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Meiotic nuclear divisions in budding yeast require PP2A^{Cdc55}-mediated antagonism of Net1 phosphorylation by Cdk

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During meiosis, one round of deoxyribonucleic acid replication is followed by two rounds of nuclear division. 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 *n*ull (*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.

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* (budding yeast), this is achieved by engaging the Cdc14 early anaphase release (FEAR) network of genes (Buonomo et al., 2003; Marston et al., 2003; Kamieniecki et al., 2005).

The FEAR network causes a transient release of Cdk-antagonizing phosphatase Cdc14 from the nucleolus during early anaphase (Stegmeier and Amon, 2004). At the heart of FEAR is the physical interaction between Cdc14 and Cfi1/Net1, its competitive inhibitor in the nucleolus (Shou et al., 1999; Visintin et al., 1999). When cells reach metaphase, phosphorylation of Net1 by mitotic Cdk is counteracted by the protein phosphatase PP2A^{Cdc55} (Queralt et al., 2006; Yellman and Burke, 2006). During anaphase, activated separase in league with Slk19, a kinetochore protein, inhibits PP2A^{Cdc55} (Queralt et al., 2006), which results in Net1 phosphorylation and Cdc14 release from the nucleolus termed as FEAR. In an independent branch of the FEAR pathway, phosphorylation of nucleolar protein Spo12 by Cdk helps in dissociation of the replication fork barrier protein (Fob1), which stabilizes the Net1–Cdc14 interaction (Stegmeier et al., 2004; Tomson et al., 2009).

FEAR regulates exit from meiosis I but is not essential for mitotic exit. Meiotic cells lacking Spo12, Slk19, or Esp1 (separase) fail to release Cdc14 from the nucleolus and are delayed in disassembly of meiosis I spindles. These cells undergo two nuclear divisions on the same meiosis I spindle, forming dyads (Buonomo et al., 2003; Marston et al., 2003).

In this paper, we investigate the role of PP2A^{Cdc55} during meiosis in budding yeast. We show that PP2A^{Cdc55} inhibits FEAR activation during meiosis, as it does during mitosis. However, unlike in mitosis, this inhibition is essential for meiotic spindle formation and nuclear division. We propose that PP2A^{Cdc55} is

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Abbreviations used in this paper: FEAR, Cdc14 early anaphase release; SC, synaptonemal complex; SPB, spindle pole body; SPM, sporulation medium.

