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Article

Regulation of Spo12 Phosphorylation and Its Essential Role in the FEAR Network

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Summary

Background: In budding yeast, the protein phosphatase Cdc14 coordinates late mitotic events and triggers exit from mitosis. During early anaphase, Cdc14 is activated by the FEAR network, but how signaling through the FEAR network occurs is poorly understood.

Results: We find that the FEAR network component Spo12 is phosphorylated on S118. This phosphorylation is essential for Spo12 function and is restricted to early anaphase, when the FEAR network is active. The anaphase-specific phosphorylation of Spo12 requires mitotic CDKs and depends on the FEAR network components Separase and Slk19. Furthermore, we find that *CDC14* is required to maintain Spo12 in the dephosphorylated state prior to anaphase.

Conclusions: Our results show that anaphase-specific phosphorylation of Spo12 is essential for FEAR network function and raise the interesting possibility that Cdc14 itself helps to prevent the FEAR network from being prematurely activated.

Introduction

Exit from mitosis requires the inactivation of mitotic cyclin-dependent kinases (CDKs). In budding yeast, the conserved protein phosphatase Cdc14 induces CDK inactivation by targeting mitotic Clb cyclins for degradation [1]. Throughout most of the cell cycle, Cdc14 is bound to its inhibitor Cfi1/Net1 in the nucleolus [2]. During anaphase, two signaling networks induce the release of Cdc14 from Cfi1/Net1 in the nucleolus, allowing Cdc14 to dephosphorylate substrates in the nucleus and cytoplasm. These are the Cdc fourteen early anaphase release (FEAR) network and the mitotic exit network (MEN) (reviewed in [3]). The FEAR network promotes the release of Cdc14 from Cfi1/Net1 during early anaphase, whereas the MEN functions later during anaphase and maintains Cdc14 in its released state.

Cdc14 released by the FEAR network contributes to spindle stability, allows for segregation of the rDNA array, and activates the MEN (reviewed in [4]). The pathway is comprised of Separase, Esp1 in budding yeast, the protease that triggers chromosome segregation; Slk19, an Esp1-associated factor; Cdc5, the Polo-like kinase; and Spo12, a protein of unknown function. How the FEAR network promotes the transient release of Cdc14 from the nucleolus during early anaphase is beginning to be understood. At the metaphase-anaphase transition, Separase/Esp1 is activated and together with Slk19 downregulates the protein phosphatase PP2A associated with its targeting subunit Cdc55 [5, 6]. This allows Clb-CDKs to phosphorylate Cfi1/Net1, which is necessary for Cdc14 release from the inhibitor [5, 7]. However, Cfi1/Net1 phosphorylation is not sufficient to maintain Cdc14 in the released state because in MEN mutants although Cfi1/Net1 remains phosphorylated, Cdc14 returns into the nucleolus [2, 7–9]. How *CDC5* and *SPO12* function within the FEAR network is not clear, but epistasis analysis placed *SPO12* and its homolog *BNS1* parallel to *ESP1* and *SLK19,* and *CDC5* downstream of, or in parallel to, the protease and its binding partner [10].

We show here that Spo12 is phosphorylated on S118 only during anaphase. Therefore, this S118 phosphorylation mirrors FEAR network activity. Additionally, this phosphorylation event is mediated by mitotic CDKs and depends on *ESP1* and *SLK19*, which places *SPO12* downstream of the Esp1-Slk19 complex in the FEAR network. We also show that *CDC14* is needed to maintain Spo12 in the dephosphorylated state before anaphase. Our results not only shed light onto how signaling through the FEAR network occurs, but also raise the interesting possibility that Cdc14 is active while sequestered in the nucleolus.

Results

S118 and S125 Phosphorylation Are Required for the FEAR Network Function of *SPO12*

Spo12 phosphorylation predominantly occurs on two conserved serine residues, serine 118 (S118) and serine 125 (S125) [11]. To determine the functional importance of this phosphorylation, we replaced endogenous *SPO12* with *SPO12* phospho mutants containing alanine substitutions for one or both serines (*spo12-S118A, spo12-S125A, spo12-SSAA*). These mutations did not affect protein stability or localization (Figure 1A, data not shown), but all three mutants behaved like *spo12Δ* mutants under all conditions examined. First, *spo12-SSAA* cells displayed an average mitotic exit delay of 13 min (SD 5.7 min), which agrees with the 10–15 min delay observed in *spo12Δ* mutants (Figure 1B, data not shown, [9]). Second, like *spo12Δ* cells, *spo12* phospho mutants exhibited synthetic lethality when combined with a deletion of the MEN activator *LTE1* (Figure 1C). Third, when induced to undergo meiosis, the *spo12* phospho mutant strains, like *spo12Δ* cells, formed only two instead of four spored asci (Figure 1D) [12]. Our results indicate that the phosphorylation of Spo12 on S118 and S125 is essential for its function.

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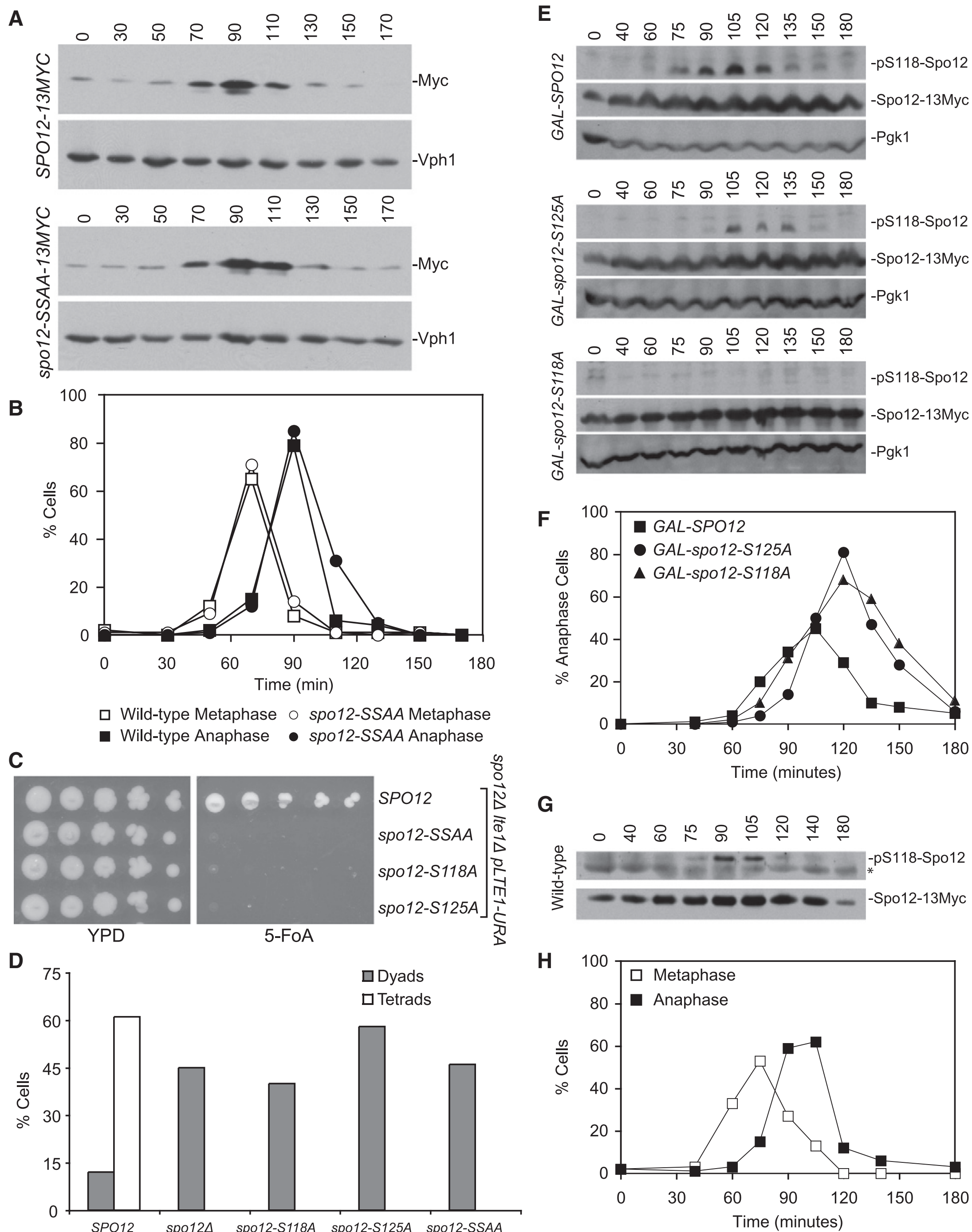


Figure 1. S118 Is Required for *SPO12* Function and Is Phosphorylated during Anaphase

(A and B) *SPO12-13MYC* (A4568; squares) and *spo12-SSAA-13MYC* (A21789; circles) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium. Spo12 levels were determined by western blot analysis at the indicated times ([A], top). Vph1 was used as a loading control ([A], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was also determined (B). The delay of *spo12-SSAA-13MYC* cells in exiting mitosis relative to wild-type, as determined by anaphase spindle disassembly, was calculated in three independent experiments, giving an average delay of 13.3 min (SD = 5.7 min).

