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Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast

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Background: Mitosis is regulated by MPF (maturation promoting factor), the active form of Cdc2/28–cyclin B complexes. Increasing levels of cyclin B abundance and the loss of inhibitory phosphates from Cdc2/28 drives cells into mitosis, whereas cyclin B destruction inactivates MPF and drives cells out of mitosis. Cells with defective spindles are arrested in mitosis by the spindle-assembly checkpoint, which prevents the destruction of mitotic cyclins and the inactivation of MPF. We have investigated the relationship between the spindle-assembly checkpoint, cyclin destruction, inhibitory phosphorylation of Cdc2/28, and exit from mitosis.

Results: The previously characterized budding yeast *mad* mutants lack the spindle-assembly checkpoint. Spindle depolymerization does not arrest them in mitosis because they cannot stabilize cyclin B. In contrast, a newly isolated mutant in the budding yeast *CDC55* gene, which encodes a protein phosphatase 2A (PP2A) regulatory subunit, shows a different checkpoint defect. In the presence of a defective spindle, these cells separate their sister chromatids and leave mitosis without inducing cyclin B destruction. Despite the persistence of B-type cyclins, *cdc55* mutant cells inactivate MPF. Two experiments show that this inactivation is due to inhibitory phosphorylation on Cdc28: phosphotyrosine accumulates on Cdc28 in *cdc55Δ* cells whose spindles have been depolymerized, and a *cdc28* mutant that lacks inhibitory phosphorylation sites on Cdc28 allows spindle defects to arrest *cdc55* mutants in mitosis with active MPF and unseparated sister chromatids.

Conclusions: We conclude that perturbations of protein phosphatase activity allow MPF to be inactivated by inhibitory phosphorylation instead of by cyclin destruction. Under these conditions, sister chromatid separation appears to be regulated by MPF activity rather than by protein degradation. We discuss the role of PP2A and Cdc28 phosphorylation in cell-cycle control, and the possibility that the novel mitotic exit pathway plays a role in adaptation to prolonged activation of the spindle-assembly checkpoint.

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Background

Cell-cycle checkpoints coordinate the cell cycle by making one step dependent upon the successful completion of previous steps (reviewed in [1–3]). Faithful transmission of genetic information depends on accurate chromosome segregation, which in turn requires a microtubule structure known as the mitotic spindle. Sister kinetochores in a replicated pair of sister chromatids are attached to opposite poles of the bipolar spindle, so that when they separate they are drawn apart, ensuring that each daughter cell receives one copy of each chromosome. The spindle-assembly checkpoint contributes to the accuracy of mitosis by delaying the onset of anaphase until the spindle has been fully assembled and each pair of sister chromatids is attached to it. Exit from mitosis depends on the proteolysis of cyclin B [4], and sister separation appears to require the destruction of other proteins [5–7]; the destruction of both sets of proteins requires a specific set of ubiquitinating enzymes [8–10]. Cyclin B destruction leads to the inactivation of MPF (maturation promoting factor or M-phase promoting factor), the Cdc2/28–cyclin B complex, whose activity is measured experimentally as histone H1 kinase [11] (Cdc28 is the budding yeast homolog of the fission yeast Cdc2 protein).

Our current understanding of the spindle-assembly checkpoint comes from a variety of approaches. Experimental manipulations of insect spermatocytes and vertebrate tissue culture cells suggest that kinetochores that have not attached to the spindle act to delay anaphase [12–14], and have identified a kinetochore-associated phosphoepitope found only on those chromosomes that are improperly

aligned on the spindle [15,16]. Biochemical analysis shows that preventing spindle assembly in *Xenopus* egg extracts blocks cyclin B degradation and MPF inactivation, mimicking the mitotic arrest seen in nocodazole-treated tissue culture cells [17]. The MAP kinase ERK2 is required for proper functioning of the frog checkpoint, but neither the signal being detected, nor the mechanism by which cyclin degradation is blocked are known [17].

Genetic analysis of budding yeast has identified multiple genes involved in the spindle assembly checkpoint (*MAD1–3*, *BUB1–3* and *MPS1*) [18–23]. Most of these genes were identified by mutations that allow cells to pass through mitosis without a fully assembled mitotic spindle. In the presence of a defective spindle assembly checkpoint, mutants fail to sustain MPF activity, allowing them to pass through mitosis and into the next cell cycle with lethal consequences [18,19]. Components of the spindle-assembly checkpoint are conserved between budding yeast, fission yeast, frogs and mammals [23–25].

Here, we show that budding yeast *cdc55* mutants define a novel class of spindle assembly checkpoint mutants. *CDC55* encodes a protein homologous to the B regulatory subunit of a type 2A protein phosphatase (PP2A) [26,27]. Comparing *cdc55* cells with the *mad* mutants reveals a novel pathway for exit from mitosis. In *mad1*, *mad2* and *mad3* mutants, spindle disruption fails to protect B-type cyclins from degradation. In contrast, *cdc55* cells do not degrade B-type cyclins, although they still inactivate MPF and separate their sister chromatids. Replacement of inhibitory phosphorylation sites in Cdc28 with nonphosphorylatable residues allows *cdc55* cells that lack spindles to sustain their MPF levels and prevent sister chromatid separation. We discuss these results with regard to the role of PP2A in cell-cycle regulation, mechanisms of escaping from mitotic arrest, and the roles of proteolysis and phosphorylation in sister chromatid cohesion.

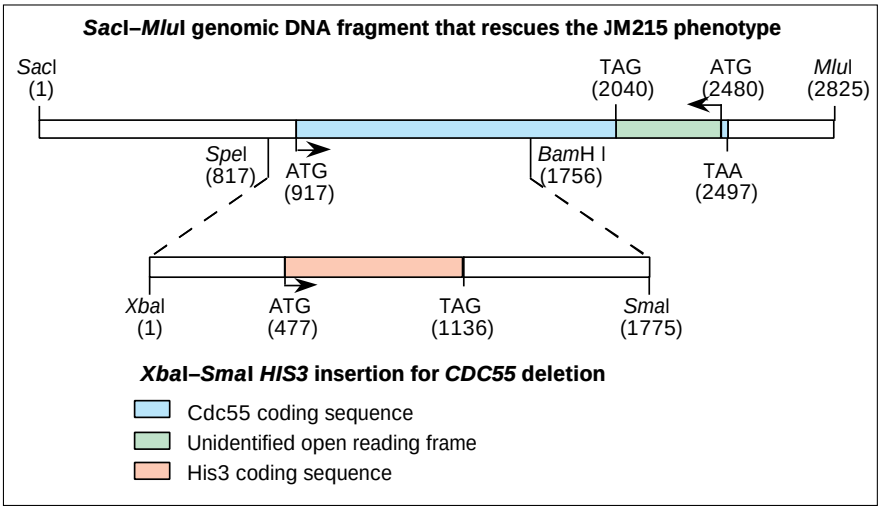
Results

cdc55 mutants lack a functional spindle assembly checkpoint

Because the original screens for mitotic checkpoint mutants in budding yeast were not saturating [18,19], we performed another screen for mutants that were hypersensitive to the microtubule-depolymerizing drugs benomyl and nocodazole. We used similar criteria to those already described [19] to isolate putative spindle-assembly checkpoint mutants: hypersensitivity to benomyl and nocodazole, and re-budding accompanied by rapid loss of cell viability when exposed to concentrations of these drugs that arrest wild-type cells in mitosis.

