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The Replication Fork Block Protein Fob1 Functions as a Negative Regulator of the FEAR Network

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Summary

Background: The protein phosphatase Cdc14 is a key regulator of exit from mitosis in budding yeast. Its activation during anaphase is characterized by dissociation from its inhibitor Cfi1/Net1 in the nucleolus and 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.
Results: 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. 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.
Conclusions: Fob1 functions as a negative regulator of the FEAR network. 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 downregulation of mitotic CDK activity is accomplished by the ubiquitin-dependent degradation of the regulatory mitotic cyclin subunit and the upregulation 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 and thereby triggers mitotic cyclin degradation and Sic1 accumulation [4, 5]. Consistent with Cdc14's essential role in exit from mitosis, its activity 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 (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; temperature-sensitive mutants defective in MEN components arrest in late anaphase. In contrast, FEAR network-induced Cdc14 activation is not essential for mitotic exit; mutations in FEAR network components cause a delay but do not preclude exit from mitosis. Although 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 yeast homolog 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 yeast homolog 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

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analyses have 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 that regulate the interaction between Cdc14 and its inhibitor within Spo12 and Spo12-associated factors. 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 and 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.

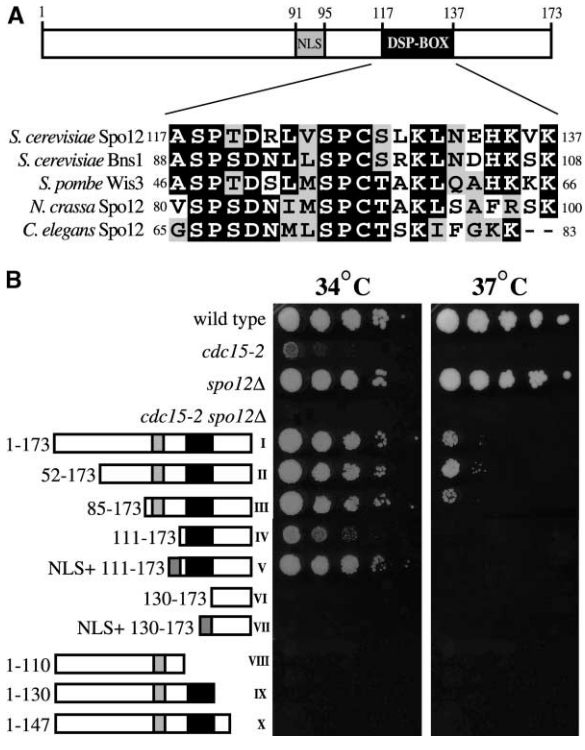

Figure 1. Spo12's C-Terminal Domain Is Required for Its Mitotic-Exit Function

(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 homologs. Identical amino acids are shaded in black, whereas amino acids with similar properties are shaded in gray. 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 spo12*Δ 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, and A7086.

Results

The Highly Conserved DSP-Box Is Essential For Spo12's Mitotic-Exit Function

To identify the regions that are important within *SPO12* 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 spo12*Δ) and that (2) overexpression of *SPO12* suppresses the growth defect of *cdc15-2 spo12*Δ cells (Figure 1B, fifth row, [5]). Individual *SPO12* truncations were introduced into *cdc15-2 spo12*Δ 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 constitute about half of the coding region of *SPO12*, were still able to suppress *cdc15-2 spo12*Δ mutants (Figure 1B, truncations II and III). Removal of the N-terminal 110 amino acids significantly reduced *SPO12*'s ability to suppress *cdc15-2 spo12*Δ 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], Figure S1 in the Supplemental Data available with this article online, truncation IV). Addition of an exogenous NLS to *GAL-SPO12(111–173)* mostly restored its nuclear localization (Figure S1, truncation V) and significantly enhanced its ability to suppress *cdc15-2 spo12*Δ mutants at 34°C (Figure 1B, compare truncations IV and V). 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, spanning amino acids 117–137, whose function is unknown (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 spo12*Δ 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 S1) or due to the inability of the protein to enter the nucleus; truncation VII carrying an exogenous NLS still failed to suppress the growth defect of *cdc15-2 spo12*Δ mutants (Figures 1B and S1, 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 S2).

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 by 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 2A), suggesting that Fob1 binds to the C-terminal domain of Spo12. The two-hybrid interaction was confirmed by coimmunoprecipitation experiments using tagged versions of Spo12 and Fob1. Immunoprecipitation of Spo12-ProA resulted in coprecipitation of Fob1-13MYC (Figure 2B) 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 2C), and Fob1 localized to the nucleolus during all cell cycle stages, as judged from costaining with the nucleolar marker Nop1 (Figure 2D). 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. 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 3A).

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 that 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 with α factor and released into nocodazole-containing medium. When Cdc14 localization was examined, a fraction of *clb5*Δ *fob1*Δ mutant cells, but not *clb5*Δ cells, transiently released Cdc14 from the nucleolus as cells entered the metaphase arrest (Figure 3B). This finding suggests that *FOB1* is necessary for preventing 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], Figures 3C and 3D). The kinetics of the release of Cdc14 from the nucleolus that resulted from deletion of *FOB1* were similar to, though not as pronounced as, those of cells lacking *MAD1* (compare Figures 3B and 3C), suggesting that *FOB1* functions as an inhibitor of the FEAR network. To conclusively distinguish between *FOB1* functioning as an inhibitor of the FEAR network versus an inhibitor of the MEN, we examined the effect of deleting *FOB1* in *mad1*Δ and *bub2*Δ mutants. Deletion of *FOB1* in *clb5*Δ *mad1*Δ mutants led to neither an increase in Cdc14

