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The Molecular Function of the Yeast Polo-like Kinase Cdc5 in Cdc14 Release during Early Anaphase

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In the budding yeast *Saccharomyces cerevisiae*, Cdc14 is sequestered within the nucleolus before anaphase entry through its association with Net1/Cfi1, a nucleolar protein. Protein phosphatase PP2A^{Cdc55} dephosphorylates Net1 and keeps it as a hypophosphorylated form before anaphase. Activation of the Cdc fourteen early anaphase release (FEAR) pathway after anaphase entry induces a brief Cdc14 release from the nucleolus. Some of the components in the FEAR pathway, including Esp1, Slk19, and Spo12, inactivate PP2A^{Cdc55}, allowing the phosphorylation of Net1 by mitotic cyclin-dependent kinase (Cdk) (Clb2-Cdk1). However, the function of another FEAR component, the Polo-like kinase Cdc5, remains elusive. Here, we show evidence indicating that Cdc5 promotes Cdc14 release primarily by stimulating the degradation of Swe1, the inhibitory kinase for mitotic Cdk. First, we found that deletion of *SWE1* partially suppresses the FEAR defects in *cdc5* mutants. In contrast, high levels of Swe1 impair FEAR activation. We also demonstrated that the accumulation of Swe1 in *cdc5* mutants is responsible for the decreased Net1 phosphorylation. Therefore, we conclude that the down-regulation of Swe1 protein levels by Cdc5 promotes FEAR activation by relieving the inhibition on Clb2-Cdk1, the kinase for Net1 protein.

INTRODUCTION

Mitotic exit pathways are responsible for the inactivation of cyclin-dependent kinase (Cdk) after chromosome segregation and a conserved phosphatase, Cdc14, plays a critical role in mitotic exit (Visintin *et al.*, 1998). The activity of Cdc14 is regulated through its subcellular localization. During most of the cell cycle stages, Cdc14 is sequestered within the nucleolus to prevent the dephosphorylation of its substrates. Two signaling cascades, Cdc fourteen early anaphase release (FEAR) and mitotic exit network (MEN), are responsible for the regulation of Cdc14 release. The FEAR network promotes a brief Cdc14 release from the nucleolus during early anaphase, and this pathway consists of Polo-like kinase Cdc5, separase Esp1, Slk19, and Spo12 (Stegmeier *et al.*, 2002). Our recent work indicates that FEAR-induced Cdc14 release during early anaphase is responsible for the reversal of protein phosphorylation imposed by S phase cyclin-associated Cdk1 (Clb5-Cdk1), which facilitates spindle stabilization and elongation (Jin *et al.*, 2008). The activation of the MEN further promotes the dissociation of Cdc14 from its inhibitor during late anaphase and telophase (Shou *et al.*, 1999; Visintin *et al.*, 1999). In addition, the phosphorylation of Cdc14 by Dbf2, a component of MEN, promotes transfer of Cdc14 to cytoplasm (Mohl *et al.*, 2009). Released Cdc14 inactivates mitotic Cdk by destroying mitotic cyclin Clb2 and stabilizing the Cdk inhibitor Sic1 (Visintin *et al.*, 1998). The complete inactivation of Cdk makes it possible for the assembly of the prereplication complex for the next round of cell cycle (Noton and Diffley, 2000).

FEAR-dependent Cdc14 release may be facilitated by the phosphorylation of Net1 that binds to and sequesters Cdc14 within the nucleolus (Shou *et al.*, 1999; Visintin *et al.*, 1999; Azzam *et al.*, 2004). Before anaphase entry, the presence of phosphatase PP2A^{Cdc55} keeps Net1 in its hypophosphorylated form, which prevents the dissociation of Cdc14 from the nucleolar localized Net1 (Queralt *et al.*, 2006; Wang and Ng, 2006; Yellman and Burke, 2006). After anaphase entry, separase Esp1 inactivates PP2A^{Cdc55} with the assistance of Slk19, Spo12, Zds1, and Zds2 (Queralt *et al.*, 2006; Queralt and Uhlmann, 2008), allowing the phosphorylation of Net1 by Clb2-Cdk1 (Azzam *et al.*, 2004). This modification may favor Cdc14 release.

Cdc5 is a conserved Polo-like kinase that plays multiple roles during the cell cycle. As a component of the MEN, Cdc5 promotes mitotic exit by phosphorylating Bfa1, the negative regulator of the MEN (Hu *et al.*, 2001). It is interesting that Cdc5 also functions in the FEAR pathway because Cdc5 is required for the release of Cdc14 from the nucleolus during early anaphase (Stegmeier *et al.*, 2002). Some evidence indicates that the direct phosphorylation of Cdc14 by Cdc5 may facilitate Cdc14 release (Visintin *et al.*, 2003; Rahal and Amon, 2008). Also, the degradation of Cdc5 protein is essential for the return of Cdc14 to the nucleolus after exit mitosis (Visintin *et al.*, 2008). Previous data indicate that Cdc5 kinase phosphorylates Swe1 to promote its degradation (Park *et al.*, 2004; Sakchaisri *et al.*, 2004; Asano *et al.*, 2005). In vitro and in vivo evidence indicates that Swe1 inhibits mitotic cyclin-associated Cdk activity by phosphorylating Cdk1 at tyrosine 19 (Booher *et al.*, 1993). Recent evidence suggests that Swe1 has little effect on S phase cyclin-associated Cdk1 (Hu and Aparicio, 2005; Liu and Wang, 2006; Keaton *et al.*, 2007). In this study, we present evidence indicating that Cdc5 regulates the FEAR network in part by inducing Swe1 degradation, which enables Clb2-Cdk1 to phosphorylate Net1.

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Table 1. Strains used in this study

