The Role of Cdc55 in the Spindle Checkpoint Is through Regulation of Mitotic Exit in *Saccharomyces cerevisiae*

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Cdc55, a B-type regulatory subunit of protein phosphatase 2A, has been implicated in mitotic spindle checkpoint activity and maintenance of sister chromatid cohesion during metaphase. The spindle checkpoint is composed of two independent pathways, one leading to inhibition of the metaphase-to-anaphase transition by checkpoint proteins, including Mad2, and the other to inhibition of mitotic exit by Bub2. We show that Cdc55 is a negative regulator of mitotic exit. A *cdc55* mutant, like a *bub2* mutant, prematurely releases Cdc14 phosphatase from the nucleolus during spindle checkpoint activation, and premature exit from mitosis indirectly leads to loss of sister chromatid cohesion and inviability in nocodazole. The role of Cdc55 is separable from Bub2 and inhibits release of Cdc14 through a mechanism independent of the known negative regulators of mitotic exit. Epistasis experiments indicate Cdc55 acts either downstream or independent of the mitotic exit network kinase Cdc15. Interestingly, the B-type cyclin Clb2 is partially stable during premature activation of mitotic exit in a *cdc55* mutant, indicating mitotic exit is incomplete.

INTRODUCTION

The critical task of mitosis is equal segregation of sister chromatids to daughter cells. Faithful chromosome transmission is required for maintenance of genomic integrity, and failures in this process lead to aneuploid states characteristic of cancer (Nowak *et al.*, 2002). Checkpoints are mechanisms that inhibit cell division in the presence of damage, allowing time for repair and subsequent reentry into the cell cycle (Hartwell and Weinert, 1989). The spindle checkpoint arrests cells in mitosis when spindle function is inadequate for faithful chromosome segregation (Lew and Burke, 2003). This checkpoint can be induced by benzimidazole drugs, which cause microtubule depolymerization and compromised attachment to kinetochores. To arrest in mitosis, cells must stabilize sister chromatid cohesion to prevent premature separation of chromosomes and the mitotic cyclin Clb2 to maintain mitotic cyclin-dependent kinase activity (Lew and Burke, 2003). Maintenance of both cohesion and mitotic cyclin-dependent kinase (CDK) activity under these conditions requires the cell to stabilize proteins whose destruction is normally initiated by an E3 ubiquitin ligase known as the anaphase-promoting complex (APC) (Harper *et al.*, 2002; Vodermaier, 2004).

The activity and substrate specificity of the APC is a key point of regulation in the cell cycle. Two distinct APC activators, Cdc20 and Cdh1, act at metaphase and mitotic exit respectively (Visintin *et al.*, 1997). These proteins direct degradation of different, but partially overlapping, substrates to

promote the irreversible passage through these cell cycle stages. APC^{Cdc20} targets Pds1 “securin”, a protein required for metaphase sister chromatid cohesion, and the S-phase cyclin Clb5, while APC^{Cdh1} targets Ase1, Cdc20 and others. Degradation of Clb2 is biphasic, with a subset of the protein being degraded by APC^{Cdc20}, and the remainder by APC^{Cdh1} upon exit from mitosis (Yeong *et al.*, 2000). Prevention of this directed proteolysis is a critical requirement for mitotic checkpoint arrest.

In response to spindle perturbations, two independent spindle checkpoint pathways are required to inhibit progression through metaphase and mitotic exit (Alexandru *et al.*, 1999; Fraschini *et al.*, 1999; Lew and Burke, 2003). Mad1, Mad2, Mad3, Bub1, Bub3 and Mps1 comprise a metaphase checkpoint that inhibits the metaphase to anaphase transition by stabilizing the APC^{Cdc20} substrate Pds1, which binds to and inhibits the protease Esp1. During a normal mitosis Esp1 degrades Mcd1, a key member of the “cohesin” complex, allowing spindle forces to irreversibly separate sister chromatids (Uhlmann *et al.*, 1999). This metaphase checkpoint requires Mad2, a direct inhibitor of Cdc20 (Hwang *et al.*, 1998). Loss of Mad2 leads to failure in chromosome biorientation and elevated rates of chromosome loss following spindle damage (Lew and Burke, 2003).

The small GTPase Tem1 promotes mitotic exit and is inhibited by a two-component GTPase-activating protein (GAP) composed of Bub2 and Bfa1. Together the GAP inhibits mitotic exit in response to spindle damage by inactivating Tem1 and comprises the mitotic exit checkpoint (Bardin *et al.*, 2000; Bloecher *et al.*, 2000; Adames *et al.*, 2001). Active Tem1 initiates a Ras-like GTPase signaling cascade known as the mitotic exit network or MEN (Shou *et al.*, 1999). Lte1, a protein that localizes to the daughter cell cortex, normally comes in contact with and activates Tem1 to initiate the MEN pathway only when the daughter-bound spindle pole, to which Tem1 preferentially localizes, has been successfully delivered to the daughter cell (Jensen *et al.*, 2002; Seshan *et al.*, 2002).

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Abbreviations used: APC, anaphase-promoting complex; FEAR, fourteen early anaphase release; GAP, GTPase-activating protein; MEN, mitotic exit network.