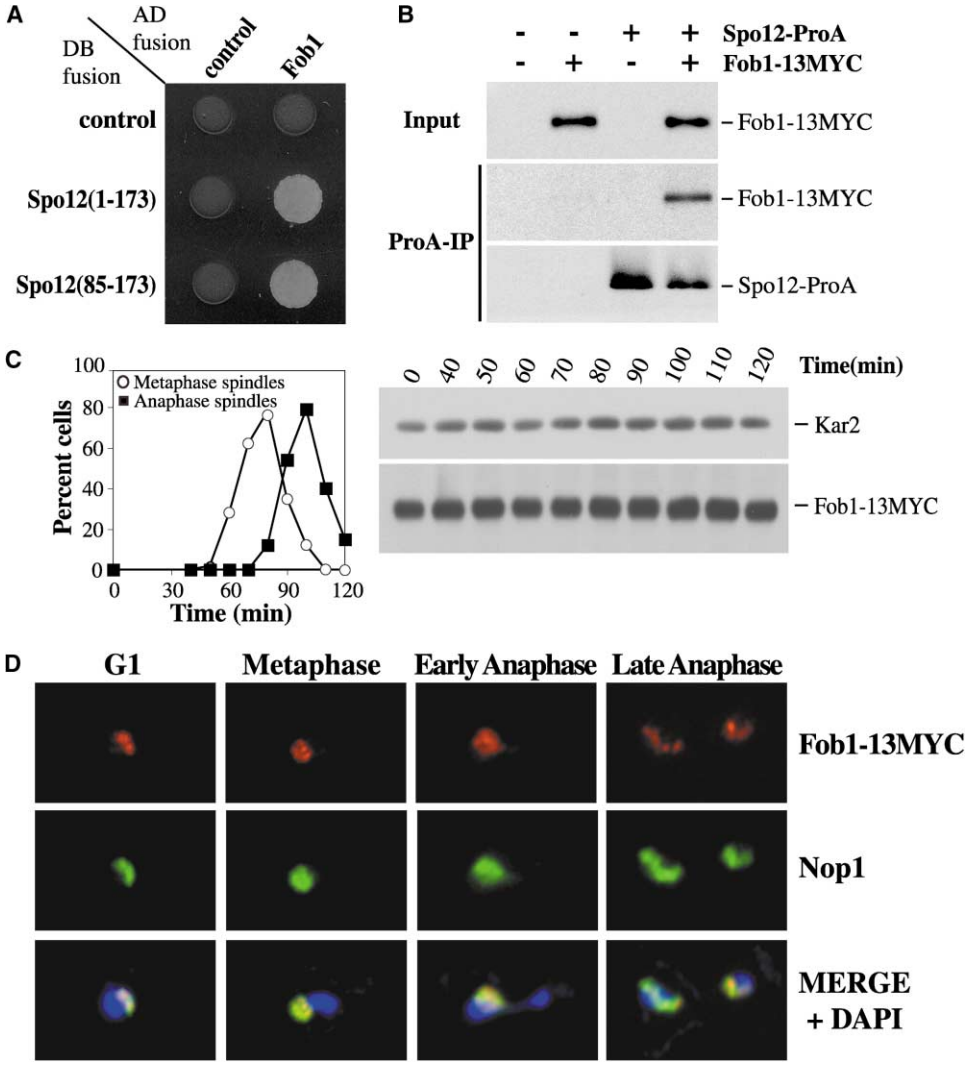

Figure 2. Fob1 Physically Associates with Spo12

(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 coimmunoprecipitates with Spo12-ProA from whole cell extracts. The following strains were used (from left to right): A2587, A8556, A5360, and 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 (5 μg/ml) and subsequently released 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.

release from the nucleolus (Figure 3C) nor an increase in exit from mitosis as judged by rebudding (Figure 3F; 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 *clb5*Δ *bub2*Δ mutants increased the proportion of cells releasing Cdc14 from the nucleolus (Figure 3D) and exiting mitosis (Figure 3G). 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* would 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 min, as indicated by the persistence of cells with anaphase spindles (Figure 4A, open squares). Consistent with the idea that *FOB1* antagonizes Cdc14 activation, we found that the

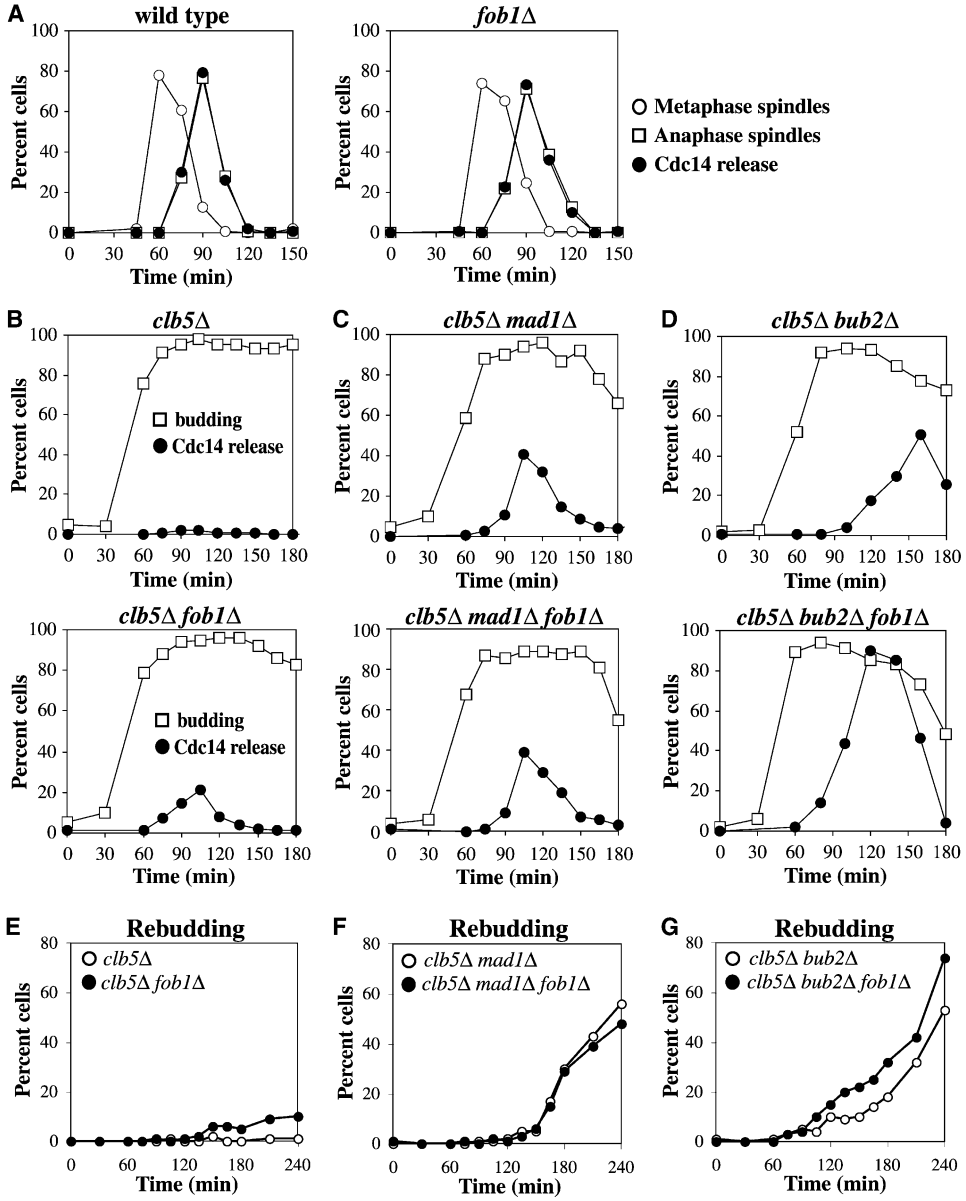

Figure 3. Deletion of *FOB1* Allows a Transient Cdc14 Release in Nocodazole-Arrested Cells

