Regulation of the Mitotic Exit Protein Kinases Cdc15 and Dbf2

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In budding yeast, the release of the protein phosphatase Cdc14 from its inhibitor Cfi1/Net1 in the nucleolus during anaphase triggers the inactivation of Clb CDKs that leads to exit from mitosis. The mitotic exit pathway controls the association between Cdc14 and Cfi1/Net1. It is comprised of the RAS-like GTP binding protein Tem1, the exchange factor Lte1, the GTPase activating protein complex Bub2-Bfa1/Byr4, and several protein kinases including Cdc15 and Dbf2. Here we investigate the regulation of the protein kinases Dbf2 and Cdc15. We find that Cdc15 is recruited to both spindle pole bodies (SPBs) during anaphase. This recruitment depends on *TEM1* but not *DBF2* or *CDC14* and is inhibited by *BUB2*. Dbf2 also localizes to SPBs during anaphase, which coincides with activation of Dbf2 kinase activity. Both events depend on the mitotic exit pathway components *TEM1* and *CDC15*. In cells lacking *BUB2*, Dbf2 localized to SPBs in cell cycle stages other than anaphase and telophase and Dbf2 kinase was prematurely active during metaphase. Our results suggest an order of function of mitotic exit pathway components with respect to SPB localization of Cdc15 and Dbf2 and activation of Dbf2 kinase. *BUB2* negatively regulates all 3 events. Loading of Cdc15 on SPBs depends on *TEM1*, whereas loading of Dbf2 on SPBs and activation of Dbf2 kinase depend on *TEM1* and *CDC15*.

INTRODUCTION

The final stage of the cell cycle is exit from mitosis (reviewed in Morgan, 1999; Zachariae, 1999). During this time, the mitotic spindle disassembles, chromosomes decondense, and cytokinesis occurs. In the budding yeast *Saccharomyces cerevisiae*, these events are brought about by inactivation of Clb cyclin-dependent kinases (Clb-CDKs), which, earlier in the cell cycle, trigger entry into mitosis (reviewed in Zachariae and Nasmyth, 1999). Inactivation of Clb-CDKs is brought about by 2 redundant mechanisms. Ubiquitin-dependent degradation of Clb cyclins by the anaphase promoting complex (APC) or cyclosome (C) complexed with the APC activator Cdh1 (APC/C^{Cdh1}) and binding of the CDK inhibitor Sic1 to the Clb-CDK complex (Schwab *et al.*, 1997; Visintin *et al.*, 1997; Fang *et al.*, 1998; Shirayama *et al.*, 1998; Kotani *et al.* 1999; Kramer *et al.*, 2000; Yudkovsky *et al.*, 2000). The protein phosphatase Cdc14 plays an important role in both stimulating APC-dependent degradation of mitotic Clb cyclins and accumulation of Sic1 (Visintin *et al.*, 1998; Jaspersen *et al.*, 1999). Cdc14 dephosphorylates the APC-specificity factor Cdh1, the CDK inhibitor Sic1 and its transcription factor Swi5, allowing for activation of Cdh1 and transcription and stabilization of Sic1, respectively.

Cdc14 is regulated by Cfi1/Net1 (Shou *et al.*, 1999; Straight *et al.*, 1999; Visintin *et al.*, 1999; Traverso *et al.*, 2001). Cfi1/Net1 inhibits Cdc14 in the nucleolus during G1, S phase, and early mitosis. During nuclear division, Cdc14 is released from Cfi1/Net1, allowing Cdc14 to reach its targets. The mitotic exit pathway is required for the release of Cdc14 from Cfi1/Net1 (Shou *et al.*, 1999; Visintin *et al.*, 1999). This pathway includes the Ras-like GTP binding protein Tem1, a putative exchange factor, Lte1; the 2 component GTPase-activating protein complex (GAP) Bub2-Bfa1/Byr4; the protein kinases Cdc5, Cdc15, Dbf2 and Dbf20; and the Dbf2-associated protein Mob1 (Kitada *et al.*, 1993; Donovan *et al.*, 1994; Toyn and Johnston, 1994; Shirayama *et al.*, 1994a, b; Charles *et al.*, 1998; Komarnitsky *et al.*, 1998; Luca and Winey, 1998; Alexandru *et al.*, 1999; Fesquet *et al.*, 1999; Fraschini *et al.*, 1999; Li, 1999). Pds1 and Esp1, both regulators of sister-chromatid separation are also required for release of Cdc14 from Cfi1/Net1 and inactivation of mitotic kinases (Cohen-Fix and Koshland, 1999; Shirayama *et al.*, 1999; Tinker-Kulberg and Morgan, 1999). In addition to release of Cdc14 from the nucleolus, Clb5-CDK1 (Cdc28) kinase, a potent antagonist of Cdc14, needs to be inactivated to allow for exit from mitosis to occur. This is achieved by APC/C^{Cdc20} which degrades Clb5 during anaphase (Shirayama *et al.*, 1999).

One of the functions of the mitotic exit pathway is to ensure that exit from mitosis does not occur before parti-

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tioning of the genetic material between mother and daughter cell. The GTPase Tem1 and Bub2-Bfa1/Byr4 localize onto spindle pole bodies (SPBs), predominantly the 1, which migrates into the daughter cell. Lte1 localizes to the bud (Fraschini *et al.*, 1999; Li 1999; Bardin *et al.*, 2000; Bloecher *et al.*, 2000; Daum *et al.*, 2000; Pereira *et al.*, 2000; Wang *et al.*, 2000). The changes of Tem1 protein levels during the cell cycle and the localization patterns of Tem1 and Lte1 result in the 2 proteins being present in the same cellular compartment only after the nucleus enters the bud during nuclear division. This mode of localization ensures that mitotic exit does not occur before chromosome partitioning. Bub2-Bfa1/Byr4 also play a critical role in preventing exit from mitosis before partitioning of the genetic material between mother and daughter cell. However, whether Bub2-Bfa1/Byr4 activity is regulated remains to be determined.

The protein kinases Cdc15, Dbf2, and Dbf20, and the Dbf2-associated protein Mob1 are thought to relay the signal generated at the cytoplasmic face of the SPB to bring about the release of Cdc14 from Cfi1/Net. Cdc15 shows a high degree of homology to the family of MAP kinase kinase kinases (Hunter and Plowman, 1997). The protein interacts with Tem1 as judged by coimmunoprecipitation (Bardin *et al.*, 2000; Asakawa *et al.*, 2001), but there is no evidence that its kinase activity is regulated by Tem1. Cdc15 protein levels and associated kinase activity are constant throughout the cell cycle (Jaspersen *et al.*, 1998); however, Cdc15 localization and its phosphorylation status change during the cell cycle. Cdc15 associates with the cytoplasmic face of SPBs during anaphase and is dephosphorylated during exit from mitosis (Cenamor *et al.*, 1999; Gruneberg *et al.*, 2000; Jaspersen and Morgan, 2000; Xu *et al.*, 2000; Menssen *et al.*, 2001). The protein levels of the related protein kinases Dbf2 and Dbf20 are also constant throughout the cell cycle (Toyn and Johnston, 1994), but Dbf2 kinase activity fluctuates during the cell cycle, being low during G1, S phase, and early mitosis, and high during anaphase and exit from mitosis (Toyn and Johnston, 1994). Dbf2, like Cdc15 localizes to SPBs and to the bud neck during cytokinesis (Frenz *et al.*, 2000). Dbf2 kinase activity is thought to be controlled at least in part by Mob1, which physically interacts with Dbf2 (Luca and Winey, 1998; Komarnitsky *et al.*, 1998).

Although the regulation of individual components of the mitotic exit pathway is the focus of intense investigation, the order in which the components of the mitotic exit pathway function is not known. To address this question, we investigated how the localization and activity of the protein kinases Cdc15 and Dbf2 is regulated during the cell cycle and how mutations impairing the mitotic exit pathway affect the 2 protein kinases. We find that Cdc15 and Dbf2 are recruited to both SPBs during anaphase. Localization of Cdc15 on SPBs depends on *TEM1*, but not on *DBF2* and *CDC14*. Loading of Dbf2 onto SPBs and activation of Dbf2 kinase activity depends on *TEM1* and *CDC15*, but not *CDC14*. These findings suggest an order of function in the recruitment of mitotic exit pathway components to the SPB and activation of Dbf2 kinase activity: *TEM1* functions upstream of *CDC15* and *CDC15* upstream of *DBF*2. Furthermore, we find that during a normal cell cycle localization of Dbf2 to SPBs and Dbf2 kinase activity correlate, suggesting that association of Dbf2 with Tem1 and Cdc15 on SPBs is a prerequisite for Dbf2 activation. In cells lacking *BUB2*, Dbf2 is localized