Strains	Relevant genotype	Source
Y300	*MATa trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	Lab stock
413-1-1	*MATa SWE1-myc-HIS2*	Lew lab
839-2-1	*MATa cdc5-1 SWE1-myc-HIS2*	This study
478-9-3	*MATa cdc5-2 SWE1-myc-HIS2*	This study
1010-11-2	*MATa cdc15-2 SWE1-myc-HIS2*	This study
1010-16-4	*MATa cdc15-2 spo12:: KanMX SWE1-myc-HIS2*	This study
2100-1-1	*MATa cdc14::CDC14-5-GFP-TRP1 TUB1-mCherry*	This study
2100-3-3	*MATa cdc15-2 cdc14::CDC14-5-GFP-TRP1 TUB1-mCherry*	This study
2099-10-2	*MATa cdc5-1 cdc14::CDC14-5-GFP-TRP1 TUB1-mCherry*	This study
2099-7-1	*MATa cdc5-1 swe1Δ::LEU2 cdc14::CDC14-5-GFP-TRP1 TUB1-mCherry*	This study
2121-2-3	*MATa cdc5-1 swe1Δ::LEU2 Δnet1::his5$^+$ net1-6Cdk-TEV-myc9::TRP1 cdc14::CDC14-5-GFP-TRP1 TUB1-mCherry*	This study
2103-17-4	*MATa PURA3::tetR::GFP::LEU2 RDN1::tetOx112::URA3 TUB1-mCherry*	This study
2103-16-4	*MATa cdc15-2 PURA3::tetR::GFP::LEU2 RDN1::tetOx112::URA3 TUB1-mCherry*	This study
2102-1-3	*MATa cdc5-1 PURA3::tetR::GFP::LEU2 RDN1::tetOx112::URA3 TUB1-mCherry*	This study
2117-11-3	*MATa cdc5-1 swe1Δ::LEU2 PURA3::tetR::GFP::LEU2 RDN1::tetOx112::URA3 TUB1-mCherry*	This study
2111-1-1	*MATa cdc5-1 cdc28Δ::URA cdc28F19::TRP1 cdc14::CDC14-5-GFP-TRP1 TUB1-mCherry*	This study
2120-1-1	*MATa cdc5-1 cdc28Δ::URA cdc28F19::TRP1 PURA3::tetR::GFP::LEU2 RDN1::tetOx112::URA3 TUB1-mCherry*	This study
702-5-3	*MATa SLD2-9myc::LEU2*	Lab stock
730-4-7	*MATa cdc15-2 SLD2-9myc::LEU2*	Lab stock
1014-9-2	*MATa cdc15-2 hsl1Δ::URA3 SLD2-9myc::LEU2*	This study
848-3-4	*MATa cdc5-1 SLD2-9myc::LEU2*	This study
1013-4-1	*MATa cdc5-2-URA3 SLD2-9myc::LEU2*	This study
861-2-4	*MATa cdc5-1 swe1Δ::LEU2 SLD2-9myc::LEU2*	This study
1017-5-3	*MATa cdc5-2-URA3 swe1Δ::LEU2 SLD2-9myc::LEU2*	This study
1057-7-3	*MATa hsl1Δ::URA3 mob1-77*	This study
2101-7-3	*MATa cdc15-2 hsl1Δ::URA3 cdc14::CDC14-5-GFP-TRP1 Tub1-mCherry*	This study
2101-10-1	*MATa cdc15-2 hsl1Δ::URA3 swe1Δ::LEU2 cdc14::CDC14-5-GFP-TRP1 Tub1-mCherry*	This study
JBY257	*MATa cdc13-1*	Lab stock
686-6-1	*MATa cdc13-1 chk1Δ::HIS3 cdc14::CDC14-5-GFP-TRP1*	Lab stock
2104-1-2	*MATa cdc13-1 chk1Δ::HIS3 hsl1Δ::URA3 cdc14::CDC14-5-GFP-TRP1*	This study
2123-13-4	*MATa cdc13-1 chk1Δ::HIS3 hsl1Δ::URA3 swe1Δ::LEU2 cdc14::CDC14-5-GFP-TRP1*	This study
426-4-3	*MATa hsl1Δ::URA3*	Lab stock
FLY63	*MATa mob1-77*	Luca Lab
405-4-2	*MATa NET1-TEV-myc9-his5$^+$*	Lab stock
2118-8-3	*MATa cdc15-2 NET1-TEV-myc9-his5$^+$*	This study
869-8-3	*MATa cdc5-1 NET1-TEV-myc9-his5$^+$*	This study
869-10-1	*MATa cdc5-1 swe1Δ::LEU2 NET1-TEV-myc9-his5$^+$*	This study

MATERIALS AND METHODS

Yeast Strains and Growth

The yeast strains used in this study are listed in Table 1. All strains are isogenic to Y300, a W303 derivative. Yeast cells were grown in yeast extract, peptone, dextrose (YPD) medium unless indicated. To arrest cells in G1 phase, 5 μg/ml α-factor was added into cell cultures (YPD, pH 3.9). After 2 h of incubation, the G1-arrested cells were washed twice with water and then released into YPD medium to start cell cycle.

Cytological Techniques

Cells with green fluorescent protein (GFP)-tagged proteins were fixed with 3.7% formaldehyde for 5 min at room temperature and then washed twice with 1× phosphate-buffered saline (PBS) buffer and resuspended in PBS buffer for fluorescence microscopy (Carl Zeiss MicroImaging, Thornwood, NY). To examine the localization of Cdc14, *CDC14-5GFP* strains were used and cells without obvious nucleolar GFP signal were counted as released Cdc14. The spindle morphology was monitored by using *TUB1-mCherry* strains, and we counted the spindles with one end within the daughter cell as anaphase spindles. For 4,6-diamidino-2-phenylindole (DAPI) staining, cells were fixed with 3.7% formaldehyde for 5 min at room temperature and then resuspended in 100% MeOH at −20°C for 30 min. The cells were incubated in DAPI solution (final concentration, 2.5 μg/ml) for 1 min at room temperature. At least 100 cells were counted for each sample.

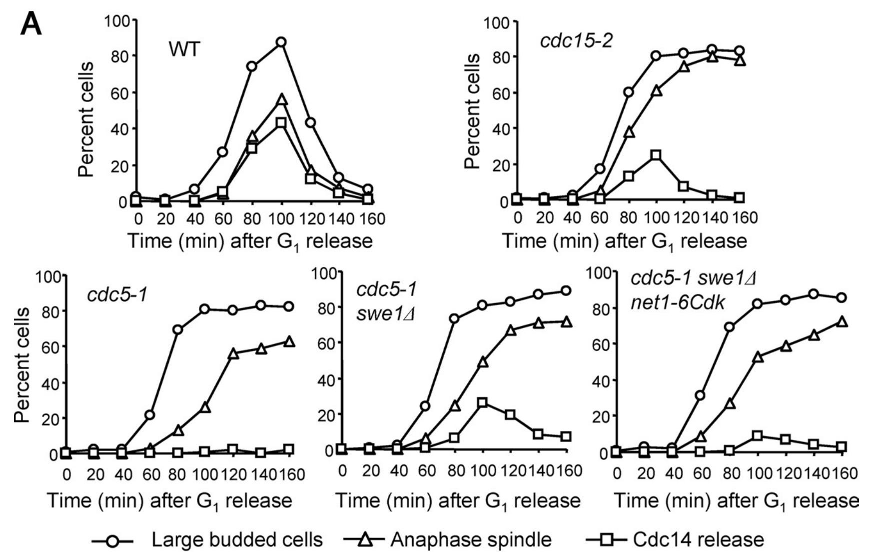
Protein Techniques

The pellets from 1.5 ml of cell cultures were resuspended in 200 μl of 0.1 N NaOH and incubated at room temperature for 5min. After centrifugation, the cells were resuspended in equal volume (30 μl) of double distilled H_2O and SDS protein-loading buffer. The samples were then boiled for 5 min and resolved with 8% SDS-polyacrylamide gel. Proteins were detected with enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences, Boston, MA) after probing with anti-myc antibody (Covance Research Products, Princeton, NJ) and horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

