Meiotic nuclear divisions in budding yeast require PP2ACdc55-mediated antagonism of Net1 phosphorylation by Cdk

Gary W. Kerr,1 Sourav Sarkar,1 Katherine L. Tibbles,1 Mark Petronczki,2 Jonathan B.A. Millar,1 and Prakash Arumugam1

1University of Warwick, Coventry CV4 7AL, England, UK
2Clare Hall Laboratories, Cancer Research UK, London Research Institute, Hertfordshire EN6 3LD, England, UK

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 PP2ACdc55 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 PP2ACdc55 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 (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 PP2ACdc55 (Queralt et al., 2006; Yellman and Burke, 2006). During anaphase, activated separase in league with Slk19, a kinetochore protein, inhibits PP2ACdc55 (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 PP2ACdc55 during meiosis in budding yeast. We show that PP2ACdc55 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 PP2ACdc55 is...
Figure 1. \textit{PP2A}^{CDC55} \textbf{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.
Results and discussion

**PP2ACdc55 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).
Figure 3. PP2A<sup>Cdc55</sup> 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 Cdc14 was monitored by fluorescence microscopy. The release of Cdc14 from the nucleolus was also monitored by immunoblotting. (C) CDC55 and cdc55-mn cells were arrested in metaphase I by transferring cells to SPM for 8 h. The nucleolar localization of Cdc14 was monitored by fluorescence microscopy. The release of Cdc14 from the nucleolus was also monitored by immunoblotting. (D) CDC55, cdc55-mn, and net1-6Cdk cells were synchronized in G<sub>1</sub> by transferring cells to SPM for 8 h. The release of Cdc14 from the nucleolus was monitored by immunoblotting. (E) Cells were treated with ethanol or estradiol and the percentage of tetranucleate cells was determined. The timing of estradiol addition was varied and the percentage of tetranucleate cells was determined.
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 <i>CDC55</i> and <i>cdc55-mn</i> cells. Expression of <i>CDC5</i> and <i>CLB3</i> during meiosis is dependent on Ndt80, the meiosis-specific transcription factor, which is activated after cells exit from pachytene (Chu et al., 1998). Although <i>CLB3</i> 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 <i>CDC55</i> and <i>cdc55-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 <i>cdc55-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 <i>CDC55</i> cells went through two rounds of nuclear division and assembled metaphase I, anaphase I, and meiosis II (metaphase II cells went through two rounds of nuclear division and <i>CDC55</i> required for the formation of metaphase I spindles. Although <i>cdc55-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 <i>cdc55-mn</i> cells, conditional <i>ccl28-ts</i> mutants arrest in meiotic prophase I with unseparated SPBs (Shuster and Byers, 1989). Failure of <i>cdc55-mn</i> 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 <i>cdc55-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 <i>CDC55</i> and <i>cdc55-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 tetrancleates with anaphase II spindles (unpublished data). Cdc14 also localized to the nucleolus in <i>cdc55-mn</i> cells. However, we observed that nucleoli began to split after 3 h in SPM, and by 6 h, 50% of mononucleate <i>cdc55-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’Amous et al., 2004), our data suggest that Cdc14 in <i>cdc55-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 <i>cdc55-mn</i> cells. Therefore, we synchronized <i>CDC55</i> and <i>cdc55-mn</i> cells in metaphase I by deletion 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 <i>cdc55-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 <i>cdc55-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 <i>cdc55-mn</i> cells, we prepared protein extracts from <i>CDC55 NET1-myc9</i> and <i>cdc55-mn NET1-myc9</i> cells arrested in metaphase I and assayed the electrophoretic mobility of Net1 by Western analysis. Net1 from <i>cdc55-mn</i> cells arrested in metaphase I had a lower electrophoretic mobility compared with Net1 from <i>CDC55</i> cells (Fig. 3 D). To test whether the hypershift of Net1 in <i>cdc55-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 <i>cdc55-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 <i>cdc55-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).

