Mol Biol Cell. 2006 Jan; 17(1): 80–89. PMCID: PMC1345648
doi: 10.1091/mbc.E04-12-1109

Phosphatase 2A Negatively Regulates Mitotic Exit in *Saccharomyces cerevisiae*

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Received 2004 Dec 22; Revised 2005 Jun 16; Accepted 2005 Jul 21.

ABSTRACT

Go to:

In budding yeast *Saccharomyces cerevisiae*, Cdc5 kinase is a component of mitotic exit network (MEN), which inactivates cyclin-dependent kinase (CDK) after chromosome segregation. *cdc5-1* mutants arrest at telophase at the nonpermissive temperature due to the failure of CDK inactivation. To identify more negative regulators of MEN, we carried out a genetic screen for genes that are toxic to *cdc5-1* mutants when overexpressed. Genes that encode the B-regulatory subunit (Cdc55) and the three catalytic subunits (Pph21, Pph22, and Pph3) of phosphatase 2A (PP2A) were isolated. In addition to *cdc5-1*, overexpression of *CDC55*, *PPH21,* or *PPH22* is also toxic to other temperature-sensitive mutants that display defects in mitotic exit. Consistently, deletion of *CDC55* partially suppresses the temperature sensitivity of these mutants. Moreover, in the presence of spindle damage, PP2A mutants display nuclear localized Cdc14, the key player in MEN pathway, indicative of MEN activation. All the evidence suggests the negative role of PP2A in mitotic exit. Finally, our genetic and biochemical data suggest that PP2A regulates the phosphorylation of Tem1, which acts at the very top of MEN pathway.

INTRODUCTION

Go to:

The key driving force for cell division in all eukaryotic cells is the conserved cyclin-dependent kinase (CDK). CDK activity fluctuates during the cell cycle, peaking at the S and M phases and dropping at the G1 phase. Although the high CDK activity is required for DNA duplication and chromosome segregation, the low CDK activity at late M and G1 phase is essential for the loading of DNA replication complexes onto replication origins (Noton and Diffley, 2000; Lengronne and Schwob, 2002). Thus, cells have developed a signal transduction pathway, named the mitotic exit network (MEN), to inactivate CDK after mitosis (Morgan, 1999). The components of the MEN pathway include protein kinases Cdc5, Cdc15, Dbf2, a GTPase Tem1, a phosphatase Cdc14, and a Dbf2 binding protein Mob1 (Jaspersen *et al.*, 1998; Luca and Winey, 1998; Lee *et al.*, 2001a). Phosphatase Cdc14, the key player in the MEN pathway, dephosphorylates Cdh1, an activator for APC (anaphase promoting complex), and subsequently activates the degradation of a B-type cyclin, Clb2. Cdc14 also enhances the stability of Sic1 protein, which acts as a CDK inhibitor (Visintin *et al.*, 1998). During most of the cell cycle, Cdc14 is localized in the nucleolus, but its translocation out of the nucleolus in anaphase and telophase allows it to encounter its substrates, such as Cdh1 and Sic1. All the

components in the MEN pathway are required for the release of Cdc14 from the nucleolus, suggesting that Cdc14 acts downstream of MEN pathway (Shou *et al.*, 1999; Visintin *et al.*, 1999).

The regulation of MEN activity is achieved through the multilayer control of Tem1, a small GTPase that localizes at the spindle pole body (SPB) and acts on the very top of the MEN pathway. Tem1's activator, the GTPase exchange factor (GEF) Lte1, exhibits daughter-cell specific localization. Thus, SPB-localized Tem1 is activated after it encounters Lte1 upon the entrance of SPB into daughter cells (Bardin *et al.*, 2000). Before that, Tem1 is kept inactive by a two-component GTPase-activating factor (GAP) composed of Bfa1 and Bub2 (Alexandru *et al.*, 1999; Geymonat *et al.*, 2002, 2003). Protein kinase Cdc5 has also been implicated in MEN signaling by regulating Bfa1/Bub2. It phosphorylates one of the GAP components, Bfa1, and frees Tem1 from the inhibition by Bfa1 (Hu *et al.*, 2001; Lee *et al.*, 2001b). Tem1-GTP is believed to activate a downstream protein kinase Cdc15, which then activates the protein kinase Dbf2 in a manner dependent on the Dbf2 associated factor Mob1 (Komarnitsky *et al.*, 1998; Mah *et al.*, 2001). We have identified a new mechanism that inactivates MEN through the induction of Amn1 protein upon MEN activation. Amn1 binds to Tem1 and abolishes its association with the downstream target Cdc15 (Wang *et al.*, 2003). Thus the cooperation of Tem1's cellular localization, Bfa1/Bub2 GAP activity, and the cell cycle regulated appearance of Amn1, limits the functional window of MEN to late M and early G1 phase.

Protein phosphatase 2A regulates a significant array of cellular events. This holoenzyme consists of a catalytic subunit, C, and two regulatory subunits, A and B (Millward *et al.*, 1999). In budding yeast, *PPH21, PPH22*, and *PPH3* encode the catalytic subunits of PP2A, and *CDC55* encodes one of the regulatory B subunits (Healy *et al.*, 1991; Ronne *et al.*, 1991; Evans and Stark, 1997). In the presence of nocodazole, a drug that disrupts spindle structures, sister chromatids separate in Δ*cdc55* mutants, suggesting a function for PP2A in anaphase entry (Minshull *et al.*, 1996; Wang and Burke, 1997). The phenotype of Δ*cdc55* mutants seems to be the result of increased Cdc28 phosphorylation. In *S. cerevisiae*, the CDK kinase activity is inhibited in G1 and early S phase by Swe1 kinase-dependent phosphorylation at tyrosine 19 of Cdc28 (Booher *et al.*, 1993). Δ*cdc55* mutants are unable to keep a high CDK activity in nocodazole-treated cells because of the inhibitory phosphorylation of Cdc28. The premature sister chromatid separation and the cold sensitivity phenotypes in Δ*cdc55* mutants are suppressed by the *CDC28F19* mutation, in which Cdc28 is resistant to the phosphorylation by Swe1 (Minshull *et al.*, 1996; Wang and Burke, 1997). Consistently, Swe1 protein level is increased in Δ*cdc55* mutants because of the compromised Swe1 protein degradation (Yang *et al.*, 2000). Thus, the accumulation of Swe1 in Δ*cdc55* mutants results in Cdc28 phosphorylation, which may contribute to the known phenotypes of Δ*cdc55* mutants.

