In *S. cerevisiae*, the inactivation of the cyclin B homolog Clb2 triggers the transition from mitosis into G1 known as exit from mitosis. This process is regulated by a small-guanine nucleoside triphosphatase (GTPase) signaling cascade known as the mitotic exit network (MEN) (Stegmeier and Amon 2004). Tem1, a small GTPase localized to spindle pole bodies (SPBs), functions at the top of the pathway and activates the downstream Hippo-like kinase Cdc15 (Bardin et al. 2000; Pereira et al. 2000; Asakawa et al. 2001; Lee et al. 2001; Visintin and Amon 2001). Cdc15 is coordinately recruited to SPBs and activated by both Tem1 and by the Polo protein kinase Cdc5 (Cenamor et al. 1999; Bardin et al. 2000; Pereira et al. 2000; Xu et al. 2000; Molk et al. 2004; Rock and Amon 2011). Cdc15 in turn activates the Ndr-family kinase Dbf2 (Mah et al. 2001; Visintin and Amon 2001). The activation of Dbf2 requires an associated factor, Mobi1, and is preceded by the recruitment of Mobi1 to the MEN scaffold Nud1 at SPBs (Komarnitsky et al. 1998; Luca and Winey 1998; Mah et al. 2001; Rock et al. 2013). The activation of Mobi1-Dbf2 at the SPB and subsequent dissociation of this complex from SPBs results in the activation of the Cdc14 phosphatase, the ultimate effector of the pathway. Cdc14 is held inactive in the nucleolus by its inhibitor Cfi1/Net1. The Mobi1-Dbf2 complex causes the release of Cdc14 from Cfi1 during late anaphase, which allows Cdc14 to spread throughout the nucleus and cytoplasm and reach its targets. Activated Cdc14 triggers mitotic CDK inactivation and hence exit from mitosis (Jaspersen et al. 1999; Shou et al. 1999; Visintin et al. 1999).

The regulation of Tem1 activity is important for the integrity of two cell cycle checkpoints: the Spindle Assembly Checkpoint (SAC) and the Spindle Position Checkpoint (SPoC). The SAC acts in metaphase to monitor mitotic spindle – chromosomal attachments before anaphase onset, thereby preventing chromosome missegregation and aneuploidy (reviewed in Musacchio and Salmon 2007). The SPoC acts in anaphase to monitor alignment of the segregating...
chromosomes along the mother-bud axis (reviewed in Caydasi et al. 2010). A key role of the two component GTPase activating (GAP) complex Bub2-Bfa1 is to restrain Tem1 activation in the presence of unattached chromosomes or in the case of mitotic spindle misalignment (Alexandru et al. 1999; Esfuet et al. 1999; Fraschini et al. 1999). This, in turn, prevents unscheduled mitotic exit. Indeed, increased levels and activation of Tem1 lead to an increased proportion of cells that bypass the SAC (Chan and Amon 2009). Therefore it is important to have a thorough understanding of how Tem1 activation is controlled.

The recruitment of Tem1 to SPBs is crucial for its activity. For example, a strain in which Tem1 is mislocalized to the plasma membrane is unable to exit from mitosis (Valerio-Santiago and Monje-Casas 2011). Conversely, a fusion between Tem1 and the SPB outer plaque component Cnm67 (Tem1-Cnm67) localizes constitutively to both SPBs and leads to bypass of the SPoC (Valerio-Santiago and Monje-Casas 2011). Thus, Tem1 localization is intertwined with its activation, but how is this process regulated?

Like Tem1, both Bub2 and Bfa1 also localize to SPBs. Bub2 localization is dependent upon Bfa1 localization and Bfa1 is asymmetrically localized to the dSPB from early anaphase through cytokinesis (Pereira et al. 2000). There is mounting evidence that Tem1 and Bfa1 localization to SPBs is interdependent. For example, in cells lacking BFA1, Tem1 localizes to a much lesser extent to both SPBs from metaphase to late anaphase, but is clearly present on both SPBs in telophase. However, this does not impair the ability of cells to exit from mitosis in a timely manner (Pereira et al. 2000; Valerio-Santiago and Monje-Casas 2011). Tem1 localization influences the residence of Bfa1 on the SPBs, although Bfa1 can localize to the dSPB in the absence of Tem1 function (Pereira et al. 2000). In the presence of a Tem1-Cnm67 fusion, Bfa1 is found symmetrically on both SPBs in metaphase and anaphase cells (Valerio-Santiago and Monje-Casas 2011). Lastly, tethering Bfa1 to the SPBs constitutively using a SPCC72-BFA1 fusion leads to partial bypass of the SPoC and suppresses the localization-defective tem1-3 allele (Scarfone et al. 2015). These results provide evidence that Bfa1 may have a positive effect on Tem1 localization and therefore, on MEN activation. However, such a role has not been clearly explored thus far.

A MEN-promoting function for Bfa1 would need to be a GAP-independent function. Interestingly, a GAP-independent role for Bfa1 in mitotic exit has been previously described. Bub2 and Bfa1 together increase the intrinsic GAP-activity of Tem1 in vitro. Paradoxically, Bfa1 alone appears to inhibit both GTP dissociation and GTP hydrolysis of Tem1 in vitro, while having no effect on GDP dissociation (Geymont et al. 2002). These in vitro data would suggest that Bfa1 positively regulates Tem1, once it becomes GTP-bound, and predict that Bfa1, when overexpressed, could activate the MEN. However, the overexpression of BFA1 instead produces a cell cycle block in anaphase. Interestingly, Ro et al. showed that this terminal arrest was independent of BUB2, suggesting that a GAP-independent function of BFA1 causes the arrest (Ro et al. 2002). Taken together, these data imply that Bfa1 primarily has a negative role in the regulation of Tem1 in vivo. However, the BUB2-independent function of BFA1 on MEN regulation is unknown.