The MEN promotes a series of downstream events, including release of the phosphatase Cdc14 from the nucleolus into the nucleus, a signature event in exit from mitosis. Released Cdc14 reverses the mitotic state by numerous downstream effects, one of which is activation of APC^{Cdh1} to target Clb2 for destruction (Stegmeier and Amon, 2004). Therefore, inhibition of the MEN by Bub2 and Bfa1 leads to stabilization of the mitotic cyclin Clb2, preventing CDK activity from advancing into the subsequent G₁ phase and resetting the cell cycle clock. An additional level of complexity in regulation of Cdc14 lies in a network known as fourteen early anaphase release (FEAR). Slk19, Spo12, Esp1, and Cdc5 are responsible for an early, transient release of Cdc14 from the nucleolus (D'Amours and Amon, 2004). This promotes completion of several events that occur between initiation of anaphase and exit from mitosis, including sub-threshold stimulation of MEN activity, but not full mitotic exit.

Cdc55 is one of two B-type subunits of protein phosphatase 2A in *Saccharomyces cerevisiae*. PP2A is a highly conserved serine/threonine phosphatase with roles in multiple cellular processes (Stark, 1996). Previous studies identified Cdc55 as a component of the spindle checkpoint in two independent screens for novel checkpoint mutants (Minshull *et al.*, 1996; Wang and Burke, 1997). It was shown that Cdc55 was required for viability and maintenance of cohesion during spindle disruption by benzimidazoles but not for stabilization of the mitotic cyclin Clb2.

Our understanding of the spindle checkpoint has advanced dramatically since the initial identification of *CDC55* as a checkpoint gene (Lew and Burke, 2003). This led us to reexamine the role of Cdc55 to determine whether it functioned in the metaphase or mitotic exit pathways of the spindle checkpoint. We examined Cdc55 in the context of the two known branches of the spindle checkpoint and show that Cdc55 is required to inhibit release of Cdc14 from the nucleolus in nocodazole, indicating a role in mitotic exit. After release of Cdc14, the anaphase inhibitor Pds1 was rapidly degraded, effectively bypassing the metaphase checkpoint and leading to failure in chromosome biorientation. We propose that Cdc55 is an inhibitor of the exit from mitosis that contributes to mitotic arrest by preventing premature release of Cdc14 from the nucleolus independently of the known spindle checkpoint pathways.

MATERIALS AND METHODS

Yeast Media and Growth

Yeast media and growth conditions followed standard techniques. Nocodazole and benomyl stock solutions were 10 mg/ml in dimethyl sulfoxide (DMSO). Cell synchronization using α -factor synchronization has been described previously (Yellman and Burke, 2004). Benomyl (Dupont, Wilmington, DE) was added to YPD medium at 12 μ g/ml in 1% DMSO. Nocodazole (Sigma-Aldrich, St. Louis, MO) was used at either 12 or 20 μ g/ml in liquid YM-1 medium with 0.2% DMSO.

Yeast Strains and Transformation

All strains used in this study are listed in Table 1. All strains were isogenic with the reference strain 8058-4-4 and congenic with the A364a background. Mutations or alleles that were crossed into our strains were backcrossed at least 10 times by standard tetrad genetics to 8058-4-4 to ensure that strains were isogenic. Yeast transformations followed the lithium acetate method (Gietz and Woods, 2002). Epitope-tagged alleles *PDS1-13MYC-HIS3MX6* and *CDC14-13MYC-HIS3MX6* were constructed by PCR-mediated one-step gene replacements by the method of Longtine *et al.* (1998). Epitope-tagged alleles replaced the endogenous copies of the respective genes. We integrated *PDS1-3HA-URA3* by endonuclease digestion of plasmid pOC52 with *SacI* and *Clal* followed by transformation. We introduced *CLB2-3FLAG-HIS3* into our strains by backcrossing. Deletion alleles were constructed by standard techniques (Longtine *et al.*, 1998).

Green Fluorescent Protein (GFP) Labeling of Chromosome III

Labeling of chromosomes with GFP has been described previously (Straight *et al.*, 1996). We integrated pAFS59 to insert the 256x array of Lac operators at the *LEU2* locus, an ~22-kb distance from the centromere of chromosome III. The plasmid pAFS135 integrated at *HIS3* expressed GFP-LacI. Detection of the GFP-labeled chromosome has been described previously (Yellman and Burke, 2004).

Protein Immunoblotting

Protein samples were collected as described by Kushnirov (2000). We normalized total protein amounts by cell number. Proteins were resolved using standard SDS-PAGE. We transferred protein to polyvinylidene difluoride membrane with a semidry transfer apparatus run at 20 V for 1–1.25 h. All antibodies were diluted into phosphate-buffered saline (PBS) containing 3% nonfat milk. Epitopes were detected using monoclonal 9E10 anti-Myc and 12CA5 anti-hemagglutinin (HA) antibodies (University of Virginia Hybridoma Facility, Charlottesville, VA) and the M2 monoclonal anti-FLAG antibody (Sigma-Aldrich). Primary antibody incubations were as follows. For detection of Myc, we used a 1:20,000 dilution of 0.5 mg/ml 9E10 monoclonal antibody (mAb) stock solution, incubated overnight at 4°C, followed by 1–2 h at room temperature. For anti-HA immunoblotting, we used a 1:6000 dilution of a 0.5 mg/ml 12CA5 mAb stock solution, incubating the blots at 4°C overnight, followed by 1–2 h at room temperature. For anti-FLAG blots, we used the M2 antibody at 1:12,500, incubated for 90 min at room temperature. Blots were washed three times in PBS for 5 min before incubation in secondary antibody. To detect β -tubulin, we used a rabbit polyclonal anti-peptide antibody (a gift from Doug Koshland, Carnegie Institution of Washington, Washington, D.C.). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) used at 1:20,000 for 45–60 min at room temperature. Quantitation of Clb2-3FLAG immunoblots was done from scanned blots using ImageQuant software (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Microscopy and Immunofluorescence

Microscopy and immunofluorescence has been described previously (Yellman and Burke, 2004).