One recessive mutant that passed all of these tests, designated JM215, was cloned by complementation using a genomic DNA library. Figure 1 shows a map of the rescuing clone. Sequencing the ends of the clone allowed us to identify the genomic DNA fragment as one containing two open reading frames: the *CDC55* gene, which encodes a yeast homologue of the B subunit of a PP2A [26]; and a sequence encoding an unidentified 147 amino-acid polypeptide. We constructed a deletion plasmid that removed the promoter and amino-terminal 280 codons of *CDC55* but not the second open reading frame, replacing them with the *HIS3* marker (Fig. 1). Integration of this plasmid at the genomic locus resulted in a strain (*cdc55Δ*) with the same spindle-assembly checkpoint defects as JM215: increased sensitivity to benomyl on plates (Fig. 2a), and rapid death in liquid media containing 10 μg ml^{-1} nocodazole (Fig. 2b). As with other *mad* mutants, this

Figure 1



Restriction map of the 2.8 kb *Sac*I–*Mlu*1 *Saccharomyces cerevisiae* genomic DNA fragment that rescued the benomyl sensitivity and sporulation defect of JM215. The ends (300 bp) of the fragment were sequenced and compared with sequences in the Genbank database. This identified the DNA as a 2830 bp segment containing *CDC55* and an unidentified open reading frame. A *CDC55* deletion construct was made by inserting the *HIS3* gene between the *Spe*I and *Bam*HI sites as indicated.

nocodazole sensitivity was rescued by the inclusion of 10 mg ml^{-1} of the DNA synthesis inhibitor hydroxyurea (Fig. 2b, [19]). Diploids formed by crossing *cdc55Δ* with strains carrying the mutation present in JM215 failed to sporulate unless they contained a plasmid carrying the wild-type *CDC55* gene. All the spores dissected from such diploids and then cured of the *CDC55* plasmid displayed the phenotypes of JM215, demonstrating that this mutant contains a loss-of-function mutation in *CDC55*, designated *cdc55-215*. All subsequent experiments were conducted on the deletion strain.

Clb proteins are stable in *cdc55* mutants but MPF is inactivated

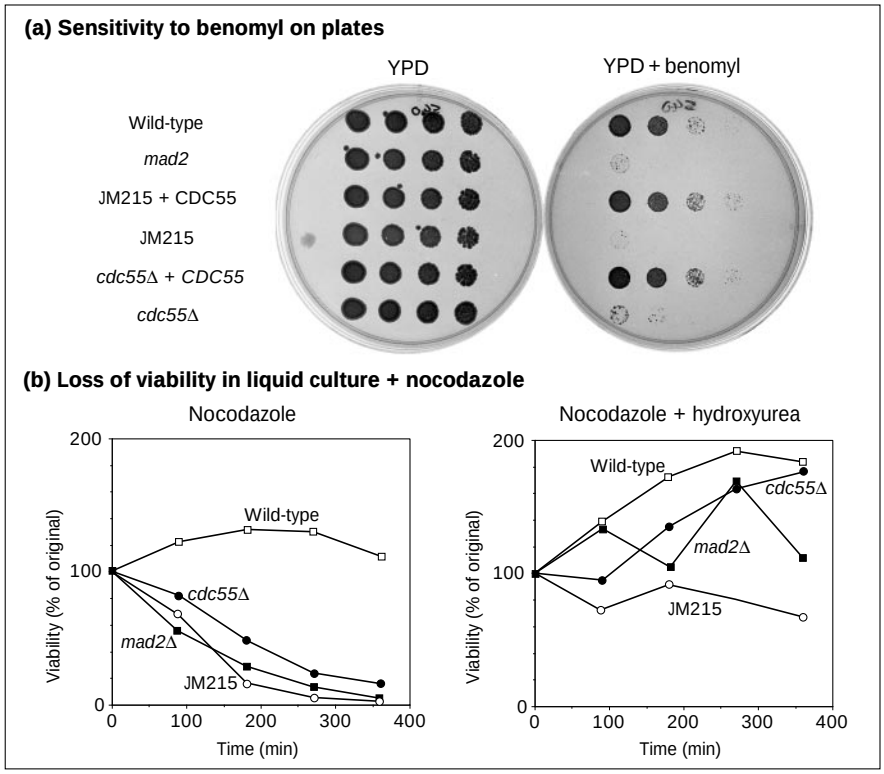
Of the six previously reported spindle-assembly checkpoint genes, *BUB1*, *BUB2* and *MAD2* have been shown to be required to sustain MPF activity when spindle microtubules are depolymerized [18,19]. MPF is normally inactivated at the end of mitosis by cyclin B proteolysis, and preventing this destruction prevents exit from mitosis [4,28]. We therefore wondered if the failure of *mad* mutants to arrest in mitosis resulted from their inability to prevent cyclin proteolysis. We looked at the levels of the yeast mitotic cyclins, Clb2 and Clb3 [29,30], in cells synchronously released from an α factor (G1-phase) block into medium containing 10 μg ml^{-1} nocodazole, a concentration that activates the spindle-assembly checkpoint in wild-type cells. Figure 3a shows that, in wild-type cells, nocodazole treatment stabilizes Clb2 and Clb3, leading to high levels of histone H1 kinase activity in Clb-associated Cdc28 (Fig. 3b). In contrast, *mad2* cells failed to prevent degradation of Clb2 and Clb3, and as a result the Clb-associated histone H1 kinase levels fell. We saw a similar fall of Clb protein levels and kinase activities in other spindle-assembly checkpoint mutants (*mad1*, *mad3* and *bub1*; data not shown).

The behavior of the mitotic cyclins in *cdc55Δ* cells differed from both wild-type and other spindle-assembly checkpoint mutants. Clb2 appeared stable in *cdc55Δ* cells released from a G1 arrest into nocodazole (Fig. 3a), but although histone H1 kinase activity initially rose (a little slower than in wild-type cells, probably resulting from its longer doubling time), the mutant was unable to sustain high kinase levels (Fig. 3b). Histone H1 kinase activity of Clb2 immunoprecipitates containing equivalent amounts of Cdc28 (as judged by western blotting with anti-PSTAIRE antibodies) was 3–5-fold lower in nocodazole-treated *cdc55Δ* cells than in wild-type cells.

