 was integrated into cd15-2SPC42-RFP::bfa1 cells, and as shown in Figure 8A, BFA1D45-GFP remained localized asymmetrically on the SPBs throughout the cell cycle, even in G2/M when wild-type Bfa1 is present on both SPBs. At G2/M, the cells with asymmetrically localized Bfa1 on the SPBs were significantly increased in cd15-2BFA1D45 cells than in cd15-2BFA1 cells; 73.9 ± 6.3% of cd15-2BFA1D45 and 19.7 ± 5.8% of cd15-2BFA1 cells (Figure 9A). When cells were arrested in late anaphase, BFA1D45-GFP was asymmetrically localized on the daughter SPB in 96.8 ± 1.5% of the cells, compared to 20.8 ± 4.3% in cd15-2BFA1D45 cells (Figure 8A and Figure 5E). These observations demonstrate that symmetric localization of Bfa1A44 is caused by loss of phosphorylation, and suggest that phosphorylation of Bfa1 by Cdc5 on 452S, 453S, 454S, and 559S is necessary for establishing Bfa1 asymmetry.

In kinase-defective cd15-2 cells, Bfa1 was unphosphorylated and localized to both SPBs (Figure 1D). The finding that Cdc5-dependent Bfa1 phosphorylation on 452S, 453S, 454S, and 559S residues regulates its asymmetric localization prompted us to ask whether the phospho-mimicking BFA1D45 is asymmetrically located in cd15-2 cells. For this, we integrated pRS304-BFA1D45-GFP into the TRP1 locus of cd15-2Aba1 cells. In late anaphase-arrested cd15-2 cells, BFA1D45 was asymmetrically localized in 72.7 ± 0.1% of the cells, while Bfa1-GFP was present on both SPBs in 90.9 ± 3.0% of cells (Figure 1E and Figure 8B). These observations further support the notion that Cdc5-dependent phosphorylation of 452S, 453S, 454S, and 559S in Bfa1 is important for its asymmetric localization.

Since Bfa1 is a target of Cdc5 phosphorylation for triggering mitotic exit, its deletion has been reported to rescue kinase-defective cd15-2 cells arrested in late anaphase at restrictive temperatures [8]. To see whether BFA1D45 can inhibit the MEN, we tested whether the viability of cd15-2 could be restored by BFA1D45. As shown in Figure S7, BFA1D45 as well as wild-type BFA1 suppressed the growth of cd15-2Aba1 cells, while knockout of BFA1 rescued the viability of cd15-2 cells. These results demonstrate that BFA1D45 is able to inhibit MEN like wild-type Bfa1.

Since BFA1D45-GFP is exclusively localized on one of the SPBs during mitosis (Figure 8A) but functions as a negative regulator of the MEN, we asked whether the lack of dynamic localization of phospho-mimetic Bfa1D45 affects cell cycle progression. cd15-2 cells expressing wild-type BFA1 or phospho-mimetic BFA1D45 were synchronized in G1 and released at room temperature, and their cell cycle progression was monitored by counting metaphase and anaphase cells. As shown in Figure 8C, BFA1D45 cells exhibited the same kinetics of cell cycle progression as wild-type BFA1 cells. In both wild-type and BFA1D45 cells, metaphase cells began to accumulate at 60 min and peaked at 100 min, while anaphase cells appeared at 100 min and reached a peak at 120 min after release (Figure 8C).

To further analyze the cell cycle progression, we measured the mitotic cyclin Clb2, which is degraded upon activation of the MEN [26]. Consistent with the above result, Clb2 began to accumulate at approximately 60 min after release in both wild-type and BFA1D45 mutant cells, peaked at 90 min, and then declined (Figure 8D and 8E). These results showed that BFA1D45 cells allow timely mitotic exit like wild-type BFA1. Together they confirm that phosphorylation of 452S, 453S, 454S, and 559S regulates the asymmetric localization of Bfa1 and timely mitotic exit in unperturbed cell cycle.

In addition, the asymmetric presence of BFA1D45 protein on SPBs in G2/M did not affect early mitotic progression (Figure 8B). Therefore we suggest that Bfa1 asymmetry is required for timely activation of the MEN but is not necessary for mitotic progression before anaphase in the unperturbed cell cycle.
Figure 7. Cell cycle progression in mutant cells with symmetric localization of Bfa1. (A–C) The indicated cells (YSK2202, 2204, 2205, 2206 and 2338) were grown to mid-log phase, synchronized in G1 with α-factor, and then released into fresh medium at 25°C. (A) Cell cycle progression. Samples were collected every 10 min. Cells were stained with DAPI and large-budded cells with divided nuclei were counted (n = 200). (B and C) Pds1 and Sic1 levels. (B) Samples were collected every 10 min. To analyze changes in Pds1 and Sic1 levels during cell cycle progression, protein extracts were prepared and subjected to western blotting with anti-HA and anti-Myc. Actin served as a loading control. (C) Calibration curves were used to...
Phospho-mimetic BFA1<sup>4D</sup> cells delay mitotic exit in response to spindle misalignment