---

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 net1-6cdc5cdc55-mn [Y2307] cells containing <i>P<sup>cdc28-2</sup>CDC20</i> 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 <i>cdc55-mn</i> cells arrested in metaphase I. A 120-kD cross-reacting band obtained with an anti-Cdc28 antibody served as a loading control. (E) <i>P<sup>cyc2-1</sup> GAL4–ER</i> [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.
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 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 nuclear segregation during mitosis (Azzam et al., 2004). To test whether nuclear 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 nuclear 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 biniucleates 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 biniucleates (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 biniucleates with prophase II spindles in net1-6Cdk cdc55-mn cells. Indeed, we found that the proportion of biniucleate 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 cdc55-mn cells. Although NET1 cdc55-mn cells failed to sporulate, the net1-6Cdk cdc55-mn 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^{Cdc55} 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. 4 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.
Materials and methods
Yeast strains and plasmids
A complete list of yeast strains and their genotypes can be found in Table S1. The Pca2p-ha3-CDC55 allele was constructed by PCR-mediated transformation using Pca2-ha3-KomMX6 as previously described (Lee and Amon, 2003). SK1 strains bearing the NET1-TEV-9 myc and net1-TEV-9 myc-Cdk2 alleles were created by backcrossing strains JD2632 and JD2626 (Azazen 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 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 potassium 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 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 five times 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 slides were sealed with nail polish. Slides were either used for microscopical 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-Nap1, rabbit anti-Cdc14 antibody (SC-33628, Santa Cruz Biotechnology, Inc.), and monoclonal mouse anti-HA (16812; 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 100x 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 exposure, 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 yis 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 MgCl2), 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% liposol, 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 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-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 (Kibbuz 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 OD500 of 0.2 and grown for 16 h. Cells were then washed with water and resuspended in 5PM (0.3% KOAc, pH 7.0) at an OD500 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 MgCl2, and 50 µg/ml propidium iodide) and 50 µg/ml RNase A for 1 h at 37°C. Cells were then 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 PP2ACdc55 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.

We would like to thank R. Deshaies (California Institute of Technology, Pasadena, CA), A. Amon (Massachusetts Institute of Technology, Cambridge, MA), K. Nasmyth (University of Oxford, Oxford, England, UK), S. Roeder (Yale University, New Haven, CT), R. Cha (National Institute of Medical Research, London, England, UK), Kyung Lee (National Cancer Institute, Bethesda, MD), and F. Uhlmann (London Research Institute, London, England, UK) for strains, antibodies, and plasmids.

Dr. Prakash Arumugam’s laboratory is funded by a research grant from the Biotechnology and Biological Sciences Research Council (BB/ G00353X/1). G.W. Kerr is supported by a Biotechnology and Biological Sciences Research Council-funded studentship.

Submitted: 2 March 2011
Accepted: 25 May 2011

Downloaded from on May 29, 2017

PP2ACdc55 prevents premature exit from meiosis I • Kerr et al.
References


Supplemental material

Kerr et al., http://www.jcb.org/cgi/content/full/jcb.201103019/DC1

Figure S1. **PP2ACdc55** is not required for SC assembly and disassembly but is required for SPB separation during meiosis. (A) Wild-type (Y1843) and cdc55-mn (Y2198) cells were induced to sporulate. Aliquots of the sporulating culture were taken every hour and subjected to chromosome spreading. The spreads were stained using anti-HA (to visualize Rec8) and anti-Zip1 (to visualize SC) antibodies. Frequencies of partial SC and full SC observed in chromosome spreads from the two cultures were plotted as a function of time. These are representative data from three experimental repeats. (B) Representative images of full SC, partial SC, dot/minimal SC, and no signal/polycomplexes are shown. (C) CDC55 (Y2111) and cdc55-mn (Y2149) cells containing SPC42-GFP were induced to sporulate. SPB separation was detected by counting the number of Spc42-GFP dots in the two sporulating cultures (n = 200). Bar, 2 µm.
Figure S2. Nuclear division defect of cdc55-mn cells is not caused by activation of pachytene/spindle assembly checkpoints or lack of Clb1 expression or hyperphosphorylation of Cdc28 at Y19 by Swe1. (A) CDC55 (Y1737), cdc55-mn (Y1738), rad24 (Y2280), cdc55-mn rad24 (Y2282), spo11 (Y2270), cdc55-mn spo11 (Y2271), mad2 (Y2289), and mad2 cdc55-mn (Y2156) strains were induced to sporulate for 24 h. Cells were harvested, and nuclear division was scored by staining with DAPI (n = 500). (B) Diploid CDC55 CLB1myc9 (Y2027) and cdc55-mn CLB1myc9 (Y2028) cells were induced to sporulate. Clb1 expression was assayed in the two sporulating cultures by immunofluorescence using anti-Myc antibodies. (C) CDC55 (Y1737), cdc55-mn (Y1738), swe1Δ (Y2102), cdc55-mn swe1Δ (Y2103), cdc28-T18A/Y19F (Y2241), and cdc55-mn cdc28-T18A/Y19F (Y2242) strains were induced to sporulate for 24 h. Cells were harvested, and nuclear division was scored by staining with DAPI (n = 500). Error bars represent SEM.
PP2ACdc55 prevents premature exit from meiosis I

