

The Role of the Polo Kinase Cdc5 in Controlling Cdc14 Localization^D

Rosella Visintin, Frank Stegmeier, and Angelika Amon*

Center for Cancer Research, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Submitted February 20, 2003; Revised June 13, 2003; Accepted July 3, 2003
Monitoring Editor: Tim Stearns

In budding yeast, the protein phosphatase Cdc14 controls exit from mitosis. Its activity is regulated by a competitive inhibitor Cfi1/Net1, which binds to and sequesters Cdc14 in the nucleolus. During anaphase, Cdc14 is released from its inhibitor by the action of two regulatory networks. The Cdc Fourteen Early Anaphase Release (FEAR) network initiates Cdc14 release from Cfi1/Net1 during early anaphase, and the Mitotic Exit Network (MEN) promotes Cdc14 release during late anaphase. Here, we investigate the relationship among FEAR network components and propose an order in which they function to promote Cdc14 release from the nucleolus. Furthermore, we examine the role of the protein kinase Cdc5, which is a component of both the FEAR network and the MEN, in Cdc14 release from the nucleolus. We find that overexpression of *CDC5* led to Cdc14 release from the nucleolus in S phase-arrested cells, which correlated with the appearance of phosphorylated forms of Cdc14 and Cfi1/Net1. Cdc5 promotes Cdc14 phosphorylation and, by stimulating the MEN, Cfi1/Net1 phosphorylation. Furthermore, we suggest that Cdc14 release from the nucleolus only occurs when Cdc14 and Cfi1/Net1 are both phosphorylated.

INTRODUCTION

During exit from mitosis, cells eliminate mitotic determinants and establish conditions permissive for budding, DNA replication, and spindle pole body duplication. In the budding yeast *Saccharomyces cerevisiae*, the protein phosphatase Cdc14 triggers this transition by reversing cyclin-dependent kinase (CDK) phosphorylation (reviewed in (Morgan, 1999; Bardin and Amon, 2001; McCollum and Gould, 2001). The activity of Cdc14 is controlled by Cfi1/Net1, which functions as a competitive inhibitor of this protein phosphatase (Shou *et al.*, 1999; Visintin *et al.*, 1999; Traverso *et al.*, 2001). The association of Cdc14 with its inhibitor is cell cycle regulated. During G1, S phase, G2, and metaphase Cfi1/Net1 sequesters and inhibits Cdc14 in the nucleolus (Shou *et al.*, 1999; Visintin *et al.*, 1999). During anaphase the phosphatase dissociates from its inhibitor in the nucleolus and spreads throughout the cell, where it is presumably active and able to dephosphorylate its targets.

Two regulatory networks have been identified that control the localization of Cdc14. The Cdc Fourteen Early Anaphase Release (FEAR) network promotes release of Cdc14 from the nucleolus during early anaphase and the Mitotic Exit Network (MEN) maintains Cdc14 in this released state during late stages of anaphase and telophase (Shou *et al.*, 1999; Visintin *et al.*, 1999; Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002). Inactivation of FEAR network components prevents Cdc14 release from the nucleolus during early anaphase, when the mitotic spindle is between 3

and 7 μm in length. In contrast, inactivation of the MEN abolishes Cdc14 release from the nucleolus during late stages of anaphase, when the mitotic spindle measures 7–10 μm in length (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002). MEN-dependent release of Cdc14 from the nucleolus is essential for exit from mitosis, because temperature-sensitive mutants in MEN components fail to exit from mitosis but arrest in telophase. The FEAR network-dependent release of Cdc14 from the nucleolus during early anaphase is required for the timely exit from mitosis but is not essential, because mutations in FEAR network components delay, but do not prevent, exit from mitosis.

The MEN resembles a Ras-like signaling cascade with the GTPase Tem1 functioning at or near the top of the MEN. Tem1 activity is thought to be regulated both by the putative GTP exchange factor (GEF) Lte1 and a two-component GTPase-activating protein (GAP) complex composed of Bub2 and Bfa1 (reviewed in Bardin and Amon, 2001; McCollum and Gould, 2001). Tem1-GTP is believed to activate the protein kinase Cdc15, which then activates the protein kinase Dbf2 in a manner dependent on the Dbf2-associated factor Mob1 (Lee *et al.*, 2001; Mah *et al.*, 2001; Visintin and Amon, 2001). The spindle pole body (SPB) component Nud1 functions as a scaffold for MEN components at the SPB (Gruneberg *et al.*, 2000). The polo kinase Cdc5 has also been implicated in MEN signaling, impinging on the pathway in at least two ways. Cdc5 phosphorylates Bfa1 and Bub2, thereby inactivating Tem1's GAP (Hu *et al.*, 2001; Hu and Elledge, 2002; Geymonat *et al.*, 2003) and functions to promote Dbf2 activity by an unknown mechanism (Lee *et al.*, 2001).

CDC5 is also a component of the FEAR network, because the gene is not only required for the release of Cdc14 from the nucleolus during late stages of anaphase but also during early anaphase (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002). Other components of the FEAR network include *ESP1*,

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-02-0095. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-02-0095.

^D Online version of this article contains supplemental material for some figures. Online version is available at www.molbiolcell.org.

* Corresponding author. E-mail address: angelika@mit.edu.

SLK19, and *SPO12* (Stegmeier *et al.*, 2002). Esp1, which is also known as Separase, encodes a protease that is responsible for triggering sister chromatid separation at the onset of anaphase (reviewed in Nasmyth, 2001; Uhlmann, 2001). *ESP1* is also required for exit from mitosis functioning upstream of *SLK19* and *CDC5* (Tinker-Kulberg and Morgan, 1999; Stegmeier *et al.*, 2002; Sullivan and Uhlmann, 2003). However, remarkably, Esp1's protease activity seems not to be required for its mitotic exit function (Sullivan and Uhlmann, 2003). The FEAR network component *Slk19* localizes to kinetochores and the spindle mid-zone during anaphase and is a substrate of Esp1 (Zeng *et al.*, 1999; Sullivan *et al.*, 2001). *Spo12* encodes a protein of unknown function.

CDC5, *ESP1*, *SLK19*, and *SPO12* have been grouped together into a network based on the observation that they are required for Cdc14 release from the nucleolus during early anaphase. Whether these genes function in a linear pathway or in parallel to bring about Cdc14 release from the nucleolus during early anaphase has not been determined. Furthermore, how the FEAR network and the MEN promote the dissociation of Cdc14 from Cfi1/Net1 is not known. Here, we investigate the relationship among FEAR network components and propose an order in which the four identified network components function. *ESP1* functions upstream of *SLK19* and *SPO12* acts in parallel to *ESP1*, *SLK19*, and *CDC5*. We further address how Cdc5 promotes Cdc14 release from the nucleolus. We show that high levels of Cdc5 cause Cdc14 release from the nucleolus in S phase-arrested cells. Furthermore, we find that Cdc5 induces Cdc14 and Cfi1/Net1 phosphorylation. Phosphorylation of Cfi1/Net1 induced by Cdc5 depends on an active mitotic exit network whereas phosphorylation of Cdc14 does not. Finally we present evidence to suggest that Cdc14 release from the nucleolus only occurs when both Cdc14 and Cfi1/Net1 are phosphorylated.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

All strains were derivatives of strain W303 (K699). The *GAL-CDC5-3MYC*, *CDC14-HA*, *CFI1-MYC*, *GAL-CDC15[1-974]* and *GAL-CDC15[1-750]* fusions were described in Taylor *et al.* (1997), Charles *et al.* (1998), Visintin *et al.* (1999), and Bardin *et al.* (2003) respectively. The *CFI1-GFP* and *DBF2-3HA* fusion were constructed by using the polymerase chain reaction-based method described in Longtine *et al.* (1998). Growth conditions for individual experiments are described in the figure legends.

Immunoblot Analysis and Dbf2 Kinase Assay

To prepare protein extracts for Western blot analysis cells were incubated for 10 min in 10% trichloroacetic acid at 4°C, pelleted, and then washed with acetone. Cells were broken in 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM p-nitrophenyl phosphate, 50 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml pepstatin with glass beads for 40 min and boiled in 1× sample buffer. Immunoblot analysis of the total amount of Clb2, Cdc14-HA, Cfi1-MYC, Cdc5-MYC, Dbf2-HA, and Kar2 was performed as described in Cohen-Fix *et al.* (1996). Dbf2 kinase activity was assayed as described in Visintin and Amon (2001).

Fluorescence Microscopy

Indirect in situ immunofluorescence methods and antibody concentrations were as described in Visintin *et al.* (1999). Cells were analyzed on an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY), and images were captured with a camera controller (Hamamatsu, Bridgewater, NJ). Openlab 3.0.2 software was used to process immunofluorescence images.

RESULTS

Overexpression of *SPO12* or *CDC5* Abolishes the Need for *SLK19* and *ESP1* in Releasing Cdc14 from the Nucleolus

Several components of the FEAR network have been identified but the order in which they function to promote Cdc14

release from the nucleolus is not known. To investigate the epistatic relationship among FEAR network components, we determined whether overexpression of one FEAR network component from the galactose-inducible *GAL1-10* promoter could restore Cdc14 release from the nucleolus in cells lacking other FEAR network components. To this end, we first characterized the effects of overexpressing various FEAR network components on Cdc14 localization in otherwise wild-type cells. We conducted this analysis in cells lacking *MAD1*, a component of the spindle checkpoint, to avoid indirect effects on FEAR network activity due to activation of the mitotic spindle checkpoint (Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002). Overexpression of *SPO12* did not significantly affect the kinetics of Cdc14 release from the nucleolus (Figure 1A). Overexpression of *CDC5* caused premature release of Cdc14 from the nucleolus during metaphase, whereas, surprisingly, overexpression of *ESP1* slightly inhibited Cdc14 release from the nucleolus (Figure 1A). We were not able to examine the consequences of overexpressing *SLK19* because high levels of *Slk19* caused spindle abnormalities precluding the reliable identification of cells with anaphase spindles (our unpublished data).

Having established the consequences of overexpressing various FEAR network components on Cdc14 localization in otherwise wild-type cells, we determined whether overexpression of *CDC5*, *ESP1*, or *SPO12* could restore Cdc14 release from the nucleolus to *slk19Δ* mutants during early anaphase. In *slk19Δ* cells, Cdc14 release from the nucleolus, in contrast to wild-type cells, occurs only during late anaphase (Figure 1B; Stegmeier *et al.*, 2002). Overexpression of *ESP1* did not restore release of Cdc14 from the nucleolus during early anaphase but overexpression of *CDC5* and *SPO12* did (Figure 1B). These findings raised the possibility that *CDC5* and *SPO12* functioned downstream of or in parallel to *SLK19* and that *ESP1* functioned upstream of *SLK19*. We could indeed show that *ESP1* functioned upstream of *SLK19* when we examined the effects of high levels of Esp1 in nocodazole arrested cells. In the nocodazole arrest, high levels of Esp1 led to premature release of Cdc14 from the nucleolus, which depended on *SLK19* (Sullivan and Uhlmann, 2003; Supplemental Figure 1).

