Tem1 localization to the spindle pole bodies is essential for mitotic exit and impairs spindle checkpoint function

Mauricio Valerio-Santiago[1,2] and Fernando Monje-Casas[1,2]

[1]Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER) and [2]Departamento de Genética, Universidad de Sevilla, E-41092 Sevilla, Spain

The mitotic exit network (MEN) is a signaling cascade that triggers inactivation of the mitotic cyclin-dependent kinases and exit from mitosis. The GTPase Tem1 localizes on the spindle pole bodies (SPBs) and initiates MEN signaling. Tem1 activity is inhibited until anaphase by Bfa1-Bub2. These proteins are also part of the spindle position checkpoint (SPOC), a surveillance mechanism that restrains mitotic exit until the spindle is correctly positioned. Here, we show that regulation of Tem1 localization is essential for the proper function of the MEN and the SPOC. We demonstrate that the dynamics of Tem1 loading onto SPBs determine the recruitment of other MEN components to this structure, and reevaluate the interdependence in the localization of Tem1, Bfa1, and Bub2. We also find that removal of Tem1 from the SPBs is critical for the SPOC to impede cell cycle progression. Finally, we demonstrate for the first time that localization of Tem1 to the SPBs is a requirement for mitotic exit.

Introduction

After the genome is duplicated and chromosomes are distributed between the mother and daughter cells during anaphase, cells prepare to exit from mitosis. Mitotic exit is determined by the inactivation of mitotic Cdks (for review see Stegmeier and Amon, 2004). In *Saccharomyces cerevisiae*, Cdk inactivation is triggered by the phosphatase Cdc14 (Visintin et al., 1998). Cdc14 is sequestered in the nucleolus from G1 to metaphase, and only at anaphase onset is it released from its inhibitor, Cfi1/Net1 (Shou et al., 1999; Visintin et al., 1999). Once Cdc14 is released, it reverses phosphorylation events promoted by mitotic Cdks, which eventually determines their inactivation. Two different signaling pathways control the release of Cdc14 from the nucleolus. The Cdc14 early anaphase release (FEAR) network promotes an initial release of the phosphatase in the first stages of anaphase (Rock and Amon, 2009). However, a second signaling cascade, the mitotic exit network (MEN), is necessary to keep a sustained release of Cdc14 and to fully inactivate mitotic Cdk activity (Jaspersen et al., 1998; Lee et al., 2001; Stegmeier and Amon, 2004).

Tem1 is a GTPase that initiates MEN signaling (Shirayama et al., 1994; Lee et al., 2001). The two-component GTPase-

activating protein (GAP) Bfa1-Bub2 is a negative regulator of Tem1 (Pereira et al., 2000; Geymonat et al., 2002). Tem1, Bfa1, and Bub2 localize to the spindle pole bodies (SPBs), the equivalent of the centrosomes in yeast (Pereira et al., 2000). Nud1, a component of the SPB, functions as an anchor for MEN components to this structure (Gruneberg et al., 2000). Bfa1 and Bub2 are thought to keep Tem1 in an inactive GDP-bound state during most of the cell cycle (Bardin et al., 2000; Pereira et al., 2000; Geymonat et al., 2002). The kinase Kin4 maintains the GAP in an active state by preventing the inhibitory phosphorylation of Bfa1 by the polo-like kinase Cdc5 (Hu et al., 2001; D'Aquino et al., 2005; Pereira and Schiebel, 2005; Maekawa et al., 2007). At anaphase onset, one of the SPBs enters the daughter cell, and inhibition of Tem1 by Bfa1-Bub2 is alleviated. At the same time, MEN signaling is stimulated by Lte1, a protein that localizes to the bud cortex (Bardin et al., 2000; Pereira et al., 2000). It is not yet known how this protein activates the MEN, but its role in mitotic exit is not mediated by a modification of Tem1 activity (Geymonat et al., 2009). Once Tem1 is activated, it initiates a signaling cascade that includes the Cdc15 and Dbf2 kinases (Jaspersen et al., 1998; Lee et al., 2001). Dbf2, together with its associated factor Mob1, promotes Cdc14 release from

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 192 No. 4 599–614
www.jcb.org/cgi/doi/10.1083/jcb.201007044

JCB **599**

THE JOURNAL OF CELL BIOLOGY

the nucleolus and maintains the phosphatase in the cytoplasm (Mohl et al., 2009).

Bfa1-Bub2 localizes asymmetrically to the SPB that enters the daughter cell during anaphase (dSPB; Bardin et al., 2000; Pereira et al., 2000). Tem1 localization to the SPBs is highly dynamic (Molk et al., 2004; Monje-Casas and Amon, 2009), and it has been shown to be dependent on both Bfa1 and Bub2 (Pereira et al., 2000). Tem1 is also enriched on the dSPB during anaphase, but it is not as asymmetric as Bfa1-Bub2 (Bardin et al., 2000; Molk et al., 2004). Based on its localization, it has been assumed that Tem1 signals mitotic exit from the SPB. However, this has not yet been formally demonstrated.

To properly distribute the genetic material, the spindle must be correctly positioned along the mother–daughter cell axis. The spindle position checkpoint (SPOC) is a surveillance mechanism that verifies the location of the mitotic spindle and restrains cell cycle progression until one SPB enters the daughter cell in anaphase (for review see Lew and Burke, 2003). Bfa1, Bub2, and Kin4 are key members of the SPOC. Upon SPOC activation, Kin4 loads onto both SPBs and increases the turnover of Bfa1-Bub2 on this structure (Caydasi and Pereira, 2009). A stable association of the GAP with the SPBs interferes with the function of the SPOC (Caydasi and Pereira, 2009). A redistribution model has been proposed by which malfunction of the SPOC could be based on a reduction of the cytoplasmic pool of Bfa1 and Bub2 that would normally spread throughout the cytoplasm, inhibiting mitotic exit (Caydasi and Pereira, 2009).

Despite numerous studies to analyze the effects of interfering with the normal localization of Bfa1-Bub2 to the SPBs, little is known about the consequences of altering the dynamics and the pattern of Tem1 localization on cell cycle progression and the SPOC function. Here, we investigate the effects of differential targeting of the GTPase and provide new insights on the regulation of the MEN and the SPOC. We find that an increase in the residence time of Tem1 on the SPBs determines the recruitment of other MEN components to this structure, whereas it does not lead to a precocious mitotic exit due to the concerted action of Bfa1 and Clb2. Additionally, we reevaluate the interdependence of Bfa1, Bub2, and Tem1 localization, and show that Tem1 can still load onto SPBs in the absence of the GAP and that Tem1 can alter the pattern of Bfa1 localization. By constitutive targeting of Tem1 to the SPBs, we also demonstrate that removal of this protein from the spindle poles is critical for SPOC function. Thus, we propose an exclusion model by which the increase observed in the turnover of Bfa1-Bub2 on the SPBs after SPOC activation contributes to impede the association of Tem1 to this structure. Finally, we demonstrate for the first time that localization of Tem1 to the SPBs is a requirement for mitotic exit.

Results

Constitutive targeting of Tem1 to SPBs

Tem1 is a highly mobile protein that localizes preferentially to the SPB that enters the daughter cell during anaphase (dSPB; Molk et al., 2004; Monje-Casas and Amon, 2009). To determine the role of Tem1 localization and dynamicity on the SPBs in

MEN signaling, we constitutively targeted Tem1 to SPBs. Cnm67 is an integral component of the outer plaque of the SPB (Schaerer et al., 2001). A fusion of Cnm67 and Tem1 forced the GTPase to constitutively load onto SPBs. Localization of this chimera was found to be symmetric from SPB duplication until the end of mitosis (Fig. 1, A and B).

Localization of Bfa1 is similar to that of Tem1, but Bfa1 is more stable on the dSPB and more asymmetric than Tem1 in late anaphase (Molk et al., 2004; Monje-Casas and Amon, 2009). To achieve a constitutive targeting of Tem1 to the SPBs but in an asymmetric manner, we also fused Tem1 and Bfa1. Localization of the Bfa1–Tem1 chimera resembled that of Bfa1: the protein started to localize asymmetrically in metaphase (Fig. 1 A), and this asymmetry was completely established by anaphase (Fig. 1 B).

Cells carrying *TEM1* fused to a degron module, and under the control of the *GAL1-10* promoter (*GAL-UPL-TEM1*; Shou et al., 1999), as the only source for Tem1, were transformed with centromeric plasmids expressing *TEM1*, *CNM67–TEM1*, or *BFA1–TEM1* under the control of the endogenous *TEM1* promoter. The levels of expression for each protein are shown in Fig. S1 A. The growth of *GAL-UPL-TEM1* cells in glucose-containing media was similar for cells carrying the plasmids with the Tem1 chimeras or a plasmid with the wild-type copy of the *TEM1* gene (Fig. 1 C). Our results show that constitutive targeting of Tem1 to the SPBs does not have a great impact on the viability of cells, regardless of whether the protein localizes in a symmetric or a mainly asymmetric manner.

Cnm67–Tem1 and Bfa1–Tem1 chimeras increase the residence time of Tem1 on SPBs

To determine the dynamicity of the Tem1 chimeras on the SPBs, we performed FRAP experiments. The half-recovery time for N-terminally eGFP-tagged Tem1 (eGFP-Tem1) on SPBs in metaphase cells was 3.4 s, and ∼62% of the total signal was recovered after photobleaching (Fig. 2 A). No difference was observed in the dynamics of eGFP-Tem1 loading onto the SPBs between metaphase and anaphase cells (Fig. 2, A and B). These results are highly similar to those obtained previously for the C-terminally tagged Tem1-eGFP (half-recovery time = 4 s; percentage recovery = 60–80%; Monje-Casas and Amon, 2009).

When Tem1 was fused to Cnm67, the dynamics of loading onto the SPBs changed dramatically. No recovery of the eGFP signal could be detected for the fusion during the extension of the FRAP experiment (2 min; Fig. 2 C). The increase in the residence time of the protein on the SPB indicated that the turnover of Cnm67–Tem1 in this structure was extremely reduced. This reduced exchange rate of Tem1 on the SPBs as a consequence of its fusion to Cnm67 was observed both in metaphase and in anaphase (Fig. 2, C and D).

