Introduction

Meiosis is a specialized form of cell division that results in the production of haploid gametes from a diploid parental cell. Meiotic cells prevent an extra round of DNA replication between the two nuclear divisions by partially inactivating Cdk between the two meiotic divisions and, thereby, inhibit relicensing of replication origins. In *Saccharomyces cerevisiae*, activation of the Cdc14 early anaphase release (FEAR) network is required for exit from meiosis I but does not lead to the activation of origins of replication. The precise mechanism of how FEAR regulates meiosis is not understood. In this paper, we report that premature activation of FEAR during meiosis caused by loss of protein phosphatase PP2A^{Cdc55} activity blocks bipolar spindle assembly and nuclear divisions. In *cdc55* meiotic null (*cdc55-mn*) cells, the cyclin-dependent kinase (Cdk)–counteracting phosphatase Cdc14 was released prematurely from the nucleolus concomitant with hyperphosphorylation of its nucleolar anchor protein Net1. Crucially, a mutant form of Net1 that lacks six Cdk phosphorylation sites rescued the meiotic defect of *cdc55-mn* cells. Expression of a dominant mutant allele of *CDC14* mimicked the *cdc55-mn* phenotype. We propose that phosphoregulation of Net1 by PP2A^{Cdc55} is essential for preventing precocious exit from meiosis I.
Figure 1. **PP2A<sup>Cdc55</sup> is required for meiotic nuclear divisions.** CDC55 (Y1843) and cdc55-mn (Y2198) cells containing REC8-ha3 and PDS1-myc18 were induced to sporulate. (A) Hourly aliquots of the sporulating CDC55 and cdc55-mn cultures were taken after transfer to SPM, and the DNA content was monitored by flow cytometry. Flow cytometric profile of cells before transfer to SPM contains a purple fill. (B) Cell samples were taken for in situ immunofluorescence.
To test whether PP2A Cdc55 is required for premeiotic DNA replication, we analyzed the DNA content of sporulating CDC55 and cdc55-mn cells by flow cytometry. Both CDC55 and cdc55-mn cells accumulate a 4C DNA peak after transfer into SPM, indicating that PP2A Cdc55 is not required for premeiotic DNA replication (Fig. 1 A). Note that CDC55 and cdc55-mn cells initiated premeiotic DNA replication after 3 and 1 h in SPM, respectively. We designated the timing of replication initiation as a reference point (start) for the two cultures.

Failure to progress through meiotic nuclear divisions could be caused by an inability to degrade securin and/or cleave cohesin. To test this possibility, we followed the levels of securin (Pds1) and meiosis-specific cohesin subunit Rec8 in sporulating CDC55 and cdc55-mn cells by immunofluorescence and immunoblotting. Degradation of Pds1 and Rec8 in wild-type cells was initiated after 3 h and completed by 5 h relative to start (Fig. 1 C). In cdc55-mn cells, initiation of Pds1 and Rec8 degradation was delayed by 1 h, and its completion was further delayed by another hour compared with wild-type cells (Pds1/Rec8 degradation initiated after 4 h and completed by 7 h relative to start; Fig. 1 C). Immunoblotting data also indicate that Pds1 and Rec8 are degraded in cdc55-mn cells during meiosis (Fig. 1 D).

Results and discussion

PP2A Cdc55 is required for meiotic nuclear divisions

Because cdc55Δ cells are too sick for carrying out any meiotic analyses (Rabitsch et al., 2003; Clift et al., 2009), we generated a meiotic-null allele of CDC55 (cdc55-mn) by replacing its promoter with the mitosis-specific PCLB2 (Grandin and Reed, 1993). cdc55-mn cells were indistinguishable from wild-type cells in terms of vegetative growth (unpublished data). To test whether PP2A Cdc55 is required for meiosis, we induced CDC55 and cdc55-mn cells to sporulate. The levels of Cdc55 in cdc55-mn cells were undetectable after transfer to sporulation medium (SPM; Fig. 1 D), indicating that cdc55-mn is a true meiotic-null allele. Although 63% of CDC55 cells underwent two rounds of nuclear divisions and formed tetrads after 10 h in SPM, cdc55-mn cells remained largely mononucleate (97%; Fig. 1 B).