Table 1. Strains and relevant genotypes

Strain	Relevant genotype
K699	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL
A852	MATalpha, dbf2-2
A1787	MATa CDC15-3HA
A1931	MATa, DBF2-3MYC
A2044	MATa, nud1-44::TRP1, nud1::HIS5, CDC15-HA
A2096	MATa, cdc15-2, DBF2-3MYC
A2107	MATa, cdc5-1, DBF2-3MYC
A2110	MATa, cdc28-4, DBF2-3MYC
A2111	MATalpha, dbf2-2, CDC15-3HA
A2125	MATa, tem1-3, CDC15-3HA
A2135	MATa, tem1-3, DBF2-3MYC
A2143	MATa, cdc20-3, DBF2-3MYC
A2152	MATa, cdc5-1, CDC15-3HA
A2156	MATa, cfi1::URA3, DBF2-3MYC
A2179	MATa, cdc14-3, DBF2-3MYC
A2181	MATalpha, cdc13-1, DBF2-3MYC
A2184	MATa, cdc23-1, DBF2-3MYC
A2184	MATa, cdc23-1, DBF2-3MYC
A2212	MATalpha, cdc14-3, CDC15-3HA
A2339	MATa, nud1-44::TRP1, nud1::HIS5, DBF2-3MYC
A2340	MATalpha, nud1-44::TRP1, nud1::HIS5, DBF2-3MYC
A2472	MATa, bub2::HIS3, DBF2-3MYC
A2840	MATa, bub2::HIS3, CDC15-3HA
A3044	MATa, YCplac22/dbf2KI→RT-MYC
A3045	MATa, dbf2-2, YCplac22/dbf2KI→RT-MYC
A3046	MATa, dbf2-2, YCplac22/DBF2-3MYC

to SPBs throughout the cell cycle, but Dbf2 kinase activity was prematurely activated only during metaphase. This finding indicates that SPB localization is not sufficient for Dbf2 activation and that other factors are necessary for Dbf2 to be active as a protein kinase.

MATERIALS AND METHODS

All strains were derivatives of strain W303 (K699) and are listed with relevant genotypes in Table 1. *MYC* tags were introduced at the N-terminus of endogenous *DBF2* or *DBF20* according to Schneider *et al.* (1995). The conserved lysine (amino acid 206) and the after isoleucine (amino acid 207) residues within the Dbf2 kinase were mutated by PCR to arginine and threonine, respectively, thereby creating a BsiWI site. Strains carrying a *CDC15-HA* fusion were as described in Jaspersen *et al.* (1998). Conditions for growth and release of synchronous cultures from arrest by α-factor were as described by Surana *et al.* (1993). For synchronous release of cells from a hydroxyurea (HU) arrest cells were arrested with 10 mg/ml HU. When arrest was complete, cells were washed and releases into medium lacking the drug. Cells were arrested with hydroxyurea and nocodazole by adding to the cultures 10 mg/ml hydroxyurea or 15 μg/ml nocodazole, respectively. Immunoblot analysis of the total amount of Clb2, Kar2 and Dbf2-Myc was performed as described in Cohen-Fix *et al.* (1996). Antibody dilutions were used as in Visintin *et al.* (1997). Anti-Myc antibodies were used at 1:50 dilution to immunoprecipitate Dbf2 either for phosphatase treatment or to measure Dbf2 kinase activity. Dbf2 kinase activity was assayed as Clb2 kinase activity. The method is described in Surana *et al.* (1993). Indirect in situ immunofluorescence methods and antibody concentrations were as described in Visintin *et al.* (1999).

RESULTS

Cdc15 Localizes to Both Spindle Pole Bodies during Anaphase

Several reports had shown that Cdc15 localizes to SPBs (Cenamor *et al.*, 1999; Xu *et al.*, 2000; Gruneberg *et al.*, 2000; Menssen *et al.*, 2001). However, slight differences in the dynamics of Cdc15 localization had been observed, depending on the epitope tag used. The localization pattern we observed was identical to that previously observed by Xu *et al.* (2000). Cdc15, whose chromosomal copy was tagged with 3 HA epitopes (Jaspersen *et al.*, 1998), was not detectable in G1 cells, in cells that had not yet formed a mitotic spindle and cells with metaphase spindles. Cdc15 was present as a dot at both ends of mitotic spindles in cells with anaphase and telophase spindles (Figure 1B). Similar results were obtained when Cdc15 was analyzed in synchronous cultures. Cells were arrested in G1 with the pheromone α-factor followed by release into the cell cycle. Cdc15 was found on SPBs in cells containing anaphase and telophase spindles but not in cells in other cell cycle stages (Figure 1C).

Previous studies have shown that Cdc15 localizes to the outer plaque of the SPB, as Cdc15 localization is impaired in *cnm67* mutants in which the outer plaque of the SPB is disrupted (Cenamor *et al.*, 1999). However, Cdc15 localization was not affected in *nud1–2* mutants (Gruneberg *et al.*, 2000). As localization of Tem1 is impaired in temperature sensitive *nud1–44* mutants (Bardin *et al.*, 2000) and Cdc15 localization to SPBs depends on *TEM1* (Figure 1E, Table 2) we wished to examine the localization of Cdc15 in *nud1–44* mutants. In *nud1–44* mutants the outer plaque of the SPB dissociates from the rest of the organelle (Adams and Kilmartin, 1999). *nud1–44* mutants were arrested in G1 with the use of α-factor pheromone and released into the cell cycle at 37°C. Cdc15 was detected on SPBs in only 15% of anaphase and telophase cells (Figure 1D). In 30 percent of cells, Cdc15 localized to a dot in the cytoplasm, indicating that Cdc15 dissociated from SPBs along with the rest of the outer plaque of the SPB (Figure 1D). These results indicate that Tem1 and Cdc15 localize to the outer plaque of the SPB in a *NUD1*-dependent manner.

Cdc15 Localization to SPBs Depends on **TEM1** *and Is Inhibited by* **BUB2**

Next we analyzed whether SPB localization of Cdc15 depended on components of the mitotic exit pathway. Cdc15 was not even transiently detected on SPBs in *tem1–3* cells as they progressed through the cell cycle (Figure 1E), but was present on SPBs in *dbf2–2* and *cdc14–3* mutants and on 50 percent of *cdc5–1* mutants (Table 2). In exponentially growing *bub2*Δ cells, Cdc15 was present on SPBs throughout the cell cycle (data not shown). To examine the effects of *BUB2* on Cdc15 localization in more detail, we first analyzed Cdc15 localization in cells released from an α-factor block. We noticed, however, that prolonged incubation of cells in G1 led to a dramatic decline of Cdc15 signal in the α-factor–arrested cells, whereas Cdc15 could be detected on SPBs in the next G1 phase of the cell cycle (data not shown). Similar results were obtained when cells were released from a hydroxyurea block. Cdc15 was not detected in cells that had not yet formed a mitotic spindle, whereas it was detected in

cells entering the next G1 phase (Figure 1F). These findings indicate that the association of Cdc15 with SPBs during interphase is not stable in *bub2*Δ cells and disrupted by prolonged cell cycle arrest. However, the association of Cdc15 with SPBs organizing mitotic spindles was more stable. During the hydroxyurea arrest, Cdc15 localized to at least 1 SPB in 75% of cells that had formed a mitotic spindle. During mitosis Cdc15 localized to SPBs in the majority of cells and remained localized at SPBs during the after G1 stage (Figure 1F). We confirmed that the Cdc15 indeed localized to SPBs in *bub2*Δ cells, as the Cdc15 staining overlapped with the staining pattern observed with the SPB component Tub4 (Figure 1G). Our data suggest that localization of Cdc15 to SPBs is inhibited by *BUB2* and depends on *TEM1* but not on *DBF2* and *CDC14*.

Dbf2 Localizes to SPBs during Anaphase

The chromosomal copies of *DBF2* and *DBF20* were tagged with 3 Myc epitopes We could not detect Dbf20 by indirect in situ immunofluorescence (data not shown); however, the subcellular localization of Dbf2 was similar to that of Cdc15 (Figure 2B). The protein was undetectable in G1, S phase, metaphase, and early anaphase cells. In most, although not all, late anaphase and telophase cells, Dbf2 was present as a dot at 1 or both ends of mitotic spindles (Figures 2B and 7A). Dbf2 staining overlapped with Tub4, indicating that Dbf2 also localizes to SPBs or a structure associated with it (Figure 2C). Dbf2 localization on SPBs also depended on *NUD1*, as Dbf2 was undetectable by indirect immunofluorescence in *nud1–44* mutants (Figures 2D and 7F). These results suggest that Dbf2 localizes to the outer plaque of the SPB or a structure associated with it.

Next we determined the fraction of cells containing Dbf2 on both SPBs versus 1 SPB and investigated whether Dbf2 localized to mother or daughter SPB in those cells containing Dbf2 on only 1 SPB. Cells were treated with α-factor pheromone until they formed a mating projection (shmoo). When cells were released into the cell cycle, the newly formed bud was spherical whereas the mother cell remained shmoo-shaped. In 60% to 70% of cells, Dbf2 was detected on both SPBs; in 30% to 40% of cells, Dbf2 was found on the SPB present in the mother cell (Figure 7A). Our results indicate that Dbf2 associates with the outer plaque of both SPBs during anaphase in the majority cells.