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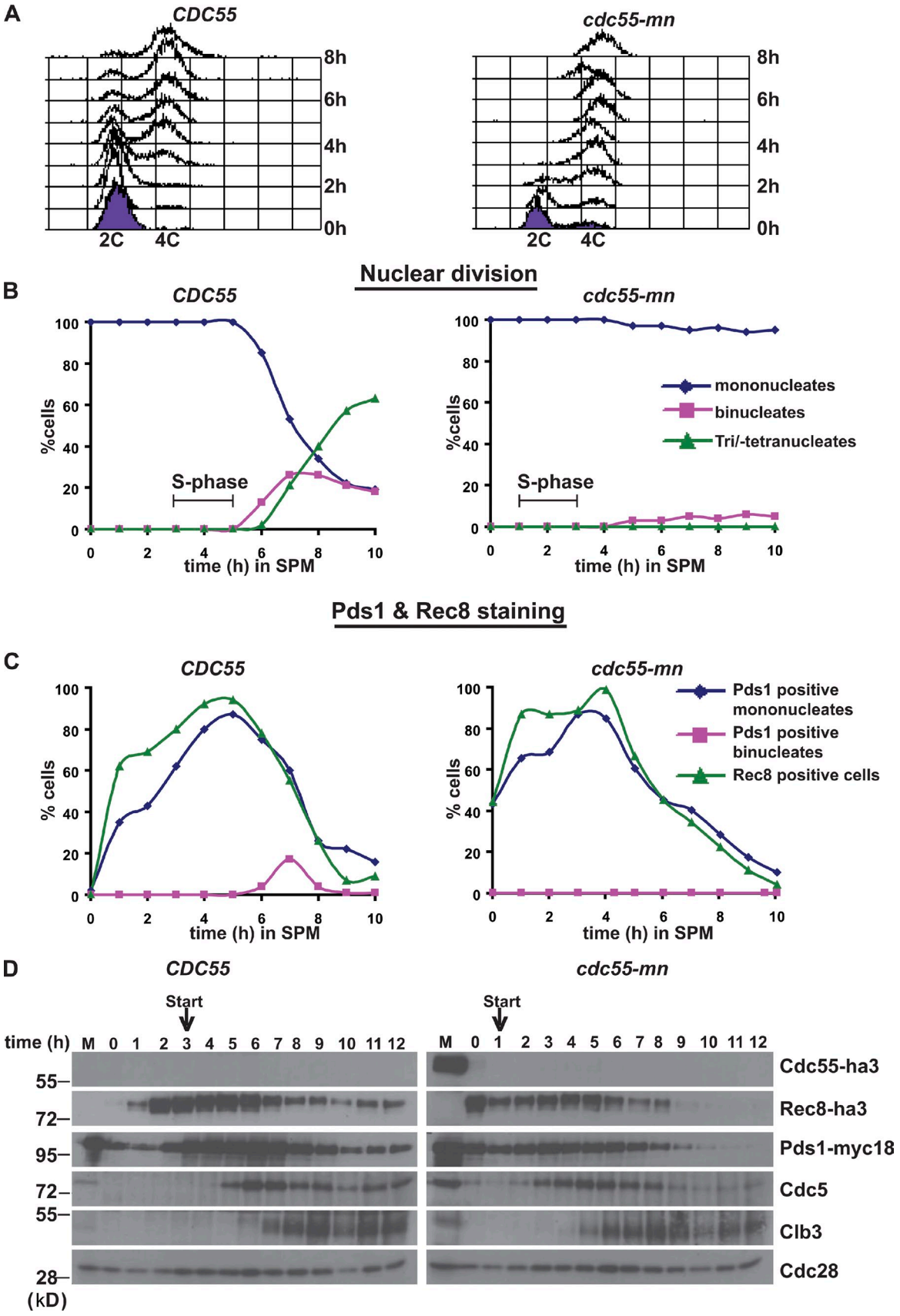

Figure 1. **PP2A^{Cdc55} is required for meiotic nuclear divisions.** *CDC55* (Y1843) and *cdc55-mn* (Y2198) cells containing *REC8-ha3* and *PDS1-myc18* were induced to