Next, we determined whether overexpression of FEAR network components rendered *ESP1* dispensable for Cdc14 release from the nucleolus during early anaphase. Because *ESP1* is also required for sister-chromatid separation (Ciosk *et al.*, 1998), we examined the kinetics of Cdc14 release from the nucleolus in *esp1-1* mutants carrying a temperature-sensitive *mcd1-1* mutation, which makes *ESP1* dispensable for chromosome segregation and spindle elongation (Guacci *et al.*, 1997). Overexpression of *CDC5* or *SPO12* restored release of Cdc14 from the nucleolus during early anaphase in *esp1-1 mcd1-1* mutants (Figure 2). These results indicate that Cdc5 and *Spo12*, when overproduced, do not require *SLK19* or *ESP1* function to promote Cdc14 release from the nucleolus during early anaphase.

High Levels of Cdc5 Suppress the Requirement for *SPO12* in Promoting Release of Cdc14 during Early Anaphase

To further investigate the relationship between FEAR network components, we determined whether overexpression of *CDC5* or *ESP1* could restore Cdc14 release from the nucleolus to *spo12Δ bns1Δ* double mutants during early anaphase. *BNS1* shares 55% similarity with *SPO12* (Grether and Herskowitz, 1999) and when inactivated further impairs the release of Cdc14 from the nucleolus during early anaphase in *spo12Δ* cells (Supplemental Figure 2). Thus, *BNS1*

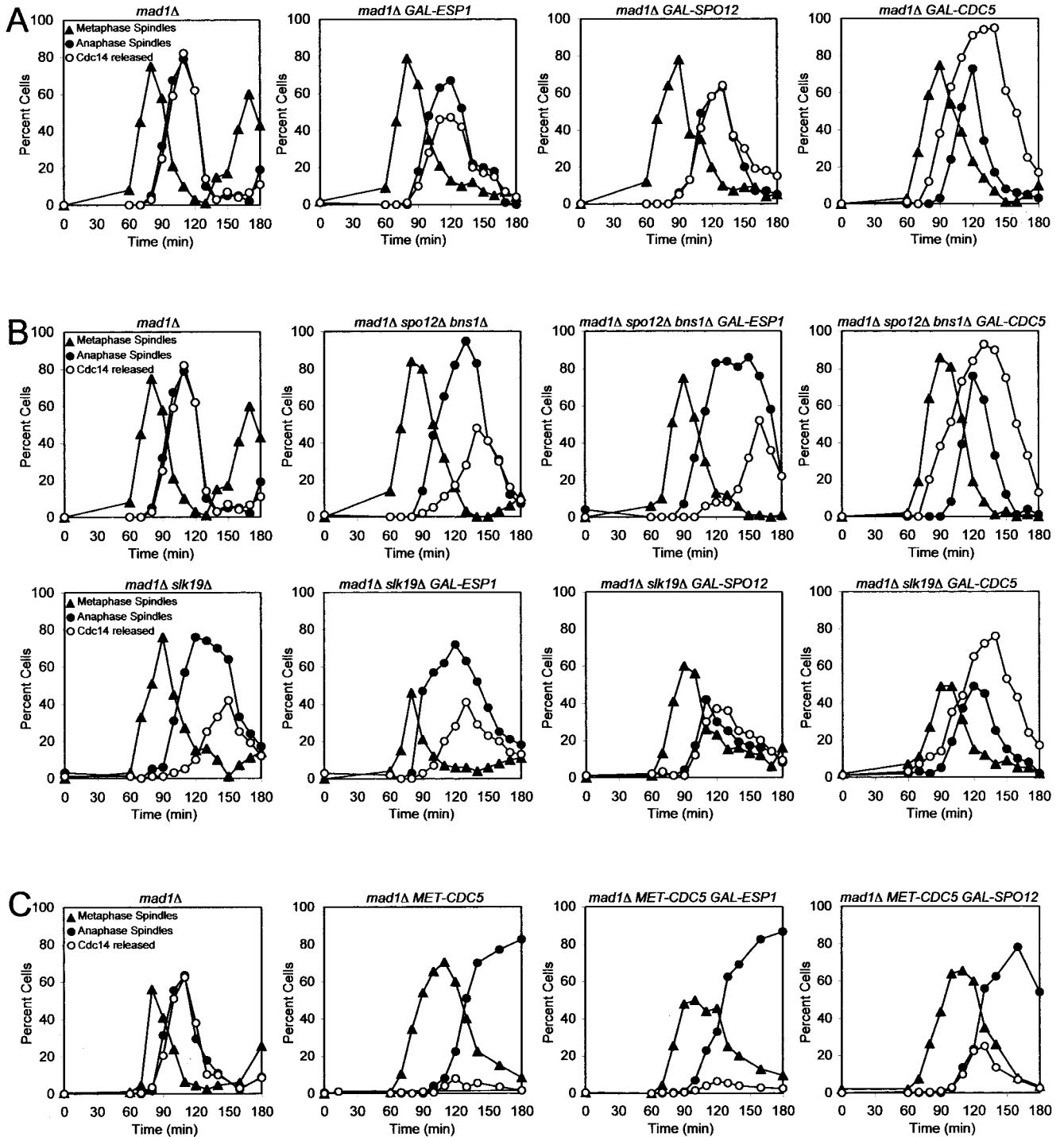


Figure 1. Epistatic relationships among FEAR network components. (A) *mad1Δ* (A2853), *mad1Δ GAL-ESP1* (A7619), *mad1Δ GAL-SPO12* (A6490), and *mad1Δ GAL-CDC5* (A7297) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEP medium containing 2% raffinose (YEPR) with α -factor (5 μ g/ml) for 3 h. Cells were then released into YEPR medium containing 2% galactose (YEPRG) lacking pheromone at 25°C. The percentage of cells with metaphase spindles (close triangles), anaphase/telophase spindles (close circles) as well as the percentage of cells with Cdc14-HA released from the nucleolus (open circles) was determined at the indicated times. (B) *mad1Δ* (A2853) *mad1Δ spo12Δ bns1Δ* (A5408), *mad1Δ spo12Δ bns1Δ GAL-ESP1* (A7619) *mad1Δ spo12Δ bns1Δ GAL-CDC5* (A7578), *mad1Δ slk19Δ* (A4302), *mad1Δ slk19Δ GAL-ESP1* (A7302), *mad1Δ slk19Δ GAL-SPO12* (A7581), *mad1Δ slk19Δ GAL-CDC5* (A8032) cells carrying a *CDC14-3HA* fusion were grown and analyzed as described in (A). (C) *mad1Δ* (A2853), *mad1Δ MET-CDC5* (A6560), *mad1Δ MET-CDC5 GAL-ESP1* (A6562), and *mad1Δ MET-CDC5 GAL-SPO12* (A6630) cells carrying a *CDC14-3HA* fusion were grown in medium lacking methionine and arrested in G1 with α -factor (5 μ g/ml). Galactose (2%) was added 1 h before release, and cells were released into YEPRG medium containing 8 mM methionine at 25°C. The percentage of cells with metaphase spindles (closed triangles), anaphase/telophase spindles (closed circles) as well as the percentage of cells with Cdc14-HA released from the nucleolus (open circles) was determined at the indicated times.

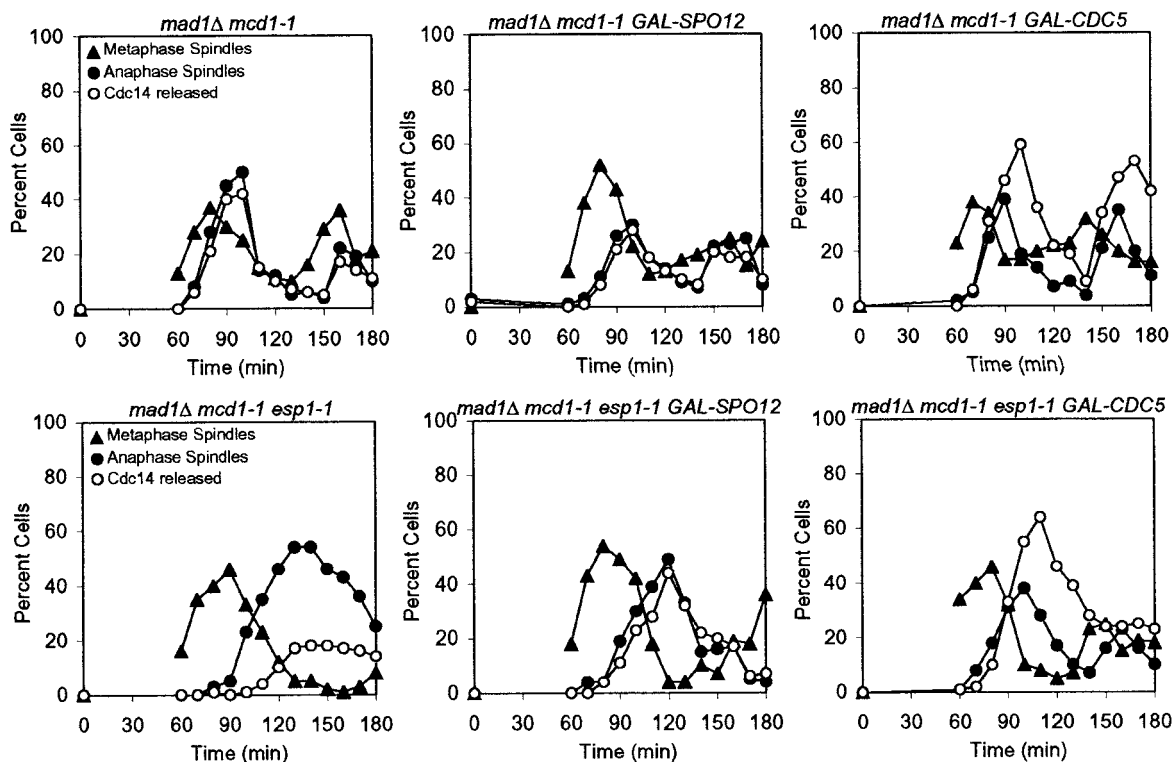


Figure 2. High levels of Cdc5 and Spo12 allow *esp1-1* to release Cdc14 from the nucleolus during early anaphase. *mad1Δ mcd1-1* (A2784), *mad1Δ mcd1-1 GAL-SPO12* (A6528), *mad1Δ mcd1-1 GAL-CDC5* (A7300), *mad1Δ mcd1-1 esp1-1* (A3023), *mad1Δ mcd1-1 esp1-1 GAL-SPO12* (A7711), and *mad1Δ mcd1-1 esp1-1 GAL-CDC5* (A7580) all carrying the *CDC14-3HA* fusion were grown and analyzed as described in Figure 1A, except cells were released from the G1 block at 37°C.

and *SPO12* perform at least partially overlapping functions in promoting Cdc14 release from the nucleolus. Overexpression of *ESP1* did not promote release of Cdc14 from the nucleolus during early anaphase in *spo12Δ bns1Δ* double mutants but overexpression of *CDC5* restored Cdc14 release to the extent observed in wild-type cells overexpressing *CDC5* (Figure 1B). This finding indicates that high levels of Cdc5 can promote Cdc14 release from the nucleolus independently of *SPO12* and *BNS1* but that *Esp1* cannot.