The mobility of Bfa1 on the SPBs differs from that of Tem1 (Caydasi and Pereira, 2009; Monje-Casas and Amon, 2009). Bfa1-eGFP is largely immobile on the dSPB. Likewise, the Bfa1–Tem1 fusion showed an increased residence time on the dSPB in comparison to Tem1 (Fig. 2, E and F). Again, no differences in the dynamics of protein loading onto the SPBs
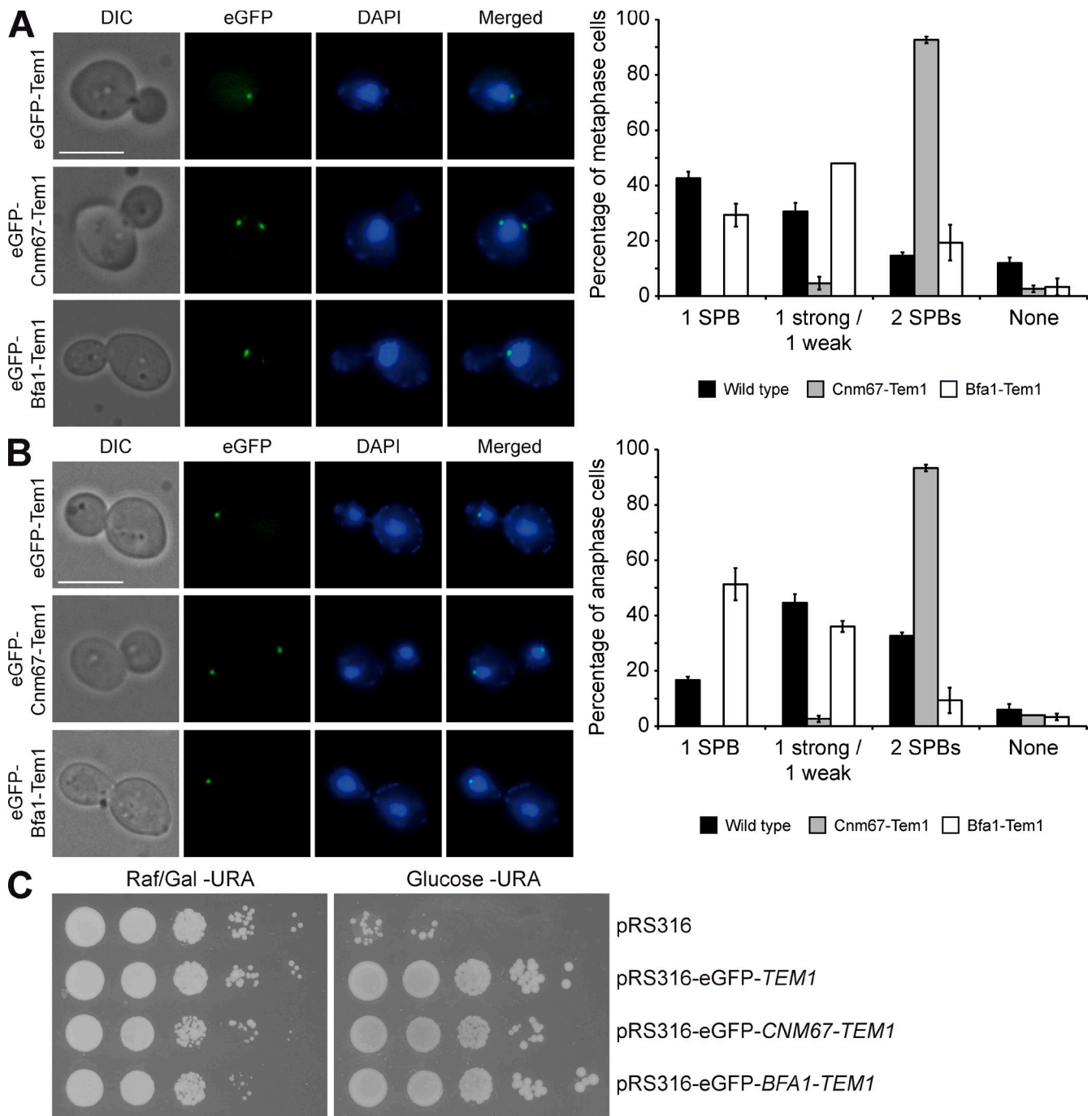
Figure 1. **Constitutive targeting of Tem1 to the SPBs.** *GAL-UPL-TEM1* cells expressing *eGFP-TEM1* (F567), *eGFP-BFA1–TEM1* (F577), or *eGFP-CNM67–TEM1* (F575) from a CEN plasmid were grown on 2% raffinose/2% galactose. (A and B) Localization of Tem1 and the chimeras (eGFP, green) in metaphase (A) and in anaphase (B) after cells were transferred to medium with 2% glucose. Nuclear morphology was assessed by DAPI (blue). A differential interference contrast (DIC) image is also shown. Loading of the eGFP-tagged protein to only one, to both (either asymmetrically [1 strong /1 weak] or symmetrically), or to none of the SPBs was quantified for each strain. Error bars represent SD (*n* = 3). Bars, 5 μm. (C) Viability of the cells was determined by spotting 10-fold serial dilutions of the original culture on plates without uracil and with either 2% glucose or 2% galactose/2% raffinose. Plates were incubated at 25°C. Cells carrying an empty vector (F797) were used as a control.

could be observed between metaphase and anaphase (Fig. 2, E and F). As previously shown for Bfa1 (Monje-Casas and Amon, 2009), the turnover of Bfa1–Tem1 on the SPBs increased in *kar9Δ* cells with mispositioned spindles (half recovery time = 24.4 s; percentage recovery = 41.2; Fig. S1 C). In agreement with the increased residence time observed for

both Cnm67–Tem1 and Bfa1–Tem1, the amounts of Tem1 on the SPBs increased compared with wild-type cells (Fig. S1 B). Thus, our results show that constitutive targeting of Tem1 to the SPBs by its fusion to Cnm67 or Bfa1 increases the residence time of the protein on the SPB and reduces its turnover in this structure.
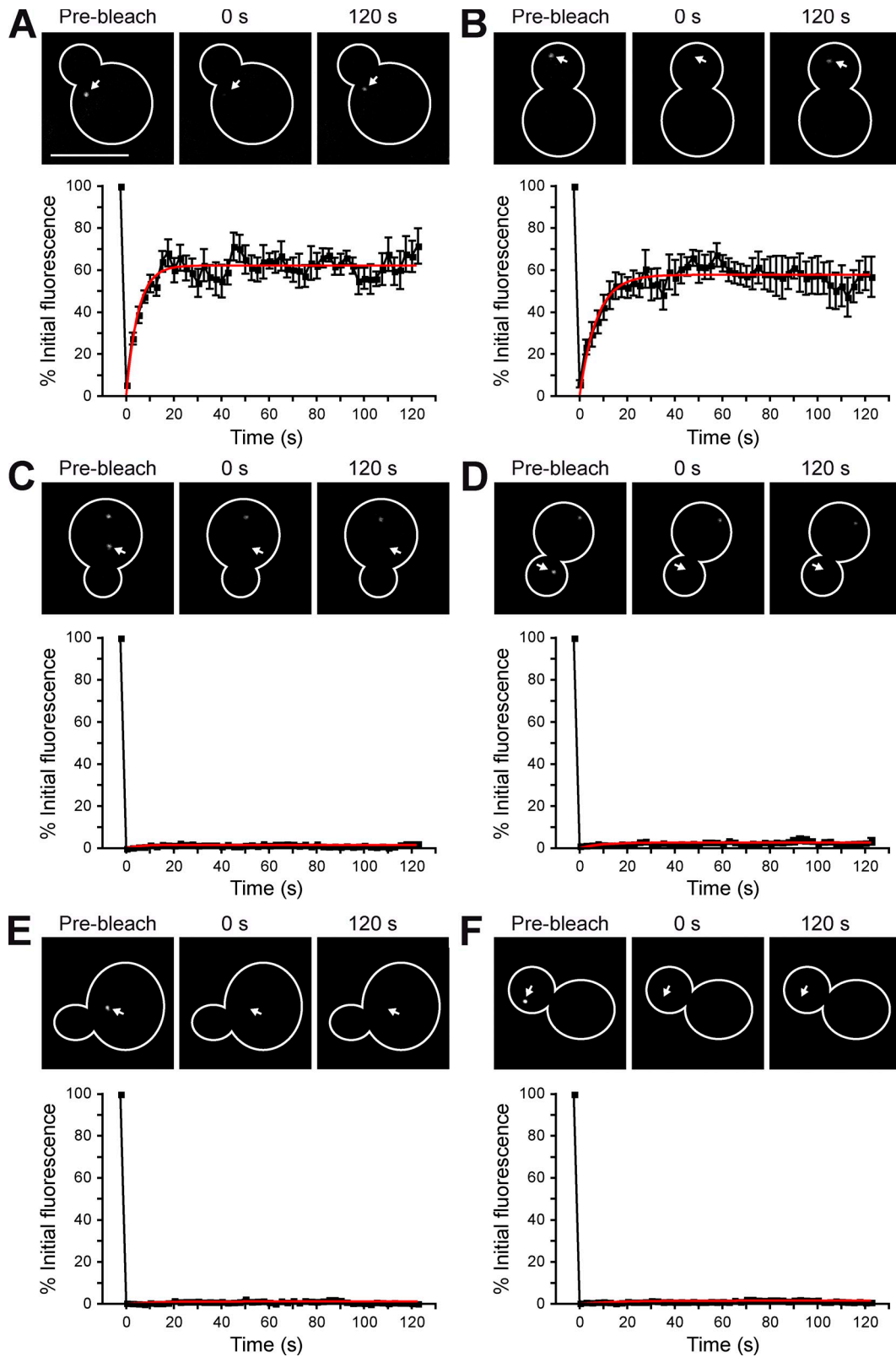
Figure 2. **The Tem1 chimeras increase the residence time of Tem1 on SPBs.** FRAP analysis in *GAL-UPL-TEM1* cells expressing *eGFP-TEM1* (A and B; F567), *eGFP-CNM67–TEM1* (C and D; F575), or *eGFP-BFA1–TEM1* (E and F; F577) and growing on 2% glucose. Images of representative experiments are shown before (Pre-bleach), immediately after (0 s), and 120 s after (120 s) the laser pulse. The cell shape is outlined in white and an arrow indicates the bleached SPB. Graphs show the percentage of initial eGFP signal recovered with time in metaphase (A, C, and E) and anaphase (B, D, and F). Error bars indicate SD (*n* = 10). The red line represents fitting of the data to an exponential function. Bar, 5 µm.

Increased residence time of Tem1 on SPBs leads to premature Cdc15 loading but not to an early Cdc14 release

Constitutive targeting of Tem1 to the SPBs, both in a symmetric (Cnm67–Tem1) and in a mainly asymmetric (Bfa1–Tem1) manner, greatly modifies the dynamics of Tem1 loading onto SPBs without having a major impact on the viability of the cells. To further determine the effects of the constitutive loading of Tem1 onto SPBs, we examined cell cycle progression in cells carrying the Tem1 fusions as the only source of Tem1. We constructed cells with the endogenous *TEM1* gene deleted and the *CNM67–TEM1* or *BFA1–TEM1* fusions integrated into the genome. The integration of the gene fusions in a single copy was checked by Southern blotting (unpublished data). As previously shown for *GAL-UPL1-TEM1* cells transformed with plasmids encoding the chimeras (Fig. 1 C), integration of *CNM67–TEM1* or *BFA1–TEM1* allowed for the growth of *tem1Δ* cells with no apparent loss of viability (Fig. S2 A).

Wild-type and *tem1Δ* cells expressing Cnm67–Tem1 or Bfa1–Tem1 were synchronized in G1 using a pheromone. After removal of the pheromone, cells were allowed to enter the cell cycle in a synchronous manner. The cells carrying the Tem1 chimeras progressed through the cell cycle as wild-type cells, and no premature entry into anaphase or mitotic exit delay could be observed (Fig. 3 A). To further characterize these cells, we checked the localization of other MEN components. Previous data suggest that loading of Cdc15 onto the SPBs triggers activation of the MEN (Visintin and Amon, 2001). In wild-type cells carrying a 3HA-tagged version of Cdc15, no signal could be observed for this protein on the SPBs until cells entered anaphase (Fig. 3, B and C; Visintin and Amon, 2001). However, cells carrying either of the Tem1 fusions showed premature Cdc15 loading onto the SPBs during metaphase (Fig. 3, B and C). Localization of Cdc15 at the SPBs followed the expected symmetric or asymmetric pattern according to Tem1 localization. Because Cdc15 localized to SPBs in metaphase cells expressing either of the chimeras, its premature loading must be caused by the increased residence time of Tem1 on the SPBs observed for both.