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Figure 3. PP2A_{CDC55} is required for preventing premature release of Cdc14 from the nucleolus. (A) Cells from the experiment described in Fig. 2 were used for assaying nucleolar localization of Cdc14 by immunofluorescence using anti-Cdc14 and anti-Nop1 antibodies (n = 200). (B) CDC55 (Y2120) and cdc55-mn (Y2119) cells containing NET1-TEV-myc9 were arrested in metaphase I by transferring cells to SPM for 8 h. The nucleolar localization of
To test whether PP2A<sup>Cdc55</sup> is required for expression of middle meiotic genes, we compared the levels of cyclin Clb3 and polo kinase Cdc5 by immunoblotting in sporulating CDC55 and cdc55-<i>mn</i> cells. Expression of CDC55 and CLB3 during meiosis is dependent on Ndt80, the meiosis-specific transcription factor, which is activated after cells exit from pachytene (Chu et al., 1998). Although CLB3 is transcribed after pachytene, it is translated only during meiosis II (Carlile and Amon, 2008). Cdc5 and Clb3 were expressed after 2 and 4 h, respectively, relative to start in CDC55 and cdc55-<i>mn</i> cells (Fig. 1 D). These results suggest that meiotic cell cycle events proceed normally in the absence of PP2A<sup>Cdc55</sup> activity but occur in the absence of any nuclear division.

**PP2A<sup>Cdc55</sup> is required for bipolar spindle assembly during meiosis**

The inability of cdc55-<i>mn</i> cells to divide is not caused by a failure to assemble/disassemble synaptonemal complexes (SCs) or by activation of the pachytene/spindle assembly checkpoints (Figs. S1, A and B; and S2 A). Because a bipolar spindle is required for nuclear division, we tested whether PP2A<sup>Cdc55</sup> is required for the formation of metaphase I spindles. Although CDC55 cells went through two rounds of nuclear division and assembled metaphase I, anaphase I, and meiosis II (metaphase II plus anaphase II) spindles, cdc55-<i>mn</i> cells formed either very short or no spindles and did not separate their spindle pole bodies (SPBs; Figs. 2 and S1 C).

Like cdc55-<i>mn</i> cells, conditional cdc28-ts mutants arrest in meiotic prophase I with unseparated SPBs (Shuster and Byers, 1989). Failure of cdc55-<i>mn</i> cells to form a bipolar spindle is not caused by hyperphosphorylation of Cdc28 at Y19 by Sve1 or lack of Clb1 expression (Fig. S2, B and C).

**Premature nucleolar splitting and Cdc14 release in cdc55-<i>mn</i> cells**

During vegetative growth, PP2A<sup>Cdc55</sup> prevents the premature activation of the Cdk-antagonizing phosphatase Cdc14 until metaphase. We therefore tested whether PP2A<sup>Cdc55</sup> was required for preventing premature release of Cdc14 during meiosis by following the localization of Cdc14 and nucleolar protein Nop1 in sporulating CDC55 and cdc55-<i>mn</i> cells by immunofluorescence. In wild-type cells, Cdc14 was largely nucleolar and released from the nucleolus in either binucleates with anaphase I spindles or tetranucleates with anaphase II spindles (unpublished data). Cdc14 also localized to the nucleolus in cdc55-<i>mn</i> cells. However, we observed that nucleoli began to split after 3 h in SPM, and by 6 h, 50% of mononucleate cdc55-<i>mn</i> cells had multiple (two to four) nucleoli (Fig. 3 A).

Because ectopic activation of Cdc14 is sufficient to resolve nucleoli during mitosis (D’Amours et al., 2004), our data suggest that Cdc14 in cdc55-<i>mn</i> cells is released albeit partially/transiently from the nucleolus.

