Regulation of Mitotic Exit in *Saccharomyces cerevisiae*

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Abstract

Successful cell division requires the coordination of several mitotic processes, such as chromosome segregation, spindle disassembly, and cytokinesis. Progression into mitosis is driven by mitotic cyclin dependent kinase (CDKs) activity. In order for cells to exit from mitosis, mitotic CDKs must be inactivated. In the budding yeast, *Saccharomyces cerevisiae*, the Cdc14 phosphatase promotes mitotic exit by antagonizing mitotic CDKs. The activation of this phosphatase is controlled by the mitotic exit network (MEN), a Ras-like signaling cascade essential for exit from mitosis. The work presented herein describes the identification and characterization of a novel regulatory pathway, termed the Cdc fourteen early anaphase release (FEAR) network, which regulates the activation of Cdc14 during early anaphase. I found that at the onset of anaphase, the FEAR network initiates the release of Cdc14 from its inhibitor. During later stages of anaphase, the MEN promotes further release of Cdc14 and maintains the phosphatase in its released state. The FEAR network is comprised of the separase Esp1, the polo-kinase Cdc5, the kinetochore protein Slk19, the replication fork block protein Fob1, and the small nuclear proteins Spo12 and Bns1. Genetic epistasis analyses revealed that the FEAR network consists of at least two branches. *ESP1* and *SLK19* function in parallel to *SPO12* and *FOB1*. I then characterized the Spo12-Fob1 branch in detail. My results suggest that Fob1 prevents Cdc14 activation prior to anaphase. Spo12, in turn, promotes the activation of Cdc14 during early anaphase at least in part by antagonizing Fob1 function. The finding that two important regulators of sister-chromatid separation, the separase Esp1 and the polo-kinase Cdc5, also promote the activation of Cdc14 provides a molecular explanation as to how cells ensure that exit from mitosis does not occur prior to the onset of sister-chromatid separation.

Thesis Supervisor: Angelika Amon
Title: Associate Professor of Biology

Dedicated to my wife and family
for their love and support

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Chapter I

Introduction

The transmission of genetic information from one generation to the next requires the accurate replication of the DNA during S phase and the faithful partitioning of chromosomes during mitosis. Accomplishing this difficult task requires the coordination of several cellular events. Successful completion of mitosis, for example, depends on the temporal and spatial coordination of many mitotic processes such as chromosome segregation, spindle disassembly, and cytokinesis. Errors during mitosis, most notably chromosome mis-segregation, lead to genetic instability, a molecular hallmark of cancer. The fidelity of mitotic processes is in part controlled by checkpoints that monitor key cell cycle events and in case of a defect halt mitotic progression to provide time for error correction. In addition, the coordination of mitotic events is often achieved by employing the same proteins to control multiple events. The thesis presented herein describes the identification and characterization of a novel regulatory network, the Cdc fourteen early anaphase release (FEAR) network, which contributes to the temporal coordination of late mitotic events.

An overview of mitosis.

In the last two decades it has become clear that the basic cell cycle machinery regulating chromosome segregation is conserved in all eukaryotes. At the core of this machinery are protein kinases known as mitotic cyclin-dependent kinases (CDKs). CDKs, which are composed of a catalytic kinase subunit and a regulatory cyclin subunit, promote entry into mitosis (reviewed in Nasmyth, 1996; Morgan, 1997).

Figure 1

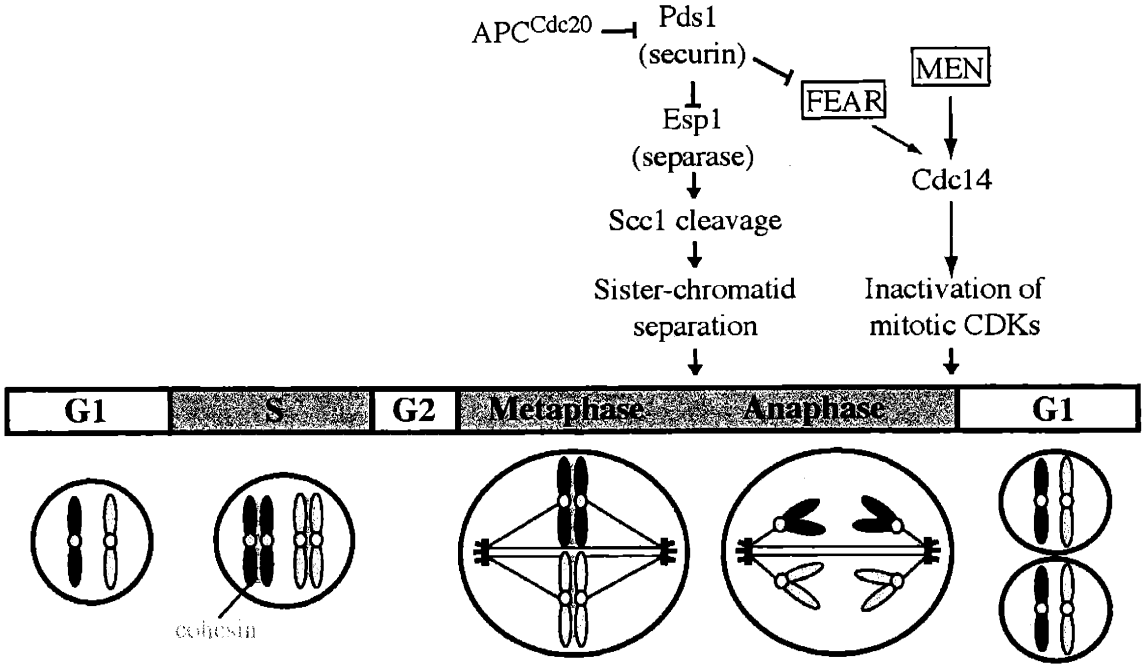

Figure 1: Regulation of mitosis in *S. cerevisiae*

After DNA replication, sister-chromatids are held together by the cohesin complex. Sister-chromatid separation is initiated when a protease called separase (Esp1 in budding yeast) cleaves the cohesin subunit Scc1/Mcd1. Until the onset of anaphase, separase is kept inactive by its inhibitor securin (Pds1 in budding yeast). Securin is inactivated at the onset of anaphase by a ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex/ Cyclosome (APC/C) together with its specificity factor Cdc20. This allows for separase to become active, thereby triggering the onset of sister-chromatid separation. Exit from mitosis requires the inactivation of mitotic CDKs. Cdc14 promotes mitotic exit by antagonizing mitotic CDK activity. The activity of Cdc14 is regulated by two signaling pathways, the Cdc fourteen early anphase release (FEAR) network and the mitotic exit network (MEN).

Preparations for chromosome segregation begin already during DNA replication, when protein complexes known as cohesins are laid down between the duplicated DNA strands (sister chromatids, reviewed in Hirano, 2000). During prophase these cohesins facilitate the bi-polar attachment of pairs of sister chromatids on the mitotic spindle (reviewed in Nasmyth, 2001). The dissolution of sister chromatids is triggered by cleavage of one cohesin subunit (Scc1/Mcd1 in budding yeast, Rad21 in *S. pombe*, Drosophila and mammals, SCC-1/COH-2 in *C. elegans*) by a CD clan family protease called separase (Esp1 in budding yeast, reviewed in Nasmyth, 2001) . Separase is kept inactive by securin (Pds1 in yeast) until all pairs of sister chromatids have attached to the mitotic spindle in a bi-polar manner. Once this is achieved, an ubiquitin ligase known as the Anaphase Promoting complex or Cyclosome (APC/C) that requires the specificity factor Cdc20 for its activation, mediates the ubiquitin-dependent proteolysis of securin (Figure 1, reviewed in Peters, 2002). Destruction of securin and hence activation of separase marks the onset of anaphase.

After the completion of chromosome segregation, cells exit from mitosis (Figure 1). This cell cycle transition is characterized by mitotic spindle disassembly, chromosomes decondensation, and the removal of mitotic determinants. The destruction of mitotic determinants creates conditions that are permissive for cytokinesis and assembly of protein complexes required for the initiation of DNA replication (pre-replicative complexes; preRCs) onto origins. In all organisms studied to date, exit from mitosis requires the inactivation of mitotic CDKs. When inactivation of mitotic CDKs is prevented, cells arrest in late anaphase/telophase with segregated DNA masses and an extended mitotic spindle (reviewed in Irniger, 2002; Wasch et al., 2002). Mitotic CDK

inactivation is brought about primarily by the ubiquitin-mediated degradation of mitotic cyclins (Clb cyclins in yeast), which is initiated at the metaphase-anaphase transition by APC/C^{Cdc20} in most organisms (reviewed in Peters, 2002).

The molecular mechanisms regulating mitotic CDK inactivation at the end of mitosis in *S. cerevisiae*.

In contrast to most other eukaryotes, in budding yeast a pool of mitotic CDK activity persists until anaphase spindle elongation is complete (Surana et al., 1993; Shirayama et al., 1994b; Jaspersen et al., 1998). This pool of mitotic CDK activity is primarily inactivated by APC/C complexed with the specificity factor Cdh1/Hct1, which targets mitotic cyclins for ubiquitin-mediated proteolysis (Schwab et al., 1997; Visintin et al., 1997; reviewed in Peters, 2002). Accumulation of a CDK inhibitor, Sic1, which directly binds to the cyclin-CDK complex, further ensures the precipitous inactivation of the mitotic CDKs at the end of mitosis (Mendenhall et al., 1987; Donovan et al., 1994; Schwob et al., 1994).

In budding yeast, the protein phosphatase Cdc14 is essential for the inactivation of mitotic CDKs. Cells lacking Cdc14 function arrest in late anaphase with high mitotic CDK activity (Wan et al., 1992; Visintin et al., 1998). Conversely, overexpression of *CDC14* results in inappropriate mitotic CDK inactivation (Visintin et al., 1998). Cdc14 promotes mitotic CDK inactivation by reversing CDK phosphorylation events. Cdc14 dephosphorylates Cdh1/Hct1, which promotes is association with the APC/C thereby activating it (Visintin et al., 1998; Zachariae et al., 1998; Jaspersen et al., 1999). Cdc14 also promotes Sic1 accumulation by dephosphorylating Sic1 and its transcription factor Swi5, which lead to the stabilization of Sic1 and upregulation of *SIC1* transcription,

respectively (Moll et al., 1991; Knapp et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Toyn et al., 1997; Verma et al., 1997; Visintin et al., 1998; Jaspersen et al., 1999). Sic1, Swi5, and Cdh1/Hct1 however are not the only substrates of Cdc14. In fact it is now clear that Cdc14 has many substrates in the cell (Figure 5) and it is likely that Cdc14 dephosphorylates many if not all Clb-Cdk substrates. This general reversal of CDK phosphorylation is likely to further contribute to the rapid resetting of the cell cycle to the G1 state.

Regulation of Cdc14 during mitosis in *S. cerevisiae*.

The activity of Cdc14 is controlled by cell-cycle dependent changes in its association with the competitive inhibitor Cfi1/Net1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999; Traverso et al., 2001). For most of the cell cycle up to metaphase, Cfi/Net1 binds to and inhibits Cdc14 activity. As Cfi1/Net1 resides in the nucleolus, Cdc14, when bound to its inhibitor, also localizes to this subnuclear structure. During anaphase, Cdc14 becomes released from its inhibitor and spreads into the nucleus and cytoplasm, allowing it to dephosphorylate its substrates (Figure 2, Shou et al., 1999; Visintin et al., 1999).

Figure 2

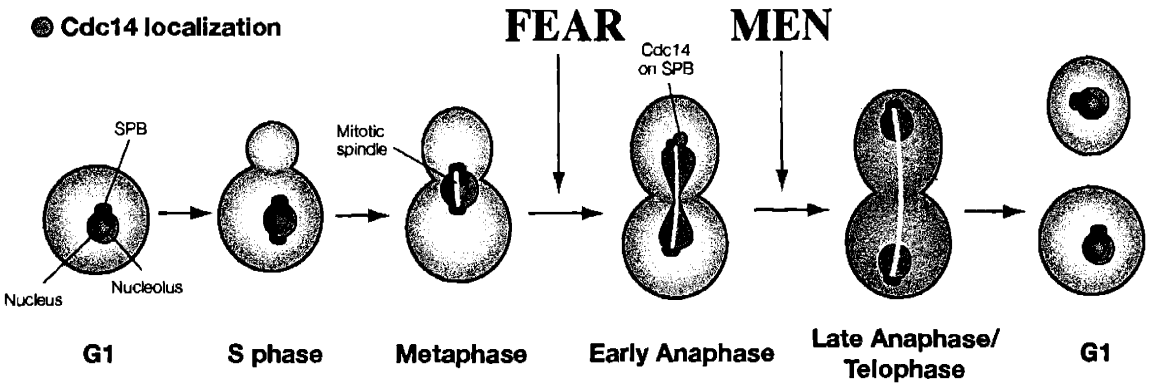

Figure 2: Localization of Cdc14 during the cell cycle.

During G1, S, G2, and metaphase, Cdc14 is sequestered within the nucleolus by its inhibitor Cfi/Net1. Activation of the FEAR network at the metaphase-anaphase transition inititates the release of Cdc14 from its inhibitor and the phosphatase spreads throughout the nucleus and associates with the daughter-bound spindle pole body (SPB). During later stages of anaphase the MEN promotes further release of Cdc14 and maintains the phosphatase in its released state. In late stages of mitosis, Cdc14 is also found in the cytoplasm.

The dissociation of Cdc14 from its inhibitor during anaphase is controlled by at least two signaling networks. At the onset of anaphase, the FEAR (Cdc fourteen early anaphase release) network initiates the release of Cdc14 from its inhibitor and the phosphatase spreads throughout the nucleus and associates with spindle pole bodies (SPBs, the yeast centrosomes) and the mitotic spindle (Figure 2, Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002a; Pereira et al., 2003). During later stages of anaphase the MEN (mitotic exit network) promotes further release of Cdc14 and maintains the phosphatase in its released state. In late stages of mitosis, when Cdc14 release is mediated by the MEN, the phosphatase is also found in the cytoplasm (Shou et al., 1999; Visintin et al., 1999). Whether the qualitative difference in release caused by the FEAR network (primarily nuclear) and by the MEN (nuclear and cytoplasmic) is relevant for Cdc14 regulation is at present unclear. It is however interesting to note that two screens aimed at identifying reduction-of-function mutants that bypass the mitotic exit defect of MEN mutants identified proteins involved in nuclear–cytoplasmic transport, such as the karyopherins Kap104 and Mtr10 (Asakawa et al., 2002; Shou et al., 2002b).

In addition to causing a qualitative difference in Cdc14 release, there are quantitative differences in the extent that the FEAR network and the MEN promote exit from mitosis. The MEN is essential for mitotic exit, as MEN mutants arrest in late anaphase with high mitotic CDK activity (Surana et al., 1993; Shirayama et al., 1994b; Jaspersen et al., 1998). In contrast, the FEAR network is not essential. Cells lacking FEAR network activity delay but do not block exit from mitosis (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002a). Furthermore, in the absence of MEN

signaling Cdc14 is not maintained in its released state, indicating that the FEAR network is active only for a brief time during early anaphase (Shou et al., 1999; Visintin et al., 1999; Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002a). These results imply that Cdc14 activated by the FEAR network does not lower mitotic CDKs enough to trigger exit from mitosis. This finding may be explained by the facts (1) that the FEAR network is not able to direct large amounts of Cdc14 to the cytoplasm where Cdh1 and Swi5 are localized (Nasmyth et al., 1990; Moll et al., 1991; Jaquenoud et al., 2002) and (2) that FEAR network activity is restricted to a brief window during early anaphase.

Despite qualitative and quantitative differences in the FEAR network and MEN-mediated Cdc14 activation, the question arises as to why budding yeast utilizes two pathways to regulate Cdc14 activation rather than one. Employing multiple pathways may allow for more elaborate regulation of exit from mitosis. Another, not mutually exclusive, possibility is that Cdc14 released by the FEAR network performs functions during mitosis that are different from that of Cdc14 released by the MEN. Recent studies underscore this idea. Cdc14 released by the FEAR network plays important roles in regulating chromosome segregation and the localization of chromosomal passenger proteins (see below, Pereira et al., 2003; D'Amours et al., 2004). Perhaps the levels of Cdc14 activity needed to accomplish these tasks are lower than that needed to promote exit from mitosis. Hence, employing a pathway to activate Cdc14 briefly during early anaphase allows the phosphatase to regulate anaphase events, whereas full activation later in the cell cycle allows it to accomplish its mitotic exit promoting function.

The Mitotic Exit Network.

The Mitotic Exit Network was the first signaling network shown to regulate the subcellular localization of Cdc14 (Shou et al., 1999; Visintin et al., 1999). The MEN resembles a Ras-like GTPase signaling cascade (Table 1, Figure 3) and is comprised of the GTPase Tem1, the putative GEF (guanine-nucleotide exchange factor) Lte1, the two-component GAP (GTPase activating protein) Bub2-Bfa1/Byr4 and its associated factor Ibd2, the protein kinases Cdc5, Cdc15, Dbf2, the Dbf2-associated factor Mob1, and a scaffold protein Nud1. A combination of genetic and biochemical investigation support the model for MEN signaling outlined in Figure 3. Tem1's GTPase activity is negatively regulated by the GAP complex Bub2-Bfa1 (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Lee et al., 1999a; Li, 1999; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Krishnan et al., 2000; Pereira et al., 2000; Wang et al., 2000; Geymonat et al., 2002; Ro et al., 2002). Lte1 positively regulates Tem1 (Shirayama et al., 1994a; Shirayama et al., 1996; Bardin et al., 2000) but in the absence of direct biochemical evidence that Lte1 functions as a GEF for Tem1, its molecular function remains controversial (Jensen et al., 2002; Yoshida et al., 2003). The activated form of Tem1, which is likely but not proven to be the GTP bound form, is thought to propagate a signal to the protein kinase Cdc15 (Asakawa et al., 2001; Lee et al., 2001a; Menssen et al., 2001; Visintin et al., 2001; Bardin et al., 2003). Cdc15 in turn activates the protein kinase Dbf2 (Mah et al., 2001), which requires the Dbf2-associated factor Mob1 (Figure 3, Komarnitsky et al., 1998; Luca et al., 1998; Mah et al., 2001).

Cdc5 has long been thought to be a component of the MEN signaling cascade, as cells lacking the polo kinase *CDC5* exhibit a phenotype similar to that of MEN mutants, arresting in late anaphase with Cdc14 sequestered in the nucleolus (Kitada et al., 1993;

Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1999; Lee et al., 2001a). Recent studies, however, suggest that Cdc5 is not a core component of the MEN signaling cascade. Instead, Cdc5 is essential for mitotic exit because it activates the MEN in multiple ways. The Bub2-Bfa1 complex is phosphorylated by Cdc5 (Hu et al., 2001; Lee et al., 2001b; Hu et al., 2002), which inactivates its GAP activity (Hu et al., 2001; Geymonat et al., 2003). Cdc5 may also regulate Lte1 (Lee et al., 2001b). Lastly, Cdc5 activates the Dbf2 kinase in a *BUB2* independent manner (Lee et al., 2001a), at least in part by promoting FEAR network-induced activation of Cdc14 (see below, Jaspersen et al., 2000; Stegmeier et al., 2002; Visintin et al., 2003). Together, these MEN activating functions of Cdc5 are likely to account for the complete loss of MEN signaling in *cdc5* mutants (Visintin et al., 2003).

Table 1: MEN and SIN components and homologues in other eukaryotes

	S.cerevisiae **MEN**	*S.pombe* **SIN**	*C. elegans*	mammals
GTPase	Tem1	Spg1	?	
putative GEF	Lte1	?	?	
GAP	Bub2 Bfa1	Cdc16 Byr4	C33F10.2 ?	GAPCenA
Protein kinase	Cdc15 Dbf2	Cdc7 Sid1 Sid2	? T19A5.2 T20F10.1 / R11G1.4	 WARTS/LATS1
Associated factors	Mob1 (w/Dbf2) Ibd2 (w/Bub2-Bfa1)	Cdc14 (w/Sid1) Mob1 (w/Sid2)	T12B3.4 / F38H4.10 / F09A5.4	mMob1
SPB scaffold	Nud1	Cdc11 Sid4	?	centriolin
Phosphatase	Cdc14	Clp1/Flp1	CeCDC-14	hCdc14A, hCdc14B

Table 2: FEAR network components and potential homologues in other eukaryotes

	S.cerevisiae **FEAR**	*S.pombe*	*C. elegans*	mammals
Separase	Esp1	Cut1	SEP-1	Separase
Polo kinase	Cdc5	Plo1	PLK-1 / PLK-2	Polo-kinase family
Spo12 family	Spo12	Wis3	4D376	?
Kinetochore protein	Slk19	?	?	?
Fork block protein	Fob1	?	?	?

Figure 3

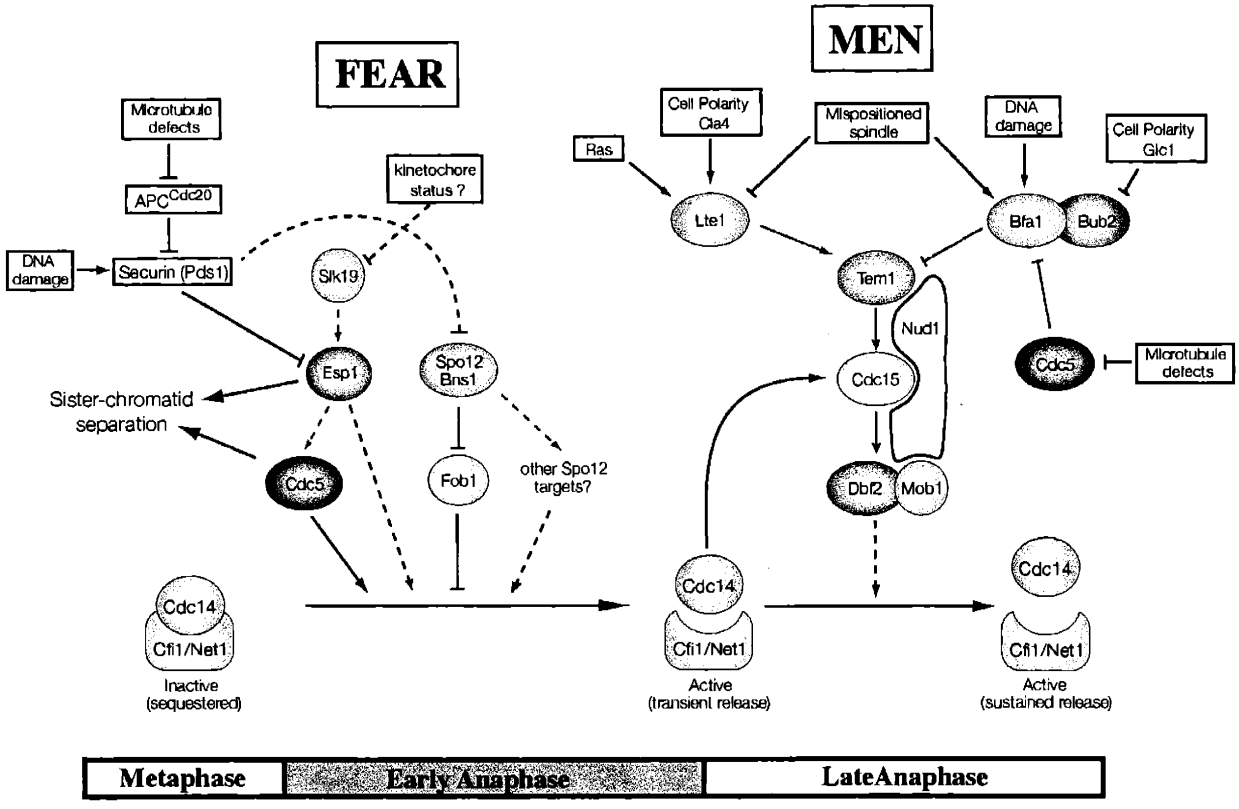

Figure 3: FEAR network and MEN components and signals controlling these pathways.

The release of Cdc14 from its inhibitor Cfi1/Net1 is initiated during early anaphase by the FEAR network. During later stages of anaphase, the MEN promotes and sustains release of the phosphatase. The signals and proteins regulating the activation of these two pathways are shown. Solid arrows indicate reasonably well established interactions; dashed arrows indicate more speculative interactions. For details refer to the main text.

Figure 4

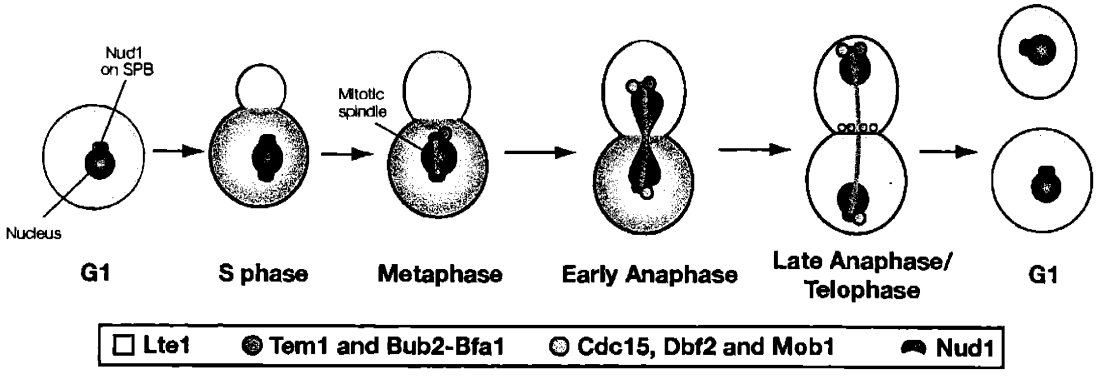

Figure 4: Localization of MEN components during the cell cycle.

Lte1 is present throughout the cell during G1, and localizes to the bud during S phase and mitosis. Nud1 functions as an anchor for MEN components. As the mitotic spindle forms, Tem1 and Bub2-Bfa1 localize to the spindle pole body (SPB) that migrates into the bud. During anaphase, Cdc15, Dbf2 and Mob1 are recruited to both SPBs. During late anaphase, Cdc15, Dbf2 and Mob1 translocate to the site of cytokinesis.

Activation of the MEN is regulated in many additional ways. Cdc15 has a potential autoinhibitory C-terminal domain (Bardin et al., 2003) but its regulation remains elusive. The spatial segregation of the MEN components Lte1 and Tem1 also contributes to the temporal coordination of MEN activation. The MEN activator Lte1 localizes to the bud cortex concomitant with bud formation while Tem1 resides on the daughter-bound SPB (Figure 4, Bardin et al., 2000; Pereira et al., 2000). Therefore, activation of Tem1 by Lte1 only occurs after the Tem1-bearing SPB has migrated into the bud (discussed in detail below, Bardin et al., 2000; Pereira et al., 2000; reviewed in Bardin et al., 2001). The subcellular localization of many other MEN components is also cell cycle regulated. Tem1, Bub2, Bfa1, Ibd2, Cdc5, Cdc15, Dbf2, and Mob1 localize to the cytoplasmic face of the SPB (Cenamor et al., 1999; Bardin et al., 2000; Gruneberg et al., 2000; Pereira et al., 2000; Xu et al., 2000; Visintin et al., 2001; Yoshida et al., 2001; Hwang et al., 2002; Bardin et al., 2003). The SPB component Nud1 functions as a scaffold for the Bub2-Bfa1-Tem1 complex and helps to recruit other MEN components onto the SPBs (Bardin et al., 2000; Gruneberg et al., 2000; Pereira et al., 2000; Visintin et al., 2001). Interestingly, while Cdc15 and Dbf2 localize to both SPBs, Tem1 and the Bub2-Bfa1 complex associate preferentially with the daughter-bound SPB (Figure 4, Cenamor et al., 1999; Bardin et al., 2000; Pereira et al., 2000; Xu et al., 2000; Menssen et al., 2001; Visintin et al., 2001; Bardin et al., 2003; Molk et al., 2004). Interestingly, the future destination (daughter versus mother cell) rather than age of the SPB (old vs. newly synthesized) determines which SPB "recruits" the Tem1-Bub2-Bfa1 complex (Pereira et al., 2001). However, the biological significance of this asymmetric localization, which curiously is conserved in fission yeast, and the molecular mechanisms that establish and/or maintain this asymmetry remain unknown. It is tempting to speculate that the

Tem1-Bub2-Bfa1 complex associates with the SPB that experiences maximal pulling force during anaphase spindle elongation. As the pulling force on the daughter bound SPB is likely to be higher during anaphase than on the SPB in the mother cell, the Tem1-Bub2-Bfa1 complex would localize to the SPB migrating into the bud. This idea is consistent with the finding that Tem1 localization on the daughter SPB is significantly decreased in cells lacking microtubule motor proteins such as *KIP2* or the dynein heavy chain *DHC1* (Molk et al., 2004). This mechanism would also predict the existence of one or multiple force sensitive factors on the SPB.

The FEAR network.

Recent studies found that the release of Cdc14 from its inhibitor during early anaphase occurs in the absence of MEN activation (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002a). Several genes, collectively referred to as the FEAR network, are required for this activation of Cdc14 during early anaphase (Stegmeier et al., 2002). The FEAR network is comprised of the separase Esp1, the polo-like kinase Cdc5, the kinetochore protein Slk19, the small nuclear protein Spo12 and its homologue Bns1, and the replication fork block protein Fob1 (Figure 3, Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002a; Visintin et al., 2003; Stegmeier et al., 2004). Genetic epistasis analyses suggest that the FEAR network consists of at least two branches (Figure 3). *ESP1* and *SLK19* appear to function in parallel to *SPO12* and *BNS1* (Sullivan et al., 2003; Visintin et al., 2003). The placement of Cdc5 within the FEAR network is complicated by the fact that Cdc5 is also an activator of the MEN. It is however likely, though not proven, that Cdc5 is the ultimate effector of the FEAR network, as its

overexpression suppresses the Cdc14 release defect of cells impaired in both the *ESP1* and *SPO12* branch (Visintin et al., 2003).

Little is known about the relationship among FEAR network components. Slk19 is cleaved by Esp1 at the metaphase-anaphase transition (Sullivan et al., 2001) but Slk19 cleavage is not required for its mitotic exit function (Stegmeier et al., 2002; Sullivan et al., 2003). Remarkably, Esp1 promotes the release of Cdc14 independently of its protease function (Buonomo et al., 2003; Sullivan et al., 2003), but the nature of this protease-independent signaling mechanism remains elusive. Rather than Slk19 being regulated by Esp1, it appears that Slk19 functions together with Esp1 to promote Cdc14 release from the nucleolus. Slk19 forms a complex with Esp1 and is required for targeting Esp1 to kinetochores and the spindle midzone (Sullivan et al., 2003). Thus it is possible that Slk19 promotes the protease-independent function of Esp1.

There is also some evidence to suggest that Esp1 and Slk19 regulate Cdc5 during anaphase. Overexpression of *ESP1* lacking its proteolytic activity induces the phosphorylation of Cfi1/Net1 in metaphase-arrested cells (Sullivan et al., 2003). As Cfi1/Net1 phosphorylation is at least in part *CDC5* dependent, it is possible that Esp1 activates Cdc5 towards its anaphase-specific substrates. Importantly, both the protease-dependent and protease-independent functions of Esp1 are inhibited by the securin Pds1 (Sullivan et al., 2003), thus enabling securin to restrain both the onset of sister-chromatid separation and FEAR-mediated activation of Cdc14.

Several studies provided first insights into the mechanism by which Spo12 promotes the release of Cdc14 from its inhibitor. Spo12 was found to physically interact with the replication fork block protein Fob1 (Stegmeier et al., 2004), which localizes to the same rDNA region as Cfi1/Net1 and Cdc14 (Huang et al., 2003; Stegmeier et al.,

2004). *FOB1* is a negative regulator within the FEAR network, functioning downstream of or in parallel to *SPO12* (Stegmeier et al., 2004). But how is the Spo12-Fob1 branch activated? The phosphorylation of two serine residues within the DSP-Box (double SP sites), a highly conserved C-terminal domain within Spo12, is required for its function and phosphorylation of these sites appears to be cell cycle regulated (Shah et al., 2001; Stegmeier et al., 2004). Thus, it is possible that phosphorylation of Spo12 during early anaphase triggers the activation of the Spo12 branch.

Regulation of the Cdc14–Cfi1/Net1 interaction by the FEAR network and the MEN.

Although it is clear that the FEAR network and the MEN promote the release of Cdc14 from its inhibitor during anaphase, the molecular mechanisms by which they break the complex apart are only partially understood. Both Cdc14 and Cfi1/Net1 are phosphorylated during anaphase, raising the possibility that complex dissociation is regulated by this post-translational modification (Shou et al., 1999; Shou et al., 2002a; Yoshida et al., 2002b; Visintin et al., 2003). Consistent with this notion, the phosphorylation of Cfi1/Net1 destabilizes the complex in vitro (Shou et al., 2002a). What kinases phosphoryate Cfi1/Net1 and Cdc14? Cdc5 appears to be at least in part responsible. Cdc5 can phosphorylate both Cdc14 and Cfi1/Net1 in vitro (Shou et al., 2002a; Yoshida et al., 2002b, R.Visintin personal communication). In vivo, the phosphorylation of Cdc14 by Cdc5 does not require the MEN, whereas Cfi1/Net1 phosphorylation is at least in part mediated by the MEN (Shou et al., 2002a; Yoshida et al., 2002b; Visintin et al., 2003). A likely candidate for the MEN-dependent Cfi1/Net1 phosphorylation is Dbf2, the most downstream kinase within the MEN.

How does the Spo12-Fob1 branch of the FEAR network contribute to the release of Cdc14 from its inhibitor? Fob1 binds to Cfi1/Net1 and was proposed to inhibit the dissociation of the Cdc14-Cfi1/Net1 complex prior to anaphase initiation (Huang et al., 2003; Stegmeier et al., 2004). As Spo12 phosphorylation decreases its binding to Fob1, it is possible that phosphorylation of Spo12 during early anaphase triggers a conformational change in the Spo12-Fob1 complex. This allosteric change in turn may help to destabilize the Cdc14-Cfi1/Net1 complex (Stegmeier et al., 2004).

We envision that the following series of events triggers the release of Cdc14 from its inhibitor. It appears that Cdc5, perhaps activated by the Esp1 branch of the FEAR network, promotes the phosphorylation of Cdc14. Together with a Spo12-dependent destabilization of the Cdc14-Cfi1/Net1 complex, this might lead to the transient release of Cdc14 from its inhibitor during early anaphase (Figure1 in ChapterV). Activation of the MEN during later stages of anaphase then might cause Dbf2 to phosphorylate Cfi1/Net1, thereby promoting further conformational changes that sustain the dissociation of this complex. Rigorous testing of such a model requires the identification of the in vivo phosphorylation sites within Cdc14 and Cfi1/Net1.

Inactivation of the MEN and FEAR network.

The inactivation of Cdc14 after mitotic exit has been completed is as important for successful cell division as its activation during anaphase. This is illustrated by the severe growth defects exhibited by cells with unconstrained Cdc14 activity (Visintin et al., 1998; Shou et al., 1999; Visintin et al., 1999). Therefore, it is important that the FEAR network and the MEN are inactivated once mitotic exit has been completed and cells have entered G1.

FEAR network activity appears to be restricted to a very brief time during early anaphase, as Cdc14 becomes re-sequestered into the nucleolus during late anaphase in cells lacking a functional MEN (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002a). How the activation of the FEAR network is restricted to the duration of early anaphase is unknown. Two FEAR network components, Cdc5 and Spo12, are targeted for degradation by APC^{Cdh1} (Charles et al., 1998; Cheng et al., 1998; Shirayama et al., 1998; Shah et al., 2001). This mechanism, although it undoubtedly contributes to inactivation of FEAR network activity after cells have exited from mitosis, cannot account for restricting FEAR network activity to early anaphase. The phosphorylation of two serine residues of Spo12 is required for its FEAR network function. Given that these phosphorylation sites conform to the Cdc14 consensus, it is possible that Cdc14 itself quenches FEAR network activation by dephosphorylating Spo12 (Shah et al., 2001; Stegmeier et al., 2004). This model also predicts that the kinase responsible for phosphorylating Spo12 is inactivated shortly after the onset of anaphase.

The inactivation of the MEN is accomplished in multiple ways. Interestingly, Cdc14 itself plants the seeds for its own demise. Cdc14 dephosphorylates both Bfa1 and Lte1, which presumably restores the GAP activity of Bub2-Bfa1 towards Tem1 (Hu et al., 2001; Pereira et al., 2002; Geymonat et al., 2003) and triggers the release of Lte1 from the bud cortex (Jensen et al., 2002; Seshan et al., 2002), respectively. The cortical release of Lte1 decreases its concentration in the bud, which is believed to be required for efficient Tem1 activation (Bardin et al., 2000; Jensen et al., 2002; Seshan et al., 2002). It is also possible that Lte1dephosphorylation decreases its activity. Furthermore, the sustained release of Cdc14 activates APC/C^{Cdh1}, which induces the degradation of the MEN activator Cdc5. Lastly, Amn1, which is expressed specifically in daughter cells

only after Cdc14 has been activated, is thought to antagonize MEN function at least in part by competing with Cdc15 for binding to Tem1 (Wang et al., 2003).

Signals controlling MEN and FEAR network activity.

Many mitotic processes, such as the onset of sister-chromatid separation, mitotic spindle disassembly, and cytokinesis are irreversible. Therefore, to ensure the successful completion of mitosis, it is essential that mitotic exit is tightly coordinated with other cell cycle events. Recent work has identified some of the cellular signals that control activation of the FEAR network and the MEN, shedding light onto the mechanisms whereby Cdc14 activation and hence the coordination of late mitotic events is accomplished.

Coordination of chromosome segregation and exit from mitosis through the FEAR network.

Faithful chromosome segregation requires that exit from mitosis is temporally coordinated with the partitioning of the genetic material between the progeny cells. The fact that two important regulators of sister-chromatid separation, the separase Esp1 and the polo-kinase Cdc5, also promote the release of Cdc14 as part of the FEAR network, provides a molecular explanation as to how cells ensure that exit from mitosis does not occur prior to the onset of sister-chromatid separation (Figure 3, Stegmeier et al., 2002; Sullivan et al., 2003). Conversely, the fact that FEAR network-induced Cdc14 activation promotes the segregation of telomeres and the rDNA region (described in detail below) ensures that sister-chromatid separation is completed before cells exit from mitosis (D'Amours et al., 2004).

Preventing mitotic exit in response to microtubule and DNA damage through inhibition of the MEN and the FEAR network.

In the event of mitotic spindle or DNA damage, cells need to delay sister-chromatid separation and mitotic exit until the damage has been repaired. Two surveillance mechanisms, known as the spindle-assembly checkpoint and the DNA-damage checkpoint, monitor defects in the attachment of microtubules to kinetochores and DNA damage, respectively (reviewed in Nyberg et al., 2002; Lew et al., 2003). Both checkpoints inhibit the degradation of Pds1, which prevents both sister-chromatid separation and FEAR network activation, and antagonize MEN function (Cohen-Fix et al., 1996; Yamamoto et al., 1996; Alexandru et al., 1999; Wang et al., 2001; Stegmeier et al., 2002; Yoshida et al., 2002a; Agarwal et al., 2003). The spindle-assembly checkpoint components Mad1, Mad2, Mad3, Bub1, and Bub3 are essential to prevent the degradation of Pds1 by inhibiting the APC^{Cdc20} (reviewed in Lew et al., 2003), whereas Bub2, Bfa1 and Ibd2 are necessary to prevent MEN activition in response to spindle damage. The current view is that these three proteins are essential to prevent MEN activation during any cell cycle arrest in mitosis (Hoyt et al., 1991; Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999; Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Krishnan et al., 2000; Pereira et al., 2000; Wang et al., 2000; Lee et al., 2001b; Hwang et al., 2002). It has been proposed that activation of the spindle checkpoint prolongs the inhibitory function of the Bub2-Bfa1 complex (Hu et al., 2002), but direct biochemical evidence (i.e. increased Bub2-Bfa1 GAP activity) is still lacking.

Coupling mitotic exit with spindle position through regulating MEN activity.

Because of its unusual division pattern (budding), *S. cerevisiae* faces the unique challenge of threading the nucleus through the mother-bud neck during chromosome segregation. A surveillance mechanism, known as the spindle position checkpoint ensures that exit from mitosis does not occur until the nucleus has moved into the bud, the future daughter cell. But how do cells "know" whether part of the nucleus has moved into the bud during anaphase? One mechanism relies on the spatial segregation of the MEN components Lte1 and Tem1. The MEN activator Lte1 localizes to the bud cortex during bud formation while Tem1 resides on the daughter-bound SPB (Figure 4, Bardin et al., 2000; Pereira et al., 2000). Thus, delivery of the Tem1 bearing SPB into the bud is likely to promote Tem1 activation during anaphase (Bardin et al., 2000; Pereira et al., 2000; reviewed in Bardin et al., 2001). Indeed, when maintenance of Lte1 in the bud is disrupted by the inactivation of septins, which form a diffusion barrier for membrane-associated proteins (Barral et al., 2000; Hofken et al., 2002; Seshan et al., 2002), cells with a mis-positioned nucleus exit from mitosis (Castillon et al., 2003). The Bub2-Bfa1 complex is also required to restrain mitotic exit in cells in which the nucleus and hence the mitotic spindle is not correctly positioned along the mother–bud axis (Bardin et al., 2000; Bloecher et al., 2000; Daum et al., 2000; Pereira et al., 2000; Wang et al., 2000; Adames et al., 2001). Interestingly, the phosphorylation of Bfa1 and its asymmetric localization to the daughter-bound SPB appear to be regulated in response to spindle orientation (Hu et al., 2001; Pereira et al., 2001). It is possible that the interaction of cytoplasmic microtubules with the bud neck controls the activity of the Bub2-Bfa1

complex (Adames et al., 2001; Pereira et al., 2001; Castillon et al., 2003), but the molecular nature of such a signaling mechanism remains elusive.

Regulation of mitotic exit by cell polarity determinants.

Several recent studies found that Lte1 and the Bub2-Bfa1 complex are also regulated by cell polarity proteins, including the Rho-like GTPase Cdc42 and its effectors Cla4, Ste20 (both PAK-like kinase), Gic1, and Gic2. (Hofken et al., 2002; Jensen et al., 2002; Seshan et al., 2002; Hofken et al., 2004). Cdc42 appears to promote MEN activation through three independent effector branches: (1) Cdc42-dependent activation of the PAK-like kinase Cla4 is both required and sufficient for phosphorylation and thus targeting of Lte1 to the bud cortex (Hofken et al., 2002; Seshan et al., 2002). Importantly, cells lacking *CDC42* or *CLA4* delay in exit from mitosis, supporting the notion that bud enrichment of Lte1 is important for the timely completion of mitotic exit (Bardin et al., 2000; Hofken et al., 2002; Seshan et al., 2002). However, it is also possible that phosphoryaltion of Lte1 is required for its activation. (2) Activation of Gic1 and Gic2 by Cdc42 is thought to promote mitotic exit by interfering with Bub2-Bfa1 GAP protein function (Hofken et al., 2004). (3) The PAK-like kinase Ste20 is also activated by Cdc42 and functions in a pathway parallel to *LTE1*, but its molecular target remains elusive (Hofken et al., 2002). Given that these cell polarity proteins help to establish Lte1's asymmetric bud localization (in the case of Cla4), and are thought to antagonize Bub2-Bfa1 function only after the mitotic spindle has been correctly positioned (in the case of Gic1), it is likely that these pathways contribute to the proper function of the spindle position checkpoint.

Regulation of mitotic exit by nutrient signaling

The effects of nutrient signaling, which is likely to be mediated at least in part by the Ras-cAMP pathway, on entry into the cell cycle are well established (Baroni et al., 1994; Tokiwa et al., 1994). Interestingly, the Ras pathway has also been implicated in controlling exit from mitosis. When the essential function of the Ras pathway in cAMP production is bypassed, cells lacking both *RAS1* and *RAS2* are impaired in mitotic exit at elevated or lower temperatures (Morishita et al., 1995). A recent study found that active Ras is required for the proper localization of Lte1 to the bud cortex (Yoshida et al., 2003), which is likely to account for the mitotic exit defect of *ras1Δ ras2Δ* mutant cells at lower temperatures. However, additional Ras effectors must regulate exit from mitosis, as *LTE1* is dispensable for mitotic exit at elevated temperatures (Adames et al., 2001). It will be interesting to determine whether Ras signaling affects the localization or the activity of the Bub2-Bfa1 complex.

The multiple roles of Cdc14 during late stages of mitosis.

The intricate regulation of Ccd14's activity is necessary because it is a key regulator of late mitotic events. This phosphatase controls these processes by reversing CDK phosphorylation events that previously promoted progression through mitosis. Recently, it became clear that Cdc14 not only promotes mitotic CDK inactivation and exit from mitosis but a variety of other cellular events such as rDNA and telomere segregation, mitotic spindle dynamics and cytokinesis. Remarkably, the execution of these diverse events relies on Cdc14 activated by different regulatory networks.

Figure 5

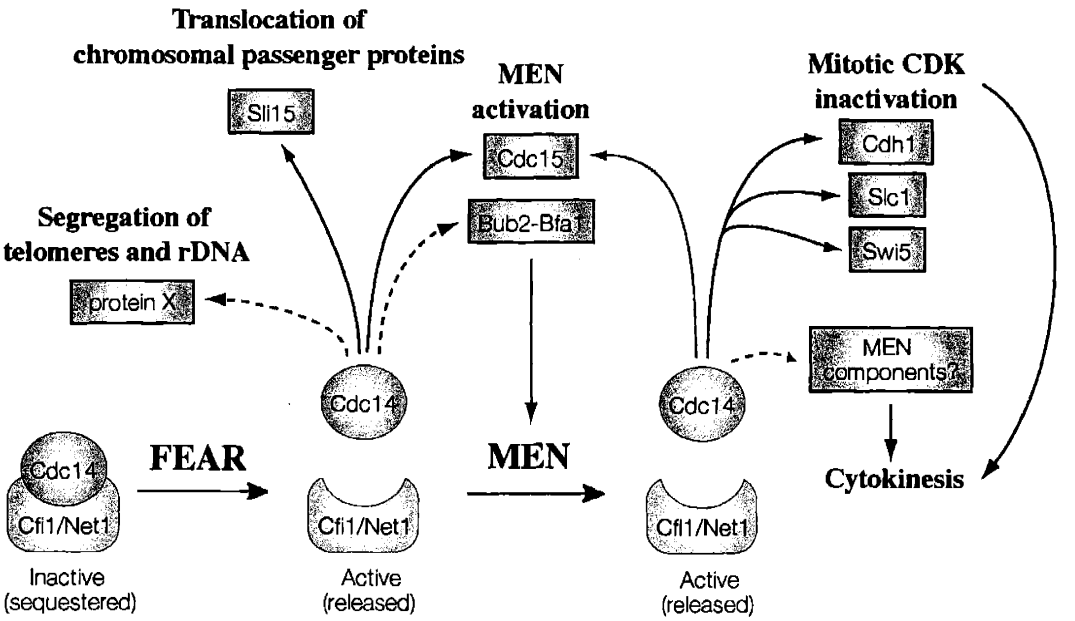

Figure 5: Substrates and functions of Cdc14 during mitosis.

