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Regulation of the Bfa1p–Bub2p complex at spindle pole bodies by the cell cycle phosphatase Cdc14p

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The budding yeast mitotic exit network (MEN) is a GTPase-driven signal transduction cascade that controls the release of the phosphatase Cdc14p from the nucleolus in anaphase and thereby drives mitotic exit. We show that Cdc14p is partially released from the nucleolus in early anaphase independent of the action of the MEN components Cdc15p, Dbf2p, and Tem1p. Upon release, Cdc14p binds to the spindle pole body (SPB) via association with the Bfa1p–Bub2p GTPase activating protein complex, which is known to regulate the activity of the G protein Tem1p. Cdc14p also interacts with this GTPase. The association of the MEN component Mob1p with the SPB acts as a marker of MEN activation. The simultaneous binding of Cdc14p and Mob1p to the SPB in early anaphase suggests that Cdc14p initially activates the MEN. In a second, later step, which coincides with mitotic exit, Cdc14p reactivates the Bfa1p–Bub2p complex by dephosphorylating Bfa1p. This inactivates the MEN and displaces Mob1p from SPBs. These data indicate that Cdc14p activates the MEN in early anaphase but later inactivates it through Bfa1p dephosphorylation and so restricts MEN activity to a short period in anaphase.

Introduction

The mitotic exit network (MEN)* is a signaling cascade that controls the transition from anaphase into the next G1 phase. This is achieved by promoting the release of the phosphatase Cdc14p from its association with the nucleolar protein Net1p (Shou et al., 1999; Visintin et al., 1999). After its release from the nucleolus, Cdc14p dephosphorylates key targets such as the mitotic cyclin-dependent kinase (Cdk–Clb) inhibitor Sic1p (Schwob et al., 1994) and the anaphase-promoting complex subunit Hct1p. Active Hct1p then promotes Clb2p degradation (Schwab et al., 1997). Clb2p degradation and Sic1p activation decrease Cdk–Clb activity and thereby drive mitotic exit (Visintin et al., 1998).

The small, Ras-like GTPase Tem1p regulates MEN activity. Tem1p activation promotes its interaction with the kinase Cdc15p (Shirayama et al., 1994b; Bardin et al., 2000), which in turn activates a protein kinase complex in which the catalytic subunit Dbf2p is bound to a regulatory subunit, Mob1p (Toyn and Johnston, 1994; Luca et al., 2001; Mah et al., 2001). The polo-like kinase Cdc5p activates the MEN by inhibiting the Tem1p GTPase activating protein (GAP) complex. This bipartite GAP complex is composed of Bfa1p and Bub2p, and Cdc5p phosphorylates Bfa1p (Hoyt et al., 1991; Li, 1999). Because overproduction of *CDC14* bypasses the requirement of all MEN proteins, it is thought that Cdc14p activation/release is the ultimate target of the MEN cascade (Jaspersen et al., 1998; Visintin et al., 1998).

In addition to regulation by the Bfa1p–Bub2p GAP complex, Tem1p activity is also modulated by the putative GDP/GTP exchange factor (GEF) Lte1p (Shirayama et al., 1994a). Tem1p forms a complex with the Bfa1p–Bub2p GAP on the spindle pole body (SPB), which leads the spindle into the bud (Pereira et al., 2000, 2001). The GEF Lte1p is retained at a distinct location on the cortex of the bud (Bardin et al., 2000; Pereira et al., 2000). It has therefore been proposed that SPB-associated Bfa1p–Bub2p GAP inactivates Tem1p until the SPB and spindle enter the bud in anaphase. This coupling of mitotic exit with nuclear migration prevents premature mitotic exit in mutants with defects in spindle orientation and has now been termed "the spindle position checkpoint."

Two recent results indicate that additional mechanisms regulate MEN activity. First, *BFA1* and *BUB2* only become essential for survival when nuclear migration is delayed (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). Second, deletion of *LTE1* does not affect the timing of mitotic

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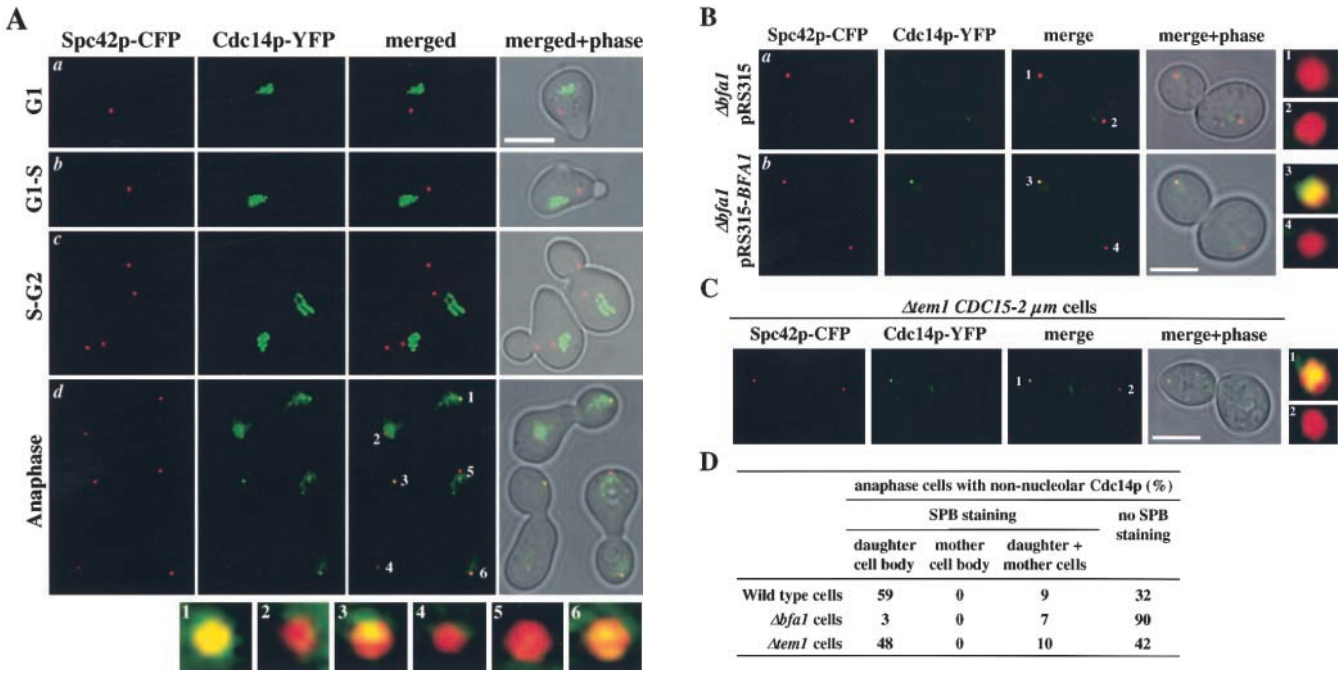
*Abbreviations used in this paper: Cdk, cyclin-dependent kinase; CFP, cyan fluorescent protein; GAP, GTPase activating protein; GEF, GDP/GTP exchange factor; GST, glutathione-*S*-transferase; HA, hemagglutinin; MEN, mitotic exit network; SIN, septum initiation network; SPB, spindle pole body; YFP, yellow fluorescent protein.

Key words: Bfa1p-Bub2p GAP; Cdc14p; MEN; polo kinase; Tem1p



D

	anaphase cells with non-nucleolar Cdc14p (%)			
	SPB staining			no SPB staining
	daughter cell body	mother cell body	daughter + mother cells	
Wild type cells	59	0	9	32
Δbfa1 cells	3	0	7	90
Δtem1 cells	48	0	10	42

Figure 1. **Cdc14p binds to SPBs via Bfa1p.** (A) Fluorescence microscopy of *CDC14–YFP SPC42–CFP* cells from an α-factor–synchronized culture: G1 (a), G1–S (b), S–G2 (c), and anaphase (d). Panels 1–6 on the bottom are magnifications of the SPBs in the anaphase cells shown above. (B) Cdc14p SPB localization is in part Bfa1p dependent. Fluorescence microscopy of anaphase cells from α-factor–synchronized *CDC14–YFP SPC42–CFP Δbfa1* cells with plasmid pRS315 (a) or pRS315-*BFA1* (b). Panels 1–4 are magnifications of the merged SPB signals. (C) Cdc14p SPB staining does not require Tem1p. Fluorescence microscopy of anaphase *Δtem1 CDC15-2μ CDC14–YFP SPC42–CFP* cells. Panels 1 and 2 are magnifications of the merged SPB signals. (D) Quantification of A–C. Cells in late anaphase with released Cdc14p ($n > 100$) were analyzed for Cdc14p–YFP SPB staining. Bars, 5 μm.

exit at 30°C (unpublished data) or 37°C (Adames et al., 2001).

The fission yeast *Schizosaccharomyces pombe* controls septum formation during cytokinesis through the activity of the septum initiation network (SIN) (Balasubramanian et al., 2000). The SIN is similar to the MEN in composition. However, in contrast to Cdc14p, the fission yeast homologue Clp1p/Flp1p is not essential and associates not only with the nucleolus but also with the SPB. Clp1p/Flp1p is released from the nucleolus very early in mitosis in a SIN-independent manner (Cueille et al., 2001; Trautmann et al., 2001). Furthermore, Clp1p/Flp1p does not regulate anaphase cyclin destruction and the accumulation of a Sic1p equivalent. Instead, Clp1p/Flp1p delays Cdk activation at the G2–M transition and is part of a cytokinesis checkpoint that arrests cells in G2 when cytokinesis is blocked (Cueille et al., 2001; Trautmann et al., 2001). The human Cdc14p homologue, hCdc14a, localizes to the centrosome but not the nucleolus and dephosphorylates hCdh1 (Bembenek and Yu, 2001). Whether the seemingly different regulatory and functional aspects of Cdc14p, hCdc14a, and Clp1p/Flp1p have a common basis is an important question.