RESULTS

The FEAR Defects in cdc5 Mutants Depend Partially on Swe1 Accumulation

Different from other MEN temperature-sensitive mutants, *cdc5-1* mutant cells do not show a transient Cdc14 release during early anaphase when grown at 37°C, suggesting that Cdc5 is also a component of FEAR pathway (Stegmeier *et al.*, 2002). Previous studies have shown that Cdc5 phosphorylates Swe1 and promotes its degradation (Asano *et al.*, 2005), and *cdc5-2* mutant cells exhibit Swe1 degradation defects when incubated at 37°C (Liu and Wang, 2006). These observations raise the possibility that the failure of Swe1 degradation might contribute to the FEAR defects in *cdc5* mutant cells. Because *cdc5-1* mutants show FEAR defects, we first examined Swe1 degradation defects in this mutant. Compared with wild-type (WT) cells, *cdc5-1* mutant cells exhibited persistent Swe1 protein levels when incubated at 37°C, but Swe1 protein degradation was not compromised in other MEN (*cdc15-2*) or FEAR (*cdc15-2 spo12Δ*) mutants (data not shown). Therefore, we believe that the Swe1 degradation



B
Cdc14-GFP
Tub1-mCherry
DAPI
cdc5-1
cdc5-1 swe1Δ
cdc5-1 swe1Δ net1-6Cdk
120 min after G_1 release

Figure 1. *swe1*Δ deletion suppresses the Cdc14 release defect in *cdc5* mutant. (A) Swe1 accumulation in *cdc5-1* mutants compromises FEAR function. *CDC14-5GFP TUB1-mCherry* cells with indicated genotypes were arrested in G1 phase at 25°C and then released into 37°C YPD medium. The localization of Cdc14 and the spindle morphology was analyzed over time by florescence microscopy. Shown here is the budding index and the percentage of cells with elongated spindle or released Cdc14. The spindle with one end that has elongated into daughter cells is counted as anaphase spindle. Cells with released Cdc14 are those without a clear nucleolar localization of Cdc14. (B) The localization of Cdc14 and spindle morphology in cells with indicated genotypes after G1 release for 120 min. The white arrow indicates the *cdc5-1 swe1*Δ cell with released Cdc14.

defect is specific to *cdc5* mutants but not to other FEAR or MEN mutant cells.

Given that Swe1 accumulates in both *cdc5-1* and *cdc5-2* mutants, it is possible that the Swe1 accumulation in these mutants contributes to FEAR defects. To test this possibility, we compared cell cycle progression and Cdc14 release in *cdc5-1* and *cdc5-1 swe1*Δ mutants incubated at 37°C. Very few *cdc5-1* mutant cells exhibited Cdc14 release as reported previously (Stegmeier *et al*., 2002), but 26% of *cdc5-1 swe1*Δ double mutant cells showed released Cdc14 after G1 release for 100 min, similar to *cdc15-2* mutant, where the FEAR, but not MEN, is active (Figure 1, A and B). We also compared the spindle elongation kinetics in these mutant cells, and the spindles with one end that has elongated into daughter cells are counted as anaphase spindle. Delayed spindle elongation was noticed in *cdc5-1* mutants at 80 and 100 min after G1 release compared with *cdc15-2*, consistent with the role of the FEAR pathway in spindle morphogenesis (Jin *et al*., 2008). The spindle elongation defect was partially suppressed by the deletion of *SWE1* (Figure 1A). Similarly, we observed Cdc14 release defect in *cdc5-2* mutant cells and deletion of *SWE1* suppressed this defect (data not shown). These observations indicate that Swe1 accumulation contributes to the Cdc14 release defect in both *cdc5-1* and *cdc5-2* mutants.

Net1 is a substrate of both Clb2-Cdk1 and phosphatase $PP2A^{Cdc55}$; its phosphorylation may facilitate Cdc14 release. Mutation of the Cdk phosphorylation sites in Net1 shows FEAR defects, as Net1 phosphorylation site mutants are compromised in the release of Cdc14 from the nucleolus during early anaphase (Azzam *et al*., 2004; Queralt *et al*., 2006). Because Swe1 exhibits specific inhibition on Clb2-Cdk1 (Booher *et al*., 1993), and Cdc5 promotes Swe1 degradation, we reason that Cdc5 induces Net1 phosphorylation by alleviating Swe1-imposed inhibition on Clb2-Cdk1. If that is the case, the impediment of Net1 phosphorylation by Cdk1 should abolish the transient Cdc14 release in *cdc5 swe1*Δ double mutants. Consistent with our prediction, *net1-6Cdk* mutants, wherein the six Cdk1 phosphorylation sites are mutated to alanine, largely suppressed Cdc14 release in *cdc5-1 swe1*Δ mutants (Figure 1A). However, a few *cdc5-1 swe1*Δ *net1-6Cdk* cells still showed released Cdc14 when grown at the restrictive temperature. One possible explanation is that *net1-6Cdk* mutant is not a mutant allele that loses FEAR function completely (Azzam *et al*., 2004) or that some mechanism other than Net1 phosphorylation also controls Cdc14 release during early anaphase.

Swe1 Degradation Is Required for Efficient rDNA Separation

FEAR-dependent Cdc14 release is important for efficient rDNA separation (D'Amours *et al*., 2004; Sullivan *et al*., 2004). Our data indicate that Cdc5-dependent Swe1 degradation promotes Cdc14 release during early anaphase, and we speculate that the failure of Swe1 degradation would lead to the rDNA separation defects in *cdc5* mutants. To test this speculation, rDNA separation in *cdc15-2*, *cdc5-1*, and *cdc5-1 swe1*Δ mutants with a tetO array integrated adjacent