Loss of MPF activity correlates with sister chromatid separation

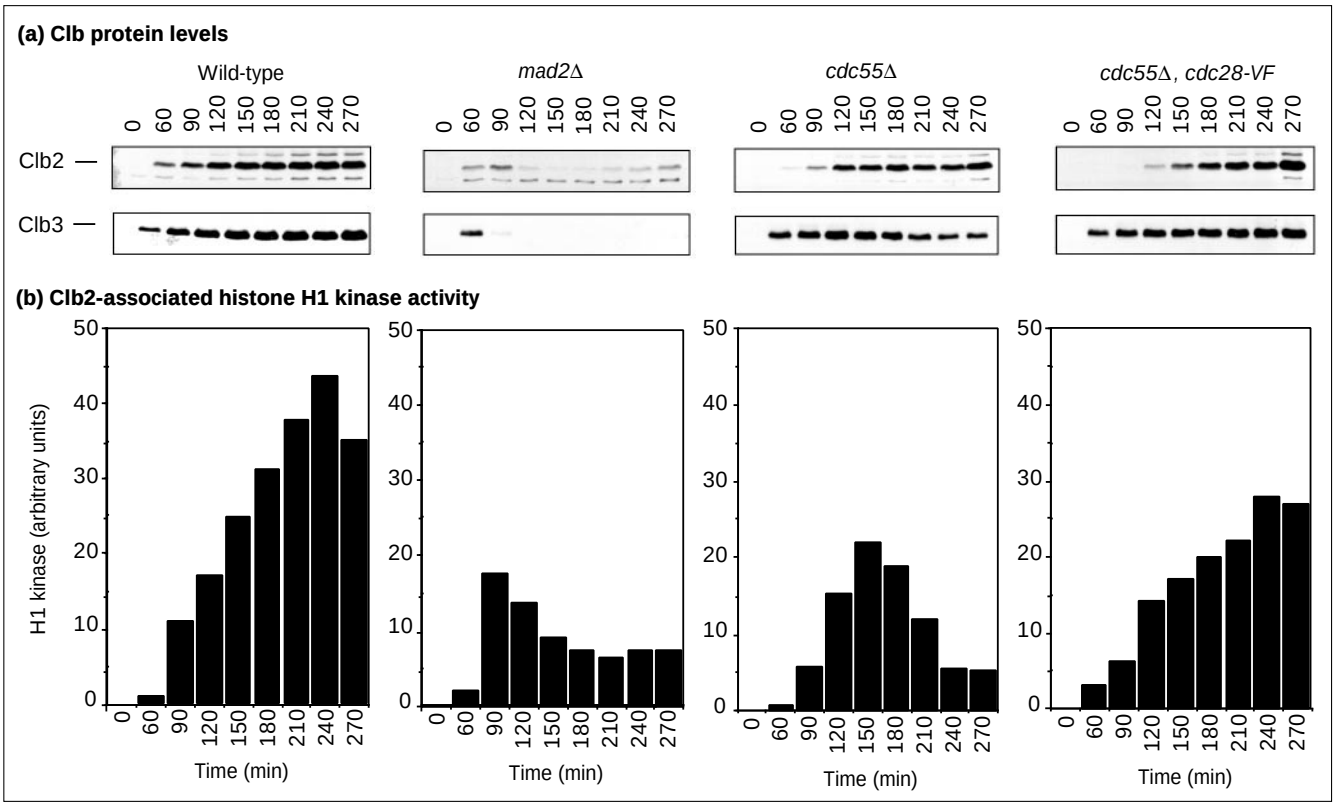
If sister chromatids separate before they have attached to the spindle, they will presumably segregate randomly with respect to each other. Thus, to prevent the generation of

Figure 2



Benomyl sensitivity of JM215, *mad2Δ* and *cdc55Δ*. **(a)** Yeast were grown in YPD overnight, diluted to A_{600} 1.0 and four serial 10-fold dilutions were replicated onto YPD plates containing no additions (left) or 7.5 μg ml^{-1} benomyl (right). The plates were incubated at 23 °C for 2 days. **(b)** Yeast were grown overnight in YPD to mid-log phase, then diluted to A_{600} 0.3 in YPD containing 10 μg ml^{-1} nocodazole ± 10 mg ml^{-1} hydroxyurea. Samples were withdrawn at 90 min intervals, diluted 5000-fold, and 200 μl was spread onto a YPD plate which was incubated at 23 °C for 3 days. The number of colonies was compared with the number immediately before nocodazole addition to determine the percent remaining viable.

Figure 3



Clb2 protein and associated kinase levels in wild-type, *mad2Δ*, *cdc55Δ* and *cdc55Δ, Cdc28-VF* cells. **(a)** The four yeast strains were grown overnight in YPD to mid-log phase, then diluted to A_{600} 0.3 in YPD containing 10 μg ml^{-1} α factor. Cells were arrested for 150 min, then washed into YPD containing 10 μg ml^{-1} nocodazole. α factor was added back to the cultures 90 min after the nocodazole to prevent reaccumulation of Clb2 in cells that had exited from mitosis. Samples were taken at 30 min intervals, lysed and immunoblotted for Clb2 and Clb3 proteins. This experiment was repeated five times with similar results. **(b)** Clb2 was immunoprecipitated from samples of the lysates prepared in (a). The immunoprecipitates were tested for their ability to phosphorylate histone H1. The kinase reactions were run on a 15 % polyacrylamide gel and radiolabeled phosphate incorporation was measured using a Molecular Dynamics Phosphorimager.

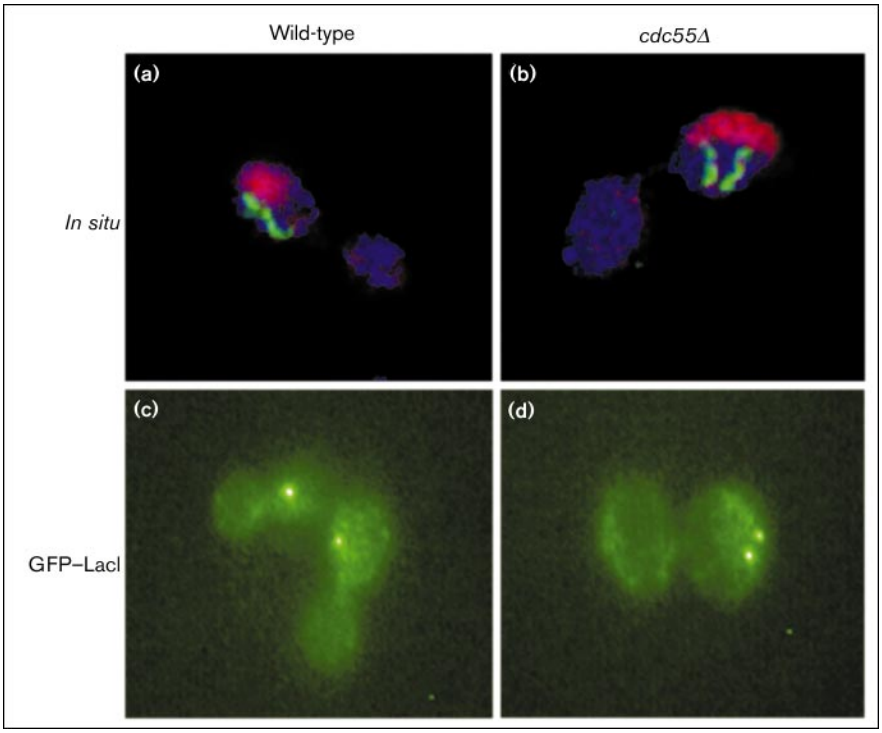
aneuploidy, the spindle assembly checkpoint should prevent sister separation as well as the inactivation of MPF. We therefore investigated whether sister separation occurred in mutants that inactivated the spindle assembly checkpoint.

