a complex by anion- and cation-exchange chromatography, and concentrated to 8 mg/ml. Purified AATGACCGGAAGTACACCGGA and TTCCGGTGTACTTCCGGTCAT 21-base oligonucleotides were added to protein in slight molar excess and dialyzed into 20 mM tris (pH 8), 1 mM EDTA, 1 mM dithiothreitol, and 0.001% sodium azide. Equal volumes of protein-DNA solution and well solution [100 mM *bis-tris* propane (pH 9), 5 mM cobaltic hexamine chloride, 9% polyethylene glycol (PEG) 1000] were mixed, and hanging drops were allowed to equilibrate by vapor diffusion at 20°C. Crystals were transferred into cryosolutions containing well solution with 12% PEG 1000 and 24% glycerol. Optimal diffraction was obtained if crystals were cross-linked by exposure to glutaraldehyde vapor for 10 min. For the 2Hg derivative, a cross-linked crystal was soaked in 1 mM EMTS (ethylmercurithiosalicylate) for 24 hours.

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Budding Yeast Cdc20: A Target of the Spindle Checkpoint

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The spindle checkpoint regulates the cell division cycle by keeping cells with defective spindles from leaving mitosis. In the two-hybrid system, three proteins that are components of the checkpoint, Mad1, Mad2, and Mad3, were shown to interact with Cdc20, a protein required for exit from mitosis. Mad2 and Mad3 coprecipitated with Cdc20 at all stages of the cell cycle. The binding of Mad2 depended on Mad1 and that of Mad3 on Mad1 and Mad2. Overexpression of Cdc20 allowed cells with a depolymerized spindle or damaged DNA to leave mitosis but did not overcome the arrest caused by unreplicated DNA. Mutants in Cdc20 that were resistant to the spindle checkpoint no longer bound Mad proteins, suggesting that Cdc20 is the target of the spindle checkpoint.

The spindle checkpoint improves the fidelity of chromosome segregation by delaying anaphase until all chromosomes are correctly aligned on the mitotic spindle (*1*, *2*). Mutants in the *MAD* (mitosis arrest deficient) and *BUB* (budding uninhibited by benzimidazole) genes inactivate the checkpoint (*3*, *4*), and overexpressing components of the checkpoint can arrest cells with normal spindles in mitosis (*5*–*7*). The checkpoint prevents ubiquitination and destruction of at least two types of protein: the B-type cyclins, which activate the protein kinase activity of cyclin-dependent kinase (Cdk1, known as Cdc28 in budding yeast and Cdc2 in fission yeast), and a protein required to maintain the linkage of sister chromatids (Pds1 in budding yeast and Cut2 in fission yeast) (*8*–*11*). Ubiquitination is catalyzed by a multiprotein complex called the cyclosome or anaphase promoting complex (APC) (*12*–*14*). The reactions that activate the APC are not understood, but cyclin B and Pds1/Cut2 destruction depends on Cdc20 and Hct1/Cdh1, two evolutionarily conserved members of the WD (Trp-Asp) repeat family of proteins. Cdc20 preferentially promotes the destruction of Pds1/Cut2, and Hct1 promotes the destruction of B-type cyclins (*15*, *16*). Unlike *HCT1*, *CDC20* is an essential gene, and temperature-sensitive *cdc20* mutants arrest in metaphase.