Our data indicate that an increased residence time of Tem1 on the SPBs leads to untimely Cdc15 loading, but not to a premature entry into anaphase. To solve this apparent discrepancy, we also checked Dbf2 and Cdc14 localization in cells carrying the Tem1 chimeras. In agreement with the dynamics of progression through the cell cycle, and in contrast to what we observed for Cdc15, no premature loading of Dbf2 was detected in Cnm67–Tem1 or Bfa1–Tem1 cells (unpublished data), and Cdc14 was released from the nucleolus with the same kinetics as in wild-type cells (Fig. 3 D). Thus, our results show that an increased residence time of Tem1 on the SPBs by its constitutive targeting to this structure leads to premature Cdc15 loading but not to an early Cdc14 release, and suggest that additional mechanisms must exist that restrain MEN activity until anaphase onset.

Viability of the Tem1 chimeras is dependent on the concerted action of Bfa1 and Clb2

The timely release of Cdc14 in cells carrying the Tem1 fusions suggests that MEN activity must be inhibited during metaphase in these cells, although Cdc15 gets prematurely loaded onto SPBs. Viability of the cells expressing Cnm67–Tem1 was not affected even when the initial release of Cdc14 by the FEAR is promoted by inactivation of the protein phosphatase 2A, its regulatory subunit Cdc55, or the Swe1 kinase (Fig. S2, B and C; Queralt et al., 2006; Liang et al., 2009).

The mechanisms that restrain untimely MEN activation in cells that constitutively load Tem1 on the SPBs could be acting either upstream of Tem1 or directly on or downstream of Cdc15. We first checked whether viability of the cells expressing Cnm67–Tem1 was dependent on the known MEN inhibitors. Deletion of either *BFA1* or *KIN4* in cells expressing Cnm67–Tem1 did not have any effect on their viability (Fig. 4 A). Cell cycle progression was not affected in the absence of Bfa1 (Fig. 4 B), and Cdc14 was also released on time (Fig. S2 D). Although Bfa1 was not required for the viability of Cnm67–Tem1 cells, we examined whether its localization was affected by constitutive loading of Tem1 onto both SPBs. We quantified the localization of Bfa1 both in wild-type cells and in cells expressing Cnm67–Tem1. Surprisingly, although Bfa1 was mainly asymmetrically localized in wild-type cells, localization of Bfa1 became mostly symmetric in Cnm67–Tem1 cells, both in metaphase (Fig. 4 C) and in anaphase (Fig. 4 D). Localization of Tem1 to SPBs has been shown to depend on both Bfa1 and Bub2 (Pereira et al., 2000). However, loading of Bfa1 and Bub2 still occurs in cells lacking Tem1 (Pereira et al., 2000). Our results demonstrate that, despite being dispensable for Bfa1 loading onto SPBs, Tem1 can influence Bfa1 localization.

It has been recently proposed that Clb2-Cdk negatively regulates the binding of Cdc15 to the mother SPB (mSPB) and inhibits Mob1, the associated factor of Dbf2 (König et al., 2010). Therefore, we tested whether viability of the cells expressing Cnm67–Tem1 was dependent on Clb2-Cdk activity. Deletion of *CLB2* had no effect on the viability of the cells when Tem1 was constitutively loaded on the SPBs (Fig. 4 E). However, when we also deleted *BFA1* in these cells, their viability was extremely impaired (Fig. 4 E), and they showed defects in cell cycle progression consistent with premature MEN activation (unpublished data). It is worth noting that deletion of *CLB2* and *BFA1* in an otherwise wild-type background had no effect on the viability of the cells (Fig. 4 E). Thus, premature mitotic exit of *clb2Δ bfa1Δ* cells is only promoted if Tem1 accumulates on the SPBs. Our results show that the viability of cells with Tem1 constitutively loaded onto SPBs is dependent on the concerted action of Bfa1-Bub2 and Clb2-Cdk. Either one of these regulators of the MEN is sufficient to fully sustain the viability of cells expressing the Tem1 fusions, but the abrogation of both activities highly impairs their growth.

Constitutive loading of Tem1 onto SPBs impairs the SPOC, but not the spindle assembly checkpoint (SAC)

Bfa1 and Bub2 also play an important role in the SPOC. The SPOC prevents exit from mitosis until the anaphase nucleus is correctly positioned (for review see Lew and Burke, 2003). Although neither progression through the cell cycle nor viability of the cells were greatly affected, we examined whether
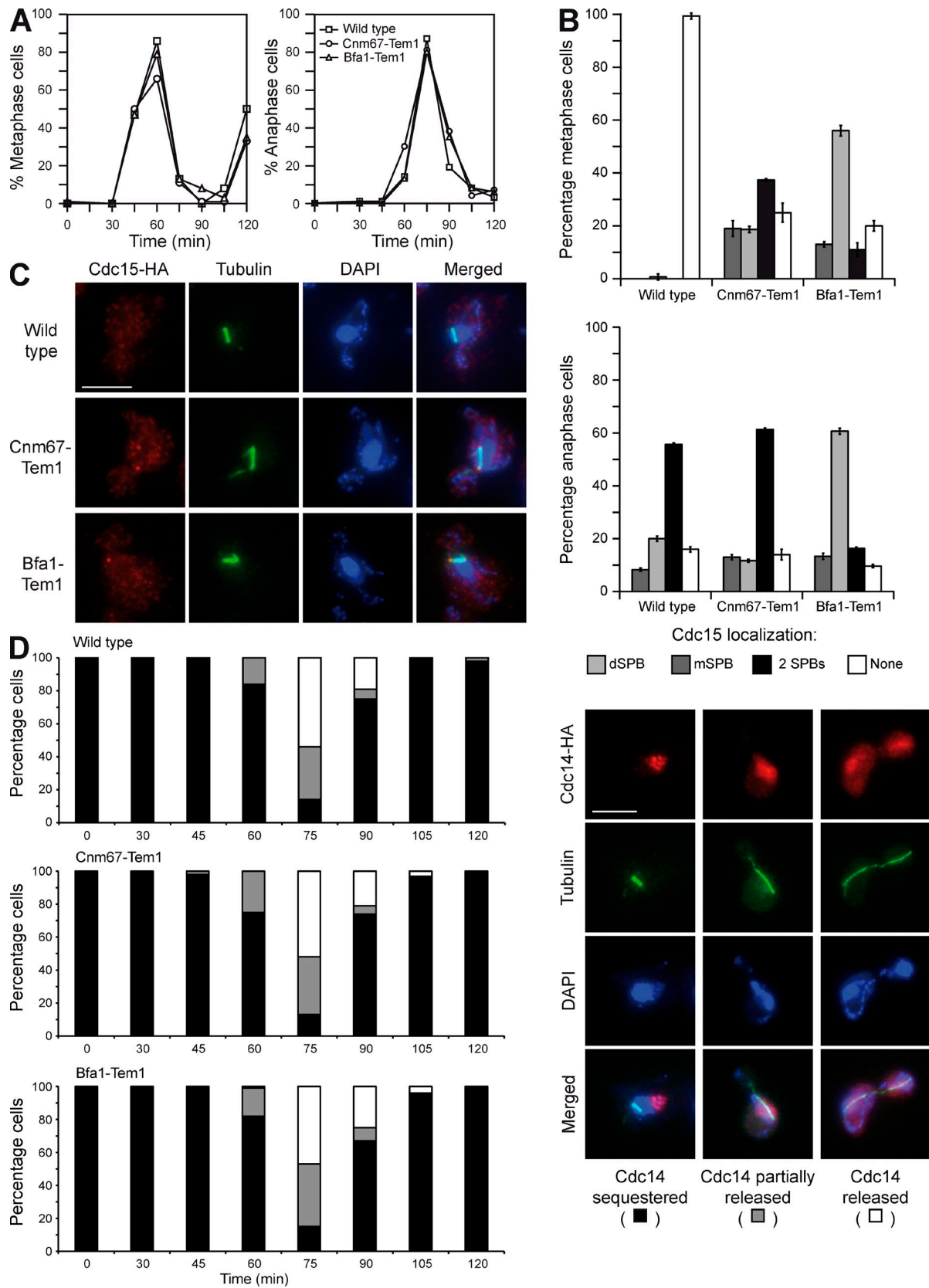
Figure 3. **Increased residence time of Tem1 on SPBs leads to premature Cdc15 loading but not to an early Cdc14 release.** (A) Wild-type (F663) and *tem1Δ* cells carrying *eGFP-BFA1–TEM1* (F679) or *eGFP-CNM67–TEM1* (F667) fusions integrated at the *URA3* locus were grown in rich media with 2% glucose (yeast peptone dextrose [YPD]) at 30°C, arrested in G1 with pheromone, and released into fresh media. Cell cycle progression was determined by spindle (tubulin) and nuclear morphology (DAPI). Percentages of metaphase and anaphase cells are shown for each time point. (B and C) Percentage of Cdc15-3HA loading onto the dSPB, mSPB, both SPBs, or none (B) for wild-type (F94) and cells expressing Bfa1–Tem1 (F678) or Cnm67–Tem1 (F677),
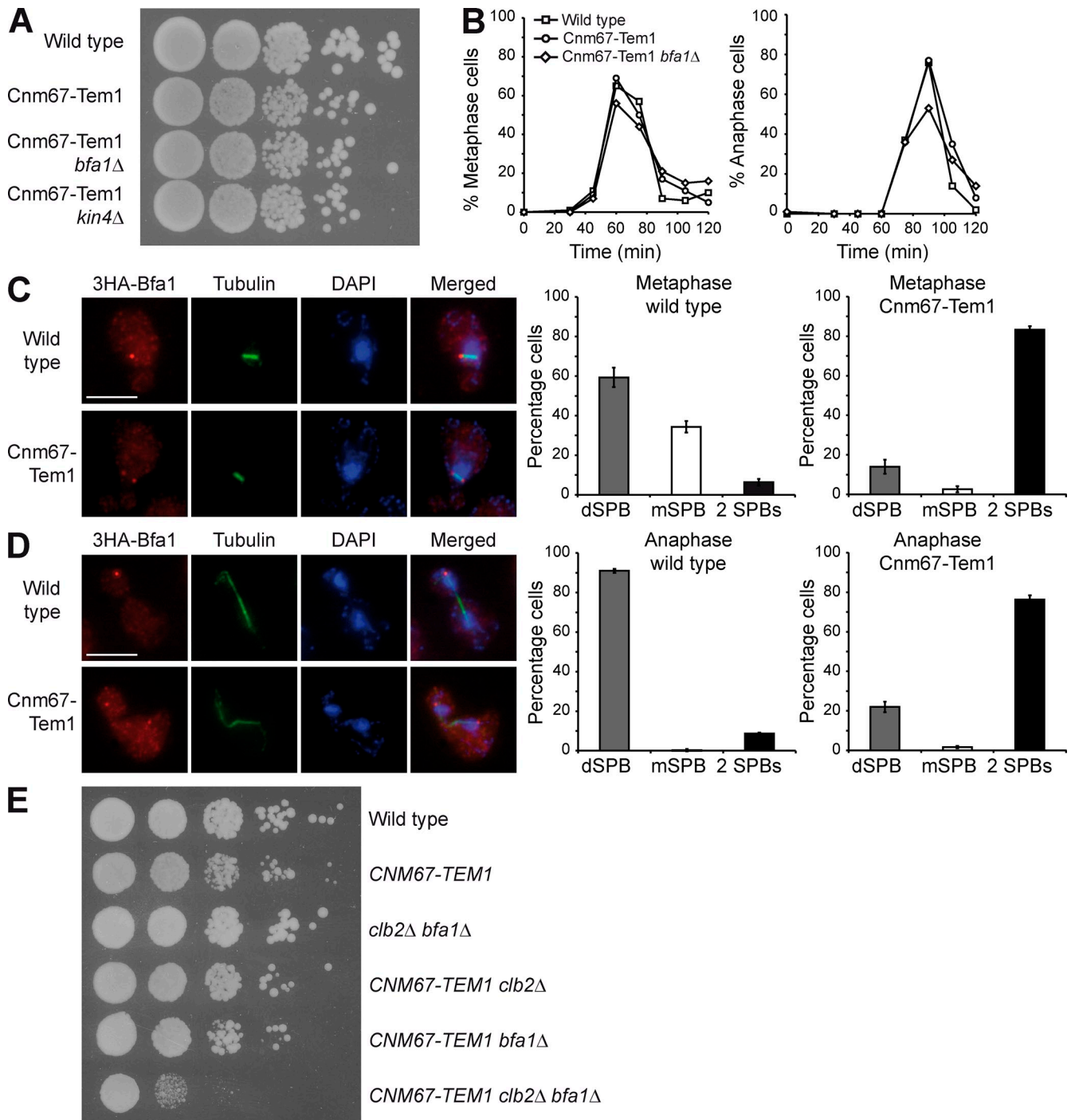
Figure 4. **Viability of the chimeras depends on Bfa1 and Clb2.** (A) Wild-type (F496) and *tem1Δ* cells expressing Cnm67–Tem1 alone (F637) or in combination with *BFA1* (F657) or *KIN4* (F745) deletions were grown on YPD at 25°C. Cells were plated on YPD in 10-fold serial dilutions spots and grown at 25°C. (B) Wild-type (F665), *tem1Δ CNM67–TEM1* (F666), and *tem1Δ CNM67–TEM1 bfa1Δ* (F786) cells were arrested in G1 and released into fresh YPD. The percentages of metaphase and anaphase cells were determined as in Fig. 3 A. (C and D) The percentage of cells showing 3HA-Bfa1 on the dSPB, mSPB, or both (2 SPBs) for wild-type (F505) and *tem1Δ* cells expressing *CNM67–TEM1* (F761) during metaphase (C) and anaphase (D). Representative images of 3HA-Bfa1 (red), tubulin (green), and DAPI (blue) are also shown. Error bars indicate SD (*n* = 3). Bars, 5 µm. (E) Wild-type (F496), *clb2Δ bfa1Δ* (F870), and *tem1Δ* cells expressing Cnm67–Tem1 in a *bfa1Δ* (F657), *clb2Δ* (F824), *clb2Δ bfa1Δ* (F875), or otherwise wild-type (F637) background were plated as in A.

