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 CDC35, PP2H21, or PP2H22 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.
Table 1. Strains used in this study

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of the inhibitory phosphorylation of Cdc28. The premature sister chromatid separation and the cold sensitivity phenotypes in Δcdc55 mutants are suppressed by the CDC28F19 mutation, in which Cdc28 is resistant to the phosphorylation by Swe1 (Minschult et al., 1996; Wang and Burke, 1997). Consistently, Swe1 protein level is increased in Δcdc55 mutants because of the compromised Swe1 protein degradation (Yang et al., 2000). Thus, the accumulation of Swe1 in Δcdc55 mutants results in Cdc28 phosphorylation, which may contribute to the known phenotypes of Δcdc55 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. Δcdc55 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 Δcdc55 Δbfa1 and Δcdc55 Δbub2 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

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 (OD600 = 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 H2O. Nocodazole was purchased from ICN (Costa Mesa, CA) and was used at 20 μg/ml in a final concentration of 1% dimethyl sulfoxide.

Cytological Techniques

Immunofluorescence staining 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/aceton 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
RESULTS

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 PGAL-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 ACP^3hi substrate and its overproduction will deteriorate the growth of cdc5-1 mutants in which the function of ACP^3hi 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; Lu 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).

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 Δcdc55, cdc15-2 Δcdc55, and cdc5-1 Δcdc55 double mutants and examined their growth at different temperatures. We found that tem1-3 Δcdc55 and cdc5-1 Δcdc55 double mutants were less temperature sensitive than the corresponding single mutants (Figure 1, B and C). But cdc15-2 and cdc15-2 Δcdc55 mutants exhibited similar growth when incubated at various temperatures. To further confirm the suppression of Ts phenotype of tem1-3 mutants by Δcdc55 deletion, we examined the plating efficiency of tem1-3 and tem1-3 Δcdc55 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 Δcdc55 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 Δbfa1 or Δbub2 mutants rebind in the presence of spindle disruption (Hoyt et al., 1991; Li, 1999). If Cdc55 plays a...
negative role in mitotic exit, we expect that Δcdc55 mutants will exhibit a similar phenotype. Thus, we examined the cell cycle progression of Δcdc55 mutants in the presence of nocodazole, a microtubule-depolymerizing drug that disrupts the spindle structure. G1-arrested wild-type and Δcdc55 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, Δcdc55 mutant cells began to rebud. After 4-h incubation in the presence of nocodazole, ~30% of Δcdc55 mutant cells exhibited extrabuds while wild-type cells were still arrested as large budded cells, indicating that Δcdc55 mutant cells might exit mitosis (Figure 2A).

We also generated Δcdc55 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 Δcdc55 mutant cells showed separated chromatids in the presence of nocodazole. After 4-h incubation, we noticed that ~10% Δcdc55 mutant cells contained more than two GFP dots, indicating that chromosomes were reduplicated (Figure 2B). The results suggest that Δcdc55 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

Δcdc55 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 Δcdc55 mutants (Minshull et al., 1996; Wang and Burke, 1997; Yang et al., 2000). Therefore, we tested if the hyperphosphorylation of Cdc28 protein in Δcdc55 mutants also contributes to its premature mitotic exit phenotype. For this purpose, we generated Δcdc55 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, Δcdc55, and Δcdc55 CDC28F19 cells were released into YPD medium containing 20 μg/ml nocodazole and the budding indexes were determined. Interestingly, similar to Δcdc55 single mutants, Δcdc55 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 Δcdc55 mutant exhibited slower cell cycle progression (Figure 2C, top). We also found that Δswe1 Δcdc55 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 Δcdc55 mutants.