Cdc14 released by the FEAR network and the MEN regulates many mitotic processes. The FEAR network-activated Cdc14 promotes the segregation of telomeres and rDNA, the translocation of chromosomal passenger proteins, and activation of the MEN. Cdc14 activated by the MEN promotes the inactivation of mitotic CDKs and cytokinesis. The known targets of Cdc14 in the regulation of these processes are shown in the grey boxes. Solid arrows indicate reasonably well established Cdc14 targets; dashed arrows indicate more speculative ones. For details refer to the main text.

Functions of Cdc14 released by the FEAR network.

Although Cdc14 activated by the FEAR network is not essential for cell proliferation, it helps to coordinate several mitotic events (Figure 5), thereby contributing to the maintenance of genomic stability. The importance of the FEAR network is illustrated by the dramatic loss of viability that cells experience when progressing through anaphase in the absence of FEAR network function (D'Amours et al., 2004), which may at least in part be due to chromosome loss (Hartwell et al., 1985).

Two recent studies found that FEAR network-induced Cdc14 activation promotes the completion of chromosome segregation (Figure 5). Cells lacking *CDC14* or FEAR network activity are impaired in the segregation of telomeres and the rDNA array (Granot et al., 1991; Buonomo et al., 2003; D'Amours et al., 2004; Sullivan et al., 2004). Given that cells lacking MEN activity do not exhibit such defects, it appears that the transient activation of Cdc14 by the FEAR network is sufficient for the separation of these late-segregating chromosomal regions. Several observations point to the possibility that Cdc14 mediates rDNA segregation by promoting the enrichment of condensins, which are protein complexes required for chromosome condensation (reviewed in Hirano, 2000; Hagstrom et al., 2003), at the rDNA locus. Cells carrying mutations in subunits of the condensin complex exhibit nucleolar segregation defects similar to *cdc14* mutants (Freeman et al., 2000; Lavoie et al., 2000; Ouspenski et al., 2000; Bhalla et al., 2002). Moreover, Cdc14 is required for rDNA condensation (Guacci et al., 1994) and the targeting of condensins to the rDNA region during anaphase (D'Amours et al., 2004). The observation that Cdc14 also induces the sumoylation of the condensin subunit Ycs4 during anaphase raises the interesting possibility that Cdc14 targets condensins to the

rDNA by promoting Ycs4 sumoylation. How Cdc14-dependent condensation facilitates individualization of these genomic regions remains unclear.

Another function of Cdc14 released by the FEAR network is to regulate the subcellular localization of chromosomal passenger proteins, which presumably contributes to the stabilization of the mitotic spindle during early anaphase (Figure 5, Pereira et al., 2003). Chromosomal passenger proteins are characterized by their change in subcellular localization during anaphase. During metaphase they reside at kinetochores but translocate to the spindle midzone during anaphase (reviewed in Carmena et al., 2003). A recent study found that the translocation of the chromosomal passenger proteins Ipl1 (Aurora B kinase) and Sli15 (INCENP) to the mitotic spindle and spindle midzone is promoted by FEAR network-induced activation of Cdc14 (Pereira et al., 2003). Cdc14 dephosphorylates Sli15, thereby inducing the change in Ipl1-Sli15 localization. This change in localization appears to be important for anaphase spindle stabilization. A *SLI15* mutant that mimics its dephosphorylated form partially rescues the premature spindle breakage observed in *esp1* mutants, possibly by recruiting other spindle stabilizing proteins, such as Slk19, to the spindle midzone (Pereira et al., 2003). However, another study reached the opposite conclusion that Ipl1 promotes mitotic spindle disassembly (Buvelot et al., 2003). One possible explanation for these contradictory observations is that the Ipl1 complex first stabilizes the mitotic spindle during early anaphase but contribute to its disassembly during telophase.

Lastly, FEAR network-released Cdc14 stimulates MEN activity. It does so in at least two ways (Figure 5). First, Cdc14 released by the FEAR network promotes the dephosphorylation of Cdc15, thus enhancing Cdc15's mitotic exit function (Jaspersen et al., 2000; Stegmeier et al., 2002). The mechanism by which dephosphorylation of Cdc15

promotes its mitotic exit function remains unknown. Dephosphorylation of Cdc15 may enhance its interaction with Tem1, its localization to SPBs, or antagonize the inhibitory function of Cdc15's C-terminal domain. Secondly, Cdc14 has been proposed to inactivate the Bub2-Bfa1 complex in a phosphatase-independent manner during early anaphase (Pereira et al., 2002). Cdc14 binds to Bub2 at the daughter SPB shortly after its release from the nucleolus during early anaphase (Pereira et al., 2002; Yoshida et al., 2002a). While the association between the Bub2-Bfa1 complex and Cdc14 has been proposed to inactivate its GAP activity during early anaphase, Bfa1's dephosphorylation by Cdc14 during later stages of mitosis is thought to re-activate the GAP complex (Pereira et al., 2002; Geymonat et al., 2003). Validation of this model will require the elucidation of Cdc14's phosphatase-independent inhibitory function on Bub2-Bfa1 and the identification of the mechanism(s) that prevent Cdc14 from dephosphorylating Bfa1 during early anaphase.

It is likely that Cdc14 released by the FEAR network regulates many other aspects of anaphase chromosome movement. What these processes may have in common is that their regulatory proteins require dephosphorylation by Cdc14 despite the presence of high mitotic CDK activity throughout the cells. Therefore, regions within the cell that contain a high local concentration of Cdc14 during early anaphase, which includes kinetochores, the mitotic spindle, and SPBs, are most likely to harbor yet unidentified targets of Cdc14 released by the FEAR-network.

Functions of Cdc14 released by the MEN.

Cdc14 activated by the MEN is mainly responsible for promoting exit from mitosis, as this transition does not occur in the absence of MEN function (Hartwell et al.,

1970; Johnston et al., 1990; Surana et al., 1993; Shirayama et al., 1994b; Jaspersen et al., 1998). Several observations, however, indicate that Cdc14 and the MEN also regulate cytokinesis, independently of their mitotic exit function. When the need for MEN function in mitotic exit is bypassed, either by weakening the Cdc14–Cfi1/Net1 interaction or by overexpression of the CDK inhibitor *SIC1*, severe cytokinesis defects become apparent (Jimenez et al., 1998; Shou et al., 1999; Lippincott et al., 2001; Luca et al., 2001; Song et al., 2001). The notion that the MEN regulates cytokinesis is also consistent with the translocation of the MEN components Cdc15, Dbf2, Mob1 and Cdc5, to the bud neck (the site of actomyosin ring constriction in budding yeast) during late anaphase and telophase (Figure 4, Frenz et al., 2000; Song et al., 2000; Xu et al., 2000; Luca et al., 2001; Song et al., 2001; Yoshida et al., 2001). Furthermore, the cytokinesis function of at least some MEN components appears to require Cdc14 function, as the accumulation of Dbf2 and Mob1 at the bud neck depends on *CDC14* (Frenz et al., 2000; Yoshida et al., 2001). We speculate that after the MEN promoted Cdc14 activation, Cdc14 itself may dephosphorylate CDK consensus sites in MEN components to promote their localization to the bud neck, where they then regulate cytokinesis (Figure 5). However, the critical Cdc14 targets within the MEN in the regulation of cytokinesis remain to be identified.

Functions of Cdc14, the FEAR network and the MEN during meiosis.

Meiosis is a specialized cell cycle during which a single round of DNA replication is followed by two chromosome segregation phases (reviewed in Nasmyth, 2001). Owing to this special segregation program, the transition from meiosis I to meiosis II has specific requirements. CDK activity needs to be lowered sufficiently to allow for the disassembly of the meiosis I spindle. On the other hand, complete CDK inactivation,

which would allow for the assembly of pre-replicative complexes (preRCs) onto the DNA, needs to be avoided to prevent an intervening S phase (Iwabuchi et al., 2000). Two studies raise the interesting possibility that meiotic cells solve this problem by employing the FEAR network rather than the MEN to promote exit from meiosis I. Cells impaired in FEAR network-mediated Cdc14 activation are severely delayed in meiosis I spindle disassembly. Given that Cdc14 activity released by the FEAR network is short lived, employing the FEAR network for Cdc14 activation may lower CDK activity sufficiently for meiosis I spindle disassembly to occur but not low enough to allow assembly of preRCs.

The requirement for Cdc14 and the FEAR network in exit from meiosis I is similar to its mitotic function, but another phenotype exhibited by *CDC14* and FEAR network mutants is unique to meiosis. Instead of arresting in anaphase I with homologous chromosomes segregated, Cdc14 and FEAR network mutants exhibit a "mixed" chromosome segregation pattern, which is characterized by some chromosomes segregating in a meiosis I-like pattern and others segregating in a meiosis II-like pattern (Klapholz et al., 1980; Sharon et al., 1990; Kamieniecki et al., 2000; Zeng et al., 2000; Buonomo et al., 2003; Marston et al., 2003). This unusual chromosome segregation pattern appears to result from meiotic events being uncoupled (Buonomo et al., 2003; Marston et al., 2003). Despite cells arresting in anaphase I, meiosis II chromosome segregation events continue to occur, leading to some chromosomes undergoing both meiotic divisions on the same anaphase I spindle. These findings suggest that Cdc14 and the FEAR network ensure that the two meiotic divisions occur on two sequentially built spindles. Presumably by antagonizing meiotic CDK activity, Cdc14 promotes not only

meiosis I spindle disassembly, but also creates conditions that preclude the onset of the second chromosome segregation phase.

The functions and regulation of Cdc14's *S. pombe* homologue Clp1/Flp1.

Like its cousin in *S. cerevisiae*, Clp1/Flp1 antagonizes mitotic CDK activity. Furthermore, the activity of Clp1/Flp1 is at least in part regulated by the septation-initiation network (SIN, Table 1), a signaling pathway homologous to the MEN that is essential for cytokinesis in fission yeast (reviewed in detail in Bardin et al., 2001; McCollum et al., 2001; Simanis, 2003). Despite these similarities, the mechanisms whereby Clp1/Flp1 and Cdc14 accomplish mitotic CDK inactivation and the cellular processes regulated by these two phosphatases appear to be quite different. In contrast to Cdc14, Clp1/Flp1 is not essential for mitotic exit but predominantly antagonizes mitotic CDK activity during G2. In the absence of *clp1+/flp1+* cells enter mitosis prematurely (reviewed in Bardin et al., 2001; Cueille et al., 2001; McCollum et al., 2001; Trautmann et al., 2001; Simanis, 2003). The importance of Clp1/Flp1 in maintaining low mitotic CDK activity during G2 is further revealed by its role in the cytokinesis checkpoint. Activation of the cytokinesis checkpoint in fission yeast leads to cell cycle arrest in G2. This cell cycle arrest requires the continuous inhibition of mitotic CDKs, which is at least in part mediated by Clp1/Flp1 (Cueille et al., 2001; Trautmann et al., 2001).

Although Clp1/Flp1's main function appears to be the regulation of mitotic CDK activity during G2, Clp1/Flp1 also regulates late mitotic events. Cells lacking *clp1+/flp1+* exhibit defects in cytokinesis and mitotic exit in response to spindle damage (Cueille et al., 2001; Trautmann et al., 2001). Mitotic CDKs inhibit cytokinesis by antagonizing the localization of the SIN components Sid1 to the SPB (Guertin et al.,

2000). Therefore, it is likely that Clp1/Flp1 regulates cytokinesis by promoting SIN activation. A recent study found that Clp1/Flp1 also regulates mitotic exit in response to spindle damage, indicating that at least under some circumstances Clp1/Flp1 is required for mitotic CDK inactivation during mitosis (Guertin et al., 2002). Mutations leading to the abrogation of the spindle assembly checkpoint such as deletion of *dma1+* lead to exit from mitosis in the presence of spindle damage. The CDK inactivation accompanying this escape from the arrest requires *clp1+/flp1+* (Guertin et al., 2002).

The observation that Clp1/Flp1 localizes to the nucleolus during some cell cycle stages and is released during others raises the possibility that some aspects of Cdc14 and Clp1/Flp1 regulation are shared between the two yeasts. During G1 and S phase, Clp1/Flp1 localizes predominantly to the nucleolus, but in contrast to its budding yeast counterpart a small portion resides at SPBs (Cueille et al., 2001; Trautmann et al., 2001). Furthermore, while Cdc14 is released from the nucleolus during anaphase, Clp1/Flp1 is released from the nucleolus already during prophase and localizes to the mitotic spindle, the SPBs, and the medial ring (Cueille et al., 2001; Trautmann et al., 2001). Whether Clp1/Flp1 is bound to an inhibitor when it resides in the nucleolus is at present unclear. However, a regulatory network homologous to the MEN termed the septation-initiation network (SIN, Table 1), appears to control Clp1/Flp1 localization in a manner similar to the MEN (reviewed in Bardin et al., 2001; Cueille et al., 2001; McCollum et al., 2001; Trautmann et al., 2001; Simanis, 2003). Although the SIN does not regulate Clp1/Flp1 localization during an unperturbed cell cycle, this signaling network is required to maintain Clp1/Flp1 in its released state when the cytokinesis checkpoint has been activated (Cueille et al., 2001; Trautmann et al., 2001). Thus, maintenance of Cdc14 and Clp1/Flp1 in its released state relies on conserved pathways. Whether a pathway similar

to the FEAR network regulates the release of Clp1/Flp1 during early stages of mitosis in *S. pombe* is unclear. However, initial observations indicate that this might not be the case. *cut1* mutants (*cut1+* encodes the *ESP1* homologue in *S.pombe*) are not impaired in the release of Clp1/Flp1 from the nucleolus (D. McCollum, personal communication). Furthermore, overexpression of the fission yeast polo-like kinase *plo1+* does not promote the release of Clp1/Flp1 from the nucleolus (D. McCollum, personal communication). Identifying the regulatory mechanisms that promote Clp1/Flp1 release from the nucleolus during early mitosis will be an important task for future studies, as this might provide hints at the mechanisms controlling the localization of metazoan homologues, which are also released from the nucleolus during prophase (see below).

The functions of Cdc14 homologues and their regulation in metazoans.

Homologues of Cdc14 exist in most if not all eukaryotes and have been characterized at least to some extent in *C. elegans* and mammals. In *C. elegans,* depletion of CeCDC14 by RNAi causes cytokinesis defects, most likely due to a failure to form an intact central spindle (Gruneberg et al., 2002). Other aspects of embryonic cell cycle progression appear normal. How CeCDC14 promotes central spindle formation is not known, but it is noteworthy that CeCDC14 is required for the localization of ZEN-4, a kinesin-related motor protein that is also required for central spindle formation, to the mitotic spindle (Gruneberg et al., 2002). Consistent with a role in cytokinesis and central spindle function, CeCDC14 localizes to the central spindle and the midbody, similar to *S.pombe* Clp1/Flp1, but the phosphatase has neither been detected in the nucleolus nor on centrosomes (Gruneberg et al., 2002). Whether CeCdc14 is regulated by signaling pathways homologous to the MEN and SIN homologs is also unclear. A recent study has

explored the function of several potential MEN and SIN homologues in *C.elegans*, using RNA-mediated interference (Gruneberg et al., 2002). Surprisingly, depletion of Dbf2, Mob1, and Sid1 homologues by RNAi did not lead to embryonic lethality (Gruneberg et al., 2002). It is possible that the RNAi-mediated depletion was incomplete or that redundant factors exist in *C. elegans*. Alternatively, the MEN/SIN components may not be necessary during embryonic divisions or these pathways evolved to fulfill functions specific to yeast. More unbiased siRNA screens will be required to identify the regulatory pathways controlling CeCdc14 activity.

The human genome encodes two Cdc14 homologues, hCdc14A and hCdc14B. The roles of these two phosphatases are poorly understood, but an involvement in mitotic exit and cytokinesis is possible. In vitro studies have shown that hCdc14A, just like its yeast counterparts, has a clear preference for substrates of proline-directed kinases (Visintin et al., 1998; Trautmann et al., 2001; Kaiser et al., 2002), which is further supported by the crystal structure of the core domain of hCdc14B (Gray et al., 2003). Furthermore hCdc14A can dephosphorylate Cdh1 and reconstitute active APC^{Cdh1} in vitro (Bembenek et al., 2001). Although this finding indicates that hCdc14A can in principle bring about mitotic CDK inactivation, this Cdc14 isoform appears to primarily regulate centrosome function in vivo. Overexpression of hCdc14A leads to premature centriole splitting in S-phase and formation of an excessive number of aberrant mitotic spindles (Kaiser et al., 2002; Mailand et al., 2002). Conversely, depletion of hCDC14A by RNAi leads to centrosome duplication, mitotic and cytokinesis defects (Mailand et al., 2002). The latter defects may, however, be an indirect consequence of the centrosome malfunction. Consistent with its proposed role in regulating centrosome function, hCdc14A localizes to this organelle (Bembenek et al., 2001; Kaiser et al., 2002; Mailand

et al., 2002). Much less is known about the functions of hCdc14B. Intriguingly, however, hCdc14B localizes to the nucleolus during interphase but not during mitosis (Bembenek et al., 2001; Kaiser et al., 2002). The dramatic differences in the localization of hCdc14A and B suggests that these isoforms perform different functions in the cell.

Potential homologues of MEN/SIN pathway components have been identified in mammals (Table 1) but their cellular functions remain largely unexplored. Interestingly, some of these putative homologues localize to centrosomes, such as GAPCenA, WARTS/LATS1 and centriolin (Cuif et al., 1999; Nishiyama et al., 1999; Gromley et al., 2003). Furthemore, the centrosome has been implicated in the regulation of cytokinesis. Ablation of centrosomes leads to defects in cytokinesis (Khodjakov et al., 2001; Piel et al., 2001). These studies raise the possibility that the centrosome, like the SPBs in budding and fission yeasts, anchors signaling networks needed for the completion of the cell cycle. In support of this hypothesis, the Nud1 homolog centriolin is important for the final stages of cytokinesis (Gromley et al., 2003).

Metazoan genomes also harbor homologues of the FEAR network components Cdc5, Esp1 and Spo12 (Table 2). Several studies suggest that polo kinases are required to control mitotic exit and cytokinesis in most if not all organisms (Descombes et al., 1998; Lee et al., 1998; Lee et al., 1999b; Moutinho-Santos et al., 1999; Hudson et al., 2001), but whether polo kinases regulate these processes through activation of Cdc14 in mammals remains unknown. Separase may also be needed for cell-cycle steps after cohesin removal in at least some metazoans. Reduction of separase function by RNAi in *C.elegans* not only prevents sister-chromatid separation but also appears to slow down subsequent cell-cycle events (Siomos et al., 2001). Clearly, further studies will be needed

to elucidate the role of homologues of Cdc14, the MEN, and the FEAR network during cell cycle progression in mammalian cells.

Conclusions and perspectives

There has been significant progress in the past few years elucidating the mechanisms that control late mitotic events in budding yeast. The Cdc14 phosphatase takes center during late mitosis and promotes several mitotic processes by reversing mitotic CDK phosphorylation events. Besides its essential role in exit from mitosis, this phosphatase also regulates sister-chromatid separation, mitotic spindle dynamics, and cytokinesis. Given its important role in coordinating several mitotic events, it is not surprising that the activation of Cdc14 is intricately controlled by at least two signaling networks, the FEAR network and the MEN, which integrate different cellular signals into the decision whether or not to activate Cdc14.

While the ability of Cdc14 homologues to antagonize mitotic CDK activity is likely to be conserved in all eukaryotes, the cellular functions of Cdc14 homologues appears to differ significantly between species. For example, the absolute requirement for Cdc14 in mitotic exit appears unique to budding yeast. We speculate that the reason for this might be founded in the different physiology of budding yeast. In contrast to most other eukaryotes, a significant pool of mitotic CDK activity persists until late anaphase in *S. cerevisiae* (Surana et al., 1993) Because of its unusual division pattern (budding), this yeast might have evolved a delay in mitotic CDK inactivation and re-engineered Cdc14's regulatory circuits to ensure that the spindle has been correctly positioned between the mother cell and the bud axis before committing to exit from mitosis. Clearly, this safeguard mechanism becomes unnecessary in cells dividing by fission.

Another conserved feature of Cdc14 homologues in all species appears to be the regulation of several mitotic events. In budding and fission yeast, for example, where the phosphatase has been most extensively studied, Cdc14 regulates chromosome segregation, spindle dynamics, cytokinesis and mitotic exit. The complex phenotype of human cells lacking or overexpressing hCdc14A and hCdc14B homologues is consistent with Cdc14 regulating many mitotic processes. Thus, we speculate that all Cdc14 homologues coordinate multiple mitotic processes by reversing CDK phosphorylation events but that each organism re-engineered its regulatory circuits according to its particular needs.

A specific example of conserved Cdc14 function is the regulation of cytokinesis in all organism studied to date (Frenz et al., 2000; Cueille et al., 2001; Trautmann et al., 2001; Yoshida et al., 2001; Gruneberg et al., 2002; Mailand et al., 2002). Could other mitotic functions of Cdc14 also be conserved across species? It is possible that Cdc14 regulates the subcellular localization of chromosomal passenger proteins in mammals. INCENP (Sli15 in yeast) is phosphorylated prior to mitosis (Bolton et al., 2002) and the translocation of the chromosomal passenger proteins aurora B (Ipl1 in yeast) and TD-60 (not conserved in yeast) from kinetochores to the spindle midzone is prevented by high levels of mitotic CDK activity (Wheatley et al., 1997; Murata-Hori et al., 2002). These findings suggest that protein dephosphorylation is necessary for this translocation to occur. However, whether the Cdc14 phosphatase regulates this process remains to be determined.

In budding and fission yeast, great strides have been made in dissecting the role of the Cdc14 phosphatase in several mitotic processes and deciphering its complex regulation by the FEAR network and the MEN/SIN. In contrast, our molecular

understanding of Cdc14 function and the regulation of late mitotic events in metazoans is still rather limited. Systematic RNAi screens in a variety of systems are likely to lead to the identification and characterization of the pathways regulating Cdc14 activity in these organisms. In addition, the identification of relevant physiological substrates of Cdc14 in diverse species will help to unearth further mitotic processes controlled by this conserved phosphatase.

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Chapter II

The FEAR network controls the localization of Cdc14 during early anaphase.

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Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase.

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Dr. Rosella Visintin contributed to work in this chapter.

Summary

In budding yeast, the phosphatase Cdc14, a key regulator of exit from mitosis, is released from its inhibitor Cfi1/Net1 in the nucleolus during anaphase. A signaling cascade known as the mitotic exit network (MEN) controls this release. We have identified a new regulatory network, the FEAR (Cdc fourteen early anaphase release) network that promotes Cdc14 release from the nucleolus during early anaphase. The FEAR network is comprised of the polo kinase Cdc5, the separase Esp1, the kinetochore-associated protein Slk19 and Spo12. We also show that the FEAR network initiates Cdc14 release from Cfi1/Net1 during early anaphase, and MEN maintains Cdc14 in the released state during late anaphase. We propose that one function of Cdc14 released by the FEAR network is to stimulate MEN activity.

Introduction

Successful cell division requires that each daughter cell receives only one copy of each chromosome. For this to occur, cells must duplicate each chromosome and segregate the duplicated genetic material equally between the two progeny cells during mitosis. In budding yeast, the onset of chromosome segregation at the metaphase- anaphase transition is controlled by the ubiquitin-protein ligase APC/C (Anaphase Promoting Complex/ Cyclosome) complexed with its activator Cdc20 (reviewed in Zachariae and Nasmyth, 1999). The APC/C^{Cdc20} initiates anaphase by triggering the ubiquitin-dependent proteolysis of the anaphase inhibitor Pds1 (also known as securin). Destruction of Pds1 liberates the protease Esp1 (also known as separase), allowing it to cleave Scc1/Mcd1, a component of a protein complex called cohesin that holds sister-chromatids together. Cleavage of Scc1/Mcd1 then leads to the onset of chromosome segregation (reviewed in Nasmyth et al., 2000; Uhlmann, 2001). After the completion of chromosome segregation, mitotic Clb cyclin-dependent kinases are inactivated and cells exit from mitosis (CDKs; reviewed in Morgan, 1999; McCollum and Gould, 2000; Bardin and Amon, 2001). The protein phosphatase Cdc14 plays an essential role in promoting Clb-CDK inactivation. Cdc14 reverses CDK phosphorylation, thereby triggering Clb cyclin degradation and accumulation of the Clb-CDK inhibitor Sic1.

During G1, S, G2, and early M phase, Cdc14 is held in the nucleolus by its inhibitor Cfi1/Net1. Cdc14 is released from its inhibitor during anaphase, allowing it to dephosphorylate its substrates (Shou et al., 1999; Visintin et al., 1999; Traverso et al., 2001). A signal transduction pathway known as the mitotic exit network (MEN) controls

the localization of Cdc14 (Shou et al., 1999; Visintin et al., 1999). Genetic evidence suggest that the GTPase Tem1 functions at or near the top of MEN and is regulated both by the GEF Lte1 and the two-component GAP complex Bub2–Bfa1. Activation of Tem1 is thought to lead to the propagation of a signal to the protein kinase Cdc15, which is then thought to activate the protein kinase Dbf2 in a manner-dependent on the Dbf2-associated factor Mob1. The polo kinase Cdc5 regulates mitotic exit in multiple ways (reviewed in Bardin and Amon 2001) and has recently been shown to activate MEN by phosphorylating Bfa1, thereby inactivating Tem1's GAP (Hu et al., 2001). How MEN promotes the dissociation of Cdc14 from Cfi1/Net1 is not known.

Yeast cells initiate anaphase before exiting from mitosis and control mechanisms exist that determine the order of these events. The regulators of sister-chromatid separation Pds1 and Esp1 are part of such a regulatory circuit (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). Overexpression of a non-degradable version of Pds1, in addition to preventing the onset of sister-chromatid separation, also delays the inactivation of mitotic CDKs. Pds1 regulates exit from mitosis by at least two mechanisms: one is dependent on *ESP1* and one is *ESP1*-independent. Esp1 has also been shown to regulate exit from mitosis. High levels of Esp1 can induce mitotic CDK inactivation in cell-cycle stages other than telophase. Conversely, cells carrying a temperature sensitive *esp1-1* allele are delayed in mitotic CDK inactivation (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). How Esp1 controls exit from mitosis is not known.

Here we show that Esp1 together with the polo kinase Cdc5, the kinetochore protein Slk19, and Spo12, a protein previously implicated in controlling exit from mitosis (Parkes and Johnston, 1992; Jaspersen et al., 1998), function in a regulatory network, termed the

FEAR (Cdc fourteen early anaphase release) network to induce release of Cdc14 from the nucleolus during early anaphase. We find that the FEAR network and MEN function independently of each other to promote Cdc14 release from the nucleolus. The FEAR network initiates Cdc14 release from the nucleolus during early anaphase, and MEN maintains Cdc14 in the released state during late anaphase and telophase. We also find that Cdc14 released by the FEAR network is capable of dephosphorylating at least one of its substrates and propose that one role of Cdc14 released by the FEAR network is to stimulate MEN activity.

Results

Cdc14 is transiently released from the nucleolus during early anaphase in MEN mutants.

In wild-type cells, Cdc14 is released from the nucleolus during early anaphase and remains in this released state until mitotic spindles are disassembled (Shou et al., 1999; Visintin et al., 1999; Figure 1A). Mutants defective in components of the mitotic exit network arrest in telophase with Cdc14 tightly sequestered in the nucleolus (Shou et al., 1999; Visintin et al., 1999). However, when we examined the localization of Cdc14 in MEN mutants progressing through the cell cycle in a synchronous manner we noticed that Cdc14 was transiently released from the nucleolus during early anaphase (Figure 1). In *cdc15-2*, *dbf2-2* or *tem1-3* mutants Cdc14 was diffuse throughout the nucleus in a small fraction (10 – 15 percent) of cells coinciding with cultures entering anaphase (Figure 1B, C, left column, Figure 1F, data not shown). At later time points, as MEN mutants reached their terminal arrest point (with fully elongated mitotic spindles and well separated DNA masses) Cdc14 was, as previously reported, tightly sequestered in the nucleolus (Figure 1B, C, 1F; data not shown; Shou et al., 1999; Visintin et al., 1999).

The release of Cdc14 from the nucleolus in *cdc15-2*, *dbf2-2*, and *tem1-3* mutants during early anaphase was particularly evident when we correlated the length of the mitotic spindle with Cdc14 localization. In budding yeast, metaphase spindles are 1.5 – 2 μm in length and elongate during anaphase to reach a length of 10 μm during telophase (e. g. Yeh et al., 1995). In wild-type cells, Cdc14 was released from the nucleolus in over 90 percent

Figure 1 A-E

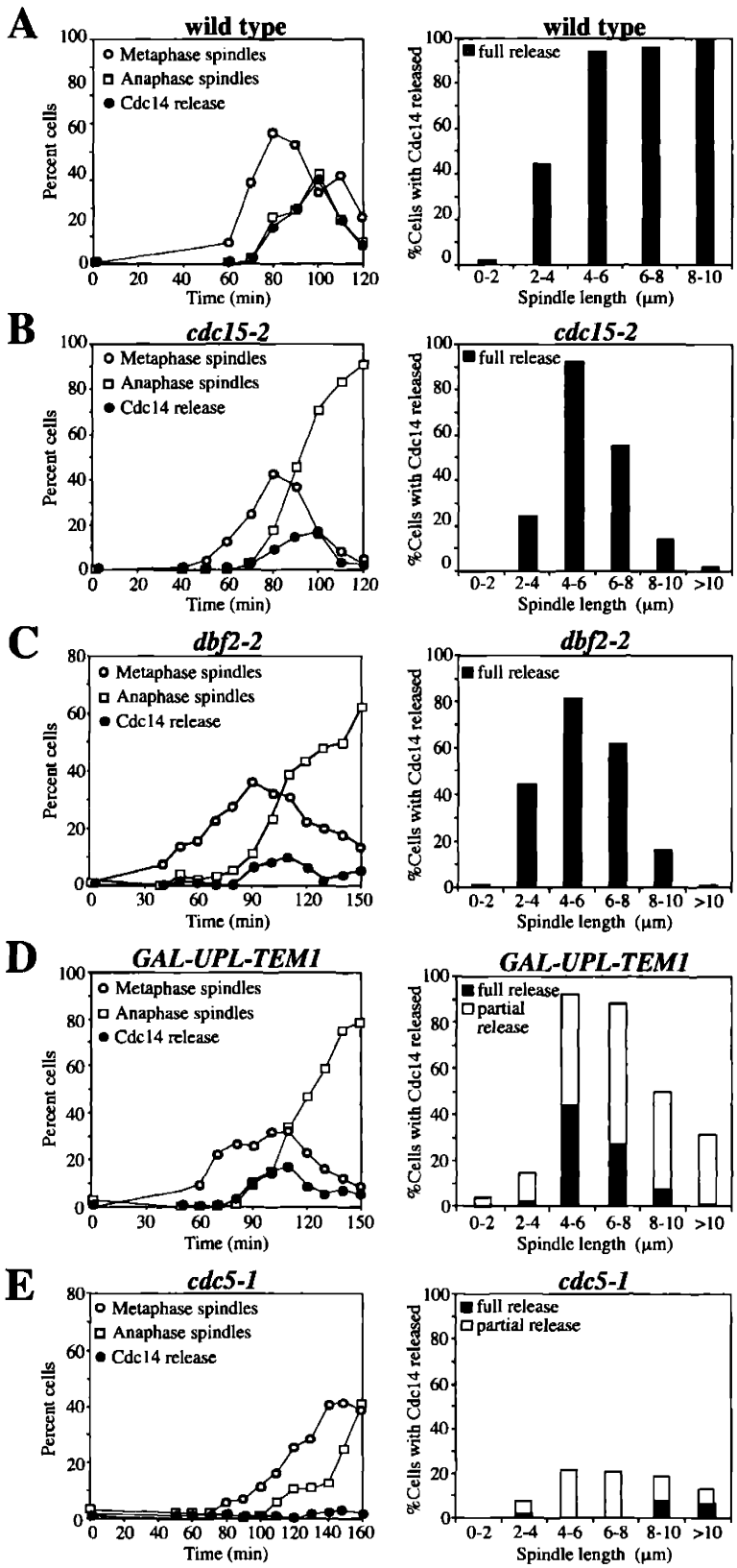
A
wild type
Metaphase spindles
Anaphase spindles
Cdc14 release
Percent cells
Time (min)
wild type
full release
%Cells with Cdc14 released
Spindle length (μm)
B
cdc15-2
cdc15-2
C
dbf2-2
dbf2-2
D
GAL-UPL-TEM1
GAL-UPL-TEM1
partial release
E
cdc5-1
cdc5-1
0-2
2-4
4-6
6-8
8-10
>10

Figure 1 F-G

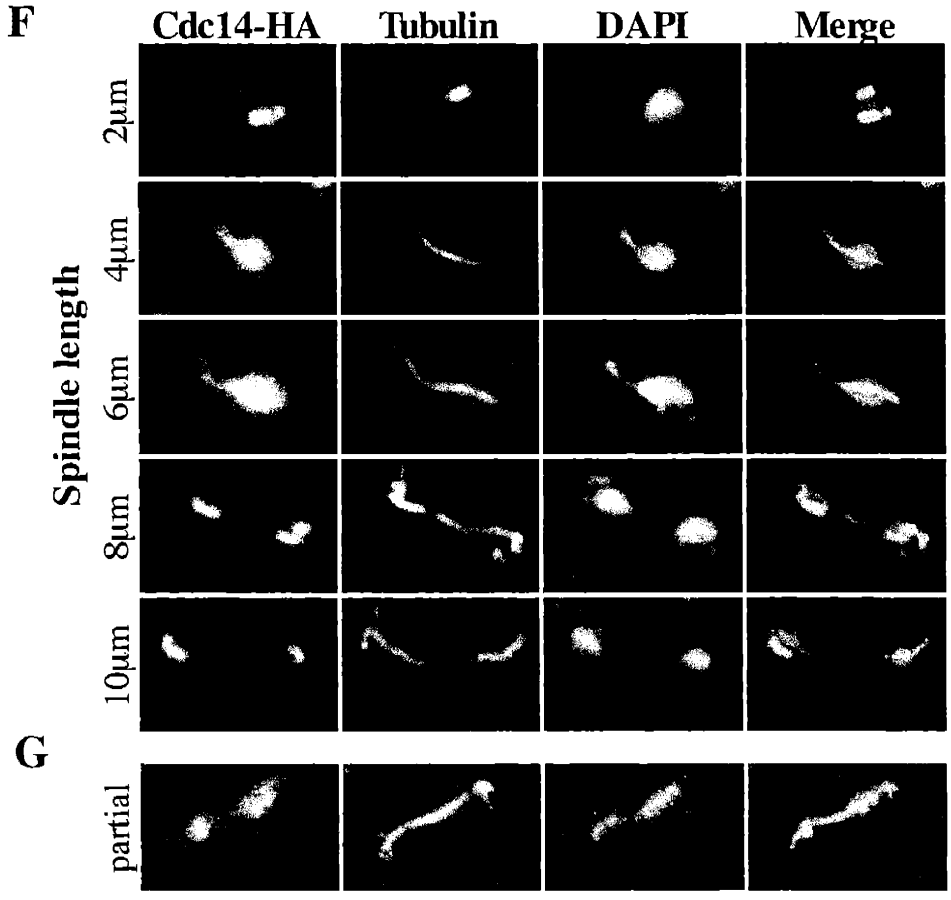

Figure 1: Cdc14 is transiently released from the nucleolus during early anaphase in MEN mutants.

(A, B, C, E) Wild-type (A1411, A), *cdc15-2* (A1674, B), *dbf2-2* (A2025, C), and *cdc5-1* (A1678, E) cells all carrying a *CDC14-HA* fusion were arrested in G1 in YEPD medium with α-factor (5µg/ml) followed by release into YEPD medium lacking pheromone at 37°C. The percentage of cells with metaphase (open circles), and anaphase spindles (open squares) as well as the percentage of cells with Cdc14-HA released from the nucleolus (closed circles) was determined at the indicated times. The graph on the right shows the percentage of cells with Cdc14 released from the nucleolus in relation to length of the mitotic spindle. Cells harvested 80 - 100 (wild-type), 80-100 (*cdc15-2*), 90-110 (*dbf2-2*), and 140-150 (*cdc5-1*) minutes after pheromone release were analyzed. For the *cdc5-1* mutant (E), both partial and full release of Cdc14 from the nucleolus was determined. In this and subsequent experiments a total of over 350 cells were analyzed for each strain.
(D) *GAL-UPL-TEM1* (A3545) cells grown in YEP Raffinose + Galactose (YEPRG) medium were shifted to YEPD medium and arrested in G1 with α-factor (5µg/ml) for 4 hours. Cells were subsequently released into YEPD medium lacking pheromone at 37°C. Samples were analyzed as in (A). Spindle length – Cdc14 correlation studies were performed on samples 90-110 minutes after pheromone release.
(F) Cdc14 localization in *cdc15-2* mutants with mitotic spindles 2, 4, 6, 8 and 10 µm in length. Cdc14 is shown in red, mitotic spindles in green and DNA in blue.
(G) Example for "partial release" of Cdc14 from the nucleolus.

of cells with mitotic spindles 4-10 μm in length, which indicates that Cdc14 is released from the nucleolus in early anaphase and remains in this released state throughout anaphase and telophase until cells exit from mitosis (Figure 1A). Live cell analysis of a Cdc14-GFP fusion protein revealed similar results (S. Yoshida and A. Toh-E, personal communications). In *cdc15-2*, *dbf2-2* and *tem1-3* mutants Cdc14 was released from the nucleolus in over 80 percent of cells with mitotic spindles 4-6 μm in length and in more than 50 percent of cells with 6-8 μm long mitotic spindles (Figure 1B, C, right column, data not shown). In cells with mitotic spindles 8 - 10 μm in length (8 - 12 μm is the spindle length observed in the terminal arrest of MEN mutants) Cdc14 was released from the nucleolus in only 10 percent of cells (Figure 1B, C and data not shown). The fact that Cdc14 is released from the nucleolus in 80 percent of cells or more with 4 – 6 μm long spindles suggest that this transient release occurs in most, if not all, cells. The reason why only a maximum of 10-15 percent of the total cell population is found to have Cdc14 released from the nucleolus is likely to be due to the fact that it takes cells only 8 minutes to elongate their mitotic spindles from 4 μm to 7 μm (e.g. Yeh et al., 1995).

To determine whether the transient release of Cdc14 from the nucleolus during early anaphase observed in MEN mutants was due to residual activity of temperature-sensitive MEN mutant alleles, we analyzed Cdc14 localization in cells depleted for *TEM1*. A strain carrying *TEM1* fused to the destabilizing *UPL* domain under the control of the *GAL1-10* promoter (*GAL-UPL-TEM1*) as the sole source of *TEM1* (Shou et al., 1999) shows a first cell-cycle arrest in telophase, when transcription of the fusion is repressed by glucose (Shou et al., 1999; Figure 1D). Cdc14 was transiently released from the nucleolus in Tem1-depleted cells with mitotic spindles 4-8 μm in length. However, we noticed that

two classes of cells with Cdc14 released from the nucleolus existed. In one class, Cdc14 was completely released from the nucleolus. In the other class Cdc14 was diffuse throughout the nucleus, but some enrichment of Cdc14 in the nucleolus was detectable. The latter localization pattern was classified as "partial release"; an example is shown in Figure 1G. The less pronounced release of Cdc14 from the nucleolus during early anaphase in Tem1-depleted cells compared to that seen in temperature sensitive MEN mutants may reflect the more effective inactivation of MEN in Tem1-depleted cells. Nevertheless, it was clear that in Tem1-depleted cells, Cdc14 was released from the nucleolus during early anaphase. Our results indicate that Cdc14 release from the nucleolus during early anaphase seen in wild-type cells also occurs in MEN mutants. But in contrast to wild-type cells, MEN mutants cannot maintain Cdc14 in its released state during later stages of mitosis. We conclude that Cdc14 release from the nucleolus is initiated during early anaphase in a MEN-independent manner. MEN activity is, however, essential for maintaining Cdc14 in this released state during late stages of anaphase and telophase. Using the same approach we found that Cfi1/Net1, which localizes to the nucleolus throughout the cell cycle, was not transiently released from the nucleolus during early anaphase (data not shown).

Cdc14 is not transiently released from the nucleolus during early anaphase in *cdc5-1* mutants.

Several studies showed that *CDC5* functions in multiple ways to control exit from mitosis (Hu et al., 2001; Lee et al., 2001a; Lee et al., 2001b; Visintin and Amon, 2001). We therefore wished to examine whether cells defective in *CDC5* released Cdc14 from the nucleolus during early anaphase. Cdc14 was sequestered in the nucleolus during early and

late anaphase in *cdc5-1* mutants (Figure 1E). This finding indicates that in contrast to *TEM1*, *CDC15* and *DBF2, CDC5* is required for the release of Cdc14 from the nucleolus during early anaphase.

ESP1 is required for MEN-independent Cdc14 release from the nucleolus during early anaphase.

Several observations raised the possibility that *ESP1* was required for release of Cdc14 from the nucleolus during early anaphase observed in MEN mutants. (1) *ESP1* was required for the timely exit from mitosis (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). (2) *esp1-1* mutants were partially defective in releasing Cdc14 from the nucleolus (Figure 3D). (3) The release of Cdc14 from the nucleolus occurred concomitantly with the activation of Esp1 at the onset of anaphase. (4) Release of Cdc14 during early anaphase required *CDC5*, which functions together with *ESP1* to trigger sister-chromatid separation (Alexandru et al., 2001). We, therefore, investigated whether *ESP1* is required for the transient release of Cdc14 from the nucleolus during early anaphase observed in MEN mutants. Indeed, the transient release of Cdc14 from the nucleolus during early anaphase observed in *cdc15-2* mutants or cells depleted for *TEM1* was largely absent in *cdc15-2* or *GAL-UPL-TEM1* cells carrying a temperature sensitive *esp1-1* allele (Figure 2A, B).

It is possible that *cdc15-2 esp1-1* mutants fail to release Cdc14 from the nucleolus during early anaphase because *esp1-1* mutants fail to separate sister chromatids and elongate their mitotic spindle. To address this possibility, we compared the kinetics of Cdc14 release from the nucleolus in *cdc15-2 esp1-1* mutants, with that of *cdc15-2* cells

Figure 2

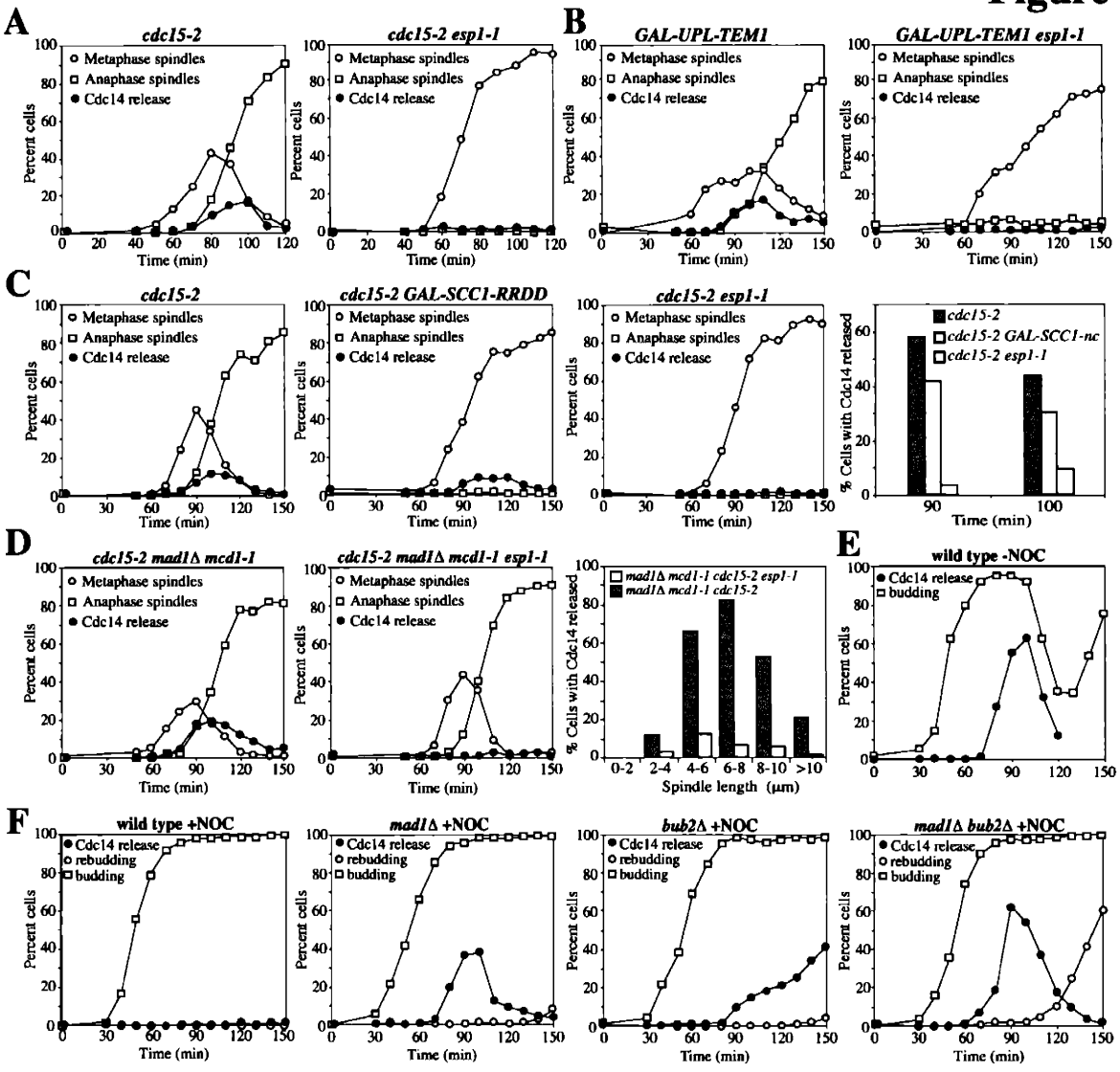

Figure 2: The transient release of Cdc14 from the nucleolus in MEN mutants depends on *ESP1*.

(A) *cdc15-2* (A1674) and *cdc15-2 esp1-1* (A3290) cells were grown and analyzed as described in Figure 1A.

(B) *GAL-UPL-TEM1* (A3545) and *GAL-UPL-TEM1 esp1-1* (A3548) cells were grown and analyzed as described in Figure 1D.

(C) *cdc15-2* (A1674), *cdc15-2 GAL-SCC1RR-DD* (A3691) and *cdc15-2 esp1-1* (A3290) cells were arrested in G1 in YEP Raffinose (YEPR) medium with α-factor (5μg/ml). One hour prior to release from the G1 block galactose was added (YEPRG). Cells were release into YEPRG medium lacking pheromone at 37°C. Spindle length measurements (graph on the right) were conducted on cells with mitotic spindles 3μm and longer 90 – 100 minutes after release from the G1 block.

(D) *cdc15-2 mad1Δ mcd1-1* (A3795) and *cdc15-2 mad1Δ mcd1-1 esp1-1* (A3797) were grown and analyzed as described in Figure 1A. Cdc14 – mitotic spindle correlation studies (graph on the right) were performed on samples harvested 90-110 minutes after pheromone release.