as determined by immunofluorescence. Error bars indicate SD (*n* = 3). (C) Representative images showing Cdc15-3HA (red), tubulin (green), and DAPI (blue) for metaphase cells are also presented. (D, left) Percentage of cells with 3HA-Cdc14 sequestered, partially released, or fully released for the same cells shown in A (key is shown on the bottom right). (D, right) Representative images showing 3HA-Cdc14 (red), tubulin (green), and DAPI (blue) are also presented. Bars, 5 µm.
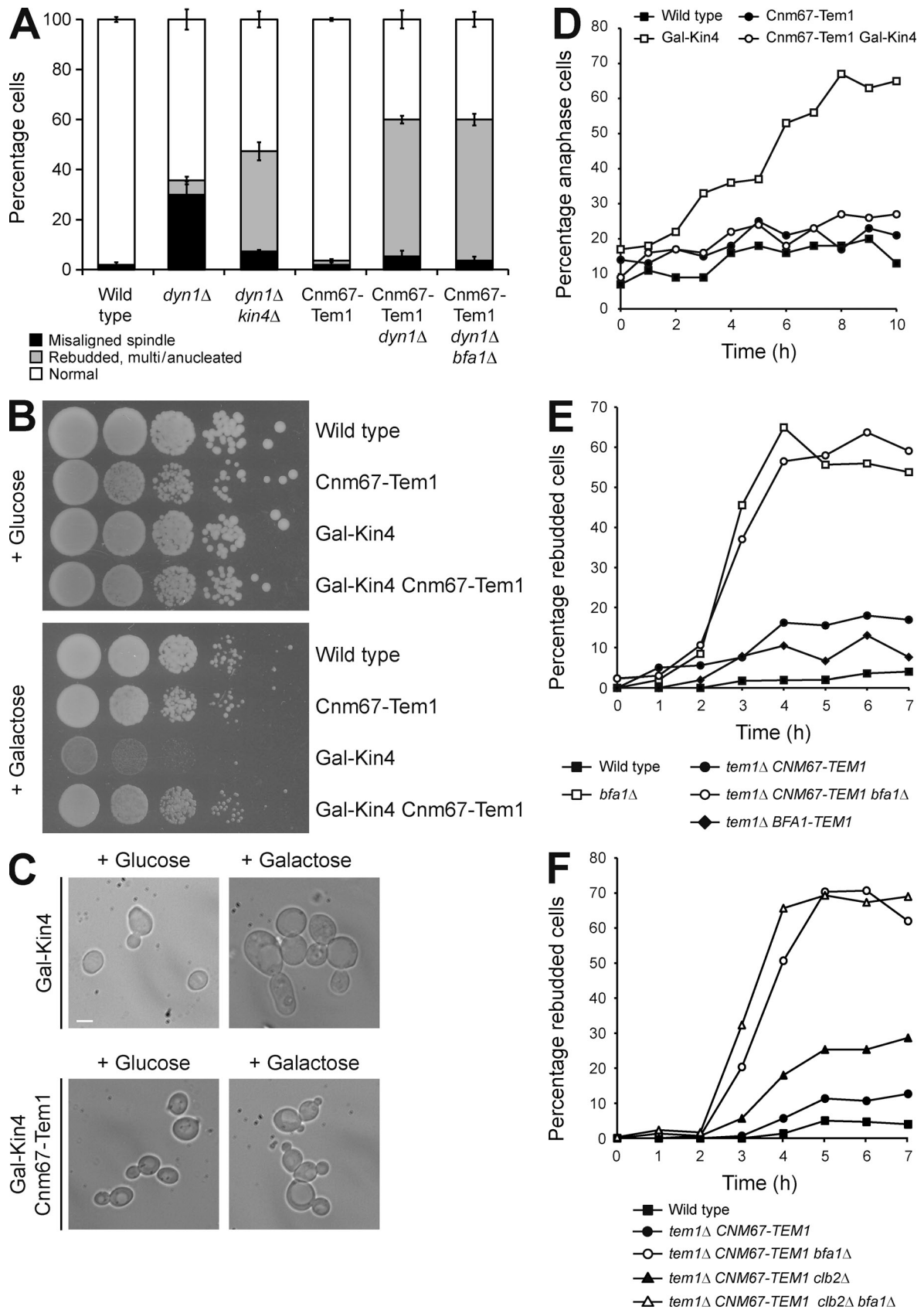
Figure 5. **Constitutive loading of Tem1 onto SPBs impairs the SPOC, but not the SAC.** (A) Wild-type (F496), *dyn1Δ* (F832), *dyn1Δ kin4Δ* (F833), *CNM67–TEM1* (F637), *CNM67–TEM1 dyn1Δ* (F659), and *CNM67–TEM1 dyn1Δ bfa1Δ* (F835) cells were grown for 24 h in YPD at 14°C. The percentage of cells with misaligned spindles, rebudded or multi/anucleated cells, and normal cells was quantified. Error bars indicate SD (*n* = 3). (B–D) Wild-type (F496), Cnm67–Tem1 (F637), Gal-Kin4 (F802), and Cnm67–Tem1 Gal-Kin4 (F803) cells were grown in YPD at 25°C. (B) Cells were plated in 10-fold

constitutive loading of Tem1 onto SPBs interferes with the SPOC. To this end, we determined the effects of expressing Cnm67–Tem1 in cells lacking cytoplasmic dynein (Dyn1), a microtubule motor protein. At low temperatures, *dyn1Δ* mutants cannot efficiently position the anaphase spindle (Yeh et al., 1995), such that their viability is highly dependent on a functional SPOC. When the SPOC is impaired, *dyn1Δ* cells inappropriately exit mitosis, which leads to an accumulation of multinucleated, anucleated, and multibudded cells (Bardin et al., 2000). Although these cells account for only 6% of *dyn1Δ* cells and 2% of Cnm67–Tem1 cells growing at 14°C, this percentage increased to 55% in *dyn1Δ* cells carrying the Cnm67–Tem1 fusion (Fig. 5 A). This percentage is similar to that shown for *dyn1Δ* cells lacking Kin4, an essential component of the SPOC (Fig. 5 A), and it indicates that the constitutive presence of Tem1 on the SPBs impairs the SPOC. Failure of the SPOC in *dyn1Δ* Cnm67–Tem1 cells was not caused by an inability of the GAP to load onto SPBs, as Bfa1 still localized to SPBs (Fig. S3). Additionally, the rebudded and multi/anucleated phenotype was not increased in *dyn1Δ bfa1Δ* cells carrying Cnm67–Tem1 (Fig. 5 A).

Kin4 promotes inhibition of mitotic exit by preventing inactivation of Bfa1-Bub2 by Cdc5 (Pereira and Schiebel, 2005). In addition, Kin4 localizes to both SPBs when the spindle is not properly aligned and blocks Tem1 loading onto this structure (D'Aquino et al., 2005). Overexpression of Kin4 also titrates Tem1 out of the SPBs and arrests cells in anaphase (D'Aquino et al., 2005). We examined the effects of constitutive targeting of Tem1 to both SPBs in cells overexpressing Kin4. Expression of Cnm67–Tem1 rescued the lethality associated with increased levels of Kin4 (Fig. 5 B). Accordingly, although overexpression of Kin4 leads to an anaphase arrest, constitutive loading of Tem1 onto both SPBs allowed cells to progress normally through the cell cycle (Fig. 5, C and D). Our results demonstrate that removal of Tem1 from the SPBs is essential for the SPOC to function. Because Bfa1 and Bub2 still load onto SPBs after SPOC activation, our results also suggest that inhibition of Tem1 by the GAP is not enough to avoid mitotic exit under these circumstances if Tem1 is not actively excluded from the SPBs.