In this study we demonstrate that BFA1 overexpression using a GAL-BFA1 allele leads to a defect in Cdc14 activation. We find that this defect stems from an inability of Tem1 to localize correctly to SPBs in the presence of GAL-BFA1. Further, we show that the GAL-BFA1 mitotic exit defect is completely suppressed by co-overexpression of TEM1. We confirm that the overexpression of BFA1 does not affect Mbi1-Dbf2 activation during mitotic exit. Interestingly, our data also suggest that the Bfa1/Bub2 GAP complex may have a MEN-dependent positive function during cytokinesis. These data underscore the positive role for Bfa1 on Tem1 localization and activation, and suggest a novel role for Bfa1/Bub2 in promoting efficient cytokinesis.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions**

All yeast strains used in this study were derivatives of W303 (AS3). The relevant genotypes of the strains used in this study are indicated in Table 1 below. The CDC15-UP construct was described in Rock and Amon (2011). The eGFP-Tem1-Cnm67 construct was described in Valerio-Santiago and Monje-Casas (2011). The Tem1-GFP, Dbi2-eGFP, Spc42-mCherry and HIS3MX6-GAL-GFP-BFA1 and HIS3MX6-GAL-BFA1 strains were constructed using standard PCR-based methods (Longtine et al. 1998; Snaithe et al. 2005). Culturing conditions were described in the figure legends.

**Fixed-cell imaging**

Indirect immunofluorescence was performed as described previously for α-tubulin (Tub1) and α-HA to detect Cdc14-HA (Visintin et al. 1999). Images for Figures 1B, 2, 4A-F, 5, and 6 were acquired using a Zeiss Axioplan 2 (Zeiss, Thornwood, NY) with a Hamamatsu Orca-R2 camera (Hamamatsu, Middlesex, NJ) and a 63x objective. Cells for Figure 4G were fixed in a 4% paraformaldehyde, 3.4% sucrose solution for 15 min. Cells were washed once in potassium phosphate/sorbitol buffer (1.2M sorbitol, 0.1M potassium phosphate, pH 7.5), and treated with 1% Triton X-100 for 5 min. Cells were washed again in potassium phosphate/sorbitol buffer and resuspended in potassium phosphate/sorbitol buffer containing 4’, 6-diamidino-2-phenylindole dihydrochloride before imaging. Cells for budding analysis and also for Figure 7B - E were fixed in 3.7% formaldehyde, 0.1M potassium phosphate, pH 6.4 prior to brief sonication and imaging. These cells were imaged using a DeltaVision Elite microscope (GE Healthcare Bio-Sciences, Pittsburgh, PA) with an InsightSSI solid-state light source, a CoolSNAP HQ2 camera, and a 60x plan-ApoN objective.

**Live-cell imaging**

Cells for Figures 3A and 7A were imaged directly from log phase cultures using a DeltaVision Elite microscope (GE Healthcare Bio-Sciences) with an InsightSSI solid-state light source, a CoolSNAP HQ2 camera, and a 60x plan-ApoN objective. Quantification of cytoplasmic Bfa1-mCherry for figure 3A was performed using Fiji software.

**Data availability**

All strains are available upon request. The authors affirm that all of the data necessary for confirming the conclusions made within this article are contained within the article and its figures.

**RESULTS**

**The overexpression of BFA1 causes a defect in Cdc14 activation**

Previous studies characterizing the effects of the GAL-BFA1 allele on mitotic exit demonstrated that these cells delay in anaphase upon galactose induction. Furthermore, it was known that this severe anaphase delay was not dependent on BUB2 (Li 1999; Ro et al. 2002; Figure 1). In order to elucidate the nature of the BUB2-independent effects of BFA1 on anaphase progression, we first sought to determine whether overexpression of BFA1 affects the release of Cdc14 from its inhibitor Cfl1. In this experiment, we analyzed the localization of Cdc14 in anaphase cells to the nucleolus, the nucleus, and the nucleus and cytoplasm. This
allowed us to distinguish between cells with inactive Cdc14 (sequestered in the nucleolus), Cdc14 released by the Cdc Fourteen Early Anaphase Release (FEAR) network (nuclear and cytoplasmic) release of Cdc14 from the nucleolus, where it is held inactive by its inhibitor Cin. This event is a marker for MEN activation (Jaspersen et al. 1999; Shou et al. 1999; Visintin et al. 1999). We arrested cells in G1 using alpha-factor pheromone and released them into a synchronous cell cycle. We monitored budding, the appearance and disappearance of metaphase and anaphase spindles, as well as Cdc14 localization at each time point. We found that synchronized GAL-BFA1 cells exhibited a severe anaphase delay and remained arrested as large budded cells (compare Figure 2A and 2C). In addition, we found that the FEAR-mediated partial activation of Cdc14 was not impaired in GAL-BFA1 cells. However, we never observed full Cdc14 release in cells containing GAL-BFA1 (compare Figure 2B and 2D). Taken together, these data demonstrate that Cdc14 activation by the MEN is blocked by the overexpression of BFA1.