(A) Wild-type (A1411) and *fob1*Δ (A8263) cells carrying a *CDC14-3HA* fusion were arrested in G1 in YEPD medium with α factor (5 μg/ml) and subsequently released 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) *clb5*Δ (A1784), *clb5*Δ *fob1*Δ (A8580), *clb5*Δ *mad1*Δ (A8604), *clb5*Δ *mad1*Δ*fob1*Δ(A9229), *clb5*Δ *bub2*Δ(A8605), and *clb5*Δ *bub2*Δ-*fob1*Δ(A8582) cells all carrying a *CDC14-3HA* fusion were arrested with α factor (5 μg/ml) and released into medium containing 15 μg/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). Adding back 7.5 μg/ml nocodazole 120 min after release from the G1 arrest prevented 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.

mitotic-exit delay of *GAL-FOB1* cells was eliminated by the introduction of *TAB6-1*, an allele of *CDC14* that suppresses the mitotic-exit defect of MEN mutants, into *fob1*Δ mutants (Figure S3, [31]). Furthermore, overexpression of *FOB1* led to a delay in the release of Cdc14 from the nucleolus, particularly during early anaphase (Figure 4A).

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

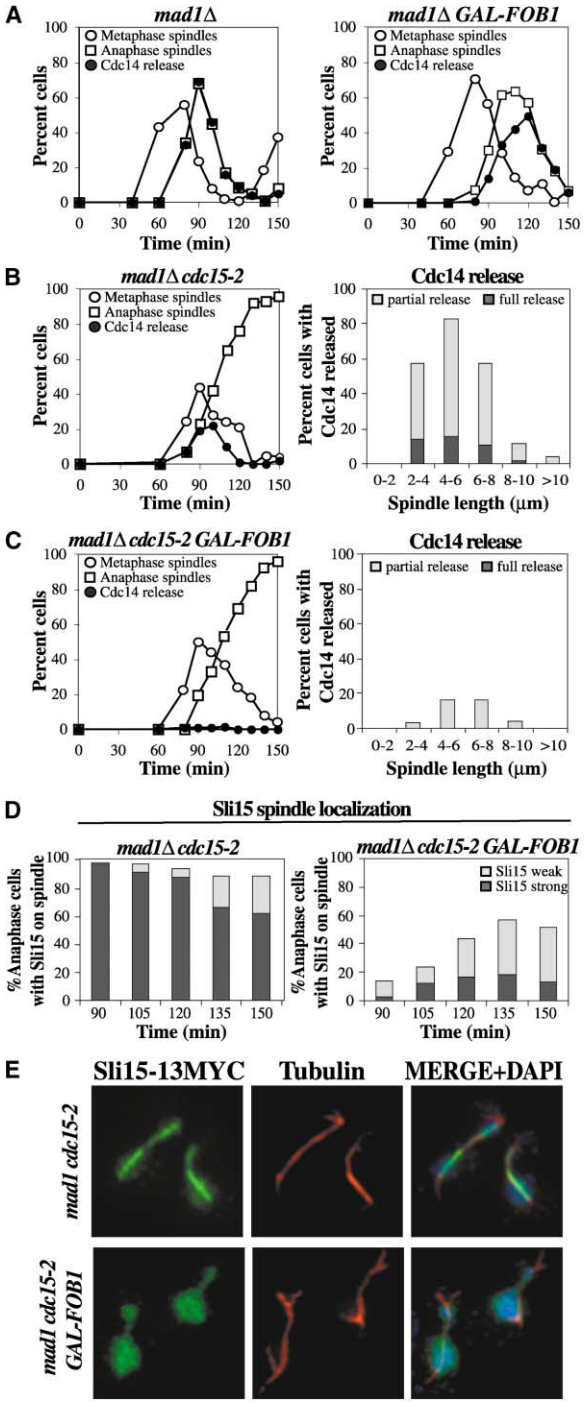

Figure 4. Fob1 Overproduction Delays Mitotic Exit

(A) *mad1*Δ (A2853) and *mad1*Δ *GAL-FOB1* (A9355) cells carrying a *CDC14-3HA* fusion were grown in YEPR medium and arrested in G1 with α factor (5 μg/ml). Expression of Fob1 was induced 2 hr prior to release by the 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 and C) *mad1*Δ *cdc15-2* (A4300) and *mad1*Δ *cdc15-2 GAL-FOB1* (A9356) cells carrying a *CDC14-3HA* fusion were grown in YEPR medium and arrested in G1 with α factor (5 μg/ml) at 25°C. Expression of Fob1 was induced 2 hr prior to release by the 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 min after pheromone release. We consistently observed that *mad1*Δ *cdc15-2* cells, when grown in raffinose and galactose instead of glucose, show mostly partial release of Cdc14 from the nucleolus. More than 400 cells were analyzed for each strain.

(D) *mad1*Δ *cdc15-2* (A9917) and *mad1*Δ *cdc15-2 GAL-FOB1* (A9918) cells carrying a *SLI15-13MYC* fusion were grown and treated as described in (B). 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 *mad1*Δ *cdc15-2* (A9917) and *mad1*Δ *cdc15-2 GAL-FOB1* (A9918) cells 105 min after release from the G1 arrest (from experiment in [D]).

spindle [11]. In *mad1*Δ *cdc15-2* mutants, the majority of early anaphase cells (mitotic spindle length of 2–7 μm) have Cdc14 released from the nucleolus, whereas most late-anaphase cells (mitotic spindle length greater than 8 μm) have Cdc14 sequestered in the nucleolus (Figure 4B, [11]). Overexpression of *FOB1* in *mad1*Δ *cdc15-2* cells largely abolished the transient release of Cdc14 from the nucleolus at intermediate spindle lengths (Figure 4C).