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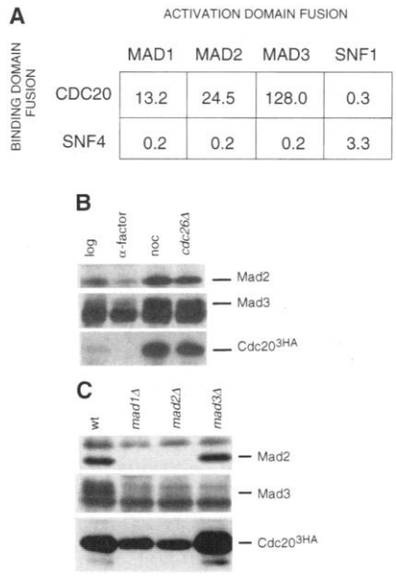
The interaction between Slp1 (the homolog of Cdc20) and Mad2 in fission yeast (*7*) prompted us to investigate the interaction between Cdc20 and components of the spindle assembly checkpoint in budding yeast. In the two-hybrid system, Mad1, Mad2, and Mad3 all showed interactions with Cdc20 (Fig. 1A), suggesting that checkpoint proteins bind to Cdc20. We confirmed this suggestion by immunoprecipitating an epitope-tagged version of Cdc20 and probing the immunoprecipitates with antibodies to Mad2 and Mad3 (*17*). We examined four conditions: cells growing asynchronously, cells arrested in G_1, cells arrested in mitosis by depolymerization of the spindle with nocodazole, and cells arrested in mitosis by *cdc26Δ*, a mutant that inactivates the APC (*18*, *19*). Both Mad2 and Mad3 were present in immunoprecipitates from strains carrying epitope-tagged Cdc20 (Fig. 1B). We were unable to monitor the physical interaction between Mad1 and Cdc20 because free Mad1 binds to antibody-coated beads in some control experiments. The amount of Mad2 and Mad3 precipitated with Cdc20 was highest in cells arrested in mitosis, lower in asynchronous cells, and still lower in cells arrested in G_1. The increased association in mitotic cells does not depend on checkpoint activation, because cells arrested by inactivation of the APC showed the same interaction between Cdc20 and Mad proteins as cells arrested in mitosis by spindle depolymerization. We suspect that the different levels of Mad-Cdc20 association between mitotic and G_1 cells reflect the level of Cdc20, which is high in mitosis and low in G_1 (*20*).

Because two Mad proteins associate with Cdc20, we asked whether they asso-

Fig. 1. Association of Mad1, Mad2, and Mad3 with Cdc20. Two-hybrid association. (**A**) Haploid strains containing fusions between the transcriptional activation domain and Cdc20 or Snf4 were crossed to strains containing fusions between the DNA-binding domain and Mad proteins or Snf1, and the resulting diploids were assayed for β-galactosidase activity. Values are shown in Miller units and are the average of three independent crosses. The fusions were constructed in the vectors pAS1-CYH2 (DNA binding domain) and pACTII (transcriptional activation domain) (*32*), and all contained the full coding region, except for the Mad1 fusion, which contains amino acids 313 to 750 of Mad1. The SNF1 and SNF4 fusions are control fusions to proteins involved in regulating sucrose metabolism. (**B**) Cell cycle regulation of Mad-Cdc20 interactions. Coimmunoprecipitation of Cdc20 with Mad2 and Mad3. Strains containing a hemagglutinin (HA) epitope–tagged version of Cdc20 were lysed, then immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by protein immunoblotting with antibodies to Mad2, Mad3, or HA. Extracts were from exponentially growing cells, cells arrested in G_1 by treatment with α-factor, cells arrested in mitosis by treatment with nocodazole, or cells arrested in mitosis by the temperature-sensitive *cdc26Δ* mutant (*18*, *19*). The polyclonal antibodies to Mad3 recognize a background protein directly below the Mad3 protein. (**C**) Effect of *mad* mutants on Mad-Cdc20 interactions. Exponentially growing *mad1Δ*, *mad2Δ*, and *mad3Δ* strains containing HA-tagged Cdc20 were lysed, and the lysates were analyzed for the interactions of Mad2 and Mad3 with Cdc20.