sporulate. (A) Hourly aliquots of the sporulating *CDC55* and *cdc55-mn* cultures were taken after transfer to SPM, and the DNA content 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.

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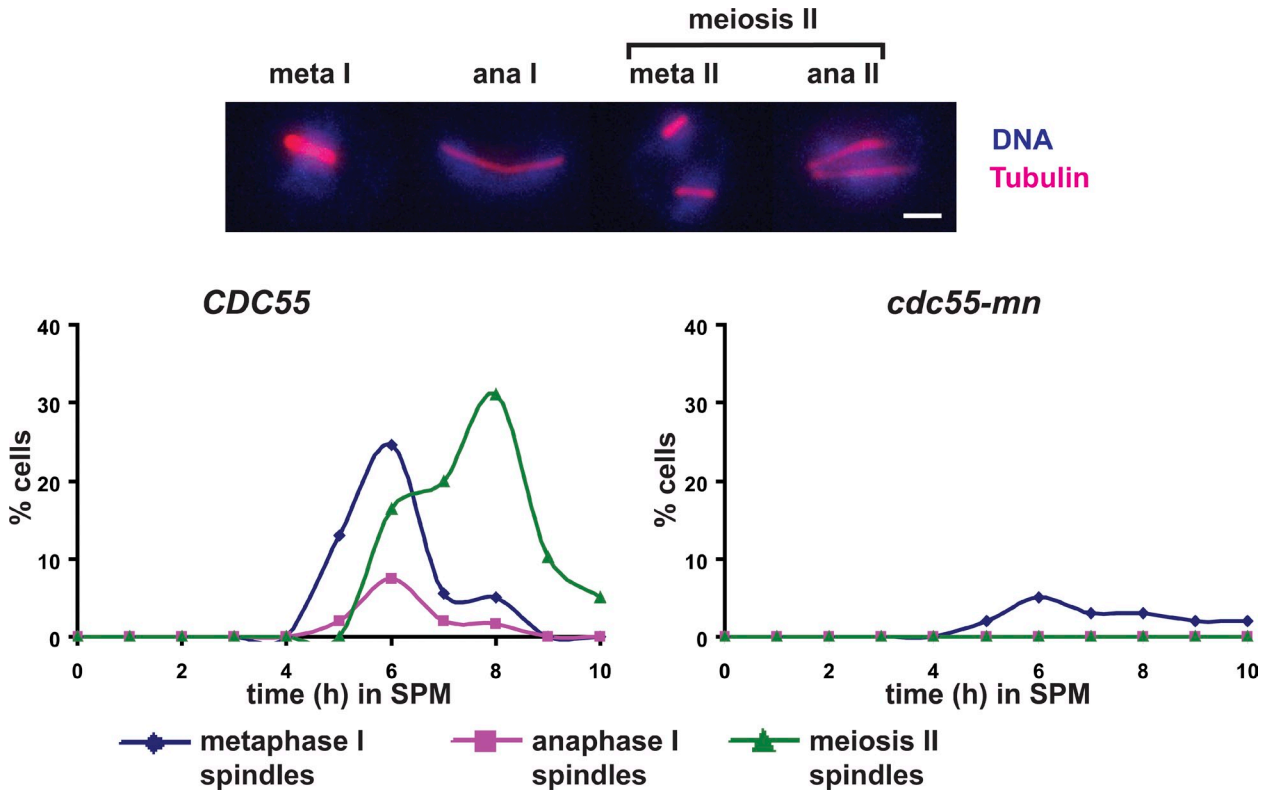