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 mad1*Δ mutants (Figures 4D and 4E) but was significantly impaired when *FOB1* was overexpressed (Figures 4D and 4E). 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 Defects of *spo12*Δ *bns1*Δ 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 5A) and Cdc14 release defect of *spo12*Δ *bns1*Δ double mutants, particularly during early stages of anaphase (Figure 5B; 70 and 80 min 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 5C). Our results suggest that *FOB1* functions down-

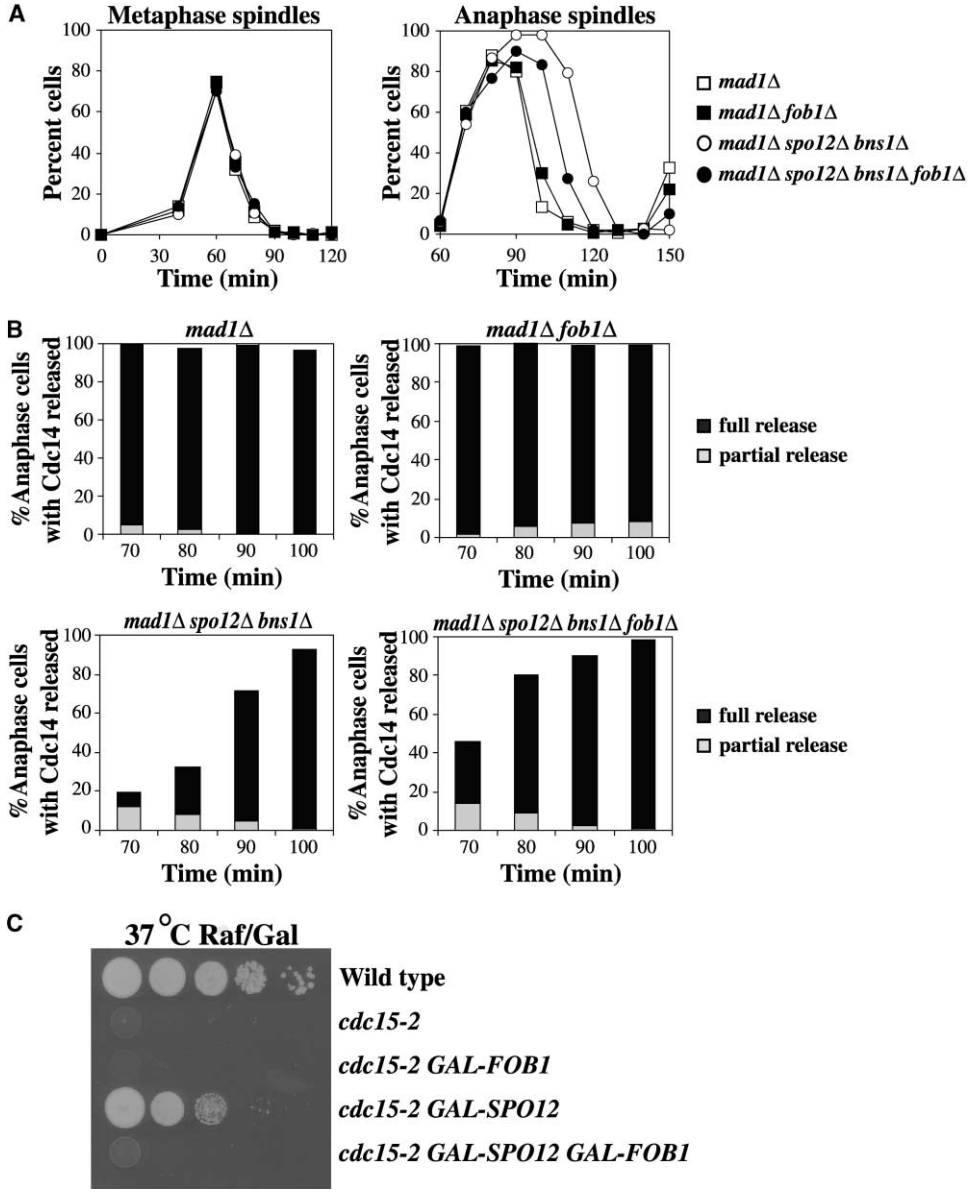

Figure 5. Deletion of *FOB1* Partially Rescues the Mitotic-Exit Defect of *spo12*Δ *bns1*Δ Mutants

(A and B) *mad1*Δ (A2853), *mad1*Δ *fob1*Δ (A8675), *mad1*Δ *spo12*Δ *bns1*Δ (A5408), and *mad1*Δ*spo12*Δ*bns1*Δ*fob1*Δ (A8603) cells all carrying a *CDC14-3HA* fusion were arrested in G1 with α factor (5 μg/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). To circumvent any indirect effects due to spindle checkpoint activation, we conducted this analysis in cells in which the essential spindle checkpoint component *MAD1* was deleted.

(C) Ten-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, and A10160.

stream of or in parallel to *SPO12*. The fact that deletion of *FOB1* only partially rescued the Cdc14 release defect of *spo12*Δ *bns1*Δ double mutants, however, also shows that *SPO12* regulates mitotic exit through mechanisms other than *FOB,* or that *FOB1*, in addition to 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 *fob1*Δ mutants do not exhibit a delay in release of Cdc14 from the nucleolus or mitotic exit (Figure 3A).