ciate with Cdc20 independently or as part of a complex. In strains lacking Mad1, neither Mad2 nor Mad3 was associated with Cdc20; in strains lacking Mad2, Mad3 failed to bind Cdc20; whereas strains lacking Mad3 had unchanged levels of Mad2 associated with Cdc20 (Fig. 1C). Deletion of any one of the three *MAD* genes does not affect the amount of the remaining two Mad proteins (*21*). These observations suggest that the association of Mad2 and Mad3 with Cdc20 depends on the presence of Mad1. For Mad2, the dependence on Mad1 probably reflects the existence of a Mad1-Mad2 complex, which has been detected in budding yeast (*22*). The dependence of Mad3 binding on Mad1 suggests that Mad3 may also form part of this complex, but we cannot exclude the possibility that Mad3 binds to an interface created by the interaction of a Mad1-Mad2 complex with Cdc20. In some experiments, the level of Cdc20 in strains that lacked Mad1 or Mad3 was greater than in wild-type strains, suggesting that association of Cdc20 with the Mad proteins may regulate the stability or synthesis of Cdc20.

The association of Mad proteins with

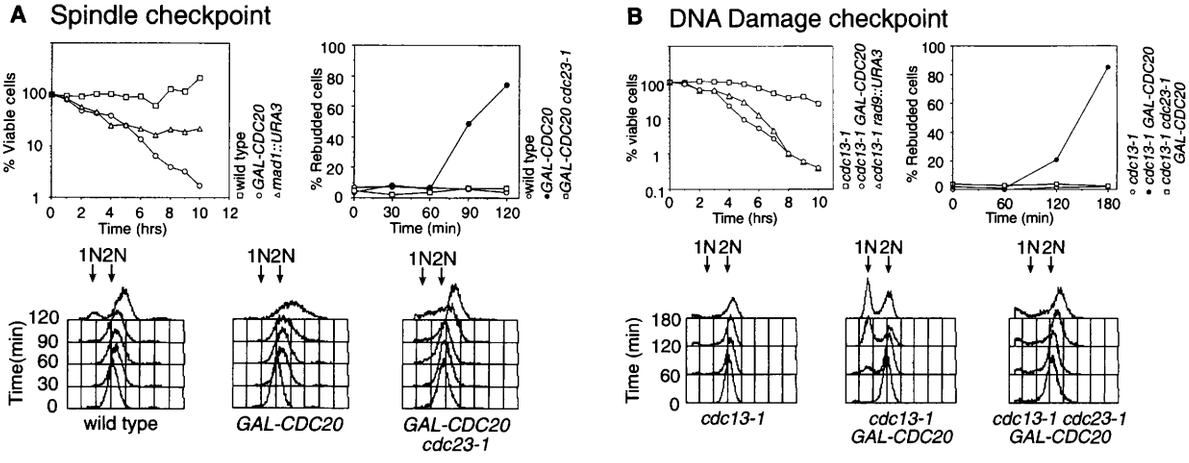


Fig. 2. Cdc20 overexpression overcomes the spindle and DNA damage checkpoints. The indicated strains were subjected to (**A**) spindle depolymerization by nocodazole, (**B**) DNA damage caused by shifting the *cdc13-1* mutant to 33°C, or (**C**) inhibition of DNA replication by hydroxyurea. For measurement of rebudding and DNA content (morphological and biochemical criteria for exit from mitosis), cells were grown in raffinose-containing medium, arrested by drug or temperature treatment for 165 min, and then treated with 2% galactose to induce expression of Cdc20 from the *GAL1* promoter. Samples were taken at the indicated times after galactose addition. Arrows indicate the 1N DNA content of G_1 cells and 2N content of G_2 cells. The slow rightward shift of the DNA peak in nocodazole-treated *cdc23-1* cells during the experiment is due to mitochondrial DNA replication. For measurements of cell viability, galactose was added to cells growing exponentially in raffinose-containing medium at the same time that nocodazole or hydroxyurea was added or the cells were shifted to 33°C. At the indicated times, cells were removed and tested for their ability to give rise to colonies on glucose-containing medium.