Here, we show that Cdc14p is initially released from the nucleolus at the beginning of anaphase (for summary see Fig. 9). This release occurs without the function of the MEN components Cdc15p, Dbf2p, and Tem1p. Cdc14p then associates with SPBs through the Bfa1p–Bub2p complex and facilitates MEN activation. In a second step, at the end of anaphase, Cdc14p dephosphorylates Bfa1p and thereby reactivates the Bfa1p–Bub2p GAP to shut down the MEN. Thus, Cdc14p shares characteristics with the human and *S. pombe* homologues, and its affinity for the Bfa1p- and Tem1p-like proteins may indicate a common function of Cdc14 proteins at SPBs and centrosomes.

Results

Cdc14p association with SPBs in anaphase is partly Bfa1p–Bub2p dependent

Cdc14p homologues are associated with the SPB or centrosome in fission yeast and mammalian cells (Cueille et al., 2001; Trautmann et al., 2001). The high degree of functional and sequence identity of Cdc14p proteins raises the possibility that the budding yeast Cdc14p may also bind to SPBs. Such SPB localization may have been missed in previous studies because of the fixation sensitivity of SPB antigens (Rout and Kilmartin, 1990). We therefore reevaluated the cellular distribution of Cdc14p using *CDC14–YFP SPC42–CFP* cells in which Cdc14p is fused to the yellow fluorescent protein (Cdc14p–YFP), and the core SPB component Spc42p (Donaldson and Kilmartin, 1996) is fused to the cyan fluorescent protein (Spc42p–CFP). Fixation was limited to 10 min to preserve SPB signals. Cdc14p–YFP localization was determined in a time course experiment with α-factor–synchronized cells, in which the mother cell body was distinguished from the daughter cell by the presence of a mating projection. During interphase, Cdc14p–YFP resided in the nucleolus (Fig. 1 A, a–c) from G1 to early anaphase, whereupon it was released from the nucleolus (Shou et al., 1999; Visintin et al., 1999) and showed costaining with the

SPB marker Spc42p–CFP (Fig. 1 A, d; SPB-1, -3, and -6). Cdc14p–YFP was associated with SPBs in the majority of anaphase cells (68%) (Fig. 1 D). In 59% of cells, Cdc14p–YFP was predominantly at the SPB in the cell body without the mating projection, the bud, and in 9%, a weaker Cdc14p–YFP signal was seen at both SPBs. In 32%, no clear SPB staining was observed. This latter class is probably due to the transient nature of Cdc14p association with SPBs in anaphase and the rapidly bleached, weak signal. In telophase, the Cdc14p signal was again seen in the nucleolus, but was no longer at the SPBs. Thus, Cdc14p binds to SPBs in a cell cycle–dependent manner.

Along with the Bfa1p–Bub2p complex and Tem1p, Cdc14p associated preferentially with the SPB in the bud rather than the SPB in the mother (Pereira et al., 2000, 2001). This similarity prompted us to ask whether Cdc14p interacts with SPBs via the Bfa1p–Bub2p complex or Tem1p. First, we addressed whether Cdc14p could bind to the SPB in the absence of Bfa1p–Bub2p and Tem1p. Deletion of *BFA1* blocked the association of Bub2p and Tem1p with the SPB (Pereira et al., 2000) and strongly diminished the association of Cdc14p–YFP with the SPB in the bud of anaphase cells (Fig. 1 B, a; SPB-1). Colocalization of Cdc14p–YFP with the SPB marker Spc42p–CFP dropped from 68 (wild type) to 10% (*Δbfa1*) (Fig. 1 D). Complementation of the *BFA1* deficiency by an episomal *BFA1* restored Cdc14p SPB staining to wild-type levels (Fig. 1 B, b; SPB-3).

We then investigated whether Tem1p alone was required for Cdc14p binding to SPBs. The essential requirement of *TEM1* can be bypassed by expression of *CDC15* from a 2μ plasmid (Shirayama et al., 1994b). As Bfa1p and Bub2p are still found on the bud-proximal SPB of *Δtem1 CDC15*-2μ anaphase cells, these cells can be used to directly address the role of Tem1p in the localization of Cdc14p (Pereira et al., 2000). In contrast to *Δbfa1* cells, Cdc14p localization was unaffected by the absence of Tem1p (Fig. 1, C and D). Thus, the reduced SPB binding of Cdc14p in *Δbfa1* cells likely results from the absence of the Bfa1p–Bub2p complex rather than the lack of Tem1p. We conclude that the Bfa1p–Bub2p complex facilitates the binding of Cdc14p to the SPB in the bud.

Cdc14p interacts with Bfa1p and Tem1p

We then asked whether Cdc14p could physically associate with Bfa1p, Bub2p, or Tem1p by testing for interactions in the yeast two-hybrid system. The Cdc14p interactor Net1p was used as a positive control (Shou et al., 1999). Cdc14p interacted with both Tem1p and Bfa1p. Cdc14p interacted most strongly with Tem1p, then Bfa1p, and then Net1p (Fig. 2 A, columns 1, 3, and 6). Interaction of Cdc14p with Bfa1p was mediated through the NH_2-terminal domain of Bfa1p (columns 3 and 4) and not the COOH-terminal portion (column 5). Cdc14p did not give a signal with Bub2p (column 2). In conclusion, Cdc14p interacts with both Tem1p and Bfa1p in the yeast two-hybrid system.

In vitro binding experiments were employed to confirm the two-hybrid interactions between the Bfa1p–Bub2p–Tem1p complex and Cdc14p. Recombinant glutathione-*S*-transferase (GST)–Cdc14p fusion proteins purified from

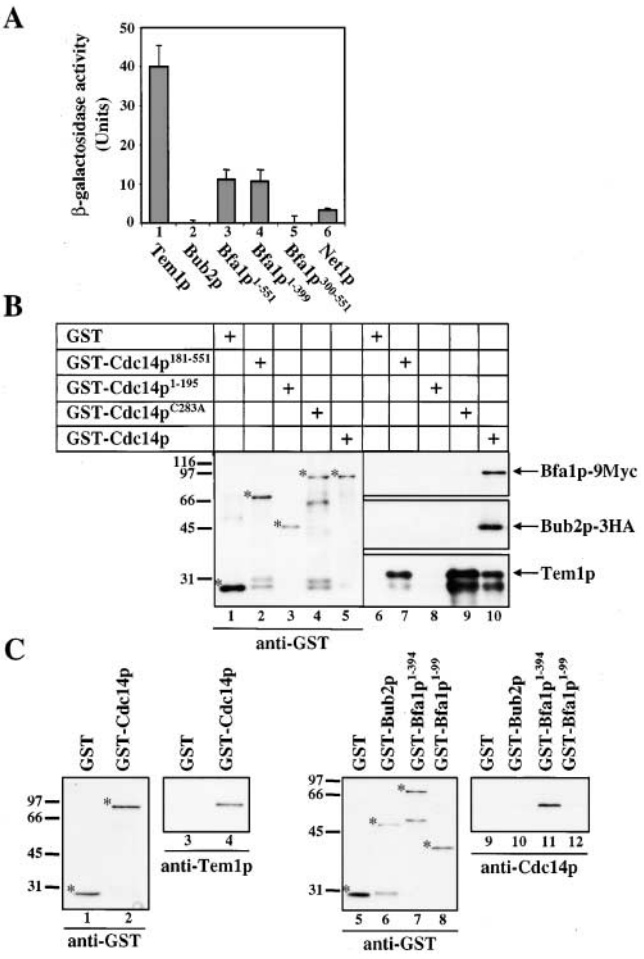


Figure 2. **Cdc14p interacts with Bfa1p and Tem1p.** (A) Two-hybrid interaction between Cdc14p and Bfa1p and Tem1p. β-Galactosidase activity of SGY37 cells with Gal4–Cdc14p and the indicated proteins fused to LexA. Average of two experiments. (B) Binding of the Bfa1p–Bub2p complex and Tem1p to GST–Cdc14p. A lysate of *BFA1–9Myc BUB2–3HA* yeast cells was incubated with GST fusion proteins expressed in *E. coli* and then bound to glutathione-Sepharose beads. Eluted proteins were analyzed by immunoblotting. (C) Bfa1p and Tem1p bind directly to Cdc14p. Recombinant 6His–Tem1p (lanes 1–4) or 6His–Cdc14p (lanes 5–12) were incubated with GST fusion proteins bound to glutathione-Sepharose beads. The eluted proteins were analyzed by immunoblotting. Asterisks in lanes 1, 2, and 5–8 highlight the GST fusion proteins. The faster migrating bands are degradation products.

Escherichia coli were incubated with an extract of *BFA1–9Myc BUB2–3HA* yeast cells to test for Cdc14p binding to Bfa1p–9Myc, Bub2p–3 hemagglutinin (HA), and Tem1p. The truncated proteins $Cdc14p^{1–195}$ and $Cdc14p^{181–551}$ and the phosphatase-dead $Cdc14p^{C283A}$, which showed little or no signal when combined with Bfa1p in the two-hybrid system (unpublished data), were used to evaluate the nonspecific binding of proteins. Bfa1p–9Myc and Bub2p–3HA associated with GST–Cdc14p (Fig. 2 B, lane 10) but not with GST or any of the mutant forms of Cdc14p (lanes 6–9). Similarly, Tem1p bound to GST–Cdc14p (Fig. 1 B, lane 10) but not to GST (lane 6) or GST–$Cdc14p^{1–195}$ (lane 8). In contrast to Bfa1p and Bub2p, Tem1p interacted with the catalytically inactive GST–$Cdc14p^{C283A}$ (lane 9) and GST–

Cdc14p$^{181–551}$ (lane 7). Posttranslational modification meant that Tem1p appeared as a doublet by SDS-PAGE (Asakawa et al., 2001). Thus, both the Bfa1p–Bub2p complex and Tem1p bind to recombinant Cdc14p. Interestingly, the failure of Cdc14p^{C283A} to interact with Bfa1p suggests that the C283A mutation not only affects the phosphatase activity of Cdc14p but also the interaction with Bfa1p.