SPB Localization of Dbf2 Coincides with Activation of Dbf2 Kinase

Dbf2 kinase activity is low during early stages of the cell cycle but high during anaphase and exit from mitosis (Toyn and Johnston, 1994). In synchronous cultures, Dbf2 kinase activity correlated well with localization of Dbf2 on SPBs of late anaphase spindles and changes in Dbf2 mobility (Toyn and Johnston, 1994; Figure 3). This change in Dbf2 mobility was due to dephosphorylation of the protein (Toyn and Johnston, 1994; Figure 3C).

To further investigate whether a correlation between the presence of Dbf2 on SPBs and active Dbf2 kinase exists, we analyzed Dbf2 in various cell cycle arrests. Dbf2 was not present on SPBs and Dbf2 kinase activity was low in G1-
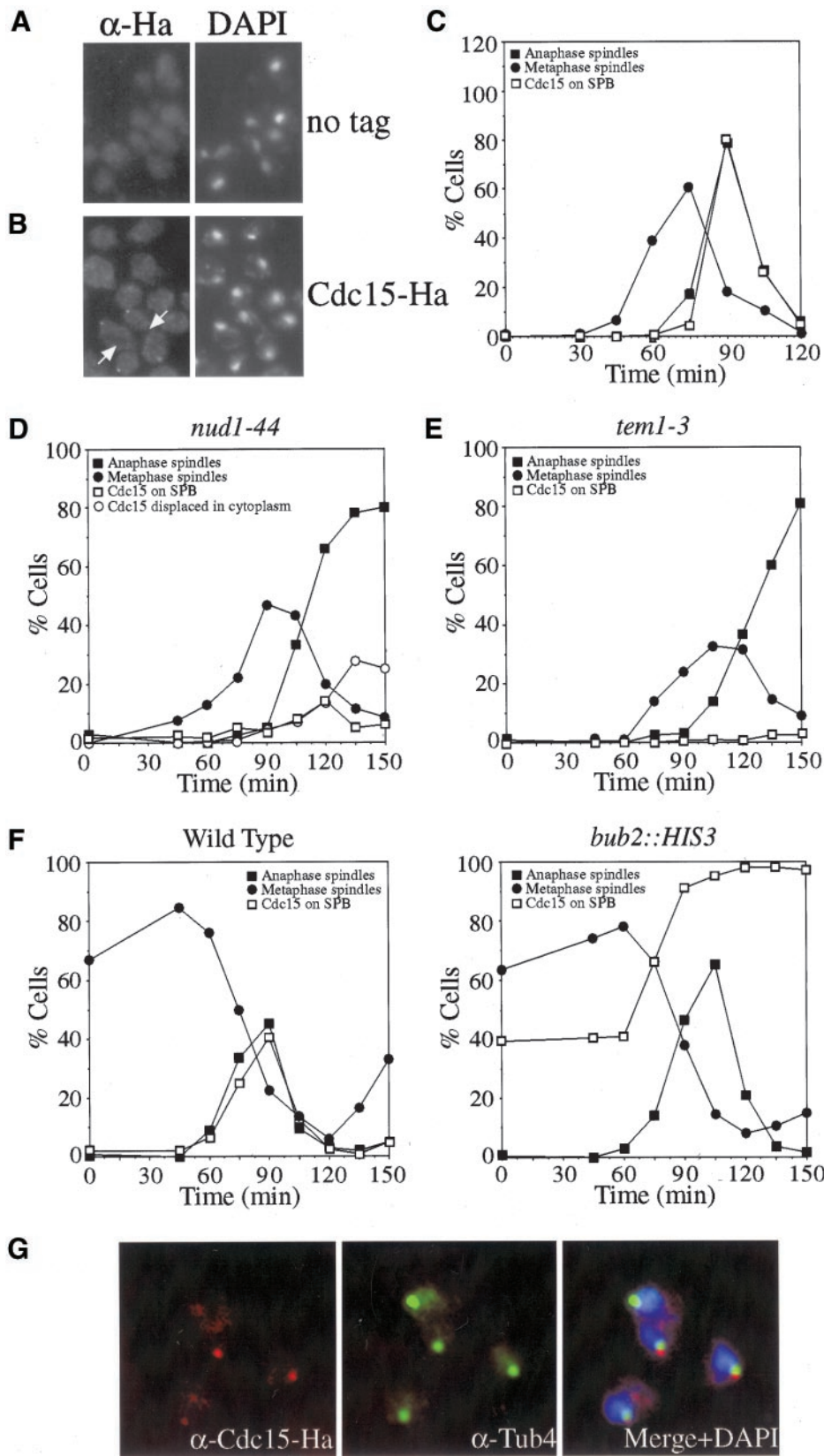
Figure 1. The subcellular localization of Cdc15-Ha during the cell cycle. Exponentially growing cells either lacking (A, no tag; K699) or carrying a *CDC15-HA* fusion (B, A1787) were fixed and subjected to indirect in situ immunofluorescence. Cdc15-Ha was visualized with the use of α-HA antibodies (BABCO). DAPI (4'6-diamidino-2-phenylindole) was used to stain DNA. (C) Cdc15-Ha localization in cells undergoing a synchronous cell cycle after an α-factor–induced G1 arrest. The percentage of cells with Cdc15-Ha on the SPB (spindle pole body) and the percentage of cells with metaphase and telophase spindles are shown. (D, E) *nud1–44* (A2044) or *tem1–3* (A2125) cells carrying a *CDC15-HA* fusion were arrested in G1 with α-factor (5 µg/ml) followed by release into medium lacking pheromone at the restrictive temperature (37°C). The percentage of cells with metaphase (closed circles) and anaphase spindles (closed squares) as well as the percentage of cells with Cdc15 on SPBs (open squares) or in the cytoplasm (open circles) was determined. (F) Wild-type (A1787) and *bub2:: HIS3* cells (A2840) were arrested in early S phase with the use of hydroxyurea (10 mg/ml) followed by release into medium lacking the drug. The percentage of cells with metaphase (closed circles) and anaphase spindles (closed squares) as well as the percentage of cells with Cdc15 on SPBs (open squares) was determined at the indicated times. (G) Colocalization of Cdc15-Ha with the spindle pole body component Tub4 in *bub2:: HIS3* cells. In all experiments, 200 cells were counted per time point (n = 200).

Table 2. Cdc15 localization in *cdc* mutants

Genotype	SPB localization
WT	+
nud1-44	−
tem1-3	−
cdc5-1	+/−
dbf2-2	+
cdc14-3	+
bub2::HIS3	prematurely*

*Prematurely indicates that Cdc15 is present on SPBs in stages of the cell cycle when it is not found on SPBs in wild-type cells.

arrested cells (α-factor, *cdc28−4*; Table 3), in S-phase-arrested cells (hydroxyurea), and G2/M phase arrested cells (nocodazole, *cdc13–1*; Table 3). In *cdc23–1* and *cdc20–1* mutants (both mutations inactivate the APC/C^{Cdc20}) Dbf2 kinase activity appeared high, yet Dbf2 was detected on SPBs in only a small fraction of cells (20% to 30%) and was present on only 1 SPB (data not shown). This finding suggested that