BFA1 overexpression causes de-localization of Tem1 from SPBs

We were interested in determining the specific step in the MEN that was perturbed by the GAL-BFA1 allele. There is prior evidence that Tem1 and Bfa1 localization to SPBs is at least partially interdependent (Pereira et al. 2000; Valerio-Santiago and Monje-Casas 2011; Scarfone et al. 2015). Therefore, we hypothesized that both Tem1 and Bfa1 localization were impaired when BFA1 was overexpressed. We first examined the localization of the GAL-GFP-BFA1 allele. We examined anaphase cells and were surprised to find that in the presence of galactose, Bfa1-GFP is concentrated at the dSPB, as it is in wild type cells (Pereira et al. 2000; Figure 3B). However, we also noted that in cells overexpressing a BFA1-mCherry construct under a GAL-inducible promoter, ten-fold more Bfa1-mCherry protein is present in the cytoplasm when compared to cells containing BFA1-mCherry under its own promoter (Figure 3A).

Given the significant cytoplasmic localization of Bfa1 in GAL-BFA1 cells, we reasoned that Tem1 could be dragged off of the SPB and into the cytoplasm in these cells. Indeed we found that Tem1-GFP was delocalized from SPBs in 100% of cells when Tem1-GFP was expressed under its own promoter (Figure 3A). Unlike Tem1, however, we noted that in cells overexpressing a Tem1-Cnm67 fusion, Bfa1 is found symmetrically localized to one or two SPBs (Figure 3C, 3D). These results demonstrate that Tem1 localization is defective in GAL-BFA1 cells.

Table 1 Strain List

| Number | Genotype |
|--------|----------|
| 3      | MAT a ade2-1, leu2-3, ura3, trp1-1, his3-1115, can1-100, GAL, [phi+] (W303) |
| 5      | MAT a GAL-GFP-BFA1::HIS3MX6 |
| 15     | MAT a TEM1-GFP:HIS3MX6 |
| 19     | MAT a URA3::pTem1-eGFP-CNMP67-TEM1::tem1::kanMX6 |
| 24     | MAT a CDC14-3HA, HIS3MX6::GAL-BFA1 |
| 36     | MAT a URA3::pTem1-eGFP-CNMP67-TEM1::tem1::kanMX6, HIS3MX6::GAL-BFA1 |
| 79     | MAT a TEM1-GFP:HIS3MX6, HIS3MX6::GAL-BFA1 |
| 109    | MAT a CDC14-3HA, URA3::pTem1-eGFP-CNMP67-TEM1::tem1::kanMX6 |
| 118    | MAT a GAL-BFA1::HIS3MX6, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6 |
| 138    | MAT a CDC14-3HA |
| 188    | MAT a CDC14-3HA, URA3::pTem1-eGFP-CNMP67-TEM1::tem1::kanMX6 |
| 206    | MAT a GAL-GFP-BFA1::HIS3MX6, bub2::HIS3MX6 |
| 207    | MAT a YEP13-LEU2 (2m) |
| 209    | MAT a YEP13-TEM1-3MYC-LEU2 (2m) |
| 211    | MAT a GAL-GFP-BFA1::HIS3MX6, YEP13-LEU2 (2m) |
| 213    | MAT a GAL-GFP-BFA1::HIS3MX6, YEP13-TEM1-3MYC-LEU2 (2m) |
| 259    | MAT a tem1::kanMX6, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6 |
| 358    | MAT a DBF2-eGFP:HIS3MX6, SPC42-mCherry::kanMX6 |
| 394    | MAT a tem1::kanMX6, HIS3MX6::GAL-BFA1, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6, DBF2-eGFP:HIS3MX6, SPC42-mCherry::kanMX6 |
| 401    | MAT a GAL-TEM1::URA3 |
| 413    | MAT a HIS3MX6::GAL-BFA1 |
| 414    | MAT a HIS3MX6::GAL-BFA1, GAL-TEM1::URA3 |
| 431    | MAT a tem1::kanMX6, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6, DBF2-eGFP::HIS3MX6, SPC42-mCherry::kanMX6 |
| 493    | MAT a tem1::kanMX6, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6, GAL-GFP-BFA1::HIS3MX6 |
| 494    | MAT a tem1::kanMX6, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6, GAL-GFP-BFA1::HIS3MX6, bub2::HIS3MX6 |
| 496    | MAT a BFA1-mCherry::kanMX6, ura3::pAFS125-TUB1p::GFPTUB1::URA3 |
| 497    | MAT a HIS3MX6::GAL-BFA1-mCherry::kanMX6, ura3::pAFS125-TUB1p::GFPTUB1::URA3 |
| 499    | MAT a tem1::kanMX6, GAL-CDC15::TRP1::pGPD-CDC15::NatMX6, bub2::HIS3MX6 |

GAL-BFA1 mitotic exit defects

Now that we had determined that Tem1 localization is affected in GAL-BFA1 cells, we hypothesized that we could suppress this defect by artificially re-localizing Tem1 to SPBs in BFA1 overexpressing cells. In the presence of a Tem1-Cnm67 fusion, Bfa1 is found symmetrically on both SPBs in metaphase and anaphase cells (Valerio-Santiago and Monje-Casas 2011). Therefore we analyzed the kinetics of Cdc14 release from Cin in the nucleolus and the kinetics of mitotic exit in GAL-BFA1 Tem1-eGFP-CNMP67 cells. Specifically, we analyzed the
did not overexpress GAL-TEM1, but not TEM1-2 localization to SPBs (Figure 4G-H). We considered two hypotheses for the partial suppression of GAL-BFA1 mitotic exit defects in Tem1-Cnm67 dSPB localization than in GAL-BFA1 served at the dSPB or at both mother and daughter SPBs regardless of the suppression was not due to de-localization of Tem1-Cnm67 in the background, as the Tem1-eGFP-Cnm67 protein was observed at the dSPB or at both mother and daughter SPBs regardless of GAL-BFA1 induction. Importantly, we observed that in the presence of the GAL-BFA1 allele, a larger fraction of anaphase cells exhibited a bias for Tem1-Cnm67 dSPB localization than in TEM1-Cnm67 cells that did not overexpress BFA1, which further suggests that when Tem1 is artificially tethered to SPBs, the overexpressed Bfa1 influences Tem1 localization to SPBs (Figure 4G-H).