Figure S3. Premature release of Cdc14 during meiosis inhibits formation of metaphase I spindles, and PP2ACdc55 acts downstream of Slk19 and Spo12 in the FEAR pathway. (A) Diploid PGAL-TAB6 GAL4-ER PCLB2-CDC20 (Y2285) and GAL4-ER PCLB2-CDC20 (Y2287) strains were arrested in metaphase I by transferring them to SPM for 8 h. Estradiol was added at 3, 4, and 5 h to the cultures, and the effect on spindle formation was determined by immunofluorescence using anti-tubulin antibodies (n = 200). These are representative data from three experimental repeats. (B) CDC55 (Y1737), cdc55-mn (Y1738), slk19 Δ (Y2267), cdc55-mn slk19 Δ (Y2269), spo12 Δ (Y2272), and cdc55-mn spo12 Δ (Y2274) strains were induced to sporulate for 24 h. Cells were harvested, and nuclear division was scored by staining with DAPI (n = 500). Error bars represent SEM.
### Table S1. List of yeast strains used in the study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Used in figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1737</td>
<td>MATa/α, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6</td>
<td>S2 and S3</td>
</tr>
<tr>
<td>Y1738</td>
<td>MATa/α, cdc55::P_CAD-ha3-CDC55::KanMX6</td>
<td>S2 and S3</td>
</tr>
<tr>
<td>Y1843</td>
<td>MATa/α, leu2::tetR-GFP/LEU2/leu2::tetR-GFP, REC8-ha3::URA3/REC8-ha3::URA3, PDS1-myc18::KTRP1/PDS1-myc18::KTRP1, leu2::tetR-GFP/URA3</td>
<td>1 and S1</td>
</tr>
<tr>
<td>Y2027</td>
<td>MATa/α, CLB1-myc9::KTRP1/CLB1-myc9::KTRP1</td>
<td>S2</td>
</tr>
<tr>
<td>Y2028</td>
<td>MATa/α, CLB1-myc9::KTRP1/CLB1-myc9::KTRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6</td>
<td>S2</td>
</tr>
<tr>
<td>Y2072</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::NET1-TEV-myc9::TRP1/trp1::NET1-TEV-myc9::TRP1, rec8::REC8-ha3::URA3/rec8::REC8-ha3::URA3, leu2::tetR-GFP/LEU2/leu2::tetR-GFP, spo11::KanMX6/ spo11::KanMX6, leu2::tetR-GFP/LEU2/leu2::tetR-GFP, spo11::KanMX6/ spo11::KanMX6</td>
<td>4 and 5</td>
</tr>
<tr>
<td>Y2075</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::NET1-TEV-myc9::TRP1/trp1::NET1-TEV-myc9::TRP1, rec8::REC8-ha3::URA3/rec8::REC8-ha3::URA3, leu2::tetR-GFP/LEU2/leu2::tetR-GFP/LEU2, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6</td>
<td>4 and 5</td>
</tr>
<tr>
<td>Y2078</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::net1-6CdK-TEVm9::TRP1/trp1::net1-6CdK-TEVm9::TRP1, rec8::REC8-ha3::URA3/rec8::REC8-ha3::URA3, leu2::tetR-GFP/LEU2/leu2::tetR-GFP/LEU2, ura3::tetO::URA3/ura3</td>
<td>4 and 5</td>
</tr>
<tr>
<td>Y2081</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::NET1-TEV-myc9::TRP1/trp1::NET1-TEV-myc9::TRP1, rec8::REC8-ha3::URA3/rec8::REC8-ha3::URA3, leu2::tetR-GFP/LEU2/leu2::tetR-GFP/LEU2, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, net1::HIS5/ net1::HIS5</td>
<td>4 and 5</td>
</tr>
<tr>
<td>Y2102</td>
<td>MATa/α, swe1::HIS3/swe1::HIS3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2103</td>
<td>MATa/α, swe1::HIS3/swe1::HIS3, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, ura3::tetO::URA3/ura3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2111</td>
<td>MATa/α, spo11::HIS5/SPC4-ha3-GFP::HIS3</td>
<td>2 and S1</td>
</tr>
<tr>