Here we report that PP2A is also involved in mitotic exit regulation. Overproduction of PP2A components is toxic to temperature-sensitive mutants that have defects in mitotic exit. Δ*cdc55* mutants exit mitosis in the presence of spindle damage, as judged by the appearance of extra buds and nuclear-localized Cdc14. PP2A and Bfa1/Bub2 may negatively regulate mitotic exit in parallel pathways because Δ*cdc55* Δ*bfa1* and Δ*cdc55* Δ*bub2* double mutants are synthetic sick and exhibit more frequent nuclear localized Cdc14. PP2A may regulate mitotic exit by promoting Tem1 protein dephosphorylation. Thus, we identified a new layer of regulation for mitotic exit, involving PP2A.

MATERIALS AND METHODS

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Yeast Strains, Growth, and Media

The genotype and sources of relevant yeast strains are listed in Table 1. All the strains listed are isogenic with W303 derived Y300 strain. All strains were constructed using standard genetic crosses. YYW28 was made by using a PCR-based method (Longtine *et al.*, 1998). To arrest yeast cells at G1 phase, 5 μg/ml α-factor was added into midlog cell cultures (OD_{600} = 0.4) and the cultures were

incubated for 2.5 h. To release them into cell cycle, the cell cultures were centrifuged and washed once with H_2O. Nocodazole was purchased from ICN (Costa Mesa, CA) and was used at 20 μg/ml in a final concentration of 1% dimethyl sulfoxide.

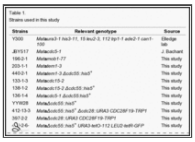


Table 1.

Strains used in this study

Cytological Techniques

Immunofluorescence straining was done after formaldehyde (3.7%) fixation for 15 min. Cells were treated with zymolase for 15 min and then stained with anti-HA antibody (1:100; CRP, Berkeley, CA) overnight at 4°C on 14-well slides after methanol/acetone treatment. Afterward, cells were stained with FITC-conjugated secondary antibody and DAPI and then visualized under immunofluorescence microscope (Zeiss, Thornwood, NY).

Protein Techniques

Two milliliters of cell culture was used to prepare protein samples for time-course experiments. Cells were collected in tubes with screw caps after being centrifuged and 50 μl of 20% TCA and glass beads were added. Cells were broken by using beads beater for 2 min. Protein was precipitated by centrifuge at 3000 rpm for 2 min after glass beads were removed. Equal volumes (50 μl) of 1 M Tris-base and protein-loading buffer were added. Dissolved protein samples were boiled for 5 min.

Two hundred milliliters of cell culture (OD_{600} = 0.4) was used for Tem1 protein immunoprecipitation. Cells were collected by centrifugation and washed once with water and then resuspended in 0.5 ml RIPA buffer supplied with protease and phosphatase inhibitors. Cells were broken with beads beater and the cell debris was removed after centrifuge at 14,000 rpm for 20 min at 4°C. Anti-myc antibody, 8 μl, (from CRP) was added into the cell extract and the tube was shaken for 1.5 h at 4°C. Then 50 μl of anti-mouse IgG agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added and shaken for 1.5 h. The beads were washed with 1× λ phosphatase buffer for three times and resuspended in 60 μl 1× phosphatase buffer. The beads were then used for λ phosphatase treatment. In our experiment, NaF, β-glyceral phosphate, and Na_3VO_4 were used as phosphatase inhibitor.

RESULTS

Go to:

Genetic Screen for Genes Lethal to cdc5-1 When Overexpressed

Cdc5 kinase promotes mitotic exit by phosphorylating Bfa1 and leading to its disassociation from Tem1 (Hu *et al.*, 2001). To further understand the regulation of mitotic exit, we carried out a genetic screen for genes that cause lethality in *cdc5-1* Ts mutants when overexpressed (Wang *et al.*, 2003). A P_{GAL}-cDNA library was introduced into *cdc5-1* mutants and the transformants were selected on URA dropout plates (Liu *et al.*, 1992). From a total of 12,000 transformants, 10 plasmids exhibited a high-dosage lethal phenotype in *cdc5-1* mutants. The recovered plasmids contain *AMN1, CLB1, CDC55, PPH21, PPH22,* and *PPH3* genes. The negative role of *AMN1* in mitotic exit regulation was reported previously (Wang *et al.*, 2003). Like Clb2, Clb1 could be another APC^{Cdh1} substrate and its overproduction will deteriorate the growth of *cdc5-1* mutants in which the function of APC^{Cdh1} is compromised (Charles *et al.*, 1998; Shirayama *et al.*, 1998). In addition to *AMN1* and *CLB1*, we also isolated several genes that encode the subunits of PP2A, including the B-regulatory subunit Cdc55 and the catalytic subunits Pph21, Pph22, and Pph3.