**GAL-TEM1, but not TEM1-2μ, can fully suppress GAL-BFA1 mitotic exit defects**

We considered two hypotheses for the partial suppression of GAL-BFA1 by TEM1-CNM67. One possibility was that the TEM1-CNM67 allele provides low levels of activated Tem1 and higher levels are needed to fully bypass GAL-BFA1. A second, non-mutually exclusive possibility was that GAL-BFA1 has TEM1-independent effects on the MEN. We decided to test this first possibility by examining the suppression of GAL-BFA1 by two different alleles of TEM1: TEM1-2μ and GAL-TEM1. We predicted that TEM1-2μ would also exhibit a partial bypass of GAL-BFA1 because this allele also has lower levels of activated Tem1. Conversely, we expected to observe complete suppression of the effects of GAL-BFA1 by the GAL-TEM1 allele because this allele exhibits high levels of activated Tem1 (Chan and Amon 2009). We synchronized cells and analyzed budding, spindle, and nuclear morphology to assess the timing of metaphase, anaphase and mitotic exit. We found that the anaphase delay phenotype observed in GAL-BFA1 was only very mildly suppressed in GAL-BFA1 TEM1-2μ cells, which contained the TEM1 gene on a multicopy plasmid (Figure 5B). In contrast, the timing of mitotic exit in GAL-BFA1 GAL-TEM1 cells overexpressing both BFA1 and TEM1 was indistinguishable from wild type cells (Figure 6). These data support the hypothesis that defects in Tem1 activation are the primary reason that GAL-BFA1 cells cannot activate the MEN.

**Dbf2 localization to the budneck is enhanced by GAL-BFA1**

Our prior data strongly suggested that the mislocalization and faulty activation of Tem1 in GAL-BFA1 cells resulted in a failure to activate the MEN. However, in order to confirm that the GAL-BFA1 allele did not impart any TEM1-independent mitotic exit defects, we sought to analyze MEN activation in GAL-BFA1 cells that lacked Tem1 activity. The deletion of TEM1 confers a lethal phenotype. In order to keep tem1Δ cells alive, the hyperactive CDC15-UP allele, in which CDC15 is overexpressed using a GAL-inducible promoter, was utilized (Rock and Amon 2011). We examined the localization of Dbf2-eGFP to SPBs as a proxy for MEN activation in tem1Δ CDC15-UP cells. Dbf2 is known to localize to SPBs during late anaphase following Cdc15 activation, but the protein also localizes to the budneck where it activates the process of cytokinesis (Stegmeier and Amon 2004; Frenz et al. 2000; Meitinger et al. 2011; Oh et al. 2012; Meitinger et al. 2013). In order to fully assess the effects of GAL-BFA1 on Dbf2 localization, we quantified the number of anaphase cells with Dbf2-eGFP localized to the budneck was restored to 47.9% (Budneck only + SPB and Budneck; Figure 7A). In large budded wild type cells containing one Scp42-mCherry SPB dot distributed in mother and daughter cells, Dbf2-eGFP was found at the SPBs in 41.2% of cells (SPB only + SPB and Budneck; Figure 7A). In tem1Δ CDC15-UP cells, Dbf2-eGFP was found at SPBs in 54.9% and 71.3% of cells respectively (SPB only + SPB and Budneck; Figure 7A). These data indicate that the overexpression of BFA1 does not have TEM1-independent mitotic exit effects.

Unexpectedly, when we examined the budneck localization of Dbf2-eGFP in these same cells, we found that Dbf2-eGFP budneck localization was impaired in tem1Δ CDC15-UP anaphase cells. Specifically, wild type cells exhibited budneck localization in 45.3% of cells, while tem1Δ CDC15-UP cells only had Dbf2-eGFP at the budneck in 24.3% of cells (Budneck only + SPB and Budneck; Figure 7A). In tem1Δ CDC15-UP GAL-BFA1 cells, however, the fraction of anaphase cells with Dbf2-eGFP localized to the budneck was restored to 47.9% (Budneck only + SPB and Budneck; Figure 7A). These data indicated that, despite the fact that tem1Δ CDC15-UP...
cells display normal cell cycle kinetics and Dbf2 kinase activity during anaphase (Rock and Amon 2011), the cytokinesis-specific role of Dbf2 was disrupted. They also suggested that the overexpression of \textit{BFA1} could suppress these defects in \textit{tem1}Δ \textit{CDC15-UP} cells.

To further explore whether cytokinesis defects were present in \textit{tem1}Δ \textit{CDC15-UP} cells, and to determine whether the deletion of \textit{TEM1} or the overexpression of \textit{CDC15} was responsible for these defects, we analyzed the cellular morphology of these cells following a brief sonication. This allows the separation of cell clumps without disrupting cell walls. We found that less than 1% of wild type cells and 5% of \textit{CDC15-UP} cells exhibited cytokinesis defects where two cell bodies remained connected after brief sonication (Figure 7B, 7C, 7D). In contrast, 33% of \textit{tem1}Δ \textit{CDC15-UP} cells exhibited chained cell morphology where two or three cell bodies remained connected (Figure 7B, 7E). Furthermore, we observed that the cytokinesis defects of \textit{tem1}Δ \textit{CDC15-UP} cells were largely suppressed in the presence of \textit{GAL-BFA1}. In \textit{tem1}Δ \textit{CDC15-UP} \textit{GAL-GFP-BFA1} cells, only 10% of cells exhibited chains of two cells, and the more severe multi-budded cell phenotypes were not observed (Figure 7B). These data imply that increased levels of \textit{BFA1} promote cytokinesis by a \textit{Tem1}-independent mechanism.