We used two assays to monitor sister chromatid linkage. The first was a modification of existing *in situ* hybridization protocols [31,32] that allowed the ribosomal DNA (rDNA) locus on chromosome XII to be localized in cells that had not been subjected to proteolysis [33]. The second was a novel technique based on the binding of a fusion protein, GFP–LacI (green fluorescent protein fused to the Lac repressor), to a 10 kb array consisting of 256 tandem repeats of the Lac operator (LacO) integrated into chromosome III [34].

Figure 4 shows chromosomes in nocodazole-treated wild-type and *cdc55Δ* cells visualized either by *in situ* hybridization or by induction of the GFP–LacI fusion. In both cases, a single signal was seen in wild-type cells and two separate signals were seen in *cdc55Δ* cells, indicating that an intact spindle-assembly checkpoint is required to maintain sister chromatid linkage in cells whose spindles have been depolymerized. The two sister separation assays gave quantitatively very similar results (data not shown).

We used the LacO-tagged chromosome to compare the effects of the two different classes of spindle-assembly checkpoint mutant on sister chromatid separation in cells whose spindle had been depolymerized by nocodazole treatment. Figure 5 shows that less than 5 % of wild-type cells arrested in nocodazole had two fluorescent spots in their nuclei, indicating that sister chromatids had not separated by more than the resolution limit of the light microscope (0.2 μm). In both *mad2Δ* and *cdc55Δ* cells, however, a dramatic increase in the fraction of nuclei containing two spots coincided with the drop in MPF activity, showing that defects in the spindle-assembly checkpoint allow both MPF inactivation and sister separation in cells that

Figure 4



Visualizing sister chromatid separation in (a,c) wild-type and (b,d) *cdc55Δ* cells treated with nocodazole by (a,b) *in situ* hybridization or (c,d) with a GFP–LacI fusion protein. The *in situ* hybridization images were prepared with a probe to the ribosomal DNA. The GFP images were prepared from strains with a Lac operator near the centromere of chromosome III and which expressed a GFP–Lac repressor fusion. Note that sister chromatids are still linked in wild-type cells, but have separated in *cdc55Δ* cells.

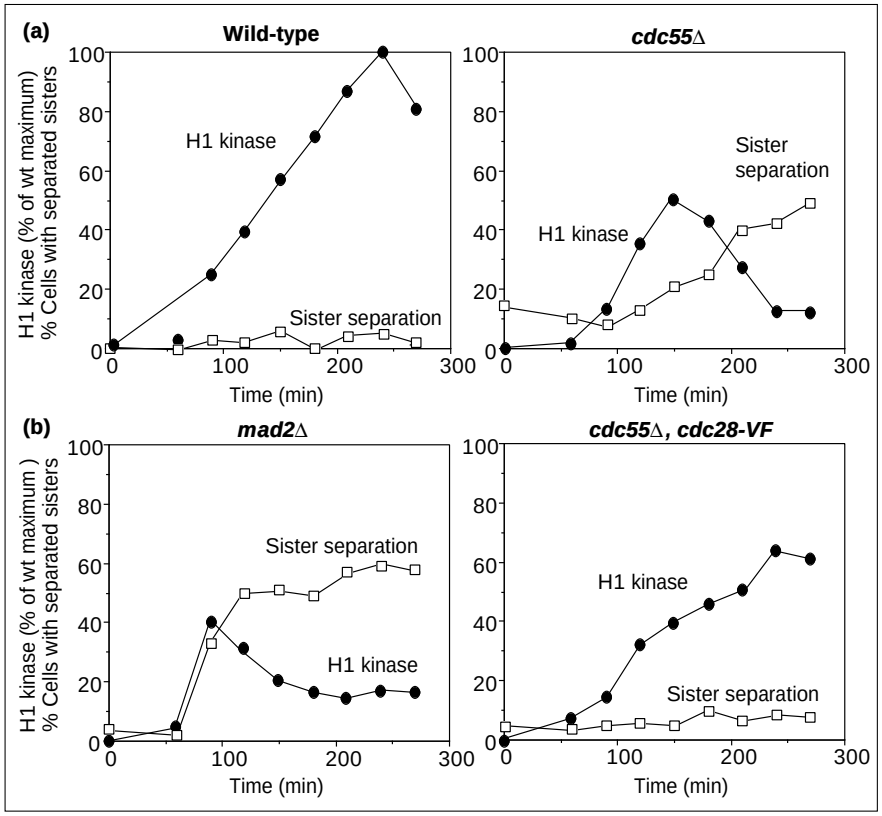
lack spindles. In the *mad2Δ* mutant, sister separation also coincided with Clb2 proteolysis, but there was no such proteolysis seen in *cdc55Δ* cells (compare Fig. 3a,b with Fig. 5). To confirm that the GFP–LacI signal was indeed measuring sister chromatid separation, we also used *in situ* hybridization to assess the degree of sister chromatid association (data not shown). The two methods gave quantitatively similar results and both showed that the fraction of nuclei containing separated sister chromatids in nocodazole-treated *mad2Δ* or *cdc55Δ* cells never exceeded 50–60 %. Nuclei with a single spot could reflect either the lack of sister chromatid separation in a fraction of the nuclei, or the presence of nuclei in which the marked sisters had separated but could not be resolved by light microscopy.