Because a crude yeast lysate was used as a source for Bfa1p, Bub2p, and Tem1p in the previous experiment, it was not clear whether the interaction with Cdc14p was direct or indirect. Therefore, all proteins were expressed in *E. coli*, purified, and mixed to test the ability of isolated proteins to physically associate. 6His–Tem1p interacted with GST–Cdc14p but not GST (Fig. 2 C, lanes 3 and 4). Anti-GST antibodies revealed that GST and GST–Cdc14p were present in about equal amounts (lanes 1 and 2). Moreover, 6His–Cdc14p also bound to GST–Bfa1p$^{1–394}$ (lane 11) but not to GST–Bfa1p$^{1–99}$ (lane 12), GST–Bub2p (lane 10), or GST (lane 9). In this experiment, similar amounts of GST–Bub2p, GST–Bfa1p$^{1–394}$, and GST–Bfa1p$^{1–99}$ (lanes 6–8) were on the beads, whereas GST (lane 5) was approximately twofold higher. Thus, Cdc14p binds directly to Bfa1p and Tem1p but not to Bub2p, suggesting that the interaction of Cdc14p to the Bfa1p–Bub2p complex (Fig. 2 B) is mediated by Bfa1p.

Cdc14p dephosphorylates Bfa1p

Because Bfa1p is a phosphoprotein (Lee et al., 2001) that interacts with the phosphatase Cdc14p (Fig. 2), we asked whether Cdc14p dephosphorylates Bfa1p. We first investigated whether Cdc14p dephosphorylates Bfa1p in vivo. For this experiment, cells deleted in the anaphase-promoting complex subunit *CDC26* were shifted to 37°C, causing a metaphase arrest, a cell cycle stage where Bfa1p is phosphorylated (Zachariae et al., 1998). Cdc14p of metaphase-arrested cells is entrapped in the nucleolus and is therefore inactive (Visintin et al., 1999). Overexpression of *CDC14* from the Gal1 promoter allows the accumulation of active Cdc14p in the cytoplasm. If Bfa1p is a Cdc14p substrate, then exposure to this excess of Cdc14p should dephosphorylate the Bfa1p of *Δcdc26* cells. The phosphatase-dead *CDC14^{C283A}* was used as a control for the specificity of the reaction.

Δcdc26 BFA1–3HA, Δcdc26 BFA1–3HA Gal1–*CDC14*, and *Δcdc26 BFA1–3HA* Gal1–*CDC14^{C283A}* cells were arrested in metaphase by incubation at 37°C. In all cell types, Bfa1p was similarly phosphorylated, as indicated by the accumulation of the slower migrating Bfa1p phosphoform (Fig. 3 A, lanes 2, 6, and 10; Bfa1p-P). Overexpression of Gal1–*CDC14* and Gal1–*CDC14^{C283A}* was then induced by the addition of galactose ($t = 0$). Bfa1p–3HA phosphorylation was not affected in *BFA1–3HA Δcdc26* and *BFA1–3HA Δcdc26* Gal1–*CDC14^{C283A}* cells (Fig. 3 A, lanes 2–5 and 10–13). However, in Gal1–*CDC14* cells, most Bfa1p–3HA became dephosphorylated within 2 h of Gal1 induction (Fig. 3 A, lanes 6–9). Immunoblotting with anti-Cdc14p antibodies revealed that Cdc14p and Cdc14p^{C283A} were expressed at similar levels (unpublished data). Moreover, we monitored the levels of Clb2p and the Cdc14p substrate Sic1p to ensure that overexpression of *CDC14* did not induce mitotic exit of the *Δcdc26* cells. In all cell types, Clb2p stayed high and Sic1p did not accumulate, indicating that *Δcdc26* remained arrested in mitosis during the course of the experiment (Fig. 3 A). We concluded that Cdc14p dephosphorylates Bfa1p in vivo.

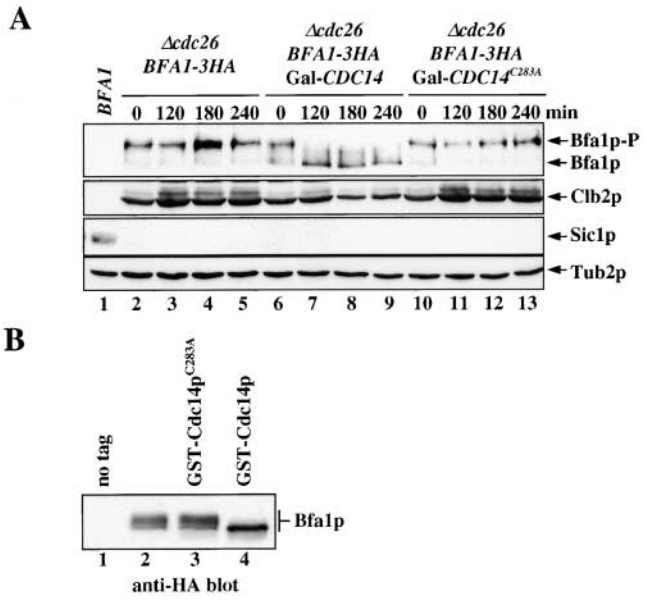


Figure 3. **Cdc14p dephosphorylated Bfa1p.** (A) Cdc14p dephosphorylates Bfa1p in vivo. α-Factor–synchronized *Δcdc26 BFA1–3HA* (lanes 2–5), *Δcdc26 BFA1–3HA* Gal1–*CDC14* (lanes 6–9), and *Δcdc26 BFA1–3HA* Gal1–*CDC14^{C283A}* cells (lanes 10–13) in YP raffinose medium were incubated for 2 h at 37°C. Galactose was then added to induce the Gal1 promoter ($t = 0$). Samples were withdrawn as indicated and analyzed for Bfa1p phosphorylation, Clb2p, and Sic1p by immunoblotting. The anti-Tub2p immunoblot was used as a loading control. *BFA1* cells (lane 1) in G1 were used as a negative control for anti-HA and as a positive control for anti-Sic1p antibodies. (B) Cdc14p dephosphorylates Bfa1p in vitro. *cdc15-1* (lane 1) and *cdc15-1 BFA1–3HA* (lanes 2–4) cells were grown for 3 h at 37°C until >95% arrested in anaphase. Cell extracts were incubated with anti-HA antibodies coupled to protein G–Sepharose to precipitate Bfa1p–3HA (Pereira et al., 2000). Immunoprecipitations were incubated with buffer (lanes 1 and 2) or with purified GST–Cdc14p^{C283A} (lane 3) or GST–Cdc14p (lane 4) for 1 h at 37°C as previously described (Jaspersen and Morgan, 2000). Proteins were analyzed by immunoblotting with anti-HA antibodies.

We then tested whether GST–Cdc14p purified from *E. coli* was able to dephosphorylate Bfa1p–3HA from yeast cells in vitro. The phosphatase-dead Cdc14p^{C283A} was used as a negative control to ensure that Bfa1p–3HA and GST–Cdc14p were not contaminated with a copurifying phosphatase. Bfa1p–3HA was immunoprecipitated from *cdc15-1 BFA1–3HA* arrested cells, in which Bfa1p was hyperphosphorylated (Fig. 3 B, lane 2). A control precipitation using *cdc15-1 BFA1* cells established that the precipitated protein was Bfa1p–3HA (lane 1). Bfa1p–3HA was efficiently dephosphorylated when incubated with GST–Cdc14p (Fig. 3 B, lane 4), whereas incubation with the inactive GST–Cdc14p^{C283A} (lane 3) or GST (unpublished data) did not affect Bfa1p phosphorylation. This shows that Cdc14p dephosphorylates Bfa1p both in vitro and in vivo.

Cdc14p binds to SPBs in early anaphase yet only dephosphorylates Bfa1p at mitotic exit

To gain a better insight into the relationship between Bfa1p dephosphorylation by Cdc14p and the association of Cdc14p with the SPB, we compared the timing of these two events. To enable the quantification of Cdc14p SPB binding, we again used a strain in which *CDC14* was fused to YFP and the SPB marker *SPC42* was fused to CFP. We generated a highly synchronized yeast culture that immediately progressed into anaphase by arresting Gal1–*CDC20 CDC14–YFP SPC42–CFP* cells in metaphase through depletion of Cdc20p and subsequently resupplying Cdc20p by the addition of galactose (Shirayama et al., 1999). Depletion of Cdc20p arrested Gal1–*CDC20 CDC14–YFP SPC42–CFP* cells in metaphase with phosphorylated Bfa1p (Fig. 4 A, compare lane 3 [metaphase arrest] with lanes 1 and 2 [nonphosphorylated Bfa1p]). However, the slowest migrating Bfa1p form in Gal1–*CDC20* arrested cells still migrated faster than the slowest migrating Bfa1p band from *cdc15-1* arrested late anaphase cells (Fig. 4 A, lanes 3 and 10). Bfa1p must therefore be subject to additional phosphorylation in anaphase before full activation of the MEN (Lee et al., 2001). This additional modification became particularly apparent when the metaphase block of Gal1–*CDC20* cells was released. Upon induction of Cdc20p, cells entered anaphase, as indicated by the appearance of binucleated cells (Fig. 4 A, bottom). At least one additional phosphoform of Bfa1p appeared at the beginning of anaphase whose migration corresponded to Bfa1p of anaphase-arrested *cdc15-1* cells (Fig. 4 A, lanes 6, 7, and 10). Phosphorylation of Bfa1p then sharply decreased at the end of anaphase as Clb2p was degraded and Sic1p accumulated (lanes 8 and 9). Because degradation of Clb2p and accumulation of Sic1p are indicators of mitotic exit (Schwab et al., 1997), we conclude that Bfa1p becomes dephosphorylated as cells exit mitosis.