(E, F) Wild-type (A1411), *mad1Δ* (A2853), *bub2Δ* (A1901) and *mad1Δ bub2Δ* (A3059) cells were arrested with α-factor (5 μg/ml) and released into medium lacking (E) or containing (F) 15 μg/ml nocodazole at 25°C to determine the percentage of Cdc14 release from the nucleolus (closed circles) and the percentage of budded (open squares) and "rebudded" (open circles) cells. Rebudding indicates that nocodazole-treated cells have exited mitosis and entered a new cell cycle.

rendered incompetent to separate sister chromatids due to expression of a non-cleavable form of *SCC1/MCD1 (GAL-SCC1RR-DD).* In cells expressing Scc1RR-DD, sister chromatids fail to separate and mitotic spindle elongation does not occur, although Esp1 is active (Uhlmann et al., 1999). In *cdc15-2 GAL-SCC1RR-DD* cells the transient release of Cdc14 from the nucleolus during early anaphase was reduced suggesting that sister-chromatid separation and/or spindle elongation were, to some extent, important for the release of Cdc14 from the nucleolus during early anaphase. The mechanisms whereby these events contribute to Cdc14 release from the nucleolus are unknown. Inactivation of *ESP1*, however, had a more dramatic effect on Cdc14 release from the nucleolus than preventing sister-chromatid separation and mitotic spindle elongation (Figure 2C). This was particularly evident when we determined the localization of Cdc14 in cells with mitotic spindles 3μm or longer 90 minutes after release from the G1 arrest. Cdc14 was released from the nucleolus in 60 percent of *cdc15-2* and 40 percent of *cdc15-2, GAL-SCC1RR-DD* but only in 3 percent of *cdc15-2, esp1-1* cells (Figure 2C). This result suggests that *ESP1* is required for the transient release of Cdc14 from the nucleolus during early anaphase and that this requirement is not solely an indirect consequence of *ESP1's* role in the initiation of anaphase.

To further establish that *ESP1* was required for the transient release of Cdc14 from the nucleolus during early anaphase in MEN mutants, we examined the kinetics of Cdc14 release from the nucleolus in *cdc15-2 esp1-1* mutants under conditions where *ESP1* activity was dispensable for mitotic spindle elongation. Scc1/Mcd1 encodes a component of cohesin, and cells carrying a mutation in this cohesin subunit, such as the *mcd1-1* mutation, do not establish cohesion between sister chromatids. Consequently, chromosome

segregation and mitotic spindle elongation occur in the absence of *ESP1* function (Severin et al., 2001). The *mcd1-1* mutation causes activation of the mitotic spindle checkpoint (Severin et al., 2001), which made it necessary to perform this experiment in cells lacking the mitotic spindle checkpoint component *MAD1*.

cdc15-2 mad1Δ mcd1-1 cells released Cdc14 from the nucleolus during early anaphase in a manner indistinguishable from that of *cdc15-2* mutants (Figure 2D). In contrast, *cdc15-2 mad1Δ mcd1-1 esp1-1* mutants failed to release Cdc14 from the nucleolus (Figure 2D). Furthermore, a large fraction of *cdc15-2 mad1Δ mcd1-1* cells with mitotic spindles 4- 10 μm in length but not *cdc15-2 mad1Δ mcd1-1 esp1-1* cells had Cdc14 released from the nucleolus (Figure 2D). Our results indicate that *ESP1* is required for the transient release of Cdc14 from the nucleolus observed in MEN mutants.

Two pathways regulate Cdc14 release from the nucleolus in nocodazole-arrested cells.

We obtained further evidence to indicate that two pathways exist that control Cdc14 localization and that *ESP1* regulates Cdc14 release from the nucleolus independently of MEN when we analyzed Cdc14 localization in *mad1Δ* or *bub2Δ* cells treated with nocodazole. Nocodazole treatment activates the mitotic spindle checkpoint causing cells to arrest in metaphase. *MAD1, MAD2, MAD3, BUB1* and *BUB3* prevent degradation of Pds1 and thus Esp1 activation when the checkpoint is activated. *BUB2* restrains MEN activity in response to checkpoint activation (reviewed in Gardner and Burke, 2000). In nocodazole-treated *mad1Δ* cells (in these cells Esp1 is active but MEN is not) Cdc14 was transiently released from the nucleolus (Figure 2F). The onset of Cdc14 release from the nucleolus in

mad1Δ cells was similar to that of wild-type cells progressing through the cell cycle in the absence of nocodazole (Figure 2E), but *mad1Δ* cells could not maintain Cdc14 in the released state. In nocodazole-treated *bub2Δ* cells (in these cells MEN is active but Esp1 is not) Cdc14 release from the nucleolus was delayed (Figure 2F), suggesting that the timely release of Cdc14 release from the nucleolus requires *ESP1* function. In *mad1Δ bub2Δ* double mutants Cdc14 release from the nucleolus occurred with kinetics similar to that of wild-type cells progressing through the cell cycle in the absence of the spindle poison (Figure 2F), confirming earlier findings that *BUB2* and the MAD/BUB1/BUB3-group of genes restrain exit from mitosis by independent mechanisms (reviewed in Gardner and Burke, 2000). Our results indicate that Cdc14 localization is regulated by two independent pathways, MEN and an *ESP1*-dependent pathway. We termed the novel regulatory circuit that controls Cdc14 localization during early anaphase the FEAR network for Cdc fourteen early anaphase release network.

esp1-1 mutants are defective in exit from mitosis.

To further establish that *ESP1* regulates the release of Cdc14 from the nucleolus and thus exit from mitosis, we characterized cell-cycle progression of *esp1-1* mutants in detail. *esp1-1* cells fail to separate sister-chromatids but cell-cycle progression continues (McGrew et al., 1992; Surana et al., 1993). After a delay, cells exit from mitosis and initiate a new cell cycle (Tinker-Kulberg and Morgan, 1999). Since cytokinesis is incomplete in *esp1-1* cells, mother and daughter cell remain connected and the daughter cell, which has received the nucleus forms a bud upon entry into the subsequent cell cycle

Figure 3 A-C

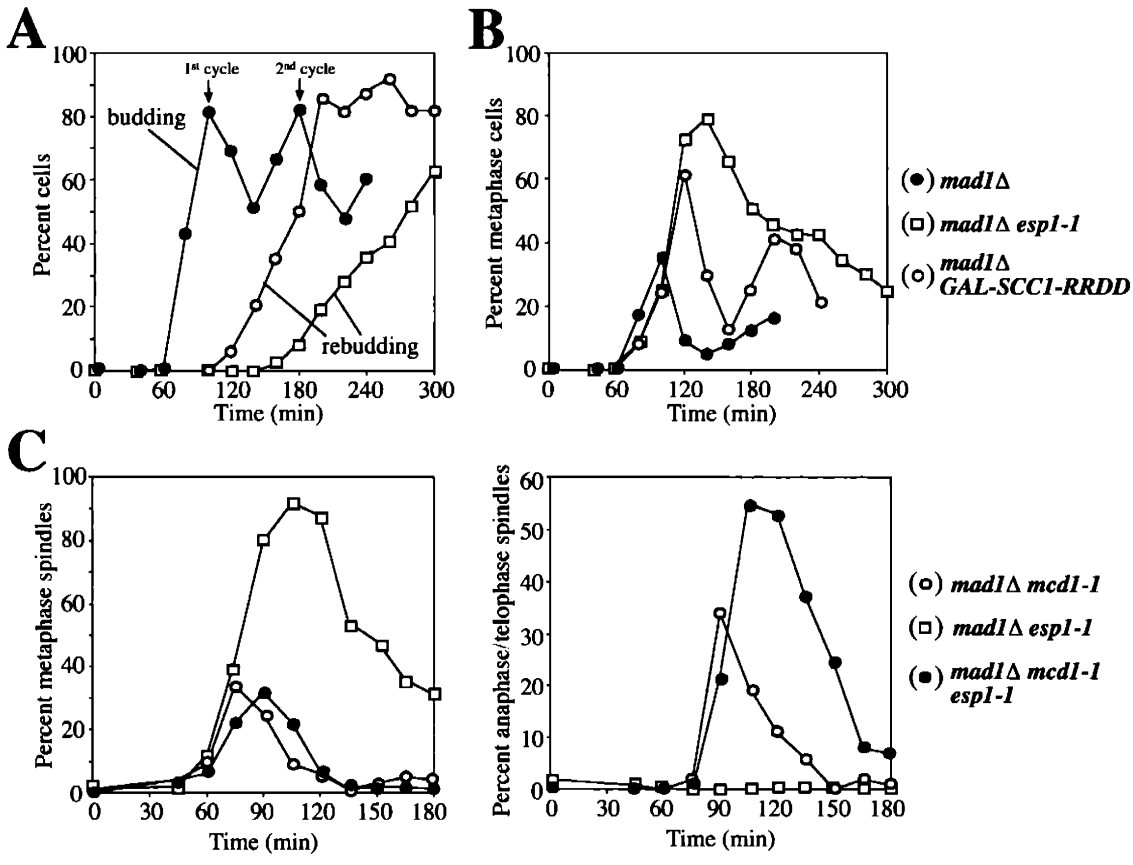
A
Percent cells
Time (min)
1st cycle
2nd cycle
budding
rebudding
B
Percent metaphase cells
Time (min)
(●) mad1Δ
(□) mad1Δ esp1-1
(○) mad1Δ GAL-SCC1-RRDD
C
Percent metaphase spindles
Time (min)
Percent anaphase/telophase spindles
Time (min)
(○) mad1Δ mcd1-1
(□) mad1Δ esp1-1
(●) mad1Δ mcd1-1 esp1-1

Figure 3 D-E

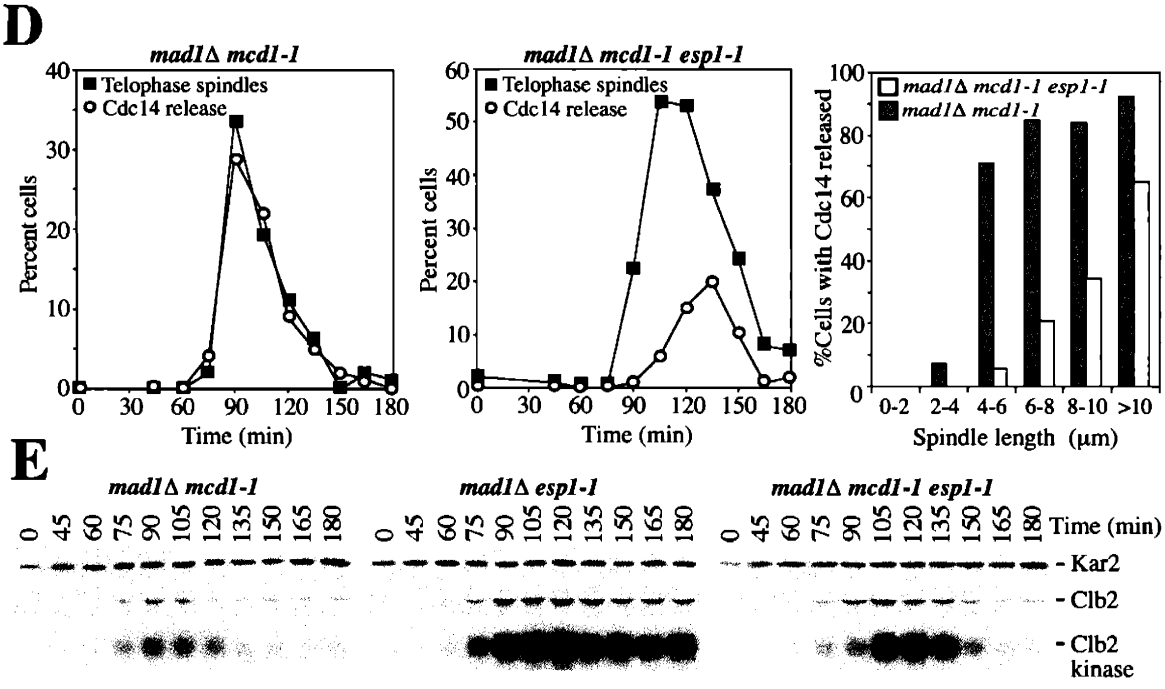

Figure 3: *esp1-1* mutants are defective in exit from mitosis.

(A, B) We analyzed the kinetics of exit from mitosis in *esp1-1* mutants lacking the spindle checkpoint component *MAD1*, to rule out the possibility that delays in exit from mitosis observed in *esp1-1* mutants were due to activation of the spindle checkpoint. *mad1*Δ (A2853), *esp1-1 mad1*Δ (A2977) and *GAL-SCC1RR-DD mad1* Δ (A3019) cells were arrested in G1 in YEPR medium with α-factor (5μg/ml) and released into YEPRG medium lacking pheromone at 37°C. The percentage of budded (A2853) and dumb-bell cells with an extra bud (rebudded cells, A; A2977 and A3019) and the percentage of cells with metaphase spindles (B) was determined. Arrows in (A) indicate when *mad1*Δ cells having produced their 1st and the 2nd bud were maximal in wild-type cultures.

(C - F) *mcd1-1 mad1*Δ (A2784), *mcd1-1 esp1-1 mad1*Δ (A3550) *and esp1-1 mad1*Δ (A2977) cells all carrying a *CDC14-HA* fusion were grown as described in Figure 1A. The percentage of cells with metaphase and anaphase/telophase spindles (C), the percentage of cells with Cdc14 released from the nucleolus (open circles) and telophase spindles (closed squares; D) and the amount of Clb2 protein and Clb2-associated kinase activity (E) was determined. Kar2 was used as a loading control in Western blots. The graph on the right in (D) shows the percentage of Cdc14 released from the nucleolus in relation to mitotic spindle length (samples 75 – 105 (A2784) and 90 – 120 (A3550) minutes after pheromone release were used for this analysis).

("rebudding"). Exit from mitosis was delayed in *esp1-1* mutants as judged by a delay in "rebudding" (Figure 3A). Whereas wild-type cells formed their second bud 90 – 120 minutes after pheromone release, *esp1-1* mutants did not do so until 180 minutes.

Next we tested whether the mitotic exit delay observed in *esp1-1* mutants was due to defects in sister-chromatid separation and/or spindle elongation. Cells expressing a non-cleavable version of the cohesin subunit Scc1/Mcd1 (Scc1RR-DD) showed a modest delay in exit from mitosis (Figure 3A), but mitotic spindle disassembly and rebudding was substantially more (90 minutes) delayed in *esp1-1* mutants (Figure 3A, B). *esp1-1* cells were also delayed in Cdc14 release from the nucleolus and exit from mitosis under conditions when *ESP1* was no longer required for chromosome segregation and mitotic spindle elongation. In *mcd1-1 mad1Δ* cells with anaphase- and telophase-like spindles, Cdc14 was released from the nucleolus when the mitotic spindle was 4 - 6 μm in length (Figure 3D). In contrast, a large fraction of *esp1-1 mcd1 mad1Δ* cells showed defects in Cdc14 release from the nucleolus with Cdc14 release occurring only when the mitotic spindle was fully extended (8 - 10 μm; Figure 3D). Clb2 protein levels and Clb2-associated kinase activity were also elevated and telophase spindles persisted for 45 minutes longer in *mad1Δ mcd1-1 esp1-1* mutants than in *mad1Δ mcd1-1* cells (Figure 3C, E). It is important to note that mitotic exit, as judged by Clb2 kinase inactivation, was delayed by more than 75 minutes in *esp1-1 mad1Δ* mutants, but by only 45 minutes in *esp1-1 mad1Δ mcd1-1* (Figure 3E). This suggests that the mitotic exit defect observed in *esp1-1* mutants is in part due to defects in mitotic spindle elongation. Our results, however, also clearly show that *ESP1* regulates exit from mitosis independently of mitotic spindle elongation, likely by regulating Cdc14 localization.

Cells depleted for *ESP1* are delayed in exit from mitosis.

esp1-1 mutants were not defective in exit from mitosis, but showed only a delay in this cell-cycle transition. It was, therefore, possible that the *esp1-1* allele was not a null allele at the restrictive temperature with respect to *ESP1*'s mitotic exit function. To test this possibility we analyzed the consequences of *ESP1* depletion on exit from mitosis. *ESP1* under the control of the methionine repressible *MET3* promoter and tagged with 18 *MYC* epitopes was fused to the heat inducible Degron cassette (*esp1-td-18Myc*), which targets proteins for degradation via the N-end rule pathway at 37°C (Dohmen et al.,1994). Upon addition of methionine and shift to 37°C, Esp1-td-Myc was degraded within two hours (Figure 4A).

esp1-1 and *esp1-td-18MYC* cells failed to separate sister chromatids (data not shown) and were similarly delayed in mitotic exit as judged by mitotic spindle disassembly and rebudding (Figure 4B). This result indicates that the *esp1-1* mutation closely resembles a complete loss-of-function mutation not only with respect to sister-chromatid separation but also exit from mitosis. As exit from mitosis was not abolished in *esp1-td-18MYC* cells, our results further indicate that *ESP1* is not absolutely essential for exit from mitosis but is required for this event to occur in a timely fashion.

Figure 4 A-C

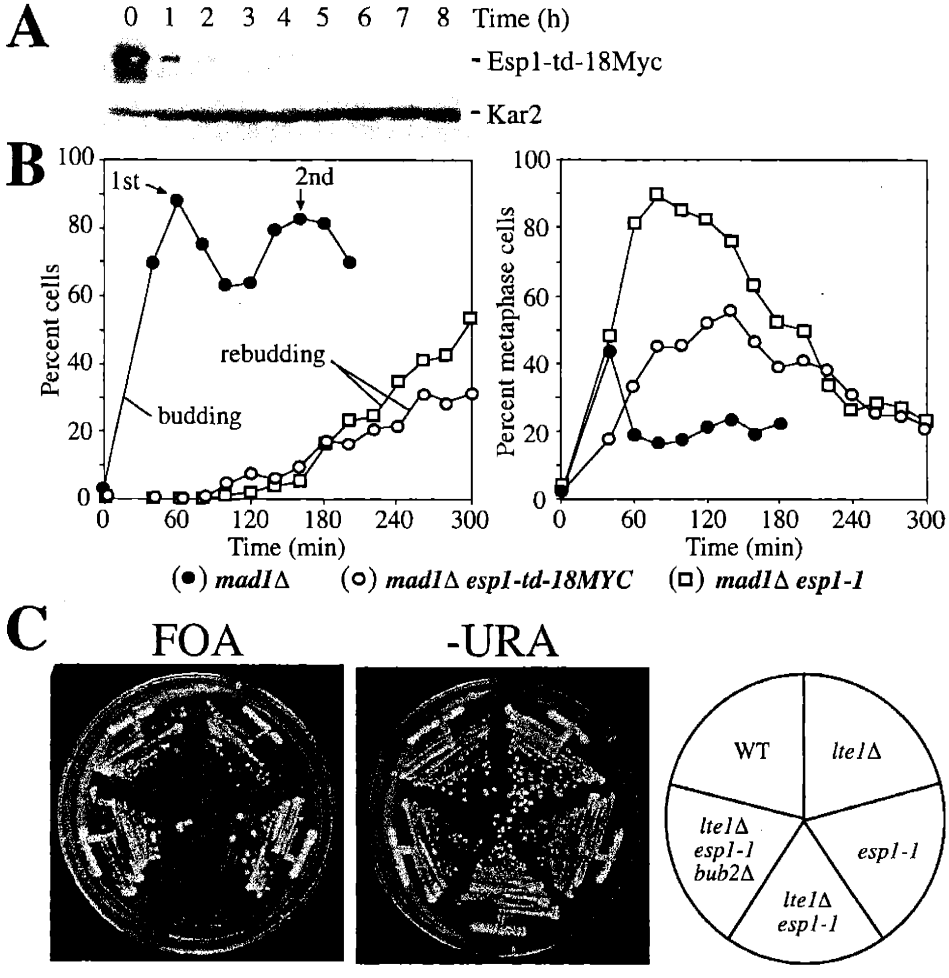
A
0 1 2 3 4 5 6 7 8 Time (h)
- Esp1-td-18Myc
- Kar2
B
Percent cells
1st
2nd
rebudding
budding
Time (min)
Percent metaphase cells
Time (min)
(•) mad1Δ (○) mad1Δ esp1-td-18MYC (□) mad1Δ esp1-1
C
FOA
-URA
WT
lte1Δ
lte1Δ esp1-1 bub2Δ
esp1-1
lte1Δ esp1-1

Figure 4 D-E

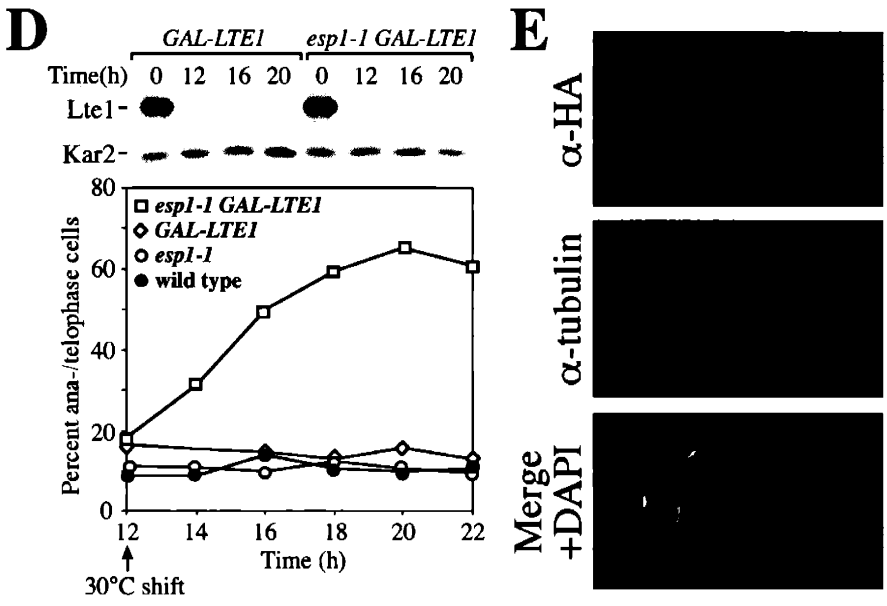

Figure 4: *ESP1*-depleted cells are delayed in exit from mitosis, but *ESP1* is essential for mitotic exit in the absence of *LTE1*.

(A) *mad1*Δ cells carrying an *esp1-td-18MYC* fusion as the sole source of *ESP1* (A3557) were transferred from medium lacking methionine (0 time point) to YEPD medium containing 4 mM methionine (37°C). Depletion of the fusion was followed by Western blot analysis.

(B) *mad1*Δ (A2853), *esp1-1 mad1*Δ (A2977) and *esp1-td-18MYC mad1*Δ cells (A3557) grown at 25°C in medium lacking methionine were shifted to YEPD medium containing 4mM methionine and arrested in G1 with α-factor (5μg/ml) for 2 hours at 25°C followed by 2 hours at 37°C. Cells were released into YEPD medium containing 4 mM methionine at 37°C. The percentage of budded cells (A2853) and rebudded cells (A2977 and A3557) and the percentage of cells with metaphase spindles was determined. As 40 percent of A3557 cells never entered the cell cycle after pheromone release and hence were unbudded (data not shown) rebudding was normalized to the total amount of budded cells rather than total cell number. Arrows in the left graph indicate when cells having produced their 1st and the 2nd bud were maximal in wild-type cultures.

(C) Wild-type (A3723), *lte1*Δ (A4101), *esp1-1* (A4102), *esp1-1 lte1*Δ (A4017) and *esp1-1 lte1*Δ *bub2*Δ (A4103) cells all carrying *LTE1* on a *CEN4-URA3* plasmid were streaked on plates either containing 5-fluororotic acid (FoA; selects against the plasmid) or plates lacking uracil (selects for the plasmid) at 30°C.

(D, E) *GAL-GFP-LTE1* (A3587) and *GAL-GFP-LTE1 esp1-1* (A4145) cells were grown in YEPD medium for 12 hours at 25°C and subsequently shifted to 30°C in YEPD (12 h time point). This regiment of medium and temperature shift was necessary to deplete cells of the GFP-Lte1 protein. Note that *esp1-1* mutants show no growth impairment at 30°C (C). Depletion of GFP-Lte1 was determined by western blot analysis and the percentage of cells with anaphase and telophase spindles was determined (D). (E) Cdc14-HA localization and mitotic spindle morphology of *GAL-GFP-LTE1 esp1-1* cells 6 hours after shift to 30°C.

ESP1 is essential for mitotic exit in cells lacking _LTE1_.

The putative exchange factor for Tem1, Lte1, is, unlike other MEN components, essential only at low temperatures (Shirayama et al., 1994), suggesting that other factors promote mitotic exit at high temperatures in the absence of *LTE1*. Since *ESP1* is also an activator of mitotic exit, we hypothesized that removal of both activators might lead to a more pronounced defect in exit from mitosis. Indeed, *esp1-1 lte1Δ* double mutants were inviable at 30°C (Figure 4C). This synthetic lethality was rescued by deletion of *BUB2*, (Figure 4C), suggesting that a defect in exit from mitosis was the cause of lethality in *esp1-1 lte1Δ* double mutants.

To analyze the terminal phenotype of *esp1-1 lte1Δ* double mutants, we constructed an *esp1-1* strain carrying a *LTE1-GFP* fusion under the control of the *GAL1-10* promoter as the sole source of *LTE1*. As Lte1 is a stable protein, depletion of Lte1 did not occur for several hours after glucose addition (data not shown). However, cells eventually ceased to divide and arrested in telophase with Cdc14 sequestered in the nucleolus (Figures 4D, E). We conclude that *ESP1* is essential for Cdc14 release from the nucleolus and mitotic exit to occur in cells lacking *LTE1*.

SLK19 is required for MEN independent release of Cdc14 from the nucleolus during early anaphase.

To date, two substrates of Esp1, the cohesin subunit Scc1/Mcd1 and the kinetochore protein Slk19 have been identified (Uhlmann et al., 1999; Sullivan et al., 2001). To determine whether these Esp1 substrates were also required for release of Cdc14 from the nucleolus during early anaphase, we examined whether deletion of any one protein causes a

delay in exit from mitosis. Cells depleted for Scc1/Mcd1 exhibited a minor (5 minute) delay in mitotic exit (data not shown). In contrast, the effect of deleting *SLK19* on mitotic exit was pronounced. Cdc14 was released from the nucleolus in only 50 percent of anaphase and telophase *slk19Δ mad1Δ* cells (Figure 5A; *MAD1* was deleted to avoid delays in mitotic exit caused by activation of the spindle checkpoint induced by the absence *SLK19*; Zeng et al., 1999). Mitotic exit was also delayed by 20 minutes as judged by a delay in Clb2 degradation, inactivation of Clb2 kinase activity and mitotic spindle disassembly (Figure 5B). This finding suggests that *SLK19* is required for the efficient release of Cdc14 from the nucleolus and exit from mitosis. Consistent with a role of *SLK19* in mitotic exit we also found that *slk19Δ lte1Δ* double mutants are inviable and that this synthetic lethality is rescued by deletion of *BUB2* (data not shown).

To determine whether *SLK19,* like *ESP1*, regulated the MEN-independent release of Cdc14 from the nucleolus during early anaphase, we analyzed Cdc14 localization in *cdc15-2 mad1Δ* mutants lacking *SLK19*. The transient release of Cdc14 from the nucleolus observed in *cdc15-2 mad1Δ* mutants was largely absent in *cdc15-2 mad1Δ slk19Δ* mutants. A large fraction of *cdc15-2 mad1Δ slk19Δ* cells with mitotic spindles 4- 8 μm in length had Cdc14 sequestered in the nucleolus (Figure 5C, D). However, whereas inactivation of *ESP1* completely abolished release of Cdc14 from the nucleolus in early anaphase, 15 percent of *slk19Δ* cells with 4 – 10 μm long spindles showed partial release of Cdc14 from the nucleolus during early anaphase (Figure 5D).

Figure 5 A-D

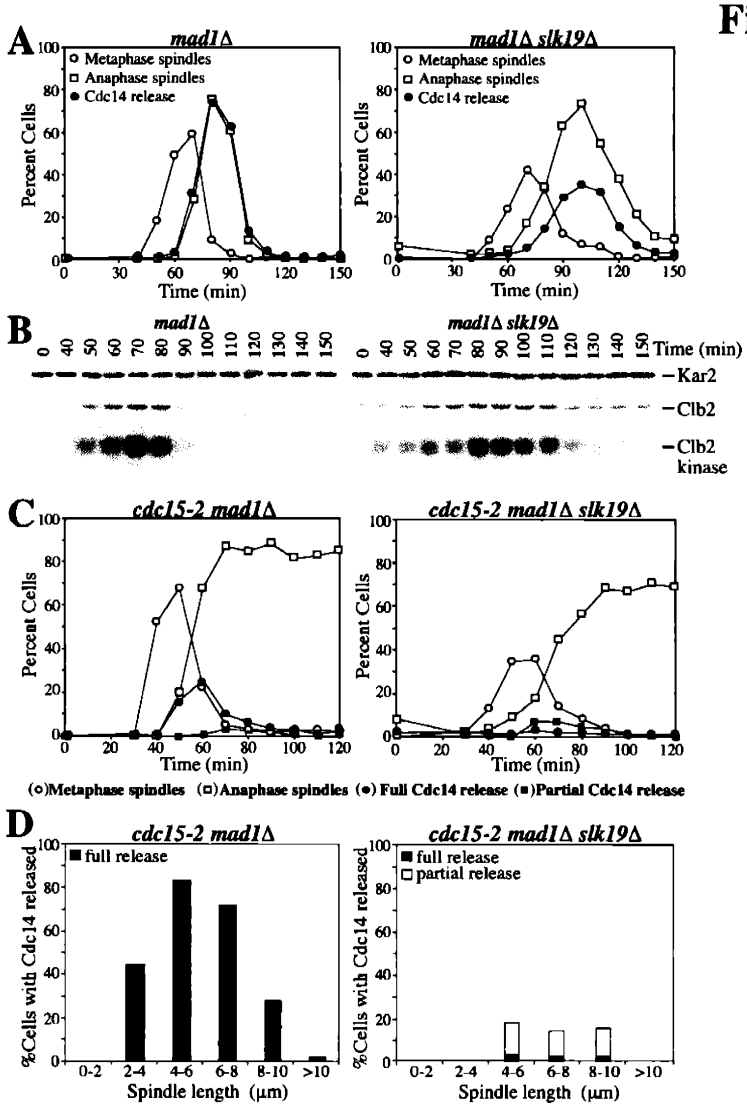

Figure 5: *SLK19* and *SPO12* are required for efficient Cdc14 release from the nucleolus during early anaphase.

(A, B) *mad1Δ* (A2853) and *mad1Δ slk19Δ* (A4302), cells were release from a pheromone block into medium lacking pheromone at 25°C. α-factor was readded 80 minutes after pheromone release. The percentage of cells with metaphase (open circles, A) and anaphase (open squares, A) spindles and Cdc14 released from the nucleolus (closed circles, A) and the amount of Clb2 protein and Clb2-associated kinase (B) was determined. (C, D) *cdc15-2 mad1Δ* (A4300) and *cdc15-2 mad1Δ slk19Δ* (A4304) were released from an α-factor into medium lacking pheromone at 35°C. The percentage of cells with metaphase (open circles), anaphase spindles (open squares) and the percentage of cells with Cdc14-HA completely (closed circles) or partially (closed squares) released from the nucleolus was determined (C). (D) Cdc14 released from the nucleolus in relation to length of the mitotic spindle. Samples 50 – 70 minutes after pheromone released were used.

Figure 5 E-H

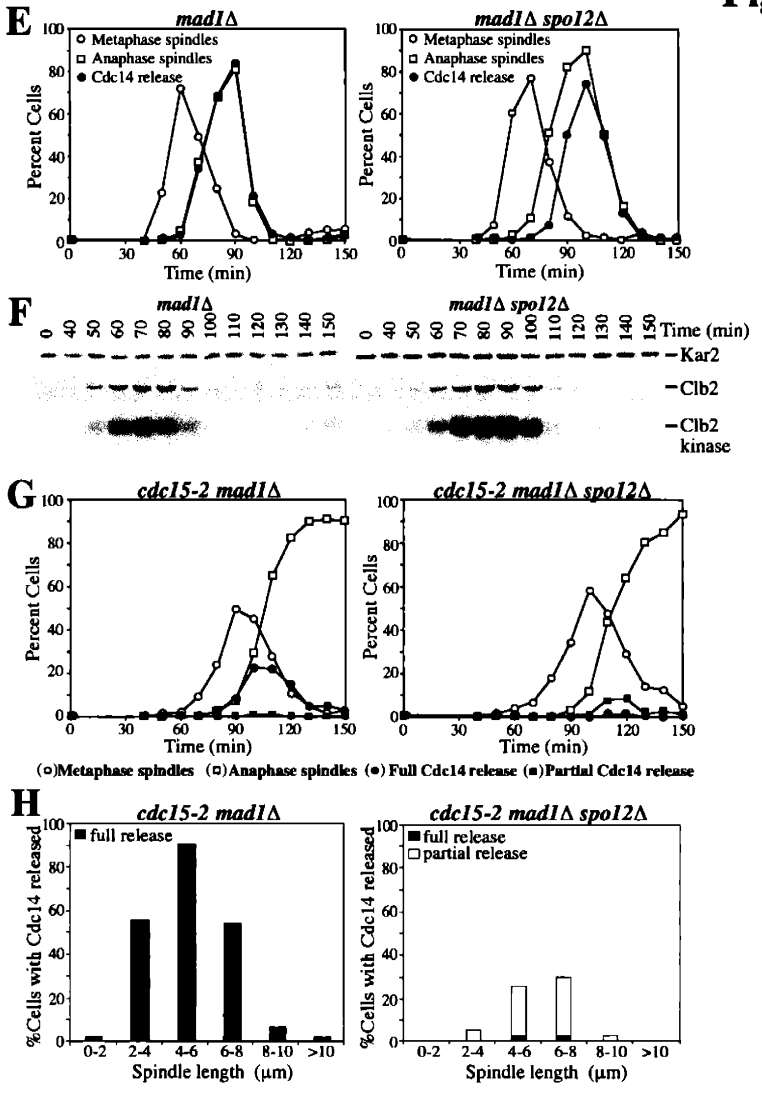

Figure 5: *SLK19* and *SPO12* are required for efficient Cdc14 release from the nucleolus during early anaphase.

(E, F) *mad1*Δ (A2853) and *mad1*Δ *spo12*Δ (A4503) cells were grown and analyzed as described in Figure 5A. The percentage of cells with metaphase (open circles, E) and anaphase (open squares, E) spindles and Cdc14 released from the nucleolus (closed circles, E), and the amount of Clb2 protein and Clb2-associated kinase (F) was determined.

(G, H) *cdc15-2 mad1*Δ (A4300) and *cdc15-2 mad1*Δ *spo12*Δ (A4500) were grown and analyzed as described in Figure 5C, except cells were released from G1 at 37°C. The percentage of cells with metaphase (open circles), anaphase spindles (open squares) and the percentage of cells with Cdc14-HA completely (closed circles) or partially (closed squares) released from the nucleolus was determined (G). (H) Cdc14 released from the nucleolus in relation to length of the mitotic spindle. Samples 100 - 120 minutes after pheromone released were used.

Figure 5 also shows that *SLK19*, like *ESP1*, is specifically required for the onset of Cdc14 release from the nucleolus during early anaphase, whereas MEN components are not. In *mad1Δ* or *cdc15-2 mad1Δ* cells, Cdc14 release from the nucleolus was maximal 10 minutes after metaphase. In contrast, peak levels of Cdc14 release from the nucleolus were seen only 30 minutes after cells with metaphase spindles were at a maximal level in *slk19Δ mad1Δ* cultures. These results suggest that *SLK19*, like *ESP1*, controls Cdc14 localization during early anaphase, but the requirement for *SLK19* to promote release of Cdc14 from the nucleolus during early anaphase is less stringent than the requirement for *ESP1*.

Esp1 cleaves Slk19 generating stable N- and C-terminal cleavage products (Sullivan et al., 2001). When the C-terminal cleavage product is rendered unstable, cells are delayed in exit from mitosis (Sullivan et al., 2001). These findings raised the possibility that Esp1 promotes release of Cdc14 from the nucleolus during early anaphase by cleaving Slk19. Several results, however, suggest that cleavage of Slk19 is not necessary for FEAR-network induced release of Cdc14 from the nucleolus. (1) Expression of neither the N-terminal nor the C-terminal cleavage product restored release of Cdc14 from the nucleolus during early anaphase in *esp1-1 cdc15-2* mutants (data not shown). (2) Expression of neither cleavage product nor coexpression of both cleavage products rescued the synthetic lethality of *esp1-1 lte1Δ* mutants (data not shown). The C-terminal *SLK19* cleavage product is however capable of rescuing the synthetic lethality of *lte1Δ slk19Δ* double mutants (data not shown) and the mitotic exit defect of *slk19Δ* cells (M. Sullivan and F. Uhlmann, personal communications) indicating that it can perform *SLK19*'s key cell-cycle functions. (3) Cells carrying a non-cleavable version of *SLK19* as the sole source of *SLK19* have no defect in exit from mitosis (M. Sullivan and F. Uhlmann, personal

communications) and expression of this non-cleavable Slk19 mutant protein rescues the synthetic lethality of the *lte1Δ slk19Δ* double mutant (data not shown). These results suggest that, although *SLK19* is a component of the FEAR network, its cleavage is not necessary for FEAR-induced Cdc14 release from the nucleolus. However, we cannot exclude the possibility that *in situ*, cleavage of the full-length protein is a prerequisite for the cleavage products to exert their function in the FEAR network.

SPO12 is required for complete release of Cdc14 from the nucleolus during early anaphase.

Overexpression of *SPO12*, a gene of unknown function, suppresses the temperature sensitive lethality of *tem1-3, cdc15-2, dbf2-2* and *cdc5-1* mutants (Parkes and Johnston, 1992; Jaspersen et al., 1998). We, therefore, investigated whether *SPO12* was required for Cdc14 release of the nucleolus during early anaphase. Cells lacking *SPO12* showed defects in Cdc14 release from the nucleolus (Figure 5E) and mitotic exit was delayed by 10 - 15 minutes as judged by a delay in Clb2 degradation, inactivation of Clb2-associated kinase activity and mitotic spindle disassembly (Figure 5F). This finding suggests that *SPO12* is required for the efficient release of Cdc14 from the nucleolus and exit from mitosis. *SPO12* was also required for the transient release of Cdc14 from the nucleolus observed in *cdc15-2* mutants (Figure 5G, H). The release of Cdc14 from the nucleolus observed in *cdc15-2 mad1Δ* mutants was diminished but not abolished in *cdc15-2 spo12Δ mad1Δ* mutants. These results suggest that *SPO12*, like *SLK19,* is required for the efficient release of Cdc14 from the nucleolus during early anaphase. Consistent with this idea we found that *spo12Δ lte1Δ* double mutants are inviable and that this synthetic lethality is rescued by

deletion of *BUB2* (data not shown).

Cdc15 is dephosphorylated in a manner dependent on Cdc14 released by the FEAR network.

When the FEAR network-dependent release of Cdc14 from the nucleolus is abolished, mitotic exit is delayed, indicating that Cdc14, released from the nucleolus during early anaphase, is important for the timely exit from mitosis. But is Cdc14 released by the FEAR network active? To address this question we examined the phosphorylation status of one Cdc14 substrate, Cdc15, under conditions where FEAR network-dependent release of Cdc14 from the nucleolus occurred, but MEN-dependent release did not, in a *dbf2-2* mutant. Cdc15, as opposed to other Cdc14 substrates, was chosen for analysis, because dephosphorylation of Cdc15 has been shown to stimulate Cdc15 activity, suggesting the existence of a feedback loop where Cdc14 stimulated MEN activity (Jaspersen and Morgan, 2000). Thus, analyzing whether Cdc15 is dephosphorylated by Cdc14 released by the FEAR network also enabled us to test whether Cdc14 released by the FEAR network could, in principle, promote Cdc15 activation.

Cdc15 phosphorylation can be assessed by analyzing its mobility by SDS-PAGE. Dephosphorylated Cdc15 migrates faster in SDS-PAGE than phosphorylated Cdc15 (Jaspersen and Morgan 2000; Xu et al., 2000). Cdc15 was transiently dephosphorylated during early anaphase in *dbf2-2* mutants, as judged by the appearance of faster migrating forms of Cdc15 as *dbf2-2* mutants entered anaphase 70 minutes after release from the G1 arrest (Figure 6B, C). The dephosphorylation of Cdc15 observed in *dbf2-2* mutants was not as pronounced as in wild-type cells (Figure 6A; Jaspersen and Morgan 2000; Xu et al.,

2000), indicating that MEN also contributes to the complete dephosphorylation of Cdc15. Nevertheless, the transient dephosphorylation that is observed in *dbf2-2* mutants was completely absent in *cdc14-3* mutants and decreased in *esp1-1* mutants (Figure 6B), suggesting that Cdc14 released by the FEAR network during early anaphase is active. Consistent with the idea that dephosphorylation of Cdc15 is an important function of Cdc14 released by the FEAR network, we found that a *cdc15* mutant that can no longer be phosphorylated (*cdc15-7A;* Jaspersen and Morgan, 2000) rescued the synthetic lethality of *esp1-1 lte1Δ* mutants (Figure 6D). Our results suggest that Cdc14 released during early anaphase by the FEAR network dephosphorylates Cdc15, thereby stimulating its ability to promote mitotic exit.

Cdc14 is required for the timely activation of MEN

Our results suggest that Cdc14 released from the nucleolus by the FEAR network, dephosphorylates Cdc15. If dephosphorylation of Cdc15 by Cdc14 were important for full activation of MEN, then components of MEN, such as Dbf2, should be less active in *cdc14-3* mutants than in wild-type cells. In wild-type cells, the specific activity of the protein kinase Dbf2 (Dbf2-associated kinase activity/Dbf2 protein) was low during G1, S phase and early mitosis, but high during anaphase and telophase (Figure 7A, Toyn and Johnston, 1994). In *cdc14-3* mutants the specific activity of Dbf2 was somewhat slow to

Figure 6 A-C

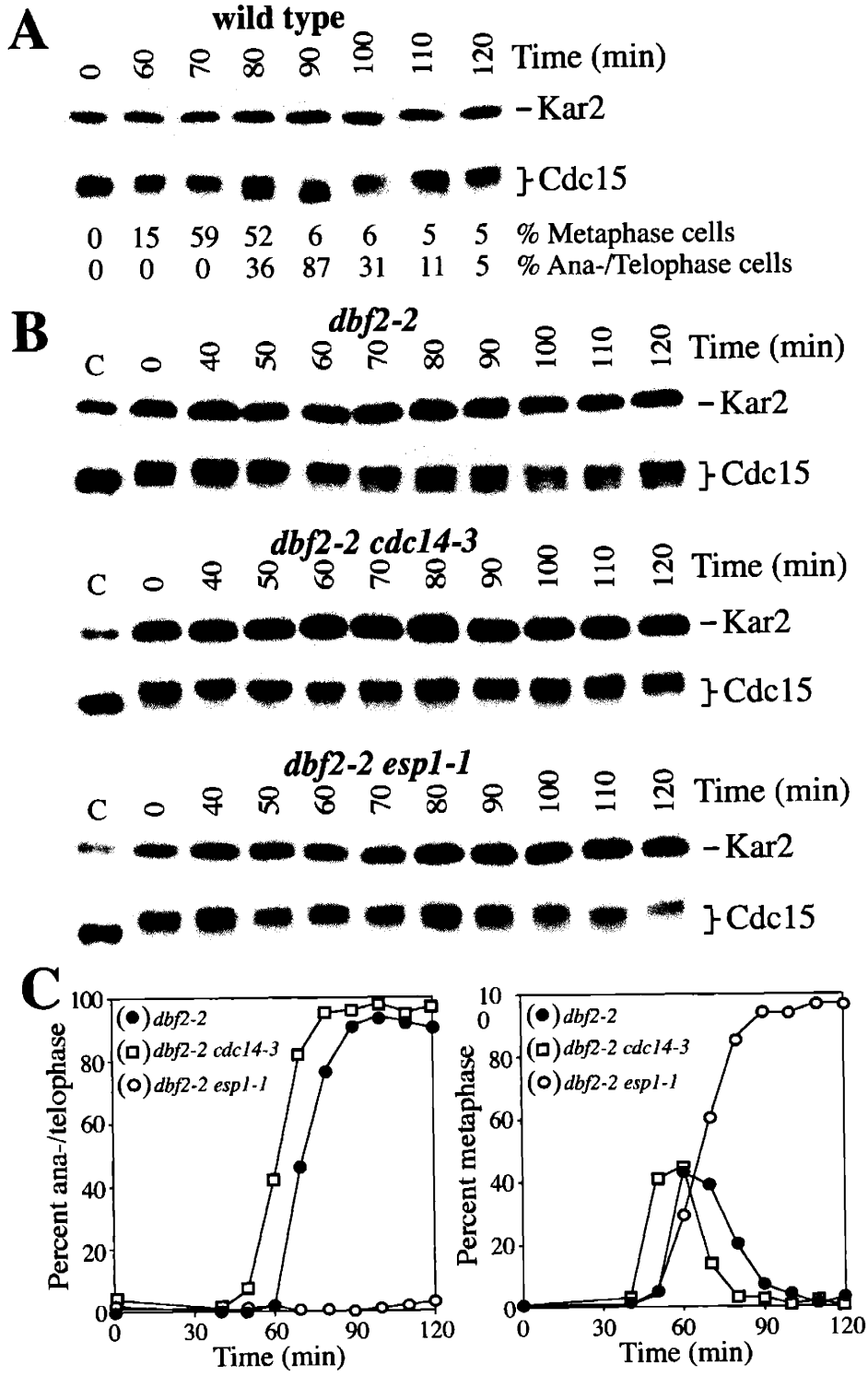
A
wild type
0 60 70 80 90 100 110 120 Time (min)
-Kar2
}Cdc15
0 15 59 52 6 6 5 5 % Metaphase cells
0 0 0 36 87 31 11 5 % Ana-/Telophase cells
B
dbf2-2
C 0 40 50 60 70 80 90 100 110 120 Time (min)
-Kar2
}Cdc15
dbf2-2 cdc14-3
C 0 40 50 60 70 80 90 100 110 120 Time (min)
-Kar2
}Cdc15
dbf2-2 esp1-1
C 0 40 50 60 70 80 90 100 110 120 Time (min)
-Kar2
}Cdc15
C
Percent ana-/telophase
(●) dbf2-2
(□) dbf2-2 cdc14-3
(○) dbf2-2 esp1-1
Time (min)
Percent metaphase
(●) dbf2-2
(□) dbf2-2 cdc14-3
(○) dbf2-2 esp1-1
Time (min)

Figure 6D

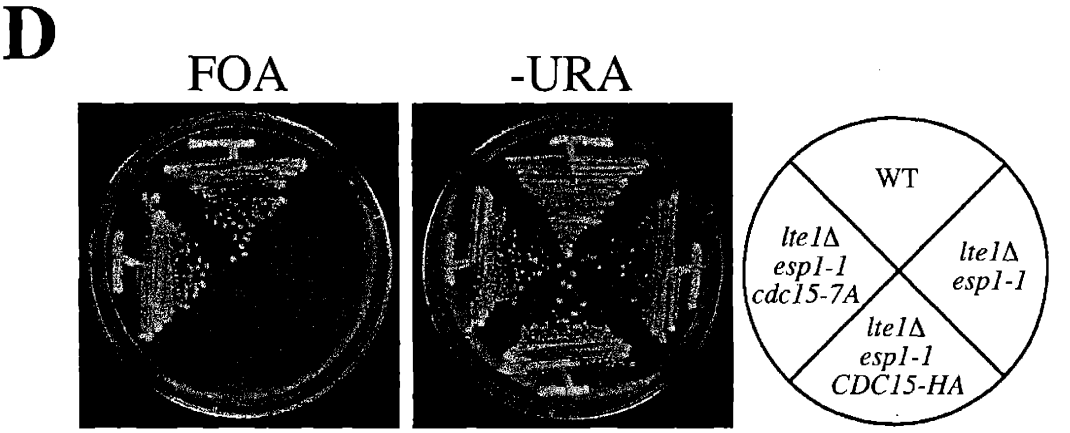

Figure 6: Cdc15 is transiently dephosphorylated during early anaphase in a *CDC14*-dependent manner.