We were next interested to study whether these novel effects on cytokinesis were dependent or independent of Bfa1 GAP activity. To test this, we determined the presence of multi-budded cells in \textit{tem1}Δ \textit{CDC15-UP} \textit{GAL-GFP-BFA1} cells where the catalytic GAP component \textit{BUB2} was deleted. We also examined the efficiency of cytokinesis in \textit{tem1}Δ \textit{CDC15-UP} \textit{bfa1}Δ \textit{bub2}Δ cells, where both components of the GAP had been deleted. Interestingly, we found that both \textit{tem1}Δ \textit{CDC15-UP} \textit{bfa1}Δ \textit{bub2}Δ and \textit{tem1}Δ \textit{CDC15-UP} \textit{bfa1}Δ \textit{bub2}Δ cells in which Bfa1/Bub2 GAP activity had been abolished exhibited cytokinesis defects to a similar extent as seen in \textit{tem1}Δ \textit{CDC15-UP} cells. Specifically we observed that 33% of \textit{tem1}Δ \textit{CDC15-UP} \textit{GAL-GFP-BFA1 bub2}Δ cells and 29.2% of \textit{tem1}Δ \textit{CDC15-UP} \textit{bfa1}Δ \textit{bub2}Δ cells exhibited defects in cell separation. These results suggest for the first time that the Bfa1/Bub2 GAP complex promotes cytokinesis in cells lacking \textit{TEM1}.

\textbf{Figure 2} Cdc14 activation is impaired in \textit{GAL-BFA1} cells. Log phase cultures of wild type (AS138) and \textit{GAL-BFA1} (AS24) cells carrying a \textit{CDC14-3HA} fusion growing at 25°C in YEPR were arrested in G1 using alpha-factor pheromone (5 \textmu g/mL). At 1.5 hr into the arrest, galactose (GAL) was added to induce expression of \textit{GAL-BFA1}. Cells were released from the G1 arrest after 3 hr into YEPRG at 25°C. Cells were collected at the times indicated to process samples for budding analysis and for tubulin and Cdc14-3HA immunofluorescence. A and C) The percentage of wild type (A) or \textit{GAL-BFA1} (C) cells that were budded (circles) and had metaphase (squares) or anaphase (triangles) spindle morphology was quantified for each time point. B and D) The percentage of wild type (B) or \textit{GAL-BFA1} (D) cells with anaphase spindle morphology (triangles), with Cdc14 localized to the nucleus and cytoplasm (full Cdc14 release; open diamonds), or with Cdc14 localized only to the nucleus (partial Cdc14 release; closed diamonds) was quantified (N = 100 – 200 cells).
DISCUSSION

The activation of the MEN is essential for the destruction of mitotic CDK activity at the M phase to G1 transition. Several components of the MEN, as well as the MEN regulators Bfa1 and Bub2, localize to either one or both SPBs (reviewed in Stegmeier and Amon 2004). Furthermore, mutations or conditions that perturb SPB localization of Tem1, Cdc15, Mob1, and Dbf2 have been shown to affect timely mitotic exit as well as mitotic checkpoint functions (reviewed in Musacchio and Salmon 2007; reviewed in Scarfone and Piatti 2015). Therefore, it is important to deepen our understanding of how the localization of these...
components to SPBs is regulated. Our findings highlight a positive role for Bfa1 in the localization of Tem1, which is required for MEN activation. In addition, we show that the Bfa1/Bub2 GAP complex promotes efficient cytokinesis in the absence of Tem1 by an unknown mechanism. These data provide another example of how the temporal coupling between mitotic exit and cytokinesis is established in budding yeast.
The well-established role of Bfa1 is to negatively regulate the MEN by stimulating Tem1’s GTPase activity in complex with the enzymatic component Bub2 (Pereira et al. 2000; Geymonat et al. 2003). However, increasing cellular Bfa1 protein levels using a GAL-BFA1 allele prevents mitotic exit even in the absence of Bub2 (Ro et al. 2002 and this study). We showed that in GAL-BFA1 cells there was no MEN-induced release of Cdc14 observed and the cells delay in anaphase. In contrast, the overexpression of BFA1 did not impact the early anaphase FEAR-mediated activation of Cdc14 (Figure 2). These results confirm that there is a Bub2-independent role for Bfa1 in the regulation of the MEN during mitotic exit, which functions to promote Cdc14 release from the nucleus.

We observed that the extent of the anaphase delay phenotype in GAL-BFA1 cells was somewhat variable. For example, we observed that in rich media at 25°C, 100% of GAL-BFA1 cells arrested with anaphase spindles (Figure 6B). In another set of experiments using GAL-BFA1 CDC14-3HA strain in rich media at 25°C, there was a 60 min delay in anaphase spindle breakdown (Figure 2C). However, even though anaphase spindles eventually broke down, 80% of cells remained arrested as large budded cells (Figure 2C). It is not clear why the phenotype is variable, but we speculate that cell-to-cell differences in FEAR network component levels and activity produced especially high levels of FEAR-dependent Cdc14 activation, which allowed some anaphase cells to exit from mitosis despite high levels of MEN-inhibition by GAL-BFA1. A FEAR-dependent mitotic exit despite MEN-inhibition has been previously shown in cells with mispositioned anaphase spindles. Falk et al. demonstrated that a small number of otherwise wild type cells exit from mitosis inappropriately, even in the presence of mispositioned spindles. However, this inappropriate mitotic exit is completely prevented in cells where the FEAR network has been inactivated by the deletion of the FEAR components SPO12 or SLK19 (Falk et al. 2016). If FEAR activity were to be a prerequisite for a proportion of GAL-BFA1 anaphase cells to exit from mitosis, the deletion of SPO12 in these cells would be predicted to extend the anaphase delay.