The SAC is another surveillance mechanism that requires the inhibition of MEN signaling. The SAC is triggered by unattached kinetochores, and arrests cells in metaphase by inactivation of the anaphase-promoting complex/cyclosome (APC/C; Musacchio and Salmon, 2007). MEN must also be inhibited, as cells lacking Bfa1 or Bub2 are deficient for the SAC (Fraschini et al., 1999). Cells impaired for the SAC cannot arrest the cell cycle in the presence of nocodazole (a microtubule-depolymerizing drug), which leads to inappropriate mitotic exit
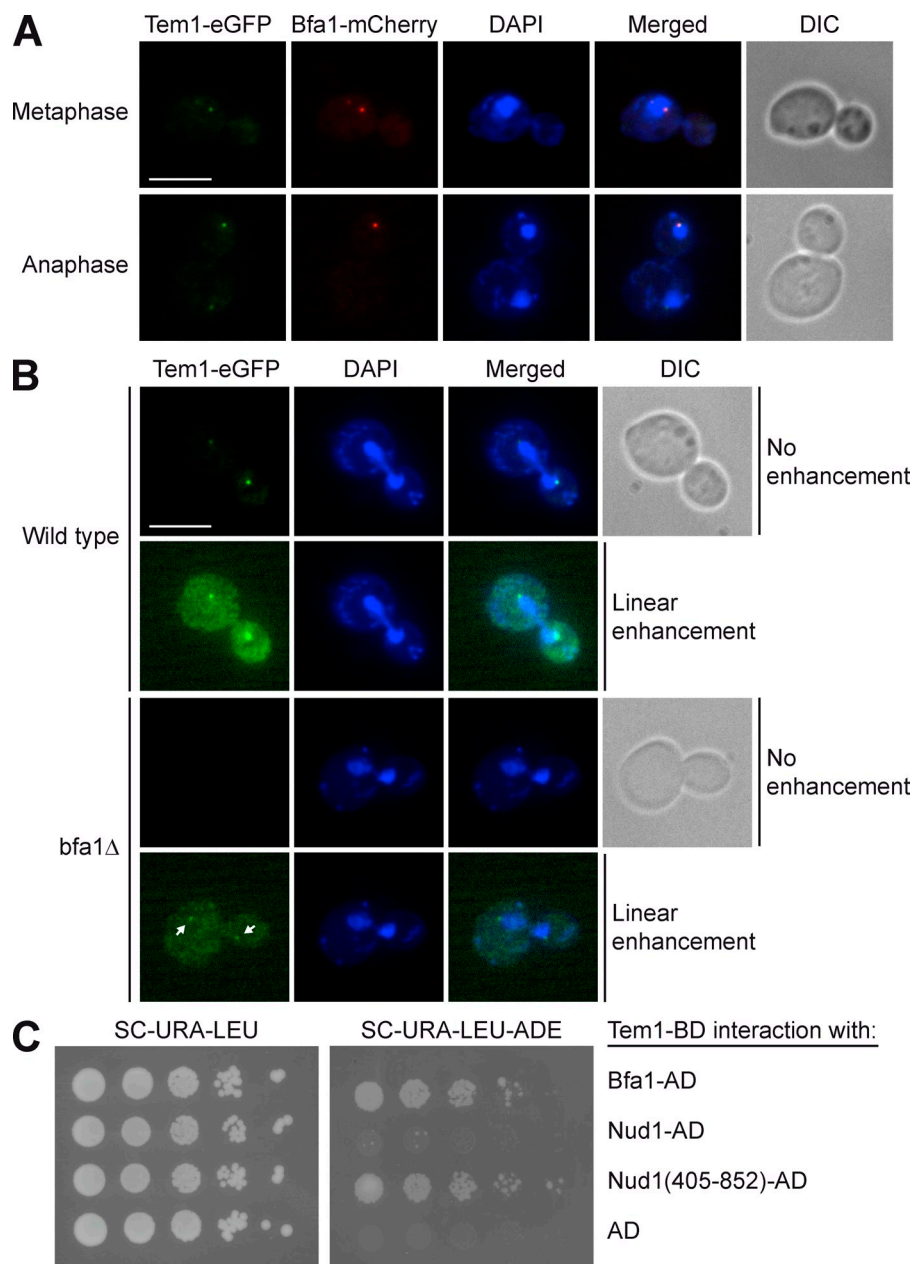
and the accumulation of multibudded cells. We tested whether cells carrying Cnm67–Tem1 or Bfa1–Tem1 as the only source for Tem1 could activate the SAC. Although there was a slight increase in cells showing the rebudded phenotype when compared with wild-type cells, both Cnm67–Tem1 and Bfa1–Tem1 cells maintained the metaphase arrest when treated with nocodazole (Fig. 5 E). The functionality of the SAC in cells expressing Cnm67–Tem1 was dependent on Bfa1, as deletion of *BFA1* in these cells increased the rebudding to the levels of a *bfa1Δ* mutant (Fig. 5 E). Cells arrested in metaphase due to activation of the SAC show high Cdk activity. However, attenuation of the Cdk activity by *CLB2* deletion in cells expressing Cnm67–Tem1 only led to a limited increase in rebudding. Furthermore, *clb2Δ bfa1Δ CNM67–TEM1* cells behaved as *bfa1Δ CNM67–TEM1* cells after nocodazole treatment (Fig. 5 F). Therefore, the differences in the functionality of the SAC and the SPOC cannot be exclusively attributed to differences in Cdk levels. Our results demonstrate that constitutive loading of Tem1 onto SPBs does not greatly disturb the SAC function, and suggest that the mechanisms by which Bfa1 restrains mitotic exit must differ for the SAC and the SPOC.

Tem1 can load onto SPBs in the absence of Bfa1

Our data demonstrate that regulation of Tem1 loading onto SPBs is critical to avoid mitotic exit when the spindle is not properly aligned. But, is Tem1 loading onto the SPBs necessary for mitotic exit? Localization of Tem1 to the SPBs is dependent on both Bfa1 and Bub2 (Pereira et al., 2000), but nevertheless the *bfa1Δ* and *bub2Δ* mutants are viable. If Tem1 loading onto the SPBs is a requirement for mitotic exit, this can only be explained if Tem1 could still load to a certain level on the SPBs in the absence of Bfa1-Bub2. In fact, it has been previously shown that Tem1 can load onto the SPBs in late anaphase in a Bfa1- and Bub2-independent manner (Pereira et al., 2000). Thus, we reevaluated the dependence of Bfa1 for Tem1 loading. First, we confirmed that Tem1 could load independently of Bfa1 in late anaphase in wild-type cells. We tagged Tem1 with eGFP and Bfa1 with mCherry in otherwise wild-type cells. Although most cells showed Tem1 on both SPBs during late anaphase, Bfa1-mCherry was mainly asymmetric (Figs. 6 A and S4 A). The lack of signal for Bfa1-mCherry on the mSPB was not caused by differences in the level of detection for both tags, as Bfa1-mCherry could be easily detected on the mSPB in metaphase cells where the Tem1-eGFP signal was weak (Fig. 6 A). Tem1-eGFP localization mimicked that of Bfa1-mCherry during the rest of the cell cycle. Thus, our results confirm that Tem1 can load independently of Bfa1 in late anaphase and that Tem1 localization is more symmetric than that of Bfa1 in this cell cycle stage.

Figure 6. Tem1 can load onto SPBs in the absence of Bfa1. (A) Localization of Tem1-eGFP and Bfa1-mCherry in an otherwise wild-type background (F544). DAPI and DIC are also shown. (B) Localization of Tem1-eGFP in wild-type (F521) and *bfa1Δ* (F539) anaphase cells. A linear enhancement of the fluorescence intensity, as well as DAPI and DIC images, is also shown. Arrows indicate the eGFP signal on the SPBs. Bars, 5 µm. (C) Two-hybrid analysis in cells expressing Tem1 fused to the DNA-binding domain of *GAL4* (Tem1-BD) and the activation domain of *GAL4* (AD), either alone (F739) or fused to Bfa1 (Bfa1-AD; F673), Nud1 (Nud1-AD; F672), or the C-terminal 447 aa of Nud1 (Nud1(405–852)-AD; F690). Cells were spotted in 10-fold serial dilutions and grown at 25°C in plates without uracil and leucine and with or without adenine.

A

| Tem1-eGFP | Bfa1-mCherry | DAPI | Merged | DIC |

Metaphase

Anaphase

B

| Tem1-eGFP | DAPI | Merged | DIC |

Wild type — No enhancement / Linear enhancement

bfa1Δ — No enhancement / Linear enhancement

C

SC-URA-LEU   SC-URA-LEU-ADE   Tem1-BD interaction with:

Bfa1-AD

Nud1-AD

Nud1(405-852)-AD

AD

We next examined the localization of Tem1-eGFP in cells lacking Bfa1. In *bfa1Δ* cells, Tem1-eGFP loading onto the SPBs was severely affected when compared with wild-type cells (Fig. 6 B). However, we could detect a signal for Tem1-eGFP on the SPBs when we applied a linear enhancement of the intensity for the eGFP signal, both in early anaphase (Fig. 6 B) and in metaphase cells (Fig. S4 B). This residual localization is not dependent on Cdc15 (Fig. S4, C and D). Our results demonstrate that, although to a much lower extent, Tem1 can load onto the SPBs in the absence of Bfa1, and this is in agreement with a requirement for Tem1 loading onto the SPBs in mitotic exit.

What could be the anchor for Tem1 on the SPB when Bfa1 is not present? One possibility is that Nud1, the anchor for Bfa1 and Bub2 (Gruneberg et al., 2000), could also serve as the protein that allows Tem1 loading onto the SPBs. We checked whether Tem1 and Nud1 interacted in a two-hybrid assay using a highly sensitive system (James et al., 1996). To validate the system, we recapitulated the previously characterized interaction between Tem1 and Bfa1 (Fig. 6 C; Kim et al., 2008). Bub2 interacts with the C-terminal region of Nud1 (aa 405–852) in a two-hybrid assay (Gruneberg et al., 2000). Similarly, we could detect a two-hybrid interaction between Tem1 and Nud1 (aa 405–802; Fig. 6 C). This interaction is not mediated by Bfa1 (Fig. S4 E). Surprisingly, we did not detect an interaction when full-length Nud1 was used (Fig. 6 C). This could be caused by an incorrect folding of the protein or a constitutive targeting of the fusion to the SPB, which could impede its ability to activate transcription of the reporter genes. In accordance, no interaction between Bfa1 or Bub2 and full-length Nud1 has been described previously with a two-hybrid. Our results suggest that Nud1 could be the anchor for Tem1 in the SPB when Bfa1 or Bub2 are not present on this structure.

Loading of Tem1 onto the SPBs is essential for mitotic exit

To determine whether loading of Tem1 onto the SPBs is a requirement for mitotic exit, we examined the effects of targeting the GTPase away from this structure. The CAAX motif from Ras2 can be used for protein targeting to the cytoplasmic side of the membrane (Srinivasa et al., 1998; Tang et al., 2009). We introduced the CAAX motif in the C terminus of Tem1 (Tem1-CAAX), and the protein was eGFP-tagged at the N terminus for detection. Tem1-CAAX showed the same intrinsic GTPase activity as Tem1, which indicates that the CAAX domain does not affect Tem1's ability to hydrolyze GTP (Fig. S5 C). Expression of Tem1-CAAX in *GAL-UPL-TEM1* cells could complement the lack of endogenous Tem1 (Fig. S5, A and B). However, and even though insertion of the CAAX motif efficiently directed Tem1 to the cell membrane, the protein still retained some ability to bind to the SPBs (Fig. S5, D and E). This result demonstrates that Tem1-CAAX is functional when loaded on the SPBs.