(C) Serial dilutions of *spo12Δ, lte1Δ, pLTE1-URA3* cells carrying a *SPO12-13MYC* (A21779), *spo12-SSAA-13MYC* (A21780), *spo12-S118A-13MYC* (A21782), or *spo12-S125A-13MYC* (A21784) allele were grown on YEPD plates (left) or plates containing 5-fluorootic acid (*5-FoA*, right) at 30°C for 3 days. The presence of 5-FoA selects against cells carrying the *LTE1-URA3* plasmid.

(D) *SPO12-13MYC* (A21797), *spo12Δ* (A21798), *spo12-S118A-13MYC* (A21799), *spo12-S125A-13MYC* (A21800), and *spo12-SSAA-13MYC* (A21801) diploid cells were induced to sporulate. After 48 hr, DNA was stained with 4′-6-Diamidino-2-phenylindole (DAPI) to determine the percentage of dyads and tetrads.

(E and F) *GAL-SPO12-13MYC* (A18282; squares) *GAL-spo12-S125A-13MYC* (A7084; circles) *GAL-spo12-S118A-13MYC* (7080; triangles) cells were arrested in G1 with α-factor (5 μg/ml) in YEPR medium at 25°C and released into galactose-containing medium. Expression of Spo12 was induced 2 hr prior to this release by addition of 2% galactose. The pS118 antibody was used to detect phosphorylated Spo12 by indicated times ([E], top). Spo12 protein levels are shown in the middle panels. Pgk1 was used as a loading control ([E], bottom). The percentage of cells with anaphase spindles was also determined (F).

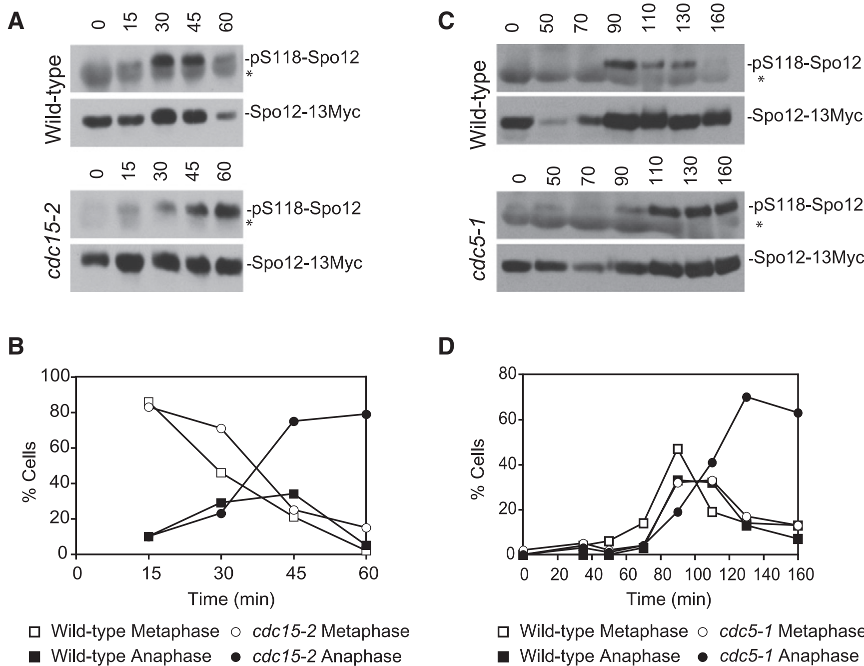


Figure 2. Spo12 Phosphorylation Is Not Dependent on the Mitotic Exit Network

(A and B) *SPO12-13MYC* (A4568; squares) and *cdc15-2 SPO12-13MYC* (A5170; circles) cells were arrested in metaphase at 25°C with nocodazole and released into fresh medium at 37°C. Spo12 protein was immunoprecipitated at each time point and probed with the pS118 antibody ([A], top) or the Myc antibody ([A], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was also determined (B). Microtubule repolymerization did not occur until 15 min after release from the nocodazole block.

(C and D) *SPO12-13MYC* (A4568; squares) and *cdc5-1 SPO12-13MYC* (A18641; circles) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium at 37°C. Spo12 protein was immunoprecipitated at each time point and probed with the pS118 antibody ([C], top) or the Myc antibody ([C], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was also determined (D).

Phosphorylation on S118 of Spo12 Occurs during Anaphase

To determine whether phosphorylation of Spo12 was cell cycle regulated, antibodies were raised against peptides containing phosphorylated S118 or S125 (see Experimental Procedures). Only antibodies against the peptide containing phospho S118 (henceforth pS118 antibody) exhibited phospho-specificity in western blots. The pS118 antibody recognized overexpressed wild-type Spo12 and Spo12-S125A (although with somewhat decreased efficiency than wild-type protein), but not Spo12-S118A (Figure 1E). The phosphospecificity of the antibody was further confirmed by treatment of Spo12 immunoprecipitates with alkaline phosphatase, after which the pS118 antibody no longer recognized Spo12 (Figure S1 available online). Interestingly, phosphorylation on S118 was not constant throughout the cell cycle, but was restricted to anaphase (Figures 1E and 1F).

We next examined S118 phosphorylation in cells expressing *SPO12* from its native promoter. Spo12 protein levels fluctuate during the cell cycle and are maximal during anaphase (Figure 1A) [13], making it difficult to discern whether the increase in pS118 signal in anaphase was due to increased phosphorylation or reflected higher protein levels. Therefore, we developed a method to probe more equal amounts of Spo12 for S118 phosphorylation across the cell cycle. We first immunoprecipitated Spo12-13Myc and determined the amount of Spo12 in each time point (Figure S2A, top panel). In the experimental western blot, the amount of Spo12 immunoprecipitate loaded was adjusted accordingly so that equal amounts of Spo12 were present across time points (Figure S2A, middle panel). For most experiments, this normalization allowed us to examine Spo12 phosphorylation independently of Spo12 protein levels across the cell cycle. However, in some experiments we were not able to compensate for the low levels of Spo12 in early stages of the cell cycle by adjusting the loading (see for example Figures 2C and 3E). With this method, we found that S118 phosphorylation fluctuates during the cell cycle. S118 phosphorylation was absent during G1, S phase, and metaphase, but are present during early anaphase (Figures 1G and 1H). As cells exited mitosis, S118 phosphorylation declined again. Our results demonstrate that Spo12 phosphorylation on S118 is restricted to anaphase.