Dbf2 kinase activity was high despite Dbf2 being largely absent from SPBs. To investigate this apparent noncorrelation between the presence of Dbf2 on SPBs and active Dbf2 kinase in these mutants in more detail, we analyzed Dbf2 in *cdc23–1* mutants undergoing a synchronous cell cycle. *cdc23–1* mutants were arrested in G1 at the permissive temperature and released into the cell cycle at the restrictive temperature. Dbf2 kinase activity was found to be unusually high but so were Dbf2 protein levels (Figure 4). Similar results were found in *cdc20–1* mutants (data not shown). A subsequent analysis of the amount of Dbf2 kinase activity per Dbf2 protein revealed that Dbf2 kinase remained low in *cdc23–1* mutants (Figure 4). Only after long periods of incubation at 37°C did Dbf2 kinase activity increase (Figure 4) indicating that Dbf2 kinase is eventually activated. We noticed that in these later time points, Dbf2 associated with 1 SPB in ~30% of the cells (data not shown). These results show that Dbf2 protein is not active as a protein kinase in APC/C^{Cdc20} mutants and that APC/C^{Cdc20} is required to keep Dbf2 protein levels low. The high levels of Dbf2 protein observed in APC/C^{Cdc20} mutants may arise from Dbf2 itself being targeted for degradation by APC/C^{Cdc20}. As Dbf2 protein levels do not fluctuate during the cell cycle (as is
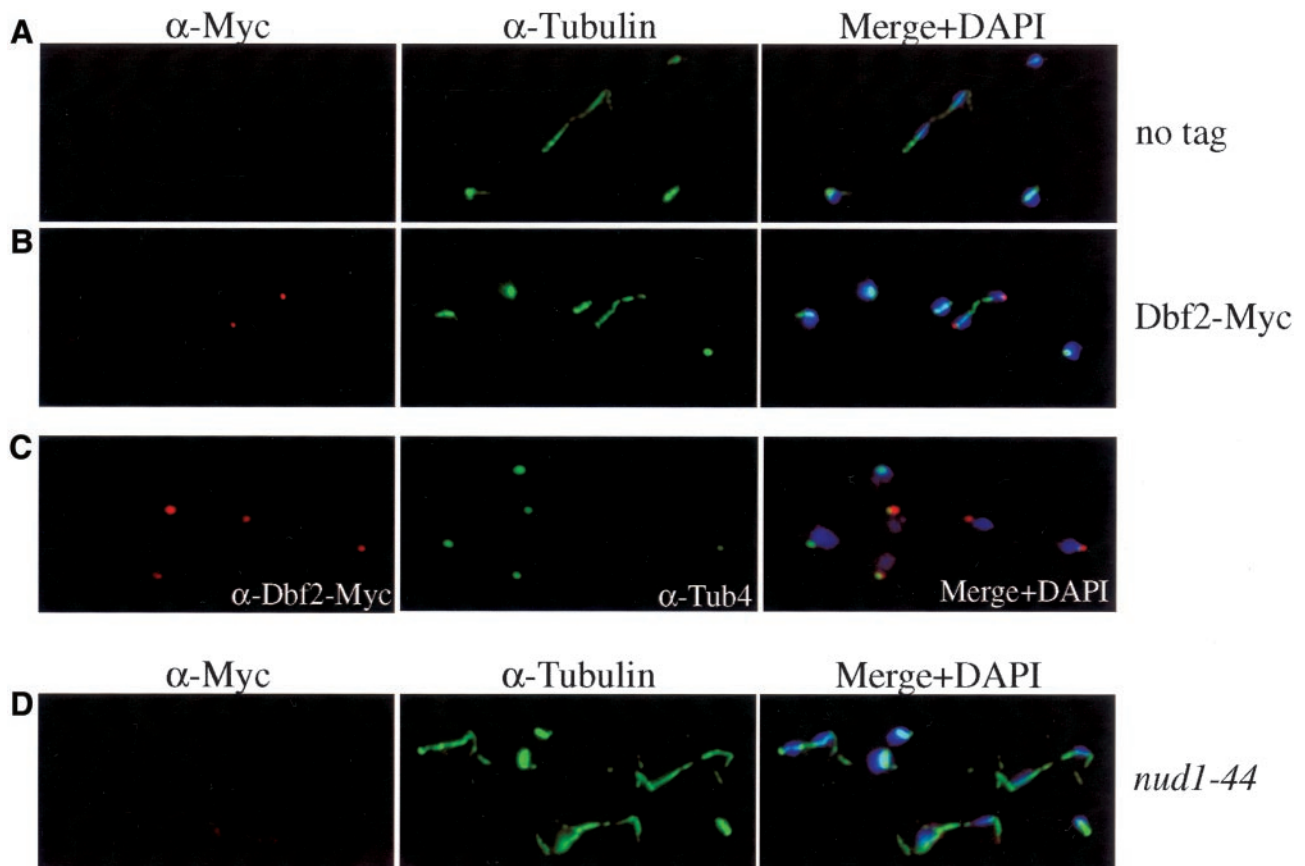

Figure 2. The subcellular localization of Dbf2-Myc during the cell cycle. (A) Exponential growing cells lacking a Dbf2-Myc fusion (no tag; K699). (B) Dbf2-Myc and microtubules of strain A1931 were visualized with the use of anti-Myc antibodies (BABCO) and antitubulin antibodies (α-tubulin), respectively. DAPI was used to stain DNA (DAPI). (C) Colocalization of Dbf2-Myc with the spindle pole body component Tub4. (D) Dbf2-Myc localization in *nud1–44* mutant (A2340) 120 min after shift to the restrictive temperature (37°C).
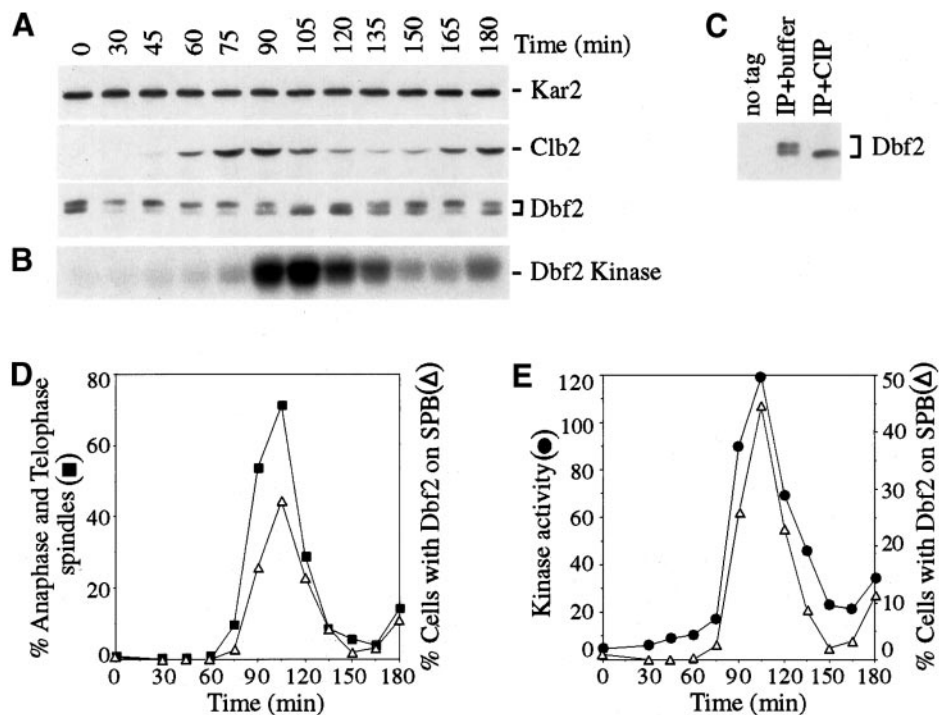
Figure 3. Localization of Dbf2 to SPBs and activation of Dbf2 kinase activity occur concomitantly. Cells carrying a *DBF2-MYC* tag (A1931) were arrested in G1 with α-factor (5 µg/ml) followed by release into medium lacking pheromone. The amount of Dbf2-Myc protein (A), Dbf2 kinase activity (B), the percentage of cells carrying Dbf2 on SPBs (n = 200; D, E) and cells containing anaphase or telophase spindles (n = 200; D) were analyzed. Clb2 protein levels (A) were examined to assess cell cycle synchrony; Kar2 was used as an internal loading control in Western blots. (C) Dbf2-myc was immunoprecipitated with the use of anti-Myc antibodies and incubated with calf intestinal alkaline phosphatase (CIP) buffer alone (IP+buffer) or with 80 U of CIP (IP+CIP). An immunoprecipitation from cells without a *DBF2-MYC* fusion is shown in the lane labeled "no tag".

characteristic for APC/C^{Cdc20} substrates), it is more likely that proteins required for Dbf2 synthesis are more abundant in APC/C^{Cdc20} mutants. In summary, our results show that in synchronous cultures as well as cell cycle arrests, Dbf2 localization to SPBs correlates with activation of Dbf2 kinase suggesting that these 2 processes are tightly linked.

Dbf2 Lacking Kinase Activity Associates with SPBs during Anaphase

Owing to the correlation between activation of Dbf2 kinase activity and localization of Dbf2 to SPBs, we were interested in determining whether Dbf2 kinase activity was required

Table 3. Dbf2 localization, phosphorylation, and kinase activity in *cdc* mutants

Genotype	SPB localization	Kinase activity	Dbf2 phosphorylation°
WT	+	+	+
nud1-44	−	−	+
α-factor	−	−	+
cdc28-4	−	−	−
hydroxyurea	−	−	++
cdc13-1	−	−	++
nocodazole	−	−	++
cdc23-1	−/+**	−/+***	++
cdc20-3	−/+**	−/+***	++
cfi1::URA3	+	+	−
cdc5-1	+/−	+/−	+
tem1-3	−	−	+
cdc15-2	−	−	+
cdc14-3	+	+	++
bub2::HIS3	prematurely*	++	+

* Indicates that Dbf2 is present on SPBs in stages of the cell cycle when it is not found on SPBs in wild-type cells.

** In approximately 30% of cells Dbf2 is present on one SPB after prolonged incubation at 37°C.

*** Dbf2 kinase activity is low in these mutants but after prolonged incubation at the restrictive temperature becomes active.