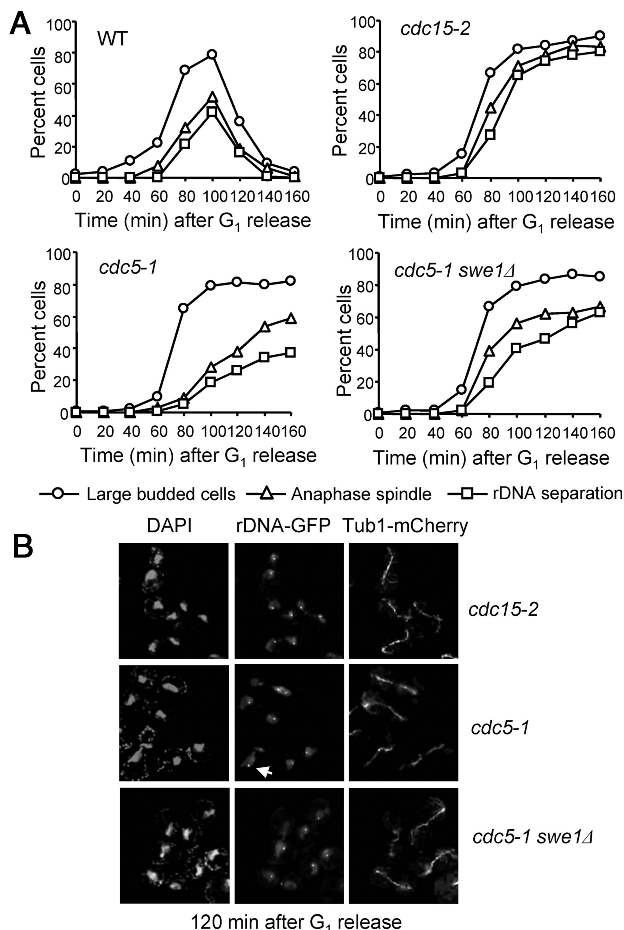


Figure 2. The partial suppression of the nucleolar separation defect in *cdc5-1* mutants by *swe1*Δ deletion. (A) Deletion of *SWE1* partially suppresses the delayed rDNA separation in *cdc5-1* mutant cells. Cells with GFP-marked rDNA and *TUB1-mCherry* were arrested in G1 phase at 25°C and then released into 37°C YPD medium. The spindle elongation and rDNA separation kinetics are shown. (B) The spindle morphology and localization of rDNA in some representative cells after 2 h release. The arrow indicates the *cdc5-1* mutant cell with a single rDNA-GFP dot.

to the rDNA locus (rDNA-GFP) was analyzed (D'Amours *et al.*, 2004). After G1 release for 2 h at 37°C, 74% of *cdc15-2* cells showed separated rDNA-GFP dots, but only 26% of *cdc5-1* mutant cells exhibited rDNA separation. In contrast, 47% of *cdc5-1 swe1*Δ double mutant cells exhibited separated rDNA (Figure 2, A and B), indicating that *swe1*Δ deletion could partially suppress the rDNA separation defect in *cdc5-1* mutants. Similar results were obtained using *cdc5-2* and *cdc5-2 swe1*Δ cells (data not shown). To further confirm the suppression of the rDNA separation defect in *cdc5-1* mutants by *swe1*Δ, we also analyzed the distribution of Net1-GFP, which localizes within the nucleolus throughout the cell cycle (Shou *et al.*, 1999; Visintin *et al.*, 1999; Machin *et al.*, 2006). *swe1*Δ deletion suppressed the Net1 separation defects in both *cdc5-1* and *cdc5-2* mutants (data not shown), suggesting that the FEAR defects in *cdc5* mutants are attributable to the failure of Swe1 protein degradation.

Swe1 Accumulation Contributes to the Delay of FEAR-dependent Dephosphorylation of Clb5-Cdk1 Substrates

We have shown that an active FEAR pathway promotes the dephosphorylation of a Clb5-Cdk substrate, Sld2, during early anaphase and inactivation of both FEAR and MEN blocks Sld2 dephosphorylation (Jin *et al.*, 2008). If the compromised FEAR function in *cdc5* mutants is a result of Swe1 accumulation, we expect defective Sld2 dephosphorylation in *cdc5* mutants and deletion of *SWE1* should suppress this defect. Therefore, we compared the dephosphorylation kinetics of Sld2 in *cdc5* and *cdc5 swe1*Δ mutants. G1-arrested WT, *cdc15-2*, *cdc5-1*, *cdc5-1 swe1*Δ, *cdc5-2*, and *cdc5-2 swe1*Δ mutants carrying *SLD2-myc* were released into YPD medium at 37°C. After G1 release for 40 min, Sld2 became phosphorylated in all the tested cells as indicated by the appearance of slow migrating bands. Although *cdc15-2* cells showed Sld2 dephosphorylation after G1 release for 80 min, both *cdc5-1* and *cdc5-2* mutant cells exhibited dramatically delayed Sld2 dephosphorylation, supporting the role of Cdc5 in both FEAR and MEN pathways. However, *swe1*Δ deletion alleviated this delay in both *cdc5-1* and *cdc5-2* mutants, although the suppression is not complete (Figure 3B). Together, the defects in Cdc14 release, rDNA separation, and Sld2 dephosphorylation observed in *cdc5* mutants can be partially suppressed by the deletion of *SWE1*, supporting the conclusion that Cdc5 kinase promotes FEAR function in part by stimulating Swe1 degradation.

Down-Regulation of Cdk1 Activity by Swe1 Is Responsible for the FEAR Defects in cdc5 Mutant Cells

Swe1 inhibits Clb2-Cdk1 activity by phosphorylating Y19 on Cdk1 (Cdc28) (Amon *et al.*, 1992; Sorger and Murray, 1992; Booher *et al.*, 1993). To test whether increased Cdk1 phosphorylation by Swe1 is responsible the FEAR defects in *cdc5* mutants, we compared Cdc14 release and rDNA separation in *cdc5* single and *cdc5 cdc28F19* double mutants, where the Swe1 phosphorylation site in Cdk1 is mutated. It was obvious that *cdc28F19* mutation suppressed the defects of Cdc14 release and rDNA separation in *cdc5-1* mutants, although the suppression is not complete (Figure 4, A and B), suggesting that the FEAR defects in *cdc5* mutants is due to the increased inhibitory phosphorylation of Cdk1 by Swe1 kinase.

The Separable FEAR and MEN Functions of Cdc5 Kinase

The identified substrates of Cdc5 kinase include Bfa1 and Swe1, and the phosphorylation of Bfa1 leads to the activation of the MEN (Hu *et al.*, 2001). The localization of Cdc5 at the spindle pole bodies is essential for Bfa1 phosphorylation, whereas the bud-neck localization of Cdc5 is responsible for the phosphorylation of Swe1 (Park *et al.*, 2004). *cdc5* mutant cells are defective for both FEAR and MEN. If the FEAR defect in *cdc5* is a result of Swe1 accumulation, the expression of *CDC5* that specifically localized at the bud neck will suppress the defects of *cdc5* mutants in FEAR but not in MEN. Therefore, we introduced a plasmid pKL2438 containing *CDC5*Δ*C-CDC12* into *cdc5-1* mutant. This Cdc5ΔC-Cdc12 fusion protein has been shown to exclusively localize at the bud neck and is competent for Swe1 phosphorylation and degradation (Park *et al.*, 2004). The introduction of this plasmid partially suppressed the Swe1 degradation defects in *cdc5-1* mutants, but the transformants were still arrested as large-budded cells when incubated at 37°C, indicating the defective MEN (Figure 5A). The expression of Cdc5ΔC-Cdc12 protein also caused Cdc14 release from the nucleolus in some *cdc5-1* mutant cells, but the suppression by Cdc5ΔC-Cdc12 was not as efficient as *swe1*Δ (Figure 5B). We reason that the slower Swe1 degradation kinetics in *cdc5-1* mutant cells containing Cdc5ΔC-Cdc12 contributes to the less efficient suppression of the FEAR defects in *cdc5-1* mutants. Thus, the bud-neck localization of Cdc5 and the subsequent