Overexpression of *SPO12* Partially Restores Cdc14 Release from the Nucleolus in *CDC5*-depleted Cells

To determine whether overexpression of FEAR network components can bypass the requirement for *CDC5* in promoting Cdc14 release from the nucleolus during early anaphase, we overexpressed various FEAR network components in cells depleted for Cdc5. We chose to inactivate Cdc5 by depletion rather than by using a temperature sensitive *cdc5* allele to ensure complete inactivation of *CDC5* function. *CDC5* was cloned under the control of the methionine-repressible *MET3* promoter (Mountain and Korch, 1991). Because Cdc5 is unstable during G1 (Charles *et al.*, 1998; Shirayama *et al.*, 1998), the protein is depleted from cells during a pheromone-induced G1 arrest. *MET-CDC5* strains were arrested in G1 by using α -factor pheromone and transcription of *CDC5* was repressed by methionine addition 1 h before release of cells from the G1 block. Under these conditions, cells arrested in telophase with Cdc14 sequestered in the nucleolus (Figure 1C). Furthermore, the telophase arrest of Cdc5-depleted cells was bypassed by the *TAB6-1* muta-

tion, a dominant allele of *CDC14* (Supplemental Figure 3; Shou *et al.*, 1999), indicating that promoting Cdc14 release from the nucleolus is *CDC5*'s essential function during exit from mitosis.

Overexpression of *ESP1* did not restore Cdc14 release from the nucleolus during early anaphase, but overexpression of *SPO12* did to some extent (Figure 1C). We also observed that 30% of Cdc5-depleted cells overexpressing *SPO12* disassembled their mitotic spindles and entered a new cell cycle, as judged by their ability to form a new bud (Supplemental Figure 4). However these cells failed to form colonies, perhaps due to the severe cytokinesis defects observed in these cells (our unpublished data). Our findings suggest that *SPO12* can to some extent promote Cdc14 release from the nucleolus in *CDC5*-depleted cells. This suppression could be due to overproduced Spo12 being able to promote Cdc14 release from the nucleolus in the absence of *CDC5* function. However, we cannot exclude the possibility that Cdc5-depleted cells contain residual amounts of Cdc5, which though unable to promote Cdc14 release from the nucleolus and exit from mitosis together with high levels of Spo12 promote Cdc14 release from the nucleolus and exit from mitosis.

Inactivation of *SPO12* and *BNS1* Enhances the Mitotic Exit Defect of *esp1-1* and *slk19Δ* Mutants

The observation that high levels of *SPO12* at least partially suppress the defect in Cdc14 release from the nucleolus caused by inactivation of *ESP1*, *SLK19*, or *CDC5* indicates that *SPO12* either functions downstream of or in parallel to

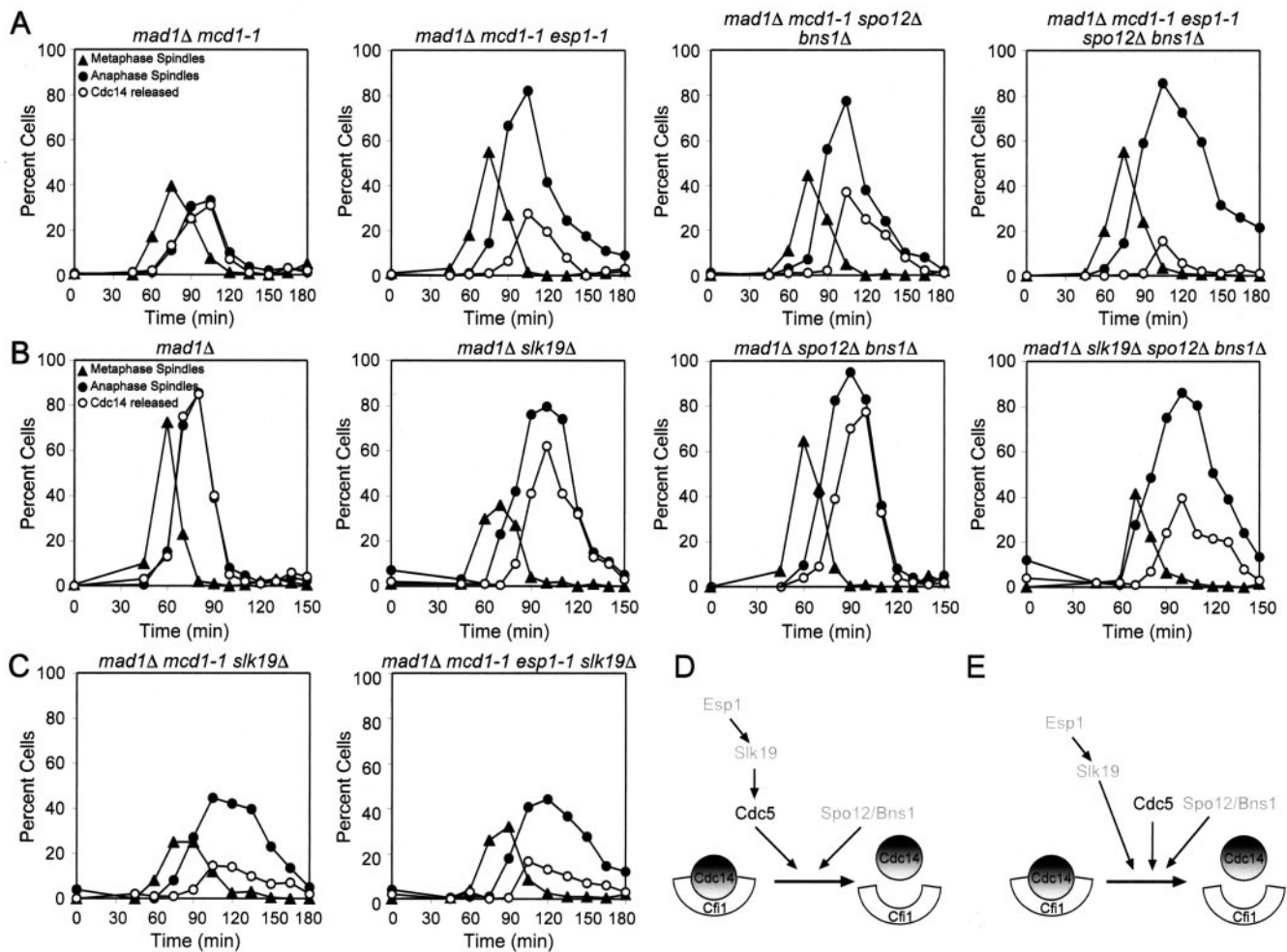


Figure 3. *SPO12* and *BNS1* function in parallel to *ESP1* and *SLK19*. (A) *mad1Δ mcd1-1* (A2784), *mad1Δ mcd1-1 esp1-1* (A3550), *mad1Δ mcd1-1 spo12Δ bns1Δ* (A8129), and *mad1Δ mcd1-1 esp1-1 spo12Δ bns1Δ* (A8130) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEPD medium with α -factor (5 μ g/ml) and subsequently released into YEPD lacking pheromone at 37°C. α -Factor was readded 90 min after release to prevent entry into a subsequent cell cycle. The percentage of cells with metaphase spindles (closed triangles), anaphase/telophase spindles (closed circles) as well as the percentage of cells with *Cdc14*-HA released from the nucleolus (open circles) was determined at the indicated times. (B) *mad1Δ* (A2853), *mad1Δ slk19Δ* (A4302), *mad1Δ spo12Δ bns1Δ* (A5408), and *mad1Δ spo12Δ bns1Δ slk19Δ* (A5515) cells all carrying a *CDC14-3HA* fusion were arrested in G1 in YEPD medium with α -factor (5 μ g/ml) and subsequently released in a synchronous manner into YEPD lacking pheromone at 25°C. α -Factor pheromone was readded 80 min after release to prevent entry into a subsequent cell-cycle. Cells were analyzed as described in (A). (C) *mad1Δ mcd1-1 slk19Δ* (A8132) and *mad1Δ mcd1-1 esp1-1 slk19Δ* (A8132) cells carrying a *CDC14-3HA* grown and analyzed as described in A. (D and E) Possible order of function of FEAR network components. *SLK19* functions downstream of *ESP1* (D, E). *SPO12* and *BNS1* function in parallel to *ESP1*, *SLK19*, and *CDC5* (D and E). *CDC5* could function in either of two ways in the FEAR network. *CDC5* could act downstream of *ESP1* and *SLK19* (D) or in parallel to the two genes (E).

these genes. To distinguish between these possibilities, we compared the kinetics of *Cdc14* release from the nucleolus and mitotic exit of *esp1-1* or *slk19Δ* mutants with that of *esp1-1* or *slk19Δ* mutants lacking *SPO12* and *BNS1*. We could not test whether deletion of *SPO12* and *BNS1* enhanced the phenotype of cells lacking *CDC5* as *Cdc14* release from the nucleolus is completely abolished in such cells (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002). Inactivation of *SPO12* and *BNS1* enhanced the defect in *Cdc14* release from the nucleolus and mitotic spindle disassembly of, both, *esp1-1* and *slk19Δ* mutants (Figure 3, A and B). In contrast, the mitotic exit defect of *esp1-1 slk19Δ* double mutants was similar to that of the *slk19Δ* and *esp1-1* single mutants (Figure 3C).

In summary our epistasis analyses indicate that *ESP1* and *SLK19* function in the same pathway and that *SLK19* functions downstream of *ESP1* (Figure 3, D and E; Sullivan and Uhlmann, 2003). *CDC5* either acts downstream of and/or in parallel to *ESP1* and *SLK19* and in parallel to *SPO12/BNS1* (Figure 3, D and E) because overexpression of *CDC5* suppresses the defect in *Cdc14* release from the nucleolus of *esp1-1* or *slk19Δ* or *spo12Δ bns1Δ* mutants as well as of *spo12Δ bns1Δ slk19Δ* triple mutants (Figures 1 and 2; Supplemental Figure 5). The findings that 1) inactivation of *SPO12* and *BNS1* enhances the mitotic exit defect of *slk19Δ* and *esp1-1* mutants; 2) overexpression of *SPO12* at least partially bypasses the requirement of *CDC5*, *ESP1*, and *SLK19* in *Cdc14* release from the nucleolus; and 3) overexpression of *CDC5*

can promote Cdc14 release from the nucleolus during early anaphase in *spo12Δ bns1Δ* mutants further suggests that *SPO12* and presumably *BNS1* function in a branch of the FEAR network that is parallel to that of *CDC5*, *ESP1*, and *SLK19* (Figure 3, D and E).