**BFA1 overexpression inhibits MEN activity but not FEAR activity**

**BFA1 overexpression does not inhibit MEN through symmetric SPB localization**

Bfa1, when present in the cell at endogenous levels, localizes preferentially to the dSPB (Pereira et al. 2000). When the protein is overexpressed, we see that a significant proportion of tagged Bfa1 is present at the dSPB and also in the cytoplasm (Figure 3A, 3B). Previous studies have suggested that a shift from symmetric Bfa1-Bub2 SPB localization to an asymmetric localization pattern precedes the activation of Tem1 and mitotic exit (Pereira et al. 2000; Pereira and Schiebel 2001; Molk et al. 2004; Fraschini et al. 2006; Maekawa et al. 2007; Geymonat et al. 2009; Kim et al. 2012). In addition, Cdc5 inactivates Bfa1 in anaphase by phosphorylating Bfa1 (Hu et al. 2001; Geymonat et al. 2003). Therefore, the overexpression of BFA1 could potentially overwhelm the Cdc5-dependent inhibitory phosphorylation of the protein, and thereby contribute to Bfa1 hyperactivity. However, our finding that GFP-Bfa1 localized asymmetrically to the dSPB in GAL-BFA1 cells argues against this, as the Bfa1-4A mutant, in which the four Cdc5 phosphorylation sites in Bfa1 are mutated, displays a symmetric localization pattern (Kim et al. 2012). This observation further suggested that the block in MEN activation in the presence of increased Bfa1 protein was not due to increased Bfa1 inhibitory activity.

**Bfa1 promotes Tem1 SPB localization**

Several lines of evidence indicate that BFA1 overexpression deters mitotic exit by preventing proper localization of Tem1 to the dSPB. First, we show that Tem1 is delocalized from SPBs in GAL-BFA1 strains (Figure 3B, 3C). Tem1 localization to SPBs is likely to be a pre-requisite for mitotic exit. Tethering the protein to the plasma membrane using a Tem1-CAAX fusion blocks mitotic exit. On the other hand, the fusion of TEM1 to CNM67, which encodes a component of the outer plaque of...
the SPB, causes mitotic to occur even in cells that have mispositioned anaphase spindles (Valerio-Santiago and Monje-Casas 2011). We demonstrated that artificially tethering Tem1 to SPBs constitutively using the TEM1-CNM67 allele leads to a partial suppression of GAL-BFA1 mitotic exit defects (Figure 4). This finding confirms that removal of Tem1 from the SPBs is a key mechanism by which the overexpression of BFA1 blocks mitotic exit and argues for a positive function for Bfa1 in mitotic exit regulation.

Although mitotic exit did proceed to some extent in GAL-BFA1 TEM1-CNM67 cells, a significant proportion of anaphase cells (31%) still had Cdc14 sequestered in the nucleolus (Figure 4E). We propose that this is due to the weak allele strength of the TEM1-CNM67 allele. Using a checkpoint bypass assay, Chan and Amon previously determined that hyperactive alleles of TEM1 confer differing levels of MEN hyperactivation. Specifically, the GAL-TEM1 allele produced the highest MEN activation level in this assay, while both TEM1-CNM67 and

![Figure 6](image-url)
TEM1-2micron alleles produced lower levels of MEN activation (Chan and Amon 2009). In keeping with this, we observed that only the presence of GAL-TEM1 completely abolished GAL-BFA1 inhibition of mitotic exit (Figure 6). In addition, we found that GAL-BFA1 posed no impact on the SPB localization of the downstream MEN kinase Dbf2 when the effects on Tem1 were bypassed. Specifically, cells lacking
TEM1 and kept alive using a hyperactive CDC15-UP allele showed normal recruitment of Dbf2 to SPBs both in the presence and absence of overexpressed BFA1 (Figure 7A). Together, these results support the conclusion that Tem1 mislocalization is the central mitotic exit defect in GAL-BFA1 cells.

Why does the TEM1-CNM67 allele display lower levels of activity, despite the fact that Tem1 localization to SPBs is a prerequisite for its activation? One reason could be that the Tem1-Cnm67 fusion at the SPBs is subject to increased GAP activity, which prevents the protein from being highly active. While we acknowledge that this is possible, we do not favor this explanation because TEM1-CNM67 bfa1Δ cells, in which the GAP is inactivated, display the same spoC bypass phenotype as TEM1-CNM67 cells (Valerio-Santiago and Monje-Casas 2011). These data suggest that GAP inactivation does not enhance the activity of TEM1-CNM67 allele.

The TEM1-CNM67 allele displays a significantly more symmetric SPB localization than the wild type TEM1, and causes endogenous Bfa1 protein to localize in a symmetric manner (Valerio-Santiago and Monje-Casas 2011; Figure 4G). Here, we showed that when BFA1 is overexpressed, Bfa1, which is concentrated in high amounts at the dSPB, led to a largely asymmetric localization of the Tem1-Cnm67 chimeric protein to the dSPB (Figure 4G). This confirms that Tem1-Cnm67 and Bfa1 interact at the SPB even when Bfa1 is present at high levels. This also provides further evidence that Bfa1 acts as a receptor for Tem1 at the dSPB.