Δcdc55 Mutant Suppresses AMNI Overexpression Phenotype

Overexpression of AMNI gene slows down cell cycle progression because of its inhibition of mitotic exit (Wang et al., 2003). We predicted that AMNI overexpression phenotype would be alleviated in mutants with hyperactive MEN. To test this, a vector and a pGAL-AMNI plasmid were trans-
formed into wild-type, Δbfa1, Δbub2, Δmad1, and Δcdc55 mutants. As expected, cells with P\textsubscript{GAL}-AMN1 plasmid grew slowly on the plates with galactose because of the slower mitotic exit. The AMN1 overexpression phenotype was suppressed by Δbfa1 or Δbub2 deletion, consistent with the negative role of Bfa1/Bub2 complex in mitotic exit regulation (Figure 3A). Similarly, we found that the Δcdc55 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\textsubscript{Cdc20} (Hwang \textit{et al.}, 1998), whereas Bfa1/Bub2 complex inhibits mitotic exit by keeping Tem1 from activation (Alexandru \textit{et al.}, 1999). Because Δbfa1, Δbub2, and Δcdc55 mutants are all able to suppress the AMN1 overexpression phenotype, it is likely that mitotic exit pathways are up-regulated in Δcdc55 mutants.

### 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 Sw1 protein in Δcdc55 mutants is suppressed when PPH21 and PPH22 are deleted (Yang \textit{et al.}, 2000). Therefore, it is not clear whether the loss or the gain of function of PP2A in Δcdc55 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 Δcdc55-like phenotype. Previous results indicate that Δcdc55 is sensitive to microtubule disassembly drugs. We therefore examined the growth of Δpph21 single and Δpph21 Δpph22 double mutants on plates containing 15 μg/ml benomyl, a microtubule depolymerizing drug like nocodazole. Δpph21 single mutants exhibited benomyl sensitivity similar to that of wild-type cells, but Δpph21 Δpph22 double mutants failed to form colonies on benomyl plates (Figure 3B). Because both Δcdc55 and Δpph21 Δpph22 mutants are sensitive to microtubule-disassembling drugs, the inactive PP2A in Δcdc55 mutants might contribute to its sensitivity to benomyl.

We have shown that Δcdc55 mutants suppress the AMN1 overexpression phenotype, presumably because of the hyperactive mitotic exit pathways. Δpph21 Δpph22 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\textsubscript{GAL}-AMN1 were introduced into Δpph21 Δpph22 double mutants and the growth of the transformants was examined on plates containing either glucose or galactose. Δpph21 Δpph22 mutants containing P\textsubscript{GAL}-AMN1 grew much better than wild-type cells (Figure 3C). Similarly, we examined if loss of the A regulatory subunit (Tp3) 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 Δcdc55 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 \textit{et al.}, 1999; Visintin \textit{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 \textit{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 Δcdc55 mutant cells was examined in the presence of nocodazole. CDC14-HA and Δcdc55 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, Δcdc55 mutant cells showed nuclear localized Cdc14 beginning at 90 min after G1 release. After 120 min, almost all of the Δcdc55 mutant cells showed nuclear localized Cdc14 in the presence of nocodazole (Figure 4, B and C). However, in the absence of nocodazole, Δcdc55 mutant exhibited normal cell cycle-regulated Cdc14 localization, except that Δcdc55 mutants showed slower cell cycle progression (Figure 4A). The nuclear localized Cdc14 in Δcdc55 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