Hyperactivation of the MEN Suppresses the Defect of FEAR Network Mutants

Our epistasis analysis thus far has not been able to distinguish between the possibilities that *CDC5* functions downstream of or in parallel to *Esp1* and *Slk19*. *Cdc5*, besides being a FEAR network component, is also a member of the Mitotic Exit Network (Shou *et al.*, 1999; Visintin *et al.*, 1999; Hu *et al.*, 2001; Lee *et al.*, 2001) that functions in parallel to the FEAR network to promote Cdc14 release from the nucleolus (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002). The finding that overexpression of *CDC5* led to ectopic activation of *Dbf2* kinase (Figure 7A) raised the possibility that the suppression of FEAR network mutants by high levels of *Cdc5* was due to *Cdc5*'s parallel activating function in the MEN. We, therefore, asked whether overactivation of the MEN could suppress the defect of FEAR network mutants in releasing Cdc14 from the nucleolus during early anaphase. Overexpression of a truncated version of *CDC15* from the *GAL1-10* promoter (*GAL-CDC15* [1-750]) leads to pronounced hyperactivation of the MEN (Bardin *et al.*, 2003) and suppressed the defect in Cdc14 release from the nucleolus in *spo12Δ bns1Δ* mutants (Figure 4A). This finding suggests that hyperactivation of the MEN can suppress the need for the FEAR network in Cdc14 release during early anaphase.

Does overexpression of *CDC5* only activate the MEN or does it also activate the FEAR network? If high levels of *Cdc5* were solely activating the MEN, inactivation of this pathway should preclude Cdc14 release from the nucleolus in *GAL-CDC5* cells. However, *GAL-CDC5 cdc15-2* mutants exited mitosis with almost the same kinetics as wild-type cells (Figure 4B). Suppression of the mitotic exit defect of the *cdc15-2* mutant by overexpressed *CDC5* was not due to *CDC5* functioning downstream of *CDC15* in the MEN. Hyperactivation of the MEN caused by overexpression of a truncated version of *CDC15* from the *GAL1-10* promoter (*GAL-CDC15* [1-750]; Bardin *et al.*, 2003) promoted Cdc14 release from the nucleolus during late stages of anaphase in the absence of *CDC5* function (Figure 4C; note that Cdc14 release from the nucleolus did not occur in early anaphase, consistent with *Cdc5*'s role in the FEAR network). We conclude that overexpression of *CDC5* can promote Cdc14 release from the nucleolus and exit from mitosis in the absence of the MEN, suggesting that high levels of *Cdc5* also cause hyperactivation of the FEAR network. However, owing to the dual role of *Cdc5* in the FEAR network and the MEN, it was not possible to place *Cdc5* within the FEAR network. Thus, *CDC5* either functions downstream of and/or in parallel to *ESP1* and *SLK19* and in parallel to *SPO12/BNS1*. This parallel function of *CDC5* could be in the MEN (Figure 3, D and E).

Overexpression of *CDC5* Causes Premature Release of Cdc14 from the Nucleolus

A key question concerning the regulation of Cdc14 activity is how Cdc14 release from its inhibitor is regulated at the molecular level. *Cdc5* seemed to play a central role in promoting the release of Cdc14 from the nucleolus because *CDC5* was the only gene, which when overexpressed, caused premature release of Cdc14 from the nucleolus dur-

ing metaphase (Shou *et al.*, 2002; Figure 1). We, therefore, investigated *Cdc5*'s role in promoting Cdc14 release from the nucleolus in more detail and asked whether overexpression of *CDC5* could induce Cdc14 release from the nucleolus in cell cycle stages other than anaphase. Overexpression of *CDC5* caused release of Cdc14 from the nucleolus in S phase- and metaphase-arrested cells (Figures 5A and 6C), suggesting that *CDC5* when present at high levels is sufficient to induce release of Cdc14 from the nucleolus. However, the kinetics of this release was sluggish, suggesting that other factors were necessary to promote efficient release of Cdc14 from the nucleolus. To identify factors that aid *Cdc5* in promoting Cdc14 release from the nucleolus, we first determined whether *Spo12*, which functions in parallel to *Cdc5* in the FEAR network could enhance *Cdc5*'s Cdc14 release-promoting activity. Overexpression of *SPO12* did not enhance the ability of *CDC5* to promote release of Cdc14 from the nucleolus nor did it cause release of Cdc14 from the nucleolus in S phase- or metaphase-arrested cells on its own (Supplemental Figure 6; our unpublished data).

Next, we determined whether activation of the MEN enhanced *Cdc5*'s ability to promote Cdc14 release from the nucleolus in S phase-arrested cells. Deletion of *BUB2* has been shown to cause a slight hyperactivation of the MEN kinase *Dbf2* (Fesquet *et al.*, 1999; Lee *et al.*, 2001; Visintin and Amon, 2001). Cells deleted for *BUB2* did not release Cdc14 from the nucleolus in a hydroxyurea (HU)-arrest, but inactivation of *BUB2* enhanced the ability of *CDC5* to promote Cdc14 release from the nucleolus (Figure 5A). In *GAL-CDC5 bub2Δ* cells, Cdc14 release from the nucleolus occurred very efficiently and as soon as *Cdc5* protein accumulated, 40 min after galactose addition (Figure 5, A and C). The release of Cdc14 from the nucleolus was not due to disruption of this organelle, or the release of the Cdc14-Cfi1/Net1 complex from the nucleolus, as the localization of both the nucleolar protein *Nop1* and *Cfi1/Net1* was not affected by high levels of *Cdc5* and inactivation of *BUB2* (Supplemental Figure 7). Overexpression of other MEN or FEAR network components did not cause Cdc14 release from the nucleolus in S phase-arrested cells (our unpublished data). Our results show that overexpression of *CDC5* and the simultaneous overactivation of the MEN is sufficient to cause efficient Cdc14 release from the nucleolus and that *Cdc5* is unique in its ability to promote Cdc14 release from the nucleolus in all cell cycle stages analyzed.

The MEN Is Required for *CDC5* to Promote Ectopic Release of Cdc14 from the Nucleolus

High levels of *Cdc5* can only bring about efficient ectopic release of Cdc14 from the nucleolus when *BUB2* is deleted indicating that *CDC5* requires MEN function to effectively release Cdc14 from the nucleolus. Consistent with this idea we found that inactivation of *TEM1* prevented the ectopic release of Cdc14 from the nucleolus in S phase-arrested *GAL-CDC5 bub2Δ* cells or nocodazole-arrested *GAL-CDC5* cells (Figure 6, A and C). In contrast, inactivation of the FEAR network component *ESP1* did not hamper *Cdc5*'s ability to promote Cdc14 release from the nucleolus (Figure 6E). These data indicate that the activity of other FEAR network components is not necessary for overexpressed *CDC5* to bring about Cdc14 release from the nucleolus but MEN activity is. This need for the MEN was, however, not universal. During anaphase, high levels of *Cdc5* could promote exit from mitosis in the absence of an active MEN. Overexpression of *CDC5* restored Cdc14 release from the nucleolus during anaphase and exit from mitosis in *cdc15-2*