Cdc28-VF prevents MPF inactivation and sister chromatid separation

How is MPF inactivated in the absence of cyclin proteolysis in *cdc55Δ* cells? We failed to detect dissociation of Cdc28 from Clb2, or the presence of non-covalently bound Cdc28 inhibitors in *cdc55Δ* cells (data not shown). In fission yeast and vertebrate cells, the kinase activity of $p34^{cdc2}$ (the Cdc28 homolog) is inhibited during G2 by phosphorylation of threonine and tyrosine (Thr14 and Tyr15) residues [35–38] in the ATP-binding site of the enzyme [39]. The homologous residues in Cdc28 are Thr18 and Tyr19, and phosphorylation of Tyr19 has been shown to inhibit Cdc28 H1 kinase activity in *Saccharomyces cerevisiae* [40]. To test whether *cdc55Δ* cells inactivate MPF by inducing inhibitory phosphorylations on Cdc28, we made a *cdc55Δ* strain containing a mutant *cdc28* gene in which Thr18 was changed to Val18 and Tyr19 to Phe19 (*cdc28-VF*) [40]. Figure 3b shows that the H1 kinase activity of Clb2 immunoprecipitates prepared from nocodazole-treated cultures of the *cdc55Δ cdc28-VF* double mutant strain was sustained rather than falling as it did in *cdc55Δ*. Thus, *cdc55Δ* mutants are able to prevent Clb degradation in response to nocodazole, but inactivate Cdc28-associated H1 kinase activity of MPF through a pathway that requires the presence of phosphorylatable residues at Thr18 and Tyr19. By contrast, in *mad1* and *mad2* mutants, replacement of the normal *CDC28* gene by *cdc28-VF* failed to prevent the loss of Clb-associated H1 kinase activity in nocodazole-treated cells (data not shown), demonstrating that the primary defect in these mutants is the inability of spindle defects to prevent activation of the cyclin proteolysis machinery. Although the *cdc55Δ cdc28-VF* double mutant arrests in mitosis when treated with nocodazole, the cells still die. We do not know whether this lethality is due to the role of Cdc55 in the spindle-assembly checkpoint or to its involvement in other processes such as cell-wall formation and morphogenesis [26].

Examination of sister chromatid separation in nocodazole-treated *cdc55Δ cdc28-VF* cells revealed that, in addition to preventing a loss of MPF activity, the non-phosphorylatable residues in Cdc28 also prevented sister chromatid separation (Fig. 5). *cdc55Δ* cells in which Cdc28 was

Figure 5



Sister chromatid separation in spindle-assembly checkpoint mutants. Four yeast strains were grown overnight in YPD to mid-log phase, then diluted to A_{600} 0.3 in YPD containing 10 μg ml^{-1} α factor. Cells were arrested for 120 min, then washed into complete minimal media lacking histidine and containing 10 μg ml^{-1} α factor and 10 mM aminotriazole to induce expression of LacI–GFP. After a further 30 min, cells were washed into media containing 10 μg ml^{-1} nocodazole. α factor was added 90 min after the nocodazole to prevent reaccumulation of Clbs in cells that had exited from mitosis. Duplicate samples were taken at 30 min intervals. One set was lysed, Clb2 immunoprecipitated from the samples and immunoprecipitates tested for their ability to phosphorylate histone H1. The other set was washed, fixed and scored for the presence of one or two GFP-staining spots in the yeast nucleus. The data shown in this figure comes from the same experiment shown in Fig. 3.

mutated at only Thr18 or Tyr19 had intermediate phenotypes: MPF activity fell and sister chromatids separated, but more slowly and to a lesser extent than in *cdc55Δ* cells with wild-type Cdc28 (data not shown).

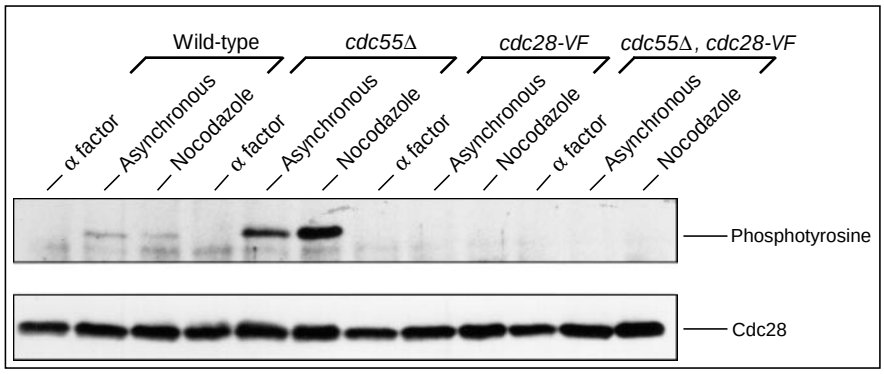
Cdc28 is tyrosine-phosphorylated in *cdc55Δ* cells treated with nocodazole

The results described above suggest that *cdc55Δ* cells treated with nocodazole escape from mitosis by phosphorylating Cdc28 on sites that inhibit its protein kinase activity. To test this idea directly, we used western blotting with anti-phosphotyrosine antibodies to assay the tyrosine phosphorylation of Cdc28 in exponentially growing and nocodazole-treated cells. Figure 6 shows that asynchronous or nocodazole-treated wild-type cells had very little phosphotyrosine in Cdc28. By contrast, Cdc28 from nocodazole-treated or asynchronous *cdc55Δ* cells contained easily detectable phosphotyrosine. As expected, nocodazole-treated *cdc55Δ* cells whose wild-type *CDC28* gene had been replaced by the *cdc28-VF* gene had no detectable phosphotyrosine on Cdc28. We conclude that the mechanism of MPF inactivation in nocodazole-treated *cdc55Δ* cells is inhibitory phosphorylation of Cdc28. The observation that no phosphotyrosine is seen on Cdc28 in α factor-treated cells is consistent with the inability of Wee1 to phosphorylate Cdc2 that is not complexed with cyclin B [41].

Cytoplasmic PP2A activity is unaltered in mitotically arrested cells lacking Cdc55

As Cdc55 is homologous to the mammalian B-regulatory subunit of PP2A [26], we wished to know whether PP2A activity differed between wild-type and *cdc55Δ* cells. Using MPF-phosphorylated histone H1 as a substrate [42], we measured the PP2A activity of yeast cell lysates as the protein phosphatase activity that was insensitive to inhibitors of protein phosphatase I but was sensitive to okadaic acid, a potent inhibitor of PP2A [27,42,43]. As shown in Figure 7, we could detect only a small difference between PP2A activity in nocodazole-treated wild-type and *cdc55Δ* cells. We wondered if the lack of difference could be because wild-type cells were arrested in mitosis, whereas the *cdc55Δ* mutants were not, but we saw no change in PP2A activity in *cdc55Δ* cells 150–240 minutes after addition of nocodazole, during which time H1 kinase activity was reduced 4-fold (Fig. 3b and data not shown). We conclude that the bulk cytoplasmic levels of PP2A are indistinguishable in wild-type and *cdc55Δ* cells, consistent with previous observations using lysates from asynchronous wild-type and *cdc55Δ* cultures [27].