Cdc5 kinase phosphorylates Bfa1 and promotes mitotic exit (Hu *et al.*, 2001). Also it phosphorylates cohesin Scc1 and facilitates its cleavage by the separase Esp1 (Alexandru *et al.*, 2001). The lethality in *cdc5-1* mutants caused by the overexpression of PP2A subunits may result from the negative effects of PP2A on mitotic exit or on other Cdc5 related cell cycle processes. Thus we examined if overexpression of PP2A components is toxic to other temperature-sensitive mutants of the MEN pathway. P_{GAL}-*CDC55*, P_{GAL}-*PPH21*, P_{GAL}-*PPH22*, P_{GAL}-*PPH3,* and a control vector were introduced into *tem1-3* and *mob1-77* mutants that have defects in mitotic exit (Shirayama *et al.*, 1994; Luca and Winey, 1998). The growth of the transformants was examined after incubation on both glucose and galactose plates at the permissive temperature. Overexpression of *CDC55, PPH21,* and *PPH22* were lethal not only to *cdc5-1*, but also to *tem1-3* and *mob1-77* (Figure 1A and unpublished data). As overexpression of PP2A components is toxic to the Ts mutants that have defects in mitotic exit, it is likely that PP2A plays a negative role in mitotic exit. In our assays, overexpression of *PPH3* was toxic to *cdc5-1* mutants, but not to *tem1-3* and *mob1-77* (unpublished data). We also noticed that high dosages of *CDC55, PPH21, PPH22*, but not *PPH3* resulted in slow growth of wild-type cells (Figure 1A).

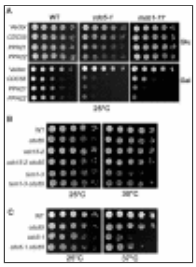


Figure 1.

(A) Overexpression of PP2A components is lethal to mutants defective in mitotic exit. Saturated cell cultures were 10-time serial diluted and then spotted onto plates containing either glucose (Glu) or galactose (Gal). The plates were incubated at 25°C **...**

If PP2A negatively regulates mitotic exit, inactivation of PP2A might suppress the defects in MEN temperature-sensitive mutants. To test this possibility, we generated *tem1-3* Δ*cdc55, cdc15-2* Δ*cdc55,* and *cdc5-1* Δ*cdc55* double mutants and examined their growth at different temperatures. We found that *tem1-3* Δ*cdc55* and *cdc5-1* Δ*cdc55* double mutants were less temperature sensitive than the corresponding single mutants (Figure 1, B and C). But *cdc15-2* and *cdc15-2* Δ*cdc55* mutants exhibited similar growth when incubated at various temperatures. To further confirm the suppression of Ts phenotype of *tem1-3* mutants by Δ*cdc55* deletion, we examined the plating efficiency of *tem1-3* and *tem1-3* Δ*cdc55* mutants. The saturated cultures of the two strains were spread onto YPD plates and incubated at 33°C for 14 h. We found that 98% of the *tem1-3* single mutants were arrested as large budded cells, whereas 48% of *tem1-3* Δ*cdc55,* mutants formed minicolonies (>4 cells). Because high dosages of PP2A regulatory and catalytic subunits are lethal in Ts mutants that are defective in mitotic exit and deletion of *CDC55* partially suppresses the temperature sensitivity of some MEN Ts mutants, we conclude that PP2A plays a negative role in mitotic exit.

Δcdc55 Mutants Exhibit Premature Mitotic Exit Phenotype

Bfa1 and Bub2 are required for preventing mitotic exit, and Δ*bfa1* or Δ*bub2* mutants rebud in the presence of spindle disruption (Hoyt *et al.*, 1991; Li, 1999). If Cdc55 plays a negative role in mitotic exit, we expect that Δ*cdc55* mutants will exhibit a similar phenotype. Thus, we examined the cell cycle progression of Δ*cdc55* mutants in the presence of nocodazole, a microtubule-depolymerizing drug that disrupts the spindle structure. G1-arrested wild-type and Δ*cdc55* mutant cells were released into 30°C YPD medium containing 20 μg/ml nocodazole. In wild-type cells, disruption of the spindle structure activates the spindle checkpoint and arrests cells at metaphase. After incubation for 3 h, however, Δ*cdc55* mutant cells began to rebud. After 4-h incubation in the presence of nocodazole, ~30% of Δ*cdc55* mutant cells exhibited extrabuds while wild-type cells were still arrested as large budded cells, indicating that Δ*cdc55* mutant cells might exit mitosis (Figure 2A).

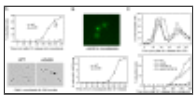


Figure 2.

(A) Δ*cdc55* mutant cells exit mitosis in the presence of nocodazole. (Y300) and Δ*cdc55* mutant (YYW28) cells in midlog phase were a phase with α-factor and then released into 30°C YPD medium cont

We also generated Δ*cdc55* mutants with GFP marked chromosome V to examine sister chromatid separation and chromosome reduplication (Michaelis *et al.*, 1997). As reported previously, a significant portion of Δ*cdc55* mutant cells showed separated chromatids in the presence of nocodazole. After 4-h incubation, we noticed that ~10% Δ*cdc55* mutant cells contained more than two GFP dots, indicating that chromosomes were reduplicated (Figure 2B). The results suggest that Δ*cdc55* mutant cells are able to exit mitosis and finish the second round of DNA replication in the presence of nocodazole.