Flow Cytometry

Flow cytometry has been described previously (Yellman and Burke, 2004). Flow cytometry analysis was performed at the University of Virginia core fluorescence-activated cell sorting facility.

RESULTS

Timing of Mitotic Chromosome Segregation in the *cdc55Δ* Mutant Is Normal

CDC55 was identified in two independent screens as a member of the spindle checkpoint required for metaphase arrest and maintenance of sister chromatid cohesion when cells are grown in the presence of nocodazole (Minshull *et al.*, 1996; Wang and Burke, 1997). It is not known whether Cdc55 has a role in the regulation of mitosis in an unperturbed cell cycle. Deleting either *MAD2*, a component of the metaphase checkpoint, or *BUB2*, a mitotic exit checkpoint gene, has little effect on the timing of mitotic events during growth in rich medium, but both pathways are required to arrest cells in response to spindle disruption by benzimidazoles (Lew and Burke, 2003). We tested whether deletion of *CDC55* affected the kinetics of chromosome segregation in an unperturbed cell cycle. We synchronized wild-type, *mad2Δ*, *bub2Δ*, and *cdc55Δ* cells in G₁ phase with mating pheromone α -factor and released them into the cell cycle into rich medium. We added α -factor after 90 min to arrest them in the subsequent G₁ phase, limiting the experiment to a single cell cycle. We used budding index and flow cytometry to monitor cell cycle progression, GFP chromosome tagging to assay the physical association of sister chromatids, and Pds1-13Myc to follow degradation of the anaphase inhibitor Pds1 (Yamamoto *et al.*, 1996).

Cell cycle progression was slightly delayed in the *cdc55Δ* mutant early in the cell cycle as indicated by a 20- to 30-min

Table 1. Yeast strains used in this study

Name	Genotype
8058-4-4 reference strain	<i>MATa bar1::loxP can1 cyh2 gal1 his3Δ1 leu2-3,112 trp1-289 ura3-52</i>
8715-10-2	<i>his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8715-6-4	<i>mad2::loxP-kanMX-loxP his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8579-4-4	<i>bub2::kanMX4 his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8766-25-4	<i>cdc55::loxP-kanMX-loxP his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8778-12-4	<i>cdc55::loxP-kanMX-loxP his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8905-4-3	<i>CDC14-13MYC-HIS3MX6 CLB2-3FLAG-HIS3 PDS1-3HA-URA3</i>
8905-1-3	<i>mad2::loxP-kanMX-loxP CDC14-13MYC-HIS3MX6 CLB2-3FLAG-HIS3 PDS1-3HA-URA3</i>
8906-1-3	<i>bub2::kanMX4 CDC14-13MYC-HIS3MX6 CLB2-3FLAG-HIS3 PDS1-3HA-URA3</i>
8866-30-2	<i>cdc55::loxP-kanMX-loxP CDC14-13MYC-HIS3MX6 CLB2-3FLAG-HIS3 PDS1-3HA-URA3</i>
8933-1-3	<i>slk19::kanMX4 spo12::CaURA3MX4</i>
8933-5-4	<i>slk19::kanMX4 spo12::CaURA3MX4 cdc55::loxP-natMX4-loxP</i>
8505-12-4	<i>lte1::kanMX4</i>
8665-17-3	<i>cdc55::loxP-natMX4-loxP lte1::kanMX4</i>
8487-7-4	<i>bub2::kanMX4</i>
8694-2-4	<i>cdc55::loxP-kanMX-loxP</i>
8914-11-3	<i>cdc55::loxP-kanMX-loxP his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8827-13-4	<i>cdc15-2 his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8913-16-4	<i>mad2::loxP-kanMX-loxP cdc15-2 his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8827-23-4	<i>bub2::kanMX4 cdc15-2 his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8914-17-3	<i>cdc55::loxP-kanMX-loxP cdc15-2 his3:pHIS3-GFP-LacI-HIS3 leu2:256LacO-LEU2 PDS1-13MYC-HIS3MX6</i>
8834-4-2	<i>bub2::kanMX4 cdc15-2</i>
8660-1-4	<i>cdc55::loxP-kanMX-loxP cdc15-2</i>
8426-9-4	<i>mad2::loxP-kanMX-loxP</i>
8862-10-2	<i>bub2::URA3</i>
8615-1-4	<i>cdc55::loxP-natMX4-loxP</i>
8862-3-3	<i>bub2::URA3 mad2::loxP-kanMX-loxP</i>
8667-2-3	<i>cdc55::loxP-natMX4-loxP mad2::loxP-kanMX-loxP</i>
8818-9-3	<i>bub2::URA3 cdc55::loxP-natMX4-loxP</i>
8870-6-3	<i>CDC14-13MYC-HIS3MX6 PDS1-3HA-URA3</i>
8870-2-4	<i>cdc55::loxP-kanMX-loxP CDC14-13MYC-HIS3MX6 PDS1-3HA-URA3</i>
8993-25-3	<i>cdc15-2 CDC14-13MYC-HIS3MX6 PDS1-3HA-URA3</i>
8993-2-3	<i>cdc55::loxP-kanMX-loxP cdc15-2 CDC14-13MYC-HIS3MX6 PDS1-3HA-URA3</i>

All strains were generated during this study.

delay in bud emergence (Figure 1A). The *cdc55Δ* mutant had a pronounced delay in G₂/M as determined by flow cytometry (Figure 1D), consistent with previous reports indicating a role for Cdc55 in cytokinesis (Healy *et al.*, 1991). Pds1 degradation and separation of sister chromatids were slightly delayed in *cdc55Δ* mutants, but the extent of the delay was equivalent to the delay in bud emergence (Figure 1, B and C). Therefore, the mitotic events of Pds1 degradation and sister chromatid separation proceeded with kinetics similar to wild-type, *mad2Δ*, and *bub2Δ* cells. The loss of Cdc55 did not disrupt the timing of mitosis in an unperturbed cell cycle.