Figure 6

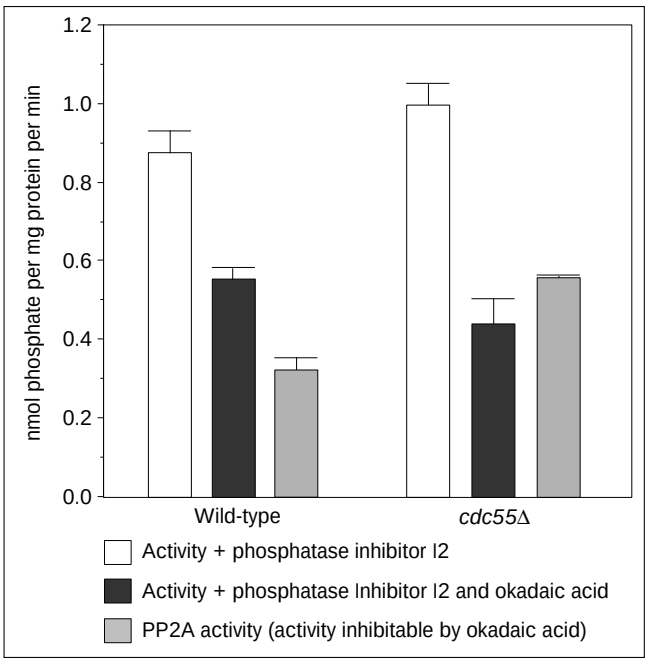


Tyrosine phosphorylation of Cdc28 in *cdc55Δ* mutants. Extracts of the indicated strains growing asynchronously or treated for 2.5 h with 10 μg ml^{-1} of α factor or 15 μg ml^{-1} of nocodazole were analyzed for tyrosine phosphorylation of Cdc28. All strains carried hemagglutinin (HA)-tagged Cdc28, which was immunoprecipitated with an anti-HA antibody (12CA5). The immunoprecipitates were western blotted with an anti-phosphotyrosine antibody, before stripping the blots and reprobing them with the anti-HA antibody (16B12) as a control for equal loading.

Discussion

In this paper, we have shown that Cdc55, a homolog of a PP2A regulatory subunit, is required for proper functioning of the spindle-assembly checkpoint. Unlike the Mad1, Mad2 and Mad3 proteins, Cdc55 is not involved in regulating the stability of B-type cyclins. Nocodazole-treated *cdc55Δ* cells contain high levels of Cdc28-associated Clb2, but these complexes have little protein kinase activity. The Cdc28 protein in these cells is tyrosine phosphorylated to a much higher level than the Cdc28 in wild-type cells treated with nocodazole. MPF remains active and sister chromatids remain linked in nocodazole-treated *cdc55Δ* mutants if amino acids Thr18 and Tyr19 of Cdc28 are mutated to non-phosphorylatable analogs, demonstrating that the absence of Cdc55 leads to MPF inactivation *via* inhibitory phosphorylation of Cdc28.

Figure 7



Phosphatase 2A activity in wild-type and *cdc55Δ* cells. Wild-type and *cdc55Δ* cells were released from α factor into nocodazole for 150 min. Lysates were prepared and assayed for phosphatase activity (see Materials and methods). Both samples were incubated in the presence of inhibitor I2 to inhibit phosphatase 1 activity. The samples in the open bars contained no other inhibitor, whereas those in the dark grey bars also contained okadaic acid, an inhibitor of protein phosphatase 2A (PP2A). The light gray bars represent the difference between the activities measured in the presence and absence of okadaic acid. We attribute this difference to PP2A activity. Duplicate samples were taken at 0, 15 and 30 min, during which time inorganic phosphate release (measured as described in [42]) was linear, and less than 10 % of the substrate was dephosphorylated. The bars indicate half of the range between the duplicate measurements.

The homologs of Thr18 and Tyr 19 (Thr14 and Tyr15) are located within the ATP-binding site of Cdk2 [39], and phosphorylation of these sites been shown to inhibit kinase activity [40,44]. Inhibitory phosphorylation in the ATP-binding site of p34^{cdc2} (the Cdc28 homolog in other eukaryotes) has been implicated in the checkpoint that prevents premature MPF activation in both fission yeast and frogs ([37,38,45], but see also [46]). Studies in budding yeast, however, have shown that mutation of amino acids Thr18 and Tyr19 does not affect the ability of DNA synthesis inhibitors to arrest the cell cycle [47,48]. Phosphorylation of Cdc28 is involved in a checkpoint that inhibits passage through mitosis in cells that have not budded, and which may be analogous to the G2/M transition in other eukaryotes [49]. Here, we have shown that, as well as preventing entry into mitosis, phosphorylation of Thr18 and Tyr19 can cause a mitotic cell to progress to interphase when cyclin proteolysis has been prevented by the spindle-assembly checkpoint. Thus, the same set of modifications can both prevent entry into mitosis and induce exit from mitosis.

Sister chromatids separate when MPF activity falls

We have shown that budding yeast mutants with defects in the spindle-assembly checkpoint separate their sister chromatids despite their failure to assemble a spindle. Cells lacking the *MAD2* gene separate their sisters at

about the same time that they degrade the Clb2 B-type cyclins. The destruction of the mitotic cyclins is mediated by a cyclin-ubiquitinating complex that contains the Cdc16, Cdc23 and Cdc27 proteins [8–10]. Experimental modifications of cyclin structure or expression that prevent MPF inactivation do not prevent sister separation, as long as the cyclin-proteolysis machinery remains active [5,50]. These observations have suggested that non-cyclin proteins must be degraded to allow sister separation, and recent experiments in fission yeast show that Cut2 destruction by the cyclin proteolysis machinery is required for sister separation [6]. We were therefore surprised to find that nocodazole-treated *cdc55Δ* cells also separate their sisters as MPF levels fall, despite the lack of significant Clb proteolysis. Furthermore, a strain that was both *cdc55Δ* and contained Cdc28-VF was not only able to sustain MPF activity when treated with nocodazole, but also maintained sister chromatid cohesion.

Although we observed no significant cyclin proteolysis in *cdc55Δ* cells, we have not been able to show conclusively that exit from mitosis can occur in the absence of the cyclin-proteolysis machinery. A simple test of this hypothesis would be to combine *cdc55Δ* with a mutant, such as *cdc16*, *cdc23* or *cdc27*, which inactivates the cyclin proteolysis machinery [9,10,51,52]. Unfortunately, these latter mutations produce a large increase in the Clb concentrations at the non-permissive temperature (data not shown). As a result, in double mutants, the 3–5-fold decrease in Cdc28-associated kinase induced by removal of Cdc55 does not reduce MPF activity below that of wild-type mitotic cells.

Until a rigorous test of the role of the cyclin-proteolysis machinery can be performed, there are three hypotheses that can account for sister separation in nocodazole-treated *cdc55Δ* cells. In the first model, proteolysis is required for separation, but cells regulate access of substrates to the cyclin-proteolysis machinery, thus allowing mitotic cyclins to remain stable, while proteins that prevent sister separation, such as Cut2 [6] are destroyed. In this model, however, it is unclear why the *cdc28-VF* mutant should prevent sister separation in nocodazole-treated *cdc55Δ* cells. In the second model, the phosphorylation of chromosomal proteins by chromosome-associated MPF maintains sister cohesion until MPF inactivation induces anaphase. Manipulations that prevent the overall level of cyclin from falling allow sisters to separate because they do not prevent the local inactivation of MPF on the chromosome. In this model, however, it is unclear why the overproduction of wild-type Clb2 in budding yeast (the sole mitotic cyclin required for viability) should disrupt putative chromosome-associated MPF and lead to sister separation.