(A) Phosphorylation status of Cdc15-HA in wild-type cells (A3392) progressing through the cell cycle after release from a pheromone arrest.
(B, C) *dbf2-2* (A4078), *dbf2-2 cdc14-3* (A4173) and *dbf2-2 esp1-1* (A4228) cells all carrying a *CDC15-HA* fusion were release from a pheromone arrest into medium lacking pheromone at 35°C. The electrophoretic mobility of Cdc15-HA (B) and the percentage of cells with metaphase and anaphase spindles (C) was determined. Lane "C" in (B) shows the electrophoretic mobility of the nonphosphorylatable Cdc15-7A protein (Jaspersen and Morgan, 2000). Since Cdc15 levels are higher in *cdc14-3* and *esp1-1* mutants than in wild-type cells, an exposure of 2 minutes is shown for wild-type and a 40 second exposure for *cdc14-3* and *esp1-1* mutants.
(D) Wild-type (A3723), *esp1-1 lte1Δ* (A4017), *esp1-1 lte1Δ cdc15Δ CDC15-3HA::LEU2*(A4105), and *esp1-1 lte1Δ cdc15Δ cdc15-7A-3HA::LEU2* (A4106) cells all carrying *LTE1* on a *CEN4-URA3* plasmid were streaked on plates either containing 5-fluororotic acid or plates lacking uracil at 30°C.

rise, and only reached 50 percent of the maximal levels observed in wild-type cells (Figure 7A). This observation is consistent with previous findings showing that Dbf2 kinase activity is not as active in *cdc14-1* or *cdc14-3* mutants as in wild-type anaphase/telophase cells (Mah et al., 2001; Visintin and Amon, 2001). We also find that *ESP1* was required for full activation of Dbf2 kinase (Figure 7A), indicating that not only *CDC14* but also *ESP1* is required for complete and timely activation of Dbf2 kinase.

If *CDC14* were required for full activation of Dbf2 kinase, high levels of Clb2 kinase, which antagonizes Cdc14 activity (Visintin et al., 1998; Jaspersen et al., 1999), should also inhibit Dbf2 kinase activity. To test this, we overexpressed a stabilized version of Clb2 (*GAL-CLB2Δdb,* Surana et al., 1993) and found that the specific activity of Dbf2 was reduced (Figure 7B). Furthermore, Cdc14 was released from the nucleolus at the onset of anaphase in these cells but many cells re-sequestered Cdc14 into the nucleolus during the prolonged telophase arrest brought about by stabilized Clb2 (Figure 7C). This was also observed in cells lacking *BUB2*, indicating that hyperactivation of MEN cannot overcome the inhibitory effects of Clb2 kinase on Dbf2 activity (data not shown). Our results indicate that *CDC14* is required for Dbf2 to be fully active and that high levels of Clb2 CDKs antagonize Dbf2 activity. Our findings further suggest that Cdc14 release from the nucleolus during early anaphase is not affected by mitotic CDK activity but maintenance of Cdc14 in the nucleus and cytoplasm during late stages of anaphase and during telophase is, to some extent, inhibited.

Figure 7

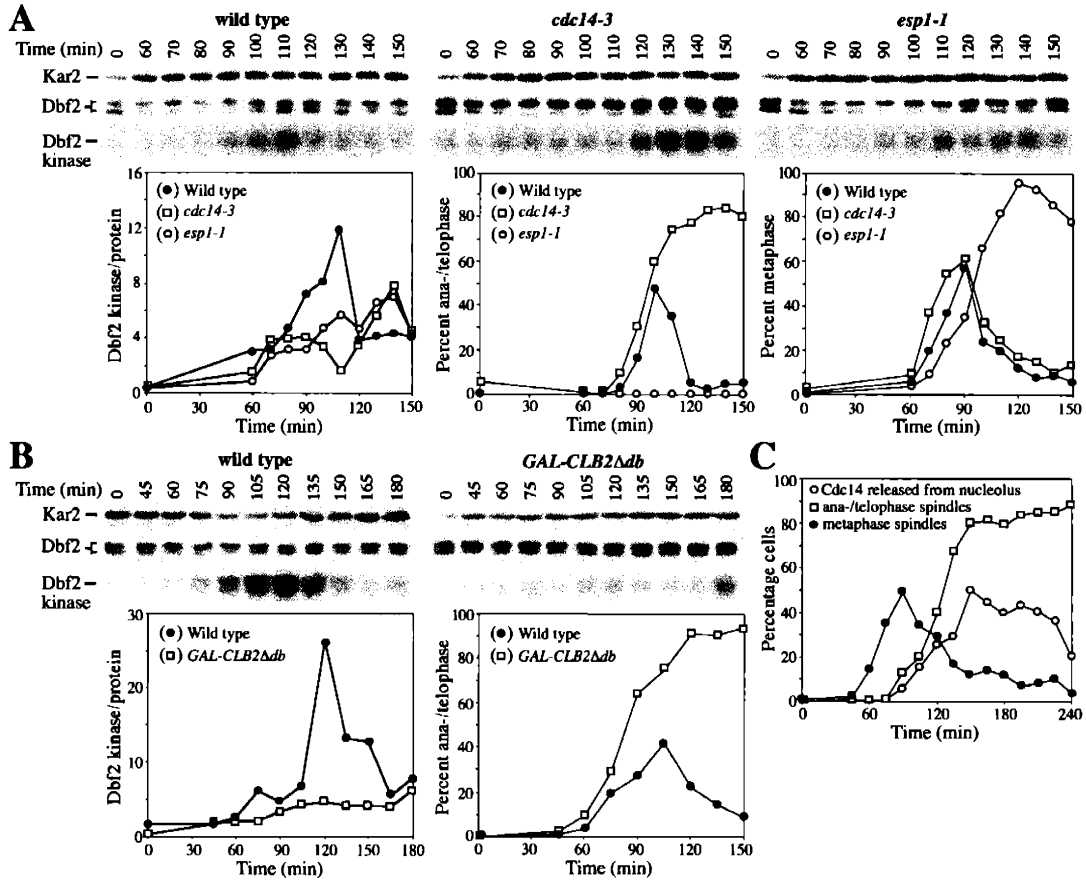

Figure 7: Dbf2 kinase activity is decreased in *cdc14-3* mutants and cells overexpressing *CLB2* lacking the destruction box.

(A) *DBF2-MYC* (A1931), *cdc14-3 DBF2-MYC* (A2179) and *esp1-1 DBF2-MYC* (A2253) cells were released from a G1 block at 37°C to determine the specific activity of Dbf2 kinase (autoradiograms and left graph), the percentage of cells with metaphase (right graph), and anaphase/telophase spindles (middle graph).

(B) *DBF2-MYC* (A1931) and *GAL-CLB2dbΔ DBF2-MYC* (A3680) cells were arrested with 3μg/ml α-factor in YEPR medium and released into YEPRG medium to determine the specific activity of Dbf2 kinase (autoradiograms and left graph) and the percentage of cells with anaphase/telophase spindles.

(C) *CDC14-HA* (A1411) and *GAL-CLB2dbΔ CDC14-HA* (A1731) cells were grown as described in Figure 7B and the percentage of cells with metaphase (closed circles), anaphase/telophase spindles (open squares), and Cdc14 released from the nucleolus (open circles) was determined.

Discussion

Given the central role of Cdc14 in promoting exit from mitosis, it is critical to understand how Cdc14 is regulated. We report here the identification of a new regulatory circuit termed the FEAR network that promotes the release of Cdc14 from the nucleolus during early anaphase, and show that it requires the activity of the separase Esp1, the polo kinase Cdc5, the kinetochore-associated protein Slk19, and Spo12, a protein of unknown function. Our findings also indicate that release of Cdc14 from the nucleolus is controlled in two steps, and by two separate networks. The FEAR network initiates Cdc14 release from Cfi1/Net1 during early anaphase, and MEN maintains Cdc14 in the released state during late anaphase and telophase. We propose that the two pathways integrate Cdc14 release from the nucleolus with different cellular events. The dual requirement for the FEAR component Esp1 in promoting both sister-chromatid separation and ensuring timely exit from mitosis ensures that Cdc14 release from the nucleolus is delayed until sister-chromatid separation has been initiated. Activation of MEN is controlled by at least one signal, the migration of the nucleus into the bud (reviewed in Bardin and Amon, 2001). By employing MEN to maintain Cdc14 in its released state, cells ensure that exit from mitosis does not occur prior to the delivery of a complement of the genetic material to the daughter cell.

A new regulatory network controlling Cdc14 activity.

A careful analysis of Cdc14 localization in MEN mutants revealed that Cdc14 was transiently released from the nucleolus during early anaphase (in cells with mitotic spindles

4–7 ∝m in length), but was rapidly re-sequestered into the nucleolus during later stages of anaphase, when the mitotic spindle extended beyond 7 ∝m. This transient release is readily detectable only when MEN mutants progress through the cell cycle in a synchronous manner and then only when Cdc14 localization is analyzed at 10 minute intervals. The finding that Cdc14 was released in more than 80 percent of MEN mutants with mitotic spindles 4-7 ∝m in length suggests that Cdc14 is released from the nucleolus during early anaphase in most, if not all, MEN mutants. The release of Cdc14 from the nucleolus during early anaphase is also not likely to be an artifact of employing MEN mutants. The onset of Cdc14 release from the nucleolus in MEN mutants occurs with similar kinetics as in wild type cells. The existence of two pathways promoting Cdc14 release from the nucleolus is not only evident in MEN mutants but also in nocodazole-arrested cells. When *MAD1* is deleted allowing *ESP1* to be active in nocodazole-treated cells, Cdc14 is released from the nucleolus as if no drug was added, but it is not maintained in the released state. Conversely, when *ESP1* activity is restrained, but MEN activity is not due to deletion of *BUB2*, Cdc14 release from the nucleolus occurs with a delay.

The transient release of Cdc14 from the nucleolus during early anaphase requires *ESP1, CDC5, SLK19* and *SPO12*. *CDC5* is essential not only for FEAR network-dependent release of Cdc14 from the nucleolus but also for maintaining Cdc14 in its released state. Cells lacking *ESP1, SLK19,* or *SPO12* are defective in releasing Cdc14 from the nucleolus only during early anaphase. Esp1's role in promoting Cdc14 release from the nucleolus appears more complex than that of Slk19 or Spo12. Our data indicate that defects in mitotic spindle elongation and/or sister-chromatid separation are in part responsible for the mitotic exit delay observed in *esp1-1* mutants. Furthermore, even under conditions where *ESP1* is

no longer required for mitotic spindle elongation and chromosome segregation, is the delay in mitotic exit more severe in *esp1-1* mutants than in *slk19Δ,* or *spo12Δ* mutants. Exit from mitosis is delayed by 40 minutes in *esp1-1* mutants, but only by 20 minutes in cells lacking *SLK19* or 10 - 15 minutes in *spo12Δ* cells. Determining whether *ESP1, SLK19, SPO12* and *CDC5* work together or in parallel to promote Cdc14 release from the nucleolus during early anaphase and identifying the mechanism(s) whereby the FEAR network (and MEN) accomplish this task are key questions that remain to be addressed.

It is important to note, that abolishing the FEAR network-dependent release of Cdc14 from the nucleolus during early anaphase, either by inactivating *ESP1* or deleting *SLK19* and/or *SPO12,* delays but does not prevent exit from mitosis. MEN eventually can promote the dissociation of Cdc14 from Cfi1/Net1 in the absence of the FEAR network. Indeed, when MEN is hyperactivated by deleting *BUB2*, the FEAR network is dispensable for exit from mitosis, as *esp1-1 mad 1Δ bub2Δ* mutants exit mitosis without delay (Shirayama et al., 1999). However, when MEN activity is decreased by deleting *LTE1*, the FEAR network becomes indispensable for this cell-cycle transition.

Functions of Cdc14 during early anaphase

Our data indicate that Cdc14 released during early anaphase by the FEAR network is active. A known substrate of Cdc14, Cdc15 is transiently dephosphorylated during early anaphase in a Cdc14-dependent manner. We also showed that this dephosphorylation was not due to MEN-dependent Cdc14 activity, as it occurred in *dbf2-2* mutants. Cdc14 is likely to dephosphorylate a range of substrates once it is released from the nucleolus during early anaphase but it cannot perform its key cell-cycle function. Cdc14 induces the switch

between mitosis, when Clb CDKs inhibit Clb cyclin degradation and accumulation of the CDK inhibitor Sic1, and G1, when Clb proteolysis and Sic1 inhibit Clb CDKs (Visintin et al., 1998; Jaspersen et al., 1999). Maintenance of Cdc14 in the nucleus and cytoplasm by MEN is needed for Cdc14 to successfully trigger this switch. In the absence of MEN this switch collapses probably because Cdc14 is not released from the nucleolus for a sufficient period of time.

One possible function of Cdc14 released by the FEAR network was that Cdc14 enhances the activity of MEN. There is good evidence that Cdc14 dephosphorylates and stimulates the activity of at least one MEN component, the protein kinase Cdc15 (Jaspersen and Morgan, 2000; Xu et al., 2000). Indeed, Cdc14 dephosphorylates Cdc15 during early anaphase in an *ESP1*-dependent but MEN-independent manner. Our data further indicate that *CDC14* is required for full activation of the MEN component Dbf2. We conclude that Cdc14 released from the nucleolus by the FEAR network is important for full MEN activity and thus for maintaining itself in the released state. Whether Cdc15 is the only MEN component activated by Cdc14 or whether other potential Cdc14 substrates in this pathway such as Lte1 and Dbf2 (Visintin and Amon, 2001; Anupama Seshan, personal communication) are activated by dephosphorylation remains to be determined.

A model for regulating mitotic exit: Two-step control of Cdc14.

At the onset of anaphase, the FEAR network is activated at least in part by the degradation of Pds1, and induces the release of Cdc14 from the nucleolus. MEN activation occurs once the Tem1-bearing SPB moves into the bud during anaphase (reviewed in McCollum and Gould, 2000; Bardin and Amon, 2001) but Cdc14 liberated by the FEAR

network further stimulates MEN activity by dephosphorylating Cdc15. Activated MEN then maintains Cdc14 in its released state during late anaphase and telophase. How this positive feedback loop is broken, so that Cdc14 returns into the nucleolus when mitotic CDKs are inactivated, is an important question that remains to be addressed.

When overexpressed *ESP1* induces premature exit from mitosis in metaphase-arrested cells (Tinker-Kulberg and Morgan, 1999). Overexpression of *CDC5* causes Cdc14 release from the nucleolus in metaphase cells and premature exit from mitosis (Charles et al. 1998; Shirayama et al., 1998; R. V. unpublished observations). High levels of *SPO12* suppress the temperature sensitive lethality of *tem1-3, cdc15-2, dbf2-2* and *cdc5-1* mutants (Jaspersen et al., 1998). Thus, overexpression of FEAR network components can induce a prolonged release of Cdc14 from the nucleolus, sufficient to induce exit from mitosis. Why the FEAR network only induces a transient release of Cdc14 from the nucleolus, that is insufficient to induce exit from mitosis is unclear. Perhaps, the activity of one or more components of the FEAR-network is restricted to anaphase causing the "release signal" generated by the FEAR network to be short lived. Alternatively, on a more speculative note, it is possible that sustained release of Cdc14 from the nucleolus requires export into the cytoplasm, which requires MEN-dependent modifications of Cdc14. In this regard it is interesting to note that Cdc14 released by the FEAR network is predominantly localized in the nucleus, but in wild-type cells or nocodazole-treated *bub2Δ* cells, Cdc14 is also found in the cytoplasm.

The two-step mode of release of Cdc14 from the nucleolus appears to be conserved at least between *S. pombe* and *S. cerevisiae*. In *S. pombe*, the Cdc14 homolog Clp1/Flp1 is released from the nucleolus during early mitosis and the septation initiation network, the

homologous pathway of MEN, is required to maintain Clp1/Flp1 in its released state (Cueille et al., 2001; Trautmann et al., 2001). Whether a pathway analogous to that of the FEAR network exists that initiates Clp1/Flp1 release during early anaphase is not known. In closing we note that the fact that *CDC5, CDC14, SLK19* and *SPO12* are required for ensuring that homologous chromosomes and not sister chromatids segregate during meiosis I (Klapholz and Esposito, 1980, Sharon and Simchen, 1990, Kamieniecki et al., 2000), raises the intriguing possibility that the FEAR network and Cdc14 are at least in part responsible for bringing about the meiotic chromosome segregation pattern.

Experimental Procedures

All strains were derivatives of strain W303 (K699). The *GAL-GFP-LTE1* fusion, the *lte1::kan*^{R}, *spo12::HIS5* and *slk19:: kan*^{R} deletion were constructed using the PCR-based method described by Longtine et al. (1998). The *esp1-td-18MYC* construct was created by fusing the heat-inducible *degron* (Dohmen et al., 1994) to the *MET3* promoter. A *URA3* gene was inserted upstream of the *MET3* promoter and served as a selectable marker during transformation. The *MET3-degron* was then introduced at the N-terminus of *ESP1* using a PCR-based method as described by Longtine et al. (1998). *CDC14-HA*, *CDC15-HA*, and *DBF2-MYC* were described in Taylor et al. (1997), Jaspersen et al. (1998) and Visintin and Amon (2001), respectively. All *SLK19* constructs are described in Sullivan et al. (2001).

Growth conditions for individual experiments are described in the figure legends. Immunoblot analysis of the total amount of Clb2, Esp1-18Myc, Cdc15-HA, Lte1-GFP, Kar2 and Dbf2-Myc was performed as described in Cohen-Fix, et al. (1996). Dbf2 and Clb2 kinase activity were assayed as described in Visintin and Amon (2001) and Surana et al. (1993), respectively. Indirect in situ immunofluorescence methods and antibody concentrations were as described in Visintin et al. (1999). The length of mitotic spindles was measured with the Openlab3.0.2 image program. 2μm long spindles were defined as metaphase spindles, 2-7 μm as early anaphase and 7-12 μm as late anaphase/telophase spindles.

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Chapter III

The role of the polo kinase Cdc5 in controlling Cdc14 localization.

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Eperiments shown in Figures 1-5 were performed by F.S. and R.V.

Experiments shown in Figures 6-11 were performed by R.V.

Summary

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 *S. 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, as 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, as 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, as 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, 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 appears not to be required for its mitotic exit function (Sullivan and Uhlmann, 2003). The FEAR network component Slk19 localizes to kinetochores and the spindle midzone 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 causes 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.

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* as high levels of Slk19 caused

Figure 1 A-B

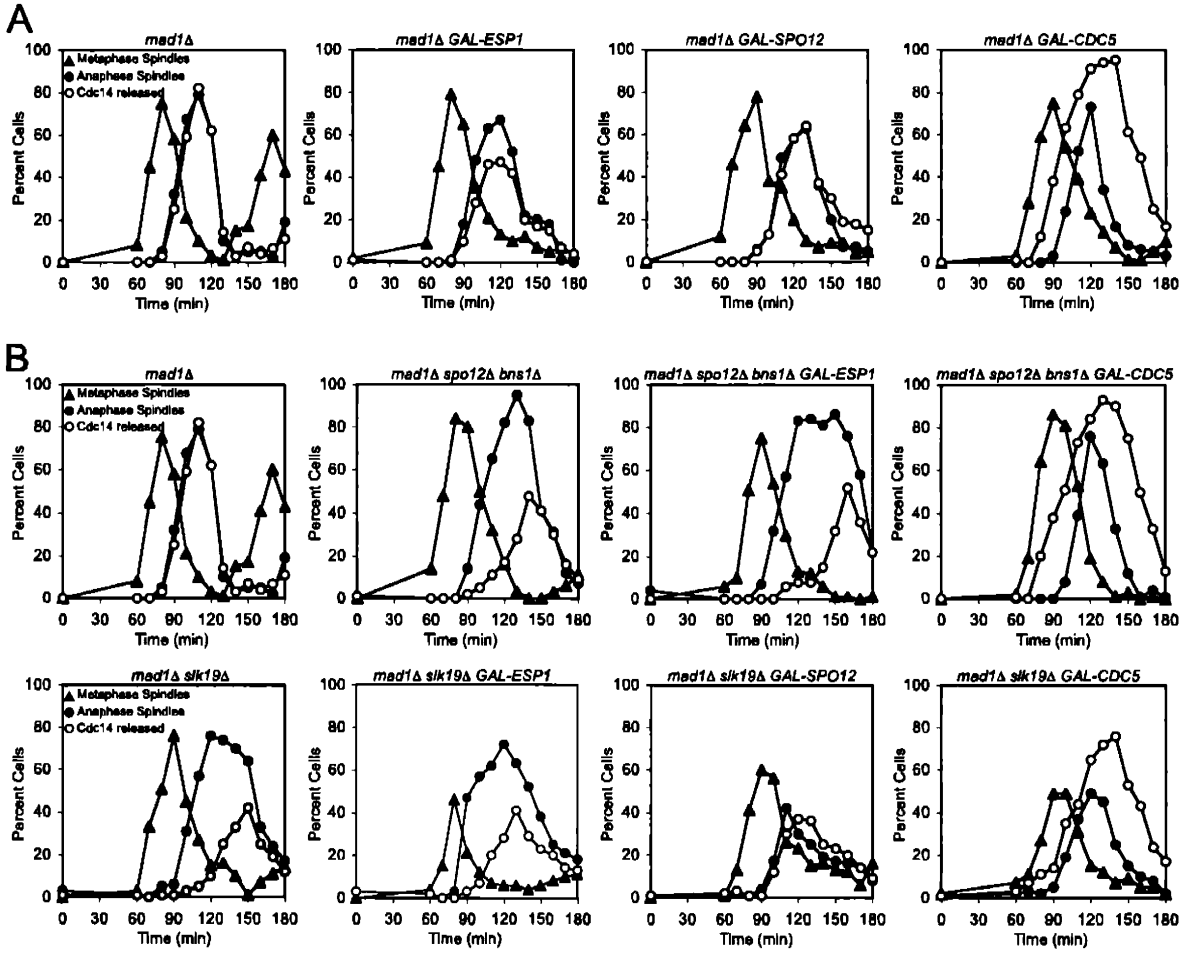
A
mad1Δ
mad1Δ GAL-ESP1
mad1Δ GAL-SPO12
mad1Δ GAL-CDC5
Metaphase Spindles
Anaphase Spindles
Cdc14 released
Percent Cells
Time (min)
B
mad1Δ
mad1Δ spo12Δ bns1Δ
mad1Δ spo12Δ bns1Δ GAL-ESP1
mad1Δ spo12Δ bns1Δ GAL-CDC5
mad1Δ slk19Δ
mad1Δ slk19Δ GAL-ESP1
mad1Δ slk19Δ GAL-SPO12
mad1Δ slk19Δ GAL-CDC5

Figure 1C

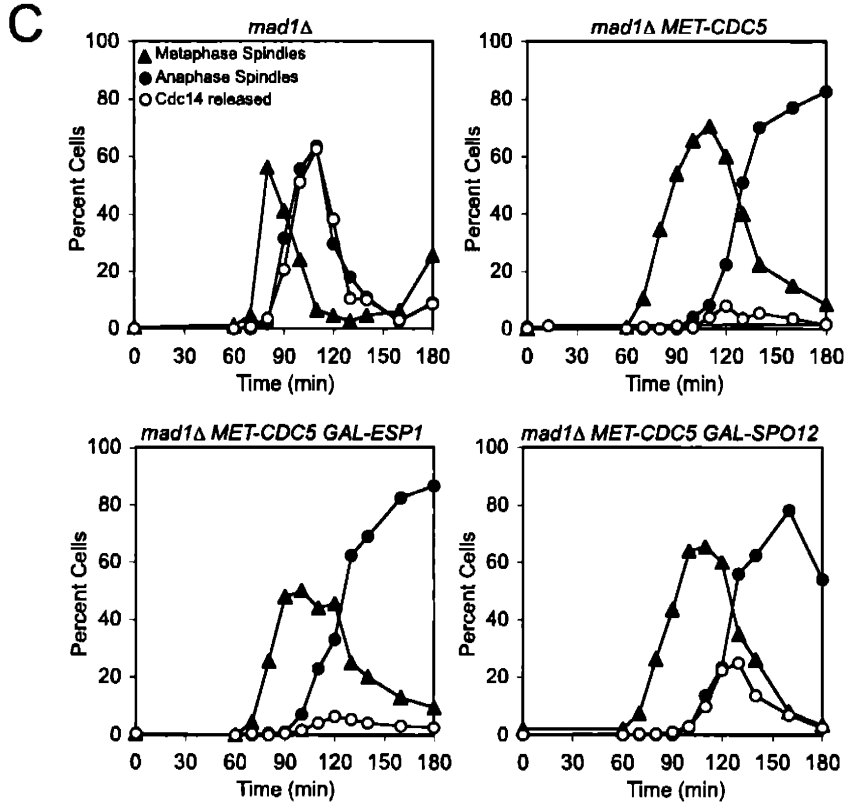

Figure 1: Epistatic relationships among FEAR network components.

(A) *mad1*Δ (A2853), *mad1*Δ *GAL-ESP1* (A7619), *mad1*Δ *GAL-SPO12* (A6490), and *mad1*Δ *GAL-CDC5* (A7297) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEP medium containing 2% raffinose (YEPR) with α-factor (5μg/ml) for 3 hours. 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) *mad1*Δ *spo12*Δ *bns1*Δ (A5408), *mad1*Δ *spo12*Δ *bns1*Δ *GAL-ESP1* (A7619) *mad1*Δ *spo12*Δ *bns1*Δ *GAL-CDC5* (A7578), *mad1*Δ *slk19* Δ (A4302), *mad1*Δ *slk19* Δ *GAL-ESP1* (A7302), *mad1*Δ *slk19* Δ *GAL-SPO12* (A7581), *mad1*Δ *slk19* Δ *GAL-CDC5* (A8032) cells carrying a *CDC14-3HA* fusion were grown and analyzed as described in (A).
(C) *mad1*Δ (A2853), *mad1*Δ *MET-CDC5* (A6560), *mad1*Δ *MET-CDC5 GAL-ESP1* (A6562), and *mad1*Δ *MET-CDC5 GAL-SPO12* (A6630) cells carrying a *CDC14-3HA* fusion were grown in medium lacking methionine and arrested in G1 with α-factor (5μg/ml). 2% galactose was added 1 hour prior to release, and cells were released into YEPRG medium containing 8mM methionine 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.

spindle abnormalities precluding the reliable identification of cells with anaphase spindles (data not shown).

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; Figure 2).

Next we determined whether overexpression of FEAR network components rendered *ESP1* dispensable for Cdc14 release from the nucleolus during early anaphase. As *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

Figure 2

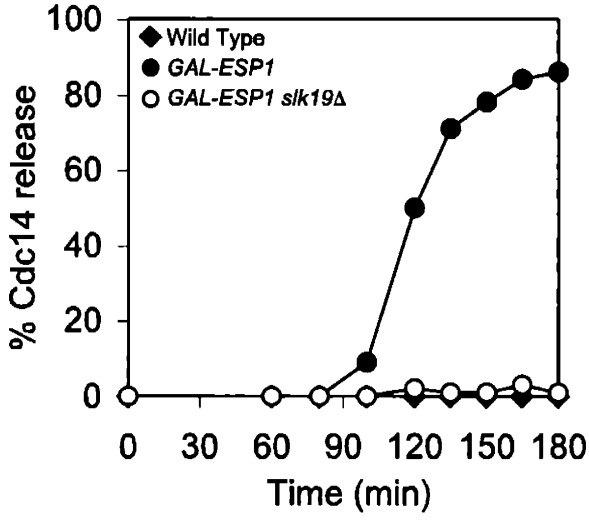

Figure 2: Cdc14 release induced by high levels of Esp1 depend on SLK19.

Wild type (A1411), *GAL-ESP1*(A4150), and *GAL-ESP1 slk19*Δ (A7234) cells all carrying a *CDC14-3HA* fusion were arrested in G1 in YEPR medium with α-factor (5μg/ml) and subsequently released in a synchronous manner into YEPRG lacking pheromone at 25°C in presence of 15μg/ml of nocodazole. The percentage of cells with Cdc14-HA released from the nucleolus was determined at the indicated times (Wild type (close diamonds), *GAL-ESP1*(close circles) and *GAL-ESP1 slk19*Δ (open circles)).

SPO12 restored release of Cdc14 from the nucleolus during early anaphase in *esp1-1 mcd1-1* mutants (Figure 3). 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 (Figure 4). Thus, *BNS1* 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.

Figure 3

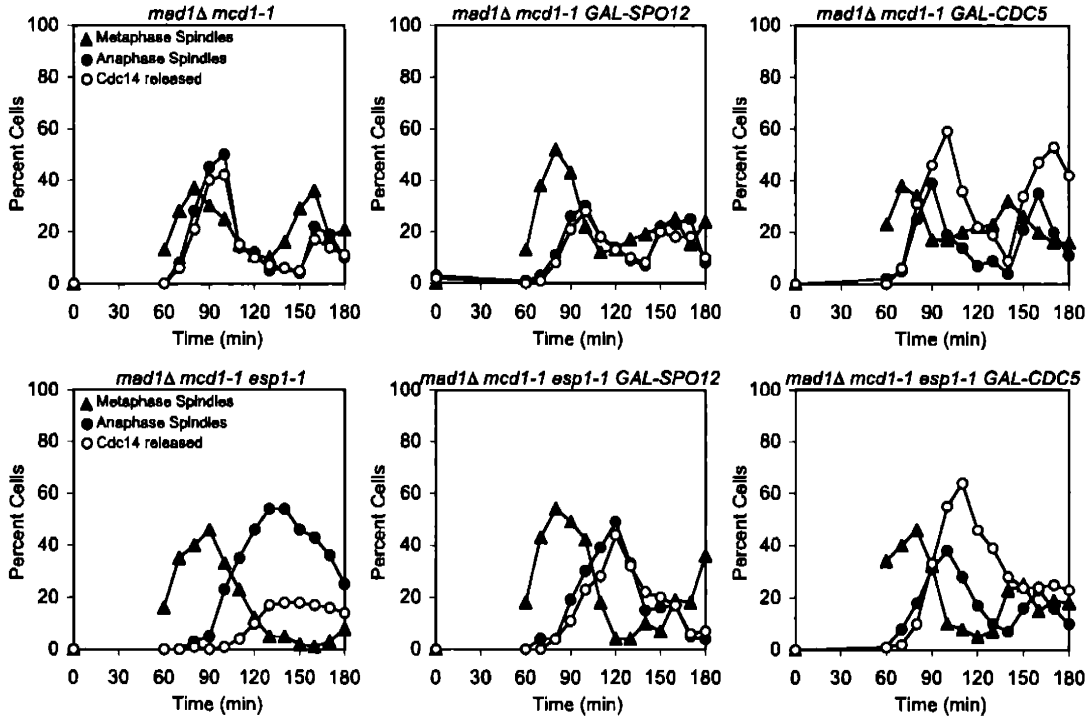

Figure 3: High levels of Cdc5 and Spo12 allow *esp1-1* to release Cdc14 from the nucleolus during early anaphase.

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.

Figure 4

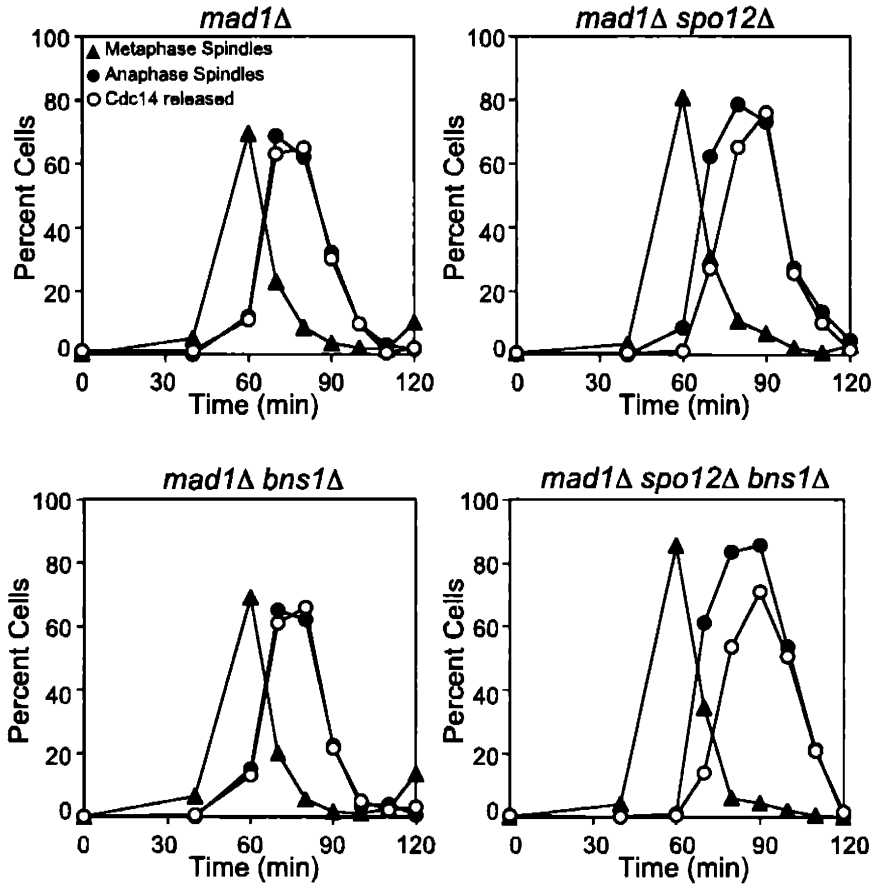

Figure 4: *BNS1* contributes to *SPO12*'s mitotic exit function.

*mad1*Δ (A2853), *mad1*Δ *spo12*Δ (A4502), *mad1*Δ *bns1*Δ (A5514), *mad1*Δ *spo12* Δ *bns1*Δ (A5408) cells all carrying a *CDC14-3HA* fusion were arrested in G1 in YEPD medium with α-factor (5μg/ml) and subsequently released in a synchronous manner into YEPD 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. We analyzed the kinetics of exit from mitosis in these mutants in a background lacking the spindle checkpoint component *MAD1*, to rule out the possibility that delays in exit from mitosis observed in the mutants was due to activation of the spindle checkpoint.

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 employing 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). As 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 using α-factor pheromone and transcription of *CDC5* was repressed by methionine addition one hour prior to 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-depeleted cells was bypassed by the *TAB6-1* mutation, a dominant allele of *CDC14* (Data not shown; 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 (Data not shown). However these cells failed to form colonies, perhaps due to the severe cytokinesis defects observed in these cells (data not shown). 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 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 5A, 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 5C).

Figure 5

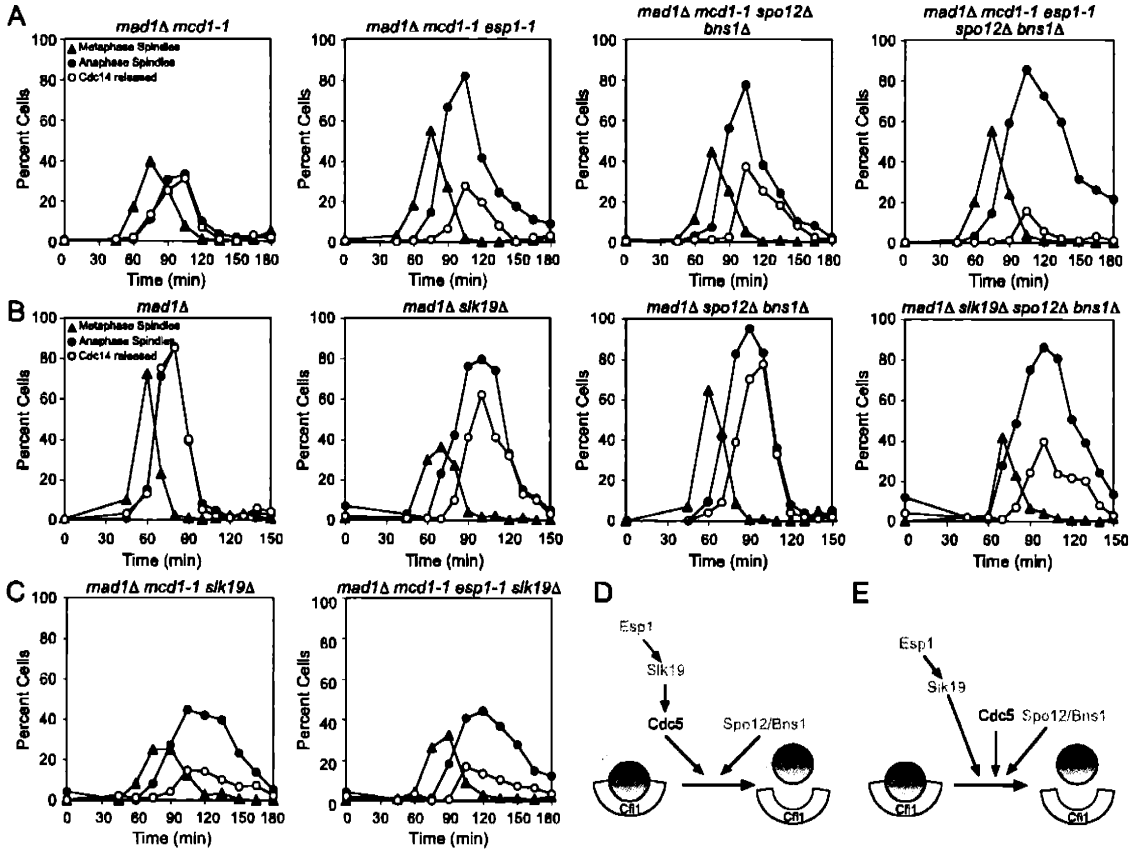

Figure 5: *SPO12* and *BNS1* function in parallel to *ESP1* and *SLK19*.

(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 (5μg/ml) and subsequently released into YEPD lacking pheromone at 37°C. α-factor was re-added 90 minutes after release to prevent entry into a subsequent cell-cycle. 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 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 (5μg/ml) and subsequently released in a synchronous manner into YEPD lacking pheromone at 25°C. α-factor pheromone was re-added 80 minutes 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,E) Two models for order of function within the FEAR network.

In summary our epistasis analyses indicate that *ESP1* and *SLK19* function in the same pathway and that *SLK19* functions downstream of *ESP1* (Figure 5D, 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 5D, 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 (Figure1, Figure 3, Data not shown). The findings that (1) inactivation of *SPO12* and *BNS1* enhances the mitotic exit defect of *slk19Δ* and *esp1-1* mutants, (2) that overexpression of *SPO12* at least partially bypasses the requirement of *CDC5, ESP1* and *SLK19* in Cdc14 release from the nucleolus and (3) that 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 5D, 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 9A) 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 over-activation 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 *GAL-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 6A). 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 6B). 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 *GAL-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 6C; 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

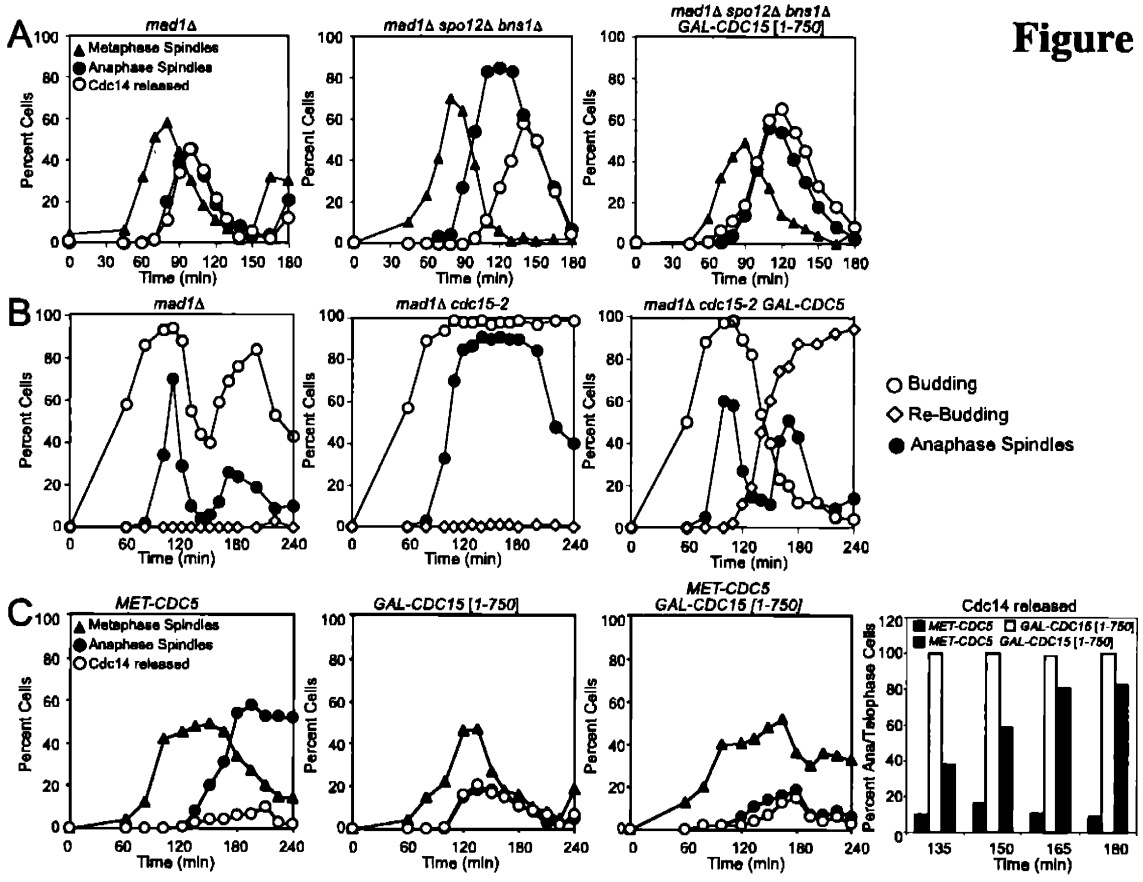

Figure 6

Figure 6: Hyperactivation of the MEN can suppress the mitotic exit defect of FEAR network mutants.

(A) *mad1Δ* (A2853), *mad1Δ spo12Δ bns1Δ* (A5408), *mad1Δ spo12Δ bns1Δ bub2Δ* (A8864), and *mad1Δspo12Δ bns1Δ GAL-CDC15[1-750]* (A8708) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEPR with α-factor (5μg/ml) followed by released in YEPRG lacking pheromone. The percentage of cells with metaphase (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 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 (5μg/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 (close 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 (5μg/ml). 8mM methionine was added 1 hour prior to release, and cells were released into YEPRG medium containing 8mM methionine at 25°C. The percentage of cells with metaphase (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. The histogram on the right shows the percentage of Cdc14 release in anaphase/telophase cells. 100 cells were scored for each time point.

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 5D, 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 appeared to play a central role in promoting the release of Cdc14 release from the nucleolus as *CDC5* was the only gene, which when overexpressed, caused premature release of Cdc14 from the nucleolus during 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 (Figure 7A, 8C) 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

Figure 7

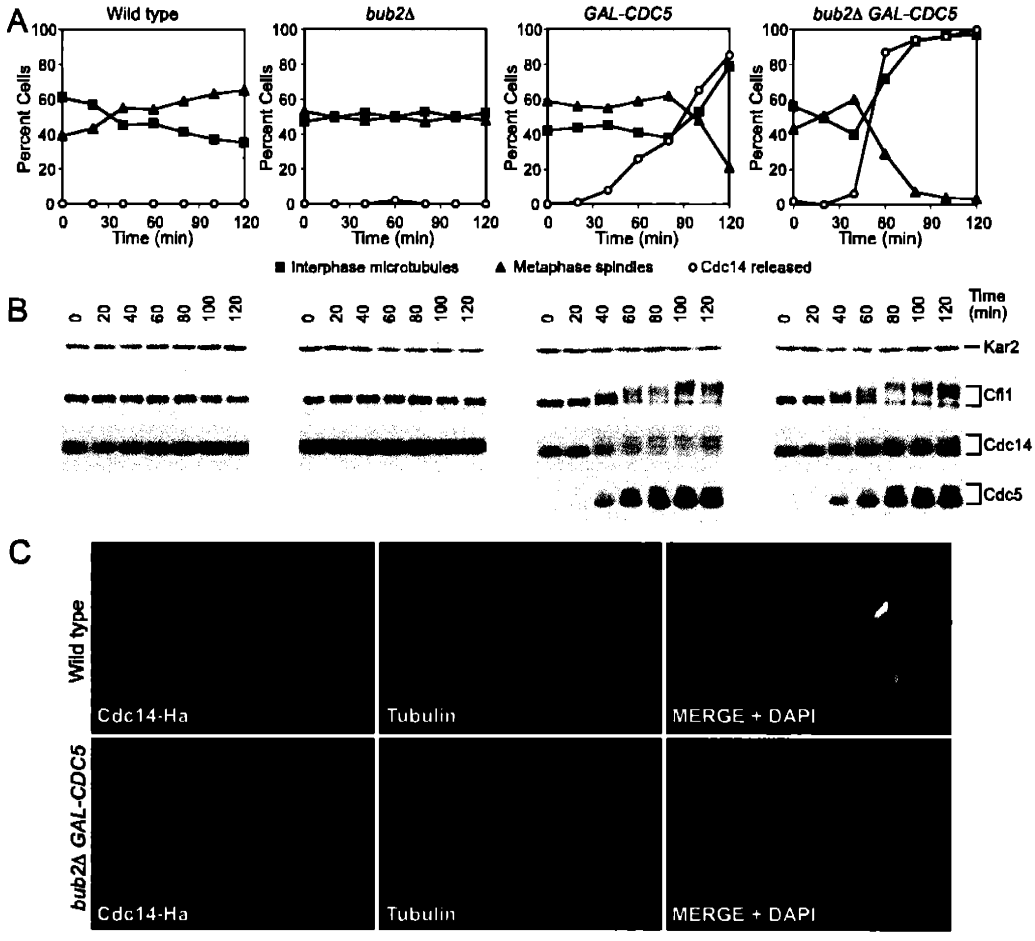

Figure 7: Overexpression of *CDC5* causes release of Cdc14 from the nucleolus in hydroxyurea-arrested cells, which is enhanced by the deletion of *BUB2*.

(A) Wild-type (A1681), *bub2*Δ (A4451), *GAL-CDC5* (A4450), and *GAL-CDC5 bub2*Δ (A4453) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion were arrested in early S phase with hydroxyurea (HU; 10 mg/ml) in YEPR medium at 25°C. After 2.5 hours 5mg/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.

(C) The pictures show Cdc14 localization in wild type and *GAL-CDC5 bub2*Δ cells 80 minutes after galactose addition. Cdc14 is shown in green, microtubules in red and nucleus in blue.

Cdc14 from the nucleolus in S phase or metaphase-arrested cells on its own (data not shown).