Monitoring Cdc14p–YFP localization in the same culture indicated that it was released from the nucleolus and started to associate with the SPB 20 min after Gal1–*CDC20* cells were released from the metaphase block (Fig. 4 B). This corresponded to the time when the leading SPB had just entered the bud (unpublished data). Cdc14p–YFP at SPBs reached a maximum between 30 and 40 min and then rapidly decreased with mitotic exit (Fig. 4 B). Thus, Bfa1p became phosphorylated at the beginning of anaphase at the same time as Cdc14p bound to SPBs and was subsequently dephosphorylated 20 min later with mitotic exit. The delay between Cdc14p SPB binding and Bfa1p dephosphorylation may indicate that Cdc14p performs a function at the SPB in early anaphase that is independent of its role in Bfa1p dephosphorylation.

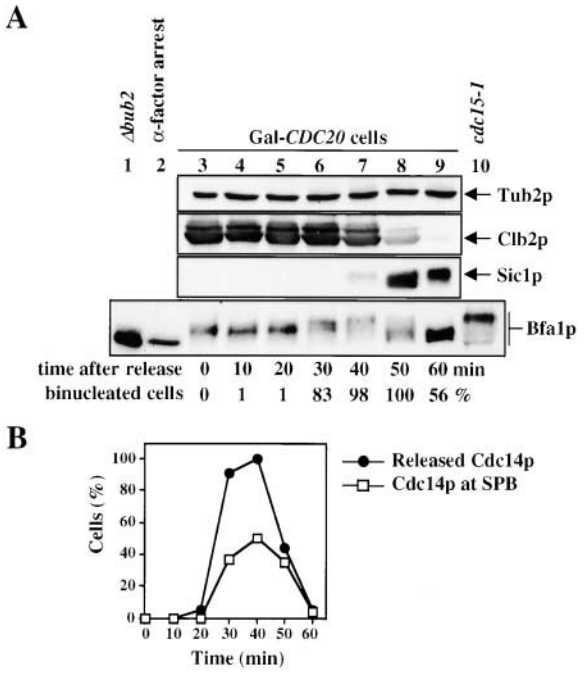


Figure 4. **Bfa1p becomes dephosphorylated with mitotic exit.** (A) Gal1–*CDC20 CDC14–YFP SPC42–CFP* cells were arrested with α-factor in YP raffinose–galactose medium (lane 2). The block was released by washing the cells with YP raffinose medium. Cells (>95%) arrested in metaphase after 3 h at 23°C. Galactose was then added ($t = 0$) to induce *CDC20* expression and to trigger cell cycle progression (lanes 3–9). Cell extracts were analyzed by immunoblotting with the indicated antibodies. Extracts of *Δbub2* (lane 1) and *cdc15-1* cells (lane 10), the latter incubated for 3 h at 37°C, were used as controls for nonphosphorylated and hyperphosphorylated forms of Bfa1p, respectively. The percentage of binucleated cells was determined by DAPI staining. (B) Cdc14p–YFP localization in the cells used in A.

Inactivation of the MEN by Bfa1p–Bub2p at the end of mitosis

The previous experiment indicated that Bfa1p became phosphorylated at the beginning of anaphase and dephosphorylated by Cdc14p during mitotic exit. Phosphorylation of Bfa1p by Cdc5p inactivates, at least partially, the Bfa1p–Bub2p GAP complex (Hu et al., 2001) (unpublished data). Thus, in a simple model, dephosphorylation of Bfa1p by Cdc14p could reactivate the Bfa1p–Bub2p complex. As a consequence, active Bfa1p–Bub2p would change Tem1p into its GDP-bound, inactive state and the MEN would be inactivated at the end of mitosis. If this is the case, dephosphorylation of Bfa1p should coincide with MEN inactivation, and cells lacking the Bfa1p–Bub2p complex should delay Tem1p inhibition at the end of mitosis. To test these predictions, we sought a simple way to monitor Bfa1p–Bub2p activity. It is established that the localization of the kinase Dbf2p to SPBs is dependent upon Tem1p activation (Fesquet et al., 1999; Visintin and Amon, 2001). Thus, when the absence of the Bfa1p–Bub2p complex promotes premature MEN activation, Dbf2p kinase binds prematurely to SPBs. Monitoring Dbf2p association with the SPB will therefore give a read out of Tem1p activity. However, the Dbf2p–YFP SPB signal is very weak (unpublished data), making it difficult to analyze. As an alternative, we investigated whether Mob1p, which forms a tight complex with Dbf2p (Komarnitsky et al., 1998), can be used as a marker for Bfa1p–Bub2p activity. We monitored Mob1p SPB localization in wild-type and *Δbfa1* cells. In α-factor–synchronized *CDC14–CFP MOB1–YFP* cells, Mob1p–YFP became associated with SPBs at the beginning of anaphase (Fig. 5 A,

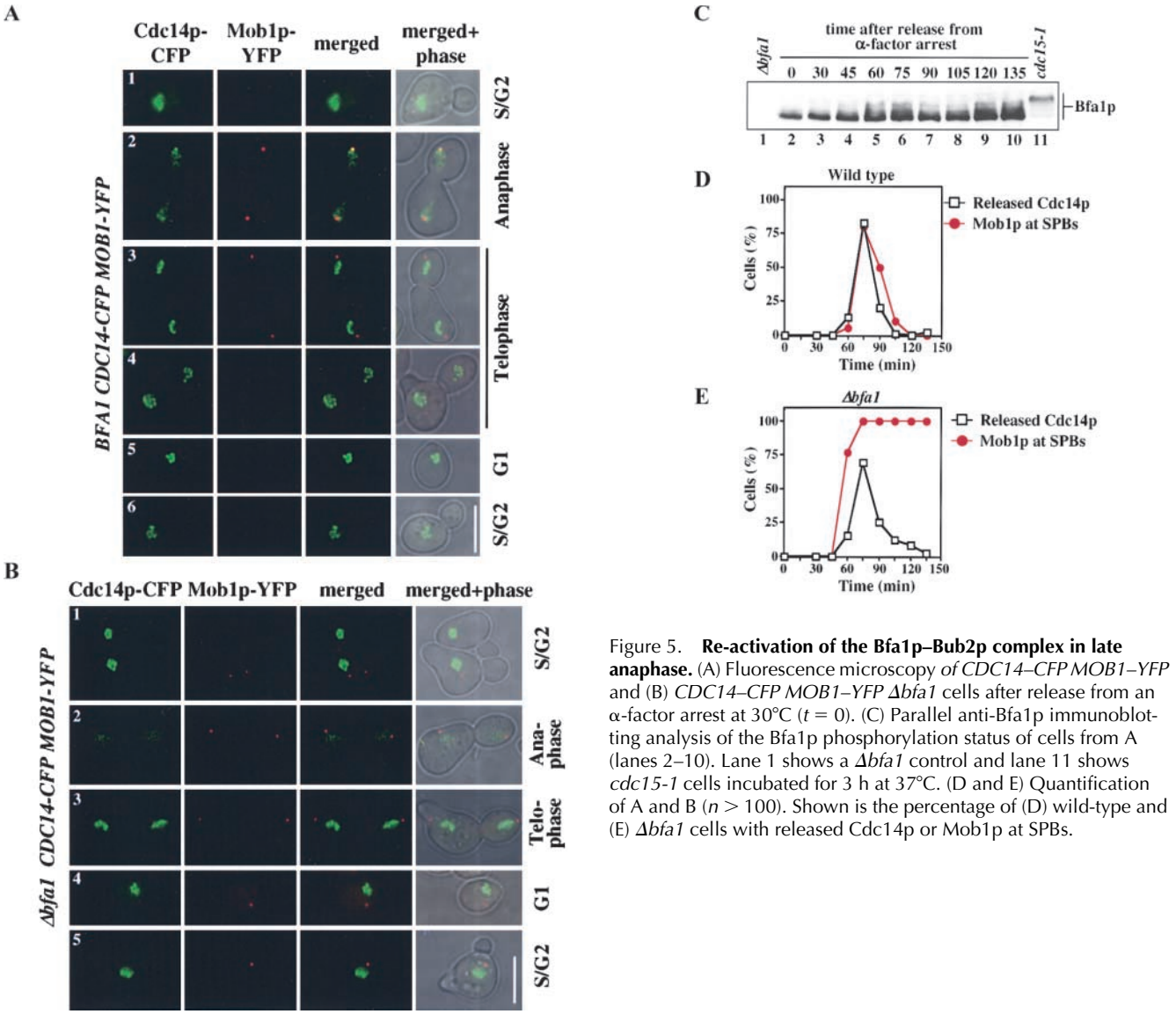


Figure 5. **Re-activation of the Bfa1p–Bub2p complex in late anaphase.** (A) Fluorescence microscopy *of CDC14–CFP MOB1–YFP* and (B) *CDC14–CFP MOB1–YFP Δbfa1* cells after release from an α-factor arrest at 30°C ($t = 0$). (C) Parallel anti-Bfa1p immunoblotting analysis of the Bfa1p phosphorylation status of cells from A (lanes 2–10). Lane 1 shows a *Δbfa1* control and lane 11 shows *cdc15-1* cells incubated for 3 h at 37°C. (D and E) Quantification of A and B ($n > 100$). Shown is the percentage of (D) wild-type and (E) *Δbfa1* cells with released Cdc14p or Mob1p at SPBs.

2, and D, $t = 60$). In contrast, in *Δbfa1* cells, Mob1p–YFP was already associated with the SPBs of metaphase cells (Fig. 5 B, 1, and E, $t = 60$). This behavior is identical to that of Dbf2p and shows that the association of Mob1p–YFP with the SPBs is a good indicator of Bfa1p–Bub2p activity.