Figure 2. **PP2A^{Cdc55} is required for bipolar spindle assembly during meiosis.** *CDC55* (Y2111) and *cdc55-mn* (Y2149) cells containing *SPC42-GFP* were induced to sporulate. Spindle formation was assayed in the two sporulating cultures by immunofluorescence using antitubulin antibodies (n = 200). Representative images of nuclei containing metaphase I (meta I), anaphase I (ana I), and meiosis II spindles are shown. Bar, 2 µm.

essential for meiotic cells to attain levels of Cdk activity required for bipolar spindle assembly and for preventing premature exit from meiosis I.

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 P_{CLB2} (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).

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).

Kinetics of nuclear division was scored by staining cells with DAPI (n = 200). Timing of premeiotic DNA replication in the two sporulating cultures is indicated. (C) Kinetics of Pds1 and Rec8 degradation was measured by immunofluorescence using anti-Myc and anti-HA antibodies, respectively (n = 200). (D) Whole-cell extracts were prepared from hourly aliquots of the sporulating cultures. Protein samples were electrophoresed on 8% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were probed using anti-Myc, anti-HA, anti-Cdc5, anti-Clb3, and anti-Cdc28 (loading control) antibodies. The lane labeled M contains mitotic extracts from the two strains.

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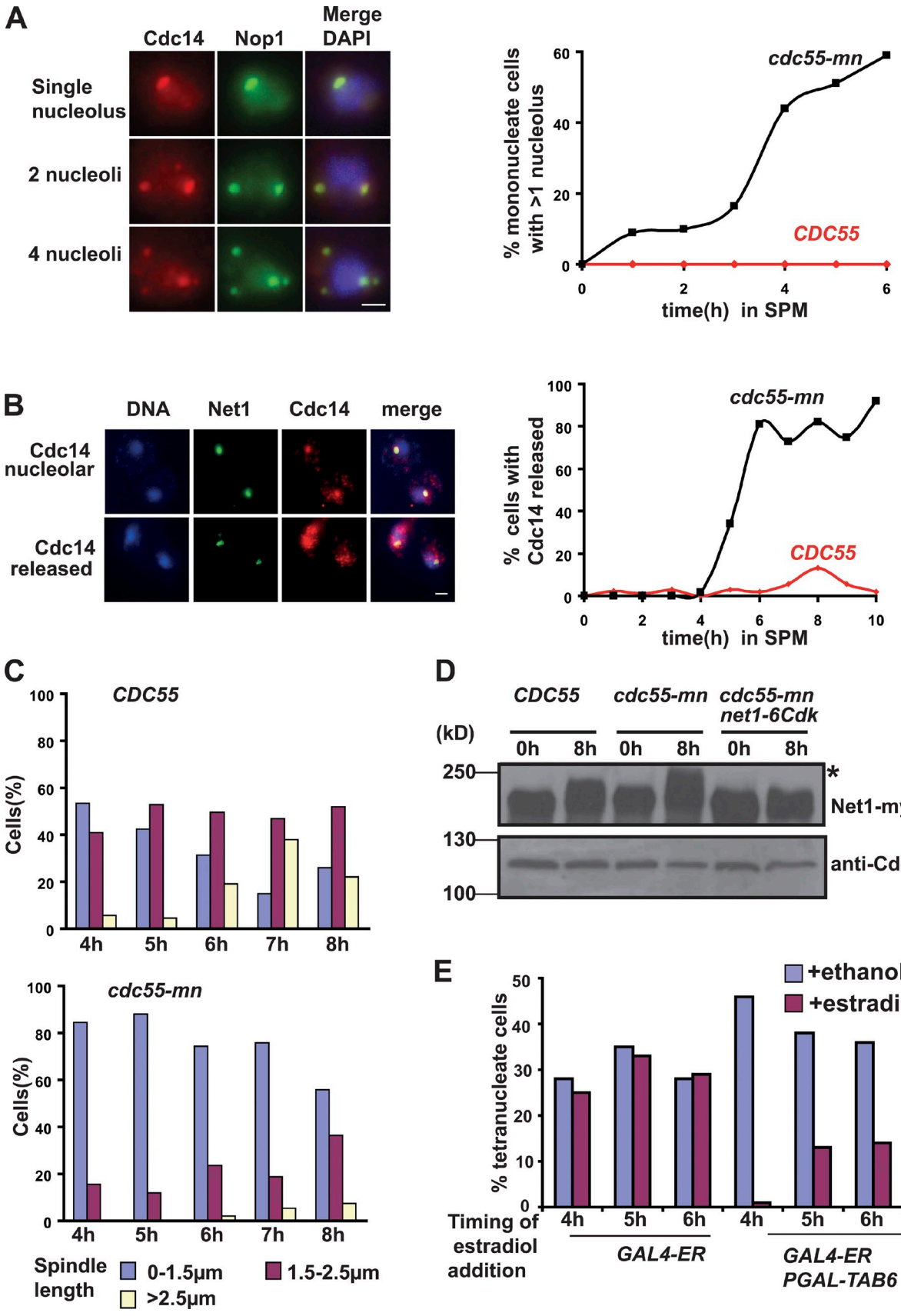

Figure 3. **PP2A^{Cdc55} is required for preventing premature release of Cdc14 from the nucleolus.** (A) Cells from the experiment described in Fig. 2 were used for assaying nucleolar localization of Cdc14 by immunofluorescence using anti-Cdc14 and anti-Nop1 antibodies (n = 200). (B) *CDC55* (Y2120) 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

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To test whether PP2A^{Cdc55} 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-mn* cells. Expression of *CDC5* 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-mn* cells (Fig. 1 D). These results suggest that meiotic cell cycle events proceed normally in the absence of PP2A^{Cdc55} activity but occur in the absence of any nuclear division.

PP2A^{Cdc55} is required for bipolar spindle assembly during meiosis

The inability of *cdc55-mn* 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^{Cdc55} 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-mn* cells formed either very short or no spindles and did not separate their spindle pole bodies (SPBs; Figs. 2 and S1 C).

Like *cdc55-mn* cells, conditional *cdc28-ts* mutants arrest in meiotic prophase I with unseparated SPBs (Shuster and Byers, 1989). Failure of *cdc55-mn* cells to form a bipolar spindle is not caused by hyperphosphorylation of Cdc28 at Y19 by Swe1 or lack of Clb1 expression (Fig. S2, B and C).