Cdc55 Is Required to Inhibit Late Mitotic Events but Also Fails in Chromosome Biorientation

Mad2 and Bub2 have clearly defined roles in spindle checkpoint inhibition of mitosis. To better define the role of Cdc55 in the checkpoint response, we compared the kinetics of Pds1 degradation and loss of sister chromatid cohesion in wild-type, *mad2Δ*, *bub2Δ*, and *cdc55Δ* mutants released synchronously into medium containing nocodazole. A comparison of bud emergence and flow cytometry profiles indicated that entry into the cell cycle was synchronous (Figure 2, A and D). Pds1 was stable, and sister chromatids remained cohesive in wild-type cells treated with nocodazole (Figure 2, B and C). Pds1 was degraded in the *mad2Δ* mutant with kinetics similar to that of wild-type cells in a normal cell cycle as described previously (Biggins and Murray,

2001). Pds1 degradation and loss of sister chromatid cohesion occurred 90 min later in the *bub2Δ* mutant compared with the *mad2Δ* mutant. The *cdc55Δ* mutant degraded Pds1 and lost sister chromatid cohesion with kinetics similar to that of *bub2Δ* (Figure 2, B and C). This suggested that the checkpoint role of Cdc55 was not direct inhibition of APC-Cdc20 as it is for Mad2. The failure of *cdc55Δ* cells to arrest in response to nocodazole resembled *bub2Δ*, suggesting that Cdc55 had a role in inhibiting mitotic exit when the spindle checkpoint was activated. We assayed cell viability to determine when cells respond to nocodazole as an independent measure of timing in the cell cycle. We synchronized cells with α -factor, released them into the cell cycle in the presence of nocodazole, and plated samples over time to determine viability (Figure 2E). Viability fell earliest in the cell cycle in the *mad2Δ* mutant and later in *cdc55Δ* and *bub2Δ*.

To determine the efficacy of chromosome segregation, we removed cells from nocodazole and grew them for 1 h, during which time they recovered and completed anaphase. *Cdc55Δ* cells segregated the GFP-labeled chromosome in an asymmetric manner, with close to 30% of cells segregating two copies of the chromosome to one spindle pole (Figure 2F). This phenotype resembled *mad2Δ*, whereas wild-type cells were able to accurately segregate the chromosome. Asymmetric segregation is characteristic of cells that fail to properly biorient sister chromatids on the mitotic spindle before segregation.

Figure 1. Mitosis in *Cdc55* Δ and checkpoint mutants in a normal cell cycle. Wild-type (8715-10-2), *mad2* Δ (8715-6-4), *bub2* Δ (8579-4-4), and *cdc55* Δ (8766-25-4) cells were synchronized with α -factor, released into the cell cycle in YM-1 medium, and sampled at 15-min intervals. Cells were limited to a single cell cycle by addition of α -factor 100 min after release. Symbols for all graphs are as indicated in A. (A) Timing of bud emergence, determined by phase contrast microscopy. (B) Timing of sister chromatid separation at *LEU2*, determined by GFP fluorescence microscopy. (C) Immunoblots of PDS1-13Myc levels to assay the onset of anaphase. (D) Flow cytometry of DNA content to determine the kinetics of replication and cell division.

Figure 2. Response of *Cdc55* Δ and checkpoint mutants to nocodazole to assay metaphase arrest. Wild-type (8715-10-2), *mad2* Δ (8715-6-4), *bub2* Δ (8579-4-4), and *cdc55* Δ (8778-12-4) cells were synchronized with α -factor, released into YM-1 medium containing 12 μ g/ml nocodazole, and sampled at 30-min intervals for a total of 5 h. Cells were limited to a single cell cycle by addition of α -factor at 100 min after release. Symbols for all graphs are as indicated in A. (A) Bud morphology, determined by phase contrast microscopy. (B) Timing of sister chromatid separation at *LEU2*, determined by GFP fluorescence microscopy. Each time point contains data from 100 cells. (C) Immunoblots of PDS1-13Myc levels to assay the onset of anaphase. (D) Flow cytometry of DNA content to determine the kinetics of replication and cell division. (E) Viability after α -factor block and release into medium containing 15 μ g/ml nocodazole at 30°C, as determined by colony-forming ability when returned to YPD agar plates. (F) Segregation fidelity was determined in the same α -factor block and release experiment at 30°C described for Figure 2E. Cells were released into medium containing 15 μ g/ml nocodazole, removed at the 2-h point, washed, and returned to liquid medium lacking nocodazole for 1 h. Cells were fixed, and GFP and 4,6-diamidino-2-phenylindole (DAPI) were visualized. Mis-segregation is the percentage cells that have undergone full nuclear division into mother and daughter cells but have segregated both copies of GFP-labeled chromosome 3 into the same nucleus.