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**Figure 3.** (A) Δcdc55 mutant suppresses AMN1 overexpression phenotype. Strains (Y300, YFH240, YYW28, and YYW81) were transformed with either a CEN-URA3 vector or a P\textsubscript{GAL}-AMN1 plasmid. Saturated cultures of the transformants were 10-fold serially diluted and spotted onto plates containing glucose or galactose and incubated at 30°C for 2 d. (B) Mutations in catalytic subunits of PP2A result in benomyl sensitivity. Saturated cultures of strains with indicated genotype (Y300, YYW28, Y2475, and Y2762) were 10-fold serially diluted and spotted onto YPD and benomyl (15 μg/ml) plates and incubated at 25°C for 3 d. (C) Δpph21 Δpph22 mutants suppress AMN1 overexpression phenotype. Y300 and Y2762 were transformed with either a CEN-URA3 vector or a P\textsubscript{GAL}-AMN1 plasmid. The transformants were treated as described in Figure 3A. (D) Δtpd3 mutants suppress AMN1 overexpression phenotype, 483-15-1 (tpd3::KAN) was transformed with either a vector or a P\textsubscript{GAL}-AMN1 plasmid and the growth of the transformants was examined as described.
the nucleolus through the cell cycle. G1 synchronized NET1-myc and Δcdc55 NET1-myc cells were released into YPD medium containing nocodazole. In contrast to Cdc14, both wild-type and Δcdc55 mutant cells exhibited nucleolar localized Net1 protein in the presence of nocodazole (Figure 4C, bottom), suggesting that the nuclear localized Cdc14 in Δcdc55 mutants is not a result of deformed nucleolus. Thus, Δcdc55 mutants fail to keep mitotic exit pathways inactive in the presence of nocodazole and the premature mitotic exit in Δcdc55 mutants is resulted from Cdc14 release from the nucleolus.

We also examined the localization of Cdc14 in Δpph21 Δpph22 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 Δcdc55 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 Δcdc55 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. HOFI-HA and Δcdc55 HOFI-HA strains were arrested at G1 and then released into YPD medium containing nocodazole. Δcdc55 mutants show more phosphorylated Hof1 protein than wild-type cells in the presence of nocodazole, supporting the notion that MEN pathway is hyperactive in Δcdc55 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 Δcdc55 BFA1-HA strain and the phosphorylation of Bfa1 protein was examined in synchronized wild-type and Δcdc55 mutant cells. As with wild-type cells, Δcdc55 mutants exhibited cell cycle-regulated Bfa1 phosphorylation, and increased hyperphosphorylated Bfa1 was not observed in Δcdc55 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.

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, Δbfa1 Δcdc55 and Δbub2 Δcdc55 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 (re budding) phenotype, indicating that the double mutant cells may exit mitosis prematurely. We then examined the localization of Cdc14 in Δbfa1 Δcdc55 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). Δcdc55 mutant exhibits SWE1-dependent abnormal morphology. The synthetic phenotype between Δcdc55 and Δbfa1, Δbub2 may come from the combination of abnormal morphology and the spindle checkpoint defects. To test this, we constructed Δcdc55 Δbub2 Δswe1 triple mutants. Even though Δswe1 suppressed the abnormal bud morphology, the synthetic slow growth phenotype of Δcdc55 Δbub2 could not be suppressed by the absence of SWE1. This observation...
indicates that SWE1-dependent abnormal morphology in Δcdc55 mutant does not contribute to the poor growth phenotype of Δcdc55 Δbfa1 and Δcdc55 Δbub2 double mutants, consistent with the notion that Swe1 accumulation in Δcdc55 mutants is not related to mitotic exit regulation. We also examined the cell cycle-regulated localization of Cdc14 in synchronized Δcdc55 Δbfa1 and Δcdc55 Δbub2 double mutants display slow-growth phenotype. Cells with indicated genotype were incubated at 30°C for 2 d. (C) The localization of Cdc14 in asynchronous Δcdc55 Δbfa1 (443-5-1) double mutants. (D) Cell cycle-regulated Cdc14 localization in Δcdc55 (173-1-3), Δbub2 (221-1-1) and Δcdc55 Δbub2 (482-7-4) mutants. Cells were arrested at G1 phase with α-factor and then released into YPD medium at 30°C. Hydroxyurea, 200 μM, was added into the medium at 80 min when majority of the cells were large budded in order to block the release of Cdc14 from the nucleolus during the next cell cycle.