Previous studies have suggested that the symmetrical distribution of Bfa1/Bub2 is directly related to the delay of mitotic exit when the spindle is not properly aligned [15,16]. We therefore asked whether Bfa1<sup>4D</sup> is able to function in the spindle position checkpoint and whether it is symmetrically localized in cells with misaligned spindles. Proper positioning of the mitotic spindle relies on two independent pathways, one involving the minus-end microtubule motor dynein, the other Bna1, a plus-end microtubule-binding protein [27,28]. The absence of Bna1 induces anaphase spindle misalignment in the mother cell and thus triggers the spindle position checkpoint [6,27]. We first examined the localization of Bfa1<sup>4D</sup> in Adyn1 cells by integrating pRS204-BFA1<sup>4D</sup>-GFP into the TRP1 locus of Adyn1-Cherry-TUB1Bfa1 cells, as described in Materials and Methods. Surprisingly, Bfa1<sup>4D</sup> was present on both SPBs in cells with misaligned spindles like wild-type Bfa1 (Figure 9A).

When the anaphase spindle is misaligned in the parent of Adyn1 cells, BFA1 deletion induces improper mitotic exit, as a result of which both multinucleate and anucleate cells accumulate [14,29]. To assess the spindle position checkpoint functioning of Bfa1<sup>4D</sup>, we monitored multinucleate and anucleate cells in Adyn1BFA1<sup>4D</sup> and compared them with Adyn1 cells with the wild-type BFA1 (Adyn1BFA1) after arrest with α-factor and release at 16°C, when the spindle orientation defect is most pronounced. As shown in Figure 9B, the improper mitotic exit seen in Adyn1Bfa1 cells was significantly decreased in both Adyn1BFA1 and Adyn1BFA1<sup>4D</sup> cells; 5.7±0.1% in Adyn1BFA1 and 4.7±0.3% in Adyn1BFA1<sup>4D</sup> cells. We also examined the spindle position checkpoint function of phospho-mimetic Bfa1<sup>4D</sup> in Abim1Abfa1 cells. Consistent with the above result, BFA1<sup>4D</sup> rescued the viability of Abim1Abfa1 cells like wild-type BFA1 (Figure 9C).

These results demonstrate that BFA1<sup>4D</sup> cells contain SPOC activity like wild-type BFA1 cells. The symmetric localization of Bfa1<sup>4D</sup> is consistent with the SPOC activity of Bfa1<sup>4D</sup> as well as previous evidence that the symmetrical localization of Bfa1 in cells with misaligned spindles is directly connected to the activation of SPOC [15,16]. Based on these observations, we suggest that the newly identified Cdc5-dependent phosphorylation residues in Bfa1, 452S, 453S, 454S, and 559S, are only important for its asymmetrical localization and the timing of mitotic exit in unperturbed cells.

**Discussion**

In budding yeast, Kar9 and dynein preferentially associate with the bud-directed SPB, from which astral microtubules emanate [30,31]. If these proteins distribute symmetrically to both SPBs, the mitotic spindle does not align properly, showing that SPB asymmetry is essential for mitosis [30,31]. König et al. showed that cyclin-dependent kinase 1 (Cdk1) is asymmetrically recruited to the SPB<sup>α</sup> in early anaphase and negatively regulates MEN activity at the SPB<sup>α</sup> [32]. Caydas and Pereira [19] reported that forced targeting of Bfa1 and Bub2 to both SPBs compromised SPOC function and Valerio-Santiago et al. demonstrated that control of Tem1 localization is essential for the proper functioning of the MEN and SPOC [13]. Recently, Bertazzi et al. showed that Lie1-promoted exclusion of Kin4 from the SPB<sup>α</sup> is essential for proper mitotic exit [33]. These previous studies mainly focused on the biological function of SPB asymmetry in cells with misaligned spindles. Here, we have demonstrated that Cdc5-dependent phosphorylation of Bfa1 contributes to its asymmetric distribution at SPB<sup>α</sup>, which is required for timely mitotic exit and, therefore, is required for the fidelity of cell division in unperturbed cells without misaligned spindles.