Cdc55 Is a Negative Regulator of Mitotic Exit and Inhibits a Subset of Events

We used a simple genetic test to determine whether *Cdc55* regulated the exit from mitosis. An *lte1 spo12* double mutant is inviable (synthetic lethal) because cells lack two independent activators of the exit from mitosis. The lethality can be suppressed by eliminating a negative regulator such as *Bub2* or *Bfa1* (Stegmeier *et al.*, 2002). We constructed a diploid strain that was heterozygous for *lte1::G418*, *spo12::URA3*, and *cdc55::NAT*, sporulated the diploid, and dissected 35 tetrads to generate all combinations of the deletion mutants. We recovered 20 tetrads that were either directly scored as tetratype for *lte1::G418* and *spo12::URA3* (four viable spores) or inferred to be tetratype for *lte1::G418* and *spo12::URA3* from the segregation of markers in the three viable spores. Twelve of the tetrads had three viable spores, and in every case the inviable spore was *lte1::G418*, *spo12::URA3* and *CDC55*, as expected for synthetic lethality of *lte1 spo12*. Eight of the tetrads gave four viable spores, and in seven cases the spore that was *lte1::G418 spo12::URA3* was also *cdc55::NAT*. A chi-square test of significance showed that we recovered equal proportions of *lte1::G418 spo12::URA3 CDC55* and *lte1::G418 spo12::URA3 cdc55::NAT* ($p < 0.05$). Deletion of *CDC55* suppressed the synthetic lethality of *lte1 spo12*, suggesting that *Cdc55* was a negative regulator of the exit from mitosis.

We assayed two other indicators of mitotic exit, release of *Cdc14* from the nucleolus and degradation of *Clb2*. In addition, we included *Pds1-3HA* to establish the timing of loss of sister chromatid cohesion relative to these events. *Cdc14* is sequestered in the nucleolus and released upon activation of the MEN (Stegmeier and Amon, 2004). Nucleolar localization and release of *Cdc14* is a simple assay for MEN activation. The relocalization of *Cdc14* promotes reversal of the mitotic state, including activation of the Cdh1-dependent form of the APC by dephosphorylation of Cdh1. APC^{Cdh1} then targets specific substrates, including *Clb2*, for ubiqui-

Figure 3. Exit from mitosis in *Cdc55*Δ and checkpoint mutants in nocodazole. Wild-type (8905-4-3), *mad2*Δ (8905-1-3), *bub2*Δ (8906-1-3), and *cdc55*Δ (8866-30-2) cells were synchronized with α -factor, released into YM-1 medium containing 12 μ g/ml nocodazole, and sampled at 30-min intervals for a total of 5 h. Cells were limited to a single cell cycle by addition of α -factor 100 min after release. Symbols for all graphs are as indicated in A. (A) Bud morphology, determined by phase contrast microscopy. (B) Timing of Cdc14-13Myc release from the nucleolus, determined by immunofluorescence microscopy. Each time point contains data from at least 100 cells. (C) Immunoblots to assay the stability of PDS1-3HA and Clb2-3FLAG proteins. (D) Quantitation of Clb2 levels from scanned immunoblots.

uitin-dependent proteolysis, promoting the transition from mitosis to G₁. We synchronized cells in α -factor and released them into medium containing nocodazole, limiting the cells to one cell cycle by readdition of pheromone. Bud emergence data indicated that there was synchronous entry into the cell cycle, with *cdc55*Δ slightly delayed as shown in Figure 3A. We assayed Cdc14-13Myc localization by immunofluorescence to determine the timing of Cdc14 release in the mutants. Examples of Cdc14-13Myc immunofluorescence in the unreleased (wild-type) and released (*cdc55*Δ cells) states can be seen in Figure 5B. Cdc14 remained nucleolar in both the wild-type and *mad2*Δ control strains for the duration of the experiment, demonstrating that these strains were able to prevent mitotic exit (Figure 3B). In contrast, both *bub2*Δ and *cdc55*Δ efficiently released Cdc14 from the nucleolus, indicating potent stimulation of this event in both mutants. The pattern of Pds1-3HA degradation was consistent with our previous data (Figures 2C and 3C). In both the *bub2*Δ and *cdc55*Δ mutants, Cdc14 release began just before Pds1 degradation, consistent with the interpretation that Cdc14 activity was the indirect cause of this degradation. We concluded that *cdc55* mutants were sensitive to benzimidazoles such as benomyl and nocodazole because of inability to restrain Cdc14 release. We also assayed Clb2 protein levels to determine whether degradation of Clb2 correlated with Cdc14 release. During normal exit from mitosis Clb2 degradation is biphasic, with a subset of

Figure 4. (A) Benomyl sensitivity and epistasis. (A) *cdc55*Δ mutant in combination with deletions of the checkpoint genes *MAD2* and *BUB2*. Stationary phase cultures were normalized to equal densities (1×10^7 cells/ml) and spotted in a 10-fold serial dilution series on YPD (rich medium) or YPD plus 12 μ g/ml benomyl. Plates were incubated at 25°C. Strains were wild-type (8058-4-4), *mad2*Δ (8426-9-4), *bub2*Δ (8862-10-2), *cdc55*Δ (8615-1-4), *mad2*Δ *cdc55*Δ (8667-2-3), and *bub2*Δ *cdc55*Δ (8818-9-3). (B) Deletion of *LTE1* in combination with *cdc55*Δ. Strains were wild type (8058-4-4), *cdc55*Δ (8615-1-4), *lte1*Δ (8505-12-4), and *cdc55*Δ *lte1*Δ (8665-17-3). (C) Deletion of the FEAR components *SLK19* and *SPO12* in combination with *cdc55*Δ. Strains were wild-type (8058-4-4), *cdc55*Δ (8615-1-4), *spo12*Δ *slk19*Δ (8933-1-3), and *cdc55*Δ *spo12*Δ *slk19*Δ (8933-5-4).