° + indicates that overall phosphorylation of Dbf2 is as in exponentially growing wild-type cells; ++ indicates that Dbf2 is primarily found in the hyperphosphorylated form; − indicates that Dbf2 is primarily found in the hypophosphorylated form.
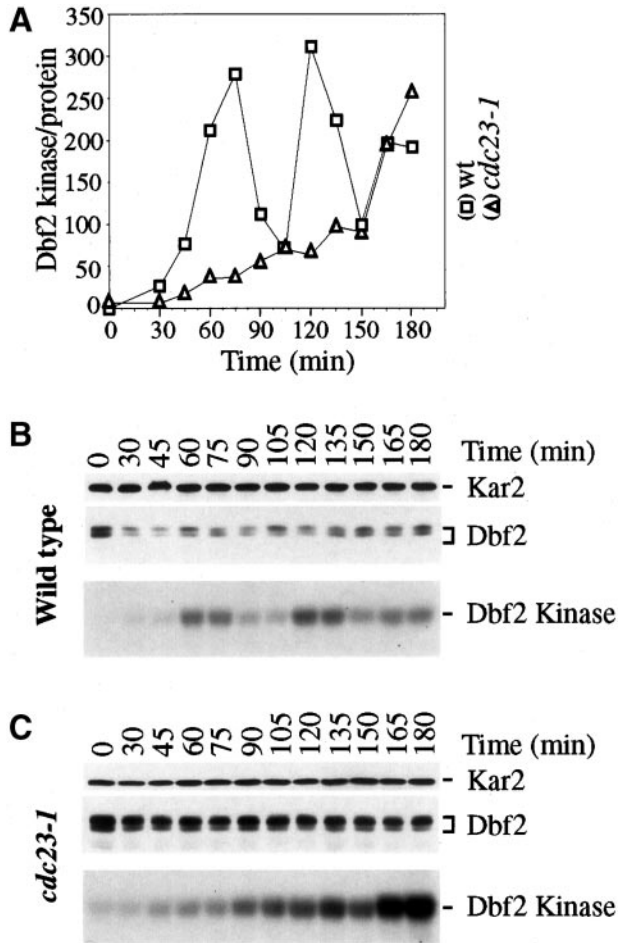
Figure 4. APC/C ^{Cdc20} is required to maintain low levels of Dbf2 protein. *DBF2-MYC* (A1931) and *cdc23–1 DBF2-MYC* (A2184) cells were arrested in G1 with the use of 5 μg/ml α-factor and released at the restrictive temperature (37°C) in medium lacking pheromone. Samples were taken at the indicated times to determine Dbf2 protein levels and Dbf2 kinase activity. The graph shows the amount of Dbf2 kinase activity per Dbf2 protein. Kar2 (top panel), Dbf2 protein (middle panel) and Dbf2 kinase activity (lower panel).

for its association with SPBs or whether Dbf2 can associate with SPBs in the absence of kinase activity. We replaced the conserved lysine residue at position 206, which is critical for kinase activity by arginine (Dbf2$^{KI ->RT}$, Materials and Methods) and analyzed the consequences on Dbf2 localization. The Dbf2$^{KI ->RT}$ mutation was inactive as a kinase in vitro (Figure 5A) and failed to complement the temperature sensitive lethality of a *dbf2–2* mutant in vivo (Figure 5B), indicating that this mutation indeed inactivates Dbf2 kinase activity in vitro and in vivo. Immunolocalization of this allele in wild-type cells and *dbf2–2* mutants showed that the Dbf2$^{KI ->RT}$ mutation did not affect Dbf2 localization (Figure 5C, data not shown). This result indicates that Dbf2 kinase activity is not required for localization of Dbf2 on SPBs.
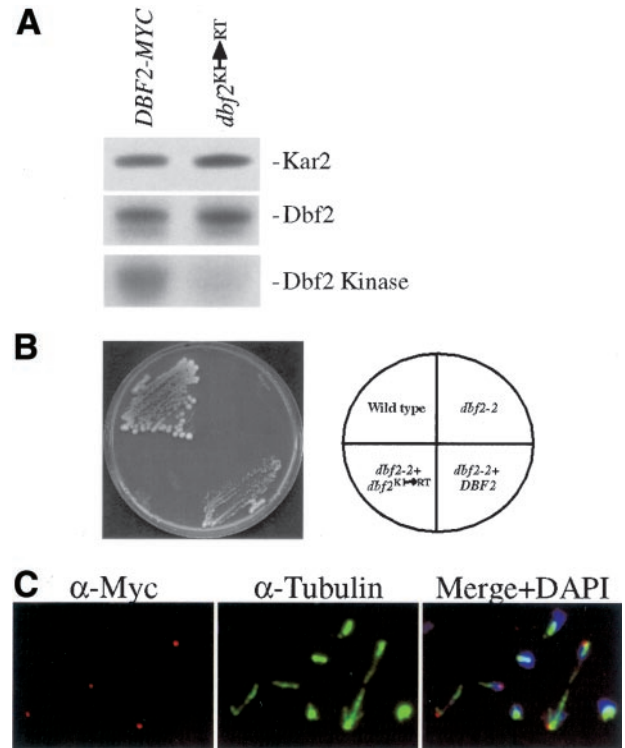

Figure 5. A kinase-dead Dbf2 localizes to SPBs. (B) Wild-type (K699), *dbf2–2* (A852), *dbf2–2 YcP DBF2-MYC* (A3046), and *dbf2–2 YcP Dbf2$^{KI ->RT}$ -MYC* (A3045) cells grown at 37°C. (A) *dbf2–2* cells carrying a *DBF2-MYC* fusion or a *DBF2-MYC* fusion with the conserved K206 mutated to R (Dbf2$^{KI ->RT}$) were grown to exponential phase followed by shift to 37°C for 2 h and Dbf2 kinase activity was determined. (C) Localization of Dbf2$^{KI ->RT}$ -Myc in exponentially growing cells (*DBF2, YcP Dbf2$^{KI ->RT}$ -MYC*; A3044).

SPB Localization and Activation of Dbf2 Require TEM1, CDC15, and NUD1

To determine whether SPB localization and kinase activity of Dbf2 were controlled by components of the mitotic exit pathway, we analyzed the localization of Dbf2 and its kinase activity in cells impaired for *TEM1, NUD1, CDC5, CDC15, CFI1* and *CDC14* (summarized in Table 3). In cells lacking *CFI1*, Dbf2 localization and Dbf2 kinase activity per Dbf2 protein was similar to that of wild-type cells but Dbf2 protein was predominantly in the dephosphorylated form (Figure 6A, Table 3). In cells defective for the polo-like kinase *CDC5*, Dbf2 was absent from SPBs in 50% of cells and found to be localized to 1 SPB in the other 50% of cells. Dbf2-associated kinase activity was 50% of the amount found in exponential growing wild-type cells (Figure 6A, Table 3). In *tem1–3* and *cdc15–2* mutants, Dbf2 was either very weakly or not at all present on SPBs. Dbf2 kinase activity was low in both mutants (Figure 6, A and B; Table 3). A detailed analysis of SPB localization in *tem1–3* mutants undergoing a synchronous cell cycle showed that the protein was never observed on SPBs (Figure 7B). Similarly, in *nud1–44* mutants progressing synchronously through the cell cycle, Dbf2 failed to localize to SPBs and Dbf2 kinase activity was low (Figure 7F).
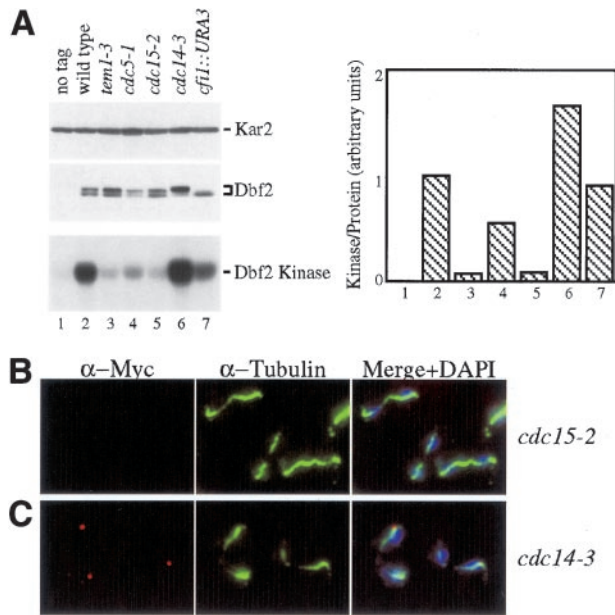
Figure 6. Dbf2 localization and kinase activity in mutants defective for the mitotic exit pathway. Cells lacking a *DBF2-MYC* fusion (K699, no tag; lane 1), *DBF2-MYC* cells (A1931, wild-type, lane 2), *tem1–3 DBF2-MYC* (A 2135, lane 3), *cdc5–1 DBF2-MYC* (A 2107, lane 4), *cdc15–2 DBF2-MYC* (A 2096, lane 5), *cdc14–3 DBF2-MYC* (A 2179, lane 6) and *cfi1:: URA3 DBF2-MYC* (A 2156, lane 7) cells were arrested at the restrictive temperature (37°C) for 2 h. Dbf2-Myc proteins levels and Dbf2 kinase activity was determined (A). The graph in (A) shows the amount of Dbf2 kinase activity/Dbf2 protein with the numbers in the graph corresponding to lane numbers. Dbf2-Myc localization was analyzed in *cdc15–2 DBF2-MYC* (B) and *cdc14–3 DBF2-MYC* (C) mutants.