Premature nucleolar splitting and Cdc14 release in *cdc55-mn* cells

During vegetative growth, PP2A^{Cdc55} prevents the premature activation of the Cdk-antagonizing phosphatase Cdc14 until metaphase. We therefore tested whether PP2A^{Cdc55} 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-mn* 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-mn* cells. However, we observed that nucleoli began to split after 3 h in SPM, and by 6 h, 50% of mononucleate *cdc55-mn* 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-mn* 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-mn* cells. Therefore, we synchronized *CDC55* and *cdc55-mn* 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 mononucleates with short thick spindles (length >1.5 µm) after 6 h in SPM (Fig. 3 C). In contrast, 74% of *cdc55-mn* 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-mn* 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-mn* cells, we prepared protein extracts from *CDC55 NET1-myc9* and *cdc55-mn NET1-myc9* cells arrested in metaphase I and assayed the electrophoretic mobility of Net1 by Western analysis. Net1 from *cdc55-mn* 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-mn* 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-mn* 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-mn* 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).

Cdc14 by immunofluorescence is shown using anti-Cdc14 and anti-Myc (Net1) antibodies. (C) Spindle formation in the two cultures in B was detected by immunofluorescence using antitubulin antibodies, and the length of the spindles was measured by analyzing the fluorescence images using MetaMorph software (n = 100). (D) *NET1 CDC55* (Y2120), *NET1 cdc55-mn* (Y2119), and *net1-6Cdk cdc55-mn* (Y2307) cells containing P_{CLB2}-*CDC20* were arrested in metaphase I by transferring cells to SPM for 8 h. Whole-cell extracts from the cultures were prepared, and protein samples were electrophoresed on 5% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were probed using anti-Myc and anti-Cdc28 antibodies. The asterisk indicates the presumptive hyperphosphorylated Net1 in *cdc55-mn* cells arrested in metaphase I. A 120-kD cross-reacting band obtained with an anti-CDC28 antibody served as a loading control. (E) P_{GAL}-*TAB6 GAL4-ER* (Y2161) and *GAL4-ER* (Y2212) strains were induced to sporulate by transferring them to SPM. Estradiol was added after 4, 5, and 6 h to the cultures, and the effect on nuclear division after 24 h was determined by staining with DAPI (n = 200). Representative data from three experimental repeats are indicated in C and E. Bars, 2 µm.

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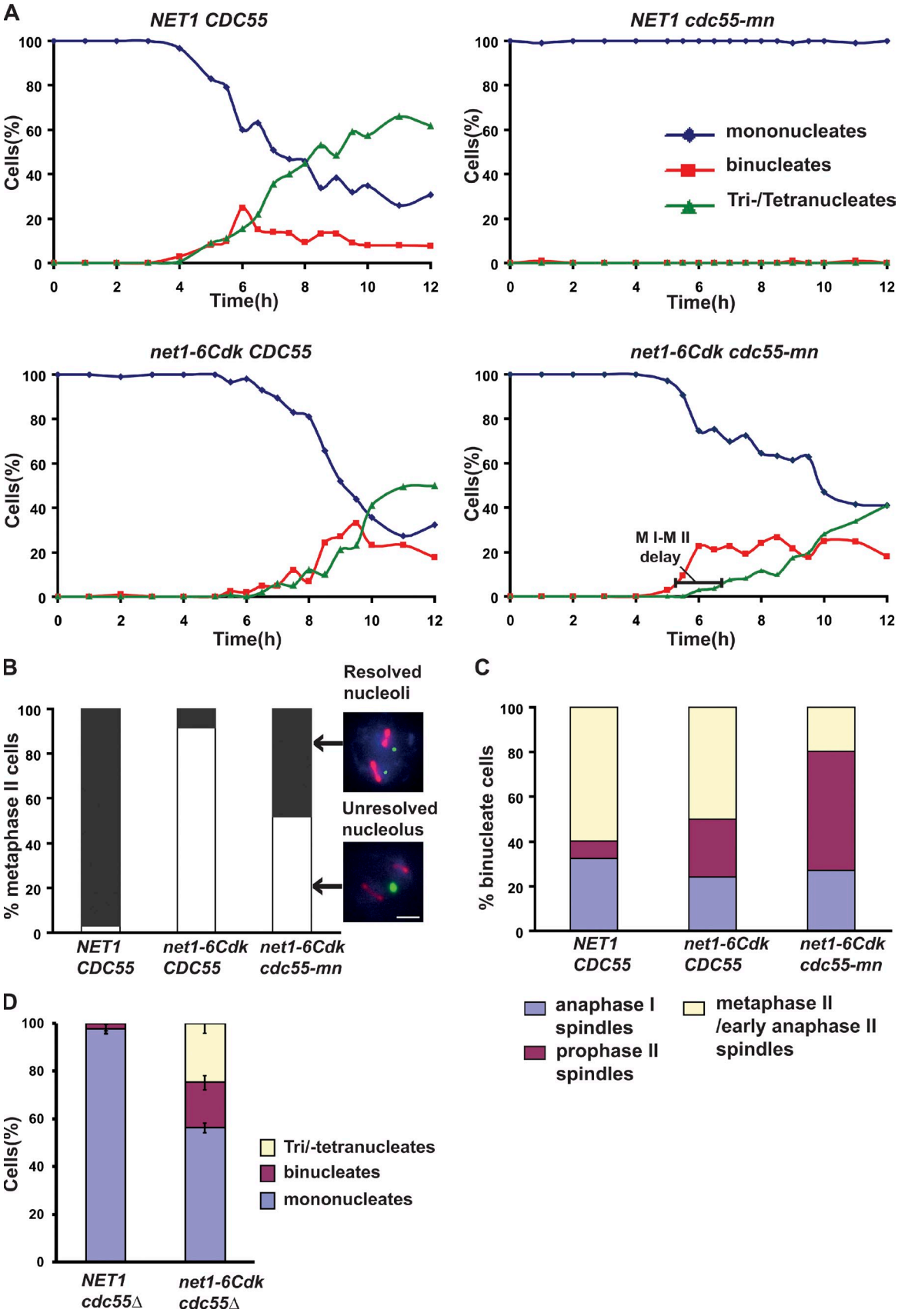