During early anaphase, destruction of mitotic cyclins by anaphase-promoting complex/Cdc20 is thought to decrease Cdk activity to an extent that is unable to sustain Net1 phosphorylation and, thereby, causing relocation of Cdc14 to the nucleolus (Queralt et al., 2006). If FEAR operated in a similar manner during meiosis, a robust nucleolar release of Cdc14 should occur in the absence of anaphase-promoting complex/Cdc20 activity in cdc55-<i>mn</i> cells. Therefore, we synchronized CDC55 and cdc55-<i>mn</i> cells in metaphase I by depletion of Cdc20 and monitored Cdc14 localization. Wild-type cells arrested in metaphase I as indicated by the presence of 68.5% of mononuclei with short thick spindles (length >1.5 µm) after 6 h in SPM (Fig. 3 C). In contrast, 74% of cdc55-<i>mn</i> cells formed either no or very short spindles (<1.5 µm) after 6 h. In wild-type cells, Cdc14 was nucleolar as measured by colocalization with Net1 (Fig. 3, B and C). However, in cdc55-<i>mn</i> cells, Cdc14 was progressively released from the nucleolus. Nucleolar release of Cdc14 initiated after 5 h, and after 8 h, >60% of cells had Cdc14 distributed all over the nucleus (Fig. 3, B and C). To test whether Net1 was hyperphosphorylated in cdc55-<i>mn</i> cells, we prepared protein extracts from CDC55 NET1-<i>myc9</i> and cdc55-<i>mn</i> NET1-<i>myc9</i> cells arrested in metaphase I and assayed the electrophoretic mobility of Net1 by Western analysis. Net1 from cdc55-<i>mn</i> cells arrested in metaphase I had a lower electrophoretic mobility compared with Net1 from CDC55 cells (Fig. 3 D). To test whether the hypershift of Net1 in cdc55-<i>mn</i> cells was caused by phosphorylation, we mutated the six Cdk consensus sites in Net1 (residues 62, 166, 212, 252, 297, and 304) that are required for Cdc14 release during early anaphase (Azzam et al., 2004). This abrogated the hypershift, suggesting that Net1 is hyperphosphorylated by Cdk in cdc55-<i>mn</i> cells (Fig. 3 D).

**Premature release of Cdc14 from the nucleolus is sufficient for inhibiting meiotic spindle assembly and nuclear divisions**

Because Cdc14 was released in cdc55-<i>mn</i> cells arrested in metaphase I, we tested whether Cdc14 release was sufficient for blocking meiotic nuclear divisions and spindle assembly. To mimic premature release of Cdc14, we expressed TAB6, a dominant mutant allele of CDC14 that binds poorly to Net1 (Shou et al., 2001). Using the Gal4-ER system (Benjamin et al., 2003), we constructed a strain that expressed TAB6 in the presence of β-estradiol. Induction of TAB6 expression in cells after 4, 5, and 6 h into sporulation affected tetrad formation (Fig. 3 E).
Figure 4. *net1-6Cdk* suppresses the nuclear division defect of *cdc55-mn* and *cdc55Δ* cells. (A) *NET1 CDC55* (Y2072), *NET1 cdc55-mn* (Y2075), *net1-6Cdk CDC55* (Y2078), and *net1-6Cdk cdc55-mn* (Y2081) cells were induced to sporulate. Kinetics of nuclear division was measured in the four sporulating cultures by staining cells with DAPI (n = 200). Lag in the appearance of tetranucleates relative to binucleates in *net1-6Cdk cdc55-mn* cells is
In contrast, addition of estradiol to the strain that lacked the \( P_{GAL1} \) TAB6 allele had little or no effect (Fig. 3 E). Expression of TAB6 also affected bipolar spindle assembly during meiosis (Fig. S3 A).

A mutant form of Net1 that lacks six Cdk phosphorylation sites suppresses the meiotic nuclear division defect of cdc55-mn cells

If the inability of cdc55-mn cells to divide their nuclei was a result of premature release of Cdc14 caused by Cdk-mediated Net1 phosphorylation, it should be overcome by a mutant allele of Net1 that lacks Cdk recognition sites. Therefore, we constructed diploid strains that contained either CDC55 or cdc55-mn allele in combination with either NET1 or the net1-6Cdk allele. We induced the four strains to undergo meiosis and analyzed the kinetics of nuclear division. Although 61% of NET1 CDC55 cells went through two rounds of nuclear division, NET1 cdc55-mn cells failed to undergo any nuclear division (Fig. 4 A). Although net1-6Cdk CDC55 cells formed 50% tetrads, a high proportion of cells (18%) formed dyads. Crucially, 41% of net1-6Cdk cdc55-mn cells went through two rounds of nuclear division and formed triads/tetrads, indicating that the nuclear division defect of cdc55-mn allele is caused by untimely phosphorylation of Net1 by Cdk and the consequent release of Cdc14 from the nucleolus (Fig. 4 A).