**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 (Cdc14 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 Δcdc55 mutants is a result of hyper-active FEAR, deletion of SLK19, which encodes one of the FEAR components, should suppress the Δcdc55 mutant phenotype. We generated Δcdc55 Δslk19 double mutants to examine their rebudding phenotype in the presence of nocodazole. It appeared that the rebudding phenotype of Δcdc55 in the presence of nocodazole was partially suppressed by the Δslk19 mutation (Figure 6B). After 5-h incubation in the presence of nocodazole, ~20% of Δcdc55 single mutant cells exhibited rebudding morphology. However, only 10% of Δcdc55 Δslk19 double mutant cells rebudded.

Because the budding index could not give us a clear-cut answer, we further analyzed mitotic exit in Δcdc55 Δslk19 mutants by examining the localization of Cdc14. G1-arrested wild-type, Δcdc55, and Δcdc55 Δslk19 mutants cells with HA-tagged CDC14 were released into YPD medium either with or without 20 μg/ml nocodazole. In the absence of nocodazole, Δslk19 and Δslk19 Δcdc55 mutants exhibited cell cycle-regulated Cdc14 localization (Figure 6A). In the presence of nocodazole, Cdc14 localized in the nucleus in wild-

![Figure 5](image)

**Figure 5.** CDC55 exhibits synthetic phenotype with BFA1 and BUB2. (A) G1-arrested BFA1-HA (YFH286) and Δcdc55 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 status of Bfa1 were determined by Western blot analysis with anti-HA antibodies. In the bottom panel, cdc14-1 BFA1-HA cells with a vector or a P_{GAL}::CDC55 plasmid were grown to midlog phase in the raffinose medium at 25°C followed by the following treatments: 1) to add galactose and incubate for 1 h at 25°C; 2) to add galactose and incubate for 1 h at 25°C and then shift to 34°C for 3 h; 3) to shift to 34°C for 3 h; and 4) to shift to 34°C for 3 h and then add galactose and incubate at 34°C for 1 h. The phosphorylation of Bfa1 protein is shown after Western blotting with anti-HA antibody. (B) Left, the budding morphology of cells with indicated genotypes was examined the cell cycle-regulated localization of Cdc14. G1-arrested Δcdc55-2 d. (C) The localization of Cdc14 in asynchronized Δcdc55 Δbfa1 (443-5-1) double mutants. (D) Cell cycle-regulated Cdc14 localization in Δcdc55 (173-1-3), Δbub2 (221-1-1) and Δcdc55 Δbub2 (482-7-4) mutants. Cells were arrested at G1 phase with α-factor and then released into YPD medium at 30°C. Hydroxyurea, 200 μM, was added into the medium at 80 min when majority of the cells were large budded in order to block the release of Cdc14 from the nucleolus during the next cell cycle.

![Figure 6](image)

**Figure 6.** Mitotic exit in Δcdc55 mutants depends on MEN pathway. (A) The localization of Cdc14 in Δslk19 and Δcdc55 Δslk19 mutants during unperturbed cell cycle progression. G1-arrested Δcdc55 Δslk19 and Δslk19 mutants were released into cell cycle at 30°C and 200 mM hydroxyurea was added into the medium at 80 min to block the next S phase. Cells were collected at 30 min interval for Cdc14 staining. (B) Left, the budding morphology of cells with indicated genotypes. Strains with indicated genotypes were arrested at G1 and released into YPD medium containing 20 μg/ml nocodazole at 30°C for the determination of budding index. Right, the localization of Cdc14 in the presence of nocodazole. Strains with indicated genotype were released into nocodazole medium after G1 arrest. Cells were collected every 30 min and subjected to immunofluorescence staining for Cdc14-HA. The percentage of cells with nucleolar localized Cdc14 in the presence of nocodazole. Strains with indicated genotype were released into nocodazole medium after G1 arrest. Cells were collected every 30 min and subjected to immunofluorescence staining for Cdc14-HA. The percentage of cells with nucleolar localized Cdc14 in the presence of nocodazole. Strains with indicated genotype were released into nocodazole medium after G1 arrest. Cells were collected every 30 min and subjected to immunofluorescence staining for Cdc14-HA. The percentage of cells with nucleolar localization of Cdc14 in the presence of nocodazole.
PP2A May Regulate Tem1 Protein Phosphorylation