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). However, Cfi1/Net1 but not Cdc14 coimmunoprecipitated with Fob1 (Figure 6A; 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; addition of

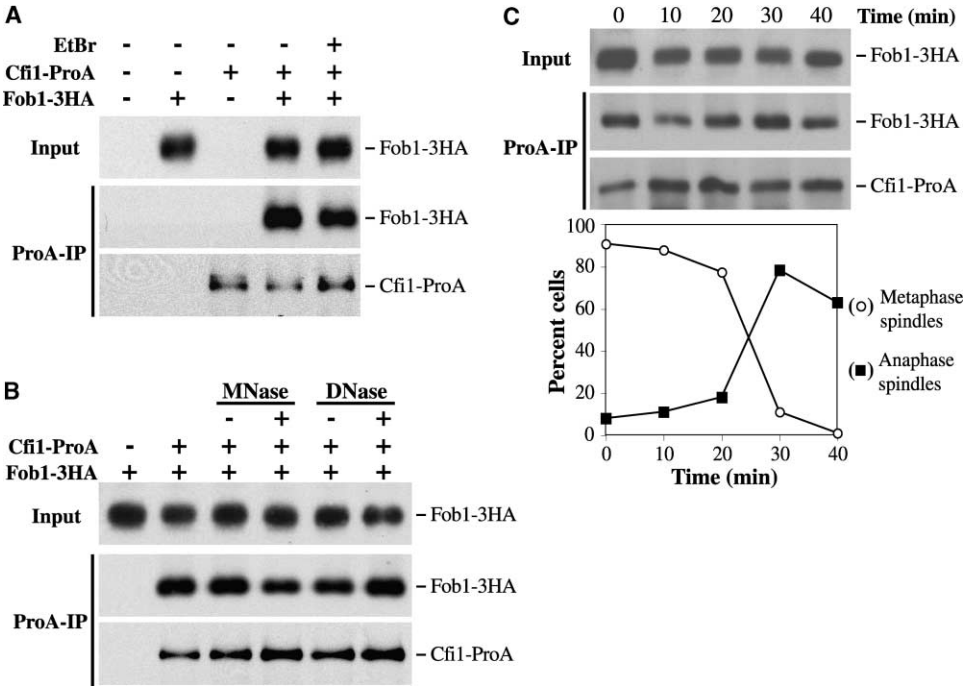

Figure 6. Fob1 Physically Associates with Cfi1/Net1

(A) Western blots showing that Fob1-3HA coimmunoprecipitates with Cfi1-ProA from whole cell extracts. Where indicated, ethidium bromide (EtBr) was added (final concentration of 0.25 mg/ml) to the extract for 30 min 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, and 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 + 0.5 mM $CaCl_2$, (4) A8656 + 0.5 mM $CaCl_2$+ 75 units MNase, (5) A8656 + 0.25 mM $CaCl_2$ + 0.5 mM $MgCl_2$, and (6) A8656 + 0.25 mM $CaCl_2$ + 0.5mM $MgCl_2$ + 25 Kunitz units DNase. Extracts were treated with MNase or DNase for 30 min on ice prior to the immunoprecipitation reaction.

(C) *MET-CDC20* (A9359) cells carrying *FOB1-3HA* and *CFI1-PROA* fusions were arrested in metaphase by depletion of *CDC20* for 2.5 hr by addition of 2 mM methionine. Cells were released from the metaphase arrest into medium lacking methionine. The graph shows the percentages of cells with metaphase (open circles) and anaphase (closed squares) spindles 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.

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 (Figures 6A and 6B). Furthermore, the amount of Fob1 associated with Cfi1/Net1 did not significantly change as cells progressed through metaphase and anaphase (Figure 6C). Our results suggest an interaction hierarchy 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 stands for nontranscribed spacers) within the rDNA repeat, respectively, as shown by chromatin immunopreciptiation analysis (CHIP; Figure 7A; [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 (Figures 7B and 7C). However, in contrast to Cfi1/Net1, which shows about 3-fold higher association with NTS2 compared to the NTS1 region [10], Cdc14 appeared to be predominantly associated with the NTS1 region (Figures 7B and 7C). 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 and that these dipeptides are essential for Spo12 function [21]. Indeed, Spo12 is a phosphoprotein; the C-terminal domain of Spo12 [Spo12(85–173)-13MYC], which is sufficient for *SPO12* function (Figure 1B and Figure S2), readily incorporated ^{32}P-orthophosphate (Figures 8C

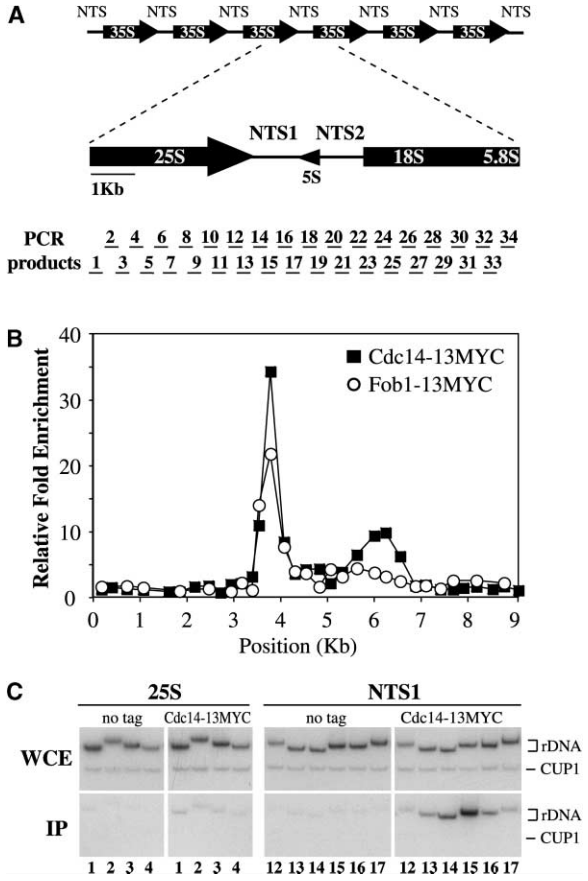

Figure 7. Fob1 and Cdc14 Bind to Overlapping Sites within the rDNA Repeats

(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.

and 8D). Phospho-amino-acid analysis showed the target amino acid to be predominantly serine (Figure 8A). 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 Thr-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, and 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 8B). These mutations did not affect protein levels or the localization pattern of Spo12 (data not shown). Furthermore, Thr-120, Cys-127, and Ser-128 were dispensable for Spo12's mitotic-exit function, as indicated by the ability of the mutant protein, when overproduced, to rescue the temperature-sensitive growth defect of *cdc15-2 spo12*Δ mutants (Figure8B, open circles, and data not shown). In contrast, substitution of Asp-121 to alanine led to loss of *SPO12* function (Figure 8B; closed circles; data not shown). In agreement with a study by Shah et al. [21], we found that the highly conserved serine proline sites (Ser-118 Pro-119 and Ser-125 Pro-126) within the DSP-Box are essential for *SPO12* function (Figure 8B, 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 Ser-118 and Ser-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 8C). This finding suggests that Ser-118 and Ser-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-arrested cells as compared to exponentially growing cells (Figure 8D). Furthermore, Spo12 phosphorylation was slightly higher in cycling than in metaphase-arrested cells (Figure 8D). Our results indicate that phosphorylation of Ser-118 and Ser-125 is cell cycle regulated; it is 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 because of the protein's instability during G1 [21], we speculate that this cell cycle stage is anaphase.