Inhibition of the FEAR pathway in net1-6Cdk strains delays nuclear segregation during mitosis (Azzam et al., 2004). To test whether nucleolar segregation is delayed in net1-6Cdk strains during meiosis, we quantified the fraction of cells with metaphase II spindles but have still not resolved their nucleolus. About 90% of net1-6Cdk cells with metaphase II spindles had an undivided nucleolus compared with 2.2% in NET1 cells (Fig. 4 B). This delay in nucleolar segregation in net1-6Cdk cells was partially suppressed by cdc55-mn (Fig. 4 B). This suggests the presence of additional phosphorylation sites in Net1 apart from the six Cdk sites, whose phosphorylation is opposed by PP2A\(^{CDC55}\). This notion is also supported by the fact that strains bearing the net1-6Cdk allele form 50% tetrads, indicative of sufficient release of Cdc14 despite the absence of six Cdk sites.

Phosphoregulation of Net1 by Cdk and PP2A\(^{CDC55}\) is required for timely onset of meiosis II after meiosis I

In NET1 and net1-6Cdk cells, the timing of appearance of binucleates coincided with that of tetrancleates (Fig. 4 A). However, in net1-6Cdk cdc55-mn cells, the appearance of tetrancleates was delayed by 2 h relative to binucleates (Fig. 4 A). After anaphase I spindle disassembly, the FEAR pathway might have to be inactivated to facilitate assembly of the metaphase II spindle. We hypothesized that lack of PP2A\(^{CDC55}\) activity might inhibit FEAR inactivation and assembly of meiosis II spindles in net1-6Cdk cdc55-mn cells, leading to a delay between meiosis I and II. If this were to be true, one would predict an increased proportion of binucleates with prophase II spindles in net1-6Cdk cdc55-mn cells. Indeed, we found that the proportion of binucleate cells with prophase II spindles was 53.4% compared with 7.8 and 25.8% in NET1 and net1-6Cdk strains, respectively (Fig. 4 C). This suggests that phosphoregulation of Net1 by Cdk and PP2A\(^{CDC55}\) is required to prevent a delay between the two successive meiotic nuclear divisions.

The major function of PP2A\(^{CDC55}\) during meiosis is to inhibit Net1 phosphorylation by Cdk

Is the inability of cdc55-mn cells to sporulate caused by premature activation of FEAR? We tested whether the net1-6Cdk allele suppresses the sporulation defect of cdc55A cells. Although NET1 cdc55A cells failed to sporulate, the net1-6Cdk cdc55A cells remarkably formed dyads (16%) and triads/tetrads (22%; Fig. 4 D). This also shows that the suppression of cdc55-mn by net1-6Cdk is not caused by activation of \( P_{Cdc23} \) in net1-6Cdk cdc55-mn cells.

Does PP2A\(^{CDC55}\) have an additional role in meiosis apart from negatively regulating the FEAR pathway? We compared the spore viabilities of NET1, net1-6Cdk, and net1-6Cdk cdc55-mn strains (Fig. 5 A). 51.95% of spores obtained from net1-6Cdk cdc55-mn cells were viable in comparison to 91.4 and 93.3% for NET1 and net1-6Cdk cells, respectively. Taking into account the partial suppression of cdc55-mn by net1-6Cdk, the delay between meiosis I and meiosis II in net1-6Cdk cdc55-mn cells (Fig. 4 A), and the pleiotropic nature of PP2A\(^{CDC55}\), the spore viability of 51.95% is strikingly high. Moreover, the segregation of GFP-tagged sister centromeres during meiosis I in cdc55-mn net1-6Cdk cells was largely reductional (86% compared with 95 and 96% in NET1 and net1-6Cdk cells, respectively). In net1-6Cdk cdc55-mn cells, 86% of sister centromeres that segregated reductionally during anaphase I retained centromeric cohesion compared with 96 and 94% in NET1 and net1-6Cdk cells, respectively (Fig. 5 A). Although we cannot exclude an auxiliary role for PP2A\(^{CDC55}\) in meiotic chromosome segregation, our results suggest that the major function of PP2A\(^{CDC55}\) during meiosis is to control the timing of FEAR activation by opposing Net1 phosphorylation by Cdk.