How does overexpressed BFA1 disrupt Tem1 localization to SPBs?
Tem1 requires Bfa1 for efficient loading onto SPBs, though the protein can be found on SPBs at low levels even in the absence of BFA1 (Pereira et al. 2000; Valerio-Santiago and Monje-Casas 2011). Although it is not known exactly how Tem1 associates with SPBs, Tem1 can associate with both Bfa1 and Nud1 in two-hybrid binding assays (Kim et al. 2008; Valerio-Santiago and Monje-Casas 2011). Bfa1 itself binds in a complex at the SPB outer plaque with both Nud1 and Spc72 (Gruneberg et al. 2000; Gryaznova et al. 2016). It has been suggested that in the absence of BFA1, Tem1 may bind to Nud1 (Valerio-Santiago and Monje-Casas 2011). Therefore, it is possible that the large amounts of Bfa1 that are present saturate the outer plaque, and specifically Nud1, and prevent the localization of Tem1 to this organelle. However, we favor the idea that the strong association between cytoplasmic Bfa1 in the GAL-BFA1 allele and Tem1 titrates Tem1 away from the SPB. We show that the levels of Bfa1 protein in the cytoplasm are ten-fold higher in GAL-BFA1-mCherry cells than in BFA1-mCherry cells (Figure 3A). In addition, it is important to note that in tem1Δ CDC15-UP GAL-BFA1 cells, Dbf2-EFP is observed at SPBs, presumably bound to its receptor phospho-Nud1 (Rock et al. 2013; Figure 7A). This indicates that increased levels of Bfa1 on the dSPB do not prevent the association of other MEN components with the Nud1 scaffold on the outer plaque.

Bfa1 serves as both an activator and an inhibitor of late M phase events
The essential mitotic exit function of Tem1 is to recruit Cdc15 to the SPB by an unknown mechanism (Rock and Amon 2011). Previous work has demonstrated that the fusion of BFA1 with TEM1 or with the SPB outer plaque component SPCC2 leads to increased, rather than decreased, MEN activation by increasing the residence time of Tem1 at SPBs (Valerio-Santiago and Monje-Casas 2011; Scarfone et al. 2015). Our results are consistent with the notion that Bfa1 can enhance Tem1 activity and MEN activation by bringing Tem1 to the SPB, and that preventing Tem1 localization by toggling the cellular levels of Bfa1 can conversely inhibit MEN.

The Septation Initiation Network (SIN) in S. pombe is homologous to the MEN, but controls septation and cytokinesis rather than mitotic cyclin inactivation (reviewed in Krapp and Simanis 2008). The Tem1 homolog Spg1p and the Bfa1 homolog Byr4p also localize to the SPB in an asymmetric manner (Cerutti and Simanis 1999; Li et al. 2000). Byr4p serves as a bridge on the SPB between Spg1p and the Bub2 homolog Cdc16p, which is the enzymatic component of the Byr4p-Cdc16p two-component GAP complex (Furge et al. 1998). It was previously shown that overexpression of Byr4p blocks SIN activation and cytokinesis due to the titration of Spg1p GTPase off the SPB (Li et al. 2000). This suggests that the cellular levels of both Bfa1 and Byr4p could similarly be important to ensure timely mitotic exit or cytokinesis respectively. Interestingly, in S. pombe the cellular levels of Byr4p and Spg1p appear to be tightly coordinated, and proteasomal degradation of Byr4p is initiated if Byr4p and Spg1p are not complexed within the cell (Krapp et al. 2008). Although the stability of Bfa1 does not appear to be regulated across the cell cycle, the phosphorylation state of the protein changes dramatically with cell cycle stage (Hu et al. 2001). Specifically, phosphorylation by both Kin4 and Cdc5 are important to activate or inhibit the GAP activity of Bfa1 respectively (Hu et al. 2001; Geymonat et al. 2003; Mackawa et al. 2007). Thus in the budding yeast system, phosphorylation status, rather than proteolysis, regulates Bfa1-Bub2 GAP activity. This allows the GAP activity of Bfa1 to be regulated independently of its positive role in mediating Tem1 localization.

Bfa1 has a positive role in cytokinesis regulation
Our findings indicate that tem1Δ CDC15-UP cells have significant cytokinesis defects. These defects appear to be caused by the absence of TEM1, rather than the hyperactivation of CDC15, since cells containing CDC15-UP alone do not exhibit chained cells (Figure 7B). Tem1 has a poorly understood role in cytokinesis that is independent of its role in promoting mitotic exit in anaphase. During cytokinesis, the septin ring splits, which allows for actomyosin ring (AMR) contraction, primary and secondary septum formation, and septum degradation in the daughter cell. This process leads to abscission between the mother and daughter cells (reviewed in Juanes and Piatti 2016). Septin ring splitting occurs before AMR contraction during cytokinesis (Lippincott et al. 2001). It was previously found that in GAL-ULP-TEM1 net1-1 cells, where the mitotic exit requirement for Tem1 has been bypassed by mutation of the Cdc14 inhibitor CFI1/NET1, both septin ring splitting and AMR contraction are defective under TEM1-depletion conditions (Lippincott et al. 2001). Another cytokinesis activator Cyk1/Iqg1 (IQGAP) is dispensable for septin ring splitting, but is required for activation of AMR contraction. Specifically, the GAP-related domain (GRD) of Cyk1/Iqg1 is required for the protein to activate AMR contraction. Interestingly, Tem1 binds specifically to the GRD of the cytokinesis activator Cyk1/Iqg1 and this raises the possibility that Tem1 regulates this protein’s activity (Shannon and Li 1999). How this regulation is achieved and where in the cell it occurs is not understood, since Cyk1/Iqg1 localizes to the AMR at the bud neck while Tem1 is not visible at this structure.
We found that the cytokinetic defects observed in tem1Δ CDC15-UP cells are suppressed by the overexpression of BFA1. Specifically, both the Dbf2 budneck localization defects, as well as the chained cell morphology defects, were largely ameliorated by GAL-BFA1 (Figure 7). Therefore, our work shows for the first time that BFA1 promotes the process of cytokinesis. We further demonstrate that the role of Bfa1 in cytokinesis occurs independently of TEM1. However, unlike the mitotic
exit defects conferred by GAL-BFA1, which are independent of BUB2 and of Bfa1/Bub2 GAP activity (Ro et al. 2002; Figure 1), the positive role for Bfa1 on efficient cytokinesis does depend upon its GAP activity. Specifically, in the absence of BUB2, tem1Δ CDC15-UP cells exhibit cytokinetic defects regardless of the presence of BFA1. These data show that the hyperactive GAP activity of GAL-BFA1 suppresses the cytokinesis defects conferred by tem1Δ. Therefore, we conclude that despite having normal Dbf2 kinase activation and cell cycle kinetics, tem1Δ CDC15-UP cells have defects in directing the active Mobi1-Dbf2 complex to the budneck to fulfill its cytokinetic role (Rock and Amon 2011; Figure 7). The accurate targeting of Mobi1-Dbf2 during cytokinesis is enhanced by GAL-BFA1 and Bub2 (Figure 7A).