We were now able to ask whether dephosphorylation of Bfa1p coincided with the inactivation of Tem1p (i.e., displacement of Mob1p from SPBs), as would be expected if dephosphorylation of Bfa1p reactivates the Bfa1p–Bub2p complex. In α-factor–synchronized *CDC14–CFP MOB1–YFP* cells, Mob1p–YFP SPB binding was maximal when Bfa1p was most heavily phosphorylated (Fig. 5, C and D, $t = 75$). This observation is consistent with the conclusion that phosphorylation of Bfa1p by Cdc5p inactivates the Bfa1p–Bub2p complex (Hu et al., 2001). The dephosphorylation of Bfa1p coincided with the dissociation of Mob1p–YFP SPB signal from SPBs during mitotic exit (Fig. 5, C and D, $t = 90$ and 105), further supporting the notion that at the end of mitosis, Cdc14p reactivates the Bfa1p–Bub2p complex and thus turns off the MEN.

In wild-type cells, Mob1p is displaced from SPBs at the same time as mitotic exit (Fig. 5 D). If Bfa1p dephosphorylation by Cdc14p is the only mechanism that triggers Mob1p–YFP dissociation from SPBs at the end of anaphase, Mob1p–YFP should stay at SPBs in cells lacking the Bfa1p–Bub2p complex. Indeed, Mob1p–YFP remained associated with SPBs in *Δbfa1* cells after the reentry of Cdc14p into the nucleolus indicated that cells had completed mitotic exit (Fig. 5 B, 3–5, and E, $t = 105$–135). Together, the correlation between Bfa1p dephosphorylation and Mob1p SPB localization and the Bfa1p–Bub2p-dependent displacement of Mob1p from SPBs at the end of mitosis suggest that the Bfa1p–Bub2p complex becomes reactivated with mitotic exit through the dephosphorylation of Bfa1p by Cdc14p.

Partial release of Cdc14p from the nucleolus is independent of Cdc15p, Dbf2p, and Tem1p

The experiments in the previous section established that in *CDC14–CFP MOB1–YFP* cells, Cdc14p was released from the nucleolus and Mob1p bound to SPBs at the same time

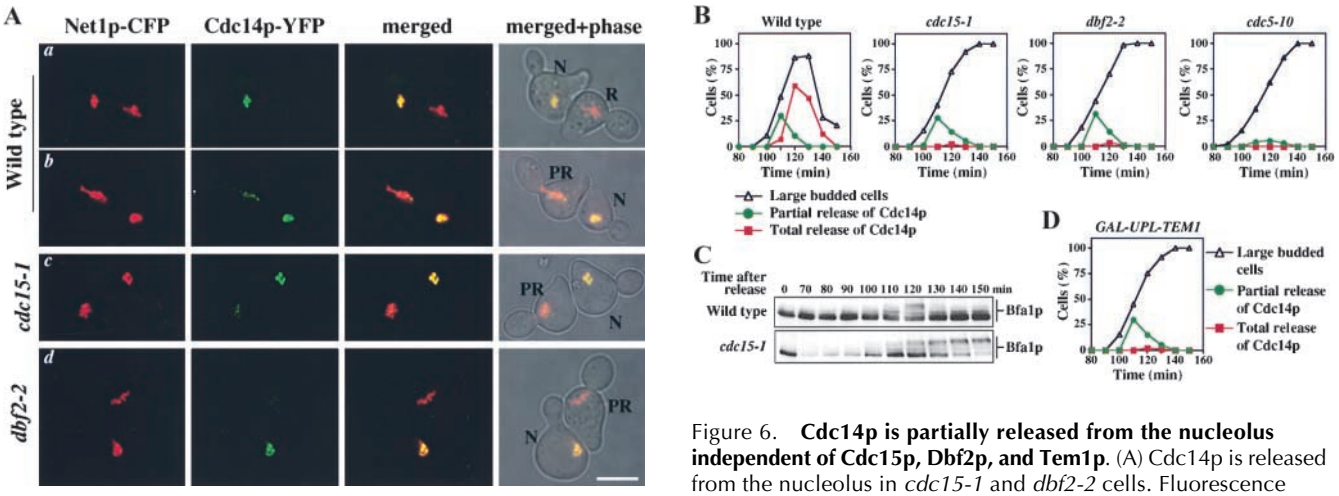


Figure 6. **Cdc14p is partially released from the nucleolus independent of Cdc15p, Dbf2p, and Tem1p**. (A) Cdc14p is released from the nucleolus in *cdc15-1* and *dbf2-2* cells. Fluorescence microscopic analysis of α-factor–synchronized wild-type (a and b), *cdc15-1* (c), and *dbf2-2* cells (d) with *NET1–CFP CDC14–YFP*. N, cells with no release of Cdc14p from the nucleolus; PR, partial release; R, full release. Bar, 5 μm. (B) Quantification of A ($n > 100$). Large budded cells with partial and total release of Cdc14p from the nucleolus were counted for each time point after α-factor release ($t = 0$). (C) Immunoblotting of wild-type and *cdc15-1* cells from A with anti-Bfa1p antibodies. (D) Gal1–*UPL-TEM1 CDC14–YFP NET1–CFP* cells were arrested in G1 with α-factor in YP raffinose–galactose medium at 30°C. Cells were washed with YPAD to remove α-factor ($t = 0$) and induce UPL-Tem1p degradation. Cells were processed as for B.

(Fig. 5 D). We did not observe any cells ($n > 200$) in which Mob1p was already at SPBs while Cdc14p was still in the nucleolus. In fact, ~5% of early anaphase cells showed Cdc14p, but not Mob1p, at SPBs (unpublished data). This observation suggests that Cdc14p dissociates from Net1p shortly before Tem1p activation. In turn, this implies that MEN activity is not essential for the initial release of Cdc14p from the nucleolus. If this is the case, then the initial release of Cdc14p from the nucleolus should still occur in cells where the MEN is defective. To test this possibility, the resident nucleolar protein Net1p, which forms a complex with Cdc14p (Shou et al., 1999; Visintin et al., 1999), was fused to CFP in wild-type, *cdc5-10*, *cdc15-1*, and *dbf2-2* cells that contained *CDC14–YFP*. We then monitored the localization of Cdc14p–YFP and Net1p–CFP as cells recovered from an α-factor block at 37°C, the restrictive temperature for these MEN mutants. In all cell types, Cdc14p–YFP and Net1p–CFP showed 100% colocalization in G1, S, and G2 cells. In wild-type cells, Cdc14p was released from the nucleolus in early anaphase. Partial release of Cdc14p–YFP (Fig. 6 A, PR, and B, green line) was followed by the complete release of the nucleolar Cdc14p–YFP (Fig. 6 A, R, and B, red line). In *cdc15-1* and *dbf2-2* cells, we saw a partial release of Cdc14p from the nucleolus in early anaphase (Fig. 6, A and B). This release had similar kinetics to wild-type cells. In contrast to wild-type cells, complete liberation of nucleolar Cdc14p was very rare in *cdc15-1* and *dbf2-2* cells. Most cells of *cdc5-10* behaved differently, i.e., they failed to show any degree of Cdc14p release (Fig. 6 B). As has been previously reported, *cdc15-1*, *cdc5-10*, and *dbf2-2* cells all eventually arrested in telophase with Cdc14p in the nucleolus (Shou et al., 1999; Visintin et al., 1999) (Fig. 6 B, $t = 140$).

The *cdc15-1* and *dbf2-2* result may indicate that the partial release of Cdc14p from the nucleolus is independent of the MEN. Alternatively, the MEN is required for the release, but the *cdc15-1* and *dbf2-2* cells still have a residual MEN activity at 37°C, which is sufficient to permit some degree of Cdc14p release. To distinguish between these two possibilities, we manipulated MEN activity with the Gal1–*UPL-TEM1* construct. Addition of glucose to the medium of Gal1–*UPL-TEM1* cells, which rely upon this construct to provide Tem1p, results in the rapid and total depletion of Tem1p (Shou et al., 1999). α-Factor–arrested cells were washed with glucose medium to simultaneously deplete UPL-Tem1p and promote synchronous cell cycle progression. The result was exactly as we had seen with *cdc15-1* and *dbf2-2* cells. Cdc14p–YFP (Fig. 6 D) was partially released from the nucleolus at the beginning of anaphase. Only a few *UPL-TEM1* cells with fully released nucleolar Cdc14p were observed. Because Tem1p was completely degraded in the Gal1–*UPL-TEM1* cells (unpublished data), an MEN-independent mechanism must be triggering the release of Cdc14p from the nucleolus at the beginning of anaphase. Given that the release of Cdc14p was virtually absent in *cdc5-10* cells, it is probable that Cdc5p plays a critical role in this MEN-independent mechanism.

Our data suggest that the Cdc14p released from the nucleolus in early anaphase is performing a distinct function from the Cdc14p liberated at the end of mitosis. If this is the case, then this early anaphase population of Cdc14p should not affect phosphorylation of Bfa1p, because the kinase responsible for phosphorylating it at this time, Cdc5p, is active until the end of anaphase (Shirayama et al., 1998). To test this possibility, we compared the accumulation of phosphorylated Bfa1p in α-factor–synchronized wild-type and *cdc15-1* cells. Phosphorylated Bfa1p appeared with similar kinetics in both cell types (Fig. 6 C). Thus, the MEN-independent release of Cdc14p did not affect Bfa1p phosphorylation.