S118 Phosphorylation Is Independent of the MEN and *CDC5*

We next wished to identify the mechanisms that restrict S118 phosphorylation to anaphase. To determine whether the MEN regulates S118 phosphorylation, we arrested *cdc15-2* cells in metaphase with the microtubule-depolymerizing drug nocodazole at the permissive temperature (23°C) and released them from the block into the restrictive temperature (37°C). This allowed cells to progress synchronously into anaphase before arresting prior to exit from mitosis resulting from failure to release Cdc14 from the nucleolus in late anaphase (Figure 2B). In wild-type cells, S118 phosphorylation appeared in anaphase and declined upon exit from mitosis (Figures 2A and 2B). In *cdc15-2* cells, S118 phosphorylation occurred concomitantly with anaphase entry and remained high throughout the arrest (Figures 2A and 3A). Similar results were obtained with other MEN mutants (*dbf2-2* and *tem1-3*) and in cells defective in the MEN and FEAR network component Cdc5 (Figures 2C and 2D; data not shown) [9, 14–16]. Our results show that neither the MEN nor *CDC5* is required for S118 phosphorylation, but that they are needed for the loss of this phosphorylation.

(G and H) *SPO12-13MYC* (A4568) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium. Spo12 protein was immunoprecipitated at each time point after release and probed with the pS118 antibody ([G]; top). The Myc antibody was used to detect total Spo12 protein ([G], bottom). A nonspecific band was seen in western blots probed with the pS118 antibody and is noted with an asterisk when present. The percentage of cells with metaphase (open squares) and anaphase (closed squares) spindles was determined at indicated times after release (H).

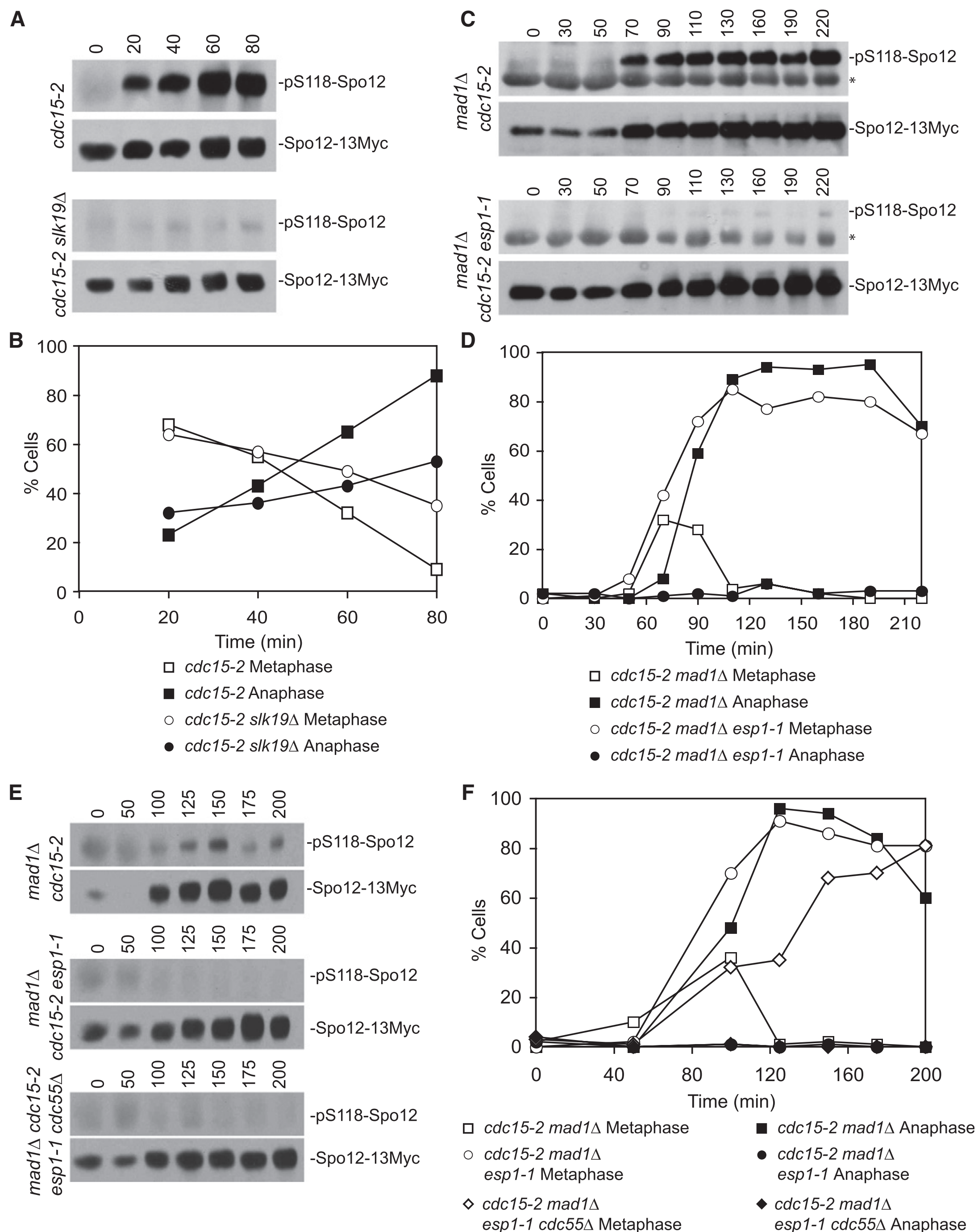


Figure 3. *SLK19* and *ESP1* Are Required for S118 Phosphorylation

(A and B) *cdc15-2 SPO12-13MYC* (A5170; squares) and *slk19Δ cdc15-2 SPO12-13MYC* (A19359; circles) cells were arrested in metaphase at 25°C with nocodazole and released into fresh medium at 37°C. Spo12 protein was immunoprecipitated at each time point after release and probed with the pS118 antibody ([A], top) or the Myc antibody ([A], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was also determined (B). The fragility of spindles at 37°C in *slk19Δ* mutants leads to an under-representation of anaphase cells in the *slk19Δ cdc15-2 SPO12-13MYC* cells.

(C and D) *cdc15-2 mad1Δ SPO12-13MYC* (A19220; squares) and *cdc15-2 mad1Δ esp1-1 SPO12-13MYC* (A19204; circles) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium at 37°C. Spo12 protein was immunoprecipitated at each time point after release and probed with the pS118 antibody ([C], top) or the Myc antibody ([C], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was also determined (D).

(E and F) *cdc15-2 mad1Δ SPO12-13MYC* (A19220; squares), *cdc15-2 mad1Δ esp1-1 SPO12-13MYC* (A19204; circles), and *cdc15-2 mad1Δ esp1-1 cdc55Δ SPO12-13MYC* (A20967; diamonds) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium at 37°C. Spo12 protein was immunoprecipitated at each time point and probed with the pS118 antibody ([E], top) or the Myc antibody ([E], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was also determined (F).