Previously, GAP activity-defective Bub2-Myc was reported to lead to localization of the Bfa1/Bub2 complex to both SPBs throughout the cell cycle. This complex inhibits mitotic exit, but only in mutant backgrounds in which the MEN is partially impaired [17]. However, it was not clear whether the inhibition of mitotic exit was due to lack of asymmetry or to the absence of GAP activity. Therefore, the importance of Bfa1 asymmetry and its specific function in normal cell cycle progression were not well understood. In this study, we identified various asymmetry-defective Bfa1 mutants that persist on both SPBs throughout the cell cycle. In particular, unlike Bub2-Myc, the Bfa1<sup>4A</sup> mutant stimulated the Tem1 GTPase (Figure 6D), and activated checkpoints for mitotic exit control (Figure S5A–S5C). These results demonstrate that wild-type Bfa1 and Bfa1<sup>4A</sup> differ only in their localization patterns. Bfa1<sup>4A</sup> delayed Sic1 accumulation and Cdc14 release by approximately 10 min relative to cells with normal localization of Bfa1 (Figure 7B–7D). On the other hand, the phospho-mimetic Bfa1<sup>4D</sup> mutant allowed timely mitotic exit like wild-type Bfa1 (Figure 9C–9E). Therefore, we suggest that Bfa1 asymmetry and its disappearance from the SPB<sup>α</sup> regulate the timing of MEN activation in unperturbed cell division cycles.

However in cells with misaligned spindles, Bfa1<sup>4D</sup> was located on both SPBs and there was full SPOC activity (Figure 9). We therefore consider that the newly identified Cdc5-dependent phosphorylation residues in Bfa1, 452S, 453S, 454S, and 559S, are only important for its asymmetrical localization and the timing of mitotic exit in unperturbed cells. The symmetric localization as well as the SPOC activity of Bfa1<sup>4D</sup> is consistent with previous studies that showed that the symmetrical localization of Bfa1 in cells with misaligned spindles is directly connected with activation of the SPOC [15,16]. We speculate that cells override the Cdc5-dependent asymmetric localization of Bfa1 in the presence of a spindle orientation defect. Thus, the previously reported mechanisms that account for the symmetric localization of Bfa1 and the arrest of mitotic exit in response to misaligned spindles may apply to Bfa1<sup>4D</sup>.

It has been suggested that a 10 min delay in the cell division cycle is not biologically significant in controlling the cell division cycle. However, considering that the entire cell cycle of budding yeast is about 90 min and mitosis takes approximately 30 min [34], a 10 min delay is not negligible. In fact, mitotic exit is only delayed by 15 minutes in the presence of constant peak levels of Clb2, which blocks spindle disassembly [35].

Valerio-Santiago et al. recently showed that localization of Tem1 to the SPBs is essential for activation of the MEN [13]. As we showed in Figure 5G, Tem1-RFP localized to both SPBs in BFA1<sup>4D</sup> cells. We also showed that Bfa1<sup>4D</sup> binds to Tem1 like wild-type Bfa1 (Figure S6C). These observations suggest that the delay of mitotic exit in BFA1<sup>4D</sup> cells is a consequence of disrupting the asymmetric localization of Tem1.
Figure 8. Localization and cell cycle progression of a phospho-mimetic Bfa14D mutant. (A,B) Localization of phospho-mimetic Bfa14D in cdc15-2 and cdc5-2 mutant cells. cdc15-2SPC42-RFPBFA1-GFP (YSK2545), cdc15-2SPC42-RFPBFA14D-GFP (YSK2858) and cdc5-2BFA14D-GFP (YSK 2907) cells were released from α-factor arrest at 35°C. (A) The localization of Bfa1-GFP were examined using fluorescence microscopy at G1 (0 min), G2/M (60 min), and anaphase (180 min) after the release. Among BFA1-GFP (YSK2545) and BFA14D-GFP (YSK2858) cells in G2/M (n = 157 for BFA1 and 132 for BFA14D) and late anaphase (n = 200 for BFA1 and 700 for BFA14D), cells with asymmetrically localized Bfa1-GFP were counted. The average percentage of cells with asymmetrically localized Bfa1-GFP in two independent counts is plotted with standard deviation. Bar, 5 μm. (B) The localization of Bfa14D Cdc5 Rules Bfa1 Asymmetry for Timely Mitotic Exit.
What molecular details underlie Bfa1 asymmetry in unperturbed mitosis? One significant contribution may come from cell polarity determinants [18]. Monje-Casas and Amon reported that the correct interaction of astral microtubules with the bud cortex alters the affinity of Bfa1 for SPBs and affects its asymmetry [18]. Consistent with this observation, Geymonat et al. showed that the activity of Lte1 in mitotic regulation depends on its localization to the bud cortex and contributes to the asymmetric localization of Bfa1 to the daughter SPB [12]. How can information in the cortex control the distribution of Bfa1 at SPBs, and how is Bfa1 able to bind to the SPBs with different affinities? When spindles misalign, Kin4 kinase activity and its localization to SPBs are reported to regulate the residence time of Bfa1 at SPBs, as well as SPOC activity [19]. However, if the spindle is correctly aligned, Kin4 begins to associate with the SPBm in mid-anaphase at a time when Bfa1 asymmetry has already been established [36]. In addition, in Akin4 cells with proper spindle positioning, Bfa1 has a normal localization pattern [19]. Furthermore, symmetric localization of Bfa14A is not caused by its defective interaction with Kin4, since Bfa14A interacted with Kin4 like wild-type Bfa1 (Figure S5D). Thus, other factors must regulate Bfa1 asymmetry, particularly in unperturbed cells. Fraschini et al. [17] proposed that the disappearance of Bfa1/Bub2 from the mother-directed SPB requires Bfa1/Bub2 GAP activity. We also observed that various GAP activity-defective Bfa1 mutants persisted at the SPBm during anaphase. However, we showed that Bfa1 asymmetry was not dependent on GAP activity. Because MEN activation requires inhibition of Bfa1 GAP activity and Bfa1 asymmetry, if Bfa1 asymmetry is regulated by its GAP activity, only a decline in GAP activity could promote Bfa1 loss from the SPBm. Nevertheless, this is probably not the case, as is shown by the symmetric localization of the GAP-defective Bub2-Myc and Bfa1DDK2 proteins. Bfa1 also localized on both SPBs in BFA14A cells with normal GAP activity, and in cdc5-2 cells where Bfa1 GAP activity is expected to be high due to lack of phosphorylation.