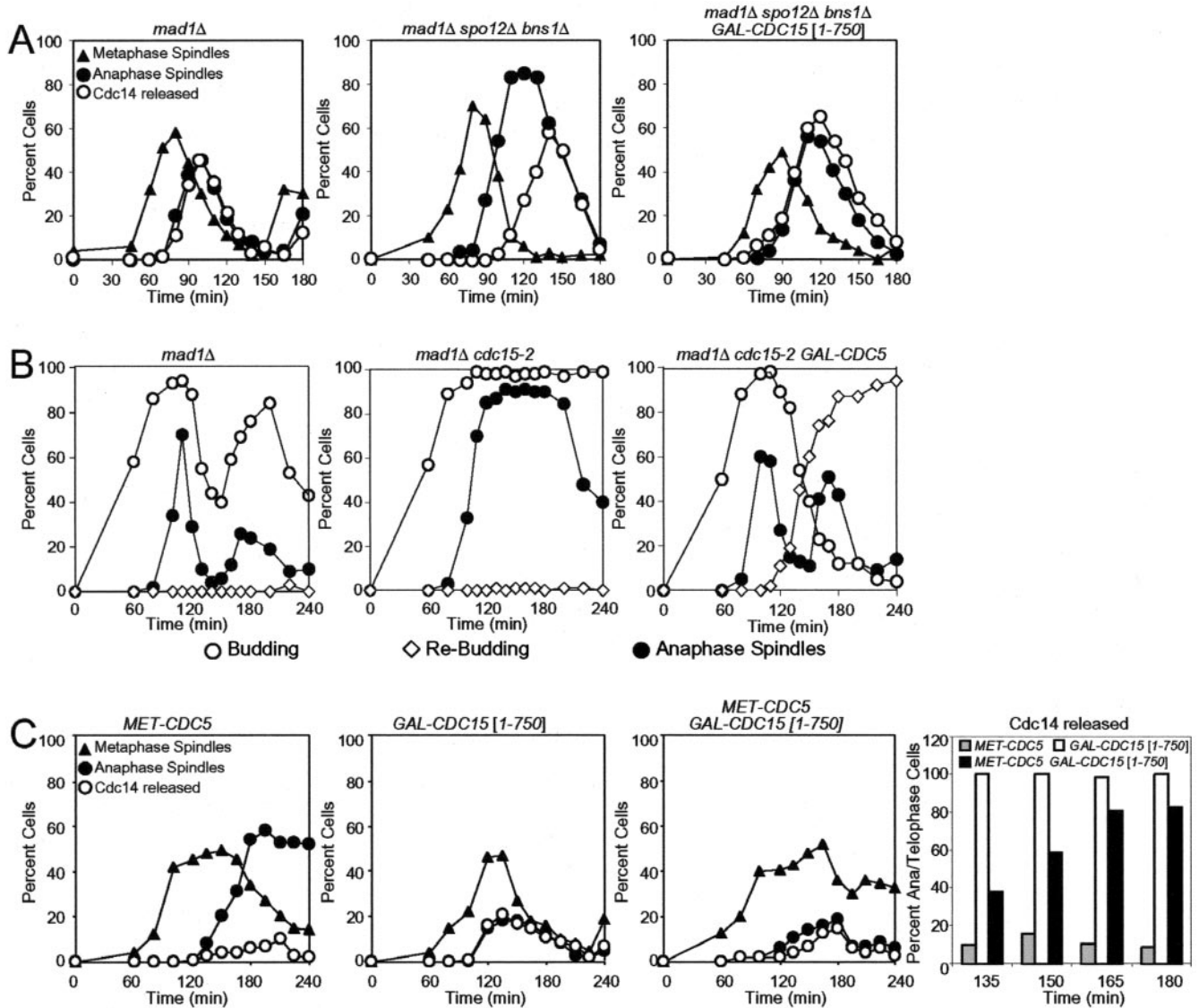


Figure 4. Hyperactivation of the MEN can suppress the mitotic exit defect of FEAR network mutants. (A) *mad1Δ* (A2853), *mad1Δ spo12Δ bns1Δ* (A5408), and *mad1Δ spo12Δ bns1Δ GAL-CDC15[1-750]* (A8708) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEPR with α -factor (5 μ g/ml) followed by released in YEPRG lacking pheromone. The percentage of cells with metaphase (closed triangles), anaphase/telophase spindles (closed circles) as well as the percentage of cells with Cdc14-HA released from the nucleolus (open circles) was determined at the indicated times. (B) *mad1Δ* (A2853), *mad1Δ cdc15-2* (A4300), and *mad1Δ cdc15-2 GAL-CDC5* (A5516) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEPR with α -factor (5 μ g/ml) followed by released in YEPRG lacking pheromone at the restrictive temperature 37°C. The percentage of budded cells (open circles), rebudded cells (open diamonds), which indicates that cells have entered the next cell cycle as well as the percentage of anaphase/telophase spindles (closed circles) was determined at the indicated times. (C) *MET-CDC5* (A9006), *GAL-CDC15[1-750]* (A8459), and *MET-CDC5 GAL-CDC15[1-750]* (A9007) cells carrying a *CDC14-3HA* fusion were grown in medium lacking methionine and arrested in G1 with α -factor (5 μ g/ml). Methionine (8 mM) was added 1 h before release, and cells were released into YEPRG medium containing 8 mM methionine at 25°C. The percentage of cells with metaphase (closed triangles), anaphase/telophase spindles (closed circles) as well as the percentage of cells with Cdc14-HA released from the nucleolus (open circles) was determined at the indicated times. The histogram on the right shows the percentage of Cdc14 release in anaphase/telophase cells. One hundred cells were scored for each time point.

cells (Figure 4A). Perhaps FEAR network components such as *SPO12* aid *CDC5* during anaphase in promoting Cdc14 release from the nucleolus but cannot do so in other stages of the cell cycle. Consistent with this idea we found that deletion of *SPO12* abolished the ability of overexpressed *CDC5* to promote exit from mitosis in *cdc15-2* mutants (our unpublished data).

Dbf2 Kinase Is Activated in *GAL-CDC5 bub2Δ* Cells in a *CDC14*-dependent Manner

Previous studies suggested that Cdc5 activated the MEN kinase Dbf2 in a *BUB2*-independent manner (Fesquet *et al.*, 1999, Lee *et al.*, 2001). We, therefore, examined the effects of overexpressing *CDC5* and deleting *BUB2* on Dbf2 kinase activity. Overexpression of *CDC5* slightly stimulated Dbf2

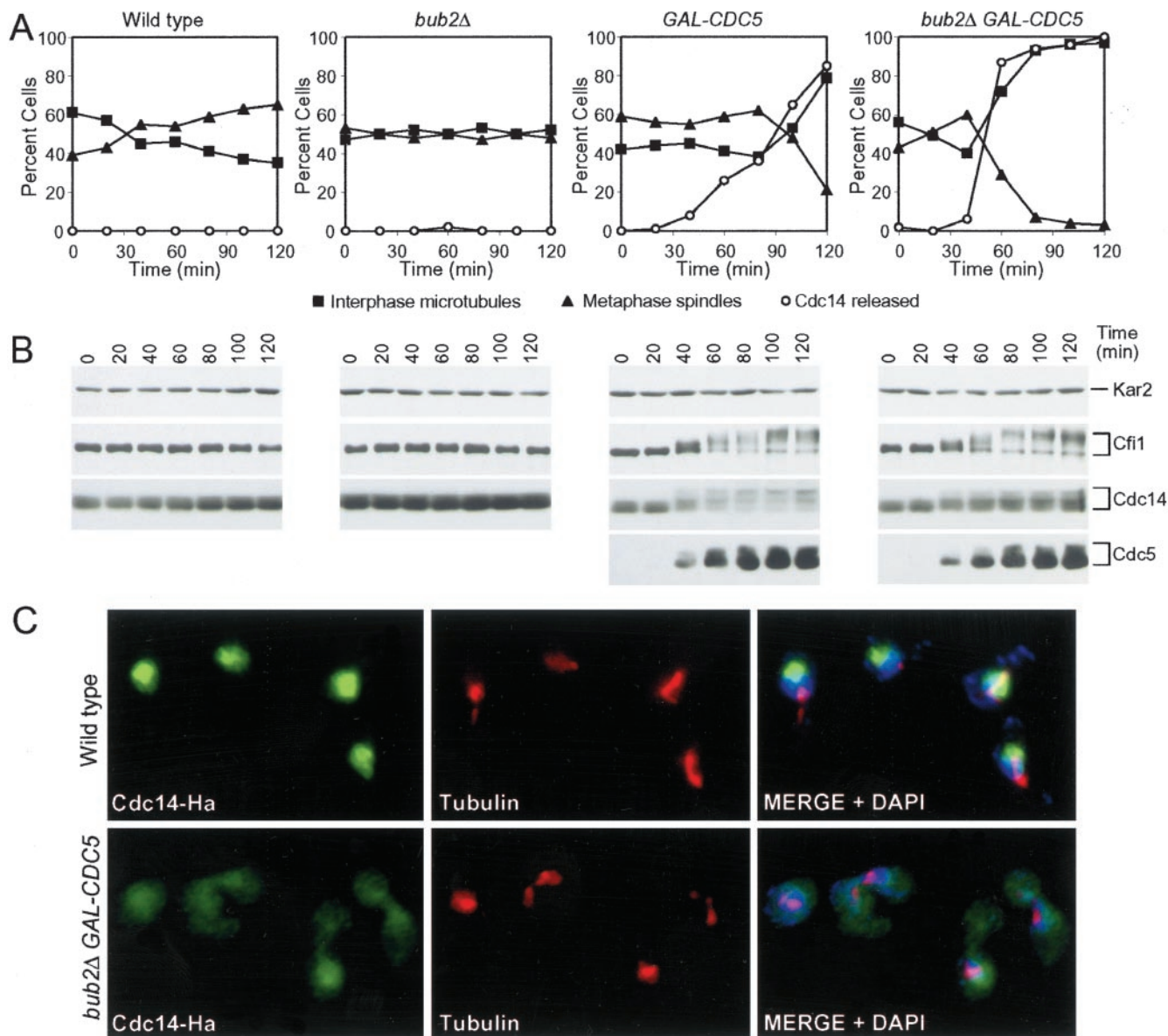


Figure 5. Overexpression of *CDC5* causes release of Cdc14 from the nucleolus in hydroxyurea-arrested cells, which is enhanced by the deletion of *BUB2*. Wild-type (A1681), *bub2Δ* (A4451), *GAL-CDC5* (A4450), and *GAL-CDC5 bub2Δ* (A4453) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion were arrested in early S phase with HU (10 mg/ml) in YEPR medium at 25°C. After 2.5 h 5 mg/ml HU and galactose (2%) were added. Samples were taken at the indicated times to analyze the percentage of cells with metaphase spindles (closed triangles; A) interphase microtubules (closed squares; A), with Cdc14 released from the nucleolus (open circles; A) and to analyze Cfi1/Net1, Cdc14, and Cdc5 protein levels and their mobility (B). Kar2 was used as an internal loading control in Western blots. The pictures in (C) show Cdc14 localization in wild-type and *GAL-CDC5 bub2Δ* cells 80 min after galactose addition. Cdc14 is shown in green, microtubules in red and nucleus in blue.

kinase activity but deletion of *BUB2* together with the overexpression of *CDC5* led to high levels of Dbf2 kinase activity (Figure 7A). To determine the mechanism whereby Dbf2 was activated in *GAL-CDC5 bub2Δ* cells, we determined whether *CDC14* was required for this activation to occur. We examined this possibility because one function of Cdc14 released by the FEAR network is to stimulate MEN activity (Jaspersen and Morgan, 2000; Stegmeier *et al.*, 2002). Inactivation of *CDC14* prevented Dbf2 kinase activation in *GAL-CDC5 bub2Δ* cells as judged by the low specific activity of Dbf2 kinase in *GAL-CDC5 bub2Δ* cells carrying a *cdc14-3*

mutation (Figure 7B). Our results show that *BUB2*-independent activation of the MEN by high levels of Cdc5 is mostly if not solely due to Cdc5's function in the FEAR network.

Overexpression of *CDC5* Induces Phosphorylation of Cdc14 and Cfi1/Net1

Release of Cdc14 from the nucleolus brought about by high levels of Cdc5 was accompanied by the appearance of slower migrating forms of Cdc14 and Cfi1/Net1 on Western blots (Figure 5B). These slower migrating forms of Cdc14