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.*, 2000; Visintin and Amon, 2001). Cells deleted for *BUB2* did not release Cdc14 from the nucleolus in a HU-arrest, but inactivation of *BUB2* enhanced the ability of *CDC5* to promote Cdc14 release from the nucleolus (Figure 7A). In *GAL-CDC5 bub2Δ* cells Cdc14 release from the nucleolus occurred very efficiently and as soon as Cdc5 protein accumulated, 40 minutes after galactose addition (Figure 7A, 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* (data not shown). Overexpression of other MEN or FEAR network components did not cause Cdc14 release from the nucleolus in S phase arrested cells (data not shown). Our results show that overexpression of *CDC5* and the simultaneous over-activation 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 8A, C). In contrast, inactivation of the FEAR network component *ESP1* did not hamper Cdc5's ability to promote Cdc14 from the nucleolus (Figure 8E). 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* cells (Figure 6A). 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 (data not shown).

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

Figure 8

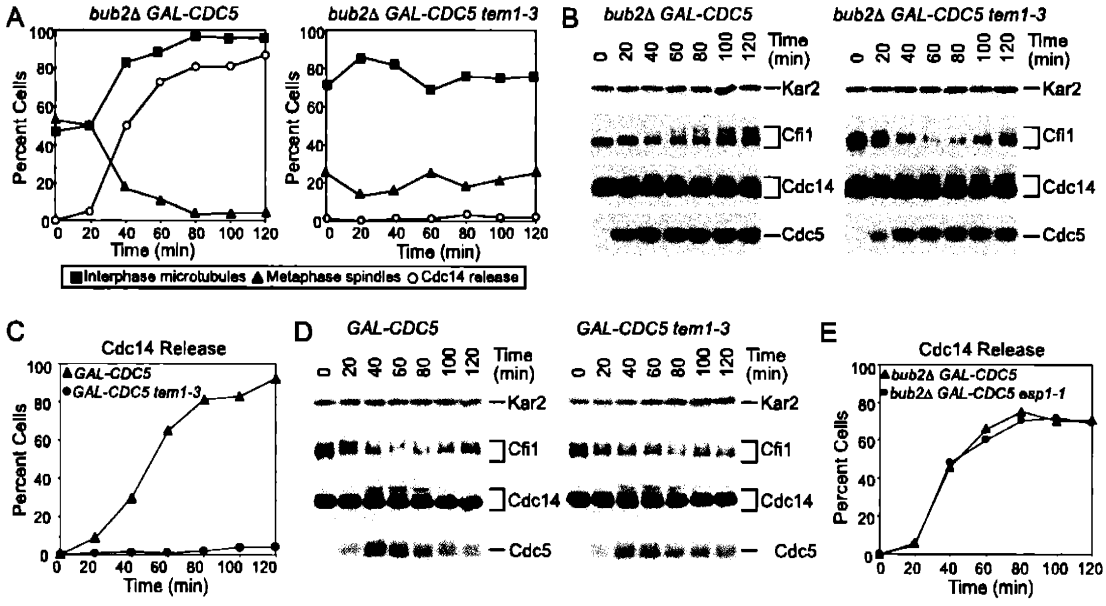

Figure 8: The ectopic release of Cdc14 from the nucleolus brought about by *GAL-CDC5* and deletion of *BUB2* depends on *TEM1*.

(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 7 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 depolymerazing drug nocodazole (15μg/ml) in YEPR at 25°C. After 2.5 hours, 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 10mg/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.

effects of overexpressing *CDC5* and deleting *BUB2* on Dbf2 kinase activity. Overexpression of *CDC5* slightly stimulated Dbf2 kinase activity but deletion of *BUB2* together with the overexpression of *CDC5* led to high levels of Dbf2 kinase activity (Figure 9A). 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 9B). 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 7B). These slower migrating forms of Cdc14 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; data not shown). 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

Figure 9

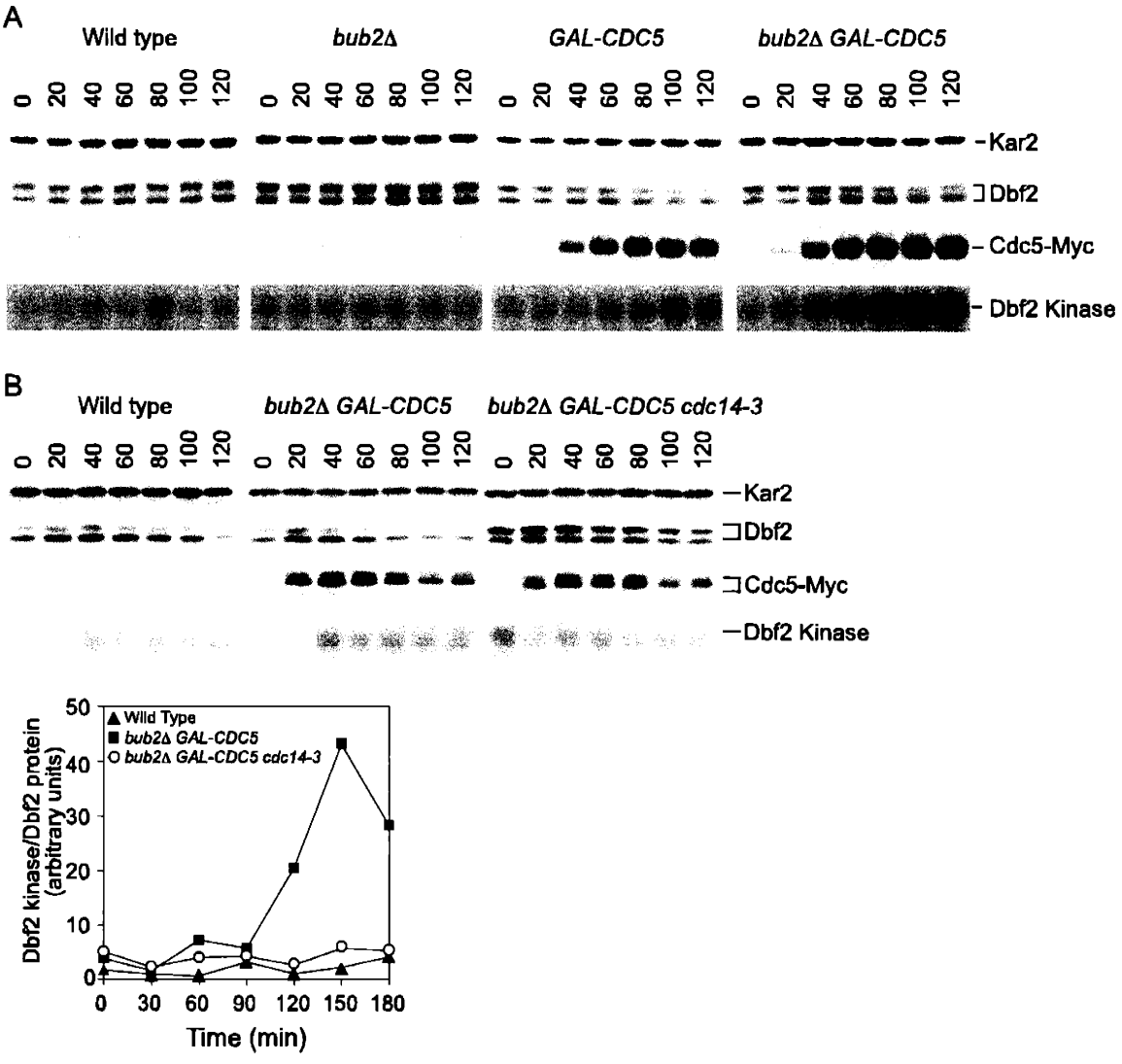

Figure 9: Overexpression of *CDC5* stimulates MEN activity.

(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 7. 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 10mg/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.

progressing through the cell cycle in a synchronous manner (Figure 10). 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 10B), 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 8B, D), phosphorylation of Cfi1/Net1 was greatly reduced in *GAL-CDC5 bub2Δ tem1-3* cells compared to *GAL-CDC5 bub2Δ* cells (Figure 8B, D). Similar results were obtained when the MEN was inactivated using a *cdc15-2* mutation (data not shown), 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 11). Phosphorylation of Cfi1/Net1 was independent of

Figure 10

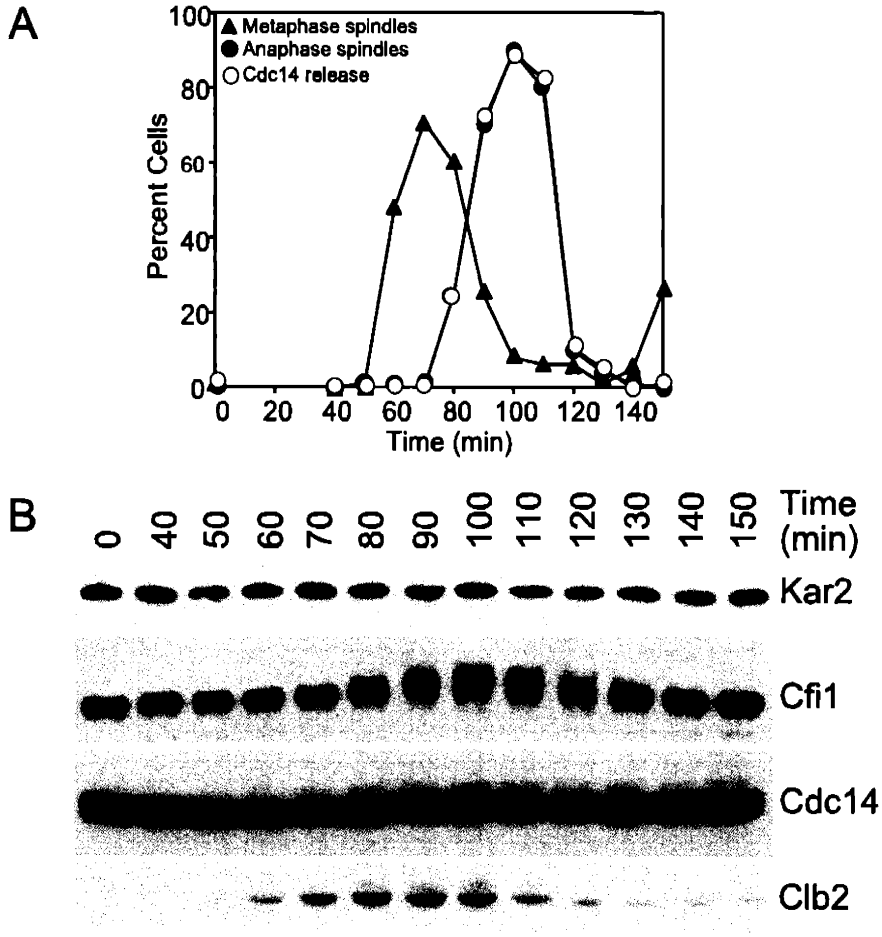

Figure 10: Cdc14 and Cfi1/Net1 are phosphorylated during mitosis.
Cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusion (A1681) were arrested in G1 in YEPD medium with α-factor (5μg/ml) followed by release into YEPD medium lacking pheromone at 25°C. The percentage of cells with metaphase (closed triangles, 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.

CDC5 (data not shown) 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 11B). 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 appeared 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 8). Thus, it appears that only when both, Cdc14 and Cfi1/Net1 are phosphorylated can the phosphatase be released from its inhibitor.

Figure 11

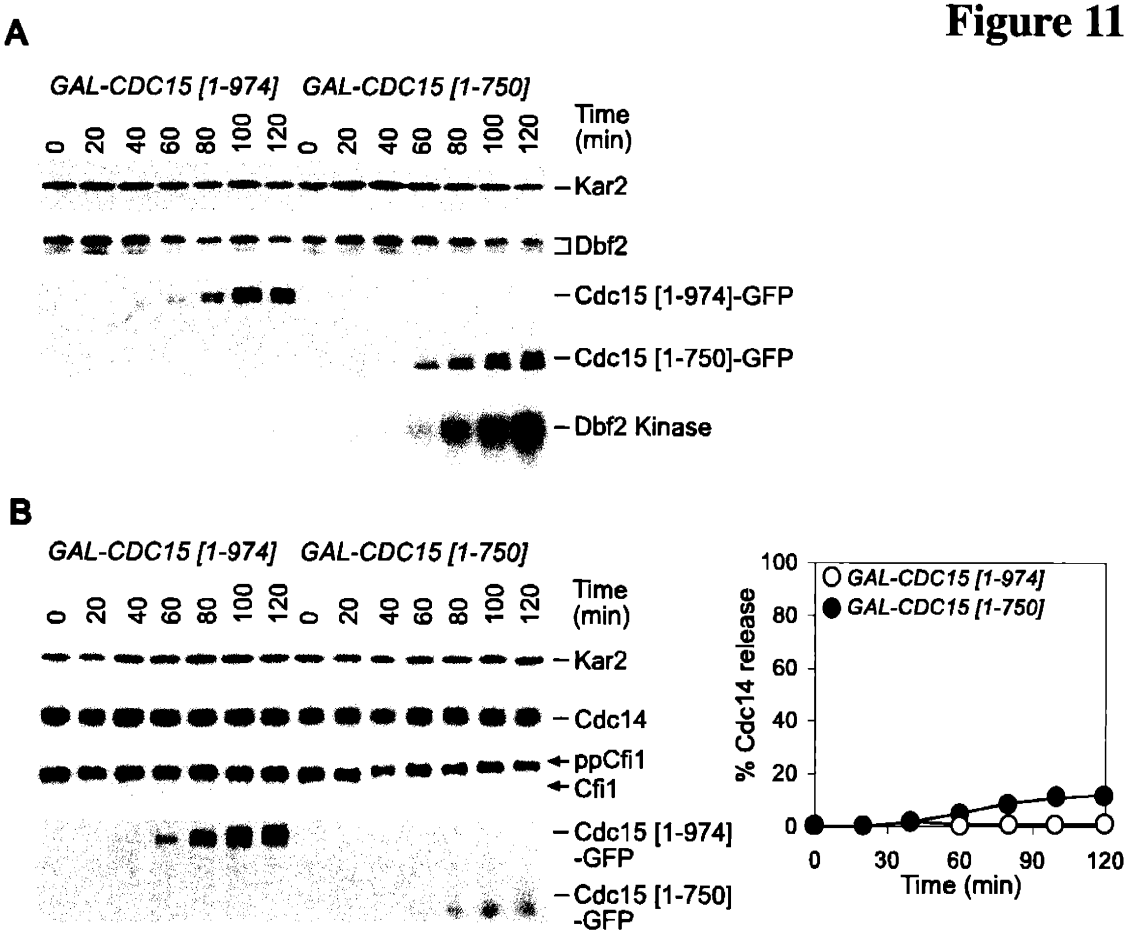

Figure 11: Hyperactivation of MEN is not sufficient to promote Cdc14 release in hydroxyurea arrested cells.

(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 hours 5mg/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 11A. 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];* close 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.

Discussion

The 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 (Figure 2; Sullivan and Uhlmann, 2003. All our data indicate that *SPO12* and *BNS1* constitute a separate branch of the FEAR network (Figure 5D, 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 appear 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* (Figure 2; 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 release from its inhibitor but is also necessary for Cdc14 re-sequestration after exit from mitosis has been completed.

Spo12's role in the FEAR network.

Inactivation of the *SPO12* homolog *BNS1*, while 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.

The 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 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; Sullivan and Uhlmann, 2003; Bardin *et al.*, 2003; R. V. 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.

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 (R. V., 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 7). 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 (R. V. unpublished observations). Thus, while it is possible that Cdc5 directly phosphorylates Cfi1/Net1, the bulk phosphorylation of the protein that occurs in vivo appears 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. 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 release 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.

Experimental Procdedures

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); Bardin *et al.*, (2003) respectively. The *CFI1-GFP* and *DBF2-3HA* fusion were constructed by using the PCR-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 minutes in 10% TCA at 4°C, pelleted and then washed with acetone. Cells were broken in 50mM TRIS pH 7.5, 1mM EDTA, 1mM PNP, 50mM DTT, 1mM PMSF and 2μg/ml pepstatin with glass beads for 40 minutes and boiled in 1x 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 a Zeiss Axioplan 2 microscope and images were captured with a Hamamatsu camera controller. Openlab 3.0.2.software was used to process immunofluorescence images.

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Chapter IV

The replication fork block protein Fob1 functions as a negative regulator of the FEAR network.

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Stegmeier F., Huang, J., Rahal R., Zmolik J., Moazed D., Amon, A.

The replication fork block protein Fob1 functions as a negative regulator of the FEAR network.

Current Biology in press.

Julie Huang conducted the experiment shown in Figure 9. Jessica Zmolik helped with the Fob1 two-hybrid screen. Rami Rahal helped with the co-IPs shown in Figure 11.

Summary

The protein phosphatase Cdc14 is a key regulator of exit from mitosis in budding yeast. Its activation during anaphase, which is characterized by Cdc14's dissociation from its inhibitor Cfi1/Net1 in the nucleolus, is controlled by two regulatory networks. The Cdc14 Early Anaphase Release (FEAR) network promotes activation of the phosphatase during early anaphase, whereas the Mitotic Exit Network (MEN) activates Cdc14 during late stages of anaphase. Here we investigate how the FEAR network component Spo12 regulates Cdc14 activation. We identify the replication fork block protein Fob1 as a Spo12-interacting factor. Our results suggest that Fob1 functions as a negative regulator of the FEAR network. Inactivation of *FOB1* leads to premature release of Cdc14 from the nucleolus in metaphase-arrested cells. Conversely, high levels of *FOB1* delay the release of Cdc14 from the nucleolus. Fob1 associates with Cfi1/Net1 and consistent with this observation we find that the bulk of Cdc14 localizes to the Fob1 binding region within the rDNA repeats. Finally, we show that Spo12 phosphorylation is cell cycle regulated and affects its binding to Fob1. We propose that Fob1 helps to prevent the dissociation of Cdc14 from Cfi1/Net1 prior to anaphase and that Spo12 activation during early anaphase promotes the release of Cdc14 from its inhibitor by antagonizing Fob1 function.

Introduction

Cell cycle progression must be carefully regulated to preserve genomic integrity. The successful completion of each cell cycle phase requires the orchestration of multiple cellular events. During exit from mitosis, for example, cells need to coordinate the completion of chromosome segregation with mitotic spindle disassembly and cytokinesis. Exit from mitosis is the transition from the mitotic state, characterized by high mitotic cyclin dependent kinase (CDK) activity, to the G1 state, when mitotic CDK activity is low. This down-regulation of mitotic CDK activity is accomplished by the ubiquitin-dependent degradation of the regulatory mitotic cyclin subunit and up-regulation of the mitotic CDK inhibitor Sic1 (reviewed in [1-3]). The protein phosphatase Cdc14 plays an essential role in promoting these events. Cdc14 reverses CDK phosphorylation, thereby triggering mitotic cyclin degradation and Sic1 accumulation [4, 5]. Consistent with its essential role in exit from mitosis, the activity of Cdc14 is tightly regulated throughout the cell cycle [6-8]. During G1, S, G2, and early M phase, Cdc14 is held inactive within the nucleolus by its competitive inhibitor Cfi1/Net1 but is released from its inhibitor during anaphase, thereby allowing it to dephosphorylate its substrates.

In addition to its mitotic exit function, Cfi1/Net1 also regulates rDNA silencing as part of a nucleolar complex termed RENT (regulator of nucleolar silencing and telophase exit) [9]. The RENT complex harbors Sir2, the only SIR protein required for rDNA silencing, in addition to Cdc14 and Cfi1/Net1 [6, 9]. Furthermore, a recent study identified a role for the replication fork block protein Fob1 in rDNA silencing [10]. Whether Sir2 and Fob1, like Cfi1/Net1, control both rDNA silencing and exit from mitosis is not known.

Two regulatory networks control the association of Cdc14 with its inhibitor. The FEAR network (Cdc Fourteen Early Anaphase Release Network) is activated at the metaphase-anaphase transition and initiates the release of Cdc14 from the nucleolus [11-13]. The MEN (Mitotic Exit Network), a GTPase signaling cascade, further promotes the release of Cdc14 and maintains the phosphatase in its released state during later stages of anaphase and telophase [6, 7]. Cdc14 activation mediated by the MEN is required for mitotic exit, as temperature sensitive mutants in MEN components arrest in late anaphase. In contrast, FEAR network-induced Cdc14 activation is not essential for mitotic exit, as mutations in FEAR network components cause a delay but do not preclude exit from mitosis. Though not essential for mitotic exit, FEAR network-induced activation of Cdc14 during early anaphase is required for full activation of the MEN [11, 12].

To date, five components of the FEAR network have been identified. These are the separase Esp1, the polo-like kinase Cdc5, the kinetochore protein Slk19, and the small nuclear/nucleolar protein Spo12 and its homologue Bns1 [11, 14]. Esp1 encodes a protease that is best known for its role in triggering sister-chromatid separation at the onset of anaphase (reviewed in [15, 16]). Slk19 belongs to the family of passenger proteins that localize to kinetochores during metaphase and to the spindle midzone during anaphase [17]. Furthermore, Slk19 is a substrate of Esp1 [18]. Surprisingly however, neither cleavage of Slk19 [11] nor Esp1's proteolytic activity [19] is required for FEAR network function. The small protein Spo12 and its homologue Bns1 localize to the nucleus and nucleolus and contain a highly conserved 20 amino acid motif [20, 21], but their molecular function remains unknown. Recent genetic epistasis analyses revealed that the FEAR network consists of two branches. One branch encompasses *ESP1* and

SLK19 [14, 19]. *SPO12* and *BNS1* function in parallel to *ESP1* and *SLK19* [14]. *CDC5* could not be placed unequivocally within the FEAR network in this genetic analysis, because *CDC5* is not only a component of the FEAR network but also a regulator of the MEN [14].

The molecular mechanisms whereby the FEAR network promotes the release of Cdc14 from its inhibitor are poorly understood. Cdc5 is, however, likely to be the ultimate effector in the FEAR network [14, 19]. The protein kinase induces the phosphorylation of both Cdc14 and Cfi1/Net1, which is thought to promote the dissociation of the complex [14, 22, 23]. How the other FEAR network components contribute to the release of Cdc14 from the nucleolus during early anaphase remains unknown. They could function to promote Cdc5 activation or contribute in parallel to the dismantling of the Cdc14 – Cfi1/Net1 complex. To begin to address how Spo12 promotes Cdc14 activation, we identified the regions within Spo12 and Spo12-associated factors that regulate the interaction between Cdc14 and its inhibitor. We show that Spo12's C-terminal domain and post-translational modifications within this region are essential for Spo12 function. Furthermore, we identify the replication fork block protein Fob1 as a Spo12-interacting factor and show that phosphorylation of Spo12 influences its binding to Fob1. Finally, we show that *FOB1* is an inhibitor of the FEAR network that forms a complex with Cfi1/Net1. We propose that Fob1 antagonizes Cdc14-Cfi1/Net1 complex dissociation prior to anaphase and that activation of Spo12 during early anaphase promotes the release of Cdc14 from its inhibitor by counteracting Fob1 function.

Results

The highly conserved DSP-Box is essential for Spo12's mitotic exit function.

To identify the regions within *SPO12* important for its mitotic exit function, we constructed truncations of *SPO12* under the control of the galactose-inducible *GAL1-10* promoter. To examine the functionality of these truncations we exploited the facts that (1) deletion of *SPO12* lowers the restrictive temperature of cells carrying a temperature sensitive allele of the MEN kinase *CDC15* (Figure 1B, *cdc15-2 spo12Δ*), and that (2) overexpression of *SPO12* suppresses the growth defect of *cdc15-2 spo12Δ* cells (Figure 1B, fifth row, [5]). Individual *SPO12* truncations were introduced into *cdc15-2 spo12Δ* mutants, and their ability to restore growth at 34°C and 37°C was assessed. *SPO12* truncations lacking the N-terminal 84 amino acids, which remove about half of the coding region of *SPO12*, were still able to suppress *cdc15-2 spo12Δ* mutants (Figure 1B, truncations II and III). Removal of the N-terminal 110 amino acids significantly reduced *SPO12's* ability to suppress *cdc15-2 spo12Δ* mutants at 34°C and completely eliminated the suppression at 37°C (Figure 1B, truncation IV). Consistent with a previous study by Chaves et al. [24], who identified a nuclear localization sequence (NLS) spanning amino acids 91-95, we found that removal of amino acids 85-110 caused Spo12 to be predominantly localized in the cytosol ([24], Supplemental Figure 1, truncation IV). Addition of an exogenous NLS to *GAL-SPO12(111-173)* mostly restored its nuclear localization (Supplemental Figure 1, truncation V) and significantly enhanced its ability to suppress *cdc15-2 spo12Δ* mutants at 34°C (Figure 1B, compare truncations IV and V).

Figure 1

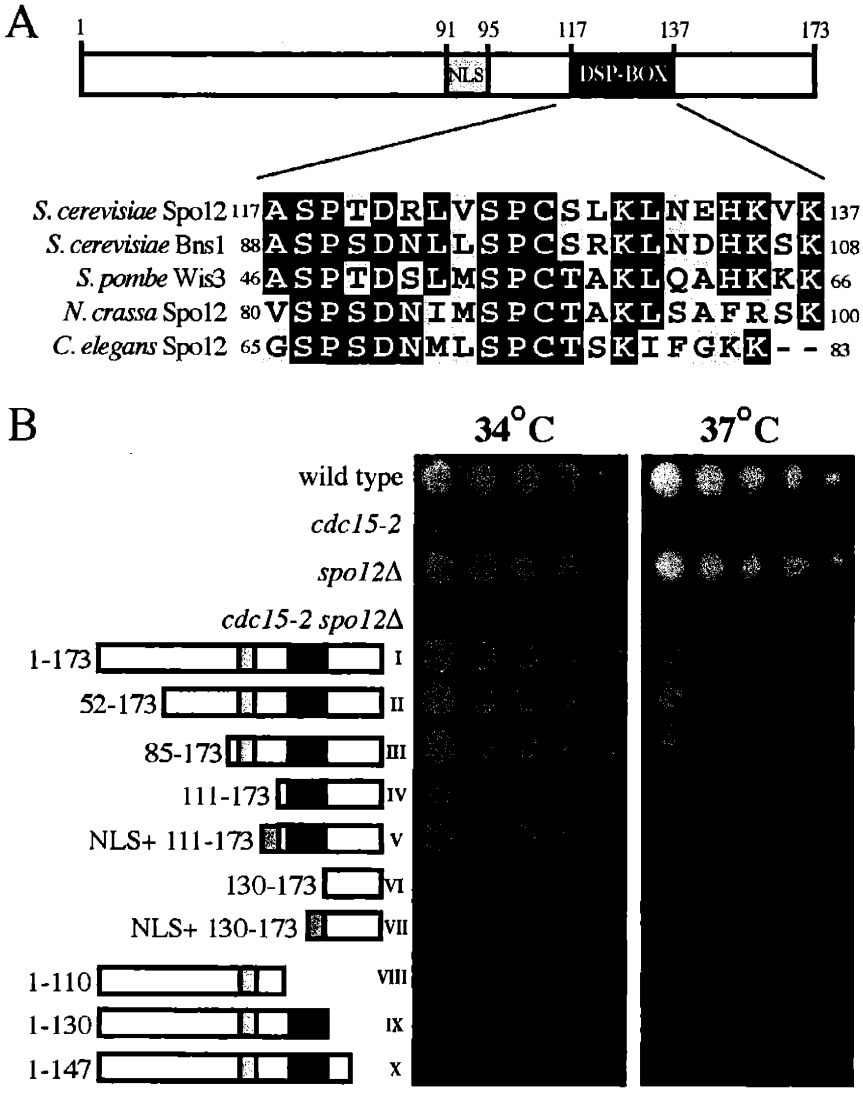

Figure 1: Spo12's C-terminal domain is required for its mitotic exit function.

(A) Schematic representation of Spo12's functional domains, including the nuclear localization sequence (NLS, spanning amino acids 91-95), and the highly conserved DSP-Box (amino acids 117-137). Below is a sequence alignment of the DSP-Box sequences of eukaryotic Spo12 homologues. Identical amino acids are shaded in black, whereas amino acids with similar properties are shaded in grey. Numbers correspond to the amino acid positions in each protein.

(B) Various Spo12 truncation constructs under the control of the *GAL1-10* promoter were tested for the ability to suppress *cdc15-2 spo12Δ* mutants at the restrictive temperature. Cells were spotted in 10-fold serial dilutions on 2% galactose-containing medium and grown for 3 days at either 34°C or 37°C. An exogenous NLS sequence (dark gray box) was added to the N-termini of constructs V and VII. The following strains were used (from top to bottom): A2587, A2596, A4874, A10010, A6895, A6897, A6899, A6900, A6901, A6903, A7416, A7090, A7088, A7086.

Figure 2

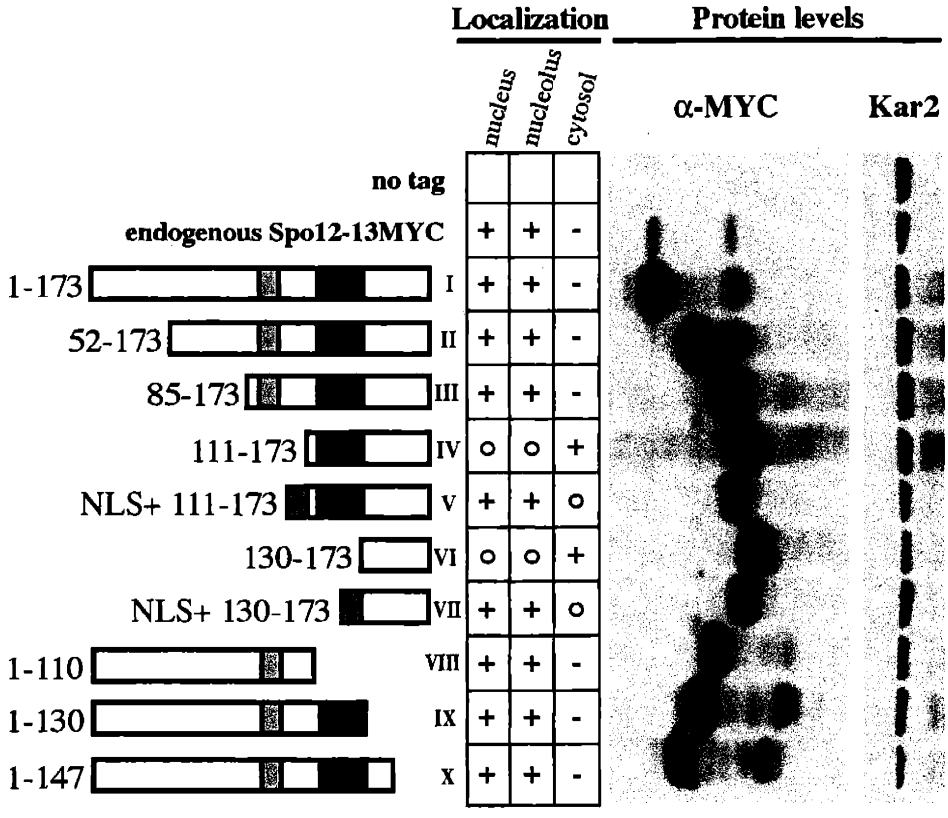

Figure 2: Expression levels and subcellular localization of various Spo12 truncation constructs.

Anti-MYC Western blot analysis from strains grown in YEPR and induced with 2% galactose for 1.5 h. Kar2 serves as a loading control. The following strains were used (from top to bottom): A2587, A4568, A7936, A7940, A7942, A7945, A7947, A7950, A7952, A7959, A7957, A7955. The localization of the Spo12 constructs was assessed by indirect immunofluoresence: (-) no signal, (O) weak signal, (+) strong signal.

Figure 3

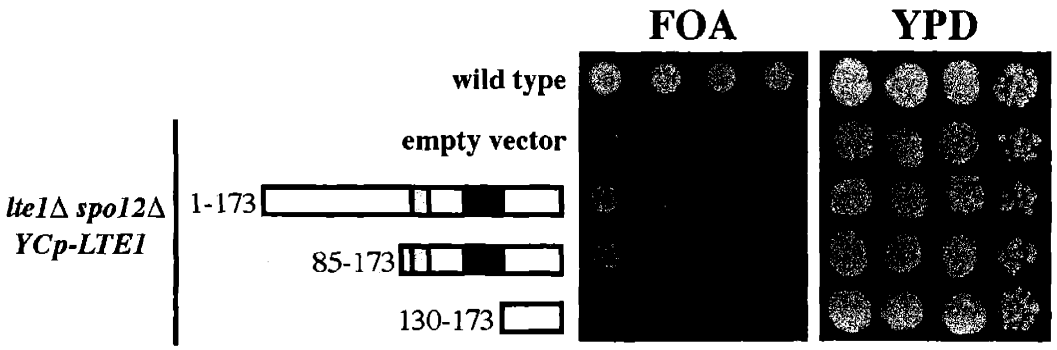

Figure 3: Spo12-CTD is sufficient for *SPO12* function

Various Spo12 truncation constructs under the control of the *SPO12* promoter were tested for the ability to suppress the synthetic lethality of *lte1Δ spo12Δ* mutants at 30°C. Serial dilutions (1:10) of *lte1Δ spo12Δ YCp50-LTE1* cells carrying the indicated *SPO12* truncations on the centromeric plasmid Ycplac111 were spotted on plates containing 5-fluororotic acid (FOA, selects against *YCp50-LTE1* covering plasmid) or on YPD plates (plating control). The following strains were used (from top to bottom): A2587, A10694, A10655, A10656, A10657.

This finding indicates that optimal *SPO12* function requires the protein to be present in the nucleus.

Spo12 contains an evolutionarily conserved 20 amino acid motif of unknown function, spanning amino acids 117 to 137 (Figure 1A, [20, 21]). We termed this highly conserved motif DSP-Box (for reasons see below). N-terminal truncations that removed the DSP-Box completely abolished *SPO12*'s ability to rescue *cdc15-2 spo12Δ* mutants at 34°C (Figure 1B, truncations VI and VII). We excluded the possibility that this loss of *SPO12* function was due to the truncated protein being unstable (Figure 2) or due to the inability of the protein to enter the nucleus, as truncation VII carrying an exogenous NLS still failed to suppress the growth defect of *cdc15-2 spo12Δ* mutants (Figure 1B and Figure 2, truncation VII). Our results show that the C-terminal domain of Spo12 (hence referred to as Spo12-CTD), which encompasses the highly conserved DSP-Box, is necessary and sufficient for *SPO12*'s mitotic exit function not only when overproduced but also when present at endogenous levels (Figure 3).

Spo12 physically interacts with Fob1.

The C-terminal domain of Spo12 (Spo12-CTD) consists of merely 88 amino acids and lacks any homology to domains with known enzymatic activities. We therefore speculated that this region of Spo12 might function as a protein-protein interaction domain. To identify potential binding partners of Spo12, we conducted a Two-Hybrid Screen using either full-length Spo12 or Spo12-CTD as a bait. Both screens recovered a single interacting protein fragment, an N-terminal fragment of Fob1 encompassing amino acids 5-410. This Fob1-Spo12 Two-hybrid interaction was also observed by Shah et al. [21]. Full-length Fob1 strongly interacted with both full-length Spo12 and Spo12-CTD in

the Two-hybrid assay (Figure 4A), suggesting that Fob1 binds to the C-terminal domain of Spo12. The Two-hybrid interaction was confirmed by co-immunoprecipitation experiments using tagged versions of Spo12 and Fob1. Immunoprecipitation of Spo12-ProA resulted in co-precipitation of Fob1-13MYC (Figure 4B) or Fob1-3HA (data not shown). Our results show that Spo12 and Fob1 form a complex that is mediated by Spo12's C-terminal domain, the region that is also essential for its mitotic exit function.

Fob1 localizes to the nucleolus throughout the cell cycle.

Fob1 localizes to the nucleolus [25] and is known to regulate many processes within this organelle. Fob1 is required for replication fork arrest in the rDNA array [26], regulates mitotic recombination within the rDNA [27, 28], rDNA silencing [10], and yeast life span [25]. To examine whether Fob1 resided in the nucleolus throughout the cell cycle, we analyzed Fob1 protein levels and localization in cells progressing through the cell cycle in a synchronous manner. Fob1 protein levels were constant throughout the cell cycle (Figure 4C) and Fob1 localized to the nucleolus during all cell cycle stages as judged by co-staining with the nucleolar marker Nop1 (Figure 4D). We conclude that Fob1 is present in the nucleolus throughout the cell cycle.

Deletion of *FOB1* allows for partial Cdc14 release in metaphase-arrested cells.

The physical interaction between Fob1 and Spo12 raised the possibility that *FOB1* functions within the *SPO12* branch of the FEAR network. To test this possibility we examined the consequences of deleting *FOB1* on Cdc14's subcellular localization.

Figure 4 A-C

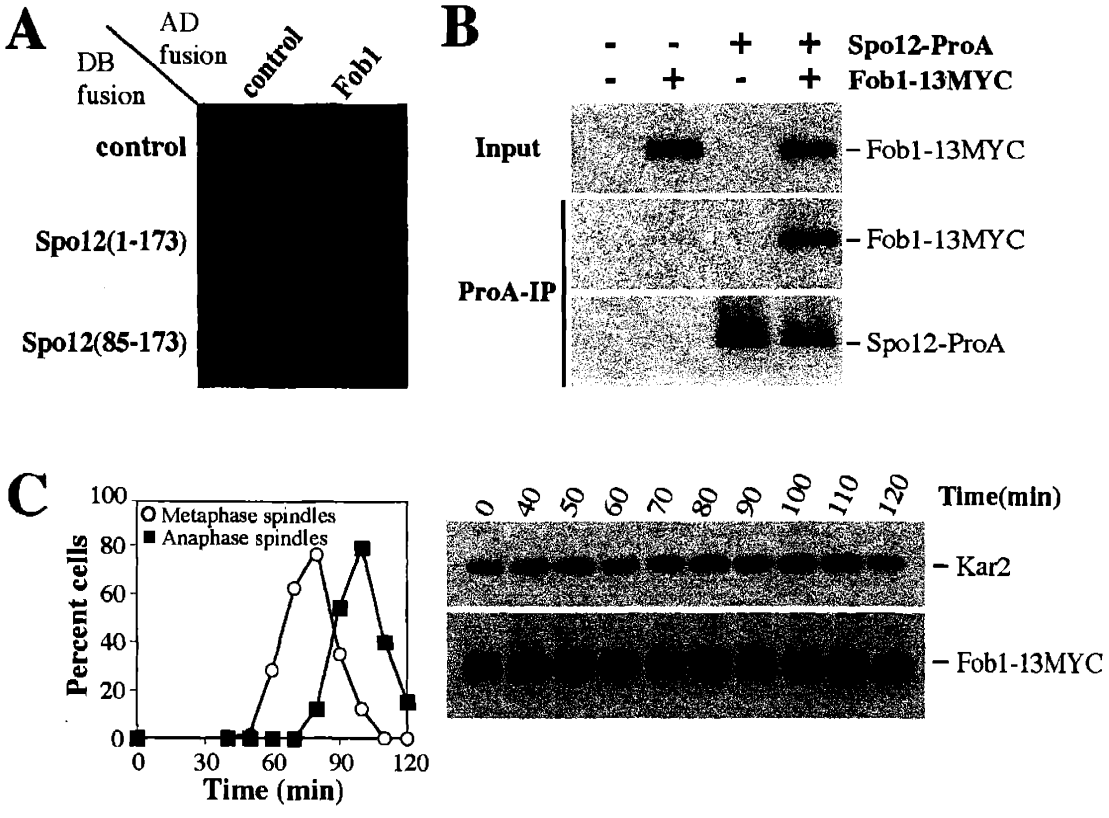
A
AD fusion
DB fusion
control
Fob1
control
Spo12(1-173)
Spo12(85-173)
B
- - + + Spo12-ProA
- + - + Fob1-13MYC
Input
Fob1-13MYC
ProA-IP
Fob1-13MYC
Spo12-ProA
C
Metaphase spindles
Anaphase spindles
Percent cells
Time (min)
0 40 50 60 70 80 90 100 110 120
Time(min)
Kar2
Fob1-13MYC

Figure 4D

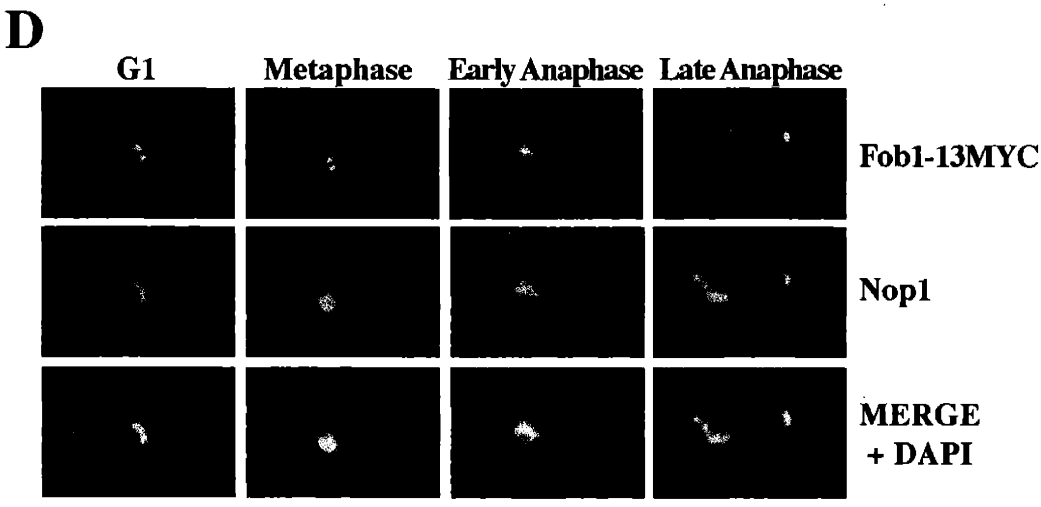

Figure 4: Fob1 physically associates with Spo12.

(**A**) Fob1 and Spo12 interact in the yeast Two-hybrid system. Both Spo12(1-173)-DB (DNA binding domain) and Spo12(85-173)-DB fusions interact with the Fob1(1-566)-AD (activation domain) fusion. An equal number of cells were spotted on medium lacking adenine.
(**B**) Western blots showing that Fob1-13MYC co-immunoprecipitates with Spo12-ProA from whole cell extracts. The following strains were used (from left to right): A2587, A8556, A5360, A9181. The Western blots show the amount of Fob1-13MYC in whole cell extract (Input, top panel), the amount of Fob1-13MYC coprecipitated with Spo12-ProA (middle panel), and the amount of Spo12-ProA immunoprecipitated (bottom panel).
(**C**) Wild-type (A8556) cells carrying a *FOB1-13MYC* fusion were arrested in G1 in YEPD (YEP plus 2% glucose) medium with α-factor (5μg/ml) followed by release into YEPD medium lacking pheromone. The graph on the left shows the percentages of cells with metaphase (open circles) and anaphase spindles (closed squares) at the indicated time points. The panel on the right shows Western blots of Fob1-13MYC at the indicated times. Kar2 was used as a loading control.
(**D**) Fob1 localizes to the nucleolus throughout the cell cycle. Fob1-13MYC localization was determined by indirect immunofluorescence at different cell cycle stages (A8556). Fob1 is shown in red, Nop1 in green, and DNA in blue.

We postulated that if *FOB1* were a positive regulator of the FEAR network then deletion of *FOB1* should cause defects in Cdc14 release from the nucleolus and a delay in mitotic exit. Conversely, if *FOB1* were to function in an inhibitory manner, Cdc14 release from the nucleolus might occur prematurely in the absence of *FOB1*. Deletion of *FOB1*, however, did not affect the kinetics of Cdc14 release from the nucleolus in cells progressing through the cell cycle in a synchronous manner (Figure 5A).

Although this finding indicates that *FOB1* is dispensable for Cdc14 regulation during an unperturbed cell cycle, *FOB1* may regulate Cdc14 release from the nucleolus under conditions when cell cycle progression is blocked and Cdc14 inhibition in the nucleolus needs to be maintained to ensure cell cycle arrest. For example, maintenance of a spindle-checkpoint induced metaphase arrest requires the continuous inhibition of both the FEAR network and the MEN (reviewed in[29]). Inhibition of the FEAR network is mediated by the *MAD1-MAD2* branch of the spindle checkpoint, whereas inhibition of the MEN requires the spindle checkpoint component *BUB2* ([11, 13], reviewed in [29]). The MEN inhibitor *BUB2* is an example of a gene that is dispensable for Cdc14 regulation during an unperturbed cell cycle but is required to prevent Cdc14 release from the nucleolus during checkpoint arrest (reviewed in [29]). To test whether *FOB1* plays a similar role, we deleted *FOB1* and monitored release of Cdc14 in cells treated with nocodazole, a microtubule-depolymerizing drug that induces spindle checkpoint arrest. In addition to *FOB1* we deleted the S phase cyclin *CLB5*, which counteracts the activity of released Cdc14 [30]. Cells were arrested in G1 using α-factor and released into nocodazole-containing medium. When Cdc14 localization was examined, a fraction of *clb5Δ fob1Δ* mutant cells, but not *clb5Δ* cells, transiently released

Figure 5

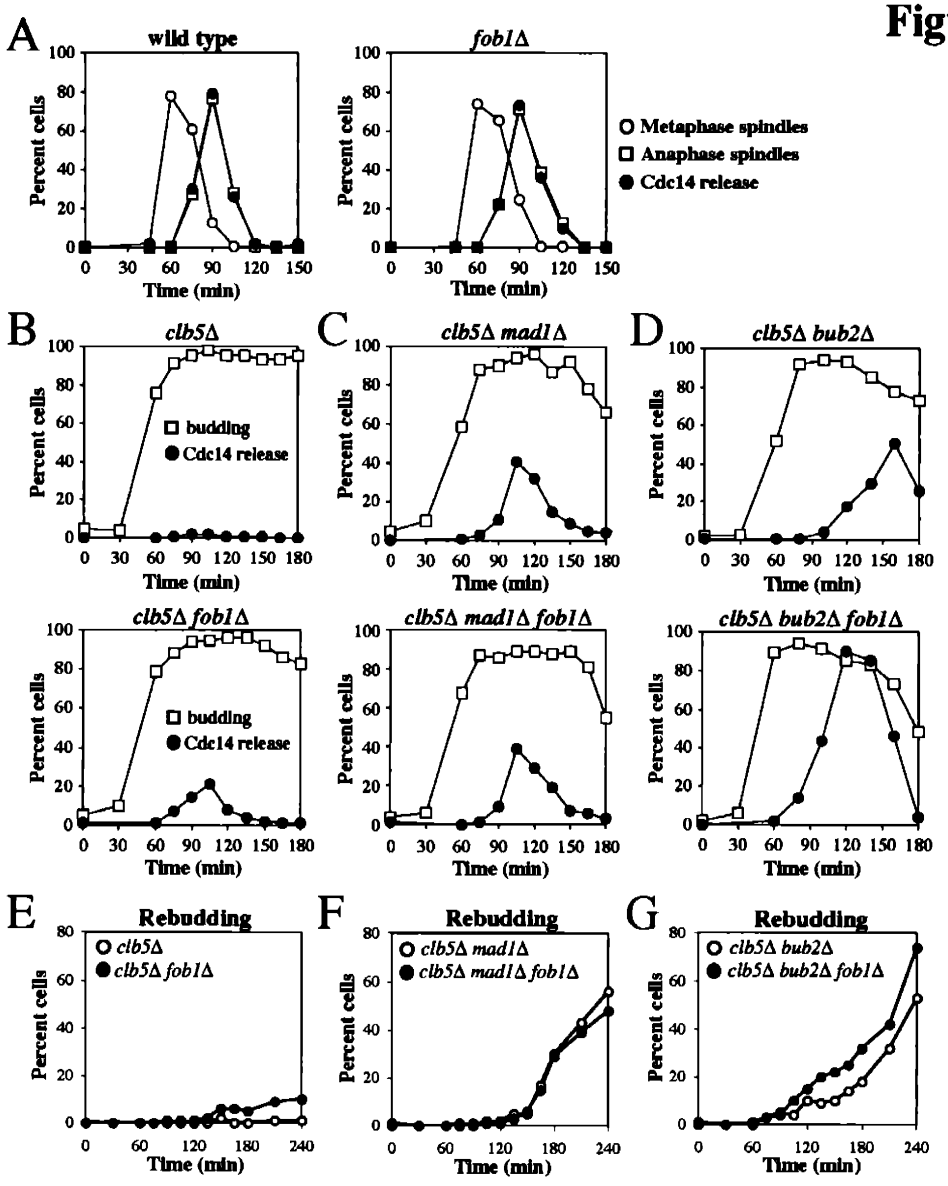

Figure 5: Deletion of *FOB1* allows a transient Cdc14 release in nocodazole-arrested cells.