Spo12 Phosphorylation Influences Fob1 Binding

Next, we examined whether phosphorylation of Spo12 affects its binding to Fob1. The interaction of Fob1 with a nonphosphorylatable form of Spo12 [Spo12(SS-AA)] was approximately 2.5-fold higher than with wild-type Spo12, as indicated by a quantitative two-hybrid assay (Figure 8E) and coimmunoprecipitation (Figure 8F). This result suggests that Spo12 phosphorylation decreases Fob1 binding.

Because phosphorylation of Spo12 is 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 coprecipitated Fob1-3HA appeared to be slightly reduced as cells entered anaphase (Figure 8G; compare 10–20 min with 30–40

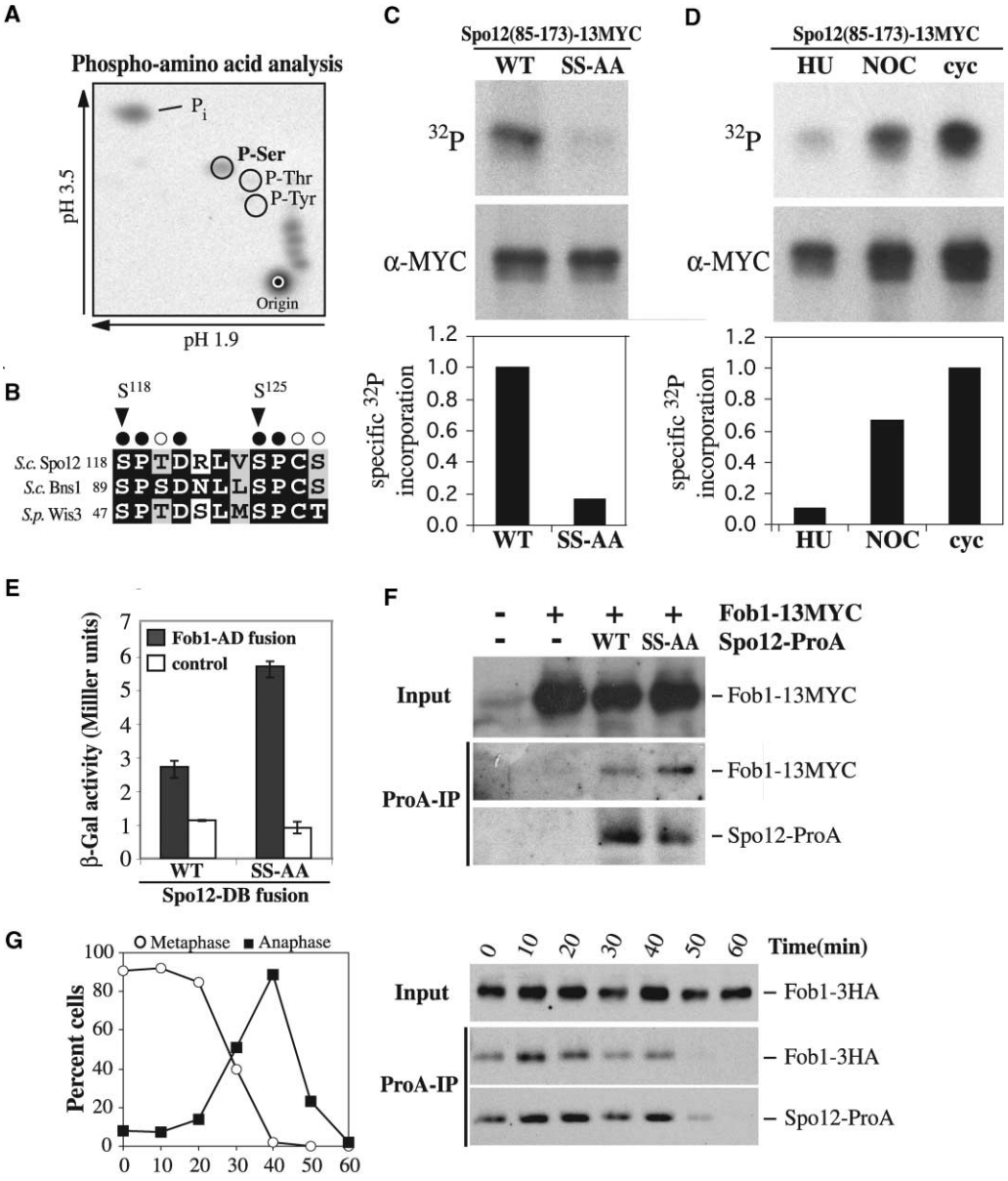

Figure 8. Spo12 Phosphorylation Is Cell-Cycle Regulated and Affects Its Binding to Fob1

(A) 10 ml of A9805 cells were grown in phosphate-depleted YEPR (YEP containing 2% raffinose) medium to an optical density at 600 nm of 0.6, and *SPO12(85-173)-13MYC* expression was induced for 45 min by addition of 2% galactose. Cells were labeled for 20 min with 0.02 mCi/ml ^{32}P-orthophosphate. Phospho-amino-acid composition was analyzed with a phosphorimager. The positions of sample loading (Origin) and the location of free phosphate (P_i) 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 Ser-118 and Ser-125 in Spo12(85–173)-13MYC were mutated to alanines for construction of Spo12[SS-AA]-13MYC. Strains (10 ml) 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 600 nm of 0.6, and expression of the constructs was induced for 45 min by addition of 2% galactose. Cells were labeled for 20 min with 0.02 mCi/ml ^{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.

(D) A9805 cells (10 ml) were grown in phosphate-depleted YEPR medium. Cultures were diluted to an optical density at 600 nm of 0.4 and either left untreated (cyc) or arrested in S-phase with 10 mg/ml hydroxyurea (HU) or in metaphase with 15 μg/ml nocodazole (NOC). After a 2 hr arrest, addition of 2% galactose induced expression of Spo12(85–173)-13MYC for 40 min, and labeling for 20 min with 0.02 mCi/ml of ^{32}P-orthophosphate followed. 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.