In the third model, either MPF inactivation or activation of the cyclin-proteolysis machinery can induce sister separation (Fig. 8). Entry into mitosis would induce activities that could cause sister separation, but these activities would be kept in check by regulatory molecules until the onset of anaphase. If these inhibitors of separation were only active when they carried MPF-induced phosphorylations, and were substrates for the cyclin proteolysis machinery, either MPF activation or activation of the cyclin proteolysis machinery would induce anaphase (Fig. 8). Thus, in unusual situations, like that of nocodazole-treated *cdc55Δ* cells, inactivation of MPF without cyclin proteolysis would induce sister separation. Pds1 in budding yeast and Cut2 in fission yeast are candidates for proteins that act during metaphase to inhibit sister separation: deletion of *Pds1* allows sister separation in cells arrested in mitosis by the spindle-assembly checkpoint or defects in the cyclin proteolysis machinery [7], and preventing Cut2 destruction at the onset of anaphase prevents sister separation [6].

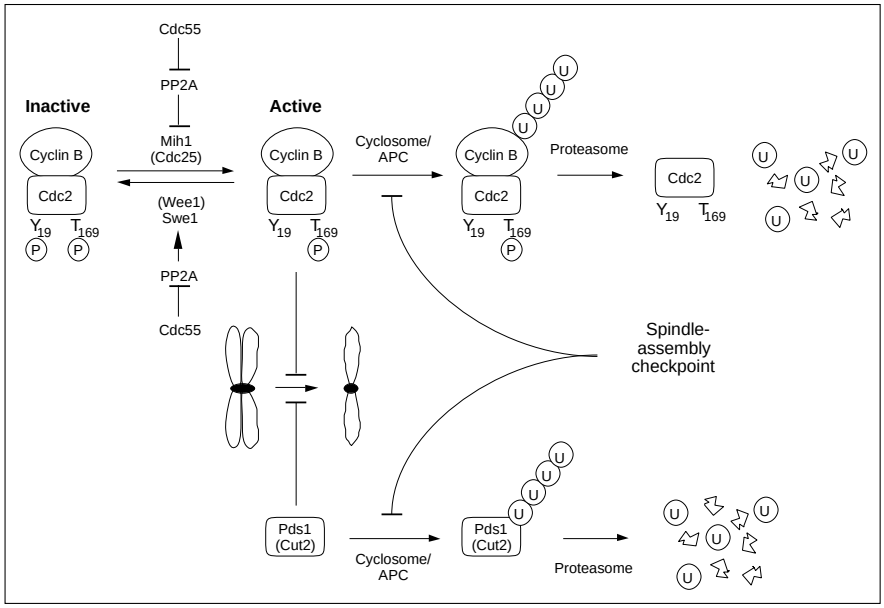
The role of PP2A in mitosis

We believe that the failure of the spindle-assembly checkpoint resulting from deletion of the *CDC55* gene is probably caused by inappropriate regulation of PP2A. Cdc55 is homologous to the PP2A regulatory subunit B from mammalian cells [26], and Cdc55 is one component of the budding yeast PP2A-like phosphatase that is activated in response to ceramide [27]. We found little difference between PP2A activities in cytoplasmic extracts made from wild-type and *cdc55Δ* cells, as measured by activity against MPF-phosphorylated histone H1. Our results are comparable with previous studies in which no difference was found in the abilities of asynchronous wild-type and *cdc55Δ* yeast extracts to dephosphorylate phosphohistone H1 [27].

Interpreting the results of these experiments is made difficult by the complex regulation of PP2A. Regulatory subunits of PP2A can either stimulate or inhibit the phosphatase activity of catalytic subunits, depending on the substrate [42,53–55]. Experiments using purified vertebrate PP2A show that phosphatase activities against CDK-phosphorylated histone H1, caldesmon and HMG-I(Y) are greatly decreased in the absence of regulatory subunits [42]. We have, so far, only been able to measure the phosphatase activities of crude yeast lysates rather than purified phosphatases. In addition, a number of PP2A catalytic subunits have been identified in budding yeast (Pph21, Pph22, Pph3 and the considerably more distantly related Sit4). To allow us to assay the activity of the purified phosphatase from yeast, we are currently trying to determine which proteins interact with Cdc55 in its role in maintaining the spindle-assembly checkpoint. Possibilities include the PP2A catalytic subunits as well as Tpd3 (a homolog of the regulatory A subunit of PP2A). Tpd3 and Sit4 have been implicated with Cdc55 as components of a ceramide-induced PP2A activity [27].

A Cdc55-regulated phosphatase could regulate the spindle-assembly checkpoint by controlling the kinases and

Figure 8



A model for the control of sister chromatid separation in budding yeast. Sister separation is inhibited by Pds1 and the presence of active MPF. Both of these inhibitory influences are required to maintain sister linkage. Activation of the cyclin proteolysis machinery induces the destruction of cyclin and Pds1, thereby removing both forms of inhibition and inducing chromosome separation. MPF can also be inactivated by inhibitory phosphorylations on Thr18 and Tyr19 (only the latter is shown). The tyrosine phosphorylation is due to Swe1, which is activated by PP2A, whereas Mih1, the tyrosine phosphatase, is inhibited by PP2A. Cdc55 reduces the activity of PP2A towards both of these substrates, thus favoring active MPF. Finally, we believe that the Mad- and Bub-dependent part of the spindle-assembly checkpoint acts to inhibit the activity of the cyclin-proteolysis machinery in cells with spindle defects. For some proteins the names of their fission yeast counterparts are indicated in parentheses.

phosphatases that modulate Cdc28 activity. The inhibitory tyrosine residue on Cdc28 is phosphorylated by Swe1 and dephosphorylated by Mih1 (homologs of the fission yeast Wee1 and Cdc25 proteins, respectively). *In vitro* evidence suggests that the vertebrate homologs of these proteins may be regulated by PP2A [56,57]. In fission yeast and frogs, PP2A has been shown to prevent MPF activation in interphase cells and to stimulate MPF inactivation in mitotic ones [58–63]. We propose that Cdc55 acts directly or indirectly to maintain Mih1 activity and to inhibit Swe1 activity in cells with defective spindles, thereby keeping MPF activity high and arresting cells in mitosis. Lack of Cdc55 would alter this balance, leading to inhibition of Mih1, activation of Swe1, inhibitory phosphorylation of Cdc28, and inactivation of MPF.

It is also possible that PP2A has a role in reversing the mitotic phosphorylations effected by MPF or its downstream kinases, in addition to its role in regulating MPF activity. Three lines of evidence support this idea: *in vitro*, PP2A dephosphorylates physiological substrates of cyclin-dependent kinases [42]; the potent PP2A inhibitor okadaic acid prevents sister chromatid separation in mammalian cells [64]; and the lack of the *Drosophila* 55 kDa PP2A B subunit results in abnormal anaphases [65]. Whether or not the same is true in *S. cerevisiae* remains to be seen.