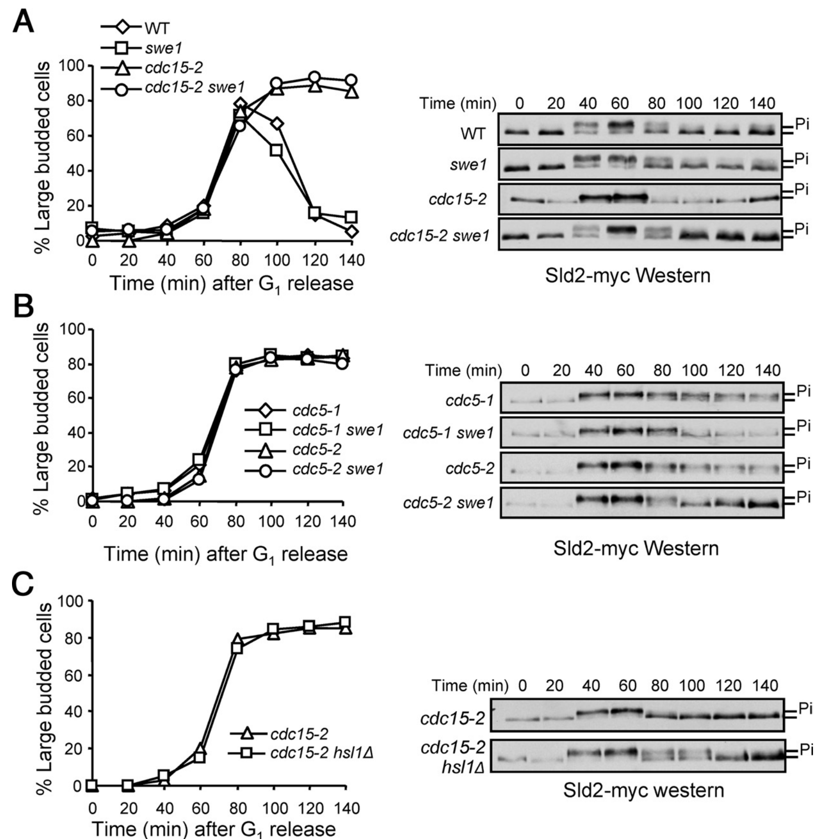


Figure 3. High levels of Swe1 protein result in delayed dephosphorylation of Sld2, a Clb5-Cdk1 substrate. *SLD2-9myc* cells with indicated genotypes were arrested in G1 phase at 25°C and then released into 37°C YPD medium. For WT and *swe1*Δ cells, α-factor was added into the medium at 40 min when the majority of cells were budded to block the initiation of next round of cell cycle. The cells were collected every 20 min for budding index and the preparation of protein samples. Sld2 protein modification is shown after Western blot analysis. The phosphorylation kinetics of Sld2 in WT, *swe1*Δ, *cdc15-2* and *cdc15-2 swe1*Δ cells is shown in A. (B) Suppression of Sld2 dephosphorylation defects in *cdc5* mutants by *swe1*Δ. The Sld2 phosphorylation status in *cdc15-2* and *cdc15-2 hsl1*Δ cells is shown in C.

Swe1 phosphorylation and degradation are required for FEAR activation.

Swe1 Plays a Negative Role in FEAR-dependent Cdc14 Release

Because Clb2-Cdk1-dependent Net1 phosphorylation plays a positive role in Cdc14 release during early anaphase (Azzam *et al*., 2004), Swe1 overexpression is expected to block the FEAR-dependent Cdc14 release. Indeed, we found that overexpression of Swe1 blocked the Cdc14 release in WT cells but not in *cdc28F19* mutant cells (data not shown), suggesting a negative role of Swe1 in Cdc14 release by inhibiting Clb2-Cdk1. However, we could not exclude the possibility that the failure of Cdc14 release in cells overexpressing *SWE1* is a result of anaphase entry block. Hsl1 is required for the recruitment of Swe1 to the bud-neck and subsequent Swe1 degradation (Shulewitz *et al*., 1999; Asano *et al*., 2005). Although *hsl1*Δ mutant cells exhibit elevated Swe1 protein levels, the mutant cells are viable. To further define the negative role of Swe1 in FEAR-dependent Cdc14 release, we examined Cdc14 localization in *cdc15-2 hsl1*Δ mutant cells. *cdc15-2 hsl1*Δ and *cdc15-2 hsl1*Δ *swe1*Δ cells with 5GFP-tagged Cdc14 were arrested in G1 phase at 25°C and then released into cell cycle at 37°C. After G1 release for 100

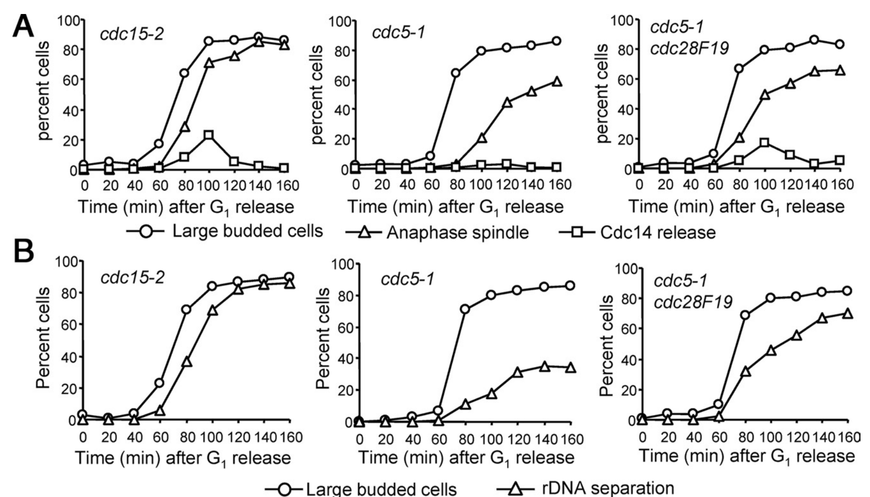


Figure 4. Down-regulation of Cdk1 is responsible for the FEAR defects in *cdc5* mutants. (A) *cdc28F19* mutant suppresses the Cdc14 release defects in *cdc5-1* mutants. *CDC14-GFP TUB1-mCherry* cells with the indicated genotypes were synchronized in G1 phase and then released into 37°C YPD medium. Budding index and the kinetics of spindle elongation and Cdc14 release are shown. (B) *cdc28F19* mutant suppresses the rDNA separation defects in *cdc5-1* mutants. G1-synchronized cells with GFP-marked rDNA were released into 37°C medium. Cells were collected at 20-min intervals to follow the kinetics of budding and rDNA separation.