In *cdc14–3* mutants, Dbf2 localized to a single SPB in the majority of cells (Figures 6C and 7C) and Dbf2-associated kinase activity was high (Figures 6A and 7E), suggesting that *CDC14* was dispensable for efficient activation of Dbf2 kinase. In synchronous cultures, Dbf2 associated with SPBs with identical kinetics as wild-type cells and activation of Dbf2 kinase correlated with localization to SPBs (Figure 7E). To determine whether Dbf2 localized to mother or daughter, SPB cells were treated with α-factor pheromone until they formed a mating projection (shmoo). When cells were released into the cell cycle, the newly formed bud was spherical whereas the mother cell remained shmoo-shaped. This analysis revealed that Dbf2 was found on the SPB that resides in the mother cell (Figure 7C). Thus, *CDC14* is required either for recruiting or maintaining Dbf2 at the daughter SPB. Alternatively, other proteins that assemble onto the SPB may mask Dbf2. Our results suggest that *CDC15* and *TEM1* are required for Dbf2 to load onto SPBs and to become active as a kinase. *CDC14* is dispensable for activation of Dbf2 kinase activity. However, *CDC14* is required for Dbf2's presence on the daughter SPB.

We also analyzed the phosphorylation status of Dbf2 in the various mitotic exit pathway mutants (summarized in Table 3). In synchronous cultures, dephosphorylation of Dbf2, the presence of Dbf2 on SPBs and Dbf2-associated kinase activity correlate suggesting that dephosphorylation

of Dbf2 may play a role in Dbf2 localization and Dbf2 kinase activation (Toyn and Johnston, 1994; Figure 3). However, this correlation was not found in *cdc14–3* mutants and nocodazole-arrested *bub2Δ* cells. In these mutants Dbf2 was hyperphosphorylated but was present at SPBs and active as a kinase (Figures 6A and 7E, data not shown). Thus, at least complete dephosphorylation of Dbf2 is not required for the protein to localize to SPBs and to be active as a protein kinase. These data, however, do not rule out the possibility that dephosphorylation at specific sites is important for Dbf2 activation.

SPB Localization and Activation of Dbf2 Are Inhibited by *BUB2*

Our results indicated that Cdc15 localization to SPBs is inhibited by *BUB2*. We therefore wished to determine whether *BUB2* had a similar effect on Dbf2 localization and Dbf2 kinase activity. Dbf2 was not only present on SPBs during anaphase and telophase but also during other cell cycle stages, and Dbf2 kinase was increased (Figure 8A and data not shown). Dbf2 colocalized with Tub4 (Figure 8B), indicating that the Dbf2 signal observed in cell cycle stages other than anaphase and telophase indeed overlapped with SPBs. To examine the effects of *BUB2* on Dbf2 in more detail, we first analyzed Dbf2 localization and activity in cells released from an α-factor block. However, as observed for Cdc15, prolonged incubation of cells in G1 led to a dramatic decline of Dbf2 signal in the G1-arrested cells, whereas Dbf2 could be easily detected on SPBs in the next G1 phase of the cell cycle (data not shown). Similarly, in cells arrested with hydroxyurea, Dbf2 was not detected on SPBs that nucleated interphase microtubules, whereas it was detected in cells entering the next G1 phase (Figure 8D). Thus, as found for Cdc15, the association of Dbf2 with interphase SPBs appears unstable and sensitive to prolonged cell cycle arrest.

In 25% of *bub2Δ* cells that had formed a mitotic spindle during the hydroxyurea arrest, Dbf2 was found to be localized to at least 1 SPB. During mitosis, Dbf2 localized to SPBs in the majority of cells and remained localized at SPBs during the after G1 stage (Figure 8D). On release from the hydroxyurea block, Dbf2 kinase activity correlated well with the presence of Dbf2 on SPBs and appeared prematurely active during early stages of mitosis (Figure 8D). However, as cells exited, mitosis Dbf2 kinase activity decreased despite Dbf2 remaining on SPBs. Our results indicate that *BUB2* is required to inhibit localization of Dbf2 on SPBs and activation of Dbf2 kinase activity during metaphase in an unperturbed cell cycle. Our findings further suggest that SPB localization is not sufficient for Dbf2 activation and that other factors are necessary for Dbf2 to be active as a protein kinase.