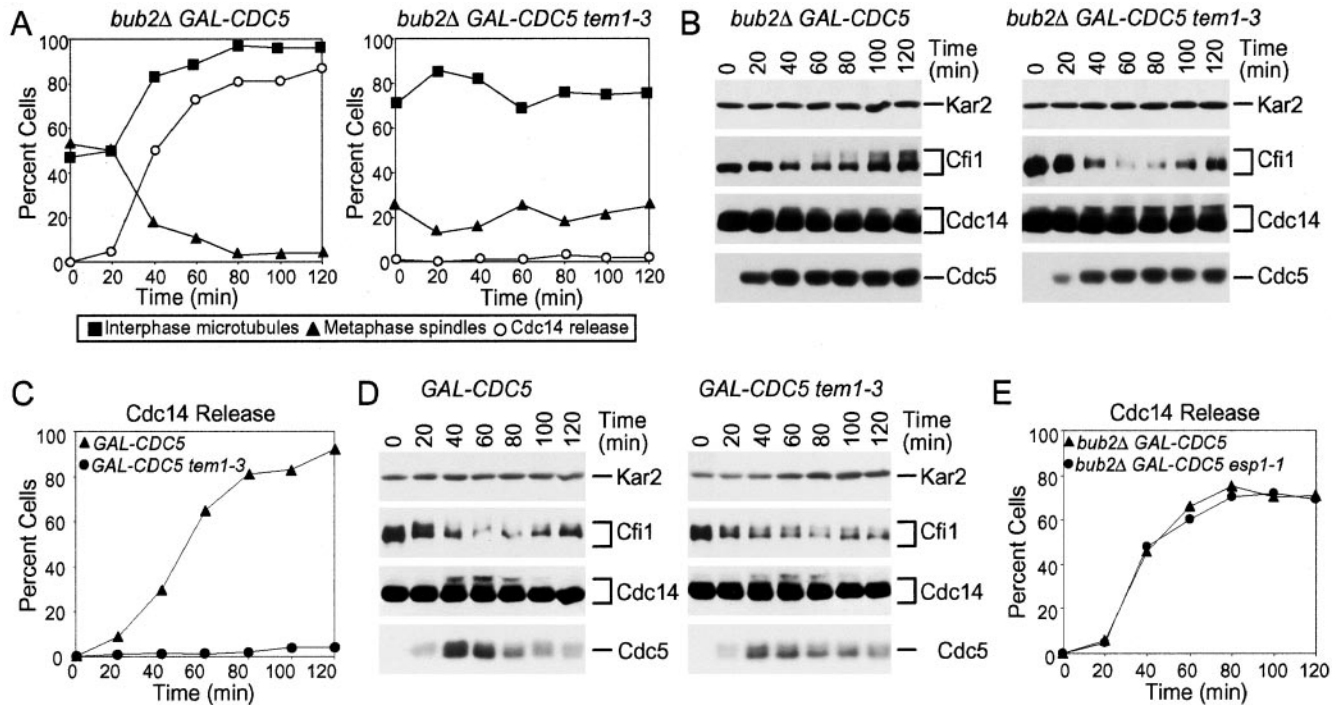


Figure 6. The ectopic release of Cdc14 from the nucleolus brought about by *GAL-CDC5* and deletion of *BUB2* depends on *TEM1*. (A, B) *GAL-CDC5 bub2Δ* (A4453) and *GAL-CDC5 bub2Δ tem1-3* (A4549) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion were grown and analyzed as described in Figure 5 except, cells were shifted to 37°C at the time of galactose addition. (C, D) *GAL-CDC5* (A4450) and *GAL-CDC5 tem1-3* (A4547) cells all carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion were arrested in early mitosis with the microtubule depolymerizing drug nocodazole (15 μg/ml) in YEPR at 25°C. After 2.5 h, cells were shifted to 37°C in the presence of nocodazole. Samples were taken at the indicated times to analyze the percentage of cells with Cdc14 released from the nucleolus (C) and Cfi1/Net1, Cdc14 and Cdc5 protein levels and mobility in SDS-PAGE (D). (E) *GAL-CDC5 bub2Δ* (A4453) and *GAL-CDC5 bub2Δ esp1-1* (A4282) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion were arrested with 10 mg/ml HU at 25°C. Galactose was then added and cells were shifted to 37°C and Cdc14 localization was analyzed at the indicated times.

and Cfi1/Net1 represent phosphorylated forms of the proteins as treatment of both proteins with alkaline phosphatase led to their disappearance (Shou *et al.*, 1999; Shou *et al.*, 2002; our unpublished data). Thus, overexpression of *CDC5* either directly or indirectly, induces phosphorylation of Cdc14 and Cfi1/Net1 and this phosphorylation occurs concomitantly with the release of Cdc14 from the nucleolus. A correlation between phosphorylation of Cdc14 and Cfi1/Net1 and Cdc14 release from the nucleolus also exists in wild-type cells progressing through the cell cycle in a synchronous manner (Figure 8). At the time of Cdc14 release from the nucleolus, Cfi1/Net1 and Cdc14 became phosphorylated as judged by the appearance of slower migrating forms of the proteins, although phosphorylation of Cdc14 was less pronounced than that seen in HU-arrested cells overexpressing *CDC5* (Figure 8B), perhaps reflecting the transient nature of this phosphorylation. We conclude that Cfi1/Net1 and Cdc14 are phosphorylated at the time when Cdc14 is released from the nucleolus and that these modifications can be brought about by the overexpression of *CDC5*.

Cdc5 Induces Cfi1/Net1 Phosphorylation by Activating the MEN

Inactivation of *TEM1* prevented the release of Cdc14 from the nucleolus in S phase-arrested *GAL-CDC5 bub2Δ* cells. To determine whether MEN activity also influenced Cdc14 and Cfi1/Net1 phosphorylation, we analyzed the mobility of the

two proteins in *GAL-CDC5 bub2Δ* cells carrying a *tem1-3* mutation. Inactivation of *TEM1* had little effect on Cdc14 phosphorylation induced by high levels of Cdc5 (Figure 6, B and D), phosphorylation of Cfi1/Net1 was greatly reduced in *GAL-CDC5 bub2Δ tem1-3* cells compared with *GAL-CDC5 bub2Δ* cells (Figure 6, B and D). Similar results were obtained when the MEN was inactivated using a *cdc15-2* mutation (our unpublished data), suggesting that the bulk of Cfi1/Net1 phosphorylation induced by high levels of Cdc5 was largely due to MEN activation. To further test this possibility, we asked whether activation of the MEN would induce Cfi1/Net1 phosphorylation. Indeed, overexpression of *CDC15[1-750]* caused hyperphosphorylation of Cfi1/Net1 in S phase-arrested cells but it did not induce Cdc14 phosphorylation (Figure 9). Phosphorylation of Cfi1/Net1 was independent of *CDC5* (our unpublished data), indicating that Cdc5 was not responsible for the phosphorylation of Cfi1/Net1 under these conditions. Remarkably, overexpression of *CDC15[1-750]* did not promote Cdc14 release from the nucleolus in S phase-arrested cells (Figure 9B). Our data indicate that phosphorylation of Cfi1/Net1 brought about by high levels of Cdc15[1-750] is not sufficient to bring about Cdc14 release from the nucleolus. Conversely, Cdc14 phosphorylation seemed not to be sufficient to cause Cdc14 release from the nucleolus in S phase-arrested cells, as Cdc14 is hyperphosphorylated in *GAL-CDC5 bub2Δ tem1-3* mutants, yet Cdc14 release from the nucleolus does not occur (Figure 6). Thus, it seems that only when both, Cdc14 and

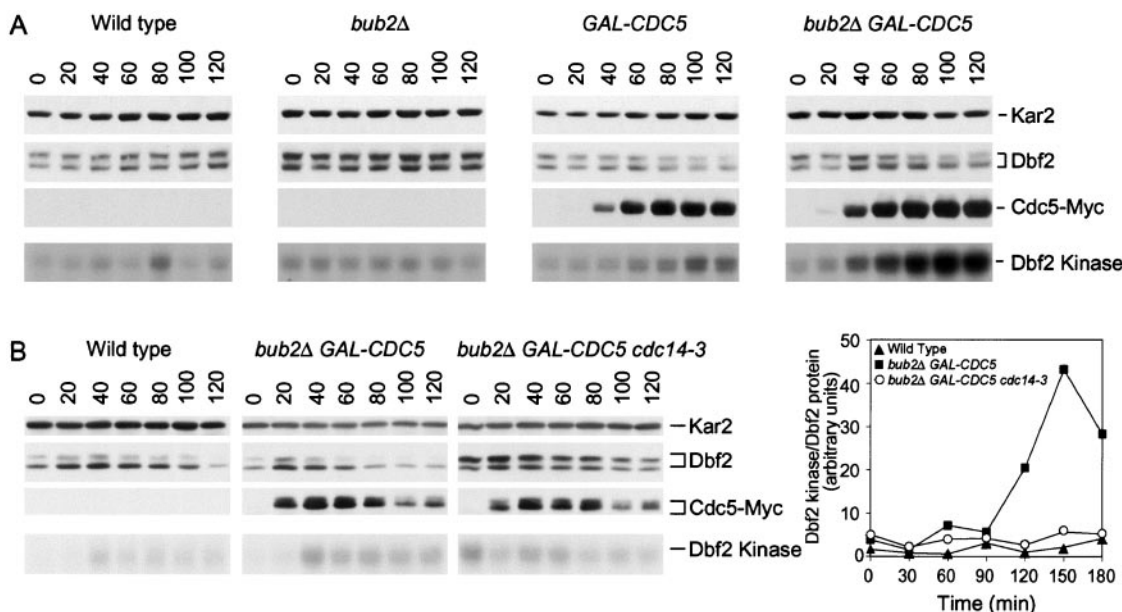


Figure 7. Overexpression of *CDC5* stimulates MEN activity. (A) Wild-type (A3912), *bub2Δ* (A6243), *GAL-CDC5* (A6207), and *GAL-CDC5 bub2Δ* (A6210) cells all carrying a *DBF2-3HA* fusion were grown as described in Figure 5. Samples were taken at the indicated times to analyze Dbf2 and Cdc5 protein levels and their mobility in SDS PAGE as well as Dbf2 associated Kinase activity. (B) Wild-type (A3912), *GAL-CDC5 bub2Δ* (A6210) and *GAL-CDC5 bub2Δ cdc14-3* (A7462) cells all carrying a *DBF2-3HA* fusion were arrested with 10 mg/ml HU at 25°C. Galactose was then added and cells were shifted to 37°C. The specific activity of Dbf2 (Dbf2 kinase/Dbf2 protein) was analyzed at the indicated times.

Cfi1/Net1 are phosphorylated can the phosphatase be released from its inhibitor.

DISCUSSION

Relationship among FEAR Network Components

Determining whether overexpression of a particular FEAR network component alleviates the need for another component to promote Cdc14 release from the nucleolus during early anaphase allowed us to characterize the epistatic relationship among FEAR network components. We propose that *ESP1* and *SLK19* function in the same branch of the FEAR network as *esp1-1 slk19Δ* double mutants exhibit the same defect in Cdc14 release from the nucleolus as *slk19Δ* single mutants. We further suggest that *SLK19* functions downstream of *ESP1* as *SLK19* is required for ectopic release of Cdc14 from the nucleolus in nocodazole-arrested cells brought about by high levels of Esp1 (Supplemental Figure 1; Sullivan and Uhlmann, 2003).

All our data indicate that *SPO12* and *BNS1* constitute a separate branch of the FEAR network (Figure 3, D and E). Overexpression of *SPO12* restored Cdc14 release from the nucleolus to *slk19Δ* and *esp1-1* mutants and inactivation of *SPO12* and *BNS1* enhanced the mitotic exit defect of *esp1-1* and *slk19Δ* mutants. The placing of *SPO12* and *BNS1* in parallel to *CDC5* is based on the observations that overexpression of *SPO12* partially suppressed the defect in Cdc14 release from the nucleolus in Cdc5-depleted cells and that high levels of *CDC5* alleviated the requirement for *SPO12* in promoting Cdc14 release from the nucleolus during early anaphase. We were not able to conclusively place *CDC5* within the FEAR network. This was due to the fact that high levels of Cdc5 activated the FEAR network and to some extent the MEN. This leads us to propose that *CDC5* either

functions downstream of and/or in parallel to *ESP1* and *SLK19* and in parallel to *SPO12/BNS1* and that this parallel function of *CDC5* could be in the MEN.

Esp1's and Slk19's Role in the FEAR Network

Esp1 is best known for its function in promoting sister-chromatid separation through cleavage of Scc1/Mcd1 (reviewed in Nasmyth, 2001) but the protease is also required for the timely exit from mitosis (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999; Stegmeier *et al.*, 2002). The *Esp1* substrate *Slk19* is also necessary for exit from mitosis but cleavage of *Slk19* by *Esp1*, does not seem to be necessary for Cdc14 release from the nucleolus (Stegmeier *et al.*, 2002). Furthermore, a recent report by Sullivan and Uhlmann (2003) indicates that *Esp1's* protease is not required for its mitotic exit promoting activity.