Although we have shown in this study that Bfa1 asymmetry is not dependent on its GAP activity, we should consider the possibility that its GAP activity influences its localization indirectly, by affecting its phosphorylation. However, Bfa1D416A, which retains approximately 50% of the GAP activity of wild-type Bfa1, is also defective in phosphorylation by Cdc5, like Bfa1 mutants completely lacking GAP activity (Figure 3). Thus, it is unlikely that...
the GAP activity of Bfa1 influences its localization indirectly by affecting its phosphorylation. It may still be possible that asymmetric localization requires a certain threshold level of Bfa1 GAP activity (which must be higher than the level in Bfa1(G14R)) as a prerequisite for phosphorylation of the four serine residues that we have identified.

Our observations that mutation of the four Cdc5-dependent phosphorylation residues, 455S, 456S, 457S, and 509S to Ala in Bfa1(D128G) significantly reduced its phosphorylation by Cdc5, as well as affecting its localization, strongly support the role of these residues in directing asymmetric localization in unperturbed mitosis. This notion was further supported by phospho-mimetic Bfa1(D128G, D128R), which was asymmetrically localized to the SPBs in cdc15-2-dependent arrested cells (Figure 8A) and even in kinase-defective cdc5-y6 (Figure 8B). However, Bfa1(D128G) were not asymmetrically localized in 100% of the cdc5-2 cells (Figure 8B) and phosphorylation of Bfa1-D128G by Cdc5 was only reduced by approximately 25% compared with Bfa1-D8 (Figure 6B). Therefore, we cannot exclude the possibility that Bfa1 contains some additional residue(s) that is/are also phosphorylated by Cdc5 and is/are involved in the asymmetric localization of Bfa1.

Bfa1(D128G) bound to both SPBs of anaphase-arrested cdc15-2 cells with properly segregated nuclei, whereas the 11 previously identified Cdc5 target sites (Bfa1-D114A) had little or no effect on Bfa1 localization. However, the association of Bfa1-D114A with the two SPBs at anaphase was not as stable as that of wild-type Bfa1 for the SPB. We also found that the Bfa1(D128G) mutant formed a stronger association with the SPBs than wild-type Bfa1 (Figure S8). Thus, further characterization of this mutant may help uncover the molecular mechanisms underlying Bfa1 dynamics. One possibility is that all Bfa1 becomes phosphorylated, and phospho-Bfa1 has different affinities for the two SPBs. Alternatively, Cdc5 may differentially phosphorylate Bfa1 at one of the two SPBs. Another possibility raised by Monje-Casas and Amon [18], is that some proteins mediating the association of Bfa1 with SPBs may control the affinity of Bfa1 for the SPBs by introducing various modifications.

When we mapped the phosphosites of Bfa1 by mass spectrometry, phosphorylation of several characterized sites, such as 7T and 424S, was detected with higher efficiency than phosphorylation of the novel residues we identified as required for Bfa1 asymmetry (Figure S9). It is tempting to speculate that the Cdc5 target sites regulating Bfa1 asymmetry in unperturbed mitosis are phosphorylated with higher fidelity, and/or that other factor(s) are involved in modulating the efficiency of phosphorylation to control Bfa1 dynamics. Due to low phosphorylation efficiency, only 435S and 436S were detected as phospho forms by MS. The low phosphorylation efficiency of these residues is consistent with the proposed biological role of their phosphorylation in controlling the timing of mitotic exit during unperturbed cell division cycles. Although phosphorylation of the newly identified phosphosites of Bfa1 by Cdc5 was not very efficient, a similar SSS534FL sequence in Claspin had been identified as the target of phosphorylation by Plk1, a frog ortholog of budding yeast Cdc5 [37].