(A) Wild-type (A1411) and *fob1Δ* (A8263) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEPD medium with α-factor (5μg/ml) followed by release into YEPD medium lacking pheromone. The percentages of cells with metaphase (open circles) and anaphase spindles (open squares), as well as the percentage of cells with Cdc14-HA released from the nucleolus (closed circles) were determined at the indicated times.

(B-G) *clb5Δ* (A1784), *clb5Δ fob1Δ* (A8580), *clb5Δ mad1Δ* (A8604), *clb5Δ mad1Δfob1Δ* (A9229), *clb5Δ bub2Δ* (A8605), and *clb5Δ bub2Δfob1Δ* (A8582) cells all carrying a *CDC14-3HA* fusion were arrested with α-factor (5μg/ml) and released into medium containing 15μg/ml nocodazole to determine the percentage of cells with Cdc14 released from the nucleolus (closed circles) and the percentage of budded cells (open squares) (B-D). 7.5μg/ml nocodazole was readded 120 minutes after release from the G1 arrest to prevent escape from the arrest. Graphs E-G show the amount of "rebudding" for the indicated strains. Rebudding indicates that nocodazole-treated cells have exited mitosis and entered a new cell cycle. Cells with two buds or unbudded cells without nuclear DNA were counted as rebudded.

Cdc14 from the nucleolus as cells entered the metaphase arrest (Figure 5B). This finding suggests that *FOB1* is necessary to prevent Cdc14 release from the nucleolus in nocodazole-arrested cells.

FOB1 functions within the FEAR network.

Activation of the FEAR network in nocodazole-arrested cells leads to a pattern of Cdc14 release from the nucleolus that is distinct from that caused by activation of the MEN [11, 13]. Activation of the FEAR network by deletion of *MAD1* leads to a transient release of Cdc14 from the nucleolus shortly after cells enter the metaphase arrest. In contrast, activation of the MEN by deletion of *BUB2* causes release of Cdc14 from the nucleolus only during later stages of the arrest ([11, 13], Figure 5C, D). The kinetics of the release of Cdc14 from the nucleolus caused by deletion of *FOB1* were similar to, though not as pronounced as, that of cells lacking *MAD1* (compare figures 3B and C), suggesting that *FOB1* functions as an inhibitor of the FEAR network. To conclusively distinguish between *FOB1* functioning as an inhibitor of the FEAR network or the MEN, we examined the effect of deleting *FOB1* in *mad1Δ* and *bub2Δ* mutants. Deletion of *FOB1* in *clb5Δ mad1Δ* mutants neither led to an increase in Cdc14 release from the nucleolus (Figure 5C) nor an increase in exit from mitosis as judged by rebudding (Figure 5F, rebudding is characterized by the formation of a new bud and requires exit from mitosis), suggesting that *MAD1* and *FOB1* function within the same pathway. In contrast, deletion of *FOB1* in *clb5Δ bub2Δ* mutants increased the proportion of cells releasing Cdc14 from the nucleolus (Figure 5D) and exiting mitosis (Figure 5G). Our results indicate that *FOB1* functions as an inhibitor of the FEAR network.

Overexpression of *FOB1* impairs FEAR network function.

If *FOB1* were indeed an inhibitor of the FEAR network, overexpression of *FOB1* should prevent Cdc14 release from the nucleolus during early anaphase. Overexpression of *FOB1* from the galactose-inducible *GAL1-10* promoter delayed mitotic exit by about 20 minutes as judged by the persistence of cells with anaphase spindles (Figure 6A, open squares). Consistent with the idea that *FOB1* antagonizes Cdc14 activation, we found that the mitotic exit delay of *GAL-FOB1* cells was eliminated by introducing *TAB6-1*, an allele of *CDC14* that suppresses the mitotic exit defect of MEN mutants, into *fob1Δ* mutants (Supplemental Figure 5, [31]). Furthermore, overexpression of *FOB1* led to a delay in the release of Cdc14 from the nucleolus, particularly during early anaphase (Figure 6A).

We also examined the effect of overexpressing *FOB1* on Cdc14 release in the absence of MEN function. In MEN mutants, such as *cdc15-2* mutants, Cdc14 is transiently released from the nucleolus during early anaphase [11-13]. This release depends on FEAR network function and becomes evident when the status of Cdc14 localization is correlated with the length of the mitotic spindle [11]. In *mad1Δ cdc15-2* mutants, the majority of early anaphase cells (mitotic spindle length of 2-7μm) have Cdc14 released from the nucleolus, whereas most late anaphase cells (mitotic spindle length greater than 8μm) have Cdc14 sequestered in the nucleolus (Figure 6B, [11]). Overexpression of *FOB1* in *mad1Δ cdc15-2* cells largely abolished the transient release of Cdc14 from the nucleolus at intermediate spindle lengths (Figure 6C).

Figure 6 A-C

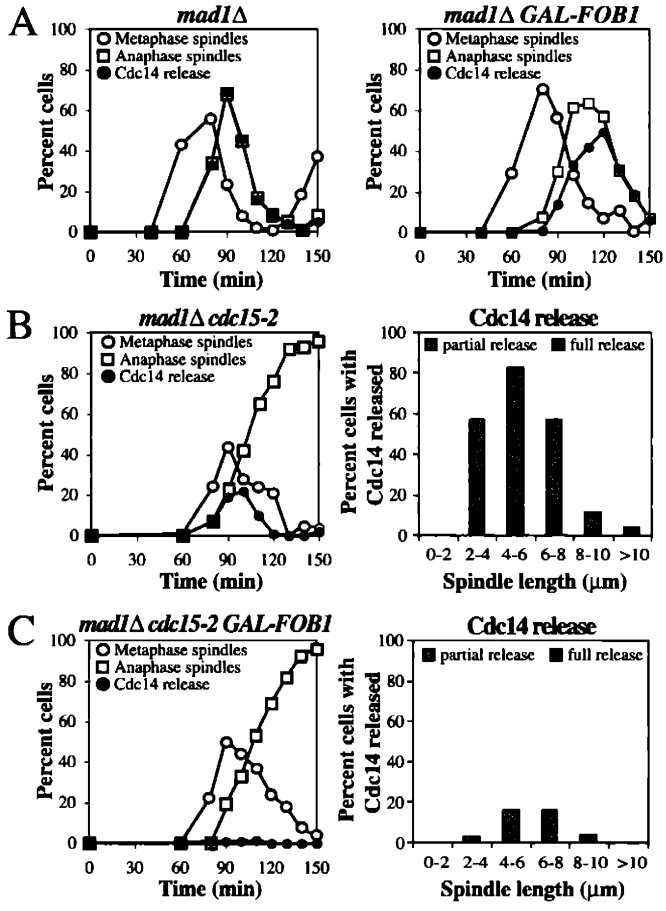

Figure 6: Fob1 overproduction delays mitotic exit.

(A) *mad1Δ* (A2853) and *mad1Δ GAL-FOB1* (A9355) cells carrying a *CDC14-3HA* fusion were grown in YEPR medium and arrested in G1 with α-factor (5μg/ml). Expression of Fob1 was induced 2h prior to release by addition of 2% galactose, and cells were released into galactose-containing medium. The percentages of cells with metaphase (open circles) and anaphase spindles (open squares), as well as the percentage of cells with Cdc14-HA released from the nucleolus (closed circles) were determined at the indicated times. We conducted this analysis in cells deleted for the spindle checkpoint component MAD1 to circumvent any indirect effects due to spindle checkpoint activation.
(B-C) *mad1Δ cdc15-2* (A4300) and *mad1Δ cdc15-2 GAL-FOB1* (A9356) cells carrying a *CDC14-3HA* fusion were grown in YEPR medium and arrested in G1 with α-factor (5μg/ml) at 25°C. Expression of Fob1 was induced 2h prior to release by addition of 2% galactose, and cells were released into galactose-containing medium at 37°C. The percentages of cells with metaphase (open circles) and anaphase spindles (open squares), as well as the percentage of cells with Cdc14-HA released from the nucleolus (closed circles) were determined at the indicated times. The graphs on the right show the percentage of cells with Cdc14 released from the nucleolus in relation to length of the mitotic spindle. Cells were analyzed 75-120 minutes after pheromone release. We consistently observed that *mad1Δ cdc15-2* cells, when grown in raffinose and galactose instead of glucose, show mostly partial release of Cdc14 from the nucleolus. A total of over 400 cells were analyzed for each strain.

Figure 6 D-E

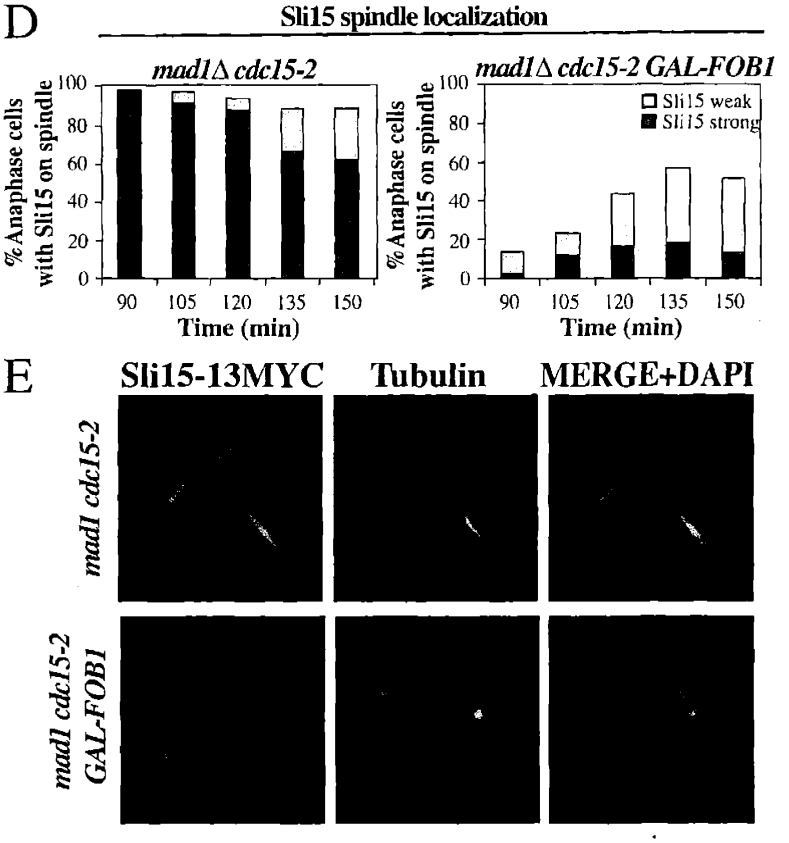

Figure 6: Fob1 overproduction delays mitotic exit.

(D) *mad1Δ cdc15-2* (A9917) and *mad1Δ cdc15-2 GAL-FOB1* (A9918) cells carrying a *SLI15-13MYC* fusion were grown and treated as described in Figure 6B. The graphs show the percentage of anaphase cells with Sli15 localized to the mitotic spindle at the indicated times after release from the G1 arrest.
(E) Examples of Sli15 localization in *mad1Δ cdc15-2* (A9917) and *mad1Δ cdc15-2 GAL-FOB1* (A9918) cells 105 minutes after release from the G1 arrest (from experiment in Figure 6D).

Pereira and Schiebel recently showed that the translocation of the chromosomal passenger protein Sli15/INCEN-P from kinetochores to the mitotic spindle and spindle midzone during early anaphase is caused by FEAR network-mediated Cdc14 activation [32]. Thus, Sli15 localization to the anaphase spindle serves as an additional marker for FEAR network function. Sli15-13MYC localization to anaphase spindles occurred readily in *cdc15-2 mad1Δ* mutants (Figure 6D, E) but was significantly impaired when *FOB1* was overexpressed (Figure 6D, E). We conclude that high levels of Fob1 inhibit FEAR network-dependent release of Cdc14 from the nucleolus.

Deletion of *FOB1* partially bypasses the mitotic exit and Cdc14 release defect of *spo12Δ bns1Δ* mutants.

Our finding that Fob1 acts as a negative regulator within the FEAR network and that Spo12 physically interacts with Fob1 suggested that *FOB1* functions within the *SPO12* branch of the FEAR network. To test this possibility we examined the epistatic relationship between *SPO12* and *FOB1*. Deletion of *FOB1* partially rescued the anaphase spindle disassembly defect (Figure 7A) and Cdc14 release defect of *spo12Δ bns1Δ* double mutants, particularly during early stages of anaphase (Figure 7B, 70 and 80 minute time points in the lower panels). Furthermore, overproduction of Spo12 no longer rescued the temperature sensitive growth defect of *cdc15-2* mutants in the presence of high levels of Fob1 (Figure 7C). Our results suggest that *FOB1* functions downstream of or in parallel to *SPO12*. The fact that deletion of *FOB1* only partially rescued the Cdc14 release defect of *spo12Δ bns1Δ* double mutants, however, also shows that *SPO12* regulates mitotic exit through mechanisms other than *FOB1*, or that *FOB1*, in addition to

Figure 7

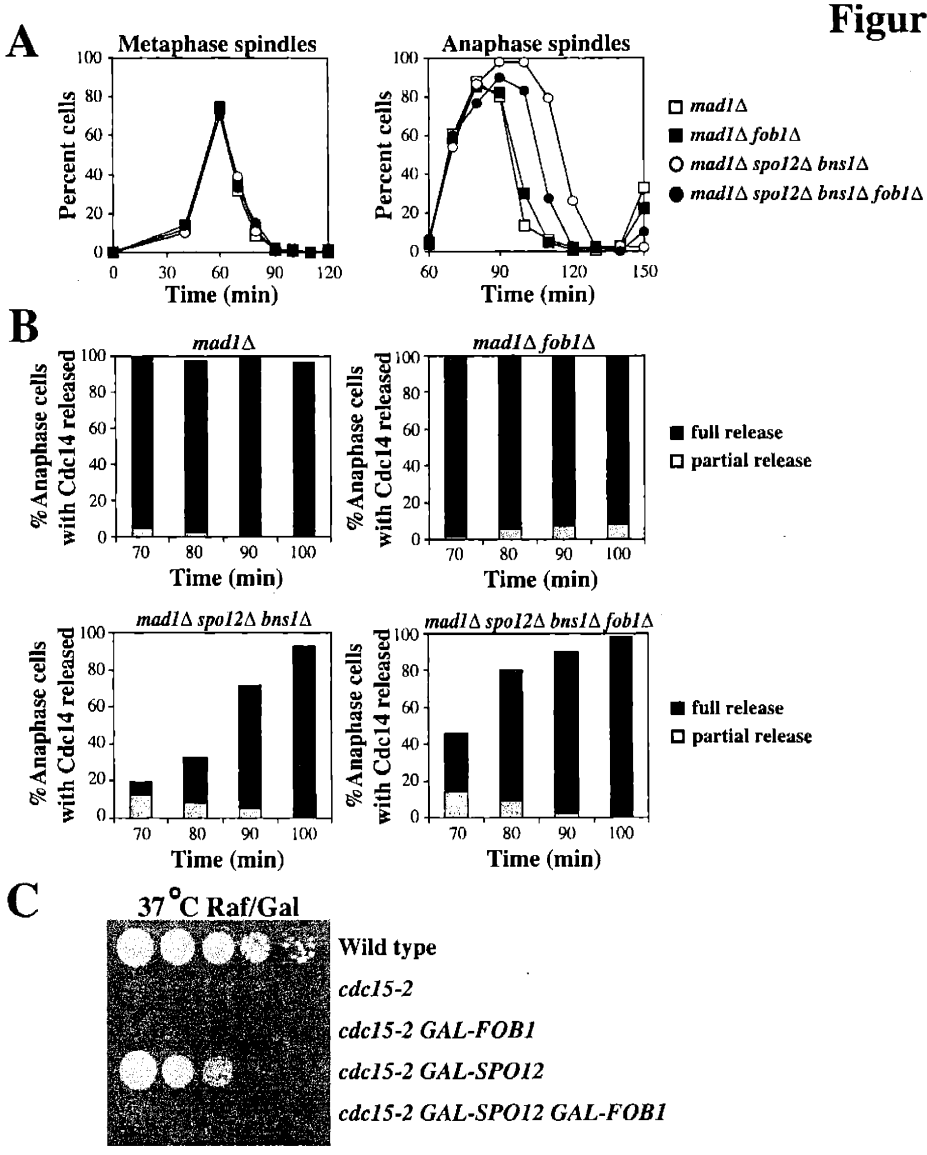

Figure 7: Deletion of *FOB1* partially rescues the mitotic exit defect of *spo12Δ bns1Δ* mutants.

(**A-B**) *mad1Δ* (A2853), *mad1Δ fob1Δ* (A8675), *mad1Δ spo12Δ bns1Δ* (A5408), and *mad1Δspo12Δbns1Δfob1Δ* (A8603) cells all carrying a *CDC14-3HA* fusion were arrested in G1 with α-factor (5μg/ml) and released into medium lacking pheromone. The percentages of cells with metaphase and anaphase spindles (A) were determined at the indicated times. The percentage of anaphase cells that have Cdc14 released from the nucleolus was determined at the indicated times (B). We conducted this analysis in cells deleted for the essential spindle checkpoint component *MAD1* to circumvent any indirect effects due to spindle checkpoint activation.

(**C**) 10-fold serial dilutions of strains with the indicated genotypes were spotted on YEPR plates containing 2% galactose and incubated for 2 days at 37°C. The following strains were used (from top to bottom): A1411, A1674, A9134, A6178, A10160.

its negative regulatory function within the FEAR network, also functions in a positive manner to promote Cdc14 release from the nucleolus. We favor the former possibility, because *fob1Δ* mutants do not exhibit a delay in release of Cdc14 from the nucleolus or mitotic exit (Figure 5A).

Fob1 physically interacts with Cfi1/Net1

To further investigate how Spo12 and Fob1 regulate the interaction of Cdc14 with its inhibitor, we examined whether the proteins interacted physically. We did not detect an association between Spo12 and Cfi1/Net1 or Cdc14 (data not shown). Cfi1/Net1 but not Cdc14 however co-immunoprecipitated with Fob1 (Figure 8A; data not shown). An interaction between Fob1 and Cfi1/Net1 was also reported recently by Huang et al. [10]. This interaction was not mediated by DNA, as addition of the DNA-intercalating agent ethidium bromide, which is commonly used to disrupt protein-DNA interactions [33], or treatment of extracts with micrococcal nuclease or DNAse prior to immunoprecipitation did not affect the interaction between Fob1 and Cfi1/Net1 (Figure 8A, B). Furthermore, the amount of Fob1 associated with Cfi1/Net1 did not significantly change as cells progressed through metaphase and anaphase (Figure 8C). Our results suggest a hierarchy of interactions that is consistent with our genetic epistasis analyses: Spo12 binds to Fob1, which in turn binds to Cfi1/Net1.

Fob1 and Cdc14 bind to overlapping sites within the rDNA repeats

Fob1 and Cfi1/Net1 have recently been shown to associate with the NTS1 and NTS1/NTS2 regions (NTS for nontranscribed spacers) within the rDNA repeat,

Figure 8

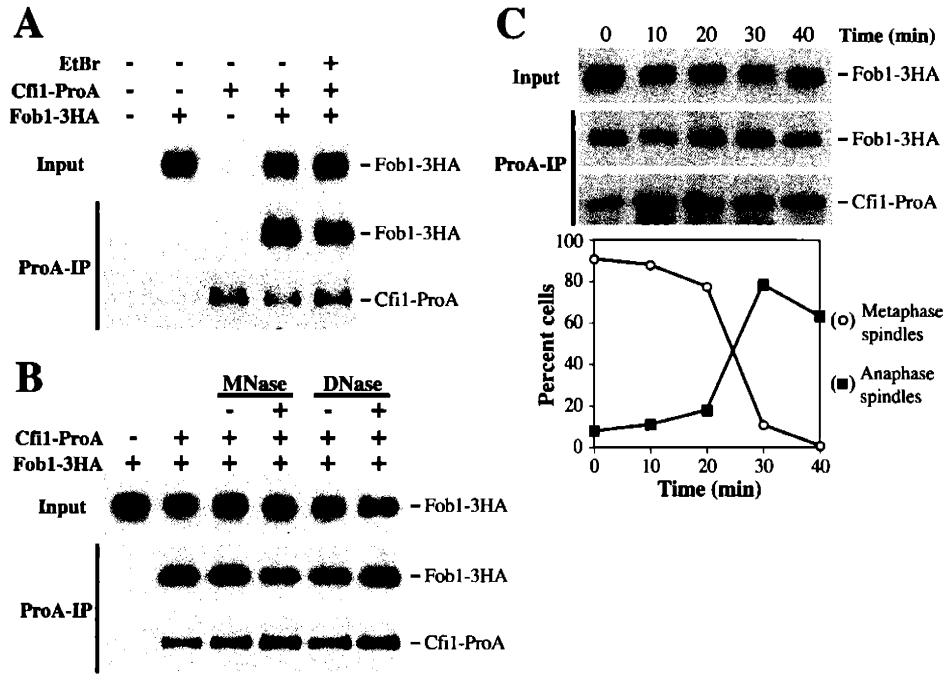

Figure 8: Fob1 physically associates with Cfi1/Net1.

(A) Western blots showing that Fob1-3HA co-immunoprecipitates with Cfi1-ProA from whole cell extracts. Where indicated, ethidium bromide (EtBr) was added (final concentration of 0.25mg/ml) to the extract for 30 minutes prior to the immunoprecipitation reaction. The Western blots show the amount of Fob1-3HA in whole cell extract (Input, top panel), the amount of Fob1-3HA coprecipitated with Cfi1-ProA (middle panel), and the amount of Cfi1-ProA immunoprecipitated (bottom panel). The following strains were used (from left to right): A2587, A8558, A8193, A8656 (last two lanes).

(B) Western blots showing that coprecipitation of Fob1-3HA with Cfi1-ProA is not DNA mediated. The following strains and conditions were used (from left to right): (1) A8558, (2) A8656, (3) A8656 + 0.5mM CaCl2, (4) A8656 + 0.5mM CaCl2+ 75 units MNase, (5) A8656 + 0.25mM CaCl2+ 0.5mM MgCl2, (6) A8656 + 0.25mM CaCl2+ 0.5mM MgCl2+ 25 Kunitz units DNase. Extracts were treated with MNase or DNase for 30 minutes on ice prior to the immunoprecipitation reaction.

(C) *MET-CDC20* (A9359) cells carrying *FOB1-3HA* and *CFI1-PROA* fusions were arrested in metaphase by depleting *CDC20* for 2.5h by addition of 2mM methionine. Cells were released from the metaphase arrest into medium lacking methionine. The graph below shows the percentages of cells with metaphase (open circles) and anaphase spindles (closed squares) at the indicated times. The Western blots above show the amount of Fob1-3HA in whole cell extract (Input, top panel), the amount of Fob1-3HA coprecipitated with Cfi1-ProA (middle panel), and the amount of Cfi1-ProA immunoprecipitated (bottom panel) at the indicated times after release from the metaphase arrest.

Figure 9

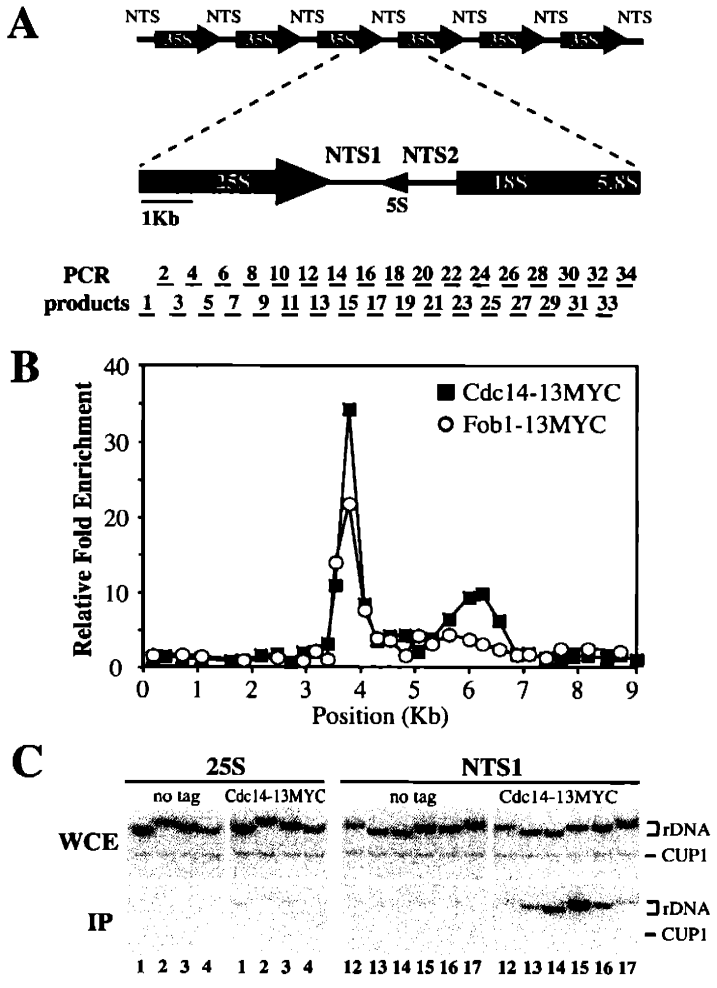

Figure 9: Fob1 and Cdc14 bind to overlapping sites within the rDNA repeats.

(A) The physical structure of the tandemly repeating rDNA of *S.cerevisiae* is shown above, and a single 9.1-Kb rDNA unit is shown expanded below. Each repeat yields a Pol I-transcribed 35S precursor rRNA (shown as a divided thick arrow), which is processed into the 25S, 18S, and 5.8S rRNAs, and a Pol III-transcribed 5S rRNA (arrowhead). The 35S coding regions are separated by a nontranscribed spacer (NTS), which is divided by the 5S gene (arrowhead) into NTS1 and NTS2. PCR products analyzed in CHIP assays are indicated below the rDNA unit.

(B) Representative graph showing the association of Fob1-13MYC (A8556) and Cdc14-13MYC (A3298) within the rDNA repeat. Relative fold enrichment refers to the relative ratio of PCR products amplified from immunoprecipitated DNA to products from whole-cell extract DNA as described previously [10].

(C) Examples of CHIP data used to calculate enrichment at the 25S and NTS1 regions of the rDNA repeat. The numbers below the panels refer to the PCR products shown in A. *CUP1* primers were used as a control. WCE and IP refer to products amplified from whole-cell extracts and immunoprecipitated DNA, respectively.

respectively, as judged by Chromatin Immunopreciptiation analysis (CHIP; Figure 9A; [10]). Furthermore, the binding of Cfi1/Net1 to the NTS1 region depends on *FOB1* [10]. Because Fob1 regulates the release of Cdc14 from its inhibitor, we examined whether Cdc14 also binds to the Fob1 binding region (NTS1) within the rDNA repeats. We found that Cdc14, like Cfi1/Net1, associated with both NTS1 and NTS2 (Figure 9B, C). However, in contrast to Cfi1/Net1, which shows about three-fold higher association with NTS2 compared to the NTS1 region [10], Cdc14 appeared to be predominantly associated with the NTS1 region (Figure 9B and C). Our data show that Fob1 and the majority of Cdc14-Cfi1/Net1 complexes localize to the same region within the rDNA repeats.

Spo12 phosphorylation is cell cycle regulated.

To begin to address how Spo12 and Fob1 control Cdc14 release from the nucleolus and how the proteins themselves are regulated we analyzed the phosphorylation status of Spo12. This analysis was prompted by the observation that Spo12 contains two conserved serine–proline dipeptides in the C-terminus of the protein that are essential for Spo12 function [21] . Indeed, Spo12 is a phosphoprotein, as the C-terminal domain of Spo12 (Spo12(85-173)-13MYC), which is sufficient for *SPO12* function (Figure 1B and Figure 5), readily incorporated ^{32}P-orthophosphate (Figure 10C,D). Phospho-amino-acid analysis showed the target amino acid to be predominantly serine (Figure 10A). Much longer exposure, however, revealed very low levels of phospho-threonine and phospho-tyrosine (data not shown). Whether this reflects genuine low-level phosphorylation of the conserved Threonine^{120}, phosphorylation of other less

well-conserved threonine and tyrosine residues within Spo12's CTD, or contamination is at present unclear.

Spo12's DSP-Box contains three highly conserved serine residues (Ser^{118}, Ser^{125}, Ser^{128}) that are potential phosphorylation sites. To test their importance for Spo12 function, we individually mutated these residues, in addition to adjacent conserved residues to alanines (Figure 10B). These mutations did not affect protein levels or the localization pattern of Spo12 (data not shown). Furthermore, $Threonine^{120}$, $Cysteine^{127}$, and $Serine^{128}$ were dispensable for Spo12's mitotic exit function, as judged by the ability of the mutant protein, when overproduced, to rescue the temperature sensitive growth defect of *cdc15-2 spo12Δ* mutants (Figure 10B, open circles, and data not shown). In contrast, substitution of $Aspartate^{121}$ to alanine led to loss of *SPO12* function (Figure 10B, data not shown). In agreement with a study by Shah et al. [21], we found that the highly conserved SP sites ($S^{118}P^{119}$ and $S^{125}P^{126}$) within the DSP-Box are essential for *SPO12* function (Figure 10B, closed circles). Because of the essential nature of the two SP sites, we termed this domain of unknown molecular function DSP-Box (double SP sites).

To determine whether serines S^{118} and S^{125} were phosphorylated in vivo, we mutated both sites to alanine (SS-AA). ^{32}P incorporation in Spo12(SS-AA) was significantly reduced, albeit not completely eliminated (Figure 10C). This finding suggests that S^{118} and S^{125} comprise the major phosphorylation sites within Spo12-CTD. To determine whether phosphorylation of Spo12-CTD was cell cycle regulated, we compared the levels of ^{32}P-orthophosphate incorporation into Spo12(85-173) in S phase (HU, hydroxyurea), metaphase (NOC, nocodazole)-arrested, and exponentially growing cells (cyc). Interestingly, specific ^{32}P incorporation was drastically reduced in S-phase

Figure 10

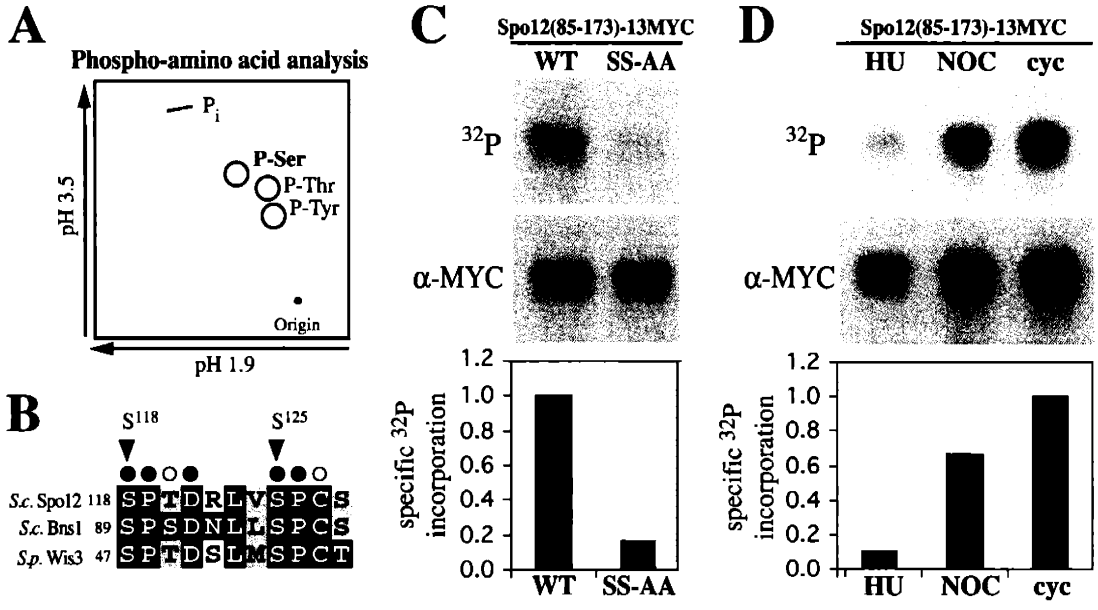

Figure 10: Spo12 phosphorylation is cell-cycle regulated.

(A) 10 ml of A9805 cells were grown in phosphate-depleted YEPR (YEP containing 2% raffinose) medium to an optical density at 600nm of 0.6, and *SPO12(85-173)-13MYC* expression was induced for 45 minutes by addition of 2% galactose. Cells were labeled for 20 min with 0.02mCi/ml of ^{32}P-orthophosphate. Phospho-amino-acid composition was analyzed using a phosphorimager. The positions of sample loading (Origin) and the location of free phosphate (Pi) are marked. Circles indicate the migration of the phospho-amino acid standards phosphoserine, phosphothreonine, and phosphotyrosine.

(B) Alignment of the N-terminal part of DSP-Box (for complete alignment see Figure 1). The residues marked by circles were changed to alanine in the *S.cerevisiae* Spo12 sequence by site-directed mutagenesis. Solid circles indicate substitutions that led to a loss of Spo12 function, whereas open circles indicate substitutions that did not impair Spo12 function.

(C) Amino acids S118 and S125 in Spo12(85-173)-13MYC were mutated to alanines to construct Spo12[SS-AA]-13MYC. 10 ml of strains carrying either wild-type Spo12[WT] (A9805), or Spo12[SS-AA] (A9807) under the control of the *GAL1-10* promoter were grown in phosphate-depleted YEPR medium to an optical density at 600nm of 0.6, and expression of the constructs was induced for 45 minutes by addition of 2% galactose. Cells were labeled for 20 min with 0.02mCi/ml of 32P-orthophosphate. The upper panel shows the amount of ^{32}P incorporation into Spo12, and the lower panel shows the amount of immunoprecipitated protein. Specific ^{32}P incorporation (^{32}P / protein) is quantitated in the graph below.

(D) 10 ml of A9805 cells were grown in phosphate-depleted YEPR medium. Cultures were diluted to an optical density at 600nm of 0.4 and arrested either in S-phase with 10mg/ml hydroxyurea (HU), in metaphase with 15µg/ml nocodazole (NOC), or left untreated (cyc). After a 2h arrest, 2% galactose was added to induce expression of Spo12(85-173)-13MYC for 40 minutes, followed by labeling for 20 minutes with 0.02mCi/ml of ^{32}P-orthophosphate. The upper panel shows the amount of ^{32}P incorporation into Spo12, and the lower panel shows the amount of immunoprecipitated protein. Specific ^{32}P incorporation (^{32}P / protein) is quantitated in the graph below.

arrested cells when compared to exponentially growing cells (Figure 10C). Furthermore, Spo12 phosphorylation was slightly higher in cycling than metaphase-arrested cells (Figure 10C). Our results indicate that phosphorylation of S^{118} and S^{125} is cell cycle regulated, being low during S phase and high in metaphase. The finding that phosphorylation in exponentially growing cells is even higher than in metaphase-arrested cells further suggests that phosphorylation must be highest in a cell cycle stage other than metaphase. Given that Spo12 levels are low in G1-arrested cells due to instability of the protein during G1 [21], we speculate that this cell cycle stage is anaphase.

Spo12 phosphorylation influences Fob1 binding.

Next, we examined whether Spo12 phosphorylation affects its binding to Fob1. The interaction of Fob1 with a non-phosphorylatable form of Spo12 (Spo12(SS-AA)) was approximately 2.5-fold higher compared to wild-type Spo12, as judged by a quantitative Two-hybrid assay (Figure 11A) and co-immunoprecipitation (Figure 11B). This result suggests that Spo12 phosphorylation decreases Fob1 binding.

Owing to phosphorylation of Spo12 being cell cycle regulated, we tested whether the association of Spo12 and Fob1 changes during the cell cycle. The interaction between Spo12 and Fob1 occurred throughout metaphase and anaphase, but the levels of co-precipitated Fob1-3HA appeared to be slightly reduced as cells entered anaphase (Figure 11C, compare 10-20 minutes with 30-40 minutes). We note that such a reduced affinity of Fob1 for Spo12 as cells enter anaphase would be consistent with the idea that Spo12 phosphorylation is maximal during anaphase.

Figure 11

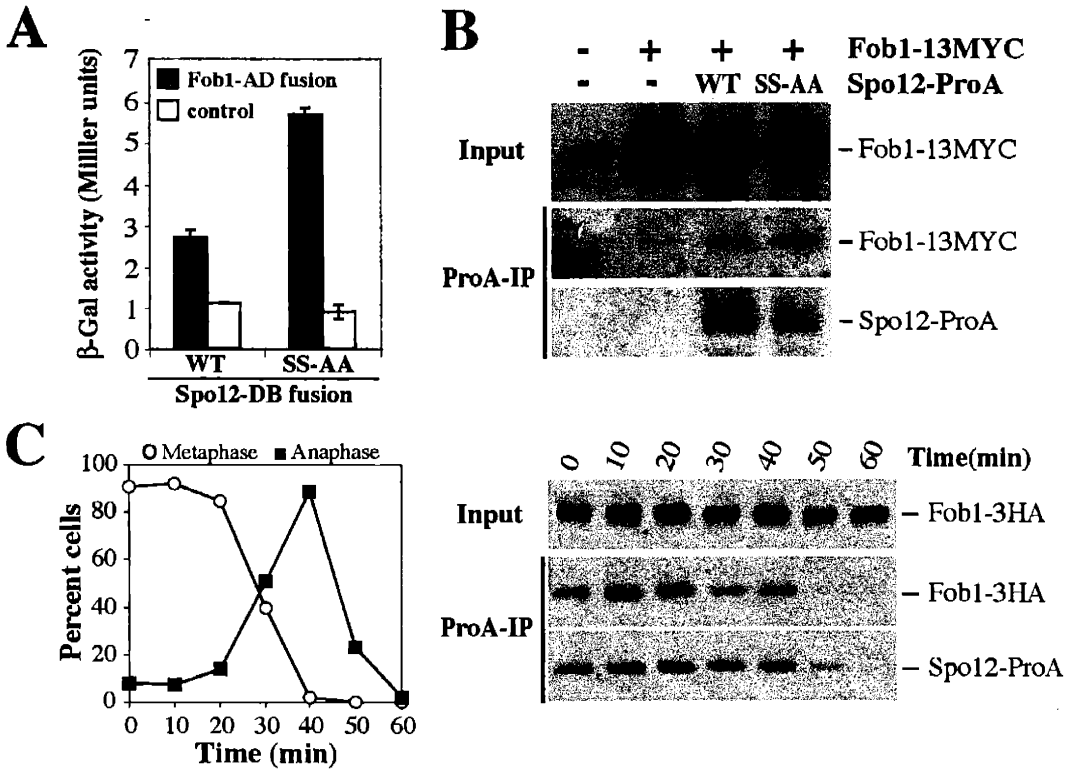

Figure 11: Spo12 phosphorylation affects its binding to Fob1

(A) Two-hybrid interactions between Fob1-AD and Spo12(WT)-DB (A10277) or Spo12(SS-AA)-DB (A10281) were estimated by measuring β-galactosidase activity. Assays were done in triplicate. The control strains lack the Fob1-AD fusion (A7569 and A10273).

(B) Western blots showing the co-immunoprecipitation of Fob1-13MYC with Spo12(WT)-ProA and Spo12(SS-AA)-ProA from whole cell extracts. The Western blots show the amount of Fob1-13MYC in whole cell extract (Input, top panel), the amount of Fob1-13MYC coprecipitated with Spo12-ProA (middle panel), and the amount of Spo12-ProA immunoprecipitated (bottom panel). The following strains were used (from left to right): A2587, A8556, A10695, A10701.

(C) *MET-CDC20* (A9792) cells carrying a *FOB1-3HA* and *SPO12-PROA* fusion were arrested in metaphase by depleting *CDC20* for 2.5h by addition of 2mM methionine. Cells were released from the metaphase arrest into medium lacking methionine. The graph on the left shows the percentages of cells with metaphase (open circles) and anaphase spindles (closed squares) at the indicated times. The Western blots on the right show the amount of Fob1-3HA in whole cell extract (Input, top panel), the amount of Fob1-3HA coprecipitated with Spo12-ProA (middle panel), and the amount of Spo12-ProA immunoprecipitated (bottom panel) at the indicated times after release from the metaphase arrest.

Discussion

Recent studies revealed that the regulation of Cdc14 by the FEAR network is critical for orchestrating mitotic and meiotic events ([11, 25, 34, 35]). However, the mechanism(s) whereby the FEAR network controls Cdc14 activity remain poorly understood. Here we define the region within the FEAR network component Spo12 important for its mitotic exit function. We identify the replication fork block protein Fob1 as a negative regulator of the FEAR network. Fob1 resides within the nucleolus throughout the cell cycle and physically interacts with both Spo12 and Cfi1/Net1. We show that two serines within the conserved DSP-Box of Spo12 are phosphorylated during mitosis but not S phase. Phosphorylation of these sites appears to decrease its binding to Fob1. We propose a model where Spo12 promotes Cdc14 release from its inhibitor during early anaphase in part by antagonizing Fob1.

Fob1 is a negative regulator of the FEAR network.

Our data show that Fob1 functions as an inhibitor of Cdc14 release from the nucleolus. Deletion of *FOB1* leads to the inappropriate release of Cdc14 from the nucleolus in cells arrested in metaphase due to activation of the spindle checkpoint. Conversely, overexpression of *FOB1* delays the release of the phosphatase from its inhibitor. Several lines of evidence further support the conclusion that *FOB1* is a negative regulator of the FEAR network. First, deletion of *FOB1* enhanced the spindle checkpoint defect of cells lacking *BUB2*, which leads to activation of the MEN, but not cells lacking *MAD1*, which allows for the activation of the FEAR network. Secondly, *FOB1*

overexpression delays the release of Cdc14 from the nucleolus specifically during early anaphase, which is characteristic of a FEAR network defect [11].

What is the role of Fob1 within the FEAR network? Fob1 localizes to the nucleolus throughout the cell cycle and can be co-immunoprecipitated with both Spo12 and Cfi1/Net1. These physical interactions raise the possibility that Fob1 functions either together with or downstream of Spo12 to control the association of Cfi1/Net1 and Cdc14. This idea is substantiated by the following observations. (1) Deletion of *FOB1* partially alleviates the mitotic exit defects of cells lacking *SPO12* and *BNS1*. (2) Overexpression of *FOB1* prevents *GAL-SPO12* from suppressing *cdc15-2* mutants at the restrictive temperature. However, if Fob1 functions downstream of Spo12, Spo12 must regulate the association of Cdc14 with its inhibitor through at least one other mechanism, as deletion of *FOB1* only partially rescues the mitotic exit defect of *spo12Δ bns1Δ* mutants. Furthermore, deletion of *FOB1* does not rescue the synthetic lethality of *spo12Δ lte1Δ* mutants, and cells overexpressing *FOB1* exhibit a slightly weaker mitotic exit delay than cells lacking both *SPO12* and *BNS1* (F. S., unpublished observations).

The spindle checkpoint inhibits both the *ESP1-SLK19* and *SPO12* branches of the FEAR network

The *ESP1* branch of the FEAR network is activated by the destruction of the Esp1 inhibitor Pds1 (also known as securin) at the metaphase-anaphase transition [11, 19]. Activation of the *MAD1* branch of the spindle checkpoint leads to the stabilization of Pds1 (reviewed in [29]) and hence inhibition of the *ESP1-SLK19* branch of the FEAR network. But how is the *SPO12-FOB1* branch of the FEAR network inhibited in response to checkpoint activation? Our data suggest that *MAD1* also inhibits the *SPO12-FOB1*

branch. Deletion of *FOB1* does not enhance the spindle checkpoint defect of *mad1Δ* cells. Furthermore, *mad1Δ* mutants exhibit a stronger spindle checkpoint defect than *fob1Δ* mutants. The observation that cells lacking *PDS1* exhibit a spindle checkpoint defect similar to that of cells lacking *MAD1* [13], suggests that *MAD1* inhibits both FEAR network branches through stabilizing Pds1. The possibility that Pds1 inhibits both FEAR network branches could also explain the observation that cells overexpressing a non-degradable version of *PDS1* display a Cdc14 release and mitotic exit defect more similar to *esp1-1 spo12Δ bns1Δ* mutants than *esp1-1* mutants [14, 36, 37].

Is Spo12 regulated by phosphorylation?

SPO12's ability to promote Cdc14 release from the nucleolus appears to be restricted to anaphase. During this cell cycle stage, Spo12 is very effective in accomplishing this task because high levels of *SPO12* render the essential MEN dispensable for mitotic exit ([5], Figure 1B). However, overproduction of *SPO12* fails to promote Cdc14 release in S phase or nocodazole-arrested cells ([14], F.S. unpublished observation), raising the question of how Spo12's mitotic exit promoting activity is restricted to anaphase. Since Spo12 localizes to the nucleus and nucleolus throughout the cell cycle ([21], F.S. unpublished observation), it is unlikely that regulation of Spo12's subcellular localization is responsible for limiting its activity to anaphase. It is possible that Spo12 requires an anaphase-specific co-factor or anaphase-specific post-translational modification. Several observations support the latter hypothesis. First, two highly conserved serine residues in Spo12 are phosphorylated in vivo. Secondly, phosphorylation of these two residues is required for Spo12's mitotic exit function (Figure 8B, [21]). Finally, phosphorylation of Spo12-CTD is cell-cycle regulated. The

protein's phosphorylation is significantly lower during S phase than in metaphase-arrested cells and even higher in exponentially growing cells. Given the fact that cycling cells contain a large fraction of G1 and S phase cells that harbor minimally phosphorylated Spo12, we speculate that phosphorylation of Spo12-CTD is maximal during anaphase. Serines S^{118} and S^{125} are part of sequences that fit the minimal consensus site for both CDKs and MAPKs. A recent study aimed at identifying CDK substrates found that Spo12 is not phosphorylated by CDKs in vitro [38]. Identifying the protein kinase(s) that phosphorylates $Serine^{118}$ and $Serine^{125}$ will be an important question to answer in the future.

A model for how the Spo12-Fob1 branch of the FEAR network promotes Cdc14 release from the nucleolus.