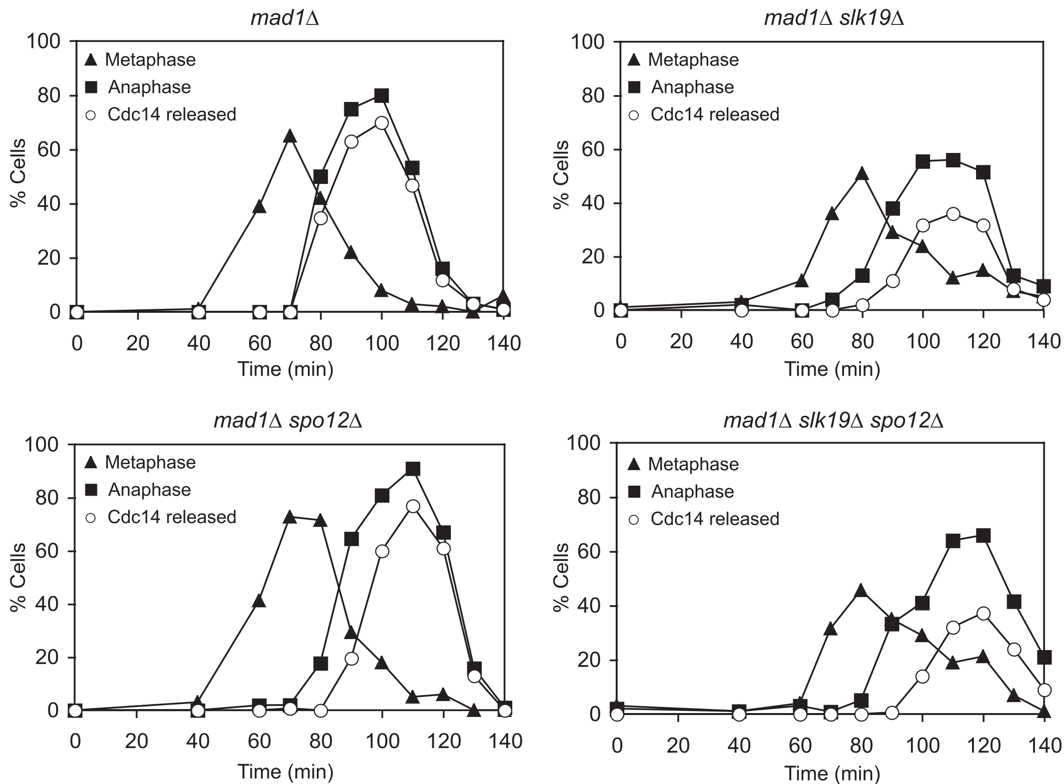


Figure 4. *SPO12* Functions Downstream of *SLK19* in the FEAR Network

mad1Δ (A2853), *mad1Δ slk19Δ* (A4302), *mad1Δ spo12Δ* (A4502), *mad1Δ slk19Δ spo12Δ* (A4560) cells carrying a *CDC14-3HA* fusion were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium at 25°C lacking pheromone. The percentage of cells with metaphase spindles (closed triangles), anaphase spindles (closed squares), and the percentage of cells with Cdc14 fully released from the nucleolus (open circles) was determined at the indicated times.

The FEAR Network Components *ESP1* and *SLK19* Are Required for Spo12 Phosphorylation in Anaphase

To determine whether FEAR network components were needed for Spo12 phosphorylation during anaphase, we examined S118 phosphorylation in various FEAR network mutants. S118 phosphorylation was not observed in *slk19Δ cdc15-2* or *slk19Δ* cells (Figures 3A and 3B, data not shown), indicating that *SLK19* is required for S118 phosphorylation. To determine whether S118 phosphorylation required *ESP1* function, we examined the consequences of inactivating the protease by using the *esp1-1* allele. We deleted *MAD1* in *esp1-1 cdc15-2* cells to avoid possible effects of an active spindle assembly checkpoint (reviewed in [17]). In *cdc15-2 mad1Δ* cells, S118 phosphorylation occurred as cells entered anaphase (Figures 3C and 3D). Cells cannot resolve the linkages between sister chromatids or undergo anaphase spindle elongation upon inactivation of *ESP1*, so although other aspects of cell cycle progression continue to occur, these cells arrest with metaphase-like spindles [18, 19]. S118 phosphorylation was barely detectable in *cdc15-2 esp1-1 mad1Δ* cells (Figures 3C and 3D). In contrast, neither deletion nor overexpression of *FOB1* affected S118 phosphorylation (Figures S3A and S3B), which is consistent with previous observations demonstrating that *FOB1* functions downstream of *SPO12* in the FEAR network [11]. Our data indicate that *ESP1* and *SLK19*, but not *FOB1*, are required for S118 phosphorylation during anaphase.

Cdc55, a regulatory subunit of PP2A, antagonizes Clb-CDK-dependent phosphorylation of Cfi1/Net1, thereby preventing the release of Cdc14 from the nucleolus during early anaphase [5]. Esp1 and Slk19 are thought to antagonize Cdc55-PP2A, thereby allowing Clb-CDKs to phosphorylate Cfi1/Net1 during anaphase [5, 7]. Although Esp1 and Slk19 regulate both Cfi1/Net1 and Spo12-S118 phosphorylation, we were surprised to find that deletion of *CDC55* did not affect either the extent or kinetics of S118 phosphorylation (data not shown). More importantly, inactivation of *CDC55* did not restore S118 phosphorylation to *esp1-1* cells (Figures 3E and 3F). FEAR network-induced phosphorylation of Cfi1/Net1 by CDKs is not required for Spo12-S118 phosphorylation either, because cells carrying a *CFI1/NET1* allele in which the 6 Clb-CDK sites were changed to residues that cannot be phosphorylated (*net1-6CDK*; [7]) did not affect phosphorylation of S118 (data not shown). Our results indicate that *CDC55* does not regulate S118 phosphorylation, nor is phosphorylation of Cfi1/Net1 by Clb-CDKs necessary for S118 phosphorylation.

Deletion of *SPO12* Does Not Enhance the Cdc14 Release Defect of *slk19Δ* Mutants

Our previous studies suggested that *SPO12* functions in a pathway parallel to *ESP1* and *SLK19* because the deletion of *SPO12* and its homolog *BNS1* (bypasses need for Spo12) enhanced the Cdc14 nucleolar release defect of *esp1-1* and *slk19Δ* mutants [10]. In this original epistasis analysis, we deleted *BNS1* in addition to *SPO12* because it is homologous to *SPO12*, and it had been shown to suppress the phenotypes associated with deleting *SPO12* in meiosis [20]. In contrast to our epistasis analysis, the finding that S118 phosphorylation is

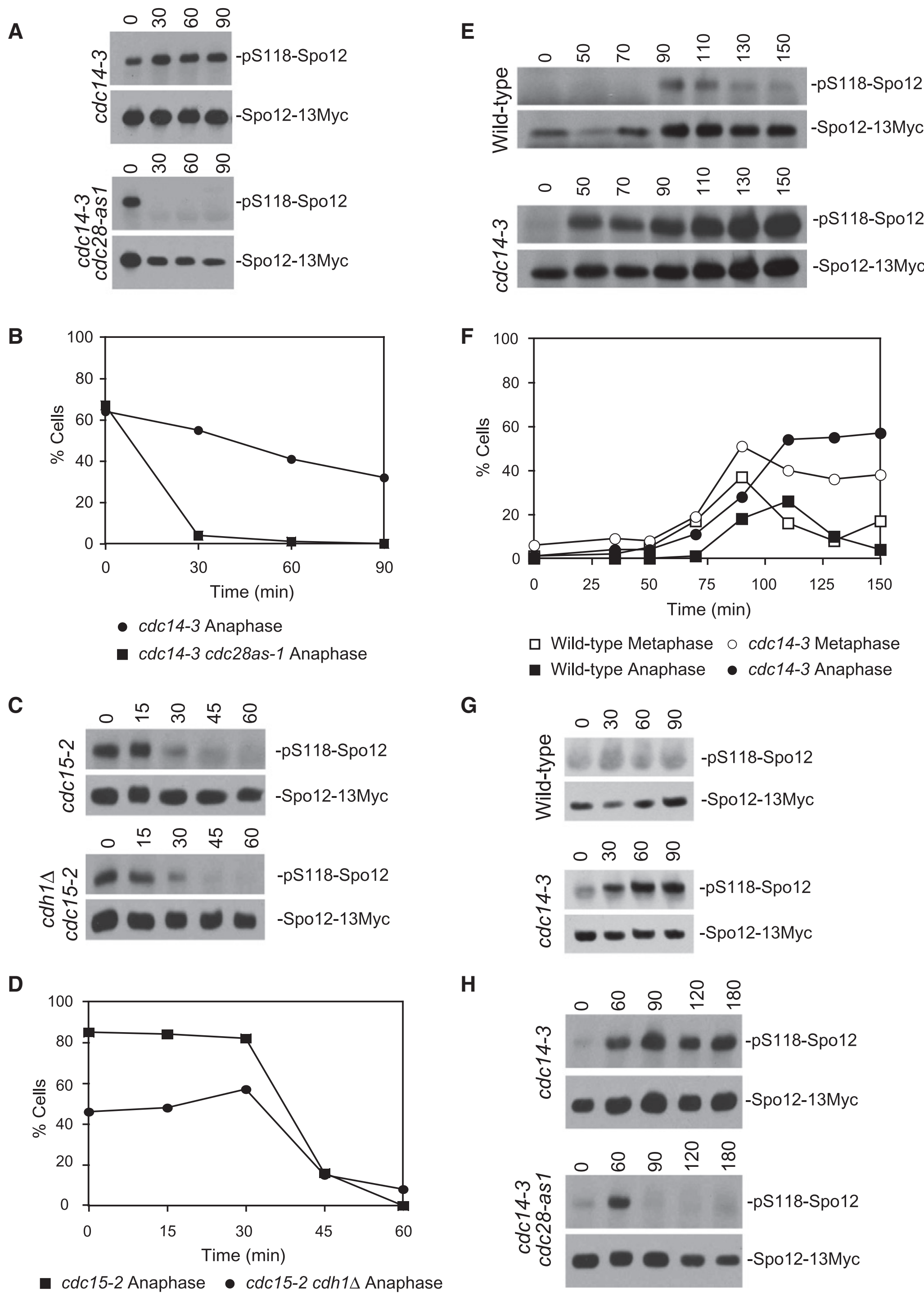


Figure 5. *CDC28* Is Required for S118 Phosphorylation, whereas S118 Dephosphorylation Depends on *CDC14*