Cdc14p binds transiently to SPBs in an MEN-independent manner

We then asked whether the partial nucleolar release of Cdc14p in *cdc15-1* and *dbf2-2* cells was coordinated with the

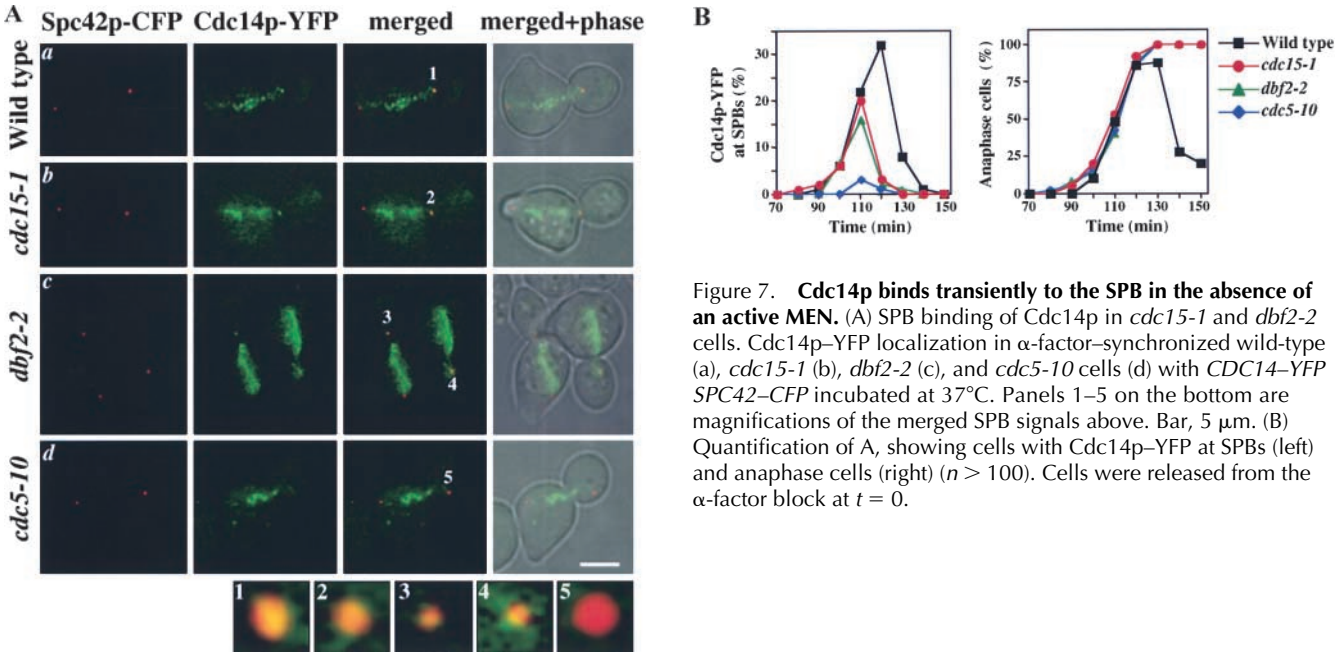


Figure 7. **Cdc14p binds transiently to the SPB in the absence of an active MEN.** (A) SPB binding of Cdc14p in *cdc15-1* and *dbf2-2* cells. Cdc14p–YFP localization in α-factor–synchronized wild-type (a), *cdc15-1* (b), *dbf2-2* (c), and *cdc5-10* cells (d) with *CDC14–YFP SPC42–CFP* incubated at 37°C. Panels 1–5 on the bottom are magnifications of the merged SPB signals above. Bar, 5 μm. (B) Quantification of A, showing cells with Cdc14p–YFP at SPBs (left) and anaphase cells (right) ($n > 100$). Cells were released from the α-factor block at $t = 0$.

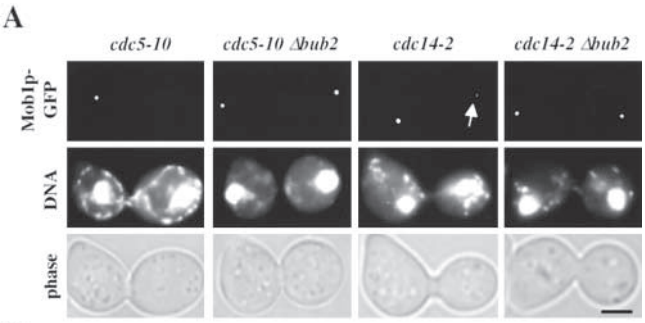
acquisition of affinity for the SPBs. Cells of *cdc5-10*, in which Cdc14p remains in the nucleolus, were used as controls. α-Factor–synchronized wild-type, *cdc5-10*, *cdc15-1*, and *dbf2-2* cells with *CDC14–YFP SPC42–CFP* were followed at 37°C. No Cdc14p–YFP SPB signal was observed from interphase to early mitosis in any of the four cell types. Localization of Cdc14p–YFP at SPBs in wild-type cells peaked at 120 min after the release of the α-factor block (Fig. 7 A, a, and B). This corresponded to the timing of the complete release of Cdc14p from the nucleolus in anaphase (Fig. 6 B). In early anaphase, Cdc14p–YFP was also found at SPBs in *cdc15-1* (Fig. 7 A, b) and *dbf2-2* cells (Fig. 7 A, c). However, the maximal SPB association was reached 15 min before the peak of wild-type cells (Fig. 7 B). The peak of Cdc14p at SPBs of the MEN mutants coincided with the maximal partial release of Cdc14p from the nucleoli of *cdc15-1* and *dbf2-2* cells (Fig. 6 B). Identical behavior was seen when the MEN was inactivated by Tem1p depletion from a Gal1–*UPL-TEM1 CDC14–YFP SPC42–CFP* strain (unpublished data). In contrast to these MEN-deficient mutants, Cdc14p–YFP was virtually absent from the anaphase SPBs of *cdc5-10* cells (Fig. 7 A, d, and B). At later time points, when *cdc15-1*, *cdc5-10*, and *dbf2-2* cells were arrested in late anaphase, no Cdc14p SPB staining was observed (Fig. 2 B, $t = 130$–150). We concluded that both the release of Cdc14p from the nucleolus and its association with the SPB in early anaphase are independent of MEN activation.

A role for Cdc14p at SPBs upstream of the MEN

The MEN-independent release of Cdc14p from the nucleolus and its interaction with Bfa1p and Tem1p, two MEN components that function on top of the pathway, suggest that Cdc14p has a role in MEN regulation at the beginning of anaphase. Cdc14p may activate the MEN, and considering the established role of the MEN in releasing Cdc14p from the nucleolus (Shou et al., 1999; Visintin et al., 1999), this would result in a positive feedback loop that triggers the rapid and complete release of Cdc14p from Net1p, which may be essential to switch the balance toward the dephosphorylation of proteins at the end of mitosis.

The interaction of Cdc14p with Bfa1p at SPBs at the beginning of anaphase (Fig. 4) raises the possibility that Cdc14p activates the MEN through inactivation of the Bfa1p–Bub2p GAP complex. If this is the case, we would expect that the Bfa1p–Bub2p GAP remains active when Cdc14p function is impaired. This failure to inactivate Bfa1p–Bub2p should be reflected in the SPB localization of Mob1p, which, as we demonstrated, can be used as a marker for Bfa1p–Bub2p activity (Fig. 5). To understand how failure to inactivate the Bfa1p–Bub2p complex affects Mob1p SPB localization in anaphase, we first studied Mob1p–GFP in α-factor–synchronized *cdc5-10* cells. In wild-type cells, Cdc5p phosphorylated Bfa1p with anaphase onset and this inactivated, at least partially, the Bfa1p–Bub2p complex (Hu et al., 2001; unpublished data) (Fig. 8 C, lane 2). In contrast, *cdc5-10* cells failed to hyperphosphorylate Bfa1p (Fig. 8 C, lane 6) and, therefore, arrested in anaphase with an active Bfa1p–Bub2p complex (unpublished data). In ∼88% of anaphase *cdc5-10* cells, Mob1p–GFP was only seen on the SPB in the mother cell body, the cell body with the mating projection, whereas in wild-type anaphase cells, both SPBs carried a Mob1p–GFP signal (Luca et al., 2001) (Fig. 8, A and B). Because in *cdc5-10* cells Bfa1p and Bub2p were only associated with the SPB in the bud (unpublished data), active Bfa1p–Bub2p complex may prevent binding of Mob1p–GFP to this SPB. Indeed, Mob1p–GFP was at both SPBs in 96% of the double *cdc5-10 Δbub2* mutant cells (Fig. 8, A and B), where the Bfa1p–Bub2p complex is absent from SPBs (unpublished data). We conclude that the presence of active Bfa1p–Bub2p complex on the bud-proximal SPB blocked Mob1p association to this SPB.