The Cdc14 and Fob1 binding regions in Cfi1/Net1 appear to be in close proximity. The N-terminal half of Cfi1/Net1 is sufficient for both Cdc14 and Fob1 binding (F.S. unpublished observations, [8]). We propose that Fob1 binds to Cfi1/Net1 throughout the cell cycle and that this interaction helps to prevent Cdc14's dissociation from Cfi1/Net1 during cell cycle stages other than anaphase. During anaphase, Spo12 might alleviate the inhibitory function of Fob1. We speculate that allosteric changes within the Spo12-Fob1 complex could lead to loss of Fob1's inhibitory function. Spo12 phosphorylation, for example, could trigger a conformational change within Spo12, thereby altering the binding surface available for Fob1. Consistent with this idea, we find that mutating serines S^{118} and S^{125} to alanines increases the stability of the Spo12-Fob1 complex.

Cdc14 release from the nucleolus during early anaphase depends on *SPO12*, *CDC5*, and the *ESP1-SLK19* branch of the FEAR network. Our results suggest that Spo12 exerts its Cdc14-activating function in part through eliminating Fob1's inhibitory function. Esp1 and Slk19 may cause Cdc5 activation [19], which in turn induces Cdc14 phosphorylation [14]. Together, these events may lead to the transient release of Cdc14 from its inhibitor during anaphase. Lastly, we note that serines S^{118} and S^{125} in Spo12 are part of sequences that could be targeted for dephosphorylation by Cdc14. This raises the intriguing possibility that Cdc14 released by the FEAR network during early anaphase leads to the dephosphorylation and hence inactivation of Spo12 by late anaphase. Thus, Spo12 might plant the seeds for its own inactivation, providing a potential means for restricting FEAR network activity to early anaphase.

Fob1 - another link between silencing and cell cycle control.

Fob1 plays a key role in regulating DNA replication in the nucleolus. It blocks movement of replication forks [26, 39], which may lead to double-strand DNA breaks that promote mitotic recombination and regulate the contraction and expansion of the rDNA array [26, 27, 39]. Furthermore, Fob1 is required for Sir2-dependent rDNA silencing [10], which down-regulates recombination [40]. Our studies show that Fob1 is also an inhibitor of exit from mitosis, at least under conditions when cell cycle progression is blocked. This dual role of Fob1 in controlling exit from mitosis and rDNA silencing is not shared by Spo12 (J. H. unpublished observations). Fob1, however, is not the only protein important for regulating exit from mitosis and rDNA silencing. The first protein shown to have such a dual role was Cfi1/Net1 [9]. It is possible that both proteins evolved to perform two independent functions - one in cell cycle regulation and one in

the maintenance of rDNA integrity. A more attractive hypothesis would be that a connection between these two events exists and that Cfi1/Net1 and Fob1 function to link them. We speculate that Fob1 and perhaps Cfi1/Net1 regulate rDNA recombination and at the same time inhibit exit from mitosis, thereby ensuring that rDNA recombination events are completed prior to entry into G1.

Experimental Procedures

Growth conditions and yeast strains.

All strains are derivatives of W303 (A2587) and are listed in Table 1. To construct N-terminal *SPO12* truncations, the *GAL1-10* promoter was inserted at an internal position within the endogenous *SPO12* coding region by a PCR-based method [41]. C-terminal truncations were created by replacing the C-terminal residues of endogenous *SPO12* with the DNA sequence encoding 13MYC epitope tag by the same PCR-based method [41]. The Cdc14-3HA and Cfi1-3MYC fusions were described previously [7]. The Fob1-13MYC, Fob1-3HA, Spo12-ProA, Cfi1-ProA, Cdc14-13MYC, and Sli15-13MYC fusions were constructed by using a PCR-based method [41]. Mutations in *SPO12* were introduced by site-directed mutagenesis and confirmed by DNA sequencing. The *GAL-FLAG-FOB1* strain was constructed by inserting a plasmid, which contained *FOB1* carrying a N-terminal FLAG epitope tag fusion under the control of the *GAL1-10* promoter, into the *URA3* locus. Further details of strains and strain construction can be obtained upon request. Growth conditions for individual experiments are described within the Figure legends. Where growth conditions are unspecified, cells were grown in yeast extract peptone (YEP) plus 2% glucose at 25°C.

^{32}P-labelling and phospho-amino-acid analysis.

Cells were grown and labeled with ^{32}P-orthophosphate as described in the Figure legends. Spo12(85-173)-13MYC was isolated by denaturing immunoprecipitation as described [42] using a 1:50 dilution of the anti-MYC 9E10 antibody (Covance). The samples were then boiled in sample buffer, and run on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. After transfer to a polyvinylidene difluoride (PVDF) membrane, samples

were hydrolyzed with 6N HCl and separated by two-dimensional thin layer electrophoresis as previously described [43].

Two-hybrid Screen.

Yeast Two-hybrid screen was performed using the Gal4-based system [44]. To construct the Spo12(1-173)-DB and Spo12(85-173)-DB bait fusions, the DNA sequence coding for Spo12(1-173) or Spo12(85-173) was PCR-amplified and inserted into SalI and BglII restriction sites of the vector pGBDU-C1. To construct the Fob1(1-566)-AD fusion, the *FOB1* coding region was PCR-amplified and inserted SalI and BglII restriction sites of the vector pGAD-C1. Mutations in *SPO12* were introduced by site-directed mutagenesis and confirmed by DNA sequencing. The β-galactosidase assay was conducted as described in [45].

CHIP assays.

CHIP assays were carried out as described [10].

Co-immunoprecipitation analysis.

For Fob1-Cfi1 co-immunoprecipitations, cells were harvested, washed with 10mM Tris (pH 7.5), and resuspended in 200μl of NP40 lysis buffer (1%NP40, 150mM NaCl, 50mM TRIS [pH7.5], 1mM dithiothreitol (DTT), 60mM β-glycerophosphate, 1mM $NaVO_3$, 2μM Microcystin-LR (EMD Biosciences), complete EDTA-free protease inhibitor cocktail [Roche]). One milligram of extract in 150μl of NP40 buffer was used for immunoprecipitations. 30μl of rabbit-IgG coupled dynabeads (Dynal Biotech) was added to each sample and incubated with rotation for 2hrs at 4°C. Samples were washed five times with NP40 buffer, boiled in sample buffer, and run on SDS-PAGE gels for subsequent Western blot analysis. Fob1-Spo12 co-immunoprecipitation reactions were

carried out in the same way, except for the substitution of NP40 lysis buffer with CHAPS lysis buffer (0.5%CHAPS, 150mM NaCl, 50mM HEPES [pH7.5], 1mM dithiothreitol, 60mM β-glycerophosphate, 1mM $NaVO_3$, 2μM Microcystin-LR (EMD Biosciences), complete EDTA-free protease inhibitor cocktail [Roche]).

Immunoblot analysis.

To prepare protein extracts for Western blot analysis, cells were incubated for 10 minutes in 5% trichlor acetic acid (TCA) at 4°C, pelleted, and then washed with acetone. Cells were broken in 100μl lysis buffer (50mM TRIS (pH7.5), 1mM EDTA, 1mM $NaVO_3$, 50mM DTT, complete EDTA-free protease inhibitor cocktail [Roche]) with glass beads for 40 minutes and boiled in sample buffer. Samples were run on a 6% SDS-PAGE gel for subsequent Western blot analysis.

Fluorescence Microscopy.

Indirect in situ immunofluorescence methods and antibody concentrations for Cdc14-3HA and Cfi1-3MYC were as described previously [7]. Primary anti-MYC 9E10 antibody (Covance) was used at 1:1000 for both Fob1-13MYC and Sli15-13 MYC. Secondary anti-mouse antibodies (Jackson Laboratories) were used at a concentration of 1:250 and 1:500 for Fob1-13MYC and Sli15-13 MYC, respectively. Cells were analyzed on a Zeiss Axioplan 2 microscope, and images were captured with a Hamamatsu camera controller. Openlab 3.0.2 software was used to process immunofluorescence images. At least 100 cells were analyzed per time point.

Table 1: Strain list

Strain	Relevant genotype
A1411	*MATa CDC14-3HA*
A1674	*MATa cdc15-2 CDC14-3HA*
A1681	*MATa CDC14-3HA CFI1-3MYC*
A1784	*MATa clb5::URA3 CDC14-3HA*
A2587	*(K699) MATa ade2-1 leu2-3 ura3 trp1-1 his3-11,15 can1-100 GAL*
A2596	*MATa cdc15-2*
A2853	*MATa mad1::URA3 CDC14-3HA*
A4300	*MATa mad1::URA3 cdc15-2 CDC14-3HA*
A3298	*MATa CDC14-3MYC*
A4568	*MATa SPO12-13MYC*
A4874	*MATa spo12::HIS3*
A5360	*MATa SPO12-TEV-ProA-7HIS*
A5408	*MATa mad1::URA3 spo12::HIS3 bns1::KanMX CDC14-3HA*
A5617	*MATa TAB6-1*
A6178	*MATa leu2::GAL-SPO12-13MYC-LEU2 CDC14-3HA*
A6895	*MATa cdc15-2 spo12::GAL-SPO12(1-173)-13MYC*
A6897	*MATa cdc15-2 spo12::GAL-SPO12(52-173)-13MYC*
A6899	*MATa cdc15-2 spo12::GAL-SPO12(85-173)-13MYC*
A6900	*MATa cdc15-2 spo12::GAL-SPO12(111-173)-13MYC*
A6901	*MATa cdc15-2 spo12::GAL-NLS-SPO12(111-173)-13MYC*
A6903	*MATa cdc15-2 spo12::GAL-SPO12(130-173)-13MYC*
A7086	*MATa cdc15-2 spo12::GAL-SPO12(1-147)-13MYC*
A7088	*MATa cdc15-2 spo12::GAL-SPO12(1-130)-13MYC*
A7090	*MATa cdc15-2 spo12::GAL-SPO12(1-110)-13MYC*
A7416	*MATa cdc15-2 spo12::GAL-NLS-SPO12(130-173)-13MYC*
A7569	*MATa, gal4D, gal80D, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ + pGBDU-C1/GAL4-BD-SPO12(WT)*
A7936	*MATa spo12::GAL-SPO12(1-173)-13MYC*
A7940	*MATa spo12::GAL-SPO12(52-173)-13MYC*
A7942	*MATa spo12::GAL-SPO12(85-173)-13MYC*
A7945	*MATa spo12::GAL-SPO12(111-173)-13MYC*
A7947	*MATa spo12::GAL-NLS-SPO12(111-173)-13MYC*
A7950	*MATa spo12::GAL-SPO12(130-173)-13MYC*
A7952	*MATa spo12::GAL-NLS-SPO12(130-173)-13MYC*
A7955	*MATa spo12::GAL-SPO12(1-147)-13MYC*
A7957	*MATa spo12::GAL-SPO12(1-130)-13MYC*
A7959	*MATa spo12::GAL-SPO12(1-110)-13MYC*
A8193	*MATa CFI1-TEV-ProA*
A8263	*MATa fob1::HIS3 CDC14-3HA*
A8556	*MATa FOB1-13MYC*
A8558	*MATa FOB1-3HA*
A8580	*MATa fob1::HIS3 clb5::URA3 CDC14-3HA*
A8582	*MATa fob1::HIS3 clb5::URA3 bub2::HIS3 CDC14-3HA*
A8603	*MATa mad1::URA3 bns1::KanMX spo12::HIS3 fob1::HIS3 CDC14-3HA*
A8604	*MATa mad1::URA3 clb5::URA3 CDC14-3HA*
A8605	*MATa bub2::HIS3 clb5::URA3 CDC14-3HA*
A8656	*MATa CFI1-TEV-ProA FOB1-3HA*
A8675	*MATa mad1::URA3 fob1::HIS3 CDC14-3HA*
A8693	*MATa fob1::HIS3 CDC14-3HA CFI1-3MYC*
A8816	*MATa ura3::GAL-FLAG-FOB1-URA3 CDC14-3HA*
A8955	*MATa TAB6-1 ura3::GAL-FLAG-FOB1-URA3*
A9134	*MATa cdc15-2 ura3::GAL-FLAG-FOB1-URA3 CDC14-3HA*
A9181	*MATa SPO12-TEV-ProA-7HIS FOB1-13MYC*
Strain	Relevant genotype

A9229	*MATa clb5::URA3 mad1::URA3 fob1::HIS3 CDC14-3HA*
A9355	*MATa mad1::URA3 ura3::GAL-FLAG-FOB1-URA3 CDC14-3HA*
A9356	*MATa mad1::URA3 cdc15-2 ura3::GAL-FLAG-FOB1-URA3 CDC14-3HA*
A9359	*MATa cdc20::MET3-CDC20-URA3 CFI1-TEV-ProA FOB1-3HA CDC14-13MYC*
A9792	*MATa cdc20::MET3-CDC20-URA3 SPO12-TEV-ProA FOB1-3HA*
A9805	*MATa spo12::HIS3 leu2::GAL-SPO12(85-173)-13MYC-LEU2*
A9807	*MATa spo12::HIS3 leu2::GAL-SPO12(S118A,S125A,85-173)-13MYC-LEU2*
A9917	*MATa cdc15-2 mad1::URA3 CDC14-3HA SLI15-13MYC*
A9918	*MATa cdc15-2 mad1::URA3 ura3::GAL-FLAG-FOB1-URA3 CDC14-3HA SLI15-13MYC*
A10010	*MATa cdc15-2 spo12::HIS3*
A10160	*MATa cdc15-2 leu2::GAL-SPO12-13MYC-LEU2 ura3::GAL-FLAG-FOB1-URA3 CDC14-3HA*
A10273	*MATa, gal4D, gal80D, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ + pGBDU-C1/GAL4-BD-SPO12(SS-AA)*
A10277	*A7569 + pGAD-C1/GAL4-AD-FOB1*
A10281	*A10273 + pGAD-C1/GAL4-AD-FOB1*
A10655	*MATa, lte1::KanMX, spo12::HIS3 +Ycp50-LTE1(URA3) + YCplac111/SPO12(1-173)-ProA*
A10656	*MATa, lte1::KanMX, spo12::HIS3 +Ycp50-LTE1(URA3) + YCplac111/SPO12(85-173)-ProA*
A10657	*MATa, lte1::KanMX, spo12::HIS3 +Ycp50-LTE1(URA3) + YCplac111/SPO12(130-173)-ProA*
A10694	*MATa, lte1::KanMX, spo12::HIS3 +Ycp50-LTE1(URA3) + YCplac111(empty vector)*
A10695	*MATa, spo12::HIS3, FOB1-13MYC +YCplac111/SPO12(WT)-ProA*
A10701	*MATa, spo12::HIS3, FOB1-13MYC +YCplac111/SPO12(SS-AA)-ProA*

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Chapter V

Discussion and Future Directions

Key Conclusions

Significant progress has been made in the past several years towards understanding the regulation of mitotic exit, mainly from studies in budding yeast. Exit from mitosis requires the inactivation of mitotic CDK activity in most if not all eukaryotic organisms (Ghiara et al., 1991; Gallant et al., 1992; Holloway et al., 1993; Surana et al., 1993; Sigrist et al., 1995; Yamano et al., 1996; Wasch et al., 2002). In budding yeast, the activation of the Cdc14 phosphatase during anaphase triggers mitotic exit by antagonizing mitotic CDK activity (Visintin et al., 1998). The activity of Cdc14 is tightly regulated throughout the cell cycle. During G1, S, G2, and metaphase, the phosphatase is sequestered in the nucleolus by its competitive inhibitor Cfi1/Net1 (Visintin et al., 1998; Shou et al., 1999; Traverso et al., 2001). Cdc14 becomes released from its inhibitor during anaphase, thus allowing it to dephosphorylate its targets. Previous studies found that activation of the MEN (mitotic exit network) is required for the release of Cdc14 from its inhibitor (Visintin et al., 1998; Shou et al., 1999).

Identification and characterization of the FEAR network.

The work in this thesis describes the identification of a novel regulatory pathway, termed the FEAR (Cdc fourteen early anaphase release) network, which regulates the activation of Cdc14 during early anaphase. I found that the FEAR network initiates the release of Cdc14 during early anaphase whereas activation of the MEN maintains Cdc14 in its released state (Chapter II, Pereira et al., 2002; Yoshida et al., 2002a). The FEAR network is comprised of the separase Esp1, the polo-like kinase Cdc5, the kinetochore

protein Slk19, the small nuclear protein Spo12 and its homologue Bns1, and the replication fork block protein Fob1 (Chapter II, III, IV).

Our molecular understanding of FEAR network signaling is still very rudimentary. Genetic epistasis analyses revealed that the FEAR network consists of two branches. *ESP1* functions together with *SLK19* in a branch parallel to *SPO12* and *FOB1* (Chapter III, Chapter IV). I found that *FOB1* is as a negative regulator within the FEAR network, functioning downstream of or in parallel to *SPO12* (Chapter IV). *CDC5* is likely to function downstream of *ESP1* and *SLK19* (Chapter III, Sullivan et al., 2003).

How the FEAR network promotes the release of Cdc14 from its inhibitor remains unknown. It is likely, however, that the dissociation of the Cfi/Net1-Cdc14 complex is regulated by phosphorylation. Both Cdc14 and Cfi1/Net1 are phosphoproteins and their phosphorylation during anaphase correlates with the timing of complex disassembly (Shou et al., 2002a; Yoshida et al., 2002b; Visintin et al., 2003). Cdc5 appears to be at least partially responsible for phosphorylating Cdc14 and Cfi1/Net1. Cdc5 is able to phosphorylate both Cdc14 and Cfi1/Net1 in vitro (Shou et al., 2002a, R. Visintin personal communication). Furthermore, phosphorylation of Cdc14 by Cdc5 does not require the MEN, whereas phosphorylation of Cfi1/Net1 by Cdc5 appears to be mediated through the MEN (Chapter III).

How does the Spo12-Fob1 branch contribute to the release of Cdc14 from its inhibitor? I found that Fob1 binds to Cfi1/Net1 and localizes to the rDNA region where the majority of the Cfi1/Net1-Cdc14 complex resides (Chapter IV), raising the possibility that Fob1's binding to Cfi1/Net1 influences the stability of the Cfi1/Net1-Cdc14 complex. As Spo12 also binds to Fob1, it is possible that Spo12 modulates the effects of

Figure 1

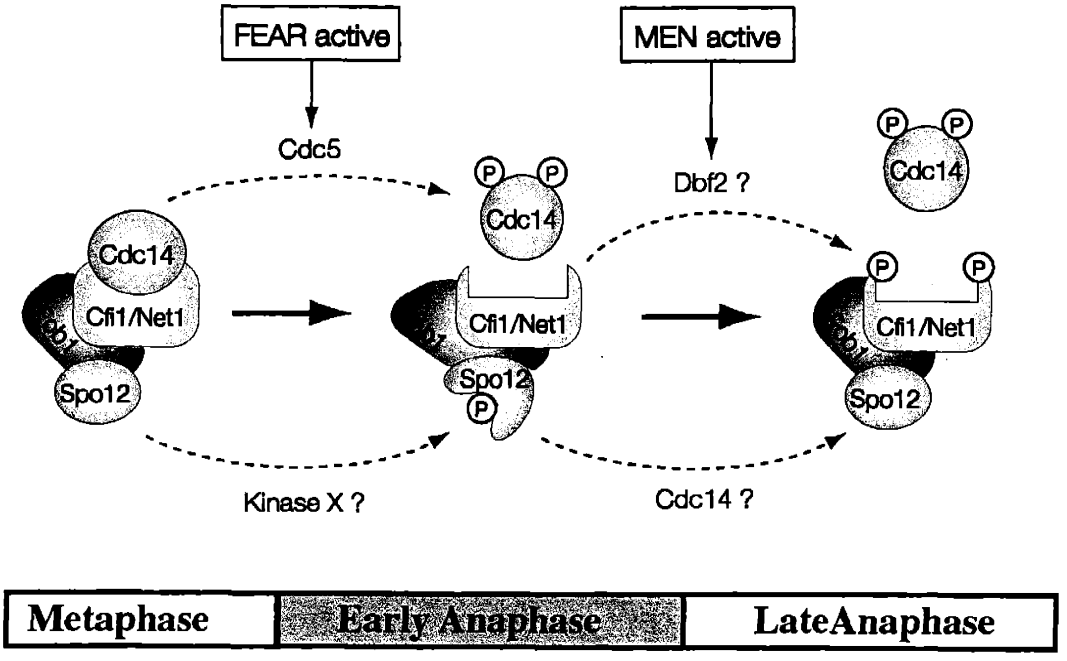

Figure 1: A model for how the FEAR network and the MEN regulate the release of Cdc14 from its inhibitor Cfi1/Net1.

During metaphase, the Spo12-Fob1 complex binds to Cfi1/Net1 and antagonizes Cdc14-Cfi1/Net1 complex disassembly. During anaphase, Spo12 is phosphorylated by an unknown kinase, which leads to a conformational change in Spo12, thereby decreasing its binding to Fob1. This structural rearrangement within the Spo12-Fob1 complex might alleviate Fob1's inhibitory function (as indicated by the altered conformation of the Cfi1/Net1-Cdc14 complex). Together with the phosphorylation of Cdc14 by Cdc5, this allosteric change in the Fob1-Cfi1/Net1 complex may lead to the transient release of Cdc14 from its inhibitor during early anaphase. Activation of the MEN during later stages of anaphase then leads to the phosphorylation of Cfi1/Net1 (perhaps mediated by the MEN kinase Dbf2), thereby promoting the sustained release of Cdc14.

Fob1 on Cfi1/Net1-Cdc14 complex stability (Chapter IV). Based on these findings, I propose the following model for the regulation of Cdc14's association with Cfi1/Net1 (FIGURE 1). Prior to anaphase, binding of Fob1 to Cfi1/Net1 and the absence of Cdc14 and Cfi1/Net1 phosphorylation, prevent the dissociation of Cdc14 from its inhibitor. During early anaphase, Cdc5 phosphorylates Cdc14 and Spo12 relieves Fob1's inhibition. Together, these events promote the transient disassembly of the Cfi1/Net1-Cdc14 complex and release of Cdc14 from its inhibitor. Phosphorylation of Cfi1/Net1 by MEN kinases (likely Dbf2) during later stages of anaphase causes further conformational changes that sustain the release of Cdc14 from its inhibitor.

Functions of the FEAR network in mitosis.

The sustained release of Cdc14 by the MEN is required for cells to exit from mitosis, as cells lacking MEN function arrest in telophase (Johnston et al., 1990; Surana et al., 1993; Shirayama et al., 1994; Jaspersen et al., 1998). In contrast, the FEAR network is not essential for mitotic exit, as cells carrying mutations in FEAR network components delay but do not block mitotic exit. This raises the question about the physiological functions of the FEAR network-mediated Cdc14 activation during early anaphase. Cdc14 released by the FEAR network appears to regulate at least three processes: (1) MEN activation, (2) segregation of telomeric and rDNA regions, and (3) aurora B complex function.

I found that Cdc14 released by the FEAR network activates the MEN, thereby feed-forward activating its own release from the nucleolus (Chapter II). But how does Cdc14 activate the MEN during early anaphase? A previous study by Jaspersen et al.

(2000) showed that a non-phosphorylatable variant of Cdc15 enhances MEN activation. I found that Cdc14 released by the FEAR network dephosphorylates Cdc15, suggesting that FEAR network-released Cdc14 activates the MEN at least in part by dephosphorylating Cdc15 (Chapter II). How the dephosphorylation of Cdc15 promotes MEN activation is unknown (discussed below). Additional mechanisms might contribute to the activation of the MEN by the FEAR network. Two studies found that the FEAR network-released Cdc14 localizes to the daughter-bound SPB during early anaphase (Pereira et al., 2002; Yoshida et al., 2002a). Cdc14 was proposed to downregulate Bfa1-Bub2 (a two-component GAP that negatively regulates the MEN) activity but the molecular mechanism remains elusive (Pereira et al., 2002). Cdc14 also dephosphorylates the MEN activator Lte1, thus promoting its release from the bud cortex (Jensen et al., 2002; Seshan et al., 2002). It is possible that the release of Lte1 from the bud cortex is required for Lte1 to contact the GTPase Tem1 at the SPBs.

The activation of Cdc14 by the FEAR network at the metaphase-anaphase transition raised the possibility that Cdc14 regulates cellular events in addition to mitotic exit. I observed that the segregation of chromosomal masses was significantly impaired in FEAR network mutants when compared to MEN mutants. Further studies in our lab revealed that FEAR network-mediated activation of Cdc14 is required for the dissolution of cohesin-independent chromosome linkages present at the rDNA locus and telomeres (D'Amours et al., 2004). The mechanism by which Cdc14 promotes the segregation of these chromosomal loci is not understood in detail. Interestingly, Cdc14 is required for the anaphase-specific nucleolar localization of the condensin subunits Ycs4 and Ycs5 (D'Amours et al., 2004), which have previously been shown to be important for efficient

segregation of the nucleolus (Bhalla et al., 2002). Furthermore, Cdc14 is required and sufficient for the anaphase-specific sumoylation of Ycs4 (D'Amours et al., 2004). Based on these findings, we proposed that Cdc14 promotes nucleolar segregation by inducing the sumoylation of Ycs4, which in turn targets it to the rDNA region. However, the target(s) of Cdc14 in the regulation of rDNA segregation remain to be identified.

Recently, the FEAR network has also been shown to promote the translocation of the aurora B kinase complex during anaphase (Pereira et al., 2003). The translocation of this complex from kinetochores to the mitotic spindle and spindle midzone has been proposed to stabilize the mitotic spindle prior to mitotic exit (discussed in more detail in Chapter I).

In summary, it appears that the FEAR network is an important regulator of mitosis, as it coordinately regulates many mitotic processes. The fact that the separase Esp1 and the polo-kinase Cdc5 regulate both sister-chromatid separation and mitotic exit also provides a molecular mechanism by which cells ensure that exit from mitosis is not initiated prior to sister-chromatid separation. The vital role of the FEAR network is illustrated by the dramatic loss in viability that cells experience when progressing through anaphase in the absence of FEAR function (D'Amours et al., 2004), which may at least in part be due to chromosome loss (Hartwell et al., 1985).

Functions of the FEAR network during meiosis.

Studies over the past several years provided significant insight into the regulatory circuits of the mitotic cell cycle. Much less is known about the regulation of meiosis, a specialized cell cycle, which is characterized by the succession of two chromosome-

segregation cycles without an intervening S phase. Previous studies suggested that many of the core mitotic regulators, such as Clb-CDKs, the APC/C, and separase, just to name a few, also function to regulate progression through meiosis (reviewed in Nasmyth, 2001). Strikingly, cells lacking various FEAR network components exhibit a similar meiotic phenotype. Cells impaired for *SPO12*, *SLK19*, *CDC5* and *CDC14* function complete only a single meiotic division in which some chromosomes segregate in a meiosis I-like pattern while others segregated in a meiosis II-like pattern, also referred to as "mixed" segregation (Klapholz et al., 1980; Sharon et al., 1990; Kamieniecki et al., 2000; Zeng et al., 2000). Given the role of the FEAR network in mitosis, these findings raised the possibility that Cdc14 activated by the FEAR network also governs meiotic processes. Based on this suspicion, two recent studies found that the FEAR-induced activation of Cdc14 is responsible for mitotic spindle disassembly during exit from the first meiotic division (Buonomo et al., 2003; Marston et al., 2003). Furthermore, similar to the situation in mitosis, the activation of Cdc14 is required for the efficient segregation of the rDNA region during the first meiotic division (Buonomo et al., 2003; Marston et al., 2003). Very unexpectedly, however, FEAR network mutants appear to uncouple meiotic events. The "mixed" segregation observed in FEAR network mutants appears to be due to the absence of surveillance mechanisms that halt the occurrence of meiosis II events when spindle disassembly is delayed. Therefore, in some cases the second meiotic chromosome segregation cycle proceeds on the anaphase I spindle. It is also noteworthy that, while the FEAR network plays an assisting role to the MEN in exit from mitosis, the FEAR network plays the major role for exit from the first meiotic division (Stegmeier et al., 2002; Buonomo et al., 2003; Marston et al., 2003). It will be interesting to determine

whether the MEN, the FEAR network, or both also govern the exit from the second meiotic division.

Unanswered questions and Future Directions

Identification of new FEAR network components and regulators of mitotic exit.

Our ability to understand FEAR network signaling at the molecular level greatly depends on having a complete inventory of its signaling components. As most of the FEAR network components have been identified based on literature search and educated guesses, a more systematic approach is likely to reveal additional components of this pathway. Here, I will discuss three genetic screens aimed at identifying further FEAR network components.

Based on the finding that *ESP1*, *SLK19*, and *SPO12* become essential for mitotic exit in cells lacking the MEN activator *LTE1* (Chapter II), I reasoned that a screen for mutants that are synthetically lethal with lte1Δ might identify potential FEAR network components. In collaboration with Lorrie Boucher in Mike Tyers' lab, we conducted a "high-throughput" SGA (synthetic genetic array) screen using the *S.cerevisiae* knockout collection (Winzeler et al., 1999; Tong et al., 2004). Over 400 candidate genes were identified in this screen, which indicated that the screen was not very selective. Surprisingly, most candidates failed to reproduce the synthetic lethal phenotype when deleted in the W303 strain background. Possible reasons for the high rate of false positives might be differences in strain background (the knock-out collection is in the S288C background) or technical difficulties with the robotics (e.g. inefficient transfer of colonies between plates). Repeating this screen using conventional approaches to

generate loss-of-function alleles might still uncover potential new regulators of mitotic exit.

We also selected for second-site mutations that rescue the synthetic lethality of either *lte1Δ spo12Δ* or *lte1Δ esp1-1*. To inactivate genes at random, we used a transposon-based mutagenesis strategy, which allows the rapid identification of the insertion site and hence affected genes (Burns et al., 1994). As this mutation strategy results mostly in loss-of-function mutants, this screen was aimed at identify negative regulators of mitotic exit. Indeed, we isolated *BFA1* and *BUB2*, two negative regulators of the MEN, validating the screening approach. In addition, the screen identified *KIN4*, which encodes a kinase of unknown function. Katie D'Aquino confirmed that complete deletion of *KIN4* rescues the synthetic lethality of both *lte1Δ spo12Δ* and *lte1Δ esp1-1*. Her data suggest that *KIN4* functions as a negative regulator within the MEN but its precise function remains to be determined.

To identify positive regulators of mitotic exit, we also screened for genes that rescue the synthetic lethality of either *lte1Δ spo12Δ* or *lte1Δ esp1-1* when overexpressed. In collaboration with Rami Rahal, I conducted an overexpression screen, using a yeast library on a high copy number plasmid. In both screens, we isolated the known mitotic exit regulators *TEM1* and *STE20* in addition to wild-type copies of the genes deleted in the starting strain. We also isolated plasmids encoding *FUN21* among other ORFs twice in both screens. Brendan Kiburz has subcloned *FUN21* and showed that its overexpression rescues the synthetic lethality of both strains. Interestingly, *FUN21* was also identified in a meiotic screen for sporulation defects (A. Marston, personal communication), where strains lacking *FUN21* accumulated dyads, a meiotic phenotype

characteristic for FEAR network mutants. Brendan Kiburz is currently in the process of further investigating the functions of *FUN21*.

So far, our genetic screens have only revealed *FUN21* as a potentially new FEAR network component. This could mean that most if not all FEAR network components have already been identified. It is important to note, however, that essential genes such as *ESP1* and *CDC5* would have been missed in our loss-of-function (the mutation strategy is strongly biased towards complete inactivation of ORFs) and overexpression screens, as both too much or too little *ESP1* and *CDC5* kills cells. Indeed, we did not recover *ESP1* or *CDC5* in any of the screens. In these cases, screens for temperature-sensitive alleles using EMS as a mutagen might be more successful. Complementary to these genetic approaches, one could try to identify FEAR network components by probing for protein-protein interaction with known FEAR network components. In fact, this approach was the basis for the identification of *FOB1* as a negative FEAR network regulator (Chapter IV). Novel protein-protein interactions could be identified either by Two-hybrid screens or by immunoprecipitating tagged FEAR network components, followed by identication of co-precipitating proteins by mass spectrometry.

Molecular basis of FEAR network signaling.

Signaling through the SLK19-ESP1 branch.

Slk19 is cleaved by Esp1 at the metaphase-anaphase transition (Sullivan et al., 2001). Therefore, it came as a surprise that neither Slk19 cleavage nor Esp1's proteolytic activity is required for FEAR network function (Chapter II, Appendix II, Sullivan et al., 2003) . Importantly, Esp1's non-proteolytic FEAR function is still regulated by the

securin Pds1 (Sullivan et al., 2003), thus coupling the onset of sister-chromatid separation with the initiation of FEAR signaling. But how do Esp1 and Slk19 promote the release of Cdc14 from the nucleolus? Slk19 binds to Esp1 and Esp1's localization to kinetochores and the spindle midzone is dependent on *SLK19* (Sullivan et al., 2003). These findings raise the possibility that Slk19 activates Esp1's protease-independent FEAR network function, possibly by targeting it to the correct subcellular locations.

Signaling through the SPO12-FOB1 branch.

Based on my findings in Chapter IV, we propose that Fob1 functions to inhibit Cdc14-Cfi1/Net1 complex disassembly prior to anaphase and that activation of Spo12 during early anaphase promotes the release of Cdc14 from its inhibitor by antagonizing Fob1 function. This model is based mostly on genetic evidence and requires further biochemical validation. We failed to detect any significant changes in Fob1-Cfi1/Net1 association during the cell cycle (Chapter IV). However, it is possible that Spo12 and Fob1 relay their signals through more subtle conformational changes. This idea is consistent with the observation that phosphorylation of Spo12 affects its binding to Fob1. However, further biochemical investigations are required to rigorously test this model.

How is Cdc5 activation regulated?

The polo kinase Cdc5 regulates several mitotic processes, such as chromosome segregation and cytokinesis, in addition to exit from mitosis (Alexandru et al., 1999; Hu et al., 2001; Song et al., 2001; Stegmeier et al., 2002; Visintin et al., 2003). Several substrates of Cdc5 have been identified, such as Scc1, Bfa1, Cfi1/Net1, and Cdc14

(Alexandru et al., 2001; Hu et al., 2001; Shou et al., 2002a; Yoshida et al., 2002b; Visintin et al., 2003). An important question is how the phosphorylation of these diverse substrates is regulated temporally. Consistent with a previous study by Cheng et al. (1999), I found that Cdc5 is already active during metaphase, at least when its activity is monitored by an in vitro kinase assay (Appendix I). However, while Cdc5 phosphorylates Scc1 already during metaphase, it does not phosphorylate Bfa1 until anaphase (Appendix I). An attractive hypothesis is that the Slk19-Esp1 complex functions to activate Cdc5 towards anaphase-specific substrates, such as Bfa1 and Cdc14. I extensively discussed this possibility and proposed experiments to test this hypothesis in Appendix I.

Activation of the FEAR network.

Activation of the Esp1 branch of the FEAR network requires the destruction of the securin Pds1, thus linking the onset of sister-chromatid separation with the initiation of FEAR network signaling (Stegmeier et al., 2002; Sullivan et al., 2003). How the activation of the Spo12 branch is controlled is less clear. Spo12 is a phosphoprotein and its phosphorylation is cell cycle regulated, being low in S phase and high in mitosis (Chapter IV). Furthermore, the phosphorylation of two serine residues (Ser^{118} and Ser^{125}) within the highly conserved DSP-Box is required for Spo12's mitotic exit function. These findings raise the possibility that activation of Spo12 is governed by phosphorylation. A useful tool to investigate Spo12 phosphorylation would be phospho-specific antibodies that recognize peptides phosphorylated on either Ser^{118} or Ser^{125}. With such antibodies in hand, one could determine the precise timing and extent of Spo12 phosphorylation on these two sites during mitosis. Furthermore, such antibodies would greatly facilitate the

identification of the kinase(s) responsible for phosphorylating Spo12. Both Ser^{118} and Ser^{125} are part of sequences that conform to the consensus phosphorylation site of both CDKs and MAPKs (mitogen activated protein kinase). A recent study aimed at identifying Clb2-CDK1 substrates found that Spo12 is not phosphorylated by this CDK, at least in vitro (Ubersax et al., 2003). It will be interesting to test whether MAPKs of the HOG pathway, a signal transduction cascade activated by high osmolarity and previously implicated in regulating mitotic exit (see below), contribute to Spo12 phosphorylation.

One could also seek to identify Spo12 activators, such as activating kinases, using a genetic approach. It is quite striking that overexpression of *SPO12* is very powerful in promoting Cdc14 release in anaphase but fails to promote the release of Cdc14 in metaphase-arrested cells (Chapters III and IV). Possibly, the absence of a Spo12 co-factor or lack of post-translational modification during metaphase prevents Spo12 from exerting its full activity. Thus, one might be able to identify Spo12 activators by screening for genes that cause benomyl-sensitivity (due to bypass of the benomyl-induced metaphase arrest) only when co-overexpressed with *SPO12*. In a secondary screen, one could test whether the co-overexpression of candidate genes with *SPO12* causes the release of Cdc14 from the nucleolus in metaphase-arrested cells.

Inactivation of the FEAR network.

The activation of the FEAR network appears to be restricted to a very brief time during early anaphase, as Cdc14 becomes re-sequestered into the nucleolus during late anaphase in cells lacking MEN activity (Chapter II, Pereira et al., 2002; Yoshida et al., 2002a). What is the molecular basis for the short-lived nature of the FEAR signal? Two

FEAR network components, the polo-kinase Cdc5 and Spo12 are targeted for degradation by APC^{Cdh1}, which becomes activated by Cdc14 as cells exit from mitosis (Charles et al., 1998; Cheng et al., 1998; Shirayama et al., 1998; Shah et al., 2001). This mechanism, although it undoubtedly contributes to the inactivation of the FEAR network after cells have exited from mitosis, cannot account for restricting FEAR activity to early anaphase, as Cdc14 becomes re-sequestered during late anaphase in MEN mutants, which maintain high levels of both Spo12 and Cdc5 (my unpublished observation). Moreover, in MEN mutant cells, the separase Esp1 should remain active, as its inhibitor Pds1 is still absent in telophase arrested cells. Intriguingly, overexpression of *SPO12* makes the MEN dispensable for mitotic exit (Chapter IV, Jaspersen et al., 1998; Grether et al., 1999), raising the possibility that Spo12 activity is the limiting factor for FEAR network activation during anaphase. The phosphorylation of two serine residues within Spo12 is required for its FEAR network function (Chapter IV, Shah et al., 2001). Given that these phosphorylation sites are part of sequences that match the Cdc14 consensus site, it is possible that the FEAR-network activated Cdc14 inhibits its own release by dephosphorylating and thus inactivating Spo12. Therefore, the initiation of FEAR network signaling at the metaphase-anaphase transition might plant the seeds for its own inactivation. This model also predicts that the kinase responsible for phosphorylating Spo12 becomes inactivated or unable to phosphorylate shortly after the initiation of anaphase. Clb5-CDK is a possible candidate, as (1) the APC^{Cdc20} targets Clb5 for degradation at the onset of anaphase, and (2) Spo12's phosphorylation sites conform to the CDK consensus.

Potential role of *STE20* and the HOG pathway in regulating mitotic exit.

Another unresolved question is the role of the PAK-like kinase Ste20 in the control of mitotic exit. Ste20 is involved in several signal transduction pathways in yeast, including the HOG pathway (high osmolarity and glycerol), the pheromone signaling cascade, and the filamentous growth pathway (reviewed in Leberer et al., 1997; O'Rourke et al., 2002). We identified *STE20* in our high-copy suppressor screen of *spo12Δ lte1Δ* and *esp1-1 lte1Δ* mutants (see above). Hofken et al. (2002) identified *STE20* as a high copy suppressor of *lte1Δ* mutants at 14°C. Furthermore, *STE20* becomes essential in cells lacking *LTE1* (Hofken et al., 2002, my unpublished observation). Together, these findings raise the possibility that *STE20* functions within the FEAR network, the MEN, or is part of a novel pathway that regulates mitotic exit. It will be important to determine the timing of Cdc14 release from the nucleolus in cells lacking *STE20.* As *STE20* mutants are insensitive to pheromone (α-factor) used for cell-cycle synchronization, it would be useful to generate a "Shokat-allele" of *STE20,* which allows targeted kinase inhibition by addition of a modified ATP analogue (Bishop et al., 2000).

STE20 functions within the HOG pathway (reviewed in O'Rourke et al., 2002), which becomes activated in response to high osmolarity, such as 1.2M Sorbitol 0.3M sodium chloride. Interestingly, conditions of high osmolarity can partially bypass the mitotic exit defect of *cdc15-2*, *dbf2-2*, and *cdc14-1* mutants (Grandin et al., 1998, my unpublished observation). Moreover, osmotic "shock" also results in a transient accumulation of cells in G1 and G2 in a *HOG1*-dependent (Hog1 is a MAPK in the HOG pathway) manner (Alexander et al., 2001). It is possible that the HOG pathway promotes

mitotic exit in order to "push" cells into G1, where cells might be better suited to cope with osmotic stress than during mitosis. Interestingly, deletion of *SPO12* prevents the growth of *cdc15-2* mutants in 1.2M sorbitol at 36°C (my unpublished observation). This could mean that the mitotic exit signal induced by the HOG pathway is transduced by *SPO12*. Alternatively, induction of the HOG pathway might fail to rescue cells lacking both MEN and FEAR network activity. The former hypothesis raises the interesting possibility that MAPKs in the HOG pathway might be responsible for phosphorylating and thus activating Spo12. Preliminary studies, however, indicate that *hog1Δ* and *pbs2Δ* mutants (two essential components of the HOG pathway) are neither delayed in mitotic exit nor in the release of Cdc14 from the nucleolus under standard conditions (my unpublished observation). While not essential during an unperturbed cell cycle, the HOG pathway might still regulate the FEAR network (or the MEN) under conditions of osmotic stress.

How does Cdc15 dephosphorylation activate the MEN?

The MEN kinase Cdc15 appears to integrate a variety of signals. It functions downstream of the GTPase Tem1 and is likely, though not proven, to be activated by GTP-bound Tem1. In addtion, Cdc15's C-terminal domain appears to inhibit Cdc15 function (Bardin et al., 2003). Lastly, it is likely that Cdc15 activation is regulated by by its subcellular localization as its kinase activity does not change throughout the cell cycle (Jaspersen et al., 1998). Cdc15 localizes to both SPBs (spindle pole bodies) from mid-anaphase to telophase (Cenamor et al., 1999; Xu et al., 2000; Menssen et al., 2001; Visintin et al., 2001). Cdc15's localization to the SPBs depends on *TEM1* but Cdc15 may

have additional SPB binding partners, as it localizes to both SPBs whereas Tem1 localizes predominantly to the daughter-bound SPB (Cenamor et al., 1999; Bardin et al., 2000; Pereira et al., 2000; Xu et al., 2000; Menssen et al., 2001; Visintin et al., 2001). Furthermore, when Cdc15 is overexpressed, it is able to localize to SPBs even in the absence of *TEM1* (Bardin et al., 2003).

As disussed above, I found that FEAR-network induced Cdc14 activation promotes the dephosphorylation of Cdc15, which in turn increases MEN signaling (Chapter II, Jaspersen et al., 2000). But how does dephosphorylation of Cdc15 increase its activity? I found that wild-type Cdc15 localizes to SPBs only in mid- to late anaphase, whereas a non-phosphorylatable version of Cdc15 localizes to both SPBs as soon as cells enter anaphase (my unpublished observation). These findings suggest that dephosphorylation of Cdc15 increases the affinity for its SPB binding partner, which in turn may facilitate MEN activation. As one of the potential CDK phosphorylation sites within Cdc15 resides in the Tem1-Cdc15 interaction domain (Jaspersen et al., 2000; Asakawa et al., 2001), it is possible that Cdc15 dephosphorylation influences its binding to Tem1. Alternatively, the dephosphorylation of Cdc15 might abolish the inhibition from its C-terminal domain.

Is there a biological significance of nuclear versus cytoplasmic release of Cdc14?

During the analysis of Cdc14 localization in wild-type and MEN mutant cells, I observed that the FEAR network-induced Cdc14 release is mostly restricted to the nucleus, whereas Cdc14 also spreads into the cytoplasm during later anaphase when the MEN becomes active (Chapter II). Furthermore, the FEAR network-induced Cdc14

release during meiosis also appears to be restricted to the nucleus (Buonomo et al., 2003; Marston et al., 2003). Two studies, however, found that FEAR network-released Cdc14 localizes to the SPBs in a *BUB2* dependent manner (Pereira et al., 2002; Yoshida et al., 2002a). As Bub2 localizes to the cytoplasmic face of the SPB (Pereira et al., 2000), this finding indicates that at least some Cdc14 has to be exported from the nucleus for it to interact with Bub2 at the SPBs. It is possible, however, that the majority of FEAR network-released Cdc14 is restricted to the nucleus and that the small amounts of Cdc14 that are exported into the cytoplasm become "sequestered" to the SPBs.

How is the FEAR network-released Cdc14 restricted to the nucleus? It is possible that the time span that Cdc14 is released by the FEAR network is too short to allow for its nuclear export before being re-sequestered into the nucleolus. A more interesting hypothesis would be that MEN signaling "actively" promotes Cdc14's export into the cytoplasm. The finding that Cdc14 localizes predominantly to the nucleus and spreads into the cytoplasm only during anaphase in *cfi1Δ* cells, which lack Cdc14's nucleolar anchor (Visintin et al., 1999, R. Visintin, personal communication), suggests that Cdc14's nuclear export is activated during anaphase (or that its nuclear import is inhibited during anaphase). Therefore, it is possible that the MEN not only promotes Cdc14's release from its inhibitor but also its nuclear export. However, whether the anaphase-specific cytoplasmic localization of Cdc14 in *cfi1Δ* mutants requires the MEN has not yet been tested. The hypothesis that the MEN promotes the "cytoplasmic release" of Cdc14 is also supported by the finding that reduction-of-function mutants in the karyopherins *KAP104* and *MTR10*, which govern nuclear transport, have been identified as suppressors of MEN mutants (Asakawa et al., 2002; Shou et al., 2002b).

How might MEN activation promote Cdc14's nuclear export? MEN kinases such as Dbf2 might phosphorylate Cdc14, which in turn could expose a cryptic NES (nuclear export sequence), or modify the nuclear export machinery, such as the karyopherins or the NPC (nuclear pore complex).

Is there a physiological reason to "restrict" the FEAR network-released Cdc14 to the nucleus? The FEAR network becomes active at the metaphase-anaphase transition. Budding yeast, however, faces the challenge of orienting the mitotic spindle through the bud neck. Thus, cells should not completely inactivate mitotic CDKs at the metaphase-anaphase transition, as the spindle has not yet elongated through the bud neck. Two major targets of Cdc14 responsible for mitotic CDK inactivation (Visintin et al., 1998), Swi5 and Cdh1, are localized to the cytoplasm when phosphorylated (Moll et al., 1991; Jaquenoud et al., 2002). Thus, by limiting the FEAR-network induced release of Cdc14 to the nucleus, cells might prevent the complete inactivation of mitotic CDKs, Indeed, Swi5 remains phosphorylated and localizes to the cytoplasm in the MEN mutant *cdc15-2* (Moll et al., 1991), indicating that the FEAR network-induced release of Cdc14 is insuffienct for Swi5 activation. This hypothesis would further predict that "forcing" Cdc14 into the cytoplasm during early anaphase leads to premature CDK inactivation and premature spindle disassembly.