(A and B) *cdc14-3 SPO12-13MYC* (A6812; squares) and *cdc14-3 cdc28-as1 SPO12-13MYC* (A20464; circles) cells were arrested in anaphase by growth at 37°C for 2 hr. Samples were taken at the indicated time after addition of 5 μM Cdc28-as1 inhibitor to cells at 37°C. Spo12 protein was immunoprecipitated at each time point and probed with the pS118 antibody ([A], top) or the Myc antibody ([A], bottom). The percentage of cells with anaphase spindles was also determined (B). The prolonged time spent at the restrictive temperature results in spindle breakage in *cdc14-3* cells, which leads to an under-representation of anaphase cells.

(C and D) *cdc15-2 SPO12-13MYC* (A5170; squares) and *cdc15-2 cdh1Δ SPO12-13MYC* (A19074; circles) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium at 37°C for 2 hr to arrest in anaphase. Cells were then shifted back down to 25°C. Samples were taken at the indicated time after shifting cells down to 25°C. Spo12 protein was immunoprecipitated at each time point and probed with the pS118 antibody ([C], top) or the Myc antibody ([C], bottom). The percentage of cells with anaphase spindles was also determined (D).

(E and F) *SPO12-13MYC* (A4568; squares) and *cdc14-3 SPO12-13MYC* (A6812; circles) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C and released into fresh medium at 37°C. Spo12 protein was immunoprecipitated at each time point after release and probed with the pS118 antibody ([E], top) or the Myc antibody ([E], bottom). The percentage of cells with metaphase (open shapes) and anaphase (closed shapes) spindles was determined in (F).

essential for *SPO12* function and is dependent on *ESP1* and *SLK19* indicate that *SPO12* functions downstream of *ESP1* and *SLK19*. To address whether this discrepancy could be due to the fact that both *BNS1* and *SPO12* were deleted in the original epistasis analysis, we examined Cdc14 localization in *slk19Δ*, *spo12Δ*, and *slk19Δ spo12Δ* mutants in the presence of the intact *BNS1* gene. This analysis revealed that inactivation of *SPO12* only marginally, if at all, enhanced the Cdc14 release defect of *slk19Δ* mutants (Figure 4). Our results indicate that *SPO12* functions downstream of *SLK19* and that a parallel contribution of *SPO12* is observed only when its homolog *BNS1* is deleted.

S118 Phosphorylation Depends on *CDC28*

Which protein kinase mediates S118 phosphorylation during anaphase? Mitotic-CDKs are good candidates, because they mediate phosphorylation of Cfi1/Net1 in the nucleolus specifically during early anaphase [7]. Furthermore, the sequences surrounding S118 and S125 of Spo12 each resemble the minimal CDK consensus phosphorylation motif with a proline in the +1 position. To examine the consequences of CDK inactivation on S118 phosphorylation, we employed the *cdc28-as1* allele, which can be specifically inhibited with the adenosine analog 1-NM-PP1 [21]. With the temperature-sensitive *cdc14-3* allele, cells were arrested in anaphase when S118 phosphorylation levels are high. Arrested cells were then treated with the *cdc28as1* inhibitor. Within 30 min of inhibitor addition, S118 phosphorylation was lost in cells carrying the *cdc28-as1* allele (Figures 5A and 5B). In contrast, other protein kinases that regulate mitotic progression such as the MEN kinases Cdc15 and Dbf2, Cdc5, the Aurora B kinase Ipl1, and the MAP kinase Hog1 were not needed for S118 phosphorylation (Figure 2, data not shown). We conclude that, like for Cfi1/Net1, the anaphase-specific phosphorylation of Spo12 depends on CDKs.

CDC14 Is Required to Maintain Spo12 in a Dephosphorylated State before Anaphase

During exit from mitosis, S118 phosphorylation is lost. The APC/C^{Cdh1} targets Spo12 for proteasomal degradation during exit from mitosis [13], raising the possibility that degradation of Spo12 eliminates S118-phosphorylated Spo12 from cells. If degradation was the sole mechanism for removing S118-phosphorylated Spo12, S118 phosphorylation should be maintained in *cdh1Δ* cells. However, this was not the case. Upon release from a *cdc15-2* anaphase arrest, Spo12 phosphorylation was lost in the absence of *CDH1*, despite the persistence of the Spo12 protein (Figures 5C and 5D). Thus, the primary cause of the loss of S118 phosphorylation during mitotic exit is likely dephosphorylation.

Cdc14 activation occurs at the time of S118 dephosphorylation. We therefore examined whether *CDC14* was required for S118 dephosphorylation. Upon release from a pheromone-mediated G1 arrest, S118 phosphorylation was restricted to anaphase in wild-type cells (Figures 5E and 5F). However, in *cdc14-3* cells, S118 phosphorylation occurred prior to anaphase. Phosphorylation was detected as early as 50 min after release, even before cells entered metaphase, and this phosphorylation remained high throughout the duration of the experiment (Figures 5E and 5F). Phosphorylation was not detected in *cdc14-3* cells carrying the *spo12-S118A* allele (Figures S4A and S4B), indicating that it was indeed S118 phosphorylation that occurred early in *cdc14-3* cells. The fact that Esp1 is not active prior to the metaphase-anaphase transition because it is bound to its inhibitor Pds1 [22] also suggests that *ESP1* is not needed for S118 phosphorylation when *CDC14* is inactive.

To determine whether *CDC14* contributes to Spo12 dephosphorylation prior to anaphase, we inactivated *CDC14* in cells that were arrested in G1 by pheromone treatment. Because of prolonged pheromone treatment, a subset of *cdc14-3* cells, and to a lesser extent wild-type cells, escape from the G1 arrest. Under these conditions, phosphorylated S118 is observed in *cdc14-3* cells (Figure 5G). This phosphorylation depends on *CDC28,* because inactivation of *CDC28* results in a rapid loss of S118 phosphorylation (Figure 5H). Inactivation of *CDC14* in nocodazole-arrested cells also led to S118 phosphorylation (data not shown). Cdc14 was also able to dephosphorylate S118 in vitro. Recombinant wild-type Cdc14, but not catalytically inactive phosphatase, dephosphorylated S118 when added to Spo12 immunoprecipitates (Figure S4C).