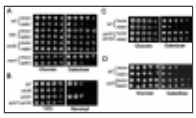
The Premature Mitotic Exit Phenotype in Δcdc55 Mutants Is Independent of Swe1

Δ*cdc55* mutant cells exhibit increased Cdc28 phosphorylation at tyrosine 19 (Minshull *et al.*, 1996). Moreover, *CDC28F19* mutant that lacks inhibitory phosphorylation site on Cdc28 suppress the cold sensitivity and premature sister separation in Δ*cdc55* mutants (Minshull *et al.*, 1996; Wang and Burke, 1997; Yang *et al.*, 2000). Therefore, we tested if the hyperphosphorylation of Cdc28 protein in Δ*cdc55* mutants also contributes to its premature mitotic exit phenotype. For this purpose, we generated Δ*cdc55 CDC28F19* double mutants. As reported earlier, the double mutants did not display abnormal bud morphology. To determine if the double mutants exit mitosis, G1-synchronized wild-type, *CDC28F19*, Δ*cdc55,* and Δ*cdc55 CDC28F19* cells were released into YPD medium containing 20 μg/ml nocodazole and the budding indexes were determined. Interestingly, similar to Δ*cdc55* single mutants, Δ*cdc55 CDC28F19* double mutants also began rebud after incubation in the presence of nocodazole for 3 h (Figure 2C, bottom). The examination of unperturbed cell cycle progression in these mutants did not show any dramatic discrepancy except that the Δ*cdc55* mutant exhibited slower cell cycle progression (Figure 2C, top). We also found that Δ*swe1* Δ*cdc55* double mutants were able to rebud in the presence of nocodazole (unpublished data). This results argue against the hypothesis that increased phosphorylation of Cdc28 by Swe1 contributes to the premature mitotic exit in Δ*cdc55* mutants.

Δcdc55 Mutant Suppresses AMN1 Overexpression Phenotype

Overexpression of *AMN1* gene slows down cell cycle progression because of its inhibition of mitotic exit (Wang *et al.*, 2003). We predicted that *AMN1* overexpression phenotype would be alleviated in mutants with hyperactive MEN. To test this, a vector and a P_{GAL}-*AMN1* plasmid were transformed into wild-type, Δ*bfa1,* Δ*bub2,* Δ*mad1,* and Δ*cdc55* mutants. As expected, cells with P_{GAL}-*AMN1* plasmid grew slowly on the plates with galactose because of the slower mitotic exit. The *AMN1* overexpression phenotype was suppressed by Δ*bfa1* or Δ*bub2* deletion, consistent with the negative role of Bfa1/Bub2 complex in mitotic exit regulation (Figure 3A). Similarly, we found that the Δ*cdc55* mutant also suppressed the *AMN1* overexpression phenotype (Figure 3A). However, deletion of *MAD1*, a spindle checkpoint gene that acts in a different branch from Bfa1/Bub2, could not suppress the *AMN1* overexpression phenotype. In response to spindle damage, Mad1, together with other spindle checkpoint components, prevents the activation of APC^{Cdc20} (Hwang *et al.*, 1998), whereas Bfa1/Bub2 complex inhibits mitotic exit by keeping Tem1 from activation (Alexandru *et al.*, 1999). Because Δ*bfa1,* Δ*bub2,* and Δ*cdc55* mutants are all able to suppress the *AMN1* overexpression phenotype, it is likely that mitotic exit pathways are up-regulated in Δ*cdc55* mutants.

Figure 3.



(A) Δ*cdc55* mutant suppresses *AMN1* overexpression phenotype. S YFH240, YYW28, and YYW81) were transformed with either a *C* or a P_{GAL}-*AMN1* plasmid. Saturated cultures of the transformants v serial diluted and **...**

Loss of Function of PP2A Leads to Mitotic Exit in Δcdc55 Mutants

Cdc55 may function as a negative regulator of PP2A, because the accumulation of Swe1 protein in Δ*cdc55* mutants is suppressed when *PPH21* and *PPH22* are deleted (Yang *et al.*, 2000). Therefore, it is not clear whether the loss or the gain of function of PP2A in Δ*cdc55* mutants leads to the premature mitotic exit. Because overexpression of either Cdc55 or catalytic subunits Pph21, Pph22 is toxic to mutants in MEN pathway, it is likely that both Cdc55 and the catalytic subunits of PP2A negatively regulates mitotic exit. If that is the case, mutations in PP2A catalytic subunits will result in a Δ*cdc55*-like phenotype. Previous results indicate that Δ*cdc55* is sensitive to microtubule disassembly drugs. We therefore examined the growth of Δ*pph21* single and Δ*pph21* Δ*pph22* double mutants on plates containing 15 μg/ml benomyl, a microtubule depolymerizing drug like nocodazole. Δ*pph21* single mutants exhibited benomyl sensitivity similar to that of wild-type cells, but Δ*pph21* Δ*pph22* double mutants failed to form colonies on benomyl plates (Figure 3B). Because both Δ*cdc55* and Δ*pph21* Δ*pph22* mutants are sensitive to microtubule-disassembling drugs, the inactive PP2A in Δ*cdc55* mutants might contribute to its sensitivity to benomyl.

We have shown that Δ*cdc55* mutants suppress the *AMN1* overexpression phenotype, presumably because of the hyperactive mitotic exit pathways. Δ*pph21* Δ*pph22* double mutants should alleviate *AMN1* overexpression phenotype as well if mitotic exit pathways are hyperactive in the double mutants. Therefore, a vector and a P_{GAL}-*AMN1* were introduced into Δ*pph21* Δ*pph22* double mutants and the growth of the transformants was examined on plates containing either glucose or galactose. Δ*pph21* Δ*pph22* mutants containing P_{GAL}-*AMN1* grew much better than wild-type cells (Figure 3C). Similarly, we examined if loss of the A regulatory subunit (Tpd3) of PP2A also exhibited hyperactive MEN activity, and we found that deletion of *TPD3* also suppressed *AMN1* overexpression phenotype (Figure 3D). Thus, we reason that the regulatory and catalytic subunits of PP2A negatively regulate mitotic exit and the premature mitotic exit phenotype in Δ*cdc55* mutants results from the loss of function of PP2A.