We observed in many independent experiments that overexpression of *ESP1* in cells progressing through the cell cycle in a synchronous manner did not promote Cdc14 release from the nucleolus but in fact slightly inhibited it. In nocodazole-arrested cells, however, overexpression of *ESP1* causes ectopic release of Cdc14 from the nucleolus and exit from mitosis, although the kinetics with which this release occurred is slower than that seen in cells overexpressing *CDC5* (Supplemental Figure 1; Sullivan and Uhlmann, 2003). This observation, however, allowed us to order *SLK19* with respect to *ESP1* function. Our results and that of Sullivan and Uhlmann (2003) suggest that *Slk19* functions downstream of *Esp1* to promote Cdc14 release from the nucleolus. Why high levels of *Esp1* promote Cdc14 release from the nucleolus in one stage of the cell cycle (metaphase) but slightly inhibit release in others (anaphase) is unknown. We speculate that *ESP1* is not only required to promote Cdc14

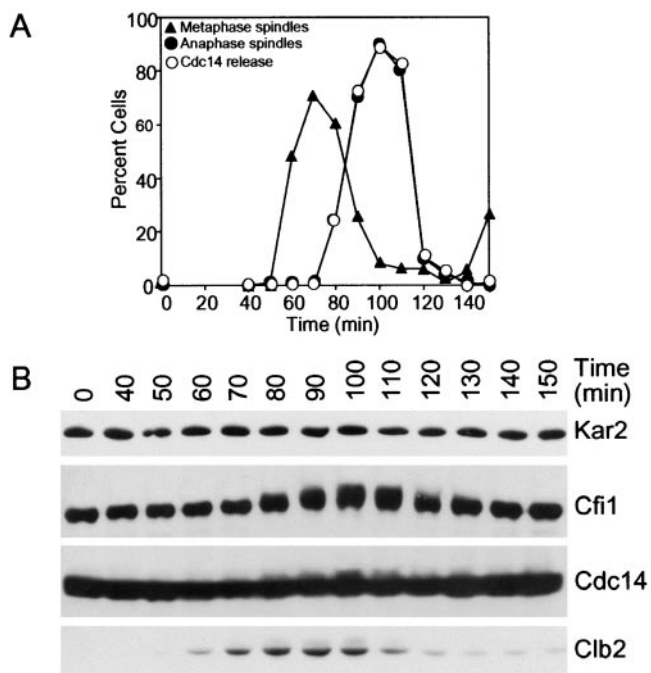


Figure 8. Cdc14 and Cfi1/Net1 are phosphorylated during mitosis. Cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion (A1681) were arrested in G1 in YEPD medium with α -factor (5 μ g/ml) followed by release into YEPD medium lacking pheromone at 25°C. The percentage of cells with metaphase (closed triangles, A) and anaphase/telophase spindles (closed circles; A) as well as the percentage of cells with Cdc14-HA released from the nucleolus (open circles; A) was determined at the indicated times. Cdc14 and Cfi1/Net1 protein levels as well as their migration in SDS-PAGE were analyzed by Western Blot analysis (B). Clb2 protein levels were examined to determine when entry and exit from mitosis occur.

release from its inhibitor but is also necessary for Cdc14 resequestration after exit from mitosis has been completed.

Spo12's Role in the FEAR Network

Inactivation of the *SPO12* homolog *BNS1*, although causing no obvious defects in Cdc14 release from the nucleolus and exit from mitosis on its own, enhances the defects associated with a deletion of *SPO12* thus adding this gene to the list of FEAR network components. However, even inactivation of both *SPO12* and *BNS1* only caused a mild defect in Cdc14 release from the nucleolus and exit from mitosis. It, thus, comes as a surprise that *Spo12* is a potent promoter of Cdc14 release from the nucleolus and exit from mitosis when overexpressed during anaphase. High levels of *Spo12* allow temperature-sensitive *tem1-3*, *cdc15-2*, *cdc5-1*, and *dbf2-2* mutants to grow with almost wild-type kinetics at the restrictive temperature (Parkes and Johnston, 1992; Jaspersen *et al.*, 1998; Shah *et al.*, 2001). To further complicate the *Spo12* puzzle, despite overproduced *Spo12* effectively promoting Cdc14 release during anaphase even in the absence of a functional MEN, *Spo12* fails to induce Cdc14 release from the nucleolus in stages of the cell cycle when Cdc14 is normally sequestered. The mechanisms that cause *Spo12* to effectively promote Cdc14 release during anaphase but not other cell cycle stages remain obscure.

Multiple Roles of Cdc5 in Mitotic Exit

Cdc5 is the only member of the FEAR network and the MEN that, when overexpressed, promoted Cdc14 release from the

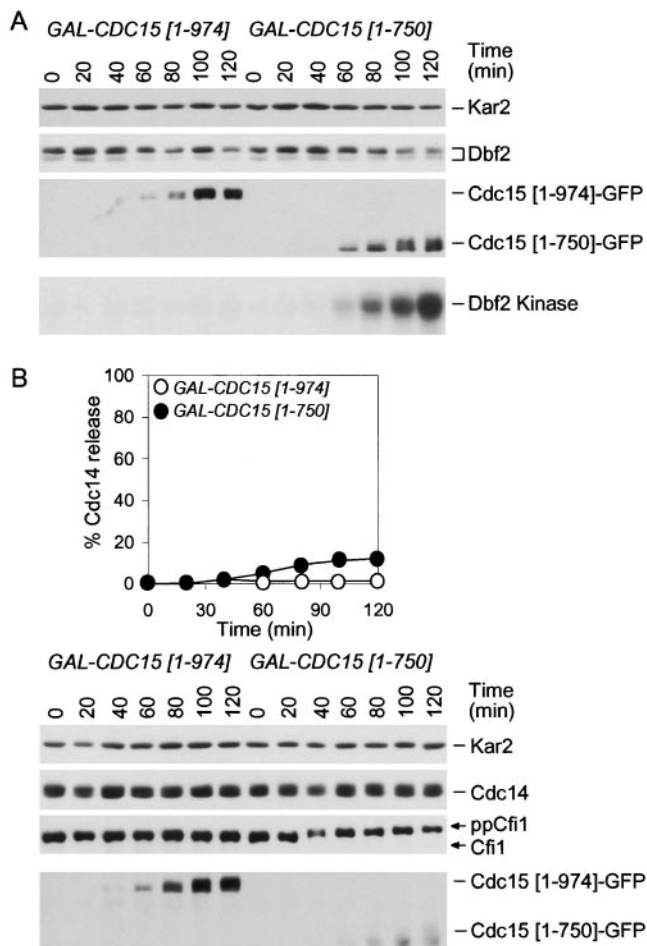


Figure 9. Hyperactivation of MEN is not sufficient to promote Cdc14 release in hydroxyurea arrested cells. (A) *GAL-CDC15[1-940]* (A5568) and *GAL-CDC15[1-750]* (A6130) cells carrying a *DBF2-3MYC* fusion were arrested in early S phase with hydroxyurea (HU; 10 mg/ml) in YEPR medium at 25°C. After 2.5 h 5 mg/ml HU and galactose (2%) were added. Dbf2 and Cdc15 protein levels and their mobility as well as Dbf2 kinase activity was analyzed at the indicated times. Kar2 was used as an internal loading control in Western blots. (B) *GAL-CDC15[1-940]* (A6460) and *GAL-CDC15[1-750]* (A6459) cells carrying a *CDC14-3HA* fusion were arrested as described in Figure 9A. Samples were taken at the indicated times to analyze the percentage of cells with Cdc14 released from the nucleolus (open circles, *GAL-CDC15[1-940]*; closed circles, *GAL-CDC15[1-750]*) and to analyze Cfi1/Net1, Cdc14, and Cdc15 protein levels and their mobility. Kar2 was used as an internal loading control in Western blots.

nucleolus in S phase-arrested cells. In contrast, several MEN and FEAR network components can promote Cdc14 release from the nucleolus in metaphase-arrested cells (Shou *et al.*, 2002; Yoshida *et al.*, 2002; Bardin *et al.*, 2003; Sullivan and Uhlmann, 2003; Visintin, unpublished observations). Our data show that Cdc5 has the unique ability to activate both the FEAR network and the MEN and this is the likely reason why Cdc5 can promote the dissociation of Cdc14 from its inhibitor in S phase-arrested cells. In metaphase-arrested cells and during anaphase, Cdc5 is active (Charles *et al.*, 1998; Shirayama *et al.*, 1998), which may be the reason why other FEAR network and MEN components can promote Cdc14 release from the nucleolus in this cell cycle stage.

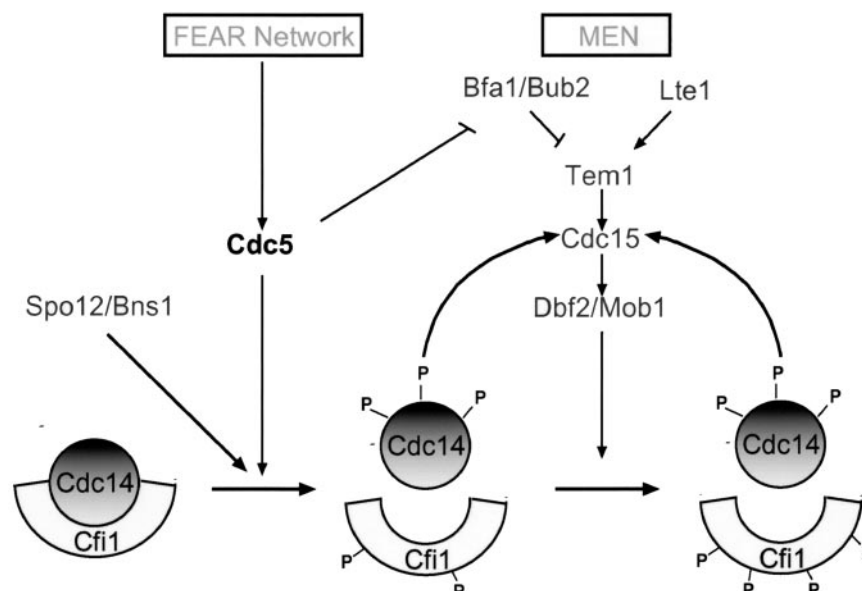


Figure 10. A model for how Cdc5 controls the release of Cdc14 from the nucleolus. At the onset of anaphase, activation of the FEAR network causes, by an unknown mechanism, Cdc5 to phosphorylate Cdc14 and Cfi1/Net1. Release of Cdc14 then promotes MEN activity by dephosphorylating Cdc15 and perhaps other proteins. Activation of the MEN then leads to further phosphorylation of Cfi1/Net1. Cdc5 also phosphorylates Bfa1 and Bub2, thereby contributing to full activation of the MEN.