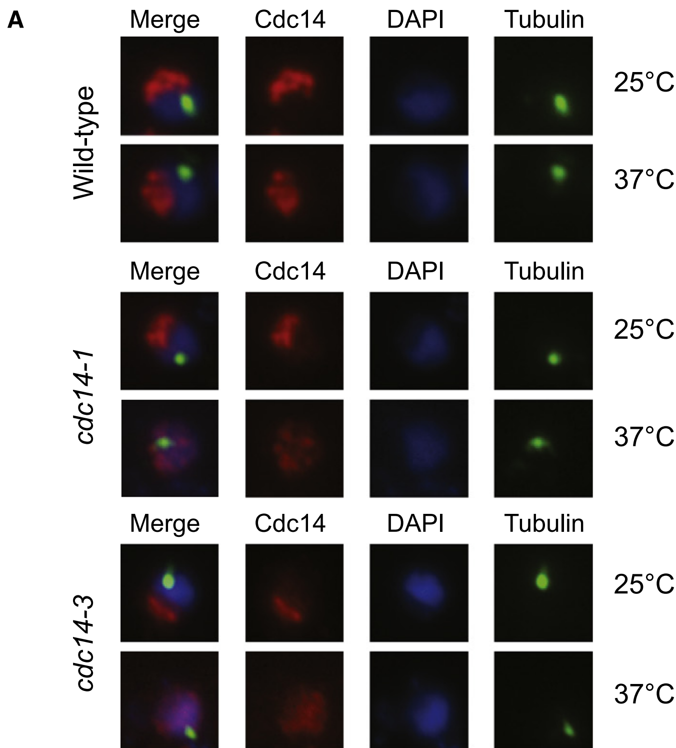
If Cdc14 antagonizes Spo12 phosphorylation, which is required to promote early anaphase release of Cdc14, *CDC14* ought to inhibit its own release from the nucleolus. Consistent with this idea, we find that two temperature-sensitive proteins, cdc14-1 and cdc14-3, are released from the nucleolus as soon as the protein is inactivated by temperature shift (Figures 6A and 6B). Spo12 localization was not affected in these mutants (data not shown). A Cdc14 protein (cdc14-C283S/R289A) that is catalytically inactive and fails to bind substrates does not localize to the nucleolus in any cell cycle stage either (Figure 6C). It is possible that the delocalization of the temperature-sensitive Cdc14 proteins is due to unfolding of the proteins at the restrictive temperature. This is, however, not likely to be the reason for delocalization of the cdc14-C283S/R289A protein because catalytically inactive Cdc14 can still bind Cfi1/Net1 [23]. Together, our results indicate that *CDC14* is required throughout the cell cycle to maintain S118 in the dephosphorylated state and may even directly dephosphorylate S118. Our data further imply that in the absence of *CDC14* and *CDC28*, other phosphatases are able to dephosphorylate S118.

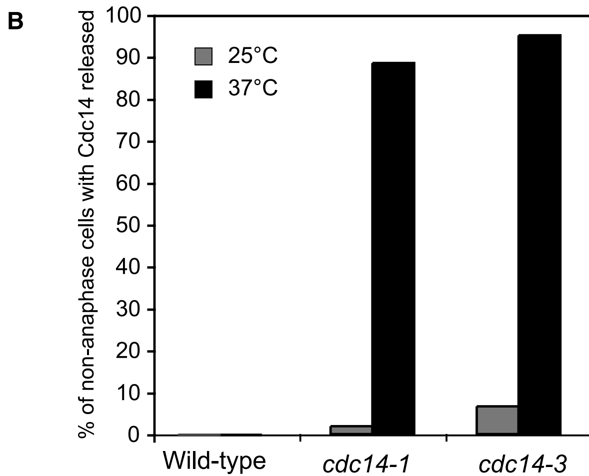
Cdc14 Promotes Dephosphorylation of S118 while in the Nucleolus

Our results implicate Cdc14 in keeping S118 in the dephosphorylated state until anaphase, thereby helping to prevent its own nucleolar release. Prior to anaphase, Cdc14 is sequestered in the nucleolus by Cfi1/Net1, a competitive inhibitor of Cdc14 in vitro (K_i = 3 nM) [23]. However, it is possible that a fraction of Cdc14 is highly mobile during these cell cycle

(G) *SPO12-13MYC* (A4568) and *cdc14-3 SPO12-13MYC* (A6812) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C for 3 hr and then shifted into medium with α-factor (5 μg/ml) at 37°C. Spo12 protein was immunoprecipitated at each time point after temperature shift to 370°C and probed with the pS118 antibody (top) or the Myc antibody (bottom). *cdc14-3* cells can escape this prolonged pheromone arrest because CDKs are no longer antagonized. This also allows S118 phosphorylation to occur.

(H) *cdc14-3 SPO12-13MYC* (A6812) and *cdc14-3 cdc28-as1 SPO12-13MYC* (A20464) cells were arrested in G1 with α-factor (5 μg/ml) at 25°C for 3 hr and then shifted into medium with α-factor (5 μg/ml) at 37°C. 60 min after temperature shift to 37°C, cells were treated with 5 μM Cdc28-as1 inhibitor. Spo12 protein was immunoprecipitated at each time point after temperature shift to 37°C and probed with the pS118 antibody (top) or the Myc antibody (bottom).





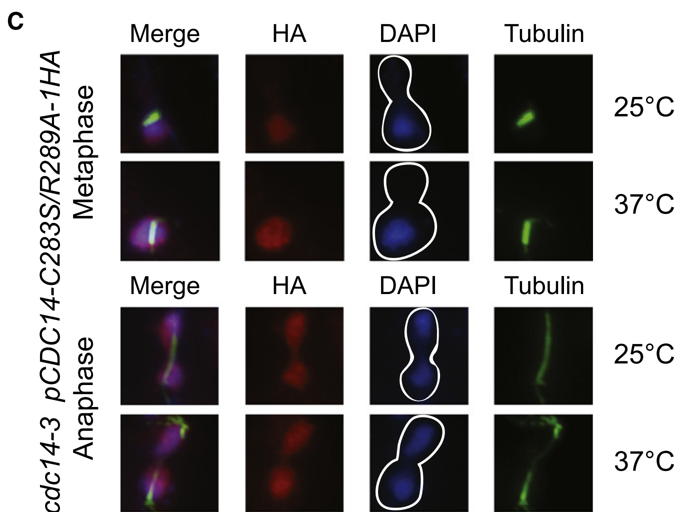


Figure 6. Localization of Cdc14 to the Nucleolus Depends on Its Own Phosphatase Activity

(A and B) Wild-type (A2587), *cdc14-1* (A3064), and *cdc14-3* (A5321) cells were grown at 25°C to OD = 0.2 and split into two and either grown at 25°C or 37°C for 60 min. The percentage of nonanaphase cells with Cdc14 released from the nucleolus is shown in (B). Examples of Cdc14 localization are shown in (A). Cdc14 is shown in red, tubulin in green, and DAPI in blue.

(C) *cdc14-3* cells carrying the plasmid *pCDC14-C283S/R289A-1HA* (A21809) were grown at either 25°C or 37°C for 2 hr. Cdc14-C283S/R289A protein was detected with mouse anti-HA and anti-mouse-Cy3 antibodies (shown in red). Cdc14-C283S/R289A is released constitutively in all cells independently of cell cycle stage and temperature.

stages and can shuttle in and out of the nucleolus. To test this possibility, we compared the mobility of nucleolar-localized Cdc14 with that of released Cdc14. We determined fluorescence recovery after photobleaching (FRAP) of Cdc14-GFP. After Cdc14-GFP was photobleached in half of the nucleolus, we did not detect any recovery of signal within the time of the experiment (>10 min; Figures 7A and 7B; n = 4), indicating that Cdc14 was exceedingly immobile when located in the nucleolus. In contrast, Cdc14 was highly mobile when present on the spindle pole body, one place where the phosphatase localizes to after its release from the nucleolus. The mean half time of recovery of the Cdc14-GFP signal at the spindle pole body was 12.5 s (SD 6.37 s) (Figures 7C and 7D; n = 4). These results indicate that when Cdc14 is located in the nucleolus, the protein is tightly anchored, arguing against the possibility that a mobile fraction of Cdc14 was responsible for dephosphorylating Spo12 during early stages of the cell cycle. Our results support a model in which nucleolar Cdc14 functions to counteract CDK-mediated phosphorylation of S118 until the onset of anaphase, when the FEAR network either modifies Spo12 or Cdc14 or upregulates Clb-CDKs in such a way that Cdc14 can no longer effectively promote the dephosphorylation of Spo12.