To further impede the localization of Tem1-CAAX to the SPBs, we deleted *BFA1*, its anchor on this structure. In *GAL-UPL-TEM1 bfa1Δ* cells, Tem1's ability to localize to the SPBs is severely decreased, but Tem1 can still load to a certain extent on this structure (Fig. 7 A). However, expression of Tem1-CAAX in these cells greatly interfered with loading of the protein (Fig. 7 A). No signal could be detected on SPBs, even after linear enhancement of the eGFP signal was applied. Thus, it is necessary to remove Bfa1 to efficiently target Tem1-CAAX away from the SPBs. To test the consequences of targeting Tem1 away from the SPBs on cell viability, we examined the growth of *GAL-UPL-TEM1 bfa1Δ* cells expressing Tem1-CAAX in glucose-containing media. The effects were striking: although *GAL-UPL-TEM1 bfa1Δ* cells expressing Tem1 from a plasmid grew normally on glucose, Tem1-CAAX could not support the growth of these cells (Fig. 7 B). This was not caused by different expression levels for Tem1 and Tem1-CAAX (Fig. 7 C). Expression of Tem1 or Tem1-CAAX had no effect on the viability when cells were grown on galactose. A detailed cell cycle progression analysis showed that mitotic exit was prevented in *GAL-UPL-TEM1 bfa1Δ* cells expressing Tem1-CAAX and growing on glucose, and the cells accumulated in anaphase (Fig. 7 D). Our results demonstrate that localization of the Tem1 to the SPBs is essential for mitotic exit.

Finally, we also checked whether the Cnm67–Tem1 or Bfa1–Tem1 fusions could suppress the lethality of a *nud1-2* mutant. The *nud1-2* cells arrest in late anaphase due to a failure to activate the MEN, but also display problems with the attachment of cytoplasmic microtubules to the outer plaque of the SPBs, which leads to nuclear migration defects (Gruneberg et al., 2000). Expression of a Spc72–Cnm67 fusion restores the problems of nuclear migration but not the cell cycle defect, as the essential function of Nud1 is related to the MEN (Gruneberg et al., 2000). If Tem1 loading on the SPBs is required for mitotic exit, then expression of the Cnm67–Tem1 fusion should recover the MEN defect of *nud1-2* cells at the restrictive temperature. Indeed, expression of Cnm67–Tem1 suppressed the lethality associated with *nud1-2* cells (Fig. 7 E). The *nud1-2* cells expressing Cnm67–Tem1, however, showed reduced viability, as they

still displayed problems positioning the spindle that were exacerbated by the impairment of the SPOC because of constitutive loading of Tem1 (Fig. 7 F). Nud1 mediates the interaction of Bfa1 with the SPBs, and the Bfa1–Tem1 fusion cannot load in *nud1-2* cells at the restrictive temperature. Accordingly, expression of the Bfa1–Tem1 fusion does not recover the lethality of a *nud1-2* mutant (Fig. 7 E). These results strongly reinforce our conclusion that loading of Tem1 onto the SPBs is a requirement for mitotic exit.

Discussion

Tem1 initiates a signaling cascade, the MEN, that maintains a sustained release of Cdc14 from the nucleolus during anaphase and determines mitotic exit (for review see Stegmeier and Amon, 2004). Tem1 localizes to the SPBs in a complex with its GAP, Bfa1-Bub2 (Pereira et al., 2000; Molk et al., 2004; Monje-Casas and Amon, 2009). Here, we investigate the consequences of altering the normal pattern of Tem1 localization on MEN signaling and spindle checkpoint function.

Constitutive targeting of Tem1 does not affect cell cycle progression

Tem1 can be constitutively targeted to the SPBs in a symmetric or in a mainly asymmetric manner by fusing the GTPase to Cnm67 (an integral component of the SPB; Schaerer et al., 2001) or Bfa1, respectively. Both fusions decrease the exchange rate of Tem1 on the SPBs, dramatically modifying its dynamics of loading onto this structure. Several lines of evidence demonstrate the functionality of the Tem1 fusions: (a) the cells expressing the chimeras do not show delayed mitotic entry into or progression through mitosis, or premature release of Cdc14, the phenotypes that characterize MEN hyperactivation (Bardin et al., 2003); (b) the fusions do not impair the SAC, and it has been shown that the GAP activity of Bfa1-Bub2 is essential for a proper SAC function, and that hyperactivation of MEN impairs the SAC (Fraschini et al., 2006; Kim et al., 2008; Geymonat et al., 2009); and (c) the simultaneous lack of Bfa1 and Clb2 highly affects the viability of cells expressing Cnm67–Tem1, but the loss of only Clb2 does not impair their growth, which suggests that the GTPase activity of the fusion and its ability to be regulated by Bfa1-Bub2 are not affected.

The increased residence time of Tem1 on the SPBs does not have a great impact on cell cycle progression, irrespective of whether Tem1 is symmetrically or asymmetrically localized. Expression of either Cnm67–Tem1 or Bfa1–Tem1 does not lead to precocious Cdc14 release, and mitotic exit occurs on time. Accordingly, Dbf2 is not prematurely loaded onto the SPBs in these cells, which is in agreement with Dbf2 localization to the SPBs and its activation being tightly linked (Visintin and Amon, 2001). Surprisingly, Cdc15 kinase precociously loads onto SPBs as a result of a reduced turnover of Tem1 in this structure. Localization of Cdc15 to the SPBs was proposed to trigger activation of the MEN and promote mitotic exit (Visintin and Amon, 2001), even though this correlation has never been demonstrated. Our results, however, are in agreement with the existence of additional regulatory mechanisms acting downstream of Tem1.
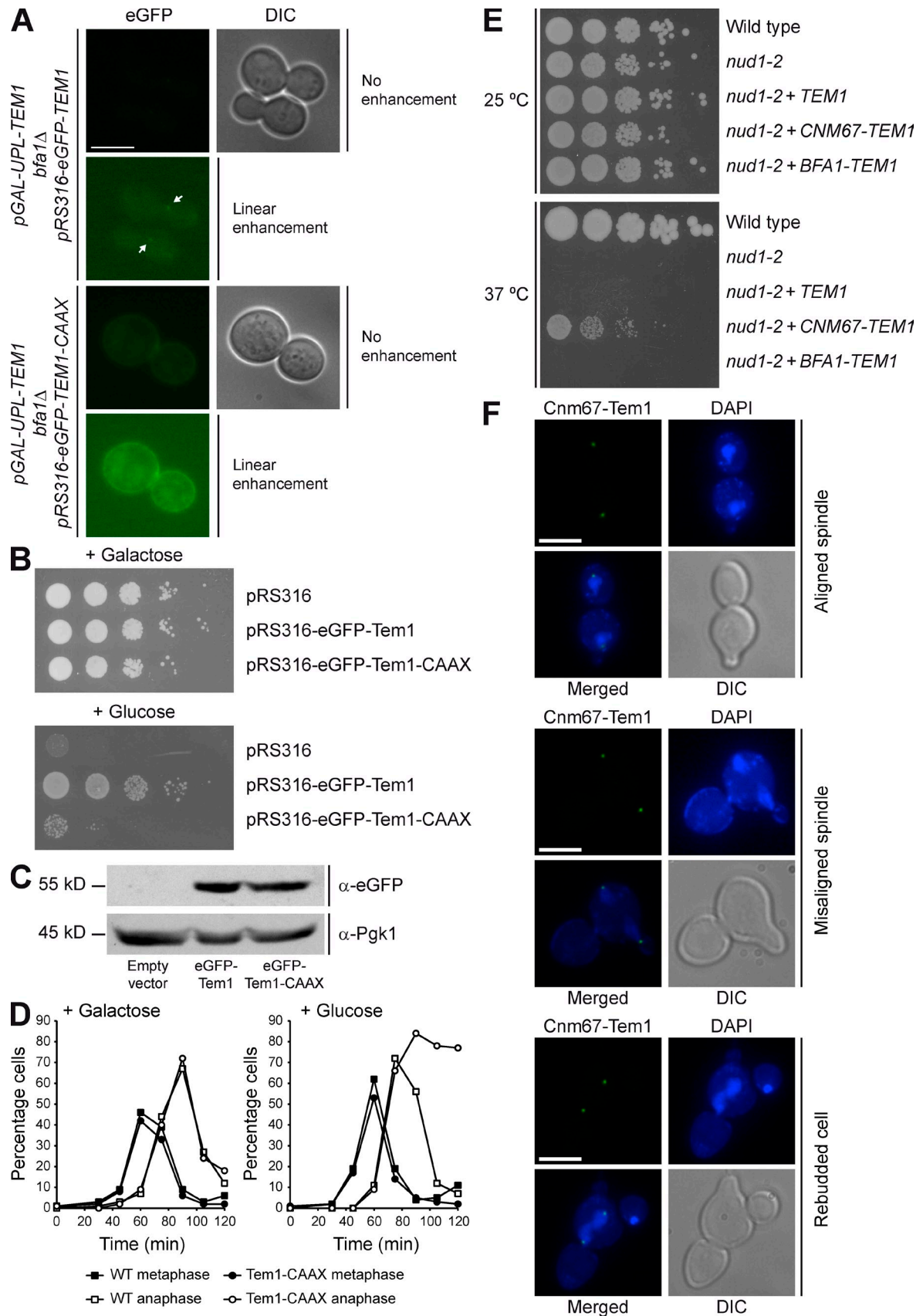
Figure 7. **Loading of Tem1 onto the SPBs is essential for mitotic exit.** (A-D) *GAL-UPL-TEM1 bfa1Δ* cells expressing *eGFP-TEM1* (F857) or *eGFP-TEM1-CAAX* (F858) from a CEN plasmid were grown on 2% galactose/2% raffinose media. (A) Localization of Tem1 and Tem1-CAAX after cells were transferred to YPD. A linear enhancement of the fluorescence intensity and a DIC image are also presented. Arrows indicate the eGFP signal on the SPBs. (B) Cells were

Premature exit from mitosis in cells expressing the Tem1 chimeras is prevented even in the absence of Bfa1-Bub2 or Kin4, which negatively regulate the GTPase (Pereira et al., 2000; Geymonat et al., 2002; D'Aquino et al., 2005). It has been recently proposed that Clb2-Cdk also controls MEN activity by inhibiting Mob1 (König et al., 2010). Surprisingly, cells lacking both Bfa1 and Clb2 are fully viable, despite lacking both negative regulators of the MEN. Our results indicate that premature mitotic exit also requires the stable association of Tem1 with the SPBs. The simultaneous lack of Bfa1 and Clb2 in cells that constitutively load Tem1 on the SPBs severely impairs cell viability. This indicates that MEN is regulated at three different levels: Tem1 activity, Tem1 localization, and Dbf2-Mob1 activity. The activity and localization of Tem1 are controlled by Bfa1-Bub2, whereas Dbf2-Mob1 is regulated by Clb2-Cdk. Therefore, a *clb2Δ bfa1Δ* mutant is viable because the lack of Bfa1 leads to a less stable association of Tem1 to the SPBs, which would impede premature mitotic exit until sufficient levels of Tem1 load on the SPBs in late anaphase by a Bfa1-independent mechanism (Molk et al., 2004; Caydasi and Pereira, 2009). The control of both the activity and the localization of Tem1 by Bfa1 is a safeguard mechanism that impedes premature activation of the MEN even in the absence of the two main regulators of the pathway. These results emphasize the importance of a proper regulation of the MEN: different levels of regulation act simultaneously to control activation of the pathway so that mitotic exit only occurs when cells ensure that the mother and daughter cell are going to receive a complete set of the genome.