the protein being degraded by APC^{Cdc20} at the transition to anaphase and the remainder by APC^{Cdh1} during mitotic exit (Yeong *et al.*, 2000). We found that Clb2 was partially stable in the *cdc55*Δ mutant, despite the release of Cdc14 from the nucleolus (Figure 3, C and D). A subset of Clb2 protein remained stable in *cdc55*Δ and *mad2*Δ mutants, whereas the protein was fully degraded in the *bub2*Δ mutant. Therefore, Cdc14 release and Clb2 degradation, two key indicators of mitotic exit, were uncoupled in the *cdc55*Δ mutant.

Inhibition of Mitotic Exit by Cdc55 Is Independent of Bub2

We performed epistasis analysis of benomyl sensitivity with *mad2*Δ, *bub2*Δ, and *cdc55*Δ mutants to determine whether Cdc55 inhibited mitotic exit through Bub2 or by a separate mechanism. The *mad2*Δ *cdc55*Δ double mutant showed a level of benomyl sensitivity similar to either *mad2*Δ or *cdc55*Δ single mutants. In contrast the *bub2*Δ *cdc55*Δ double mutant was more benomyl sensitive than either single mutant (Figure 4A). We conclude that Bub2 and Cdc55 inhibit the exit from mitosis by separate mechanisms but that Mad2 and Cdc55 checkpoint functions overlap.

Cdc55 Acts Either Downstream of Lte1 in the MEN, Downstream of Spo12 and Slk19 in the FEAR Pathway, or in an Unidentified Pathway

Additional mechanisms can contribute positively to mitotic exit. Lte1, a putative guanidine exchange factor, stimulates

Tem1 to activate the MEN, and the FEAR network stimulates the MEN by releasing Cdc14 from the nucleolus in early anaphase. Because the *cdc55Δ* mutant cannot inhibit mitotic exit appropriately in nocodazole, we wanted to know whether inappropriate stimulation of exit by either Lte1 or FEAR was the cause.

Lte1 is maintained on the bud cortex of the daughter cell and prevented from migrating into the mother cell by factors acting at the bud neck (Barral *et al.*, 2000; Castillon *et al.*, 2003). Cdc55 has been shown to localize to the septin ring surrounding the bud neck (Gentry and Hallberg, 2002). If the role of Cdc55 were to restrict Lte1 to the daughter cell, then a loss of Cdc55 could lead to inappropriate exit from mitosis. We constructed a *cdc55Δ lte1Δ* double mutant and assayed the ability of the mutant to restrain mitotic exit by determining the benomyl sensitivity. If *cdc55Δ* mutants are benomyl sensitive because of hyperactive Lte1, the benomyl sensitivity of *cdc55Δ* should be suppressed by *lte1Δ*. The *cdc55Δ lte1Δ* did not have lower benomyl sensitivity and was even slightly more sensitive to benomyl than the *cdc55Δ* single mutant (Figure 4B). We concluded that the benomyl sensitivity of *cdc55Δ* was not because of MEN activation upstream of Lte1.

To determine whether inappropriate FEAR activity was the cause of mitotic exit in *cdc55Δ* cells that were challenged with benomyl, we deleted the genes encoding Spo12 and Slk19, two positively acting members of FEAR, to make a *spo12Δ slk19Δ cdc55Δ* triple mutant. Loss of Spo12 and Slk19 specifically eliminates FEAR (D'Amours and Amon, 2004). The *spo12Δ slk19Δ* double mutant had viability and benomyl resistance comparable with wild type (Figure 4C). The *spo12Δ slk19Δ cdc55Δ* triple mutant was no less sensitive to benomyl than *cdc55Δ* alone, suggesting that activation of these FEAR components was not the reason for benomyl sensitivity in *cdc55Δ*. Together, our data show that the role of Cdc55 in mitotic arrest, as measured by nocodazole-induced lethality, must lie downstream of the MEN activator Lte1 or the FEAR activators Spo12 and Slk19 or in some unidentified pathway.

The MEN Mutant *cdc15-2* Does Not Block Mitotic Exit in *cdc55Δ* Cells

We reasoned that if the lethality of *cdc55Δ* cells in the presence of benzimidazoles was because of inappropriate activation of the MEN, then it should be suppressed by blocking the MEN pathway. Cdc15 kinase acts in the MEN and *cdc15-2* is a temperature-sensitive allele that reversibly blocks the pathway (Futcher, 1999; Lee *et al.*, 2001). We assayed Cdc14 localization, Pds1 stability, and nocodazole-induced lethality of wild-type and *cdc55Δ* cells and the corresponding double mutants with *cdc15-2* at restrictive temperature. Synchronous populations of cells were released from α -factor arrest into nocodazole at 35°C to inactivate Cdc15 and sampled for 3 h. Samples were removed at 1-h intervals to determine Cdc14-13Myc localization and Pds1-3HA levels and plated at 23°C in the absence of nocodazole to determine viability by colony formation. The immunofluorescence images show the localization of Cdc14-13Myc at the 2-h time point in each strain (Figure 5B). Wild-type cells maintained Cdc14-13Myc in the nucleolus and remained largely viable (Figure 5; our unpublished data). The viability was normalized to wild type. Partial degradation of Pds1-3HA occurred in wild-type cells, probably reflecting the reduced efficacy of nocodazole at the elevated temperature (Figure 5C). The *cdc55Δ* mutant released Cdc14-13Myc, partially degraded Pds1-3HA, and lost viability during exposure to nocodazole, indicating failure to arrest (Figure 5).