PP2A Regulates Cdc14 Localization

Phosphatase Cdc14 localizes in the nucleolus during most of the cell cycle (Shou *et al.*, 1999; Visintin *et al.*, 1999). After MEN activation, Cdc14 is released into the nucleus so that Cdc14 is able to dephosphorylate its substrates and promote the inactivation of CDK (Visintin *et al.*, 1998). Thus, the localization of Cdc14 has been used as a molecular marker for mitotic exit. We tested the possibility that PP2A inhibits mitotic exit through the regulation of Cdc14 localization. The localization of Cdc14 in wild-type and Δ*cdc55* mutant cells was examined in the presence of nocodazole. *CDC14-HA* and Δ*cdc55 CDC14-HA* strains in midlog phase were synchronized at G1 and then released into 30°C YPD medium containing 20 μg/ml nocodazole. The cells were harvested and subjected to immunofluorescence staining. As expected, the majority of wild-type cells exhibited nucleolar localized Cdc14, indicating that mitotic exit pathways were inactive. In contrast, Δ*cdc55* mutant cells showed nuclear localized Cdc14 beginning at 90 min after G1 release. After 120 min, almost all of the Δ*cdc55* mutant cells showed nuclear localized Cdc14 in the presence of nocodazole (Figure 4, B and C). However, in the absence of nocodazole, Δ*cdc55* mutant exhibited normal cell cycle-regulated Cdc14 localization, except that Δ*cdc55* mutants showed slower cell cycle progression (Figure 4A). The nuclear localized Cdc14 in Δ*cdc55* mutants in the presence of nocodazole could be a result of deformed nucleolar structure. To clear this issue, we examined the localization of Net1, a protein localized in the

nucleolus through the cell cycle. G1 synchronized *NET1-myc* and Δ*cdc55 NET1-myc* cells were released into YPD medium containing nocodazole. In contrast to Cdc14, both wild-type and Δ*cdc55* mutant cells exhibited nucleolar localized Net1 protein in the presence of nocodazole (Figure 4C, bottom), suggesting that the nuclear localized Cdc14 in Δ*cdc55* mutants is not a result of deformed nucleolus. Thus, Δ*cdc55* mutants fail to keep mitotic exit pathways inactive in the presence of nocodazole and the premature mitotic exit in Δ*cdc55* mutants is resulted from Cdc14 release from the nucleolus.

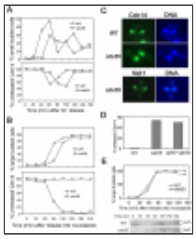


Figure 4.
Cdc14 is released from the nucleolus in Δ*cdc55* mutants in the presence of nocodazole. (A**)** A1411 (*CDC14-HA*) and 173-1-3 (Δ*cdc55 CDC14-HA*) cells were arrested at G1 phase with α-factor and then released into YPD medium. Cells were **...**

We also examined the localization of Cdc14 in Δ*pph21* Δ*pph22* double mutants in the presence of nocodazole. Asynchronized cells were incubated in YPD medium containing 20 μg/ml nocodazole for 3 h and Cdc14 localization was examined. Like Δ*cdc55* mutants, 50% of *pph21 pph22* double mutant cells exhibited nuclear localized Cdc14, whereas almost all the wild-type cells showed nucleolar localized Cdc14 (Figure 4D). These data support our conclusion that the B regulatory subunit Cdc55 and the catalytic subunits Pph21 and Pph22 act together to prevent the activation of mitotic exit pathways in the presence of nocodazole.

To determine the mitotic exit in molecular level, we analyzed the phosphorylation of Hof1 in wild-type and Δ*cdc55* mutants in the presence of nocodazole. Hof1 is a phosphoprotein required for cytokinesis. The phosphorylation of Hof1 depends on the functional MEN pathway as its phosphorylation is blocked in *dbf2-2, cdc14-1*, and *cdc15-2* mutants (Vallen *et al.*, 2000 ▸). Thus, the phosphorylation status of Hof1 protein could be used as a marker of MEN activation. *HOF1-HA* and Δ*cdc55 HOF-HA* strains were arrested at G1 and then released into YPD medium containing nocodazole. Δ*cdc55* mutants show more phosphorylated Hof1 protein than wild-type cells in the presence of nocodazole, supporting the notion that MEN pathway is hyperactive in Δ*cdc55* mutants (Figure 4E).

PP2A Controls Mitotic Exit Independent of Bfa1/Bub2

Our data indicate that PP2A plays a negative role in mitotic exit and Bfa1/Bub2 does so as well. We have demonstrated that protein kinase Cdc5 phosphorylates Bfa1 and promotes mitotic exit (Hu *et al.*, 2001 ▸). One reasonable model is that PP2A dephosphorylates Bfa1 and keeps Bfa1 active. If that is the case, mutations in PP2A will result in the hyperphosphorylation and inactivation of Bfa1. To test this model, we first constructed a Δ*cdc55 BFA1-HA* strain and the phosphorylation of Bfa1 protein was examined in synchronized wild-type and Δ*cdc55* mutant cells. As with wild-type cells, Δ*cdc55* mutants exhibited cell cycle-regulated Bfa1 phosphorylation, and increased hyperphosphorylated Bfa1 was not observed in Δ*cdc55* mutants (Figure 5A). We also examined the phosphorylation of Bfa1 in cells overexpressing *CDC55*. The results indicate that *CDC55* overexpression does not change the Bfa1 phosphorylation profiles (Figure 5A). Therefore, PP2A does not appear to inhibit mitotic exit through dephosphorylation of Bfa1.