While mapping the Cdc5 target sites, we identified p150S and p108S, which were reported to be phosphorylated by Kin4 and to prevent further modification of Bfa1 by Cdc5 (Figure S10; [38]). Although we cannot exclude the possibility that p150S and p108S were phosphorylated nonspecifically due to the extremely high Cdc5 kinase activity, the phosphorylation efficiency at these sites was comparable to that of other Cdc5 targets responsible for GAP activity, such as 7T and 424S (Figure S9). Furthermore, despite the phosphorylation of these sites, other sites were phosphorylated by Cdc5. Thus, we suspect that Bfa1 phosphorylation by Cdc5 and Kin4, and the biological functions of these modifications are far more complicated than we currently understand, and will require further study.

We found that the 455S, 456S, 457S in budding yeast Bfa1 are conserved as 505S, 506T, 509S in its fission yeast homologue by4 (Figure S11), which is also localized asymmetrically to SPBs in anaphase [39]. It would be interesting to examine whether these conserved residues of by4 are also phosphorylated by a polo-like kinase, contribute to the asymmetric localization of by4 and regulate the timing of SIN in fission yeast.

In summary, we have shown that Cdc5-mediated phosphorylation of the newly identified residues on Bfa1 modulates the affinity of Bfa1 for SPBs, and as a consequence contributes to the asymmetric distribution of Bfa1 at anaphase. The asymmetric Bfa1 distribution is required for timely mitotic exit, thus probably ensuring tight coupling of MEN activation and chromosome segregation during normal cell cycle progression. We have also uncovered a novel function of the polo kinase, Cdc5, in the control of mitotic exit. Further studies are needed to identify factors that control the Cdc5-dependent Bfa1 phosphorylation responsible for asymmetric localization in unperturbed mitosis, and how it is overridden in the presence of a misaligned spindle. These studies promise to provide crucial insights into how centrosome asymmetry is generated, and its biological importance in the asymmetric division of eukaryotic cells.

Materials and Methods

Yeast strains, culture, and cell cycle synchronization

All yeast cultures and genetic techniques were carried out as described by Kim et al. [22]. The S. cerevisiae strains used in this study are described in Table S1. Strains were generated by PCR-based methods and verified by PCR, and Southern and western blot analysis [40,41]. The integrating plasmid, pRS315-BA1-GFP, was linearized with EcoRV and integrated into the TRP1 locus, as described by Kim et al. [10]. Bfa1 mutants were constructed by PCR-based site-directed mutagenesis, as described by Kim et al. [10]. Cells were synchronized in G1 by adding 10 µg/ml or 50 ng/ml α-factor (Sigma-Aldrich) to BARI or bar1 cells, respectively, and at S phase with 0.2 M hydroxyurea (Sigma-Aldrich) for 2–3 h. CDC5 expression was driven by the GAL promoter.

Microscopy and imaging analysis

Fluorescence microscopy was performed essentially as described by Kim et al. [22]. Cellular labeling was visualized on an Axioplan2 (Zeiss) microscope with a Zeiss 100× Plan Neofluar oil immersion objective. Images were acquired using an Axiocam CCD (Zeiss) camera and AxioVision software (Zeiss). The fluorescence intensity of GFP and RFP-fused proteins was quantitatively analyzed by confocal microscopy. For confocal images, we used a Nipkov disk-based UltraVIEW RS confocal system (PerkinElmer) equipped with a Nikon microscope (TE2000-PSF). The 100× NA 1.4 oil immersion objective lens was controlled by a piezoelectric z stepper. In each experiment, 10 to 15 x sections were acquired at 0.5 µm steps with x2 w binning, the same laser power and exposure time, and projected in UltraVIEW RS software (PerkinElmer). Fluorescence intensity of selected regions of interest was quantified using UltraVIEW RS and Image J software (Version 1.38a, NIH), and the background fluorescence was subtracted by placing the same measurement circle in nearby intracellular regions without a Bfa1-GFP or Tem1-RFP signal. Since the fluorescence intensity of Bfa1-GFP...
generally increased with Spc42-RFP intensity, we normalized the intensity of Bfa1-GFP to Spc42-RFP to precisely measure the amount of Bfa1 associated with the SPB. For time-lapse experiment, a Nipkov disk-based UltraVIEW RS confocal system (PerkinElmer) equipped with a Nikon microscope (TE2000-PFS) was used. Images for cells on agar plugs were taken every 5 min and processed with Adobe Photoshop 7.0. No manipulations were added other than adjustments in brightness and contrast.