Concluding Remarks

Impressive progress has been made in understanding the regulation of mitotic exit and its coordination with other mitotic events, particularly from studies in budding yeast. Through many studies in several labs, we now have a pretty clear picture

of the molecular mechanisms underlying the MEN signal transduction cascade. In contrast, our molecular understanding of FEAR network signaling is still in its infancy. In this chapter, I have discussed how additional regulators of the FEAR network as well as parallel pathways controlling exit from mitosis might be identified. I also tried to outline some (of the many) important questions that remain to be addressed.

Very little is known about the regulation of mitotic exit and the function(s) of Cdc14 homologues in higher eukaryotes. The recent advent of siRNA technology should allow forward genetic screens to be applied in higher eukaryotes, which are likely to reveal some of the players important for the regulation of mitotic exit in these organisms. It will be interesting to see whether higher eukaryotes rely on conserved regulatory circuits, such as the FEAR network or the MEN, or evolved novel strategies to regulate their late mitotic events.

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Appendix I

Temporal regulation of Cdc5 substrate phosphorylation

Introduction

Successful completion of mitotis requires the orchestration of many cellular events such as chromosome segregation, spindle disassembly and cytokinesis. Coordination of these processes is in part achieved by employing the same proteins to regulate multiple cellular processes. In budding yeast, for example, the conserved Polo kinase Cdc5 contributes to the activation of the Anaphase Promoting Complex/Cyclosome (APC/C), removal of cohesin complexes from chromosomes, mitotic exit, and cytokinesis (Charles et al., 1998; Shirayama et al., 1998; Alexandru et al., 1999; Hu et al., 2001; Song and Lee, 2001; Stegmeier et al., 2002; Visintin et al., 2003). However, it is still a mystery how Cdc5 manages to regulate distinct processes at different times during mitosis.

The dissolution of sister-chromatids is initiated by cleavage of the cohesin subunit Scc1/Mcd1 by the protease Esp1 (reviewed in Nasmyth, 2001), thus destroying the cohesive potential of the cohesin complex. The polo kinase Cdc5 promotes, but is not essential, for sister-chromatid separation. Cdc5 phosphorylates the cohesin subunit Scc1, which is thought to render it more susceptible to cleavage by separase (Alexandru et al., 2001).

The only essential function of Cdc5 its role in mitotic exit, as cells lacking *CDC5* arrest in telophase with high mitotic CDK activity (Kitada et al., 1993; Jaspersen et al., 1998; Lee et al., 2001a; Stegmeier et al., 2002; Visintin et al., 2003). Cdc5 regulates this cell cycle transition in a complex manner, as it is a component of the FEAR network (Cdc fourteen early anaphase release) but also contributes to MEN (mitotic exit network) activation (Chapter II, Hu et al., 2001; Visintin et al., 2003). The FEAR network and the

MEN regulate the activity of the Cdc14 phosphatase, which triggers mitotic exit by reversing CDK phosphorylation (Visintin et al., 1998). During G1, S, G2, and early M phase, Cdc14 is held inactive within the nucleolus by its competitive inhibitor Cfi1/Net1 but is released from its inhibitor during anaphase, thereby allowing it to dephosphorylate its substrates (Shou et al., 1999; Visintin et al., 1999). The FEAR network is activated at the metaphase-anaphase transition and initiates the release of Cdc14 from the nucleolus (Chapter II, Pereira et al., 2002; Yoshida et al., 2002; Sullivan and Uhlmann, 2003). The MEN (Mitotic Exit Network), a GTPase signaling cascade, further promotes the release of Cdc14 and maintains the phosphatase in its released state during later stages of anaphase and telophase (Chapter II, Shou et al., 1999; Visintin et al., 1999; Pereira et al., 2002; Yoshida et al., 2002).

The FEAR network is comprised of the separase Esp1, the polo-like kinase Cdc5, the kinetochore protein Slk19, and the small nuclear/nucleolar protein Spo12 and its homologue Bns1, and Fob1 (Chapter II, III, IV). Cdc5 is likely to be the ultimate effector of the FEAR network (Chapter III, Sullivan and Uhlmann, 2003). Cdc5 induces the phosphorylation of both Cdc14 and Cfi1/Net1, which is thought to promote dissociation of the complex (Shou et al., 2002; Yoshida and Toh-e, 2002; Visintin et al., 2003). In addition, Cdc5 promotes MEN activation by phosphorylating Bfa1 (Hu et al., 2001), a subunit of the inhibitory two-component GAP (GTPase activating protein) within the MEN, which presumably inactivates its GAP activity (Hu et al., 2001; Geymonat et al., 2003).

As the FEAR network and the MEN needs to be kept inactive prior to the initiation of anaphase, it is somewhat surprising that Cdc5 is already active in metaphase,

at least as judged by in vitro kinase measurements (Cheng et al., 1998). It is possible that the in vitro kinase assays do not accurately reflect Cdc5's in vivo activity. Alternatively, Cdc5 might already be active in metaphase but unable to target its anaphase substrates. To begin to address these questions, I re-investigated Cdc5 kinase activation during the cell cycle by an in vitro kinase assay, which confirmed that Cdc5 becomes active during metaphase. I found, however, that different Cdc5 substrates are phosphorylated at different cell cycle stages in vivo. Lastly, I tested whether overexpression of upstream FEAR network components could induce Cdc5 to phosphorylate anaphase specific substrates already during metaphase.

Results

Cdc5 in vitro kinase activity is high in metaphase-arrested cells.

To determine Cdc5 kinase activity during the cell cycle, I measured its activity in an in vitro kinase assay. Cells carrying a 3HA tagged version of Cdc5 were synchronously released from a pheromone-induced G1 arrest and Cdc5 kinase activity, as well as the protein levels of Cdc5-3HA, Clb2, and Cdc28 were determined at the indicated times. I found that Cdc5's in vitro kinase activity was maximal during metaphase and anaphase (Figure 1A, B). Moreover, the activation of Cdc5 kinase occurred with similar kinetics when cells were released into nocodazole-containing medium, which causes metaphase arrest due to activation of the spindle checkpoint (Figure 1A, B). My findings suggest that Cdc5 is already active in metaphase, at least when assayed in vitro, which is in agreement with a previous study by Cheng et al. (1998).

Bfa1 phosphorylation is maximal during anaphase.

Cdc5 has been implicated in phosphorylating Scc1, Bfa1, Cfi1/Net1, and Cdc14 (Alexandru et al., 2001; Hu et al., 2001; Shou et al., 2002; Yoshida and Toh-e, 2002; Visintin et al., 2003). I wanted to investigate whether Cdc5 phosphorylates all of these substrates at similar times during mitosis. To analyze the phosphorylation status of Bfa1, I constructed 3HA, 3MYC, and 13MYC epitope tag fusions of Bfa1. Surprisingly, the location and nature of the epitope tags greatly affected the functionality of the protein and our ability to determine phosphorylation shifts by SDS-PAGE (Table1). I used the

TABLE 1: Epitope tagging of Bfa1 and Bub2.

	Terminus tagged	Cdc14 release	Nocodazole checkpoint	Phosph. shift by SDS-PAGE
Bfa1-13MYC	C	delayed	Not tested	NO
3MYC-Bfa1	N	normal	Not tested	NO
Bfa1-3MYC	C	normal	Not tested	NO
3HA-Bfa1	**N**	**normal**	**Functional**	**YES**
Bfa1-3HA	C	normal	Not tested	NO
Bub2-13MYC	C	normal	Not tested	NO
Bub2-3MYC	C	delayed	Not tested	NO
3HA-Bub2	**N**	**normal**	**Functional**	**YES (weak)**
Bub2-3HA	C	normal	Not tested	NO

I found the 3HA-Bfa1 and 3HA-Bub2 tags to be most functional and used these strains for futher experiments.

Note: Katharine D'Aquino found that the 3HA-Bfa1 and 3HA-Bub2 tags rescue the mitotic exit delay of *GAL-KIN4* strains, suggesting that the N-terminal tags reduce the function of both proteins.

Figure 1

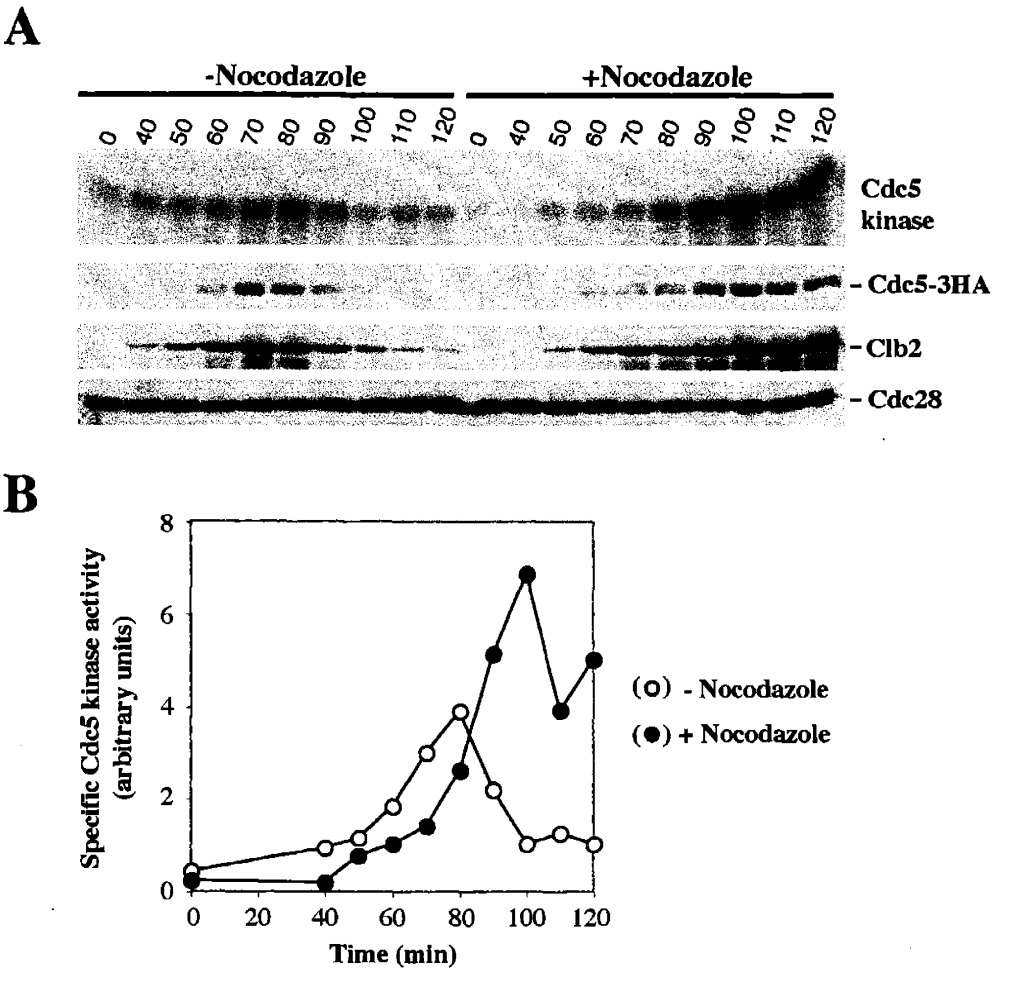

Figure 1: Cdc5 activity is maximal during metaphase.

(**A**) Wild-type cells (A2399) carrying a *3HA-CDC5* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/ml) followed by release into YEPD medium lacking (-Nocodazole) or containing 15μg/ml nocodazole (+Nocodazole). 5 μg/ml α-factor was re-added after 80 minutes to prevent entry into the next cell cycle. The Cdc5-associated kinase activity (upper panel), and the protein levels of Cdc5-3HA, Clb2, and Cdc28 protein was determined at the indicated time points.
(**B**) Quantification of the specific kinase activity of Cdc5 from the data shown in (A).

N-terminal 3HA fusion of Bfa1 for further analysis, as it appeared to be the most functional and allowed easy detection of phosphorylation shifts by Western blot analysis.

I analyzed the phosphorylation status of Bfa1 in cells synchronously released from a G1 arrest. Bfa1 was unphosphorylated during G1 and S phase and the appearance of a lower phosphorylated form corresponded to the time when cells entered metaphase (Figure 2A, Bfa1-P at 90 minutes). Bfa1 phosphorylation was maximal during anaphase (Figure 2A, Bfa1-PP at 105 and 120 minutes) and declined when cells exited from mitosis (Figure 2A, 135 and 150 minutes). To determine the phosphorylation status of Bfa1 during anaphase more precisely, cells carrying a temperature sensitive allele of the MEN kinase *CDC15* (*cdc15-2*) were synchronously released from a G1 arrest at the restrictive temperature. The hyperphosphorylated form of Bfa1 predominated in telophase-arrested cells (Figure 2B, Bfa1-PP), suggesting that Bfa1 phosphorylation is maximal during anaphase. To test whether Bfa1 phosphorylation is already initiated

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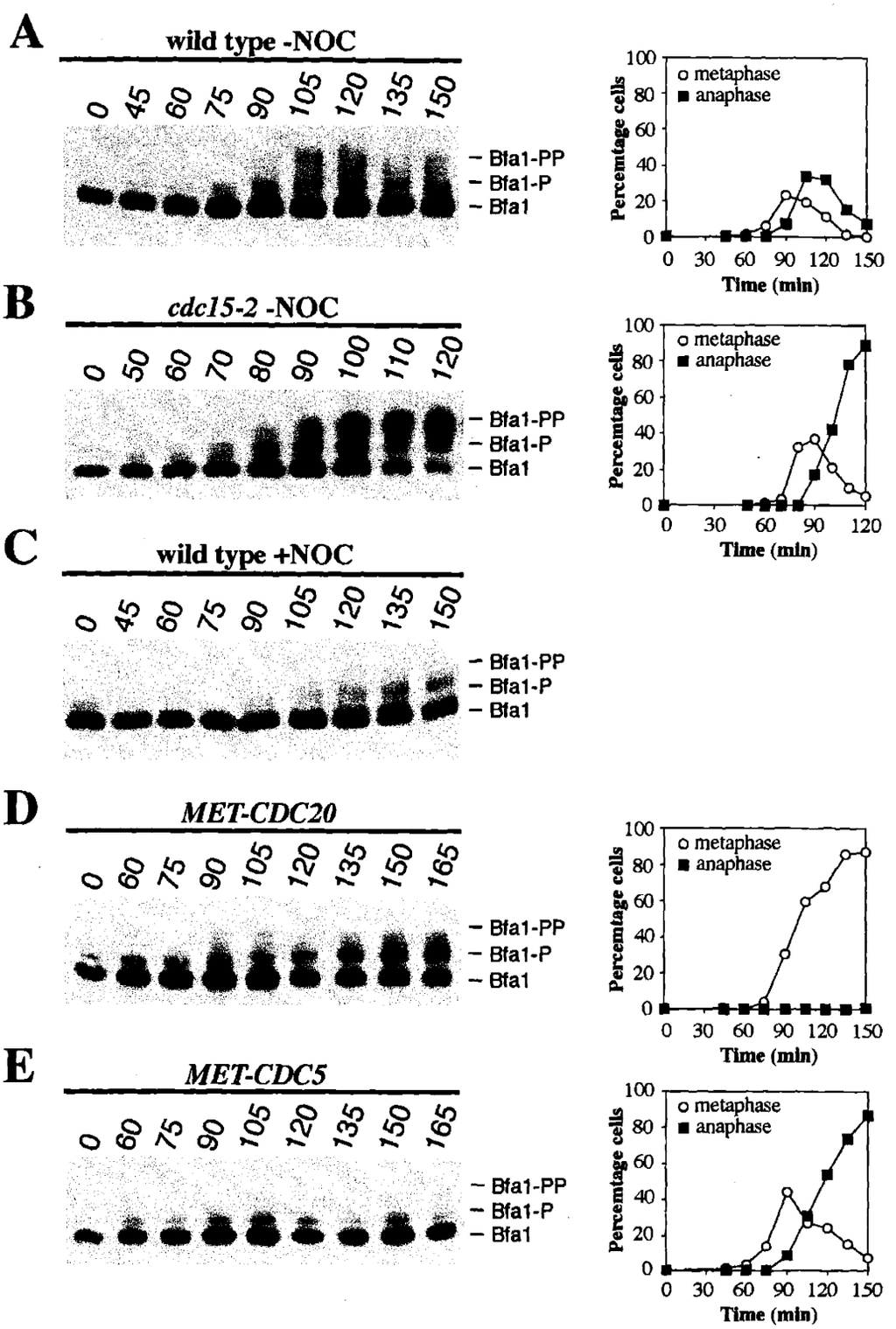
Figure 2: Bfa1 phosphorylation is maximal during anaphase

(A,C) Wild-type (A4378) cells carrying a *3HA-BFA1* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/ml) followed by release into YEPD medium lacking (-NOC) or containing 15μg/ml nocodazole (+NOC). Samples were analyzed for the migration of Bfa1-3HA (6% SDS-PAGE gels) at the indicated times. The graphs on the right show the percentage of cells in metaphase (open circles) and anaphase (closed squares) at the indicated time points.
Note: Monitoring spindle morphology is not possible in nocodazole-treated cells, as nocodazole causes the depolymerization of mitotic spindles.
(B) *cdc15-2* (A3516) cells carrying a *3HA-BFA1* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/ml) followed by release into YEPD medium at 37°C and analyzed as described in (A).
(D,E) *MET-CDC20* (A6458) or *MET-CDC5* (A6376) cells both carrying a *3HA-BFA1* fusion grown in medium lacking methionine were arrested in G1 with α-factor (5 μg/ml). 4mM methionine was added 1hr prior to release and cells were released into YEPD medium containing 8mM methionine. Samples were analyzed as described in (A).

Figure 2


A
wild type -NOC
0 45 60 75 90 105 120 135 150
Bfa1-PP
Bfa1-P
Bfa1
B
cdc15-2 -NOC
0 50 60 70 80 90 100 110 120
Bfa1-PP
Bfa1-P
Bfa1
C
wild type +NOC
0 45 60 75 90 105 120 135 150
Bfa1-PP
Bfa1-P
Bfa1
D
MET-CDC20
0 60 75 90 105 120 135 150 165
Bfa1-PP
Bfa1-P
Bfa1
E
MET-CDC5
0 60 75 90 105 120 135 150 165
Bfa1-PP
Bfa1-P
Bfa1
metaphase
anaphase
Percentage cells
Time (min)

during metaphase, wild-type cells were released from a G1 arrest into media containing the microtubule poison nocodazole, which triggers metaphase arrest through activation of the spindle checkpoint (reviewed in Lew and Burke, 2003). The lower phosphorylated form of Bfa1 appeared as cells entered metaphase. The hyperphosphorylated form of Bfa1 (Bfa1-PP), however, was not observed in nocodazole-arrested cells (compare Figure 2C and 2B). It is possible that the observed lack of Bfa1 hyperphosphorylation is a consequence of spindle checkpoint activation rather than cells being halted in metaphase. To exclude this possibility, I arrested cells in metaphase by depletion of *CDC20* (*MET-CDC20)*, an essential APC co-factor (reviewed in Peters, 2002). Similar to nocodazole-arrested cells, the lower phosphorylated form of Bfa1 (Bfa1-P) appeared as cells entered metaphase, but hyperphosphorylated forms (Bfa1-PP) where absent throughout the metaphase arrest (compare Figure 2D and 2B). Together, these findings suggest that Bfa1 phosphorylation is initiated during metaphase and that maximal phosphorylation of Bfa1 occurs not until anaphase and telophase.

Cdc5 has previously been shown to phosphorylate Bfa1 in vitro and in vivo (Hu et al., 2001; Lee et al., 2001b), but Hu et al. (2001) detected only one phosphorylated species of Bfa1. To test whether all phosporylated forms of Bfa1 depend on *CDC5,* I analyzed the phosphorylation status of Bfa1 in *CDC5* depleted cells (*MET-CDC5*). Depletion of *CDC5* prevented the accumulation of hyperphosphorylated Bfa1 (Bfa1-PP). However, the absence of *CDC5* did not completely eliminate the appearance of the lower phosphorylated form of Bfa1 (Bfa1-P in Figure 2E). My findings suggest that a priming phosphorylation of Bfa1 occurs during metaphase in a *CDC5* independent manner and that *CDC5* promotes maximal Bfa1 phosphorylation during anaphase.

Figure 3

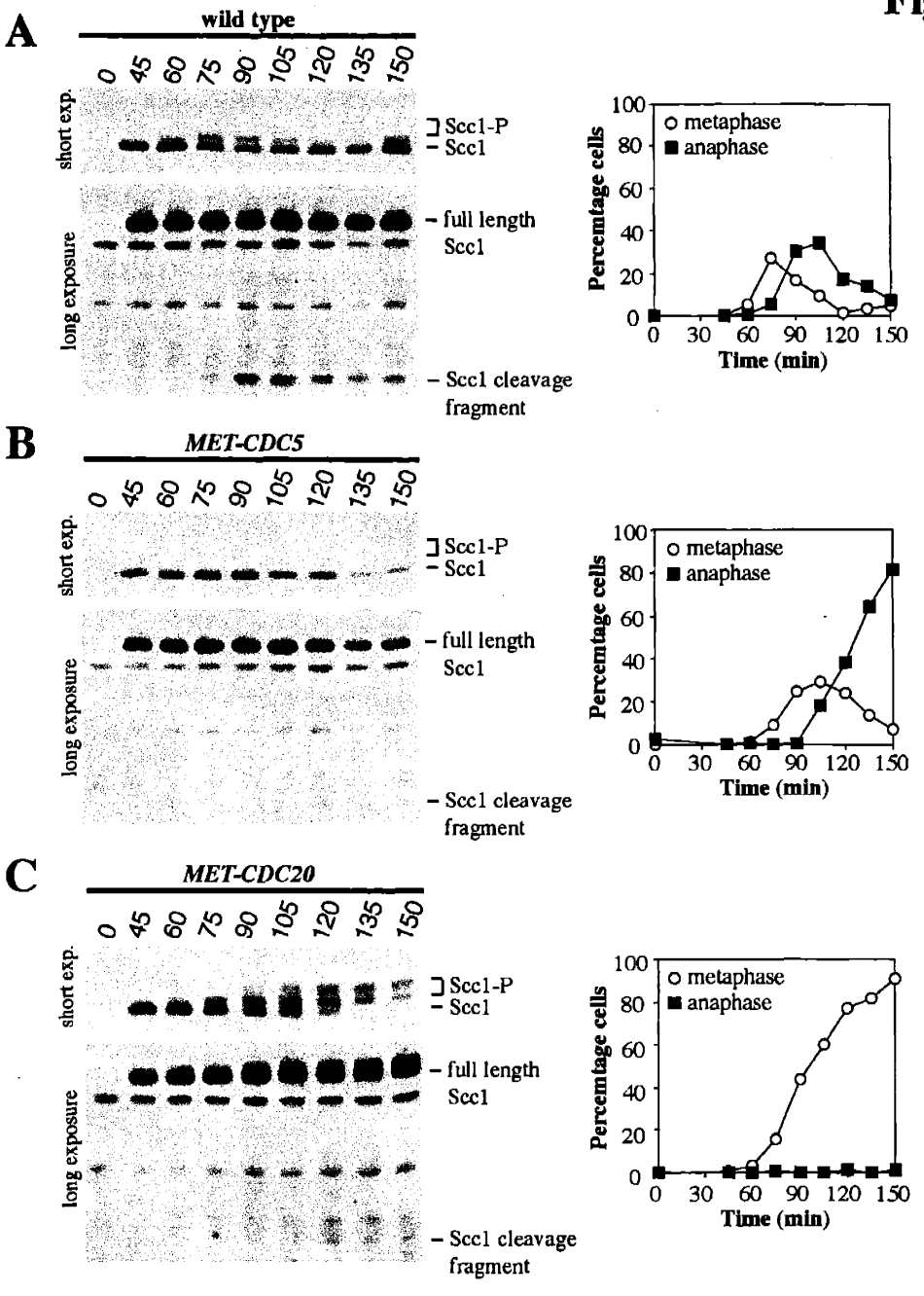


Figure 3: Scc1 is phosphorylated during metaphase

(A) Wild-type (A4072) cells carrying a *SCC1-3HA* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/ml) followed by release into YEPD medium lacking pheromone. Samples were analyzed for the migration of Scc1-3HA (6% SDS-PAGE gels) at the indicated times. The graphs on the right show the percentage of cells in metaphase (open circles) and anaphase (closed squares) at the indicated time points.

(B,C) *MET-CDC5* (A6636) or *MET-CDC20* (A6640) cells both carrying a *SCC1-3HA* fusion grown in medium lacking methionine were arrested in G1 with α-factor (5 mg/ml). 4mM methionine was added 1hr prior to release and cells were released into YEPD medium containing 8mM methionine. Samples were analyzed as described in (A).

Scc1 phosphorylation is maximal in metaphase

Next, I wanted to examine the timing of Scc1 phosphorylation, which is cleaved at the metaphase-anaphase transition by the separase Esp1 (Uhlmann et al., 1999; Alexandru et al., 2001). I analyzed the phosphorylation of Scc1 in cells synchronously released from a G1 arrest. Scc1 became phosphorylated as cells entered metaphase shortly before its cleavage (Figure 3A , Uhlmann et al., 1999; Alexandru et al., 2001). As previously reported by Alexandru et al. (2001), the phosphorylation of Scc1 was entirely dependent on *CDC5*, as the phosphorylated forms of Scc1 were absent in *CDC5* depleted cells (Figure 3B). In contrast to Bfa1, however, Scc1 was hyperphosphorylated in *CDC20* depleted cells, which arrest in metaphase (Figure 3C). Thus, my data suggest that Cdc5 phosphorylates Scc1 during metaphase.

Effects of overexpression of FEAR network components on Bfa1 phosphorylation

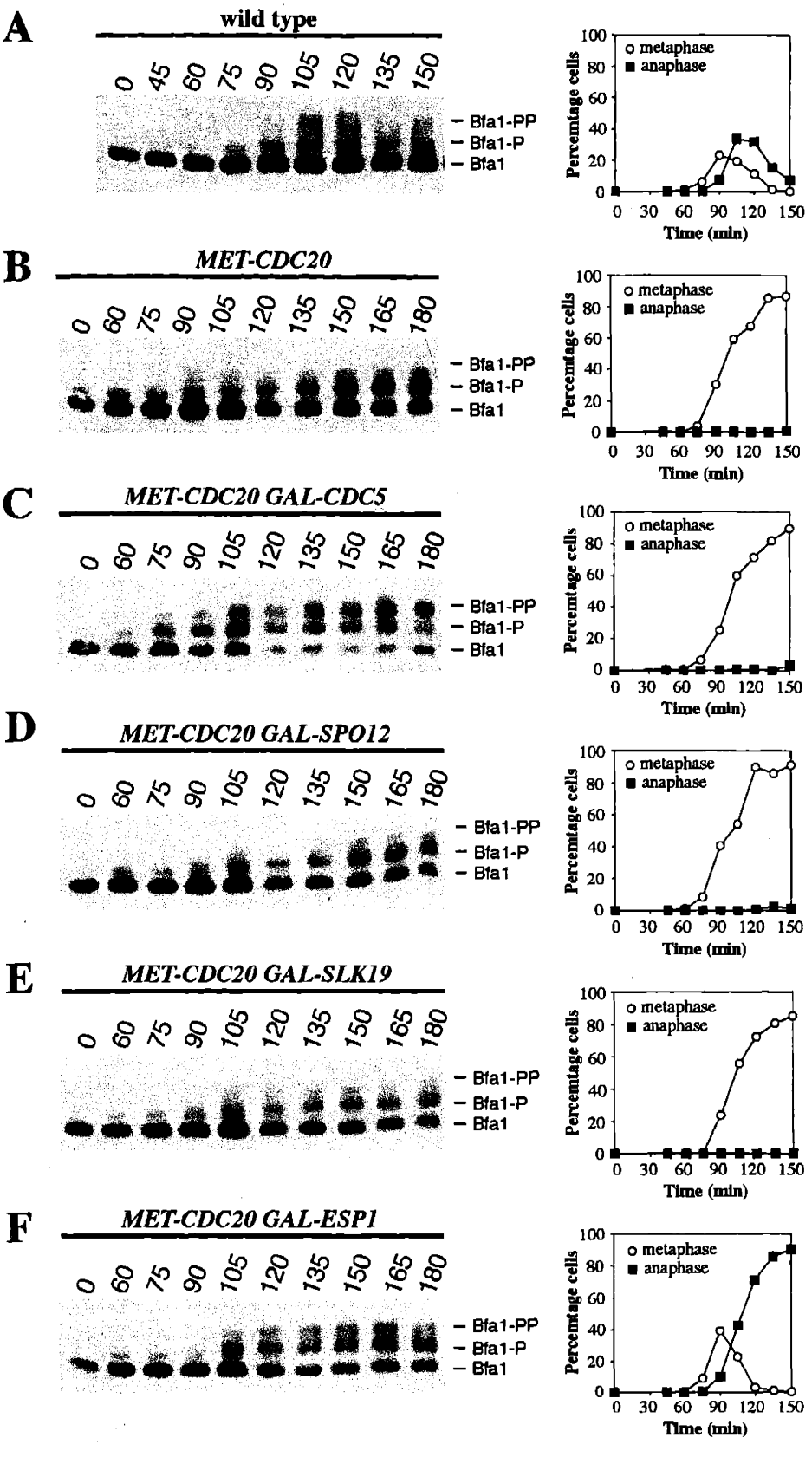
My results indicate that Cdc5 phosphorylates Scc1 in metaphase but is unable to phosphorylate Bfa1 during this cell cycle stage. Thus, Cdc5 must somehow be "activated" towards phosphorylating Bfa1 during anaphase. Cdc5 is a component of the FEAR network, and is likely to be the ultimate effector of this pathway (Chapter III, Sullivan and Uhlmann, 2003). Therefore, I wanted to test whether the overexpression of other FEAR network components can "activate" Cdc5 to phosphorylate Bfa1 during metaphase. To this end, *CDC20* depleted cells were synchronously released from a G1 arrest into galactose containing medium to induce expression of either *CDC5*, *SPO12*,

SLK19, or *ESP1*. Overexpression of *CDC5* induced the hyperphosphorylation of Bfa1 in *CDC20* depleted cells (Bfa1-PP, compare Figure 4C and 4B), suggesting that increasing Cdc5 protein levels is sufficient to promote the hyperphosphorylation of Bfa1 during metaphase. Overexpression of *SPO12* or *SLK19* did not promote the hyperphosphorylation of Bfa1 (Figure 4D,E). *ESP1*, however, was almost as potent as *CDC5* in inducing Bfa1 hyperphosphorylation when overexpressed (Bfa1-PP, Figure 4F), raising the intriguing possibility that *ESP1* could function to activate Cdc5 towards its anaphase substrates. It is important to note that overexpression of *ESP1* allows *CDC20* depleted cells to progress into anaphase (Figure 4F, graph on right). Therefore, the observed induction of Bfa1 hyperphosphorylation could be a consequence of progression into anaphase rather than *ESP1* directly promoting Cdc5 activity.

Figure 4: Effects of overexpression FEAR network components on Bfa1 phosphorylation.

(A) Experiment from Figure 2A is shown as reference.
(B-F) *MET-CDC20* (A6458), *MET-CDC20 GAL-CDC5* (A7398), *MET-CDC20 GAL-SPO12* (A7400), *MET-CDC20 GAL-SLK19* (A7402), and *MET-CDC20 GAL-ESP1* (A7404) cells all carrying a *3HA-BFA1* fusion grown in medium lacking methionine and containing raffinose were arrested in G1 with α-factor (5 μg/ml). 4mM methionine and 2% galactose was added 1hr prior to release and cells were released into YEPR medium containing 8mM methionine and 2%galactose. Samples were analyzed for the migration of Bfa1-3HA (6% SDS-PAGE gels) at the indicated times. The graphs on the right show the percentage of cells in metaphase (open circles) and anaphase (closed squares) at the indicated time points.

Figure 4


A
wild type
0 45 60 75 90 105 120 135 150
Bfa1-PP
Bfa1-P
Bfa1
B
MET-CDC20
0 60 75 90 105 120 135 150 165 180
C
MET-CDC20 GAL-CDC5
D
MET-CDC20 GAL-SPO12
E
MET-CDC20 GAL-SLK19
F
MET-CDC20 GAL-ESP1
metaphase
anaphase
Percentage cells
Time (min)

Discussion

The polo kinase Cdc5 regulates a variety of mitotic processes, which raises the questions as to how Cdc5 activity is temporally regulated. I found that Cdc5 becomes active during metaphase, as judged by its in vitro kinase activity, consistent with a previous study by Cheng et al. (1998). The *CDC5* dependent phosphorylation of Scc1 occurs during metaphase (Figure 3, Alexandru et al., 2001). In contrast, the MEN inhibitor Bfa1 does not become phosphoryated until anaphase (Figure2). A previous study by Hu et al. (2001) found that cells lacking *CDC16* (a gene encoding a component of APC/C complex required for anaphase entry) accumulated the phosphorylated form of Bfa1. As Hu et al. were only able to detect one phosphorylated form of Bfa1, some regulatory phosphorylation events on Bfa1 might have escaped their detection due to failure of separating the two phosphorylated species of Bfa1 (Bfa1-P and Bfa1-PP in Figure 2A). I found that the initial phosphorylation of Bfa1 during metaphase is mostly *CDC5* independent. I speculate that another kinase might be responsible for this "priming" phosphorylation as recently proposed by Elia and Yaffe for other Polo kinase substrates (see below, Elia et al., 2003a; Elia et al., 2003b). Whether Bfa1's priming phosphorylation is required for *CDC5* depent hyperphosphorylation of Bfa1 during anaphase is not known. Identification of the putative Bfa1 "priming" kinase(s) will be an important task for future studies.

Elia et al. (2003) showed that the Polo box, a conserved domain found in the C-terminus of all Polo-like kinases, recognizes the phospho-motif Ser pSer/pThr Pro/X. This interaction is thought to promote substrate phosphorylation by "anchoring" Polo kinases to their substrates and increasing their kinase activity (Elia et al., 2003a; Elia et

al., 2003b). These findings suggest that full Cdc5 activation towards at least some of its substrates might only occur after the substrate has been "primed" by another protein kinase. Therefore, an attractive hypothesis that could account for the temporal differences in Cdc5 substrate phosphorylation postulates that different Cdc5 substrates require "priming" phosphorylations by different kinases. Temporal regulation could be achieved if the various "priming" kinases were active at distinct stages of the cell cycle. Alternatively, Cdc5's temporal selectivity could be achieved by changes in its subcellular localization. However, the fact that both Bfa1 and Cdc5 are already present on spindle pole bodies during metaphase (my unpublished observation), argues against this possibility, at least in the case of Bfa1 phosphorylation.

Even if the "priming kinase" hypothesis for Cdc5's temporal selectivity is correct, it is possible that additional mechanism contribute to Cdc5 activation towards certain substrates or in specific cell cycle stages. My finding that high levels of *ESP1* induces Bfa1 hyperphosphorylation in *CDC20* depleted cells raises the intriguing possibility that *ESP1* activates Cdc5 to phosphorylate its anaphase specific substrates. I have not yet formally tested whether this *ESP1*-induced Bfa1 hyperphosphorylation is *CDC5* dependent. More importantly, overexpression of *ESP1* allows *CDC20* depleted cells to progress into anaphase. Thus, it is impossible to determine whether *ESP1* "directly" activates Cdc5 or whether the observed effect on Bfa1 phosphorylation is an indirect consequence of anaphase progression. The following experiments will allow me to distinguish between these two possibilities. (1) To prevent anaphase progression in the *ESP1* overexpressing cells, I can use a protease-dead version of *ESP1*, as Esp1's protease activity is required for sister-chromatid separation but dispensable for its FEAR network

function (Appendix II, Sullivan and Uhlmann, 2003). Alternatively, anaphase progression could be prevented by co-overexpression of a non-cleavable version of Scc1 (Uhlmann et al., 1999) (2) To test whether anaphase progression in the absence of *CDC20* activity is sufficient for Bfa1 phosphorylation, I will analyze Bfa1 phosphorylation in strains depleted for both *CDC20* and *SCC1 (MET-CDC20 MET-SCC1* strain). The depletion of Scc1 allows for spindle elongation in the absence of separase activity (Ciosk et al., 1998; Stegmeier et al., 2002). It will also be interesting to determine whether Esp1 physically interacts with Cdc5. No matter the outcome, these experiments will deliver some insight into the regulation of substrate recognition by Cdc5. Esp1 may "directly" activate Cdc5 towards Bfa1 phosphorylation. Alternatively, spindle elongation or spindle pole body migration into the bud might be the signal that induces Cdc5 to phosphorylate Bfa1, thus inactivating Bfa1and preparing cells to exit from mitosis.

Experimental Procedures

Cdc5 in vitro kinase assay

Cells were pelleted, washed once in 10mM Tris (pH7.5) and frozen in liquid nitrogen. Pellets were resuspended in 150µl of CHAPS lysis buffer (50mM HEPES pH7.4, 100mM NaCl, 0.5% CHAPS, 5mM $MgCl_2$ 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1mM Na_3VO_4, 50mM NaF, complete EDTA-free protease inhibitor cocktail [Roche]). Cells were lysed by adding 150µl of acid-washed glass beads and vortexing twice for 5 minutes. 400 µg of extract in 60µl of CHAPS lysis buffer was used for immunoprecipitations. 1.2 µl of α-HA.II mouse antibody (Covance, final concentration 1:50) was added and incubated for 1hr on ice. Then, 12 µl of Protein-G sepharose (Pierce) was added and incubated for 1.5 h. Immunoprecipitates were pelleted by centrifugation and washed three times with CHAPS lysis buffer and twice with 25mM MOPS (pH7.0). Samples were resuspended in 6µl HB(II) buffer (25mM MOPS pH7.0, 15 mM $MgCl_2$, 5 mM EGTA, 1mM DTT, complete EDTA-free protease inhibitor cocktail [Roche], 60mM β-glycerophosphate, 0.1mM Na_3VO_4). For determination of kinase activity, 20 µg of casein, 10 µCi of [γ-^{32}P]ATP, and cold ATP to 10 µM were added to 20µl of this suspension. The reaction proceeded fro 20 minutes at room temperature and stopped by boiling in sample buffer, and resolved by elecrophoresis on a 12.5% SDS polyacrylamide gel. Gels were dried and exposed to film.

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Appendix II

Esp1's proteolytic activity is not required for its mitotic exit function

Introduction

The separase Esp1 is a conserved protease that initiates chromosome segregation by cleaving the cohesin subunit Scc1, thus destroying the "molecular glue" that holds sister chromatids together prior to anaphase (reviewed in Nasmyth, 2001). Prior to anaphase, the protease activity of Esp1 is inhibited by the securin Pds1 (reviewed in Nasmyth, 2001). Previous studies and work presented in this thesis show that *ESP1* also promotes exit from mitosis by regulating the activity of the Cdc14 phosphatase as part of the FEAR network (Chapter II, Cohen-Fix et al., 1999; Tinker-Kulberg et al., 1999). During G1, S, G2, and early M phase, Cdc14 is held inactive within the nucleolus by its competitive inhibitor Cfi1/Net1 but is released from its inhibitor during anaphase, thereby allowing it to dephosphorylate its substrates (Shou et al., 1999; Visintin et al., 1999). The FEAR network (Cdc Fourteen Early Anaphase Release Network) is activated at the metaphase-anaphase transition and initiates the release of Cdc14 from the nucleolus (Chapter II, Pereira et al., 2002; Yoshida et al., 2002).

In addition to Esp1, FEAR network components include the polo kinase Cdc5, the small nuclear protein Spo12 and its homologue Bns1, the replication fork block protein Fob1, and the kinetochore protein Slk19, which is also a substrate of Esp1 (Sullivan et al., 2001). Slk19 cleavage, however, appears not to be required for its FEAR network function (Chapter II, Sullivan et al., 2003). This finding suggests that either Esp1 promotes mitotic exit by cleaving a substrate other than Slk19 or that Esp1's protease activity is not required for its FEAR network function. Here, I present evidence that Esp1's protease activity might not be required for its mitotic exit function.

Results

Esp1 is a member of the CD clan family of caspases. All members of this protease family contain highly conserved Histidine and Cysteine residues, which form a catalytic dyad (Uhlmann et al., 2000). Mutation of either one of these residues within Esp1 has been shown to abolish Esp1's protease activity in vivo and in vitro (Uhlmann et al., 2000). To construct a protease-dead mutant version of *ESP1* (*esp1-pd*), I mutated both catalytic residues (Cys^{1531} and His^{1505}) to alanines. I integrated a single copy of this mutant under the control of the *ESP1* promoter into the TRP locus of *esp1-1* mutant cells. Wild-type *ESP1* but not the protease dead *ESP1* (*esp1-pd*) rescued the growth defect of *esp1-1* mutants at 37°C (data not shown). To assess whether Esp1's protease activity was required for its mitotic exit function, I analyzed the kinetics of Cdc14 release from the nucleolus and mitotic spindle disassembly in wild-type and *esp1-1* cells, as well as *esp1-1* mutants carrying either an integrated wild-type copy of *ESP1* (*ESP1-WT*) or a protease-dead version of *ESP1* (*esp1-pd*). Cells were synchronously released from a G1 arrest at the restrictive temperature for *esp1-1* mutants. *esp1-1* mutants delayed both the release of Cdc14 from the nucleolus (Figure 1C, Chapter II) and exit from mitosis, as judged by mitotic spindle disassembly (Figure 1B, Chapter II). As expected, introduction of a wild-type copy of *ESP1* rescued both the mitotic exit and Cdc14 release defect of *esp1-1* mutant strains (Figure 1B,C). Surprisingly, the protease-dead version of *ESP1* rescued the Cdc14 release and mitotic exit defect to a similar extend as wild-type *ESP1* (Figure 1B,C). Importantly, the *esp1-1 esp1-pd* cells released Cdc14 although failing to progress into anaphase (Figure 1A). These findings suggest that Esp1's protease activity is not required for its mitotic exit function.

Figure 1

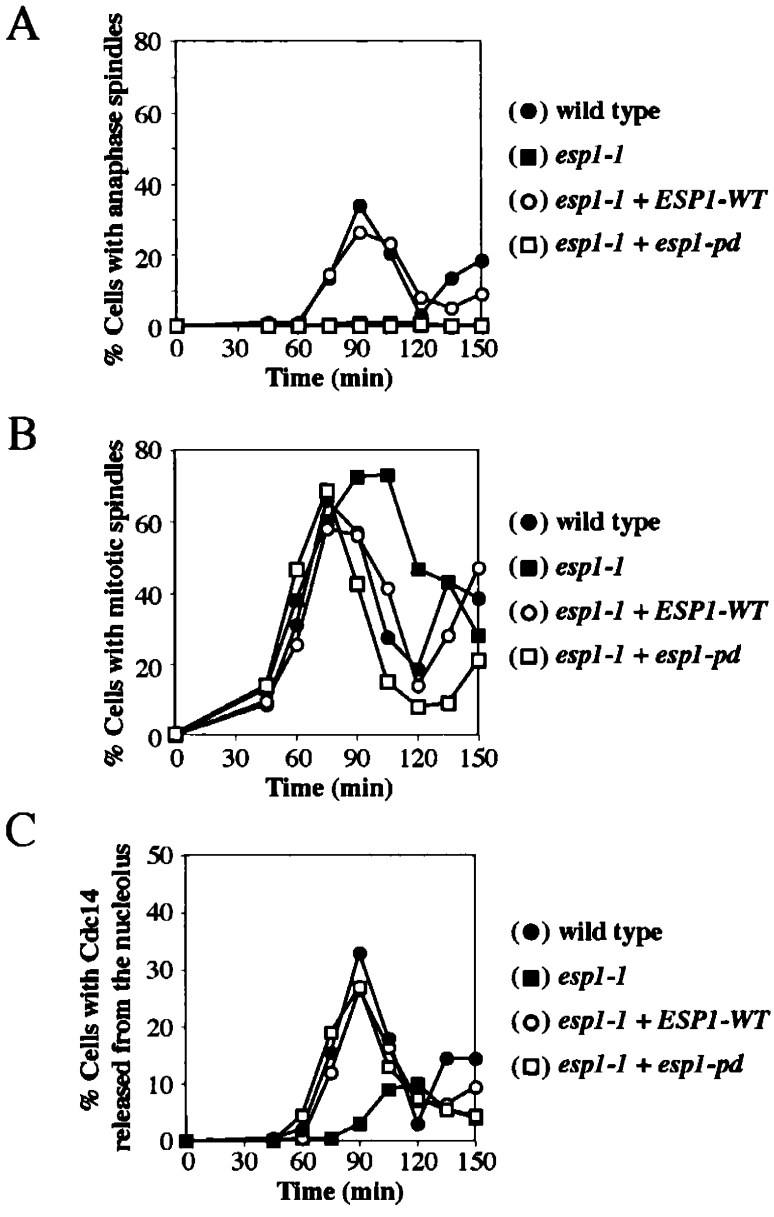

Figure 1: Esp1's proteolytic activity is dispensable for its mitotic exit function
(A-C) wild-type (A1411), *esp1-1* (A2277), *esp1-1 ESP1-WT* (A7556), and *esp1-1 esp1-td* (A7558) cells all carrying a *CDC14-3HA* fusion were arrested in G1 in YEPD medium with α-factor (5 μg/ml) and released into YEPD medium lacking pheromone at 37C. The percentage of anaphase (A) and mitotic spindles (B) and the percentage of cells with Cdc14 released from the nucleolus (C) was determined at the indicated times.

Note: Metaphase and anaphase spindles were counted together as mitotic spindles because expression of wild-type *ESP1* promotes anaphase initiation, whereas *esp1-td* cells do not enter anaphase.

Discussion

My findings suggest that Esp1's proteolytic activity is dispensable for its FEAR network function, which is in agreement with two other recent studies (Buonomo et al., 2003; Sullivan et al., 2003). An important caveat of each of these studies is the fact that they utilized the *esp1-1* mutant to inactivate endogenous *ESP1* function. The use of a temperature-sensitive allele raises the possibility that the *esp1-1* mutant has some residual activity even at its restrictive temperature that could be complemented by the *esp1-pd* mutant. Indeed, the *esp1-1* mutant has been shown to exhibit residual protease activity (Sullivan et al., 2001). Thus, it is possible that the protease-inactive form of *ESP1* stimulates the activity of the *esp1-1* mutant, perhaps by stabilizing it or by increasing its nuclear concentration. Dimers of Esp1, however, have not been detected, making this scenario less likely (Buonomo et al., 2003; Sullivan et al., 2003). Alternatively, the binding of the protease-dead Esp1 to Pds1 might lower the effective concentration of this anaphase inhibitor, thereby elevating the residual activity of *esp1-1* mutants.

The fact that the protease-inactive *ESP1* was just as effective as wild-type *ESP1* in promoting Cdc14 release from the nucleolus and mitotic exit, argues at least against the possibility that a large amount of "FEAR substrate" cleavage is required. To settle this question once and for all, one would need to conduct this experiment in a "true" *ESP1* null background. This task is quite challenging, as *ESP1* is essential for proper chromosome segregation, and too much or too little *ESP1* interferes with the faithful execution of this process. I tried to construct heat-inducible degron-alleles of *ESP1* (Dohmen et al., 1994), and although the protein can be depleted below detection levels by Western analysis, it is possible that also these alleles retain residual activity. In this light,

the best way to conclusively proof Esp1's protease independent FEAR network function will be to elucidate the protease-independent mechanism by which Esp1 promotes mitotic exit.

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