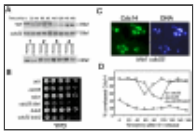


Figure 5.
CDC55 exhibits synthetic phenotype with *BFA1* and *BUB2*. (A) G1-arrested *BFA1-HA* (YFH286) and Δ*cdc55 BFA1-HA* (283-2-4) cells were released into 30°C YPD medium. Cells were taken every 20 min to prepare protein extracts. The phosphorylation **...**

If Bfa1 is not a substrate of PP2A, it is possible that PP2A regulates mitotic exit in a pathway independent of Bfa1/Bub2. If so, we would expect some synthetic phenotypes when both pathways are abolished. Thus, Δ*bfa1* Δ*cdc55* and Δ*bub2* Δ*cdc55* double mutants were constructed. Compared with the single mutants, the double mutants exhibited a poor growth phenotype (Figure 5B). Furthermore, in asynchronized cell cultures, ~20% of the double mutants showed extrabuds (rebudding) phenotype, indicating that the double mutant cells may exit mitosis prematurely. We then examined the localization of Cdc14 in Δ*bfa1* Δ*cdc55* double mutants and found that a significant portion of the double mutant cells exhibited nuclear localized Cdc14, and many double mutant cells had more than one nucleus (Figure 5C).

Δ*cdc55* mutant exhibits *SWE1*-dependent abnormal morphology. The synthetic phenotype between Δ*cdc55* and Δ*bfa1,* Δ*bub2* may come from the combination of abnormal morphology and the spindle checkpoint defects. To test this, we constructed Δ*cdc55* Δ*bub2* Δ*swe1* triple mutants. Even though Δ*swe1* suppressed the abnormal bud morphology, the synthetic slow growth phenotype of Δ*cdc55* Δ*bub2* could not be suppressed by the absence of *SWE1*. This observation indicates that *SWE1*-dependent abnormal morphology in Δ*cdc55* mutant does not contribute to the poor growth phenotype of Δ*cdc55* Δ*bfa1* and Δ*cdc55* Δ*bub2* double mutants, consistent with the notion that Swe1 accumulation in Δ*cdc55* mutants is not related to mitotic exit regulation. We also examined the cell cycle-regulated localization of Cdc14 in synchronized Δ*cdc55* Δ*bub2* Δ*swe1* triple mutants. Because the mutant cells are very sick, only some of the cells responded to α-factor treatment. Thus, only the cells with shmoo morphology were counted for this experiment. We failed to observe proper cell cycle-regulated Cdc14 localization in the triple mutants. Even in the G1-arrested cells, ~40% of the triple mutant cells exhibited nucleolar localized Cdc14, whereas almost all the Δ*cdc55* and Δ*bub2* single mutant cells exhibited nucleolar localized Cdc14 (Figure 5D). On the basis of these results, we conclude that the synthetic phenotype between *CDC55* and *BFA1/BUB2* is likely due to constitutively activated mitotic exit pathways. The results also suggest that PP2A and Bfa1/Bub2 control mitotic exit in an independent manner and the presence of either one of them is sufficient for a successful mitosis.

FEAR Pathway May Not Be Required for the Mitotic Exit in Δcdc55 Mutants

What is the target of PP2A that is related to mitotic exit? Both FEAR and MEN pathways control mitotic exit by regulating Cdc14 localization. The FEAR (Cdc fourteen early anaphase release) network promotes Cdc14 release from the nucleolus during early anaphase (Stegmeier *et al.*, 2002 ▸). We next addressed the possibility that PP2A regulates mitotic exit by inhibiting the FEAR pathway. If the premature mitotic exit phenotype in Δ*cdc55* mutants is a result of hyperactive FEAR, deletion of *SLK19,* which encodes one of the FEAR components, should suppress the Δ*cdc55* mutant phenotype. We generated Δ*cdc55* Δ*slk19* double mutants to examine their rebudding phenotype in the presence of nocodazole. It appeared that the rebudding phenotype of Δ*cdc55* in the presence of nocodazole was partially suppressed by the Δ*slk19* mutation (Figure 6B). After 5-h incubation in the presence of nocodazole, ~20% of Δ*cdc55* single mutant cells exhibited rebudding morphology. However, only 10% of Δ*cdc55* Δ*slk19* double mutant cells rebudded.

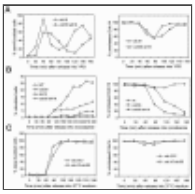


Figure 6.
Mitotic exit in Δ*cdc55* mutants depends on MEN pathway. (A) The localization of Cdc14 in Δ*slk19* and Δ*cdc55* Δ*slk19* mutants during unperturbed cell cycle progression. G1-arrested Δ464-3-2 and 450-3-1 were released **...**

Because the budding index could not give us a clearcut answer, we further analyzed mitotic exit in Δ*cdc55*Δ *slk19* mutants by examining the localization of Cdc14. G1-arrested wild-type, Δ*cdc55,* and Δ*cdc55* Δ*slk19* mutants cells with HA-tagged *CDC14* were released into YPD medium either with or

without 20 μg/ml nocodazole. In the absence of nocodazole, Δ*slk19* and Δ*slk19* Δ*cdc55* mutants exhibited cell cycle-regulated Cdc14 localization (Figure 6A). In the presence of nocodazole, Cdc14 localized in the nucleus in wild-type cells, whereas Δ*cdc55* mutants exhibited nuclear-localized Cdc14. Δ*cdc55* Δ*slk19* double mutants also showed nuclear localized Cdc14, but with delayed kinetics (Figure 6B, right). It appeared that defects in FEAR pathway delayed the mitotic exit process in Δ*cdc55* mutants. One explanation is that PP2A inhibits mitotic exit partially through its inhibition of FEAR pathway. Alternately, the premature release of Cdc14 in Δ*cdc55* mutants has nothing to do with FEAR pathway, but the defects of this pathway slow down the mitotic exit process in Δ*cdc55* mutants. Thus, PP2A must regulate mitotic exit pathway other than FEAR.