Protein analyses and preparation of recombinant proteins

For phosphatase treatment, TAP-tagged Bfa1 was precipitated with IgG Sepharose beads (Amersham) from total cellular lysates (1 mg in 700 μl modified H-buffer containing 1% NP-40) as described by Kim et al. [10] and treated with Calf Intestinal Alkaline Phosphatase (CIP, New England Biolabs) for 30 min at 37°C. For co-immunoprecipitation of TAP-tagged Bfa1-D8 and 3XHA-tagged Kin4, Kin4 was purified with anti-HA (Roche) followed by protein A-agarose (Sigma) and co-precipitates were blotted with peroxidase anti-peroxidase (PAP, Sigma) for Bfa1 or Bfa1-D8. For western blot analysis, peroxidase anti-peroxidase (PAP, Sigma), monoclonal anti-HA (Roche), monoclonal anti-MyC (Roche), polyclonal anti-gpG (Santa Cruz Biotechnology), Cb2 (produced in the lab), monoclonal anti-α-Tubulin (Sigma), and polyclonal anti-Actin (Santa Cruz Biotechnology) were used. Band intensity was quantified and analyzed using the LAS-3000 image analyzer (Fujifilm) and Image J software (Version 1.30u, NIH).

In vitro Tem1 GTPase assay and yeast two-hybrid assay

The intrinsic or GAP-stimulated GTPase activity of Tem1 was measured, as described by Kim et al. [10] with an EnzCheck Phosphate Assay Kit (E-6646; Molecular Probes). The amount of γ-P, released from Tem1-GTP was monitored by measuring the absorbance at 360 nm. Yeast two-hybrid assays were performed as described by Geymonat et al. [9]. Tem1, GST-Cdc5 and MBP-Bfa1, and MBP-D8-Bfa1 were prepared from E. coli, as described by Kim et al. [10].

In vitro kinase assay

In vitro kinase assays were performed, as described by Geymonat et al. [9]. For radioactive kinase assays, 100 ng of substrate was mixed with 10–50 ng of either GST-Cdc5 or GST-Cdc5KD in 15 μl kinase buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol) with 50 μM ATP and 0.1 μM γ-[32P]ATP (Amersham Biosciences, 370 MBq/ml, 3000 Ci/mmol). After incubation at 30°C for either 15 min for full-length Bfa1 or 30 min for Bfa1-D8, Laemmli buffer was added to stop the phosphorylation reaction. γ-[32P]-labeling was visualized by autoradiography. To better detect the phosphorylation of Bfa1-D8 by Cdc5, 1–10 μg substrates were incubated with 1–3 μg of either GST-Cdc5 or GST-Cdc5KD in the same kinase buffer with 0.5 mM non-radioactive ATP, separated on 7.5% SDS-PAGE containing 100 μM Phos-tag acrylamide (MANAC Incorporated) [42] and 200 μM MnCl2, and stained with Coomassie brilliant blue.

Determination of Cdc5-dependent phosphorylation by mass spectrometry

After in vitro phosphorylation of Bfa1 was performed with purified Cdc5 kinase and the product was digested with trypsin, liquid chromatography was carried out on a Dionex LC Packings nano HPLC system (LC-Packings) coupled to the QSTAR Pulsar ESI-hybrid Q-TOF tandem mass spectrometer (Applied Biosystems), as described in Lee et al [43]. The column outlet was coupled directly to the high voltage ESI source (typically 2.5 kV) and peptides eluting from the column were sprayed directly into the orifice of the mass spectrometer. Information-Dependent Acquisition (IDA) mode was performed to acquire MS/MS spectra based on an inclusion mass list and dynamic assessment of relative ion intensity. For MS/MS, a full mass scan range mode was m/z = 100–2000 Da. After determining the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with relative collision energy of 55%. The individual spectra from MS/MS were processed using the Analyst QS software (v1.1, Applied Biosystems) and searched against a limited database containing only the protein of interest, Bfa1, which was performed with mass tolerance 0.1 Da and with a confidence value no less.

Supporting Information

Figure S1 Verification of the single copy integration and expression levels of Bfa1 mutants. cdc15-2SPC42-RFP cells expressing the indicated BFA1-GFP mutants (YSK1165, 2545, 2547, 2549, 2551, 2553, and 2557) were analyzed. (A) A genomic Southern blot assessing the single-copy integration of BFA1 or each BFA1 mutant. Genomic DNA of indicated cells was digested with Eco RI and detected with the 836 bp Hind III-Eco RI fragment of BFA1 as a probe, as described by Kim et al. [10]. Integration into the TRPI locus of Bfa1 background cells generated a 3731 bp fragment (arrow) after the genomic DNA was digested with Eco RI. (B) The quantification of the expression levels of Bfa1 mutants. In actively growing cells, the expression levels of GFP-tagged version of each Bfa1 derivative were quantified and are plotted relative to actin. Actin was used as an internal loading control. (TIF)