Figure 5. Mitotic arrest phenotypes during MEN block by *cdc15-2*. Cells were synchronized with α -factor in YM-1 medium at 23°C. The water bath temperature was then raised to 34°C to allow a gradual temperature shift. Cells were washed twice with water, released into YM-1 medium containing 20 μ g/ml nocodazole and incubated in a 35°C water bath for 3 h. Samples were collected at the 0-, 1-, 2-, and 3-h time points. (A) Percentage of cells that have released Cdc14 from the nucleolus as determined by Cdc14-13Myc immunofluorescence and DAPI staining. Strains were wild type (8870-6-3), *cdc55Δ* (8870-2-4), *cdc15-2* (8993-25-3), and *cdc55Δ cdc15-2* (8993-2-3). (B) Cdc14-13Myc immunofluorescence of cells from the 2-h time point. (C) Pds1-13Myc protein levels during the MEN block. Strains were wild type (8715-10-2), *cdc55Δ* (8914-11-3), *cdc15-2* (8827-13-4), and *cdc55Δ cdc15-2* (8914-17-3). (D) Viability during the MEN block. The same cells sampled for B were removed and plated onto YPD plates at the permissive temperature of 23°C to determine the number of colonies formed. Viability data were normalized to wild-type and *cdc15-2* levels, respectively.

Blocking the MEN with the *cdc15-2* allele caused cells to maintain Cdc14 in the nucleolus, stabilize Pds1, and maintain viability in nocodazole. The viability data were normalized to *cdc15-2*. In contrast, the *cdc55Δ cdc15-2* double mutant cells released Cdc14-13Myc from the nucleolus, degraded Pds1-3HA, and lost viability. We concluded that premature mitotic exit in *cdc55Δ* occurred because of events that were either downstream of Cdc15 in the MEN or in a separate pathway.

DISCUSSION

We showed that Cdc55 was required for inhibiting release of Cdc14 from the nucleolus when the spindle checkpoint was

activated. Degradation of the anaphase inhibitor Pds1 was preceded by Cdc14 release from the nucleolus in *cdc55* cells treated with nocodazole. Surprisingly, Cdc55 was required for chromosome biorientation after spindle disruption, a role normally ascribed to the metaphase spindle checkpoint. Deleting *CDC55* suppressed the synthetic lethality of an *lfe1 spo12* double mutant, suggesting that Cdc55 is a negative regulator of mitotic exit. Although *cdc55* cells prematurely released Cdc14 in nocodazole, this was accompanied by only partial Clb2 degradation, indicating exit from mitosis was incomplete. Genetic data showed that Cdc55 acted downstream of Lfe1 in the MEN or downstream of Spo12 and Slk19 in the FEAR pathway to stimulate the release of Cdc14. Cdc14 release, Pds1 degradation, or lethality of *cdc55* in nocodazole was not suppressed by a mutation in the gene for the MEN component Cdc15. Therefore, Cdc55 acted after the Cdc15 step in mitotic exit or in a separate pathway.

Previous studies of Cdc55 in *S. cerevisiae* have described roles in a variety of processes, including cell morphology, cytokinesis, and entry into mitosis (Healy *et al.*, 1991; Lin and Arndt, 1995). We confirmed these observations and found that a *cdc55* mutant delayed bud emergence, indicating multiple roles in the cell cycle. However, we detected no effect on the kinetics of mitosis in an unperturbed cell cycle. The inhibitory role of Cdc55 became evident during treatment with nocodazole, which activates the spindle checkpoint. The complexity of the spindle checkpoint was not appreciated at the time that a checkpoint role was first proposed for Cdc55 (Minshull *et al.*, 1996; Wang and Burke, 1997). The mislocalization of Cdc14 in *cdc55* cells, treated with nocodazole, in addition to sister chromatid separation and Pds1 degradation seem paradoxical because the metaphase-to-anaphase transition is regulated by Mad2-dependent inhibition of APC^{Cdc20}, which is intact in a *cdc55* mutant. We propose that *cdc55* mutants (and *bub2* mutants) bypassed Cdc20 inhibition as a consequence of the release of Cdc14 from the nucleolus, leading to Pds1 degradation and premature sister chromatid separation.

The metaphase checkpoint is capable of responding to two different types of defects, loss of microtubule occupancy at the kinetochore or decreased tension across the kinetochores (Lew and Burke, 2003). Recent models of the tension-sensing branch of the metaphase checkpoint propose that it acts through Mad2 to inhibit APC^{Cdc20} and hold cells at metaphase to ensure biorientation of sister chromatids (Stern and Murray, 2001; Indjeian *et al.*, 2005). Failure in chromosome biorientation is an important assay that identifies mutants lacking the tension component of the spindle checkpoint. Our results suggest an alternative interpretation. We found that both *cdc55* and *mad2* cells taken from nocodazole and returned to permissive conditions missegregated a single labeled chromosome, indicating failure to properly biorient paired sister chromatids before losing cohesion. We propose that *cdc55* mutants failed in biorientation upon recovery from nocodazole because of premature release of Cdc14 followed by degradation of Pds1 and sister chromatids separation. Future studies of the tension-sensing branch of the spindle checkpoint must account for the possibility that failure to biorient chromosomes may be because of misregulation of Cdc14 localization.