PP2A and MEN Pathway

We then tested if MEN was a target of PP2A. If the hyperactive MEN pathway contributes to mitotic exit in Δ*cdc55* mutants, inactivation of MEN components should block the mitotic exit in Δ*cdc55* mutations. Cdc15 is a component of MEN and *cdc15-2* mutants arrest at telophase when incubated at the restrictive temperature (Visintin and Amon, 2001 ▸). Thus, we examined the cell cycle progression of *cdc15-2* single and *cdc15-2* Δ*cdc55* double mutants to see if the defective MEN could block the mitotic exit in Δ*cdc55* mutants. G1-arrested cells were released into YPD medium at 37°C and the budding indexes were determined. Both *cdc15-2* single and *cdc15-2* Δ*cdc55* double mutants arrested at large budded cells (Figure 6C). Moreover, Cdc14 localized in the nucleolus in both *cdc15-2* single and *cdc15-2* Δ*cdc55* double mutants when incubated at the restrictive temperature (Figure 6C), indicating that mitotic exit in Δ*cdc55* mutants depends on MEN function. Therefore, we reason that PP2A might negatively regulate the MEN pathway.

PP2A May Regulate Tem1 Protein Phosphorylation

To answer if the mitotic exit in Δ*cdc55* mutants depends on MEN functions, the cell cycle progression of *tem1-3* and *tem1-3* Δ*cdc55* was also examined at 37°C. Unlike *cdc15-2*, the mitotic exit defects in *tem1-3* mutants were partially suppressed by deletion of *CDC55*. After 4-h incubation, >30% of *tem1-3* Δ*cdc55* double mutants exited mitosis, as indicated by the appearance of extrabuds; however, all the *cdc15-2* Δ*cdc55* double mutants were arrested as large budded cells (Figure 7A). This result is consistent with our observation that Δ*cdc55* mutation partially suppresses the temperature sensitivity of *tem1-3* mutants when incubated at 30°C (Figure 1B). Because Δ*cdc55* suppresses the mitotic exit defects in *tem1-3* mutants, PP2A might function as a negative regulator of Tem1.

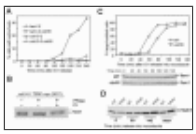


Figure 7.
PP2A regulates mitotic exit through Tem1 modification. (A) Δ*cdc55* deletion partially suppresses the mitotic exit defects of *tem1-3* mutants. G1-arrested *tem1-3, tem1-3* Δ*cdc55, cdc15-2,* and *cdc15-2* Δ*cdc55* mutants were released into **...**

It has been shown that Tem1 exhibits cell cycle-regulated modification, but the nature of this modification remains unclear. Therefore, we first examined if phosphorylation contributes to the band shift of Tem1 protein. Protein samples were prepared with *cdc14-1 TEM1-myc* strain incubated at 36°C for 2 h, as Tem1 exhibited more slow-migrating forms in *cdc14-1*-arrested cells. After immunoprecipitation with anti-myc antibody, Tem1 protein was subjected to λ protein phosphatase treatment in the presence or absence of phosphatase inhibitors. We found that the majority of the slow-migrating forms of Tem1 disappeared after λ phosphatase treatment (Figure 7B), indicating that Tem1 is a phosphoprotein.

Then we asked if the Tem1 protein phosphorylation is regulated by PP2A. We examined the phosphorylation status of Tem1 in wild-type and Δ*cdc55* mutant cells, and it was very clear that Δ*cdc55* mutants exhibited more modified Tem1 protein in asynchronized cells (Figure 7D). The phosphorylation of Tem1 was also examined in synchronized *TEM1–13myc* and Δ*cdc55 TEM1–13myc* cells. G1-arrested cells were released into YPD medium containing 20 μg/ml nocodazole at 30°C. In G1-arrested cells, we observed more phosphorylated Tem1 in Δ*cdc55* mutants. As cells entered S-phase, there were fewer phosphorylated Tem1 proteins. We noticed the appearance of a slow-migrating Tem1 band in Δ*cdc55* mutants at 100 min; however, the phosphorylated Tem1 did not appear until 140 min after G1 release in wild-type cells (Figure 7C). When we ran the protein samples from wild-type and Δ*cdc55* mutant cells side by side, it was clear that Δ*cdc55* mutants exhibited more phosphorylated Tem1 protein under various conditions (Figure 7D), indicating that loss of function of PP2A enhances Tem1 phosphorylation. Given the fact that Cdc55 is a component of phosphatase, a reasonable model is that PP2A dephosphorylates Tem1 and inhibits its functions in mitotic exit.

DISCUSSION

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Previous data indicate that budding yeast Cdc55 regulates bud morphology and sister chromatid separation. Here we report that PP2A also plays a negative role in mitotic exit and several pieces of evidence support this conclusion. First, overexpression of some of the PP2A components is toxic to mutants in which the MEN pathway is compromised. Second, Δ*cdc55* mutants exhibit extrabuds and nuclear localized Cdc14 in the presence of nocodazole. Moreover, the Δ*cdc55* deletion mutant could suppress the toxicity resulting from the overproduction of Amn1, which acts as a negative regulator of MEN. Finally, Hof1, a protein required for cytokinesis and its phosphorylation, depends on activated MEN pathway and exhibits more phosphorylated forms in Δ*cdc55* mutants in the presence of nocodazole. These results are consistent with our earlier observation that Δ*cdc55* mutants suppress the temperature sensitivity of *cdc20-1* mutants (Wang and Burke, 1997). *HCT1*/*CDH1* was identified as a high copy suppressor of *cdc20–1* mutants (Schwab *et al.*, 1997). We reason that the hyperactive MEN pathway in Δ*cdc55* mutants activates APC^{Cdh1}, which overcomes the requirement of Cdc20.