To investigate whether inactivation of the Bfa1p–Bub2p complex also requires Cdc14p, Mob1–GFP localization was monitored in *cdc14-2* and *cdc14-2 Δbub2* cells. In 67% of



B

Phenotype	Mob1p-GFP localisation in anaphase cells (%) wild type	Δbub2	cdc5-10 BUB2	cdc5-10 Δbub2	cdc14-2 BUB2	cdc14-2 Δbub2
	0	0	2	4	0	0
	0	0	88	0	5	0
	0	0	9	0	67	2
	0	0	0	0	0	0
	71	50	1	96	28	98
	29	50	0	0	0	0

DAPI • Mob1p-GFP

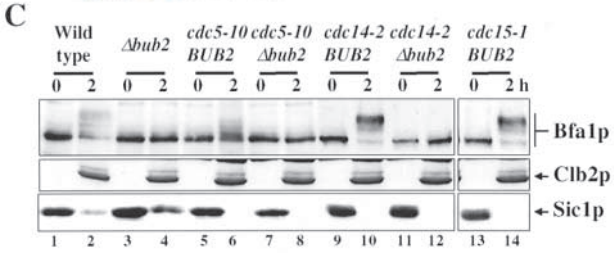


Figure 8. **Cdc14p plays a role in the inactivation of the Bfa1p–Bub2p complex in early anaphase.** (A–C) Active Bfa1p–Bub2p prevents Mob1p from binding to the SPB. The indicated cell types with *MOB1–GFP* were synchronized with α-factor at 23°C ($t = 0$). This marked the mother cell with a mating projection. After washing, cells were incubated at 37°C for 2 h until most of the cells had separated their DAPI staining regions. (A) Cells incubated for 2 h at 37°C were analyzed. After fixation, DAPI and Mob1p–GFP SPB staining were recorded. Bar, 5 μm. (B) Phenotypes of anaphase cells incubated for 2 h at 37°C ($n > 100$). Note that Mob1p was only detected at the cytokinesis site in wild-type and Δ*bub2* cells, which is a reflection of late anaphase arrest of *cdc5-10* and *cdc14-2* mutants (Luca et al., 2001). (C) Cell extracts (α-factor cells, $t = 0$; cells incubated for 2 h at 37°C, $t = 2$) were analyzed by immunoblotting for Bfa1p, Clb2p, and Sic1p. *cdc15-1* cells that had been incubated with α-factor at 23°C (lane 13) or for 2 h at 37°C (lane 14) were included as controls for Bfa1p phosphorylation. The high Clb2p and low Sic1p levels confirmed that all cell types were in late anaphase.

cdc14-2 MOB1–GFP cells, Mob1p–GFP associated most strongly with the anaphase SPB in the mother cell body (Fig. 8 A). A weaker signal was seen on the bud-proximal SPB (Fig. 8 A, arrow), which carried the Bfa1p–Bub2p complex (unpublished data). A similar result has been reported for *cdc14-1* cells (Luca et al., 2001). Deletion of *BUB2* equalized the Mob1p–GFP SPB signals (Fig. 8, A and B), suggesting that the Bfa1p–Bub2p complex is still active in *cdc14-2* cells and inhibits the SPB binding of Mob1p. Taking into account that Bfa1p is hyperphosphorylated in *cdc14-2* cells (Fig. 8 C, lane 10), phosphorylation of Bfa1p by Cdc5p is probably not the only mechanism that inactivates the Bfa1p–Bub2p complex. An additional step involving Cdc14p may play a role in the inactivation of Bfa1p–Bub2p in early anaphase.

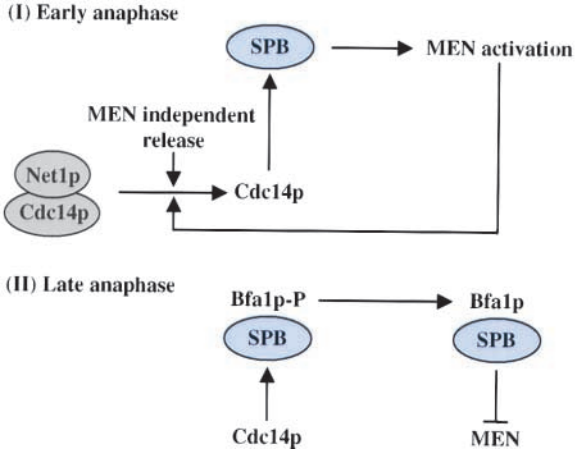


Figure 9. **Model for the function of Cdc14p**. (I) During interphase, Cdc14p is entrapped in the nucleolus through binding to Net1p (Shou et al., 1999; Visintin et al., 1999). In early anaphase, Cdc14p is partially released from the nucleolus in an MEN-independent manner. Cdc14p binds to the SPB and activates the MEN, which then triggers the complete release of Cdc14p from the nucleolus. (II) In late anaphase, Cdc14p activates the Bfa1p–Bub2p GAP through dephosphorylation of Bfa1p, which in turn inactivates the MEN.

Discussion

Previously, it has been shown that Cdc14p is released from the nucleolus in anaphase upon MEN activation and then facilitates mitotic exit by decreasing Cdk–Clb activity (Visintin et al., 1998, 1999; Shou et al., 1999). Here, we extend this model and propose that Cdc14p has a dual role in MEN regulation: it activates the MEN in early anaphase and inactivates the MEN through Bfa1p dephosphorylation in late anaphase (Fig. 9).

Cdc14p activates the MEN in early anaphase

Our results show that Cdc14p is partially released from the nucleolus in early anaphase independently of the MEN components Cdc15p, Dbf2p, and Tem1p (Fig. 9 I). Evidence for an MEN-independent step in releasing the nucleolar Cdc14p has already been obtained while studying Cdc14p in *net1-1* cells (Shou et al., 1999). In addition, during revision of our manuscript, a paper by Stegmeier et al. (2002) also reported that Cdc14p is partially released from the nucleolus in cells with an inactive MEN. These authors showed that separase, Cdc5p, the kinetochore protein Slk19p, and Spo12p all have a role in the control of Cdc14p localization during early anaphase. Consistent with this, we observed that Cdc14p remained largely in the nucleolus in *cdc5-10* cells.

What is the function of the fraction of Cdc14p that is released from the nucleolus in early anaphase in an MEN-independent manner? In *cdc14-2* cells, the Bfa1p–Bub2p complex

was still partially active, as assessed by Mob1p localization, despite phosphorylation of Bfa1p by Cdc5p. This suggests a role of Cdc14p in Bfa1p–Bub2p inactivation, resulting in the conversion of the GTPase Tem1p into its GTP-bound active form and thereby the activation of the MEN. Thus, phosphorylation of Bfa1p by Cdc5p at anaphase onset may not be sufficient to fully inactivate the Bfa1p–Bub2p GAP. A similar conclusion has been reached based on the observation that a constitutively active *BFA1-11A* does not significantly delay mitotic exit (Hu et al., 2001).

How Cdc14p regulates the MEN in early anaphase is not fully understood. However, given that Cdc14p interacts with Bfa1p and Tem1p and that Cdc14p is required to fully activate the MEN, it is possible that Cdc14p acts through Bfa1p and Tem1p. Considering that Cdc14p does not dephosphorylate Bfa1p before mitotic exit and the observation that phosphatase-independent functions of Cdc14p exist (Shirayama et al., 1996), inactivation of the Bfa1p–Bub2p complex or activation of Tem1p in early anaphase may not require the phosphatase activity of Cdc14p. Cdc14p binding to Bfa1p or Tem1p may activate the MEN by decreasing the GAP activity of the Bfa1p–Bub2p complex or by facilitating the exchange of GDP to GTP of Tem1p. Dephosphorylation of the Tem1p binding kinase Cdc15p in early anaphase by Cdc14p probably also contributes to MEN activation (Jaspersen and Morgan, 2000; Stegmeier et al., 2002).

Cdc14p inactivates the MEN at the end of anaphase through Bfa1p dephosphorylation

Although Cdc14p probably activates the MEN in early anaphase (as discussed above), it seems to have the opposite function when cells exit mitosis (Fig. 9 II). We show that Bfa1p is dephosphorylated by the phosphatase Cdc14p as cells exit mitosis. Because phosphorylation of Bfa1p by Cdc5p inactivates the Bfa1p–Bub2p GAP (Hu et al., 2001; unpublished data), it is likely that dephosphorylation of Bfa1p by Cdc14p reactivates the Bfa1p–Bub2p GAP complex in telophase/G1. Evidence for the reactivation of the Bfa1p–Bub2p complex with mitotic exit was obtained through studying the SPB localization of the MEN protein Mob1p. In wild-type cells, inactivation of the MEN coincided with Bfa1p dephosphorylation at the end of mitosis. Furthermore, in cells lacking the Bfa1p–Bub2p complex, Tem1p remained active even when cells entered G1. This suggests a Bfa1p–Bub2p-dependent inactivation of the MEN when wild-type cells exit mitosis. Taken together, these observations support a model in which the dephosphorylation of Bfa1p by Cdc14p at the end of mitosis activates the Bfa1p–Bub2p complex and thereby inhibits the MEN until the renewed phosphorylation of Bfa1p by Cdc5p during the next mitosis.

Cdc14p is part of a positive feedback loop

We propose the order of events outlined in Fig. 9. Cdc14p is released from the nucleolus in early anaphase in an MEN-independent manner and binds immediately to SPBs (Fig. 9 I). Our data are consistent with a model in which this fraction of Cdc14p activates the MEN in early anaphase together with the polo-like kinase Cdc5p (Hu et al., 2001; Lee et al., 2001; unpublished data). Thus, the partially released Cdc14p stimulates the MEN, which in turn promotes the further release of Cdc14p or prevents its nucleolar reuptake (Fig. 9 I). The latter has been suggested for SIN regulation of Clp1p/Flp1p (Cueille et al., 2001; Trautmann et al., 2001). This scenario creates a positive feedback loop that is essential to produce an efficient wave of cytoplasmic Cdc14p, which is probably required to switch the balance toward the dephosphorylation of proteins and promote exit from mitosis. Only this massive liberation of Cdc14p is sufficient to dephosphorylate Bfa1p and thereby reactivate the Bfa1p–Bub2p GAP in late anaphase, a step that will subsequently inhibit the MEN (Fig. 9 II). Cdc14p also dephosphorylates Hct1p, Sic1p, and Swi5p, which then trigger inactivation of Cdk–Clb (Visintin et al., 1998). Thus, Cdc14p first activates the MEN in early anaphase, and when sufficient Cdc14p is released from the nucleolus, it triggers mitotic exit and inactivates the MEN through the dephosphorylation of Bfa1p. This dual role of Cdc14p will restrict MEN activity to a short period, sufficient for mitotic exit.

Do Cdc14p-like proteins fulfill a common function at SPBs?