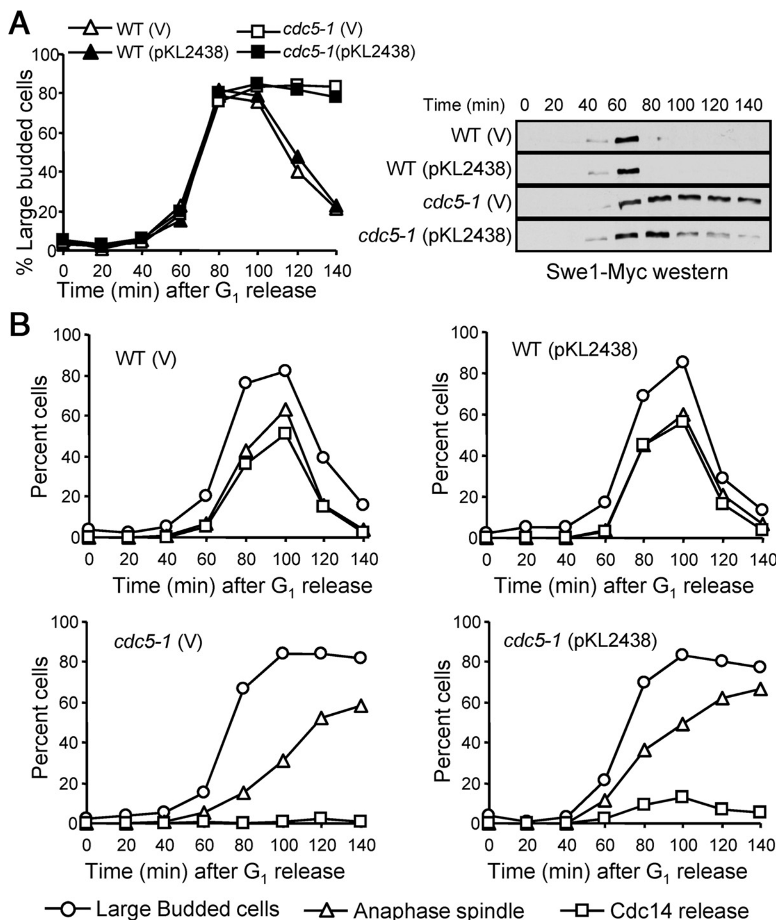


Figure 5. Expression of bud-neck–localized Cdc5 suppresses the FEAR defect in *cdc5* mutants. (A) Expression of Cdc5ΔC-Cdc12 fusion protein that localizes exclusively at the bud neck partially suppresses the Swe1 degradation defects in *cdc5-1* mutants. pKL2438 plasmid that expresses Cdc5ΔC-Cdc12 fusion protein were introduced into WT and *cdc5-1* mutants containing *SWE1-myc*. The transformants were grown and arrested in G1 phase in LEU dropout medium and then released into 37°C YPD medium. Swe1 protein levels were determined after Western blotting with anti-myc antibody. (B) *cdc5-1* cells containing pKL2438 plasmid show released Cdc14. *CDC14-GFP TUB1-mCherry* and *cdc5-1 CDC14-GFP TUB1-mCherry* cells containing either a vector or pKL2483 plasmid were synchronized in G1 phase and then released into 37°C YPD medium. The kinetics of spindle elongation and Cdc14 release are shown.

min, 22% *cdc15-2* cells showed released Cdc14, indicating that FEAR pathway is active. In contrast, *cdc15-2 hsl1*Δ mutant cells showed delayed and decreased Cdc14 release, but the defect was suppressed by introducing *swe1*Δ deletion (Figure 6A). The results suggest that high levels of Swe1 in *cdc15-2 hsl1*Δ cells contribute to the Cdc14 release defects. We also examined the kinetics of Sld2 dephosphorylation in *cdc15-2* and *cdc15-2 hsl1*Δ cells incubated at 37°C. In *cdc15-2* cells, Sld2 became dephosphorylated after G1 release for 80 min, whereas a delayed Sld2 dephosphorylation was observed in *cdc15-2 hsl1*Δ cells (Figure 3C). Together, these observations demonstrate that low level of Swe1 protein is needed for Cdc14 release during early anaphase as well as the subsequent dephosphorylation of Clb5-Cdk1 substrates.

Chk1, one of the DNA damage checkpoint components, controls mitotic exit by negatively regulating FEAR pathway. *net1-6Cdk* mutation, which is resistant to the phosphorylation by Clb2-Cdk1, is able to block the mitotic exit in *chk1*Δ mutants in the presence of DNA damage (Liang and Wang, 2007). If Swe1 plays a negative role in FEAR activation, high levels of Swe1 would block the mitotic exit in *chk1*Δ mutant cells with damaged DNA. To test this possibility, we examined the microcolony formation in *chk1*Δ and *chk1*Δ *hsl1*Δ mutants arrested with *cdc13-1*, which activates DNA damage checkpoint because of the unprotected telomeres when incubated at the restrictive temperature. Consistent with previous data, 61% of *cdc13-1 chk1*Δ cells formed microcolonies when incubated at 37°C for 8 h, indicating cells exit mitosis, but only 11% *cdc13-1 chk1*Δ *hsl1*Δ cells did, presumably due to the Swe1 degradation defects in *hsl1*Δ. Deletion of *SWE1* allowed *cdc13-1 chk1*Δ *hsl1*Δ cells to regain the ability for microcolony formation (Figure 6B), confirming that high levels of Swe1 compromise the FEAR-dependent mitotic exit.

FEAR and MEN promote Cdc14 release from the nucleolus and deletion of *LTE1*, a MEN component, is synthetically lethal with FEAR mutants *slk19*Δ and *spo12*Δ (Stegmeier *et al.*, 2002). To further determine the FEAR defects in *hsl1*Δ mutants, double mutants between *hsl1*Δ and MEN mutants, *mob1-77*, *cdc15-2* and *lte1*Δ, were generated. When incubated at 30°C, *hsl1*Δ *mob1-77* double mutants failed to grow, whereas *mob1-77* single mutant cells grew well. Similarly, the growth of *cdc15-2 hsl1*Δ mutant cells at 30°C was not as well as each single mutant. Deletion of *SWE1* suppressed the growth defects of the double mutants (Figure 6C). Also, *hsl1*Δ *lte1*Δ double mutants exhibited poor growth phenotype when incubated at 25°C (data not shown). All these observations support the notion that Swe1 plays a negative role in FEAR activation.