In wild-type cells, PP2A\(^{CDC55}\) restricts Cdc14 to the nucleolus by keeping Net1 dephosphorylated until metaphase I, which allows accumulation of the Cdk activity required for building bipolar spindles. During anaphase, activated separase inhibits PP2A\(^{CDC55}\), which results in Net1 phosphorylation, nucleolar...
release of Cdc14, and exit from meiosis I. In cdc55-mn cells, Net1 is hyperphosphorylated by Cdk, resulting in premature Cdc14 release, which antagonizes Cdk activity and prevents bipolar spindle formation (Fig. 5 B). Premature activation of FEAR blocks spindle assembly during meiosis but not during mitosis, suggesting that FEAR becomes more potent during meiosis. Differential ability of Cdc14 to dephosphorylate Cdk-Clb2 (mitosis-specific Cdk) and Cdk-Clb1 (major Cdk during meiosis I) targets might explain why FEAR is more important during meiosis than mitosis. The effect of FEAR activation on origin licensing could be limited by the meiosis-specific kinase Ime2, which is sufficient for preventing origin licensing and adds phosphates to proteins that cannot be removed by Cdc14 (Holt et al., 2007). Determining how FEAR is activated during meiosis and how replication origin licensing is prevented during FEAR activation are key challenges for the future.

Figure 5. Analyses of spore viability and sister centromere segregation during meiosis I in net1-6Cdk cdc55-mn cells and a model for PP2A<sup>Cdc55</sup>’s role in preventing premature exit from meiosis I. (A) NET1 CDC55, net1-6Cdk CDC55, and net1-6Cdk cdc55-mn cells from the same experiment described in Fig. 4 A were stained against tubulin, and the segregation of GFP-tagged sister centromeres was examined in cells containing anaphase I spindles (n = 100). Representative data from three experimental repeats are indicated. Spore viabilities of the aforementioned strains were obtained after dissecting 100 spores from tetrads onto YEPD plates followed by incubation at 25°C for 3 d. Values indicated represent means obtained from two independent experiments. (B) See the last paragraph of the Discussion for details. P, phosphorylation.
Materials and methods

Yeast strains and plasmids
A complete list of yeast strains and their genotypes can be found in Table S1. The Pcr2::pA3-CDC55 allele was constructed by PCR-mediated transformation using Pcr2::pA3-KanMX6 as previously described (Lee and Amon, 2003). SK1 strains bearing the NET1-TEV myc and NET1-TEV-myc-Cdc55 alleles were created by backcrossing strains RD2652 and RD2662 (Azzone et al., 2004) with SK1 strains at least six times. An integrative plasmid carrying the TAB6 allele was obtained as a gift from K. Lee (National Cancer Center, Plainview, NY; Park et al., 2003). The TAB6 allele was cloned downstream of the GAL1–10 promoter in pRS303. The plasmid was targeted for integration at the HIS3 locus in yeast by linearizing with PstI.

Assay for SPB separation
For visualizing Spc42-GFP, cells from 1 ml meiotic culture were pelleted and resuspended in 1 ml of 70% ETOH and kept for 10 min at room temperature. Cells were then pelleted, resuspended in 100 mM K phosphate buffer, pH 6.4, containing 200 µg/ml DAPI, and subjected to fluorescence microscopy.