DISCUSSION

Our analysis of Cdc15 localization in various mitotic exit pathway mutants showed that Cdc15 localization to SPBs was independent of *DBF2* and *CDC14*, but dependent on *TEM1*. A similar analysis of Dbf2 showed that Dbf2 localization to SPBs and Dbf2 kinase activity required *TEM1* and *CDC15* but not *CDC14* function. In cells lacking *BUB2*, Cdc15 and Dbf2 were loaded onto SPBs prematurely and Dbf2
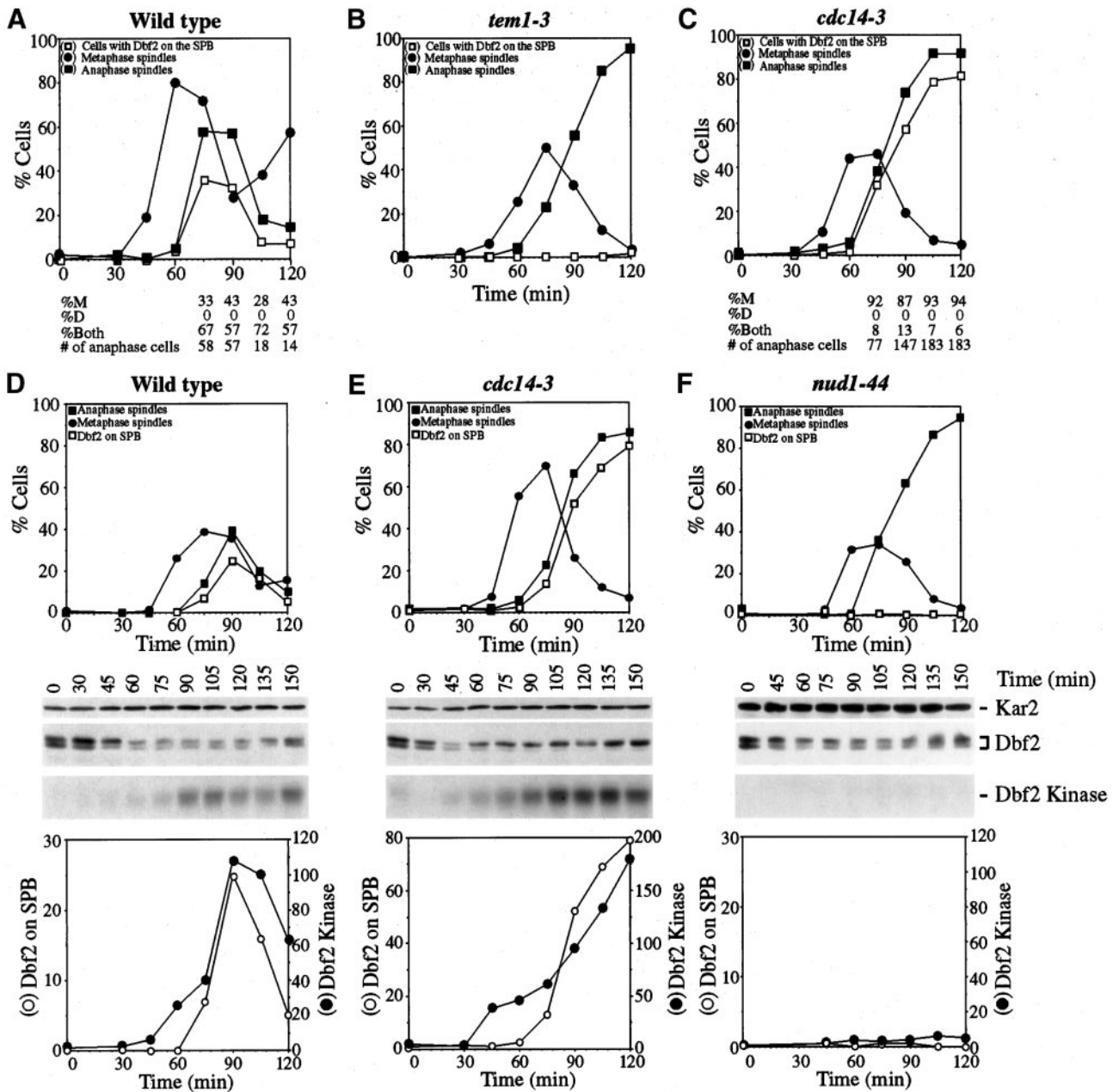
Figure 7. Dbf2 localization and kinase activity in *tem1–3* and *cdc14–3* mutants. (A-C) *DBF2-MYC* (A1931), *tem1–3 DBF2-MYC* (A2135) and *cdc14–3 DBF2-MYC* (A2179) cells were arrested in G1 with the use of 10 μg/ml (A-C) or 5 μg/ml (D-F) α-factor at 23°C in YEP medium containing glucose (YEPD). When the arrest was complete (after 2 h (D-F)) and a mating projection could be seen (after 3 h (A-C)), cells were released at the restrictive temperature (37°C) in YEPD. (A) Percentage of cells in metaphase (closed circles), anaphase (closed squares) and with Dbf2-Myc protein localized either on 1 or on both SPBs (open squares). The numbers underneath the graph represent the percentage of anaphase/telophase cells with the Dbf2-Myc protein on the SPB in the mother cell (%M), in the daughter cell (%D) or on both SPBs (%Both). "# of anaphase cells" indicates the number of anaphase/telophase cells analyzed. (B) Percentage of *tem1–3 DBF2-MYC* cells in metaphase (closed circles), anaphase (closed squares), and Dbf2-Myc protein localized either on 1 or both SPBs (open squares). (C) Percentage of *cdc14–3 DBF2-MYC* cells in metaphase (closed circles), anaphase (closed squares) and with Dbf2-Myc protein localized either on 1 or both SPBs (open squares). The numbers representing various types of Dbf2-Myc localization underneath the graph are as in (A). (D - F) Wild-type (A1931, D), *cdc14–3* (A2179, E), and *nud1–44* mutants (A2339, F), all carrying a *DBF2-MYC* fusion, were arrested in G1 with α-factor (5 μg/ml) followed by release into medium lacking pheromone. The graph at the top of each panel shows the percentage of cells with metaphase spindles (closed circles), with anaphase spindles (closed squares) and Dbf2 on SPBs (open squares). Dbf2-Myc protein levels and Dbf2 kinase activity are shown in the middle of each panel. The graphs at the bottom of each panel show a comparison between SPB localization of Dbf2 (open squares) and Dbf2 kinase/Dbf2 protein (closed squares).
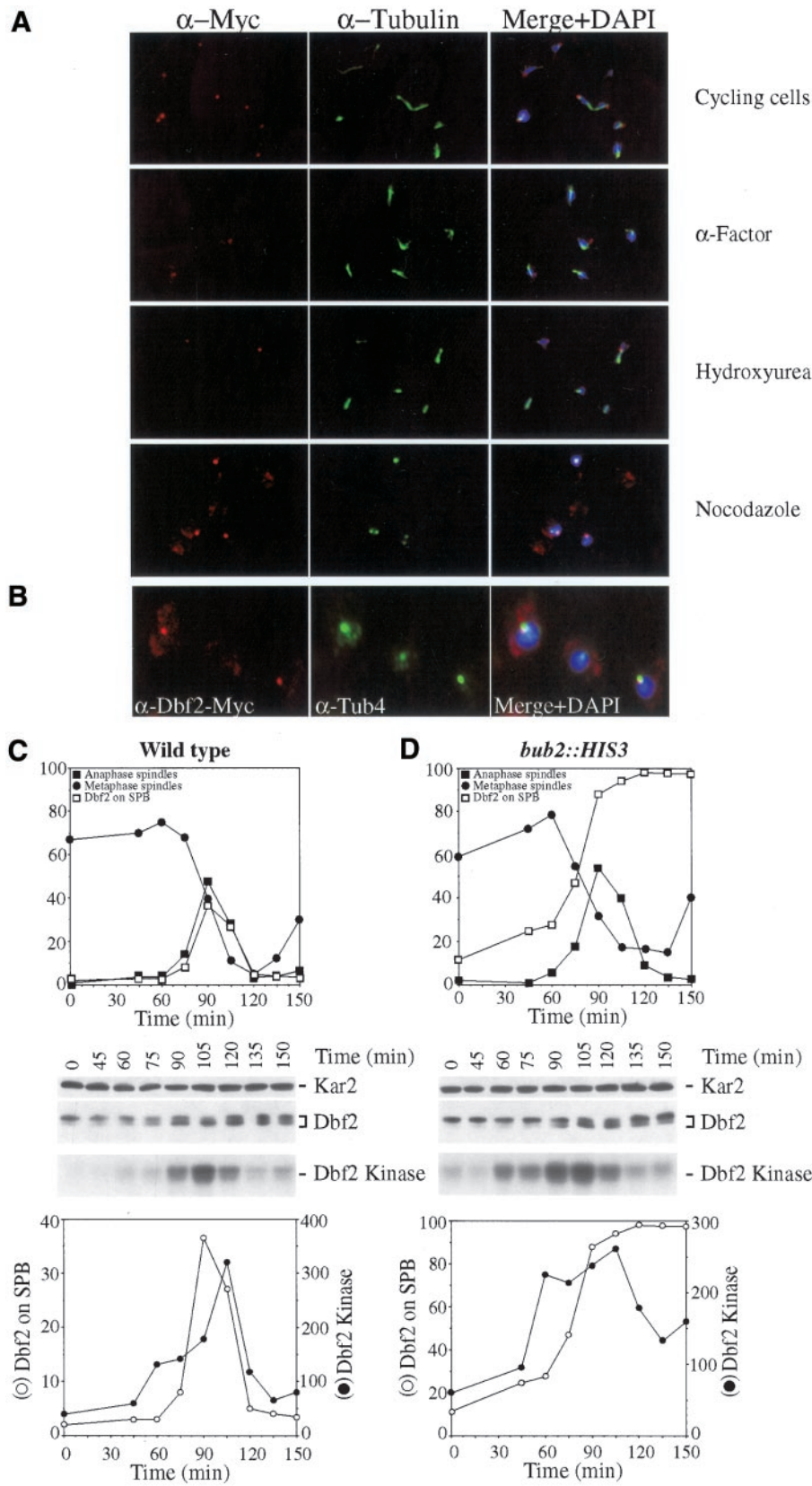
Figure 8. Dbf2 loading onto SPBs and activation of Dbf2 kinase occur prematurely in cells lacking *BUB2*. (A) *bub2:: HIS3 DBF2-MYC* (A 2472) cells were either grown to exponential phase (Cyc), or arrested with α-factor (α-F), hydroxyurea (HU), or nocodazole (Noc) and Dbf2 localization was determined. (B) Colocalization of Dbf2-Myc with the SPB component Tub4 in *bub2:: HIS3* cells. (C - D) Wild-type (A1931, C) and *bub2:: HIS3* (A2472, D) cells all carrying *a DBF2-MYC* fusion were arrested in early S phase with hydroxyurea followed by release into medium lacking pheromone. The graph at the top of each panel shows the percentage of cells with metaphase spindles (closed circles), with anaphase spindles (closed squares) and Dbf2 on SPBs (open squares). Dbf2-Myc protein levels and Dbf2 kinase activity are shown in the middle of each panel. The graphs at the bottom of each panel show a comparison between SPB localization of Dbf2 (open squares) and Dbf2 kinase/ Dbf2 protein (closed squares).
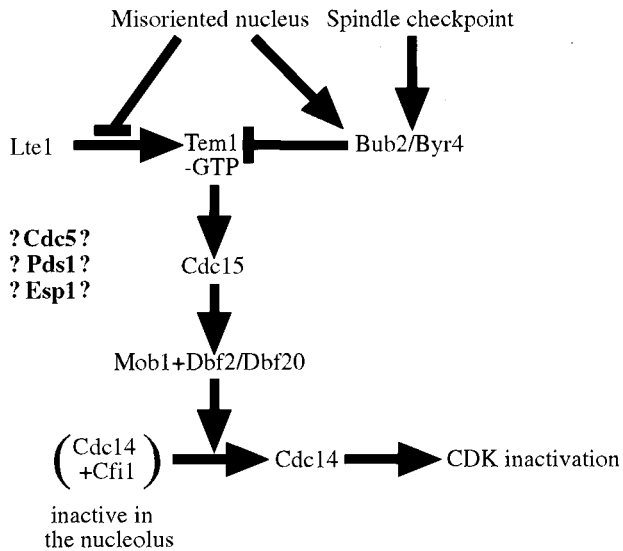
Figure 9. Order of function of the mitotic exit pathway - a model. Conversion of Tem1-GDP to Tem1-GTP causes activation of the mitotic exit pathway. The status of nucleotide binding of Tem1 is controlled by the nucleotide exchange factor (GEF) Lte1 and the 2 component GTPase activating enzyme (GAP) Bub2-Bfa1/Byr4. Mitotic spindle mis-orientation and mitotic spindle defects prevent Lte1 from activating Tem1 and protract Bub2/Byr4 activity causing Tem1 to be in the GDP-bound form. After nuclear division led to partitioning of the nucleus between mother and daughter cell, activation of Tem1 leads to recruitment of Cdc15 to SPBs which in turn causes recruitment of Dbf2 to SPBs and activation of Dbf2 kinase. Dbf2 kinase activity also requires Clb-CDK activity. How Clb-CDKs control Dbf2 activity is at present unclear. How *CDC5* and the regulators of sister-chromatid separation *PDS1* and *ESP1* participate in this signal transduction pathway is also not known. Activation of the mitotic exit pathway eventually leads to release of Cdc14 from the nucleolus leading to inactivation of mitotic CDKs.