(E) Two-hybrid interactions between Fob1-AD and Spo12(WT)-DB (A10277) or Spo12(SS-AA)-DB (A10281) were estimated from β-galactosidase

min). 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.

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 that is 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 Spo12's binding to Fob1. We propose a model in which 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 as a result of 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 led to activation of the MEN, but it did not enhance this defect in 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 coimmunoprecipitated 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*; and (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 because deletion of *FOB1* only partially rescues the mitotic-exit defect of *spo12*Δ *bns1*Δ mutants. Furthermore, deletion of *FOB1* does not rescue the synthetic lethality of *spo12*Δ *lte1*Δ mutants, and cells overexpressing *FOB1* exhibit a slightly weaker mitotic-exit delay than cells lacking both *SPO12* and *BNS1* (F.S., unpublished data).

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 *mad1*Δ cells. Furthermore, *mad1*Δ mutants exhibit a stronger spindle checkpoint defect than *fob1*Δ 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 by stabilizing Pds1. The possibility that Pds1 inhibits both FEAR network branches could also explain the observation that cells overexpressing a nondegradable version of *PDS1* display a Cdc14 release and mitotic-exit defect more similar to that of *esp1-1 spo12*Δ *bns1*Δ mutants than of *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 data), raising the question of how Spo12's mitotic exit-promoting activity is restricted to anaphase. Because Spo12 localizes to the nucleus

activity. Assays were done in triplicate. The control strains lack the Fob1-AD fusion (A7569 and A10273).

(F) Western blots showing the coimmunoprecipitation 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, and A10701

(G) *MET-CDC20* (A9792) cells carrying a *FOB1-3HA* and *SPO12-PROA* fusion were arrested in metaphase by depletion of *CDC20* for 2.5 hr by the addition of 2 mM 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 (closed squares) spindles 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.

and nucleolus throughout the cell cycle ([21]; F.S., unpublished data), 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 cofactor or anaphase-specific post-translational modification. Several observations support the latter hypothesis. First, two highly conserved serine residues in Spo12 are phosphorylated in vivo. Second, 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. Ser-118 and Ser-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 Ser^{118} and Ser^{125} will be an important future endeavor.

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 data; [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 (Figure S4). 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 and thereby alter the binding surface available for Fob1. Consistent with this idea, we find that mutating Ser-118 and Ser-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 by eliminating Fob1's inhibitory function. Esp1 and Slk19 may cause Cdc5 activation [19], which in turn would induce Cdc14 phosphorylation [14]. Together, these events may lead to the transient release of Cdc14 from its inhibitor during anaphase (Figure S4). Finally, we note that Ser-118 and Ser-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 data). 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 and that they thereby ensure 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 S1. For construction of N-terminal *SPO12* truncations, the *GAL1-10* promoter was inserted at an internal position within the endogenous *SPO12* coding region via a PCR-based method [41]. For creation of C-terminal truncations, the C-terminal residues of endogenous *SPO12* were replaced with the DNA sequence encoding the 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 via 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 are available 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-Labeling and Phospho-Amino-Acid Analysis

Cells were grown and labeled with ^{32}P-orthophosphate as described in the Figure legends. For isolation of Spo12(85-173)-13MYC, denaturing immunoprecipitation was used as described [42], with 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

A yeast two-hybrid screen was performed with the Gal4-based system [44]. For construction of 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. For construction of the

Fob1(1-566)-AD fusion, the *FOB1* coding region was PCR amplified and inserted into 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].

Coimmunoprecipitation Analysis

For Fob1-Cfi1 coimmunoprecipitations, cells were harvested, washed with 10 mM Tris (pH 7.5), and resuspended in 200 μl of NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM TRIS [pH 7.5], 1 mM dithiothreitol [DTT], 60 mM β-glycerophosphate, 1 mM $NaVO_3$, 2 μM Microcystin-LR [EMD Biosciences], and complete EDTA-free protease inhibitor cocktail [Roche]). One milligram of extract in 150 μl of NP40 buffer was used for immunoprecipitations. Rabbit-IgG coupled dynabeads (30 μl; Dynal Biotech) were added to each sample and incubated with rotation for 2 hr 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 coimmunoprecipitation reactions were carried out in the same way, except for the substitution of NP40 lysis buffer with CHAPS lysis buffer (0.5% CHAPS, 150 mM NaCl, 50 mM HEPES [pH 7.5], 1 mM dithiothreitol, 60 mM β-glycerophosphate, 1 mM $NaVO_3$, 2 μM Microcystin-LR [EMD Biosciences], and complete EDTA-free protease inhibitor cocktail [Roche]).

Immunoblot Analysis

For preparation of protein extracts for Western blot analysis, cells were incubated for 10 min in 5% trichlor acetic acid (TCA) at 4°C, pelleted, and then washed with acetone. Cells were broken in 100 μl lysis buffer (50 mM TRIS [pH 7.5], 1 mM EDTA, 1 mM $NaVO_3$, 50 mM DTT, and complete EDTA-free protease inhibitor cocktail [Roche]) with glass beads for 40 min 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 for processing immunofluorescence images. At least 100 cells were analyzed per time point.

Supplemental Data

Supplemental Data including four figures and a table are available at http://www.current-biology.com/cgi/content/full/14/6/467/DC1/.