Immunostaining
Immunostaining was performed as previously described (Pringle et al., 1991). A 1.8-ml aliquot of sporulation culture was mixed with 200 µl of 37% formaldehyde solution and incubated at 25°C in a shaker for 15 min. Cells were then pelleted and resuspended in 1 ml buffer A (0.1 M potassium phosphate, pH 6.4, and 0.5 mM MgCl2) containing 3.7% formaldehyde and incubated at 4°C overnight. Cells fixed overnight were pelleted, washed with 1 ml buffer A thrice, and resuspended in 200 µl buffer A. 1 µl of 10-mg/ml Zymolyase was then added, and the cell suspension was incubated at 25°C for 15 min. 4 µl of 10-mg/ml Zymolyase was then added, and cells were incubated at 37°C for 5–10 min. The reaction was stopped by adding 1 ml buffer B (0.1 M K phosphate buffer, pH 7.4, 1.2 M sorbitol, and 0.5 mM MgCl2). Spheroplasts were washed once with 1 ml buffer B, resuspended in 200 µl buffer B, and stored at −20°C. About 5 µl spheroplasts were added to a polylysine-coated 18-well slide and incubated for 5 min at room temperature. Excess solution was aspirated out, and the slide was treated with ice-cold methanol for 3 min followed by ice-cold acetone for 10 s. The slides were then allowed to air dry. For immunostaining, the slides were washed once with 10 µl PBS-BSA (5 mg/ml of powdered BSA in PBS). Buffer was ice-cold methanol for 3 min followed by ice-cold acetone for 10 s. The slides were then treated with 3% hydrogen peroxide in methanol for 10 min to quench endogenous peroxidase activity. Slides were then washed with PBS and then blocked with 100 µl blocking buffer (0.5% BSA and 0.2% gelatin in PBS) under a coverslip. After 1 h, the slide was incubated with 40 µl primary antibody (diluted in blocking buffer) at room temperature for 2 h. After washing with PBS for 10 min, the slide was incubated with 40 µl secondary antibody for 2 h at room temperature, washed again in PBS, and mounted in antifade solution (diluted 1:7-fold in blocking buffer). Zip1 antibody, and the 3F10 anti-HA antibody were used at a 1:500 dilution for staining chromosome spreads.

Immunoblotting
Whole-cell extracts were prepared by cell breakage with glass beads in 10% trichloroacetic acid. Cell pellets were resuspended in 2x SDS sample buffer and neutralized with 1 M Tris, and proteins were denatured by heating the samples at 95°C for 5 min. After centrifugation, protein samples were electrophoresed on 8%/5% SDS-PAGE gels. The HA epitope was detected by mouse monoclonal antibody 16B12 at 1:5,000. Anti-Cld3 (SC-7167; Santa Cruz Biotechnology, Inc.) and anti-Cdc5 (SC-6733; Santa Cruz Biotechnology, Inc.) antibodies were used at 1:1,000 dilutions. The Myc epitope was detected using the 9E10 antibody (Cambridge Bioscience).

Flow cytometry
The DNA content of sporulating cells was measured by flow cytometry as previously described (Epstein and Cross, 1992). 1 ml sporulation culture was centrifuged, and the cells were resuspended in 1 ml of 70% ETOH and incubated overnight at 4°C. Cells were then pelleted and resuspended in 1 ml of 50-mM Tris-HCl, pH 7.8, with 200 µg RNase A and incubated at 37°C for 6 h. Cells were then pelleted and resuspended in 0.5 ml buffer D (200 mM Tris-HCl, pH 7.5, 211 mM NaCl, 78 mM MgCl2, and 50 µg/ml propidium iodide) and 40 µl primary antibody (diluted in blocking buffer) at room temperature for 20-fold in 1 ml of 50-mM Tris-HCl, pH 7.8, before they were analyzed using a FACScan (BD).

Online supplemental material
Fig. S1 shows that cdc55-mn cells are proficient in SC assembly/dis-assembly but fail to separate their SPBs. Fig. S2 shows that the nuclear division defect of cdc55-mn cells is not caused by hyperphosphorylation of Cdc28 at Y19 by Swel or lack of Cbi1 expression or activation of pachytene/spindle assembly checkpoints. Fig. S3 shows that ectopic expression of a dominant mutant allele of Cdc14 blocks spindle assembly and that PPA2Δ65Δ65 works downstream of Skl19 and Spo12 in the FEAR pathway. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201103019/DC1.

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