Discussion

S118 Phosphorylation Is Essential for Spo12 Function

Spo12 promotes the release of Cdc14 from the nucleolus in part by antagonizing the Cdc14 release inhibitor Fob1 and also by additional mechanisms (Figure S5) [11]. The C terminus of Spo12 fulfills all of Spo12's FEAR network functions and contains a conserved domain with two phosphorylation sites, serine 118 and serine 125 [11]. By using a phospho-specific antibody directed against S118 on Spo12, we found that phosphorylation of this residue occurs during early anaphase, concomitant with the release of Cdc14 from the nucleolus mediated by the FEAR network, and that this phosphorylation is then lost as cells exit mitosis. This, together with the observation that S118 is essential for Spo12 function, indicates that S118 phosphorylation is required for mediating the FEAR network-dependent release of Cdc14 from the nucleolus. Therefore, S118 phosphorylation is the first modification on a FEAR network component that both mirrors FEAR network activity and is required for FEAR network function. It is not known whether S125 is phosphorylated in vivo. Given that the S125 site is located only seven residues away from S118, also resembles a CDK site, and is essential for Spo12 function, this is likely to be the case. We were unable to test whether S118 phosphorylation is sufficient to promote the transient release of Cdc14 from the nucleolus because *SPO12* mutants that mimic constitutive phosphorylation through mutation of either S118 or S125 to glutamic or aspartic acid led to a loss of *SPO12* function (B.N.T., unpublished observations). However, we believe that Spo12 phosphorylation is not likely to be sufficient because CDKs also phosphorylate Cfi1/Net1, which is required for FEAR network-mediated release of Cdc14 from the nucleolus [7].

Regulation of Spo12 Phosphorylation

Several lines of evidence indicate that Clb-CDKs are responsible for phosphorylating Spo12 on S118. The residues surrounding S118 resemble a CDK consensus sequence, and S118 phosphorylation in vivo depended on CDK activity. Currently it is unclear whether Clb-CDKs phosphorylate nuclear Spo12, which then translocates into the nucleolus to promote Cdc14 release, or whether Clb-CDKs phosphorylate Spo12 in both compartments. The pS118 antibody did not detect pS118-Spo12 in indirect immunofluorescence analyses.

CDK activity alone is not sufficient for S118 phosphorylation. Mitotic CDKs are activated in G2, yet S118 is not phosphorylated until anaphase entry. Esp1 and Slk19 are important for S118 phosphorylation, so these two proteins may be required by mitotic CDKs to phosphorylate S118 or could be preventing dephosphorylation. To do this, Esp1 and Slk19 could target Clb-CDKs to the nucleolus or transiently downregulate Cdc14 activity. The observation that *ESP1* and *SLK19* are needed for S118 phosphorylation only when *CDC14* is functional is consistent with both possibilities.

Precedent for anaphase-specific phosphorylation of Cdc14 regulators by Clb-CDKs exists. Cfi1/Net1 was previously shown to be phosphorylated by CDKs on T212 only during anaphase and this also depends on *SLK19* [7]. Therefore, Spo12-S118 phosphorylation and Cfi1/Net1-T212 phosphorylation may be coregulated. However, the mechanisms whereby mitotic CDKs bring about these phosphorylation events only during anaphase appear to be different. In the case of Cfi1/Net1 phosphorylation, Esp1 and Slk19 are thought to downregulate PP2A via Cdc55 at the onset of anaphase, thereby causing a transient net increase in Clb-CDK phosphorylation [5]. However, deletion of *CDC55* did not alter the phosphorylation pattern of S118 in Spo12, nor did it restore S118 phosphorylation in an *esp1-1* mutant. Furthermore, deletion of *CDC55* rescues the lethality of *spo12Δ lte1Δ* double mutants [24], indicating that *CDC55* functions in parallel to *SPO12*. Thus, although Spo12 and Cfi1/Net1 are found together at the rDNA and are phosphorylated by the same kinase at the same time, their mode of regulation appears to differ, because one includes downregulation of Cdc55 whereas the other does not. It will be important to characterize this difference further.

Order of Function within the FEAR Network

Several lines of evidence indicate that *SPO12* functions largely downstream of *ESP1* and *SLK19*. Spo12-S118 phosphorylation depends on *ESP1* and *SLK19*. Furthermore, overexpression of *SPO12* can rescue the Cdc14 release defects of *slk19Δ* cells, whereas overexpression of *ESP1* cannot rescue the Cdc14 release defects in either *slk19Δ* cells or *spo12Δ* cells [10]. Finally, examination of Cdc14 release from the nucleolus in *slk19Δ*, *spo12Δ*, and *slk19Δ spo12Δ* cells revealed no significant additive effects on the release of Cdc14 from the nucleolus in the double mutant (Figure 4). Thus, *SPO12* functions largely downstream of *ESP1* and *SLK19*, although a minor *SLK19*-independent effect on Cdc14 release exists that is shared with its homolog *BNS1*.

Esp1 and Slk19 do not solely function through Spo12 to bring about the release of Cdc14 from the nucleolus. Loss of either *ESP1* or *SLK19* function has a much more severe effect on Cdc14 nucleolar release than does loss of *SPO12* function [9, 10]. *ESP1* and *SLK19* also promote CDK-dependent phosphorylation of Cfi1/Net1 by antagonizing Cdc55 (Figure S6). Thus, Esp1, through Slk19, directs Clb-CDKs to phosphorylate multiple components of the RENT complex. This, in turn, leads to the partial disassembly of the RENT complex and release of Cdc14 from its inhibitor (Figure S6). In addition, Cdc5 phosphorylation of one or more components of the FEAR network is necessary to bring about this event [10, 25].