Previous data and our results implicate Cdc5 in multiple aspects of mitotic exit. Cdc5 as a component of the FEAR network promotes Cdc14 release from the nucleolus (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002). Cdc5 has also been shown to regulate the MEN. Cdc5 phosphorylation inhibits Bub2-Bfa1 (Hu *et al.*, 2001; Hu and Elledge, 2002, Geymonat *et al.*, 2003) and activates Dbf2 activity independently of Bub2-Bfa1 (Lee *et al.*, 2001). How Cdc5 controlled the activity of Dbf2 was not known. We show that the ability of overexpressed *CDC5* to activate Dbf2 in the absence of *BUB2* depends on *CDC14*. Our data further indicate that Cdc5 does not function downstream of *CDC15*. Thus, our findings together with that of others suggest that Cdc5 functions in two ways to promote the activation of the MEN. Cdc5 directly activates the MEN by inhibiting Bub2-Bfa1 (Hu *et al.*, 2001; Hu and Elledge, 2002; Geymonat *et al.*, 2003). Cdc5's *BUB2*-independent role in promoting MEN activation is through releasing Cdc14 from the nucleolus as a component of the FEAR network (Pereira *et al.*, 2002; Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002; this study).

A Model for How Cdc14 Is Released from Its Inhibitor Cfi1/Net1 during Anaphase

In vitro studies suggest that phosphorylation is responsible for the dissociation of Cdc14 from its inhibitor Cfi1/Net1 (Shou *et al.*, 1999; Shou *et al.*, 2002). Furthermore, in vivo phosphorylation of Cfi1/Net1 and Cdc14 correlates with the release of Cdc14 from the nucleolus. Two observations suggest that Cdc5 directly phosphorylates Cdc14. Overexpression of *CDC5* induced Cdc14 phosphorylation. Furthermore, Cdc5 can phosphorylate Cdc14 in vitro (Visintin, unpublished observations). Previous studies suggested that Cfi1/Net1 is a direct target of Cdc5. Cfi1/Net1 is a substrate of the protein kinase in vitro (Shou *et al.*, 2002), Cfi1/Net1 phosphorylation depends on *CDC5* activity in vivo (Shou *et al.*, 2002; Yoshida and Toh-e, 2002) and high levels of Cdc5 induce Cfi1/Net1 phosphorylation (Figure 5). However, at least in S phase-arrested cells, the bulk of Cfi1/Net1 phosphorylation induced by *CDC5* depended on an active MEN, indicating that Cdc5 induced Cfi1/Net1 phosphorylation indirectly, by activating the MEN. Consistent with this idea

is the finding that Cfi1/Net1 phosphorylation depends on the MEN kinase Dbf2 (Visintin, unpublished observations). Thus, although it is possible that Cdc5 directly phosphorylates Cfi1/Net1, the bulk phosphorylation of the protein that occurs in vivo seems to be mediated by the MEN.

Our studies on Cdc14 and Cfi1/Net1 phosphorylation also revealed that the dissociation of Cdc14 from its inhibitor correlated only with the phosphorylation of both proteins. In *GAL-CDC5 tem1-3* mutants, Cdc14 was hyperphosphorylated but Cfi1/Net1 was not and Cdc14 was not released from the nucleolus. In cells overexpressing *CDC15[1-750]* Cfi1/Net1 was hyperphosphorylated but Cdc14 was not and no Cdc14 release from the nucleolus was observed. Based on this correlation and results described above, we propose a two-step model for how Cdc14 release from the nucleolus is controlled. During early anaphase Cdc5 phosphorylates Cdc14 and perhaps to some extent Cfi1/Net1. We speculate that Spo12/Bns1 then aids in the dissociation of phosphorylated Cdc14 from Cfi1/Net1 (Figure 10). Activation of the MEN, in part through Cdc5 and Cdc14 then leads to phosphorylation of Cfi1/Net1. These phosphorylation events are necessary for maintaining Cdc14 in the released state during late stages of anaphase and telophase. Thus, Cdc5 initiates a positive feedback loop that brings about the rapid activation of Cdc14 at the end of mitosis.

ACKNOWLEDGMENTS

We are grateful to Frank Uhlmann for strains overexpressing *ESPI* from the *GAL1-10* promoter and for communicating results before publication and to Ray Deshaies for providing the *TAB6-1* allele. We thank Frank Solomon and members of the Amon laboratory for critical reading of the manuscript. R.V. is a postdoctoral fellow of the Merck foundation and F.S. is a predoctoral fellow of the National Science Foundation. This research was supported by a National Institute of Health grant GM-56800 to A.A. A.A. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

Bardin, A.J., and Amon, A. (2001). Men and sin: what's the difference? *Nat. Rev. Mol. Cell. Biol.* 2, 815-826.

- Bardin, A.J., Boselli, M.G., and Amon, A. (2003). Mitotic exit regulation through distinct domains within the protein kinase Cdc15. *Mol. Biol. Cell* 23, 5018–5030.
- Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A., and Morgan, D.O. (1998). The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr. Biol.* 8, 497–507.
- Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* 93, 1067–1076.
- Cohen-Fix, O., and Koshland, D. (1999). Pds1p of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit. *Genes Dev.* 13, 1950–1959.
- Cohen-Fix, O., Peters, J.M., Kirschner, M.W., Koshland, D. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* 10, 3081–3093.
- Fesquet, D., Fitzpatrick, P.J., Johnson, A.L., Kramer, K.M., Toyn, J.H., and Johnston, L.H. (1999). A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. *EMBO J.* 18, 2424–2434.
- Geymonat, M., Spanos, A., Walker, P.A., Johnston, L.H., and Sedgwick, S.G. (2003). In vitro regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5. *J. Biol. Chem.* 278, 14591–14594.
- Grether, M.E., and Herskowitz, I. (1999). Genetic and biochemical characterization of the yeast spo12 protein. *Mol. Biol. Cell* 10, 3689–3703.
- Gruneberg, U., Campbell, K., Simpson, C., Grindlay, J., and Schiebel, E. (2000). Nud1p links astral microtubule organization and the control of exit from mitosis. *EMBO J.* 19, 6475–6488.
- Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*. *Cell* 91, 47–57.
- Hu, F., and Elledge, S.J. (2002). Bub2 is a cell cycle regulated phospho-protein controlled by multiple checkpoints. *Cell Cycle* 1, 351–355.
- Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell* 107, 655–665.
- Jaspersen, S.L., Charles, J.F., Tinker-Kulberg, R.L., and Morgan, D.O. (1998). A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 9, 2803–2817.
- Jaspersen, S.L., and Morgan, D.O. (2000). Cdc14 activates cdc15 to promote mitotic exit in budding yeast. *Curr. Biol.* 10, 615–618.
- Lee, S.E., Frenz, L.M., Wells, N.J., Johnson, A.L., and Johnston, L.H. (2001). Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. *Curr. Biol.* 11, 784–788.
- Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Mah, A.S., Jang, J., and Deshaies, R.J. (2001). Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. *Proc. Natl. Acad. Sci. USA* 98, 7325–7330.
- McCollum, D., and Gould, K.L. (2001). Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol.* 11, 89–95.
- Morgan, D.O. (1999). Regulation of the APC and the exit from mitosis. *Nat. Cell Biol.* 1, E47–E53.
- Mountain, H.A., and Korch, C. (1991). TDH2 is linked to MET3 on chromosome X of *Saccharomyces cerevisiae*. *Yeast* 7, 873–880.
- Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annu. Rev. Genet.* 35, 673–745.
- Parkes, V., and Johnston, L.H. (1992). SPO12 and SIT4 suppress mutations in DBF2, which encodes a cell cycle protein kinase that is periodically expressed. *Nucleic Acids Res.* 20, 5617–5623.
- Pereira, G., Manson, C., Grindlay, J., and Schiebel, E. (2002). Regulation of the Bfa1p-Bub2p complex at spindle pole bodies by the cell cycle phosphatase Cdc14p. *J. Cell Biol.* 157, 367–379.
- Shah, R., Jensen, S., Frenz, L.M., Johnson, A.L., and Johnston, L.H. (2001). The Spo12 protein of *Saccharomyces cerevisiae*: a regulator of mitotic exit whose cell cycle-dependent degradation is mediated by the anaphase-promoting complex. *Genetics* 159, 965–980.
- Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.* 17, 1336–1349.
- Shou, W., Azzam, R., Chen, S.L., Huddleston, M.J., Baskerville, C., Charbonneau, H., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2002). Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. *BMC Mol. Biol.* 3, 3.
- Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* 97, 233–244.
- Stegmeier, F., Visintin, R., and Amon, A. (2002). Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. *Cell* 108, 207–220.
- Sullivan, M., Lehane, C., and Uhlmann, F. (2001). Orchestrating anaphase and mitotic exit: separase cleavage and localization of Slk19. *Nat. Cell Biol.* 3, 771–777.
- Sullivan, M., and Uhlmann, F. (2003). A non-proteolytic function of separase links the onset of anaphase to mitotic exit. *Nat. Cell Biol.* 5, 249–254.
- Taylor, G.S., Liu, Y., Baskerville, C., and Charbonneau, H. (1997). The activity of Cdc14p, an oligomeric dual specificity protein phosphatase from *Saccharomyces cerevisiae*, is required for cell cycle progression. *J. Biol. Chem.* 272, 24054–24063.
- Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. *Genes Dev.* 13, 1936–1949.
- Traverso, E.E., Baskerville, C., Liu, Y., Shou, W., James, P., Deshaies, R.J., and Charbonneau, H. (2001). Characterization of the Net1 cell cycle-dependent regulator of the Cdc14 phosphatase from budding yeast. *J. Biol. Chem.* 276, 21924–21931.
- Uhlmann, F. (2001). Secured cutting: controlling separase at the metaphase to anaphase transition. *EMBO Rep.* 2, 487–492.
- Visintin, R., and Amon, A. (2001). Regulation of the mitotic exit pathway protein kinases Cdc15 and Dbf2. *Mol. Biol. Cell* 12, 2961–2974.
- Visintin, R., Hwang, E.S., and Amon, A. (1999). Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* 398, 818–823.
- Yoshida, S., Asakawa, K., and Toh-e, A. (2002). Mitotic exit network controls the localization of Cdc14 to the spindle pole body in *Saccharomyces cerevisiae*. *Curr. Biol.* 12, 944–950.
- Yoshida, S., and Toh-e, A. (2002). Budding yeast Cdc5 phosphorylates Net1 and assists Cdc14 release from the nucleolus. *Biochem. Biophys. Res. Commun.* 294, 687–691.
- Zeng, X., Kahana, J.A., Silver, P.A., Morphew, M.K., McIntosh, J.R., Fitch, I.T., Carbon, J., and Saunders, W.S. (1999). Slk19p is a centromere protein that functions to stabilize mitotic spindles. *J. Cell Biol.* 146, 415–425.