Swe1 Accumulation Is Responsible for the Decreased Net1 Phosphorylation in cdc5 Mutants

The phosphorylation of Net1 by Clb2-Cdk1 promotes FEAR activation (Azzam *et al.*, 2004; Queralt and Uhlmann, 2008). Given that Swe1 negatively regulates Clb2-Cdk1 and that *cdc5* mutants exhibit failure in Swe1 degradation, it is likely that the FEAR defect in *cdc5* mutants is due to the inability of Net1 phosphorylation. To confirm this speculation, we examined the phosphorylation status of Net1 protein in synchronous *cdc5-1* and *cdc5-1 swe1*Δ mutants incubated at

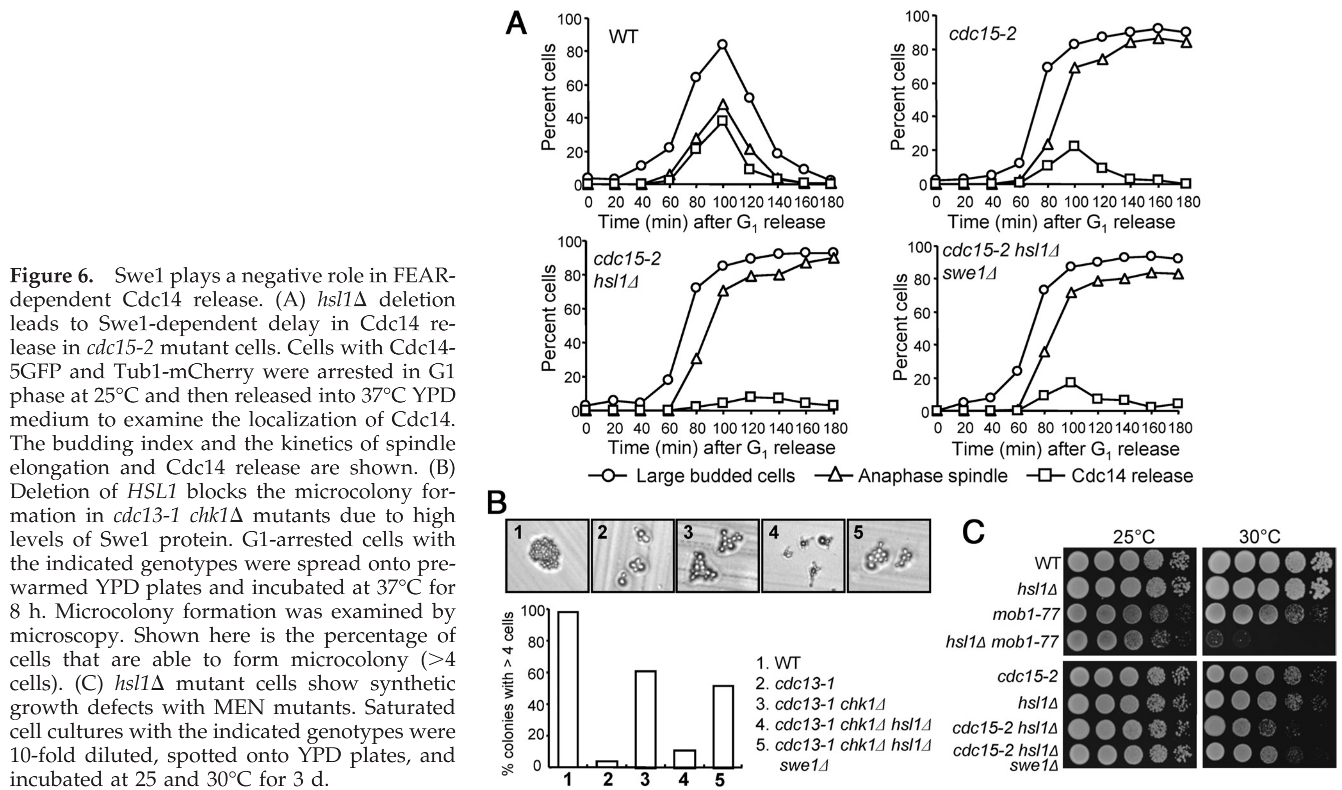
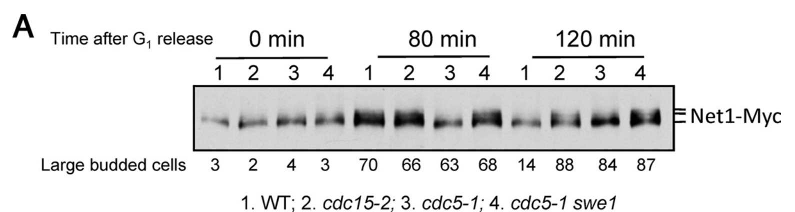


Figure 6. Swe1 plays a negative role in FEAR-dependent Cdc14 release. (A) *hsl1*Δ deletion leads to Swe1-dependent delay in Cdc14 release in *cdc15-2* mutant cells. Cells with Cdc14-5GFP and Tub1-mCherry were arrested in G1 phase at 25°C and then released into 37°C YPD medium to examine the localization of Cdc14. The budding index and the kinetics of spindle elongation and Cdc14 release are shown. (B) Deletion of *HSL1* blocks the microcolony formation in *cdc13-1 chk1*Δ mutants due to high levels of Swe1 protein. G1-arrested cells with the indicated genotypes were spread onto prewarmed YPD plates and incubated at 37°C for 8 h. Microcolony formation was examined by microscopy. Shown here is the percentage of cells that are able to form microcolony (>4 cells). (C) *hsl1*Δ mutant cells show synthetic growth defects with MEN mutants. Saturated cell cultures with the indicated genotypes were 10-fold diluted, spotted onto YPD plates, and incubated at 25 and 30°C for 3 d.

37°C. After G1 release for 80 min, both WT and *cdc15-2* mutants showed slow migrating forms of Net1, presumably due to phosphorylation (Azzam *et al*., 2004; Queralt and Uhlmann, 2008). In contrast, only hypophosphorylated Net1 was observed in *cdc5-1* mutant cells and deletion of *SWE1* suppressed the Net1 phosphorylation defects in *cdc5-1* mutants (Figure 7A). This is a strong indication that Cdc5 regulates Net1 phosphorylation through Swe1. In *cdc15-2* mutant cells, we noticed persistent Net1 phosphorylation even after G1 release for 2 h, but fewer *cdc15-2* mutant cells exhibited released Cdc14 after G1 release for 2 h (Figure 1A). This observation indicates that Net1 phosphorylation might be essential, but not sufficient, for Cdc14 release during early anaphase.