It has been shown that Swe1 protein accumulates in Δ*cdc55* mutants and either Δ*swe1* deletion or *CDC28F19* mutation can suppress the abnormal bud morphology in Δ*cdc55* mutants (Wang and Burke, 1997; Yang *et al.*, 2000). However, neither Δ*swe1* deletion nor *CDC28F19* mutation could suppress the mitotic exit in Δ*cdc55* mutants in the presence of nocodazole. Other observations also argue against the role of Swe1 in mitotic exit. We noticed that Δ*cdc55* Δ*swe1* double mutant cells are capable of reduplicating their chromosomes in the presence of nocodazole (Wang, personal observation). Moreover, the sickness of Δ*cdc55* Δ*bub2* double mutants could not be rescued by Δ*swe1* deletion. Also, Δ*pph21* Δ*pph22* double mutants are capable of exiting mitosis without accumulating Swe1. Apparently, the premature mitotic exit phenotype in Δ*cdc55* mutants has nothing to do with Swe1-dependent Cdc28 phosphorylation. It is likely that PP2A has many substrates and the defects in the phosphorylation of different substrates contribute to the complex phenotype of Δ*cdc55* mutants.

Swe1 protein accumulates in Δ*cdc55* mutants and deletion of *PPH21* and *PPH22* suppress Swe1 accumulation, suggesting that Cdc55 deregulates PP2A activity. But that is not the case for Cdc55 in the regulation of mitotic exit. First, Δ*pph21*Δ *pph22* double mutants exhibit benomyl sensitivity, as do Δ*cdc55* mutants. Moreover, either Δ*cdc55* or Δ*pph21*Δ *pphh22* mutants suppress the *AMN1* overexpression phenotype and both mutants exhibit nuclear localized Cdc14 in the presence of nocodazole, suggesting that mitotic exit pathways are hyperactive in both Δ*cdc55* and Δ*pph21* Δ*pph22* mutants. Consistently, deletion of *TPD3*, the A regulatory subunit of PP2A, also leads to less sensitivity to *AMN1* overexpression. All these results indicate that both regulatory and catalytic subunits of PP2A are required for the negative regulation of mitotic exit pathways. It is the loss of function of PP2A in Δ*cdc55* mutants that causes premature mitotic exit.

Bfa1 and Bub2 also negatively regulate mitotic exit by forming a complex with Tem1, a key player in the MEN pathway (Pereira *et al.*, 2000). Because Bfa1 is a phosphoprotein and its phosphorylation promotes mitotic exit (Hu *et al.*, 2001), a reasonable model is that PP2A dephosphorylates Bfa1 to inhibit mitotic exit. However, our results argue against this model. The synthetic phenotype of Δ*cdc55* Δ*bfa1* and Δ*cdc55* Δ*bub2* double mutants indicates that PP2A and Bfa1/Bub2 might act in concert and have additive effects on mitotic exit. Inactivation of both pathways results in deregulated Cdc14 localization and premature mitotic exit, which leads to the aberrant mitosis and sickness of the double mutants.

Both FEAR and MEN pathways promote mitotic exit by stimulating Cdc14 release from the nucleolus. Our result indicates that defective FEAR pathway fails to block the mitotic exit completely in Δ*cdc55* mutants, because Δ*cdc55* Δ*slk19* double mutants are still able to rebud and release Cdc14 from the nucleolus in the presence of nocodazole. It is unlikely that PP2A negatively regulates mitotic exit by inhibiting FEAR pathway. MEN could be the target of PP2A, based on the following observations. We have shown that *AMN1* inhibits mitotic exit by binding to Tem1. Deletion of either *CDC55* or *PPH21 PPH22* suppresses *AMN1* overexpression phenotype. Deletion of *CDC55* also partially suppresses the temperature sensitivity of *cdc5-1* and *tem1-3,* indicating that MEN is hyperactive in Δ*cdc55*. However, *cdc15-2* mutation completely suppresses the mitotic exit in Δ*cdc55* mutants when incubated at the restrictive temperature, suggesting that MEN is indispensable for the mitotic exit in Δ*cdc55* mutants. Importantly, Δ*cdc55* mutants show increased Tem1 protein phosphorylation. Therefore, the hyperactive MEN in Δ*cdc55* mutants may result from the change of Tem1 protein modification. It is possible that a protein kinase phosphorylates Tem1 and activates MEN pathway, whereas PP2A dephosphorylates Tem1 and keeps it inactive. Defective PP2A will result in the increase of hyperphosphorylated Tem1, which promotes mitotic exit. In summary, we have identified a new layer of regulation of MEN, involving PP2A. In collaboration with Bfa1/Bub2 and Amn1, PP2A ensures cell cycle-regulated localization of Cdc14, which is essential for a successful mitosis.

ACKNOWLEDGMENTS

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We thank Drs. Stephen Elledge, Angelica Amon, Jeff Bahant, Kyung Lee, Raymond Deshaies, Erfei Bi, James Broach, and John Pringle for plasmids and strains. We thank Dr. Daniel Burke for sharing the unpublished data. We thank Yanping Sun for preparing the media and buffers for the experiments. We also thank Dr. Stephen Elledge for his comments and Dr. Lloyd Epstein for thorough reading of this manuscript. This work was support by American Heart Association Scientist Development Grant 0435276B to Y.W. Y.W. was also supported by The Leukemia and Lymphoma Society Special Fellowship.

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This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–12–1109) on August 3, 2005.

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