Comparing the MEN and SIN, it becomes apparent that the regulatory aspects of the two pathways are similar. A common property of Clp1p/Flp1p and Cdc14p is their association with the SPB (Cueille et al., 2001; Trautmann et al., 2001). Although it is unclear how Clp1p/Flp1p binds to SPBs, our data indicate that the association of the budding yeast Cdc14p with the SPB is mediated through an association with Bfa1p. Cdc14p also interacts with Tem1p. It will be interesting to see whether the fission yeast Clp1p/Flp1p binds to the Bfa1p–Bub2p complex and Tem1p homologues, named Byr4p–Cdc16p and Spg1p, respectively. At least, localization of Clp1p/Flp1p with SPBs was in part dependent on Cdc16p (Cueille et al., 2001). It is important to note that Cdc14p must bind to additional SPB or SPB-associated MEN components, because colocalization of Cdc14p–YFP and the SPB marker Spc42p–CFP was observed in 10% of anaphase *Δbfa1* cells in which Bub2p, Bfa1p, and Tem1p all fail to associate with the SPB (Pereira et al., 2000). Thus, additional SPB components or cell cycle regulators associated with the SPB may be the targets of Cdc14p and Clp1p/Flp1p (Bridge et al., 1998).

A further common feature is the MEN/SIN-independent release of Cdc14p/Clp1p/Flp1p from the nucleolus (Cueille et al., 2001; Trautmann et al., 2001). A third conserved mechanism is the inactivation of Byr4p–Cdc16p by polo kinase (Balasubramanian et al., 2000; Hu et al., 2001; Tanaka et al., 2001). The data from fission yeast are also consistent with an activation of the SIN by Clp1p/Flp1p through Byr4p–Cdc16p. The fission yeast cytokinesis checkpoint requires SIN activity but is impaired in the absence of Clp1p/Flp1p (Cueille et al., 2001; Trautmann et al., 2001). In addition, it was found that SPB localization of Sid1p–GFP, which is dependent on an active SIN (Guertin et al., 2000), required the presence of Clp1p/Flp1p. Thus, the activation of the MEN and SIN via Bfa1p–Bub2p or Byr4p–Cdc16p may be a conserved feature of spindle pole–associated Cdc14-related proteins.

Table I. **Yeast strains**

Name	Genotype	Source or reference
CLY254	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 cdc5-10 MOB1-GFP-KanMX6*	This study
CLY278	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δbub2::URA3 MOB1-GFP-KanMX6*	This study
CLY279	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δbub2::URA3 cdc5-10 MOB1-GFP-KanMX6*	This study
ESM356-1	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1*	Pereira et al., 2001
ESM1278	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 cdc15-1*	This study
ESM1379	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 MOB1-GFP-KanMX6*	This study
ESM1387	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 CDC14-YFP-His3MX6 SPC42-CFP-KanMX6 Δbfa1::klTRP1*	This study
ESM1393	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 cdc14-2 MOB1-GFP-KanMX6*	This study
ESM1462	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 CDC14-CFP-KanMX6 MOB1-YFP-His3MX6*	This study
ESM1463	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 dbf2-2 CDC14-YFP-His3MX6 SPC42-CFP-KanMX6*	This study
ESM1466	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ cdc15-1 CDC14-YFP-His3MX6 SPC42-CFP-KanMX6*	This study
ESM1476	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δbub2::URA3 cdc14-2 MOB1-GFP-KanMX6*	This study
ESM1581	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 Δtem1::KanMX6 2μ-CDC15 SPC42-CFP-klTRP1 CDC14-YFP-His3MX6*	This study
ESM1625	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ CDC14-YFP-His3MX6 NET1-CFP-KanMX6*	This study
ESM1626	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ dbf2-2 CDC14-YFP-His3MX6 NET1-CFP-KanMX6*	This study
ESM1627	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ cdc15-1 CDC14-YFP-His3MX6 NET1-CFP-KanMX6*	This study
ESM1628	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ cdc5-10 CDC14-YFP-His3MX6 NET1-CFP-KanMX6*	This study
GPY190	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 BFA1-3HA-KanMX6*	This study
GPY196	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 BFA1-9Myc-klTRP1 BUB2-3HA-KanMX6*	This study
GPY247	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 BFA1-3HA-KanMX6 Δcdc26::klTRP1*	This study
GPY436	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 CDC14-YFP-His3MX6 SPC42-CFP-klTRP1*	This study
GPY464	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 cdc15-1 BFA1-3HA-His3MX6*	This study
GPY470	*MATa ura3-52::Gal1-CDC14 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 BFA1-3HA-KanMX6*	This study
GPY471	*MATa ura3-52::Gal1-CDC14 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 BFA1-3HA-KanMX6 Δcdc26::klTRP1*	This study
GPY548	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 Δbfa1::klTRP1 CDC14-CFP-KanMX6 MOB1-YFP-His3MX6*	This study
GPY602	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 CDC14-YFP-His3MX6 SPC42-CFP-klTRP1 Δtem1::Gal1-UPL-TEM1*	This study
GPY604	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 CDC14-YFP-His3MX6 SPC42-CFP-klTRP1*	This study
GPY606	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 cdc5-10 CDC14-YFP-His3MX6 SPC42-CFP-klTRP1*	This study
GPY622	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 CDC14-YFP-His3MX6 SPC42-CFP-klTRP1 KanMX6-Gal1-CDC20*	This study
GPY623	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::Gal1-CDC14^{C283A} BFA1-3HA-KanMX6 Δcdc26::klTRP1*	This study
SGY37	*MATa ura3-52::URA3-lexA-op-LacZ trp1 his3 leu2*	Geissler et al., 1996
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter, 1989

Materials and methods

Growth conditions, strain constructions, two-hybrid analysis, and conditional lethal alleles of *CDC5*

Basic yeast methods and growth media were as previously described (Sherman, 1991). Yeast strains were grown in yeast extract, peptone, dextrose medium containing 100 mg/liter adenine (YPAD medium). Synthetic complete medium was used to select for plasmids. To induce the Gal1 promoter, galactose (2%) was added to yeast extract, peptone, 3% raffinose (YP raffinose) medium. Yeast cells were synchronized by α-factor (10 μg/ml) block and released. Yeast strains are listed in Table I. All strains are derived from YPH499 and plasmids were pRS derivatives (Sikorski and

Hieter, 1989). *cdc15-1*, *cdc14-2*, and *dbf2-2* were cloned by gap repair using conditional lethal mutants (Jaspersen et al., 1998). *CDC5* was mutagenized by PCR and conditional lethal alleles of *CDC5* were selected as previously described (Geissler et al., 1996). Analysis of *cdc14-2* and *cdc5-10* by sequencing identified C309Y and F614L substitutions, respectively. *cdc15-1*, *cdc14-2*, *cdc5-10*, and *dbf2-2* were introduced into YPH499 by pop-in-pop-out. Gal1–*UPL-TEM1* was introduced with plasmid pWS103 (Shou et al., 1999). Strains were constructed using PCR-based methods (Knop et al., 1999). Plasmids pDH3 and pDH5 (provided by T. Davis, University of Washington, Seattle, WA) were used to construct CFP and YFP gene fusions. Two-hybrid interactions were determined in strain SGY37 with *CDC14*, *BFA1*, *BUB2*, and *NET1* subcloned into pMM5 and pMM6 (Geissler et al., 1996). β-Galactosidase activity was determined after cell lysis (Ausubel et al., 1994).

Antibodies and fluorescence microscopy

Bfa1p, Cdc14p, Clb2p, and Sic1p antibodies were raised in rabbits against the recombinant GST fusion proteins purified from *E. coli*. Anti-Tem1p antibodies have been described by Pereira et al. (2000). Antibodies were affinity purified with recombinant protein coupled to CNBr-Sepharose (Amersham Pharmacia Biotech). Mouse monoclonal anti-GST and rabbit anti-Tub2p antibodies were gifts from Z. Yazici (Beatson Institute for Technology, Glasgow, UK) and F. Solomon (Massachusetts Institute of Technology, Cambridge, MA), respectively. Anti-Myc and -HA antibodies were from Boehringer. Secondary antibodies for immunoblotting were goat anti–rabbit and goat anti–mouse IgGs coupled to HRP (Jackson ImmunoResearch Laboratories). Yeast cells with CFP-, GFP-, or YFP-tagged proteins were analyzed by fluorescence microscopy after fixation with 4% paraformaldehyde in 150 mM phosphate buffer, pH 6.5, for 10 min at 20°C. In the case of GFP-tagged proteins, fixed cells were incubated with 1 μg/ml DAPI for 10 min at 20°C to stain DNA.

In vitro binding experiments

GST and GST–Cdc14p fusion proteins, produced in *E. coli,* were incubated with glutathione-Sepharose beads as recommended by Amersham Pharmacia Biotech. Proteins bound to beads were washed with UB buffer (0.05 M Hepes, pH 7.5, 0.1 M KCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.2% Triton X-100, 1 mM GTP, EDTA-free protease inhibitors cocktail tablets [Roche]). A yeast lysate of *BFA1–9MYC BUB2–3HA* cells was prepared using glass beads (Knop et al., 1999). In brief, yeast cells were resuspended in UB buffer and acid-washed glass beads (Sigma-Aldrich) were added. Cells were lysed using a vortex mixer until >90% of the cells were sheared. The lysate was cleared by centrifugation (5,100 *g*, 10 min, 4°C). The supernatant was incubated for 60 min at 4°C with the purified GST proteins bound to the glutathione-Sepharose beads. After three washes with UB buffer, the associated proteins were resuspended in HU-DTT (200 mM Tris, pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 15 mg/ml DTT, bromophenol blue) and incubated for 15 min at 65°C. Samples were analyzed by immunoblotting. Alternatively, recombinant 6His–Tem1p or 6His–Cdc14p, produced in *E. coli*, were presented to Ni^{2+}-NTA-agarose and affinity purified as recommended by the manufacturer (QIAGEN). The purified 6His–Tem1p or 6His–Cdc14p were incubated for 60 min at 4°C with recombinant GST, GST–Cdc14p, GST–Bfa1p, and GST–Bub2p, produced in *E. coli*, and bound to glutathione-Sepharose. Subsequent washes and immunoblotting were performed as described above.

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