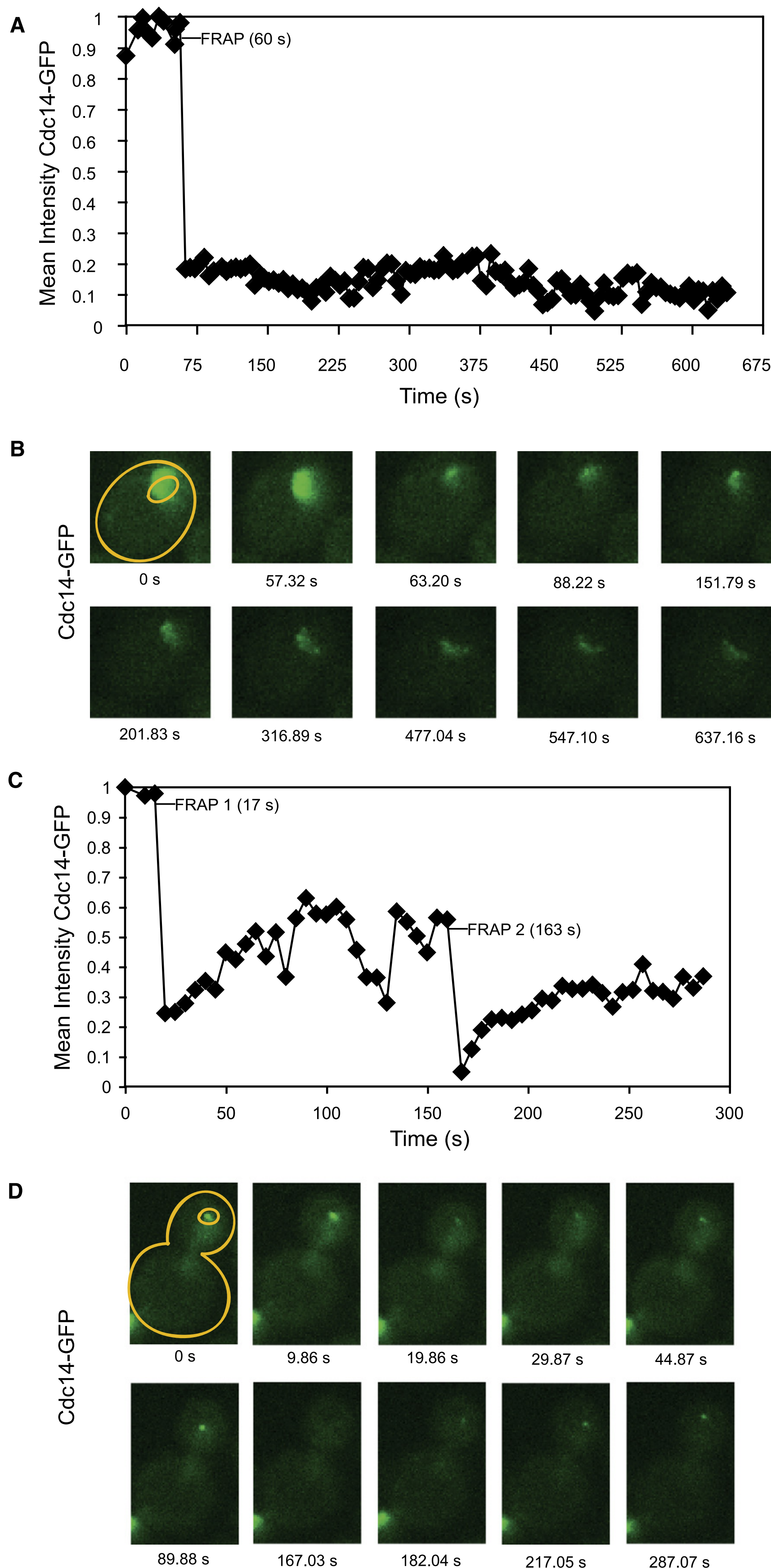


Figure 7. Cdc14 Mobility before and after Release from the Nucleolus

(A and B) Cells carrying a *CDC14-GFP* (A4645) fusion were grown at 25°C. Half the Cdc14-GFP signal in the nucleolus was photobleached at 60 s after the start of image acquisition (as noted in [A]) and GFP fluorescence intensity relative to background (cytoplasmic fluorescence signal) was quantified (A). Representative images of Movie S1 are shown in (B). The large circle in the first image depicts the outline of the cell, and the small circle indicates the area that was photobleached. Signal recovery was never observed within the time of the experiment (n = 4; image acquisition 10 min).

(C and D) Cells carrying a *CDC14-GFP* (A4645) fusion were grown at 25°C. Cdc14-GFP located at the spindle pole body (SPB) was photobleached at 17 s after the start of the image acquisition (FRAP 1). At 163 s, Cdc14-GFP was again photobleached (FRAP2) (as noted in [C]). GFP fluorescence intensity relative to background (cytoplasmic fluorescence signal) was quantified (C). Representative images of Movie S2 are shown in (D). The large circle in the first image depicts the outline of the cell, and the small circle indicates the area that was photobleached. For FRAP 1, the half-time of recovery was 21.96 s and the percentage of recovery was 52.98% (calculated as indicated in Experimental Procedures). For FRAP 2, the half-time of recovery was 10.80 s and the percentage of recovery was 62.21%. For Cdc14-GFP at the SPB (n = 4), the mean half time of recovery was 12.52 s (SD 6.37 s) and the mean percentage of recovery was 61.27% (SD 6.18%). Because of the dynamicity of SPBs during anaphase, the SPB on occasion moves out of focus during image acquisition (see Movie S2). This is reflected in the staggered nature of the curve in (C). Even with this complication, it is clear that signal recovery is exponential.

Is Cdc14 Active in the Nucleolus?

Spo12 levels are low, but not absent, during G1 and S phase (Figure 1A) [13]. Our results show that *CDC14* is required to maintain this pool of Spo12 in the dephosphorylated state. In the absence of *CDC14*, S118 phosphorylation occurs prematurely as soon as Clb-CDKs accumulate in cells. Given that Cdc14 is highly immobile when located in the nucleolus, the most likely explanation for these results is that Cdc14 functions in the nucleolus until anaphase to bring about dephosphorylation of S118. Cdc14 may dephosphorylate S118 directly (S118 can be dephosphorylated by Cdc14 in vitro), though we cannot exclude a more complicated scenario in which Cdc14 activates another phosphatase.

Irrespective of how Cdc14 promotes Spo12 dephosphorylation, the observation that *CDC14* maintains S118 in the dephosphorylated state while bound to Cfi1/Net1 in the nucleolus is at odds with the observation that Cfi1/Net1 functions as a competitive inhibitor of Cdc14 in vitro with high affinity [23]. The recent discovery of Tof2, which directly binds to nucleolar Cdc14 and enhances its phosphatase activity in vitro, could explain this apparent discrepancy [26]. Cdc14 and Tof2 preferentially associate with *NTS1*, whereas Cfi1/Net1 predominantly associates with *NTS2* regions of the rDNA [11, 27, 28]. It is thus possible that a fraction of Cdc14 bound to Tof2 is actually active in the nucleolus. Besides dephosphorylating S118 within Spo12, Cdc14 may also maintain Cfi1/Net1 in the dephosphorylated state. Like S118 in Spo12, Cfi1/Net1 is highly phosphorylated when cells carrying a temperature-sensitive *cdc14-1* allele are grown at the restrictive temperature [7].

Our analysis of S118 phosphorylation revealed another interesting result. In MEN mutants, Cdc14 returns to the nucleolus after being briefly released, yet S118 phosphorylation is maintained in these mutants. Why is Cdc14 resequestered after FEAR network-mediated release no longer capable of promoting dephosphorylation of S118? It is unlikely that high-mitotic CDK activity outcompetes Cdc14 because in late anaphase, mitotic CDK activity has declined by at least 50% from its maximal levels in metaphase [19]. Could the localization of Spo12 and Cdc14 be changed in such a way that Cdc14 can no longer dephosphorylate S118? Spo12 localization is not altered in MEN mutants, because the protein is found in both the nucleus and nucleolus (B.N.T., unpublished observations). We also tested the possibility that Cdc14 localization within the rDNA repeat is changed, such that it may be prevented from dephosphorylating S118. However, chromatin immunoprecipitation analyses showed that the distribution of Cdc14 within *NTS1* and *NTS2* did not differ between wild-type and MEN mutants (Figure S7). Perhaps phosphorylated Spo12 is shielded from Cdc14 during anaphase or Cdc14 is prevented from dephosphorylating the protein. It is also possible that despite an overall decrease in Clb-CDK levels, kinase activity could remain high in the nucleolus. Addressing these possibilities will be essential if we are to understand the molecular mechanisms of Cdc14 control.

Finally, our results reveal a novel function of Cdc14 in controlling its own release from the nucleolus and promoting mitotic exit. Previous studies showed that during late anaphase, Cdc14 promotes its own release by promoting MEN activity [29]. Our studies indicate that during early anaphase, Cdc14 inhibits its own release by antagonizing FEAR network activity. Why would Cdc14 first inhibit its mitotic exit-promoting function but later promote it? By inhibiting its own release during early stages of anaphase, Cdc14 could ensure that nucleolar release of the phosphatase occurs only when Clb-CDK levels are extremely high. This system would provide a simple mechanism to guarantee that initiating exit from mitosis occurs only once high Clb-CDK levels have been reached and hence cells have progressed well into mitosis. During later stages of anaphase, Cdc14 promotes its mitotic exit function by activating the MEN, a pathway that can promote the release of Cdc14 from the nucleolus in the absence of high Clb-CDK activity. In this way, Cdc14 ensures that it remains released in the face of declining Clb-CDK levels. Our analysis of Spo12 phosphorylation, therefore, has not only revealed an unexpected function of Cdc14 outside of anaphase, but also points toward Cdc14 itself having a central role in controlling its own mitotic exit-promoting activity.

Experimental Procedures

Yeast Strains

All strains are isogenic with W303 (A2587) and are listed in Table S1.

Growth Conditions

Growth conditions are described within the figure legends and cell cycle arrests were conducted as previously described [30] (see Supplemental Experimental Procedures for more details).

Immunofluorescence and Western Blot Analysis

Indirect in situ immunofluorescence on whole cells and western blot analysis was carried out as previously described [1, 2] (see Supplemental Experimental Procedures for antibody information).

Phospho-Antibody Production and Detection

A phospho-specific antibody was custom-made by Abgent Technologies against phospho-S118 Spo12 with peptide QLQQRFA(pS)PTDRLVSC (see Figure 1 and Figures S1 and S2). See Supplemental Experimental Procedures for details on detecting phosphorylated S118.

FRAP

FRAP experiments were conducted at 25°C. Details regarding instrumentation and data analysis were as described [31].

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, one table, and two movies and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00730-1.

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