Figure 4. ***net1-6Cdk* suppresses the nuclear division defect of *cdc55-mn* and *cdc55Δ* cells.** (A) *NET1 CDC55* (Y2072), *NET1 cdc55-mn* (Y2075), *net1-6Cdk CDC55* (Y2078), and *net1-6Cdk cdc55-mn* (Y2081) cells were induced to sporulate. Kinetics of nuclear division was measured in the four sporulating cultures by staining cells with DAPI (n = 200). Lag in the appearance of tetranucleates relative to binucleates in *net1-6Cdk cdc55-mn* cells is

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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* cells 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 nucleolar 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 nucleoli. 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 tetranucleates (Fig. 4 A). However, in *net1-6Cdk cdc55-mn* cells, the appearance of tetranucleates 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 *cdc55Δ* cells to sporulate caused by premature activation of FEAR? We tested whether the *net1-6Cdk* allele suppresses the sporulation defect of *cdc55Δ* cells. Although *NET1 cdc55Δ* cells failed to sporulate, the *net1-6Cdk cdc55Δ* 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_{CLB2} 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

indicated. (B) Nucleolar resolution in binucleate cells was scored by immunofluorescence using anti-Nop1 and antitubulin antibodies (n = 200). Sample images indicating resolved and unresolved nucleoli are shown (DNA in blue, spindles in red, and Nop1 in green). (C) Spindle formation was detected by immunofluorescence using antitubulin antibodies. Binucleate cells were classified into those that contain anaphase I spindles, prophase II spindles, or metaphase II/early anaphase II spindles (n = 200). (D) *NET1 cdc55Δ* (Y2278) and *net1-6Cdk cdc55Δ* (Y2276) strains were induced to sporulate for 24 h. Cells were harvested, and nuclear division was scored by staining DNA with DAPI (n = 200). Error bars represent SEM. Representative data from three experimental repeats are indicated in B and C. Bar, 2 µm.