Tem1 exclusion from the SPB is essential for SPOC function

The activation of the SPOC in cells with a misaligned spindle leads to a decreased residence time of Bfa1-Bub2 on the SPBs (Caydasi and Pereira, 2009; Monje-Casas and Amon, 2009). A redistribution model has been recently suggested according to which removal of Bfa1-Bub2 from the SPBs upon SPOC activation would inhibit mitotic exit in the cytoplasm by inactivating Tem1 throughout the cell (Caydasi and Pereira, 2009). Based on our results, we instead favor an exclusion model that proposes that keeping Bfa1-Bub2 away from the SPBs contributes to impeding Tem1 localization to this structure, as Bfa1 and Bub2 are the main determinants for localization of the GTPase (Pereira et al., 2000; Monje-Casas and Amon, 2009). Constitutive loading of Tem1 onto the SPBs does not present a problem for the cells until anaphase onset because other mechanisms exist that avoid an untimely release of Cdc14, as previously discussed. However, once cells enter the metaphase-to-anaphase transition, these mechanisms cannot prevent the full release of Cdc14, and it becomes essential to regulate Tem1 localization to SPBs. This cell cycle stage also corresponds with an increase in the

asymmetric localization of Tem1 (Molk et al., 2004). When the spindle is not properly aligned, phosphorylation of Bfa1 by Kin4 reduces the residence time of the GAP on the SPBs (Caydasi and Pereira, 2009), which not only prevents inactivation of the GAP activity (Maekawa et al., 2007) but also determines an exclusion of Tem1 from the SPBs (D'Aquino et al., 2005; Caydasi and Pereira, 2009). Constitutive loading of Tem1 on the SPBs causes inappropriate mitotic exit in cells with misaligned spindles, even though Bfa1 is also loaded. Thus, removal of the GTPase from the SPBs is critical for SPOC function, as the inhibitory action of the GAP alone is not sufficient to restrain cell cycle progression. Accordingly, constitutive loading of Tem1 onto the SPBs rescues the anaphase arrest induced by overexpression of Kin4, which also causes a removal of Tem1 from the SPBs (D'Aquino et al., 2005). Our results demonstrate that a proper SPOC function relies on the regulation of Tem1's localization.

The SAC, however, does not depend on this exclusion of Tem1 from the SPBs. Cells carrying the Tem1 fusions can still hold cell cycle progression after nocodazole treatment. The metaphase arrest induced by microtubule depolymerization depends on Bfa1, as Cnm67–Tem1 cells carrying a *BFA1* deletion cannot restrain cell cycle progression after nocodazole treatment. Thus, the mechanism by which Bfa1 promotes cell cycle arrest when the SAC is activated must differ from that taking place after SPOC activation. The inhibitory action of Bfa1 on Tem1 activity could be sufficient after SAC activation to restrain cell progression even when the residence time of the GTPase on the SPBs is increased, in contrast to the SPOC. Accordingly, a previous study suggests that although Bfa1-Bub2 GAP activity is not sufficient to delay exit from mitosis after SPOC activation, it is essential for a proper SAC function (Kim et al., 2008).

Interdependence on the localization of Bfa1, Bub2, and Tem1 to the SPBs

The results presented here reevaluate the interdependence of Tem1, Bfa1, and Bub2 on their localization to the SPBs. Bfa1 and Bub2 loading onto SPBs is thought to be interdependent and independent of Tem1, whereas Tem1 localization depends on both Bfa1 and Bub2 (Pereira et al., 2000). However, we have demonstrated that Tem1 can still load onto the SPBs in the absence of Bfa1, albeit inefficiently. The localization of Tem1 to the SPBs is essential for the viability of *bfa1Δ* cells, as discussed later. How does Tem1 localize to the SPBs when Bfa1 is not present? We propose that, in this situation, Nud1 becomes the anchor for Tem1 on the SPBs. Tem1 and the C-terminal region of Nud1 (aa 405–852) can interact in a two-hybrid assay. Interestingly, the Tem1 and Nud1 homologues in fission yeast have also been shown to directly interact (Morrell et al., 2004). We were not able to consistently coimmunoprecipitate Tem1 and Nud1 (unpublished data), but this may reflect a weak or

transient nature of their interaction, as previously suggested (Gruneberg et al., 2000). Alternatively, Tem1 could bind other proteins on the SPB. The direct interaction of Tem1 with the SPBs in the absence of Bfa1 is only strong enough as to accumulate normal levels of the GTPase on the spindle poles in telophase (Molk et al., 2004).

We have also found that Tem1 can influence Bfa1 loading onto SPBs, even though Bfa1 does not normally need Tem1 to localize to this structure. Bfa1 localization becomes more symmetric in cells expressing Cnm67–Tem1. Bfa1 and Tem1 interaction is essential for the proper regulation of mitotic exit. We favor the idea that Bfa1 and Tem1 tend to preferentially interact with each other so that under normal circumstances, Bfa1 controls both the activity and the localization of Tem1, based on a higher affinity of the GTPase for Bfa1 than for its alternative anchor on the SPB. This affinity would bring Bfa1 to the SPBs when Tem1 is constitutively targeted to this structure. Conversely, a higher residence time of Bfa1 on the SPBs would determine an increased presence of Tem1 on the spindle poles. This could explain why constitutive targeting of Bfa1 and Bub2 to the SPB impairs the SPOC (Caydasi and Pereira, 2009), in accordance with the exclusion model that we have proposed.

Tem1 signaling from the SPBs is required for MEN function

It is clear from our results that regulation of the localization of MEN components to the SPBs is important for the proper function of this signaling cascade. However, it has never been formally tested whether loading of Tem1 to the SPBs is essential for mitotic exit. Even though it has been recently suggested that some MEN regulation could take place at the cytoplasm (Caydasi and Pereira, 2009; König et al., 2010), we aimed to determine whether Tem1 signaling out of the SPB could trigger mitotic exit. Addition of the CAAX membrane-targeting domain to Tem1 in the absence of Bfa1, which regulates binding of the GTPase to the SPBs, impairs its ability to load onto the spindle poles. The expression of Tem1-CAAX as the only source for the GTPase is not sufficient to maintain the viability of *bfa1Δ* cells, which indicates that Tem1 must load onto the SPBs to initiate the signaling cascade that triggers mitotic exit. The anaphase arrest observed for these cells clearly demonstrates that the lethality is not caused by Tem1-CAAX being hyperactive in the absence of its negative regulator, as in this case cells would prematurely exit from mitosis. Additional support for the requirement of Tem1 loading on the SPBs in mitotic exit comes from expression of our Tem1 fusions in the *nud1-2* mutant. Bfa1, Bub2, and, hence, Tem1 do not localize to the SPBs in this mutant. Although Cnm67–Tem1, which allows for loading of Tem1 on the SPBs in *nud1-2* cells, recovered the lethality associated with this mutant, expression of Bfa1–Tem1 did not. Therefore, even though the MEN could be regulated at some level by events taking place in the cytoplasm of the cells, our results clearly demonstrate that loading of Tem1 to the SPBs is crucial for termination of mitosis.

The results presented here highlight the importance of the SPB as a signaling center during the cell cycle. A signal that is generated in this microtubule-organizing center is necessary for cells to exit mitosis, which, combined with the asymmetric localization of the proteins that trigger the signaling cascade determining mitotic exit, ensures that the cells do not enter the final stages of the cell cycle until the spindle is correctly positioned and consequently prepared to equally distribute the duplicated genome between both cells. Because the SPB is the equivalent of the centrosome in yeast cells, it will be interesting to determine whether similar signaling mechanisms operate in higher eukaryotes, especially in cells that exhibit some polarity during cell division.

Materials and methods

Strains and plasmids
All strains, except for those used for the two-hybrid assay, are derivatives of W303 and are described in Table S1. *GAL-KIN4* was generated as described in Longtine et al. (1998). Plasmids used were derivatives of pRS306 and pRS316 (Sikorski and Hieter, 1989). The *TEM1* open reading frame (ORF) under the control of its own promoter was N-terminally tagged with eGFP by a series of overlapping PCRs, adding a flexible linker of 8 aa in-between to increase the functionality of the chimeras (Sheff and Thorn, 2004). The Tem1 fusions were constructed by inserting the *CNM67* or *BFA1* ORFs within a PacI site in the linker, maintaining the linker sequence at both sides. Strains F698 (AM5271) and F699 (AM3164) were a gift from A. Marston (Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, Scotland, UK).

Immunolocalization and fluorescence microscopy
Immunofluorescence was performed as described in Visintin et al. (1999). In brief, cells were fixed for 15 min in 3.7% formaldehyde and 0.1 M potassium phosphate buffer, pH 6.4. Cells were then washed twice with 0.1 M potassium phosphate buffer, pH 6.4, and resuspended in 1.2 M sorbitol in 0.12 M K_2HPO_4/0.033 M citric acid, pH 5.9. Fixed cells were digested with 0.1 mg/ml zymolyase-100T (US Biological) and 1/10 volume of glusulase (PerkinElmer) at 30°C for 15 min, washed once, and resuspended in 1.2 M sorbitol in 0.12 M K_2HPO_4/0.033 M citric acid, pH 5.9. 3HA-Bfa1 and 3HA-Cdc15 were detected using anti-HA antibody (HA.11; Covance) at 1:500 and anti–mouse Cy3 antibody (Jackson ImmunoResearch Laboratories, Inc.) at 1:2,000. The same antibodies were used to detect 3HA-Cdc14, but at 1:200 for the primary antibody and at 1:1,000 for the secondary antibody. Anti-tubulin (Abcam) and anti–rat FITC (Jackson ImmunoResearch Laboratories, Inc.) antibodies were used at 1:500. Samples for eGFP imaging were prepared as described in Monje-Casas and Amon (2009). In brief, cells were fixed in 2.5% formaldehyde for 10 min, washed twice, and resuspended in 0.1 M potassium phosphate buffer, pH 6.4. Cells were then fixed for 10 min in 80% ethanol and resuspended in 1 mg/ml DAPI. Microscope preparations were analyzed and imaged at 25°C using a microscope (DM6000; Leica) equipped with a 100×/1.40 NA oil immersion objective lens, A4, L5, and TX2 filters, and a digital charge-coupled device camera (DFC350; Leica). Pictures were processed with LAS AF (Leica) and ImageJ (http://rsbweb.nih.gov/ij/) software.

Western blot analysis
Protein extracts were prepared using the TCA precipitation method as described in D'Aquino et al. (2005). eGFP-tagged proteins were detected with JL-8 Living colors monoclonal antibody (Takara Bio, Inc.) at 1:1,000 and anti–mouse HRP-linked antibody (GE Healthcare) at 1:2,000, and the ECL Plus reagent (GE Healthcare) was used for fluorescence imaging. Pgk1 levels were also detected using anti-Pgk1 antibody (Invitrogen) at 1:10,000 and anti–mouse HRP-linked antibody (GE Healthcare) at 1:2,000.