Finally, we would like to know what function Cdc55 has in exit from the spindle-assembly checkpoint. Exit from mitosis normally requires cyclin degradation, and non-degradable cyclins arrest cells in mitosis [4,28]. In the checkpoint arrest, however, *cdc55Δ* cells inactivate MPF and return to interphase despite the presence of high levels of B-type cyclins associated with Cdc28. This may reflect a difference between normal mitotic control and the control of aberrant mitoses. Exit from the mitotic checkpoint occurs once the chromosomes have successfully attached to the spindle (recovery) [12–14,16]. It has also been observed that cells that are unable to assemble a spindle will eventually exit from mitosis, despite presumably never receiving the signal that the mitotic apparatus has been successfully assembled (adaptation) [66–68]. In either of these cases, it is possible that rather than reversing the stabilization of B-type cyclins effected by the spindle assembly checkpoint, cells simply bypass the proteolytic mechanisms and inactivate MPF by phosphorylating the inhibitory sites on Cdc28.

Conclusions

We conclude that absence of the Cdc55 protein perturbs PP2A activity in a way that allows MPF to be inactivated by inhibitory phosphorylation instead of by cyclin destruction. Preventing the inhibitory phosphorylation of Cdc28 blocks sister chromatid separation. Thus, in *cdc55Δ* cells treated with nocodazole, sister chromatid separation appears to be regulated by MPF activity rather than protein degradation.

Materials and methods

Strains and media

All strains used in this paper were derived from strain AFS34, an isogenic derivative of W303a. Gene transplacements and deletions or disruptions were performed by the one-step gene disruption method [69]. *MAD2* was deleted using a disruption construct that removed the entire open reading frame (R-H. Chen and A.W.M., unpublished results). *CDC55* was deleted by insertion of the *HIS3* gene between

the *Spe*I site (nucleotide 817) and an end-filled *Bam*HI site nucleotide 1756) as shown in Fig. 1. The *Sac*I–*Mlu*I fragment was then used to transform wild-type W303 and HIS$^+$ transformants were selected. Transformants were checked for integration of the *HIS3* into the genomic *CDC55* gene by the polymerase chain reaction. Mutants of *CDC28* in which Thr18 was replaced by Val and Tyr19 by Phe were made as described previously [40]. *GFP–LacI* under the control of the *HIS3* promoter was integrated at the *HIS3* locus by integration of plasmid pAFS78 after *Nhe*I digestion [34]. Lac operator repeats [70] were integrated at the *LEU2* locus by integration of plasmid pAFS59 after *Eco*RV digestion [34]. All cells used were *MAT**a***.

Yeast media, α factor, benomyl and hydroxyurea were prepared and used as described previously [19,21,68]. Nocodazole was purchased from Sigma, dissolved to 10 mg ml^{-1} in dimethylsulfoxide and this stock was diluted 1000-fold into media. All yeast were grown at 23 °C to ensure consistent effects of microtubule-depolymerizing drugs.

Cloning and sequencing

CDC55 was cloned using a yeast genomic DNA library in YCp50 [21] to rescue the benomyl sensitivity of JM215 on YPD plates. A 10 kb rescuing fragment was restriction mapped and subcloned, and subclones were tested for their ability to rescue the JM215 benomyl sensitive phenotype. The ends of a positive 2.8 kb *Sac*I–*Mlu*I fragment in pRS303 were sequenced by the chain termination method [71] using Sequenase from USB, following the manufacturers' directions. The sequences were compared with sequences in Genbank and found to be identical with the ends of a 2830 bp *Sac*I–*Mlu*I piece of the *S. cerevisiae* genome located on chromosome VIII, containing the gene for *CDC55*, and an unidentified open reading frame. Restriction mapping of our subclone with a number of enzymes gave the patterns predicted by the yeast genomic sequence.

Cell death in nocodazole

The rate of death of strains with depolymerized microtubules were measured by a modification of the method described in [19], as described in the legend to Figure 2b.

Western blotting: Clb2 and anti-phosphotyrosine

Yeast lysates were prepared as previously described [72]. Proteins were separated by polyacrylamide gel electrophoresis [73] and immunoblotted for Clb2 and Clb3 using previously described antibodies [74]. Phosphotyrosine was detected by probing the blots with a rabbit polyclonal antibody from Zymed Laboratories.

Histone H1 kinase assay

Clb2 was immunoprecipitated from yeast lysates and used to phosphorylate histone H1 as described previously [72].

Chromosome staining: in situ *hybridization and GFP–LacI*

Log-phase cultures of strains – AFS173 (wild-type), AFS176 (*mad2*), JM480 (*cdc55Δ*) and JM477 (*cdc55Δ cdc28-VF*) – containing GFP–LacI under control of the *HIS3* promoter and Lac operator repeats integrated at the *LEU2* locus on chromosome 3, were induced for 30 min in CSM-HIS plus 10 mM 3-aminotriazole. After removal of 3-aminotriazole, cells were resuspended in YPD with or without 10 μg ml^{-1} nocodazole. Cultures were fixed for 30 min in 3.7 % formaldehyde and the GFP–LacI staining was visualized using conventional fluorescence microscopy. For further details, see [34]. The fraction of cells with separated sister chromatids was then calculated for each cell population.

In situ hybridization was performed to intact, fixed yeast cells after enzymatic removal of the cell wall. Cultures were grown to mid log phase, fixed with paraformaldehyde, spheroplasted with Zymolyase 100-T (Seikagaku), and the cells adhered to polylysine-coated coverslips. In these experiments, the probe was a cloned yeast rDNA repeat; the plasmid was fragmented and 3′-end labeled with Rhodamine-4-dUTP (FluoroRed, Amersham) using terminal deoxynucleotidyl transferase (TdT, Promega). Fixation and hybridization to *S. cerevisiae* cells are described in greater detail elsewhere [33].

Phosphatase assay

Calf histone H1 (Boehringer Mannheim) was phosphorylated to 3.5 mol phosphate per mol H1 by *Xenopus* MPF as follows. MPF was bound to p13^{suc1} beads and washed as described previously [75,76]. The bead-bound MPF was incubated in 80 mM Na β-glycerophosphate (pH 7.4), 15 mM $MgCl_2$, 20 mM EGTA, 1 mM ATP (0.8 mCi ml^{-1}), 2 mM DTT, 60 μM histone H1 for 6 h at 30 °C. Histone H1 was separated from bead-bound MPF and free label by loading 100 μl aliquots of the entire phosphorylation reaction onto 1 ml Sepharose G-50 spin columns equilibrated with 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 15 mM 2-mercaptoethanol. Yeast lysates were made as described previously [43]. Lysates were diluted to 0.1 mg ml^{-1} protein in 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 15 mM 2-mercaptoethanol, 0.3 mg ml^{-1} bovine serum albumin (Boehringer Mannheim), 100 nM inhibitor I2 and, in some cases, 2 nM okadaic acid [43,77]. Phosphohistone H1 was added to 5 μM and phosphatase activity was measured by the release of inorganic phosphate from histone H1 [42].

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