Two independent measures, Cdc14 release in *cdc55* cells treated with nocodazole and suppression of synthetic lethality of *spo12 lfe1* by *cdc55* triple mutant show that Cdc55 is an inhibitor of mitotic exit. However, some Clb2 was stabilized in *cdc55* cells, indicating that full mitotic exit had not occurred as it had in *bub2* cells. Cdc14 release from the nucleolus at the exit from mitosis requires the protein kinase

Cdc15, a member of the MEN pathway (Stegmeier and Amon, 2004). The failure of *cdc15-2* arrest to prevent Cdc14 release, Pds1 degradation, and benzimidazole-sensitivity in the *cdc55* mutant indicated that Cdc55 acted either downstream of the Cdc15 step in mitotic exit or in a separate pathway. Epistasis experiments such as these do not allow us to distinguish between these alternatives.

Despite the release of Cdc14 from the nucleolus and degradation of Pds1 in nocodazole-treated *cdc55* cells, at least a portion of Clb2, an important substrate of APC^{Cdh1}, remained stable. In addition, Cdc20, an APC^{Cdh1} substrate, remained stable in *cdc55* mutants during exposure to nocodazole, indicating that APC^{Cdh1} was inactive (our unpublished data). The stability of these two APC^{Cdh1} substrates and the instability of Pds1 suggested that only APC^{Cdc20} was active in the *cdc55* mutant in nocodazole. This was consistent with the genetic data that the *mad2 cdc55* double mutant was no more benomyl sensitive than either single mutant, because both Mad2 and Cdc55 inhibited APC^{Cdc20} during mitotic arrest.

We propose two models for the role of Cdc55 in the spindle checkpoint. The first is that Cdc55 acts in the MEN downstream of Cdc15 to inhibit Cdc14 release, but it has a separate role in promoting APC^{Cdh1} activation. It is possible that the phosphorylation status of APC^{Cdh1} substrates is important for degradation by APC^{Cdh1}. The second model is that Cdc55 acts downstream of the known FEAR components to inhibit Cdc14 release. It is known that FEAR-dependent release of Cdc14 does not lead to sustained exit from mitosis, consistent with partial stabilization of Clb2 in *cdc55* (D'Amours and Amon, 2004). This model is attractive because it would explain the independence of Cdc55 and Bub2 in regulating mitotic exit and because a recent report has shown that Cdc14 is released from its inhibitory binding partner Net1 by FEAR-activated CDK phosphorylation during mitosis (Azzam *et al.*, 2004). Net1 is also an *in vitro* target of phosphorylation by the polo-like kinase Cdc5, which promotes disassembly of recombinant Cdc14–Net1 complexes (Loughrey *et al.*, 2002). In our model, PP2A, acting through Cdc55, would reverse phosphorylation of Net1 to inhibit Cdc14 release during mitosis. This model explains the checkpoint nature of Cdc55 phenotypes, because maintaining the cell in mitosis is the key function of mitotic checkpoint arrest, and explains why loss of Cdc55 cannot be suppressed by deleting the known upstream activators of FEAR. Although we did not test any of the other components of PP2A, previous data show that the other subunits of the enzyme are required for spindle checkpoint arrest, implicating the enzyme complex in regulation of mitotic exit (Wang and Burke, 1997).

Our data differ from a recent study that also identified Cdc55 as a negative regulator of mitotic exit (Wang and Ng, 2006). The authors suggest that Cdc55, through PP2A, dephosphorylates the MEN activator Tem1 to prevent mitotic exit. Our data indicate that the effect of Cdc55 is either downstream or independent of both Tem1 and Cdc15. In addition, our finding that Clb2 protein is partially stabilized in the *cdc55* mutant indicates that regardless of misregulation of Tem1, MEN activity did not reach the level required for full mitotic exit. In our experiments, the only clear indicator of mitotic exit was the release of Cdc14 from the nucleolus. For this reason, we conclude that Cdc55 inhibits only a subset of mitotic exit events. It is possible that Cdc55 has roles in both Tem1 and FEAR regulation, and this is not unreasonable given that Cdc55 encodes a subunit of PP2A and has multiple targets within the cell.

A mutation in Par1, a B'-type subunit of PP2A of *Schizosaccharomyces pombe* suppresses a temperature-sensitive mutation in *spg1*, the *S. pombe* homologue of *S. cerevisiae* Tem1 (Le Goff *et al.*, 2001). Therefore, Par1 plays a role in initiation of cell division through the septin initiation network, a network analogous to the MEN (Simanis, 2003). Together with our results, this suggests that PP2A regulation is a conserved feature of the exit from mitosis. There is evidence that Cdc55 regulates mitotic exit in a cell cycle in which the checkpoint is not active. Loss of Cdc55, similar to loss of Bub2, is able to bypass the mitotic arrest of a *cdc20-1* mutant (Tavormina and Burke, 1998). Furthermore, we show in this study that *cdc55* suppressed the synthetic lethality of a *spo12 lte1* double mutant. This is evidence that Cdc55 has a function, apart from checkpoint response, and is likely to act during every cell cycle. This is an exciting observation suggesting that there are roles of Cdc55 and potential targets of PP2A in mitosis that remain to be identified and that the regulation will be more complex and interesting than previously thought.

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