<td>Y2119</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::NET1-TEV-myc9::TRP1/trp1::NET1-TEV-myc9::TRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, cdc20::P_CAD-ha3-CDC55::KanMX6/cdc20::P_CAD-ha3-CDC55::KanMX6</td>
<td>3</td>
</tr>
<tr>
<td>Y2120</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::NET1-TEV-myc9::TRP1/trp1::NET1-TEV-myc9::TRP1, cdc20::P_CAD-ha3-CDC55::KanMX6/cdc20::P_CAD-ha3-CDC55::KanMX6, rad24::X::URA3/ rad24::X::URA3</td>
<td>3</td>
</tr>
<tr>
<td>Y2149</td>
<td>MATa/α, spo11::HIS5/SPC4-ha3-GFP::HIS3, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, rad24::X::URA3/ rad24::X::URA3</td>
<td>2</td>
</tr>
<tr>
<td>Y2156</td>
<td>MATa/α, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, mad2::HIS3/ mad2::HIS3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2161</td>
<td>MATa/α, rad24::X::GAL4(848)::ER::URA3/ura3::GAL4(848)::ER::URA3</td>
<td>3</td>
</tr>
<tr>
<td>Y2198</td>
<td>MATa/α, leu2::tetR-GFP/LEU2/leu2::tetR-GFP/LEU2, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, leu2::tetR-GFP/LEU2/leu2::tetR-GFP/LEU2, ura3::tetO::URA3/ura3</td>
<td>1 and S1</td>
</tr>
<tr>
<td>Y2212</td>
<td>MATa/α, ura3::GPD1/URA3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2244</td>
<td>MATa/α, cdc28::GPD1/URA3</td>
<td>3</td>
</tr>
<tr>
<td>Y2242</td>
<td>MATa/α, cdc28::GPD1/URA3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2267</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::net1-6CdK-TEVm9::TRP1/trp1::net1-6CdK-TEVm9::TRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, spo11::HIS5/ spo11::HIS5</td>
<td>S3</td>
</tr>
<tr>
<td>Y2271</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::net1-6CdK-TEVm9::TRP1/trp1::net1-6CdK-TEVm9::TRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, spo11::HIS5/ spo11::HIS5</td>
<td>S3</td>
</tr>
<tr>
<td>Y2272</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::net1-6CdK-TEVm9::TRP1/trp1::net1-6CdK-TEVm9::TRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, spo11::HIS5/ spo11::HIS5</td>
<td>S3</td>
</tr>
<tr>
<td>Y2277</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::net1-6CdK-TEVm9::TRP1/trp1::net1-6CdK-TEVm9::TRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, spo11::HIS5/ spo11::HIS5</td>
<td>S3</td>
</tr>
<tr>
<td>Y2278</td>
<td>MATa/α, net1::HIS5/net1::HIS5, trp1::NET1-TEV-myc9::TRP1/trp1::NET1-TEV-myc9::TRP1, cdc55::P_CAD-ha3-CDC55::KanMX6/cdc55::P_CAD-ha3-CDC55::KanMX6, spo11::HIS5/ spo11::HIS5</td>
<td>S3</td>
</tr>
<tr>
<td>Y2280</td>
<td>MATa/α, rad24::X::GPD1/URA3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2282</td>
<td>MATa/α, rad24::X::GPD1/URA3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2288</td>
<td>MATa/α, rad24::X::GPD1/URA3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2285</td>
<td>MATa/α, ura3::GPD1/URA3</td>
<td>S3</td>
</tr>
<tr>
<td>Y2287</td>
<td>MATa/α, ura3::GPD1/URA3</td>
<td>S3</td>
</tr>
<tr>
<td>Y2289</td>
<td>MATa/α, rad24::X::GPD1/URA3</td>
<td>S2</td>
</tr>
<tr>
<td>Y2307</td>
<td>MATa/α, ura3::GPD1/URA3</td>
<td>3</td>
</tr>
</tbody>
</table>

All yeast strains are derivatives of SK1 and have the following markers, unless otherwise stated: ha::YLS2/ha::YLS2, ura3::ura3, leu2::hisG/leu2::hisG, trp1::hisG/trp1::hisG, his3::hisG/his3::hisG, and lys2/lys2.