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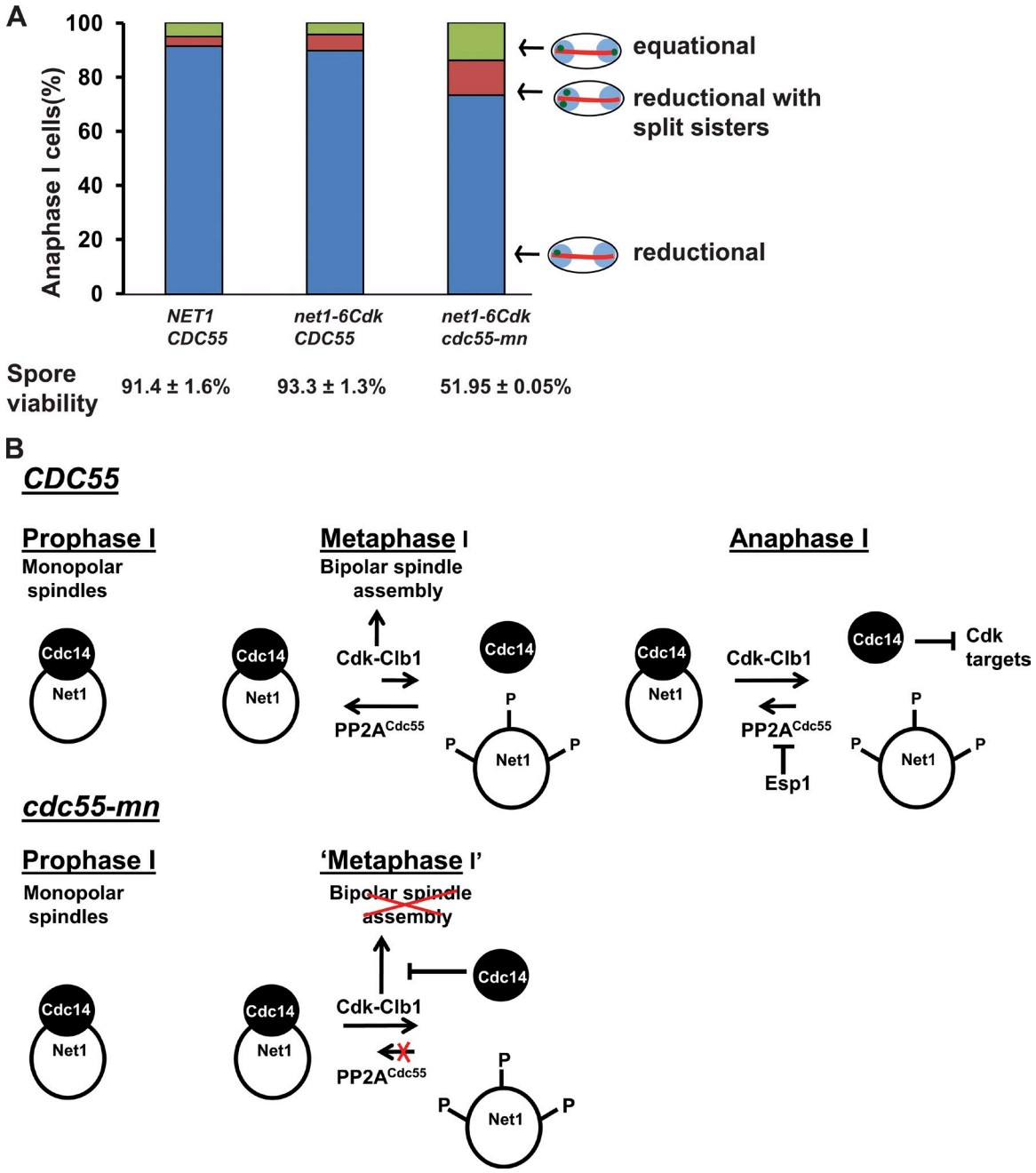

Figure 5. **Analyses of spore viability and sister centromere segregation during meiosis I in *net1-6Cdk cdc55-mn* cells and a model for PP2A^{Cdc55}'s role in preventing premature exit from meiosis I.** (A) *NET1 CDC55*, *net1-6Cdk CDC55*, and *net1-6Cdk cdc55-mn* cells from the same experiment described 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.

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.