Figure S2 The function of Bfa1DDR2 in mitotic exit. Bfa1DDR2 is unable to prevent mitotic exit in mitotic exit-defective cells, such as ste20BFA1 (low temperature) and ste20BFA1ste20, and in response to various checkpoint-activating signals, such as DNA damage, spindle damage, and spindle misorientation. The expression of Bfa1-GFP and Bfa1DDR2-GFP was verified by western blot analysis. (A) The ability of Bfa1-DDR2 to suppress the growth rate of Alte1Bfa1 cells. Alte1BFA1 (YSK2052), Alte1Abfa1 (YSK2051), and Alte1BBFA1 (YSK2218) cells were serially diluted on YPAD and incubated at either 25°C for 2 days or 13°C for 10 days. (B) The growth rate of Alte1Abfa1BFA1DDR2 mutant. Alte1Abfa1BFA1DDR2 cells with pURA3-LTE1 plasmid were transformed with BFA1 or the BFA1DDR2 mutant. Indicated cells (YSK2063, 2062, and 2257) were serially diluted on either YPAD or YPAD containing 5-FOA and incubated at 25°C for 2–3 days. (C) The ability of Bfa1-DDR2 to prevent mitotic exit when DNA is damaged. cdc13-1 (YSK2073), cdc13-1Abfa1 (YSK1138), and cdc13-1BFA1DDR2 (YSK2317) were synchronized with mNop1 and released into YPAD at 34°C. At each time point, the cells were collected to analyze DNA content by FACS (n = 50,000). (D) The ability of Bfa1-DDR2 to prevent mitotic exit when spindles are improperly positioned. Abim1 (YSK2093), Abim1Abfa1 (YSK1867), and Abim1BFA1DDR2 (TIF)
BFA1G411E was followed by the appearance of phosphorylated forms in Figure 1A. No Bfa1 phosphorylation was observed (t = 0) in cells arrested in S-phase with hydroxyurea. However, Cdc5 induction was followed by the appearance of phosphorylated forms in BFA1 and BFA1G411E cells, see the accumulation of slowly migrating bands. In contrast, no slower migrating forms of Bfa1 were detected in other BFA1 mutants, even after 8 h of CDC5 overexpression.

Figure S3 Phosphorylation of GAP activity-defective Bfa1 mutants in cells overexpressing CDC5. The indicated cells (YSK2121, 2142, 2143, 2144, 2145, and 2472) were transformed with pGAL (vector only) or pGAL-CDC5, arrested with 0.2 M hydroxyurea in raffinose medium, and then treated with galactose (t = 0) to induce CDC5 overexpression. Cells were harvested at each indicated time point. P is a positive control used as in Figure 1A. No Bfa1 phosphorylation was observed (t = 0) in cells arrested in S-phase with hydroxyurea. However, Cdc5 induction was followed by the appearance of phosphorylated forms in BFA1 and BFA1G411E cells, see the accumulation of slowly migrating bands. In contrast, no slower migrating forms of Bfa1 were detected in other BFA1 mutants, even after 8 h of CDC5 overexpression.

Figure S4 Phosphorylation of Bfa1 mutants in cells overexpressing CDC5. (A) Phosphorylation of Bfa1-11A, Bfa14A-11A, Bfa1M413I-11A, Bfa13416A-11A, Bfa13418A-11A, and Bfa14A-11A in cells overexpressing CDC5. Indicated cells (YSK2121, 2142, 2144, 2468, 2470, and 2336) were transformed with pGAL or pGAL-CDC5, arrested with 0.2 M hydroxyurea in raffinose medium, and then treated with galactose (t = 0) to induce CDC5 overexpression. Cells were harvested at each indicated time point. P is a positive control used as in Figure 1A. (B) Condition of BFA1, BFA1-11A, and BFA1G411E cells after Cdc5 was overexpressed in S-phase arrest cells for 10 h in (A). The morphology of BFA1, BFA1-11A, and BFA1G411E cells were shown to show the viability of these cells, when Cdc5 was overexpressed in S-phase arrest cells for 10 h. Cells without CDC5 overexpression (t = 0) were shown as controls. Bar, 20 μm.

Figure S5 The ability of the Bfa14A and Bfa1-11A mutants to prevent mitotic exit in response to checkpoint-activating signals. The GAP activity of Bfa1 mutants for Tem1 was analyzed in vitro by the ability to suppress mitotic exit [10]. The expression of GFP-fused Bfa1 mutants was verified by western blot with anti-GFP. Actin was used as a loading control. (A) Spindle damage. The indicated cells (YSK1077, 2083, 2131, 2149, 2455, and 2152) were synchronized with α-factor and released into YPAD containing 15 μg/ml nocodazole at 25°C. At each time point, cells with either large buds or new bud formation were scored (n = 200). (B) DNA damage. Indicated cells (YSK1138, 2073, 2314, 2315, 2484, and 2313) were grown at 25°C, synchronized with α-factor, and released into fresh YPAD at 34°C. At each time point, the percentage of cells with either large buds or new buds was determined (n = 200). (C) Spindle orientation defects. The indicated cells (YSK1129, 2103, 2485, 2486, 2487, and 2488) were synchronized with α-factor at 30°C and released into YPAD at 16°C for 24 h. Cells were stained with DAPI, and cells with indicated phenotypes were quantified (n = 200). The average of three independent counts is plotted with standard deviations. (D) The physical interaction of wild-type Bfa1 and Bfa14A with Kin4. KIN4-3HA::Bfa1 (YSK2910), KIN4-3HA::BFA1-TAP (YSK9911) and KIN4-3HA::BFA1G411E-TAP (YSK2912) cells were grown at 25°C to mid-log phase and harvested. Crude extracts were prepared, and Kin4 was purified with anti-HA followed by protein A-agarose as described in Materials and Methods. Bfa1 and Kin4 were detected with PAP and anti-HA, respectively.