What could be the role of Bfa1/Bub2 in promoting proper cytokinesis in the absence of TEM1 function? In tem1Δ CDC15-UP cells, the polo kinase Cdc5 is required for mitotic exit (Rock and Amon 2011). Recently it was shown that Bfa1 is required for the outer plaque dSPB localization of Cdc5 (Botchkarev et al. 2017). Therefore, one attractive possibility is that Bfa1/Bub2 are important to efficiently recruit Cdc5 to the dSPB. The role of Cdc5 localization to dSPBs is likely threefold: 1) the phosphorylation and inhibition of Bfa1 GAP activity (Hu et al. 2001; Geymonat et al. 2003); 2) the phosphorylation and activation of the essential Cdc15 kinase (Rock and Amon 2011); and 3) the initiation of septum formation during cytokinesis. To highlight the evidence in support of the role of Cdc5 dSPB localization in the activation of septum formation, previous studies showed that the CDC5Δ CDC12 allele produced a mutant fusion protein that localized to the budneck, but not to SPBs (Park et al. 2004). This mutant allele was capable of initiating mitotic exit in cells lacking BFA1. However, the Cdc5ΔCdc12 mutant protein was defective in initiating septum formation and cytokinesis, exhibiting chained cell growth. In contrast, the CDC5ΔCNM67 allele, which localized to SPBs but not to the budneck, exhibited proper mitotic exit, as well as efficient septum formation and cytokinesis. The authors concluded that Cdc5 SPB localization was required for efficient cytokinesis (Park et al. 2004).

If Bfa1/Bub2 are needed to promote Cdc5 association with dSPBs particularly in the absence of TEM1, then what is the purpose of Cdc5 dSPB localization in promoting cytokinesis? More recently, it was shown that Cdc5 inhibits the GTPase Cdc42 in late anaphase, which was also required for efficient septum formation (Atkins et al. 2013). However, it is not yet known whether the outer plaque SPB localization of Cdc5 is necessary for inhibition of Cdc42. In addition, it is not yet known whether the effects of Cdc5 on cytokinesis occur in a Mobi1-Dbf2 dependent manner. In this context, it would be important to examine whether GAL-BFA1 cells display either decreased or increased recruitment of Cdc5 to the dSPB in order to determine whether the effects of GAL-BFA1 on cytokinesis are mediated through Cdc5. While our results clearly demonstrate that Dbf2 recruitment to the primary septum is reduced in the absence of TEM1, the links between Bfa1, Cdc5, and Mobi1-Dbf2 function in cytokinesis have not been explored.

What is the significance of this dual positive and negative role for Bfa1 on Cdc14 regulation and mitotic exit? Why does Bfa1 have an additional positive function in cytokinesis regulation? One possible explanation is that the use of these same cellular components for multiple cell cycle functions allows for the temporal coupling of mitotic exit and cytokinesis (reviewed in Seshan and Amon 2004). This is critical in order for cells to restrain cytokinesis until the completion of chromosome segregation. In the S. pombe system, the SIN pathway regulates septation and cytokinesis, but not mitotic exit (reviewed in Krapp and Simanis 2008). Consequently, the deletion of Byr4p, the Bfa1 homolog in fission yeast, causes lethality due to resulting hyperactivity of Spg1p followed by multiple rounds of septation. Thus, in the absence of byr4, the process of septation is completely uncoordinated with other mitotic events (Song et al. 1996). Importantly, this is because cells lacking Byr4p are still capable of localizing Spg1p to SPBs, which allows for hyperactivation of the SIN (Sohrmann et al. 1998; Cerutti and Simanis 1999). In the absence of BFA1 in S. cerevisiae, Tem1 localizes to both SPBs later in anaphase than in wild type cells, and cell cycle progression is normal in an unperturbed cell cycle (Pereira et al. 2000; Valerio-Santiago and Monje-Casas 2011). Perhaps this is due to the fact that, as we have shown here, Bfa1 is a key anchor for Tem1 at SPBs. The weaker localization of Tem1 in the absence of BFA1 may safeguard budding yeast cells from MEN hyperactivity and prevent bfa1Δ lethality. Further investigation of the role of Bfa1 as a scaffold at the dSPB will lead to clarification of the role of this multi-functional protein in the efficient and accurate execution of late M phase events.

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