kinase activity was elevated. Previous studies have shown that Tem1 localization to SPBs is independent of *CDC15*, *CDC5*, *DBF2*, and *CDC14* (Bardin *et al.*, 2000). Together these findings suggest an order of function as to how Cdc15 is loaded onto SPBs and how Dbf2 localization to SPBs and Dbf2 kinase are controlled. *BUB2* negatively regulates all 3 events. Furthermore, Cdc15 localization depends on *TEM1* but not other mitotic exit pathway components, whereas activation of Dbf2 kinase activity and SPB localization depend on *TEM1* and *CDC15* but not *CDC14*. It is tempting to speculate that the dependencies observed for the loading of Cdc15 and Dbf2 onto SPBs and activation of Dbf2 kinase reflect the order of function in which these proteins function in promoting release of Cdc14 from Cfi1/Net1. In Figure 9, we propose a model where *BUB2* negatively regulates the activity of the mitotic exit pathway and where *TEM1* acts upstream of *CDC15* and *CDC15* upstream of *DBF2*. Similar dependencies have been found for the homologous pathway in *S. pombe* (Sparks *et al.*, 1999). This pathway is termed septation initiation network and controls cytokinesis (reviewed in Balasubramanian *et al.*, 2000). It is important to note that our findings do not imply direct activation of Cdc15 by Tem1-GTP or Dbf2 by Cdc15. Additional components of the mitotic exit pathway may await identification. It

is also important to note that while our results clearly demonstrate an order of function in which Cdc15 and Dbf2 assemble onto SPBs and in which Dbf2 kinase is activated, we do not yet know whether association of these protein kinases with SPBs is critical for promoting release of Cdc14 from Cfi1/Net1. The analysis of mutants in Cdc15 and Dbf2 that can no longer associate with SPBs will be necessary to address this question.

Our attempts to place *CDC5* with respect to Cdc15 and Dbf2 localization onto SPBs were inconclusive, as Cdc15 and Dbf2 were localized to SPBs in 50% of *cdc5–1* cells, but not in the other 50%. Dbf2 kinase activity was low in *cdc5–1* mutants, however not as low as in *tem1–3* mutants. These findings raise the possibility that *CDC5* function is required at multiple steps during mitotic exit.

The Localization of Cdc15

Despite Cdc15 protein being present at constant levels throughout the cell cycle (Jaspersen *et al.*, 1998), we and others found that Cdc15 only localized to SPBs during anaphase and telophase (Cenamor *et al.*, 1999; Gruneberg *et al.*, 2000; Xu *et al.*, 2000; Menssen *et al.*, 2001). However, there are slight variations in the localization pattern observed for Cdc15, which appears to depend on the epitope tag used in the different studies. Our localization data on endogenous Cdc15 are in agreement with previously published reports showing that Cdc15 localizes to both SPBs during anaphase and telophase (Xu *et al.*, 2000). We also find that localization of Cdc15 to SPBs depends on the outer plaque component *NUD1*. In *nud1–44* mutants, Cdc15 is largely delocalized from SPBs, as are Tem1 and Dbf2 (Bardin *et al.*, 2000; Gruneberg *et al.*, 2000; this study). Cdc15 localization was not affected in *nud1–2* mutants (Gruneberg *et al.*, 2000), indicating that different *NUD1* alleles affect Cdc15 localization differently.

Regulation of Dbf2 Localization and Activity

Contrary to our observations, Frenz *et al.* (2000) showed that Dbf2 localizes to SPBs throughout the cell cycle. In this study, mutations in components of the mitotic exit pathway did not affect Dbf2 localization. We can envision several reasons or a combination thereof for these differences. (1) While Frenz *et al.* (2000) used strains containing 3 copies of a Dbf2-GFP fusion, we analyzed strains carrying a single copy of Dbf2 with 3 Myc epitopes. The employment of strains containing higher amounts of Dbf2 may cause constitutive association of Dbf2 with SPBs. This idea is consistent with the finding that in strains carrying a single copy of the *DBF2-GFP* fusion, the protein is found on SPBs only during anaphase and telophase (Frenz *et al.*, 2000). (2) It is also possible that the signal generated by the Dbf2-Myc fusion is too weak or the Myc epitope is masked and, thus, escapes detection in cell cycle stages other than anaphase and telophase. However, we should note that we do detect Dbf2 on SPBs in cell cycle stages other than anaphase and telophase in cells lacking *BUB2*. Dbf2-GFP was also shown to localize to the bud neck during cytokinesis (Frenz *et al.*, 2000). Why we fail to detect Dbf2 at the bud neck is at present unclear. However, it is worth noting that several proteins, such as Cap2, shown to be localized to the bud neck with the use of GFP fusion protein, cannot be detected

with the use of conventional immunofluorescence protocols (Waddle *et al.*, 1996; Lippincott and Li, 1998).

Although Dbf2 protein levels do not fluctuate during the cell cycle Dbf2 protein is only present at 1 or both SPBs during anaphase and telophase. The SPB localization of Dbf2 correlates with activation of Dbf2 kinase in synchronous cultures and various cell cycle arrests and mutant backgrounds. We also find that loading of Dbf2 onto SPBs does not require Dbf2 kinase activity. While these data suggest that localization of Dbf2 to SPBs, where Tem1 and Cdc15 localize, is important for Dbf2 kinase activation, it is also clear that it is not sufficient for Dbf2 kinase to be active. In cells lacking *BUB2*, Dbf2 protein is present on SPBs in G1 but Dbf2 is not active as a protein kinase. Only during metaphase is Dbf2 prematurely active in *bub2Δ* cells. The identity of these other factor(s) controlling Dbf2 activity is unclear. However, our results lead us to believe that 2 steps are necessary for Dbf2 to be active as a protein kinase. When the SPB enters the bud, the mitotic exit pathway components *TEM1* and *CDC15* not only recruit Dbf2 to SPBs but are also required for Dbf2 kinase to be active. In addition, a yet to be identified factor is required for Dbf2 to be active as a protein kinase.

The Spindle Pole Body—a Signaling Beacon

The GTPase Tem1 predominantly localizes to the SPB that migrates into the bud (Bardin *et al.*, 2000; Pereira *et al.*, 2000). It was, therefore, surprising that Cdc15 and Dbf2 localized to both SPBs and that localization to both SPBs depended on *TEM1*. It was also surprising that Dbf2 was present on only the mother cell SPB in some wild-type cells and almost exclusively present on the mother cell SPB in *cdc14–3* mutants. How can we explain these observations? It is possible that Tem1 protein is present on the mother cell SPB but undetectable by indirect in situ immunofluorescence or by with the use of a Tem1-GFP fusion. It is also possible that Cdc15 and Dbf2 are somehow modified in a *TEM1*-dependent manner on the daughter cell SPB, which allows them to associate with the mother cell SPB. We also need to explain the finding that in some wild-type cells and *cdc14–3* mutants, Dbf2 is found only on the mother cell SPB. One or more of the following ideas can explain this observation. (1) Dbf2 at the daughter cell SPB is more sensitive to fixation procedures or masked by other proteins. (2) In *cdc14–3* mutants, the absence of Dbf2 from the daughter cell SPB may indicate that Cdc14 is required for efficient maintenance of Dbf2 localization on the daughter SPB. (3) We favor the idea that once Dbf2 is activated at the daughter SPB, it dissociates from this organelle and associates with its targets in other regions of the cell. This idea is consistent with the localization pattern of Sid2, the Dbf2 homolog in *S. pombe*, which is critical for the cytokinesis (Sparks *et al.*, 1999). Sid2 first localizes to SPBs during mitosis and then localizes to the site of septation during septum formation.

Control of the Mitotic Exit Pathway

The activity of the mitotic exit pathway is sensitive to the status of a variety of different cellular events, such as nuclear position, integrity of the mitotic spindle, and onset of sister-chromatid separation (Figure 9; Alexandru *et al.*, 1999; Cohen-Fix and Koshland, 1999; Fesquet *et al.*, 1999; Fraschini

et al., 1999; Li, 1999; Shirayama *et al.*, 1999; Tinker-Kulberg and Morgan, 1999; Bardin *et al.*, 2000; Bloecher *et al.*, 2000; Daum *et al.*, 2000; Pereira *et al.*, 2000; Wang *et al.*, 2000). Most of these signals are thought to control the nucleotide binding status of Tem1. Our analysis of Dbf2 kinase activity in cells lacking *BUB2* suggests that Dbf2 activity is not only controlled by Tem1 and Cdc15 but by an as yet unknown factor. Furthermore, our and previous analyses of Dbf2 activity and localization in cells lacking *BUB2* showed that there is at least 1 other factor controlling Cdc14 release from Cfi1/Net1, which functions downstream or in parallel to Dbf2. In *bub2Δ* cells, Dbf2 is present on SPBs already during metaphase and Dbf2 kinase activity is unusually high (Fesquet *et al.*, 1999). However, only a small percentage of *bub2Δ* cells release Cdc14 prematurely during metaphase (Bardin *et al.*, 2000). This result indicates that active Dbf2 kinase is not sufficient to trigger release of Cdc14 from Cfi1/Net1 and raises the interesting possibility that Cdc14 release for Cfi1/Net1 is regulated by factors other than mitotic exit pathway components. Determining the identity of these factors and the nature of signals they sense to regulate release of Cdc14 from Cfi1/Net1 will be critically important to understand how exit from mitosis is integrated with other cell cycle events.

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