Figure S6 The physical interaction of asymmetry-defective Bfa114 and GAP activity-defective Bfa1 mutants with Cdc5, Tem1, and Bub2 by yeast two-hybrid assays. Cdc5, Tem1 and Bub2 were fused to the DNA activation domain in pG4-5, and Bfa1 and each Bfa1 mutant were fused to the DNA binding domain in pGlda. The yeast strain EGY40 was co-transformed with these constructs and the reporter plasmid pSH18-34. Western blots show that the similar amount of proteins was included in each assay. (A, C and D) The interaction of wild-type Bfa1 and Bfa14A mutant with (A) cdc5, (C) Tem1 or (D) Bub2 was measured quantitatively. (B) The interaction of wild-type Bfa1 and GAP activity-defective Bfa1 mutants with Cdc5 was measured quantitatively.

Figure S7 The ability of Bfa14D to suppress the growth of cdc5-2Δbfa1 cells. cdc5-2Δbfa1 (YSK2526), cdc5-2ΔBFA1 (YSK2606), and cdc5-2ΔBEA14D (YSK2907) were grown to mid-log phase, then serially diluted on YPAD and incubated at either 25°C or 37°C.

Figure S8 The fluorescence intensity of Bfa1DDR2-GFP at SPBs. The box plots compare the fluorescence intensities of Bfa1-GFP at SPBs. cdc5-2ΔSPC42-RFPBFA1-GFP (YSK2545) and cdc5-2ΔSPC42-RFPBFA1DDR2-GFP (YSK2557) cells were released for 3 h at 35°C from α-factor synchronization. GFP fluorescence signals were analyzed as described in Materials and Methods (n = 30 for Bfa1 and 41 for Bfa1DDR2). The line inside the box indicates the median. D, GFP signal at the daughter SPB. M, GFP signal at the mother SPB. Note that this experiment was accomplished in parallel with Figure 5F, and the wild-type Bfa1 control is the same in both cases.

Figure S9 Analysis of Cdc5-dependent phosphorylation by mass spectrometry. In-gel trypptic digests of the in vitro phosphorylated Bfa1 with purified Cdc5 kinase were analyzed by LC-MS/MS. The Cdc5 phosphorylation efficiency at known Bfa1 phospho-residues and the novel residues responsible for asymmetry that we identified in this study is plotted. Among the eight peptides with 'T' residue, peptide including p'T' (phosphorylated 'T') was detected twice with 25% efficiency (2/8). 150S, 180S, 424S, and 454S were detected in phospho-form with 25% (3/12), 25% (5/20), 25% (5/20), and 16.7% (2/12) efficiency respectively.

Figure S10 The Cdc5-dependent phosphorylation of 150S and 180S. The MS/MS spectra of doubly charged mass/charge (m/z) = 437.1862 were used to search against a limited database containing only the protein of interest, Bfa1, and corresponds to (A) a Bfa1 peptide pSMEMELKPK 157 with a phosphorylated 150Ser and (B) 180pSMPNLALVNPAIR 192 with a phosphorylated 180Ser. The b and y ions detected are marked on the peaks. The mass of 98 on the peaks was derived from neutral losses (−97,9769 Da) of phosphoric acid from the precursor ion. Peaks are seen for ions which have water (−18 Da) denoted yΔ and b§.

Figure S11 Sequence alignment Bfa1-D8 with its fission yeast homologue byr4. (A) 125S, 453S, and 454S residues of Bfa1 that are necessary for asymmetric localization of Bfa1 to the SPBs are conserved in byr4. The amino acid sequence similarity between Bfa1 and byr4 was analyzed by BLAST program (NCBI). Conserved residues are shown in red.
Table S1 Yeast strains used in this study. Yeast strains constructed and used in this study were listed in the following table.

(DOCX)

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Author Contributions

Conceived and designed the experiments: KS. Performed the experiments: JK GL. Analyzed the data: KS JK GL. Contributed reagents/materials/analysis tools: KS YYB. Wrote the paper: KS JK GL.

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