Protein expression, purification, and GTPase assays
Escherichia coli BL21-CodonPlus (Agilent Technologies) expressing His-Tag-*TEM1* and His-Tag-*TEM1-CAAX* from the pET28a plasmid (EMD) were grown in lysogeny broth (LB) containing kanamycin (Sigma-Aldrich) at 37°C, transferred to 25°C, and induced with 1 mM isopropyl-1-thio-β-galactopyranoside for 8 h. Cells expressing the proteins were resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, 1 mM PMSF, 10% glycerol, 0.5% NP-40, 1 mM DTT, and Complete EDTA-free protease inhibitor cocktail [Roche]) and frozen with liquid N_2. After thawing, cells were incubated with 0.5 mg/ml lysozyme (Sigma-Aldrich) and

20 µg/ml DNase I (Roche) for 30 min at 25°C, then sonicated. The extract was clarified by centrifugation at 15,000 rpm for 30 min at 4°C. The proteins were purified by affinity chromatography using the ÄKTA explorer system (GE Healthcare) and HisTrap FF Crude columns (GE Healthcare). After elution, the proteins were dialyzed against 20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM MgCl$_2$, and 0.1 mM DTT. GTPase assays were performed with 1 µg of the purified protein as detailed in Geymonat et al. (2009), but the GTPase reaction was performed at 25°C.

FRAP

FRAP experiments were performed at 25°C using a confocal microscope (TCS SP5; Leica) equipped with a 100×/1.45 NA oil immersion objective lens. Fluorescence recovery was analyzed using the equation $[F_{inf} − F(t)]/[F_{inf} − F(0)] = 1 − e^{−kt}$, where F_{inf} is the mean intensity of the bleached region after maximum recovery, $F(t)$ is the fluorescence intensity at each point, $F(0)$ is the fluorescence immediately after bleaching, k is the rate constant for exponential decay, and t is time (Monje-Casas and Amon, 2009). The slope of the straight line obtained when $1 − \{[F_{inf} − F(t)]/[F_{inf} − F(0)]\}$ is plotted versus time represents the rate constant k. The half recovery time was estimated as $t_{1/2} = \ln2/k$, whereas the percentage of recovery is given by the equation $\%R = [F_{inf} − F(0)]/[F_{initial} − F(0)]$, where $F_{initial}$ is the initial fluorescence intensity before photobleaching. Fluorescence intensity relative to background (cytoplasmic fluorescence signal) was quantified to correct for bleaching. The eGFP signal was quantified using the ImageJ software.

Online supplemental material

Fig. S1 shows additional experiments to characterize the properties of the Tem1 chimeras. Fig. S2 shows that the viability of cells expressing the Tem1 fusions is maintained in FEAR or Bfa1/Bub2 mutants. Fig. S3 shows the localization of Bfa1 in a *dyn1Δ* mutant when the spindle is mispositioned. Fig. S4 shows different experiments to demonstrate that Tem1 can still localize to SPBs in *bfa1Δ* cells. Fig. S5 shows the localization and activity of Tem1-CAAX in wild-type cells. Table S1 lists of all the strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201007044/DC1.

References

Bardin, A.J., R. Visintin, and A. Amon. 2000. A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell*. 102:21–31. doi: 10.1016/S0092-8674(00)00007-6

Bardin, A.J., M.G. Boselli, and A. Amon. 2003. Mitotic exit regulation through distinct domains within the protein kinase Cdc15. *Mol. Cell. Biol.* 23:5018–5030. doi:10.1128/MCB.23.14.5018-5030.2003

Caydasi, A.K., and G. Pereira. 2009. Spindle alignment regulates the dynamic association of checkpoint proteins with yeast spindle pole bodies. *Dev . Cell*. 16:146–156. doi:10.1016/j.devcel.2008.10.013

D'Aquino, K.E., F. Monje-Casas, J. Paulson, V. Reiser, G.M. Charles, L. Lai, K.M. Shokat, and A. Amon. 2005. The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects. *Mol. Cell*. 19:223–234. doi:10.1016/j.molcel.2005.06.005

Fraschini, R., E. Formenti, G. Lucchini, and S. Piatti. 1999. Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *J. Cell Biol*. 145:979–991. doi: 10.1083/jcb.145.5.979

Fraschini, R., C. D'Ambrosio, M. Venturetti, G. Lucchini, and S. Piatti. 2006. Disappearance of the budding yeast Bub2-Bfa1 complex from the mother-bound spindle pole contributes to mitotic exit. *J. Cell Biol*. 172:335–346. doi:10.1083/jcb.200507162

Geymonat, M., A. Spanos, S.J. Smith, E. Wheatley, K. Rittinger, L.H. Johnston, and S.G. Sedgwick. 2002. Control of mitotic exit in budding yeast. In vitro regulation of Tem1 GTPase by Bub2 and Bfa1. *J. Biol. Chem*. 277:28439–28445. doi:10.1074/jbc.M202540200

Geymonat, M., A. Spanos, G. de Bettignies, and S.G. Sedgwick. 2009. Lte1 contributes to Bfa1 localization rather than stimulating nucleotide exchange by Tem1. *J. Cell Biol*. 187:497–511. doi:10.1083/jcb.200905114

Gruneberg, U., K. Campbell, C. Simpson, J. Grindlay, and E. Schiebel. 2000. Nud1p links astral microtubule organization and the control of exit from mitosis. *EMBO J*. 19:6475–6488. doi:10.1093/emboj/19.23.6475

Hu, F., Y. Wang, D. Liu, Y. Li, J. Qin, and S.J. Elledge. 2001. Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell*. 107:655–665. doi:10.1016/S0092-8674(01)00580-3

James, P., J. Halladay, and E.A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*. 144:1425–1436.

Jaspersen, S.L., J.F. Charles, R.L. Tinker-Kulberg, and D.O. Morgan. 1998. A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*. 9:2803–2817.

Kim, J., S.S. Jang, and K. Song. 2008. Different levels of Bfa1/Bub2 GAP activity are required to prevent mitotic exit of budding yeast depending on the type of perturbations. *Mol. Biol. Cell*. 19:4328–4340. doi:10.1091/mbc.E08-02-0149

König, C., H. Maekawa, and E. Schiebel. 2010. Mutual regulation of cyclin-dependent kinase and the mitotic exit network. *J. Cell Biol*. 188:351–368. doi:10.1083/jcb.200911128

Lee, S.E., L.M. Frenz, N.J. Wells, A.L. Johnson, and L.H. Johnston. 2001. Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. *Curr. Biol*. 11:784–788. doi:10.1016/S0960-9822(01)00228-7

Lew, D.J., and D.J. Burke. 2003. The spindle assembly and spindle position checkpoints. *Annu. Rev. Genet*. 37:251–282. doi:10.1146/annurev .genet.37.042203.120656

Liang, F., F. Jin, H. Liu, and Y. Wang. 2009. The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. *Mol. Biol. Cell*. 20:3671–3679. doi:10.1091/mbc.E08-10-1049

Longtine, M.S., A. McKenzie III, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast*. 14:953–961. doi:10.1002/(SICI)1097-0061(199807)14:10<953::AID-YEA293>3.0.CO;2-U

Maekawa, H., C. Priest, J. Lechner, G. Pereira, and E. Schiebel. 2007. The yeast centrosome translates the positional information of the anaphase spindle into a cell cycle signal. *J. Cell Biol*. 179:423–436. doi:10.1083/jcb.200705197

Molk, J.N., S.C. Schuyler, J.Y. Liu, J.G. Evans, E.D. Salmon, D. Pellman, and K. Bloom. 2004. The differential roles of budding yeast Tem1p, Cdc15p, and Bub2p protein dynamics in mitotic exit. *Mol. Biol. Cell*. 15:1519–1532. doi:10.1091/mbc.E03-09-0708

Mohl, D.A., M.J. Huddleston, T.S. Collingwood, R.S. Annan, and R.J. Deshaies. 2009. Dbf2-Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. *J. Cell Biol*. 184:527–539. doi:10.1083/jcb.200812022

Monje-Casas, F., and A. Amon. 2009. Cell polarity determinants establish asymmetry in MEN signaling. *Dev. Cell*. 16:132–145. doi:10.1016/ j.devcel.2008.11.002

Morrell, J.L., G.C. Tomlin, S. Rajagopalan, S. Venkatram, A.S. Feoktistova, J.J. Tasto, S. Mehta, J.L. Jennings, A. Link, M.K. Balasubramanian, and K.L. Gould. 2004. Sid4p-Cdc11p assembles the septation initiation network and its regulators at the *S. pombe* SPB. *Curr. Biol*. 14:579–584. doi:10.1016/j.cub.2004.03.036

Musacchio, A., and E.D. Salmon. 2007. The spindle-assembly checkpoint in space and time. *Nat. Rev. Mol. Cell Biol*. 8:379–393. doi:10.1038/nrm2163

Pereira, G., and E. Schiebel. 2005. Kin4 kinase delays mitotic exit in response to spindle alignment defects. *Mol. Cell*. 19:209–221. doi:10.1016/j.molcel .2005.05.030

Pereira, G., T. Höfken, J. Grindlay, C. Manson, and E. Schiebel. 2000. The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Mol. Cell*. 6:1–10. doi:10.1016/S1097-2765(00)00002-2

Queralt, E., C. Lehane, B. Novak, and F. Uhlmann. 2006. Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. *Cell*. 125:719–732. doi:10.1016/j.cell.2006.03.038

Rock, J.M., and A. Amon. 2009. The FEAR network. *Curr. Biol*. 19:R1063–R1068. doi:10.1016/j.cub.2009.10.002

Schaerer, F., G. Morgan, M. Winey, and P. Philippsen. 2001. Cnm67p is a spacer protein of the *Saccharomyces cerevisiae* spindle pole body outer plaque. *Mol. Biol. Cell*. 12:2519–2533.

Sheff, M.A., and K.S. Thorn. 2004. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast*. 21:661–670. doi:10.1002/yea.1130

Shirayama, M., Y. Matsui, and A. Toh-E. 1994. The yeast TEM1 gene, which encodes a GTP-binding protein, is involved in termination of M phase. *Mol. Cell. Biol*. 14:7476–7482.

Shou, W., J.H. Seol, A. Shevchenko, C. Baskerville, D. Moazed, Z.W. Chen, J. Jang, A. Shevchenko, H. Charbonneau, and R.J. Deshaies. 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell*. 97:233–244. doi:10.1016/S0092-8674(00)80733-3

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 122:19–27.

Srinivasa, S.P., L.S. Bernstein, K.J. Blumer, and M.E. Linder. 1998. Plasma membrane localization is required for RGS4 function in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*. 95:5584–5589. doi:10.1073/pnas .95.10.5584

Stegmeier, F., and A. Amon. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annu. Rev. Genet.* 38:203–232. doi:10.1146/annurev.genet.38.072902.093051

Tang, X., J.J. Punch, and W.L. Lee. 2009. A CAAX motif can compensate for the PH domain of Num1 for cortical dynein attachment. *Cell Cycle*. 8:3182–3190. doi:10.4161/cc.8.19.9731

Visintin, R., and A. Amon. 2001. Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. *Mol. Biol. Cell*. 12:2961–2974.

Visintin, R., K. Craig, E.S. Hwang, S. Prinz, M. Tyers, and A. Amon. 1998. The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell*. 2:709–718. doi:10.1016/S1097-2765(00)80286-5

Visintin, R., E.S. Hwang, and A. Amon. 1999. Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature*. 398:818–823. doi:10.1038/19775

Yeh, E., R.V. Skibbens, J.W. Cheng, E.D. Salmon, and K. Bloom. 1995. Spindle dynamics and cell cycle regulation of dynein in the budding yeast, *Saccharomyces cerevisiae*. *J. Cell Biol.* 130:687–700. doi:10.1083/jcb.130.3.687