DISCUSSION

In budding yeast *S. cerevisiae*, the Polo-like kinase Cdc5 functions as a component of both FEAR and MEN pathways to promote Cdc14 release from the nucleolus. As a component of the MEN pathway, Cdc5 promotes Cdc14 release by phosphorylating Bfa1, the negative regulator of the MEN (Hu *et al*., 2001). We believe that Cdc5 executes its FEAR function in part by promoting Swe1 degradation on the basis of the following observations. First, we found that both *cdc5-1* and *cdc5-2* mutants exhibited Swe1 degradation defects. Deletion of *SWE1* partially alleviated the defects in Cdc14 release and rDNA separation in *cdc5* mutants. In contrast, *hsl1*Δ mutant strains, wherein Swe1 degradation is



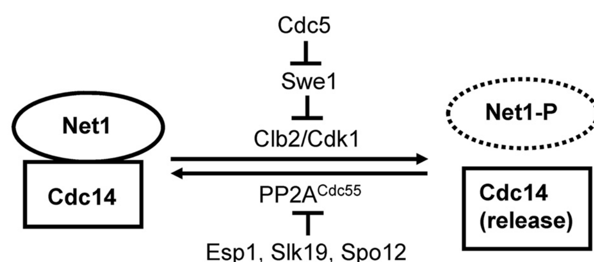


Figure 7. Accumulation of Swe1 in *cdc5* mutants causes compromised Net1 phosphorylation. (A) G1-synchronized *NET1-myc* cells with indicated genotypes were released into 37°C YPD medium. Cell samples were collected at 0, 80, and 120 min for protein preparation. The protein samples were resolved with 8% SDS-polyacrylamide gel electrophoresis to determine the phosphorylation status of Net1. The percentage of large-budded cells is shown at the bottom. (B) Model for the regulation of Cdc14 release by Cdc5 kinase and other FEAR components. Cdc5-dependent Swe1 degradation promotes the Net1 phosphorylation by Clb2-Cdk1, which may facilitate Cdc14 release.

compromised, exhibited FEAR defects, as indicated by the delayed Cdc14 release and the synthetic growth defects with MEN mutants. More importantly, *cdc5-1* mutants exhibited Swe1-dependent defect in Net1 phosphorylation.

Our evidence suggests that Swe1 acts as a negative regulator of FEAR. S phase-expressed Swe1 is likely to prevent the premature activation of Clb2-Cdk1, whereas the activity of Clb5-Cdk1 remains unaffected. Because we have shown that the activation of FEAR facilitates spindle stabilization and elongation (Jin *et al.*, 2008), Swe1 accumulation in S phase cells could be an important mechanism to prevent mitotic activities when DNA replication is underway. Consistent with this speculation, we have demonstrated previously that the block of DNA replication stabilizes Swe1 (Liu and Wang, 2006). Surprisingly, no premature mitosis is observed in *swe1*Δ mutant cells when DNA synthesis is blocked (Amon *et al.*, 1992; Sorger and Murray, 1992), indicating that other mechanisms might be present to prevent premature activation of Clb2-Cdk1. For example, the low transcription levels of *CLB2* gene during S phase could avoid premature mitosis induced by Clb2-Cdk1.

Although *swe1*Δ deletion can suppress the FEAR defects in *cdc5* mutants, the kinetics of rDNA separation and Sld2 dephosphorylation in *cdc5 swe1*Δ double mutant cells is a little slower than that in *cdc15-2* mutants, indicating that additional defects in *cdc5* mutants may also contribute to the failure of FEAR activation. Previous studies suggest that overexpression of Cdc5 leads to hyperphosphorylated Cdc14 and that this modification may play a positive role in Cdc14 release (Visintin *et al.*, 2003). Recently, Cdc5 has been shown to interact with Cdc14 directly, but the significance of this interaction remains to be defined (Rahal and Amon, 2008). Thus, Cdc5 may regulate Cdc14 release in multiple ways and Cdc5-dependent Swe1 degradation could be one of the functions of Cdc5 in mitotic exit.

We and others have demonstrated that FEAR components Esp1, Slk19, and Spo12 promote FEAR function by inhibiting $PP2A^{Cdc55}$-dependent Net1 dephosphorylation (Queralt *et al.*, 2006; Wang and Ng, 2006; Yellman and Burke, 2006). Unlike other FEAR components, the Polo-like kinase Cdc5 regulates Net1 phosphorylation through Swe1. Cdc5-induced Swe1 degradation enables the activation of mitotic cyclin-associated Cdk1, which phosphorylates Net1. Therefore, two different mechanisms control FEAR activation through the regulation of Net1 phosphorylation. The activation of Cdc5 kinase before metaphase results in the degradation of Swe1, which allows the activation of Clb2-Cdk1. However, the presence of the phosphatase $PP2A^{Cdc55}$ keeps Net1 protein from being phosphorylated by Clb2-Cdk1. On anaphase onset, the degradation of the anaphase inhibitor Pds1 frees the separase Esp1 that inactivates $PP2A^{Cdc55}$ and shifts the equilibrium toward Net1 phosphorylation. Recent works from the Amon laboratory indicate that Spo12 is also a substrate of Clb2-Cdk1 (Tomson *et al.*, 2009), raising the possibility that Cdc5-dependent Swe1 degradation may also lead to Spo12 phosphorylation and further activate FEAR. In conclusion, both Cdc5-dependent Clb2-Cdk1 activation and Esp1-induced inactivation of $PP2A^{Cdc55}$ promote FEAR activation by stimulating the phosphorylation of Net1 (Figure 7B).

Mammalian cells express Cdc14A and Cdc14B, which are the functional homologues of budding yeast Cdc14 (Trinkle-Mulcahy and Lamond, 2006). Cdc14A is located at centrosomes, whereas Cdc14B resides in the nucleolus during interphase but not during mitosis (Bembenek and Yu, 2001; Kaiser *et al.*, 2002). We found that S phase cyclin substrates, including Ase1, a spindle midzone component, are subjected to FEAR-dependent dephosphorylation after anaphase entry (Jin *et al.*, 2008). PRC1, the mammalian homologue of Ase1, also becomes dephosphorylated during metaphase-to-anaphase transition, and this dephosphorylation is essential for the spindle midzone formation (Zhu *et al.*, 2006), raising the possibility that the dephosphorylation of some Cdk substrates occurs after anaphase entry in mammalian cells. However, further experiments are needed to determine whether PRC1 is a substrate of Cdc14A or B. So far, there is no solid evidence indicating the presence of FEAR and MEN networks that regulate Cdc14 activity in mammalian cells. Much more research work is needed to determine whether mammalian cells dephosphorylate some Cdk substrates after anaphase entry through the activation of phosphatase Cdc14.

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