To answer if the mitotic exit in Δcdc55 mutants depends on MEN functions, the cell cycle progression of tem1-3 and tem1-3 Δcdc55 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 Δcdc55 double mutants exited mitosis, as indicated by the appearance of extrabuds; however, all the cdc15-2 Δcdc55 double mutants were arrested as large budded cells (Figure 7A). This result is consistent with our observation that Δcdc55 mutation partially suppresses the temperature sensitivity of tem1-3 mutants when incubated at 30°C (Figure 1B). Because Δcdc55 suppresses the mitotic exit defects in tem1-3 mutants, PP2A might function as a negative regulator of Tem1.

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 phosphoprotein. Tem1-myc fusion protein was immunoprecipitated from the cell lysate of cdc14-1 TEM1-myc incubated at 36°C for 2 h. The precipitates were incubated with λ phosphatase at 30°C for 30 min in the presence or absence of phosphatase inhibitors. Proteins were separated with 10% SDS-PAGE and then subjected to Western blot analysis. Top, the percentage of large budded cells; bottom, the modification of Tem1 protein. (D) Protein samples from 0-, 80-, and 140-min time points in C were loaded side by side and the band shift of Tem1 protein is shown after Western blot analysis.

PP2A and MEN Pathway

We then tested if MEN was a target of PP2A. If the hyperactive MEN pathway contributes to mitotic exit in Δcdc55 mutants, inactivation of MEN components should block the mitotic exit in Δcdc55 mutants. 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 Δcdc55 double mutants to see if the defective MEN could block the mitotic exit in Δcdc55 mutants. G1-arrested cells were released intoYPD medium at 37°C, and the budding indexes were determined. Both cdc15-2 single and cdc15-2 Δcdc55 double mutants arrested at large budded cells (Figure 6C). Moreover, Cdc14 localized in the nucleolus in both cdc15-2 single and cdc15-2 Δcdc55 double mutants when incubated at the restrictive temperature (Figure 6C), indicating that mitotic exit in Δcdc55 mutants depends on MEN function. Therefore, we reason that PP2A might negatively regulate the MEN pathway.

PP2A Inhibits Mitotic Exit

To answer if the mitotic exit in Δcdc55 mutants depends on MEN functions, the cell cycle progression of tem1-3 and tem1-3 Δcdc55 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 Δcdc55 double mutants exited mitosis, as indicated by the appearance of extrabuds; however, all the cdc15-2 Δcdc55 double mutants were arrested as large budded cells (Figure 7A). This result is consistent with our observation that Δcdc55 mutation partially suppresses the temperature sensitivity of tem1-3 mutants when incubated at 30°C (Figure 1B). Because Δcdc55 suppresses the mitotic exit defects in tem1-3 mutants, PP2A might function as a negative regulator of Tem1.

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 Δcdc55 mutant cells, and it was very clear that Δcdc55 mutants exhibited more modified Tem1 protein in asynchronized cells (Figure 7D). The phosphorylation of Tem1 was also examined in synchronized TEM1–13myc and Δcdc55 TEM1–13myc cells. G1-arrested cells were released intoYPD medium containing 20 µg/ml nocodazole at 30°C. In G1-arrested cells, we observed more phosphorylated Tem1 in Δcdc55 mutants. As cells entered S-phase, there were fewer phosphorylated Tem1 proteins. We noticed the appearance of a slow-migrating Tem1 band in Δcdc55 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 Δcdc55 mutant cells side by side, it was clear that Δcdc55 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

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 and several pieces of evidence support this conclusion. First, a report that PP2A also plays a negative role in mitotic exit. Previous data indicate that budding yeast Cdc55 regulates

**REFERENCES**


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