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Materials and methods

Yeast strains and plasmids

A complete list of yeast strains and their genotypes can be found in Table S1. The P_{CLB2}-*ha3-CDC55* allele was constructed by PCR-mediated transformation using P_{CLB2}-*ha3-KanMX6* as previously described (Lee and Amon, 2003). SK1 strains bearing the *NET1-TEV-9 myc* and *net1-TEV-9 myc-6Cdk* alleles were created by backcrossing strains RJD2632 and RJD2862 (Azzam 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 $MgCl_2$) 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 β-mercaptoethanol was 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 $MgCl_2$). Spheroplasts were washed once with 1 ml buffer B, resuspended in 200 µl buffer B, and stored at −20°C. About 5 µl spheroplasts was 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 aspirated out, and the slide was incubated with 10 µl primary antibody (diluted in PBS-BSA) for 2 h in a moist chamber. Slides were washed five times with 10 µl PBS-BSA and then incubated with 10 µl secondary antibody (diluted in PBS-BSA) for 2 h. Slides were washed five times with PBS-BSA, and then 5 µl antifade solution (0.05% *p*-phenylenediamine in PBS with 90% glycerol and 0.4 µg/ml DAPI) was added to the slide. A coverslip was placed on the slide, and the edges were sealed with nail polish. Slides were either used for microscopic analysis or stored at −20°C.

The following primary antibodies were used: monoclonal rat anti–α-tubulin (1:500; AbD Serotec), monoclonal mouse anti-Myc (9E10; 1:5), polyclonal rabbit anti-Myc (1:500; Gramsch), monoclonal rat anti-HA (3F10; 1:500; Eurogentec), mouse anti-Nop1, rabbit anti-Cdc14 antibody (SC-33628; Santa Cruz Biotechnology, Inc.), and monoclonal mouse anti-HA (16B12; 1:500; Eurogentec). Secondary antibodies, preabsorbed against sera from other species used in labeling were conjugated with Cy3 or Cy5 (Millipore) or Alexa Fluor 488 (Invitrogen) and diluted 1:500 (Cy3 and Alexa Fluor 488) or 1:50 (Cy5). DNA was visualized by staining with DAPI.

Image acquisition and analysis

Images were acquired using an inverted microscope (TE-2000; Nikon) with a 100× 1.49 NA objective lens equipped with a liquid cooled charge-coupled device camera (CoolSNAP HQ2; Photometrics). 16 z-stack (spacing = 0.2 µm) exposure times were used at 1 s for Cy3, Cy5, and Alexa Fluor 488/GFP and 0.25 s for DAPI. Images were analyzed using MetaMorph (version 7.5.2.0; MAG Biosystems Software). Apart from changing brightness and contrast, no other changes were made to the images.

Chromosome spreading

Chromosome spreading was performed as described previously (Nairz and Klein, 1997). Cells from 5 ml sporulation culture were pelleted and resuspended in 1 ml spheroplasting solution (2% potassium acetate, 0.8 M sorbitol, and 10 mM DTT) including 7 µl Zymolyase (10 mg/ml) and incubated at 37°C . Spheroplasting was monitored by testing for lysis in 1% sarkosyl. The reaction was stopped by adding 0.5 ml of ice-cold buffer C (0.1 M 2-(*N*-morpholino) ethane sulfonic acid, pH 6.4, 1 M sorbitol, 1 mM EDTA, and 0.5 mM $MgCl_2$), and the spheroplasts were spun down at 2,000 rpm and resuspended in 0.5 ml buffer C. On a clean slide, 20 µl cell suspension was prefixed with 40 µl fixative (4% paraformaldehyde and 3.4% sucrose), lysed with 80 µl of 1% lipsol, and fixed with 80 µl fixative. Slides were allowed to dry overnight. For immunostaining, slides were 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 2× 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-Clb3 (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).

Sporulation

Induction of sporulation was performed as previously described (Kiburz et al., 2008). In brief, cells were grown to saturation in YPD (1% yeast extract, 2% bactopeptone, and 2% glucose) at 30°C for 24 h. Cells were then diluted into YPA (1% yeast extract, 2% bactopeptone, and 2% KOAc) at an OD_{600} of 0.2 and grown for 16 h. Cells were then washed with water and resuspended in SPM (0.3% KOAc, pH 7.0) at an OD_{600} of 3 at 30°C (275 rpm) to induce sporulation. To induce *TAB6* expression, β-estradiol was added to the cultures at the final concentration of 1 µM.

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 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 $MgCl_2$, and 50 µg/ml propidium iodide). Cells were sonicated for 5 s and diluted 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/disassembly 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 Swe1 or lack of Clb1 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 $PP2A^{Cdc55}$ works downstream of Slk19 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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