Acknowledgments

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References

1. Morgan, D.O. (1999). Regulation of the APC and the exit from mitosis. Nat. Cell Biol. *1*, E47–E53.
2. Bardin, A.J., and Amon, A. (2001). Men and sin: what's the difference? Nat. Rev. Mol. Cell Biol. *2*, 815–826.
3. McCollum, D., and Gould, K.L. (2001). Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. Trends Cell Biol. *11*, 89–95.
4. Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol. Cell *2*, 709–718.
5. Jaspersen, S.L., Charles, J.F., Tinker-Kulberg, R.L., and Morgan, D.O. (1998). A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. Mol. Biol. Cell *9*, 2803–2817.
6. Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell *97*, 233–244.
7. Visintin, R., Hwang, E.S., and Amon, A. (1999). Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. Nature *398*, 818–823.
8. Traverso, E.E., Baskerville, C., Liu, Y., Shou, W., James, P., Deshaies, R.J., and Charbonneau, H. (2001). Characterization of the Net1 cell cycle-dependent regulator of the Cdc14 phosphatase from budding yeast. J. Biol. Chem. *276*, 21924–21931.
9. Straight, A.F., Shou, W., Dowd, G.J., Turck, C.W., Deshaies, R.J., Johnson, A.D., and Moazed, D. (1999). Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell *97*, 245–256.
10. Huang, J., and Moazed, D. (2003). Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. Genes Dev. *17*, 2162–2176.
11. Stegmeier, F., Visintin, R., and Amon, A. (2002). Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. Cell *108*, 207–220.
12. Pereira, G., Manson, C., Grindlay, J., and Schiebel, E. (2002). Regulation of the Bfa1p-Bub2p complex at spindle pole bodies by the cell cycle phosphatase Cdc14p. J. Cell Biol. *157*, 367–379.
13. Yoshida, S., Asakawa, K., and Toh-e, A. (2002). Mitotic exit network controls the localization of Cdc14 to the spindle pole body in Saccharomyces cerevisiae. Curr. Biol. *12*, 944–950.
14. Visintin, R., Stegmeier, F., and Amon, A. (2003). The role of the polo kinase cdc5 in controlling cdc14 localization. Mol. Biol. Cell *14*, 4486–4498.
15. Uhlmann, F. (2001). Secured cutting: controlling separase at the metaphase to anaphase transition. EMBO Rep. *2*, 487–492.
16. Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. Annu. Rev. Genet. *35*, 673–745.
17. Zeng, X., Kahana, J.A., Silver, P.A., Morphew, M.K., McIntosh, J.R., Fitch, I.T., Carbon, J., and Saunders, W.S. (1999). Slk19p is a centromere protein that functions to stabilize mitotic spindles. J. Cell Biol. *146*, 415–425.
18. Sullivan, M., Lehane, C., and Uhlmann, F. (2001). Orchestrating anaphase and mitotic exit: separase cleavage and localization of Slk19. Nat. Cell Biol. *3*, 771–777.
19. Sullivan, M., and Uhlmann, F. (2003). A non-proteolytic function of separase links the onset of anaphase to mitotic exit. Nat. Cell Biol. *5*, 249–254.
20. Grether, M.E., and Herskowitz, I. (1999). Genetic and biochemical characterization of the yeast spo12 protein. Mol. Biol. Cell *10*, 3689–3703.
21. Shah, R., Jensen, S., Frenz, L.M., Johnson, A.L., and Johnston, L.H. (2001). The Spo12 protein of Saccharomyces cerevisiae: a regulator of mitotic exit whose cell cycle-dependent degradation is mediated by the anaphase-promoting complex. Genetics *159*, 965–980.
22. Yoshida, S., and Toh-e, A. (2002). Budding yeast Cdc5 phos-

phorylates Net1 and assists Cdc14 release from the nucleolus. Biochem. Biophys. Res. Commun. *294*, 687–691.
23. Shou, W., Azzam, R., Chen, S.L., Huddleston, M.J., Baskerville, C., Charbonneau, H., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2002). Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. BMC Mol. Biol. *3*, 3.
24. Chaves, S.R., and Blobel, G. (2001). Nuclear import of Spo12p, a protein essential for meiosis. J. Biol. Chem. *276*, 17712–17717.
25. Defossez, P.A., Prusty, R., Kaeberlein, M., Lin, S.J., Ferrigno, P., Silver, P.A., Keil, R.L., and Guarente, L. (1999). Elimination of replication block protein Fob1 extends the life span of yeast mother cells. Mol. Cell *3*, 447–455.
26. Kobayashi, T., and Horiuchi, T. (1996). A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. Genes Cells *1*, 465–474.
27. Kobayashi, T., Heck, D.J., Nomura, M., and Horiuchi, T. (1998). Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. Genes Dev. *12*, 3821–3830.
28. Johzuka, K., and Horiuchi, T. (2002). Replication fork block protein, Fob1, acts as an rDNA region specific recombinator in S. cerevisiae. Genes Cells *7*, 99–113.
29. Burke, D.J. (2000). Complexity in the spindle checkpoint. Curr. Opin. Genet. Dev. *10*, 26–31.
30. Shirayama, M., Toth, A., Galova, M., and Nasmyth, K. (1999). APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. Nature *402*, 203–207.
31. Shou, W., Sakamoto, K.M., Keener, J., Morimoto, K.W., Traverso, E.E., Azzam, R., Hoppe, G.J., Feldman, R.M., DeModena, J., Moazed, D., et al. (2001). Net1 stimulates RNA polymerase I transcription and regulates nucleolar structure independently of controlling mitotic exit. Mol. Cell *8*, 45–55.
32. Pereira, G., and Schiebel, E. (2003). Separase regulates INCENP-Aurora B anaphase spindle function through Cdc14. Science 302, 2120-2124, published online November 6, 2003. 10.1126/science.1091936.
33. Lai, J.S., and Herr, W. (1992). Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. Proc. Natl. Acad. Sci. USA *89*, 6958–6962.
34. Buonomo, S.B., Rabitsch, K.P., Fuchs, J., Gruber, S., Sullivan, M., Uhlmann, F., Petronczki, M., Toth, A., and Nasmyth, K. (2003). Division of the nucleolus and its release of CDC14 during anaphase of meiosis I depends on separase, SPO12, and SLK19. Dev. Cell *4*, 727–739.
35. Marston, A.L., Lee, B.H., and Amon, A. (2003). The Cdc14 phosphatase and the FEAR network control meiotic spindle disassembly and chromosome segregation. Dev. Cell *4*, 711–726.
36. Cohen-Fix, O., and Koshland, D. (1999). Pds1p of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit. Genes Dev. *13*, 1950–1959.
37. Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev. *13*, 1936–1949.
38. Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859–864.
39. Takeuchi, Y., Horiuchi, T., and Kobayashi, T. (2003). Transcription-dependent recombination and the role of fork collision in yeast rDNA. Genes Dev. *17*, 1497–1506.
40. Gottlieb, S., and Esposito, R.E. (1989). A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. Cell *56*, 771–776.
41. Longtine, M.S., McKenzie, A., III, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.
42. Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5. Cell *66*, 743–758.
43. Cooper, J.A., and Hunter, T. (1983). Identification and characterization of cellular targets for tyrosine protein kinases. J. Biol. Chem. *258*, 1108–1115.
44. James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425–1436.
45. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: John Wiley & Sons, Inc.).