Cdc20 suggests that Cdc20 is the target for the spindle checkpoint. We therefore tested whether overexpressing Cdc20 overcame any cell cycle checkpoints (*23*). Cells overexpressing Cdc20 from the *GAL1* promoter were defective in two checkpoints: They no longer arrested in mitosis in response to spindle depolymerization (Fig. 2A) or to the DNA damage caused by the *cdc13-1* mutation [Fig. 2B and (*24*, *25*)]. Overexpression of Cdc20 bypasses checkpoints by activating the APC, because combining the *cdc23–1* mutation, which disrupts APC activity, with *GAL-CDC20* restores the mitotic arrest to cells with damaged spindles or DNA. Overexpressing Cdc20 does not overcome the cell cycle arrest caused by hydroxyurea, an inhibitor of DNA synthesis (Fig. 2C). These results are consistent with the idea that the spindle and DNA damage checkpoints arrest cells in mitosis by inhibiting Cdc20 and suggest that the DNA replication checkpoint uses another mechanism to arrest the cell cycle.

If Cdc20 is the target of the spindle checkpoint, it should be possible to isolate dominant mutations in Cdc20 that no longer respond to the checkpoint. Overexpression of *MPS1*, a component of the checkpoint, arrests cells in mitosis even though their spindle is still fully functional (*5*). We mutagenized the *CDC20* gene, selected for mutants that overcame the mitotic arrest caused by Mps1 overexpression, and analyzed four of these mutants in detail (*26*). Mutant forms of Cdc20, like *mad* and *bub* mutants (*5*), allowed cells overexpressing Mps1 to proliferate more rapidly than control cells (Fig. 3A). The Cdc20 mutants also increased the rate at which cells exited mitosis and died after treatment with nocodazole (Fig. 3B). The checkpoint-resistant Cdc20 mutants also weaken the DNA damage checkpoint, although this effect is somewhat variable (*21*). The checkpoint-resistant mutants complement the temperature-sensitive growth defect of *cdc20-1* strains. In the cdc20-*1* strains that carry the mutant plasmids, the spindle checkpoint is inactive at 37°C, demonstrating that the checkpoint defect is not due to a dominant negative activity of the Cdc20 mutants (*21*). The checkpoint-resistant Cdc20 mutants greatly diminished the binding of Mad2 and Mad3 to Cdc20 (Fig. 3C) but had no effect on the overall level of Mad1, Mad2, or Mad3 in the cell (*21*). For all four mutants, the checkpoint-resistant phenotype is conferred by the mutations in the region of Cdc20 shown by Kim *et al.* to be required for interaction with Mad2 in fission yeast (*7*). Sequencing this region revealed that all the mutants had changes in a short region of Cdc20 that corresponds to the region that contains the checkpoint-resistant mutation in Slp1 (Fig. 3D). Hoyt and Schott have also isolated a dominant allele of *CDC20* that bypasses the spindle assembly checkpoint but not the DNA damage checkpoint (*25*). It is not known if this mutant affects the interaction between Cdc20 and the Mad proteins.

The idea that the spindle checkpoint specifically inhibits Cdc20 is strengthened by comparing the sequences of Cdc20 and Hct1. These proteins have homologs in fission yeast, plants, and animals, and probably conserve the functional difference between Cdc20 and Hct1; Cdc20 is required for the proteolysis that drives the metaphase-to-anaphase transition, and Hct1 is required for cyclin B proteolysis during G_1 (*15*, *16*). Members of the Cdc20 family have conserved the region defined in fission yeast as the Mad2 interaction region (*7*), but members of the Hct1 family lack this homology. It has been suggested that Mad2 inhibits exit from mitosis by binding directly to the APC (*6*), but we have been unable to detect binding of Mad2 to the APC in yeast (*21*).

We do not yet understand how the checkpoint regulates Cdc20 activity. Although Mad2 and Mad3 are bound to Cdc20, this association does not change on activation of the checkpoint. Perhaps

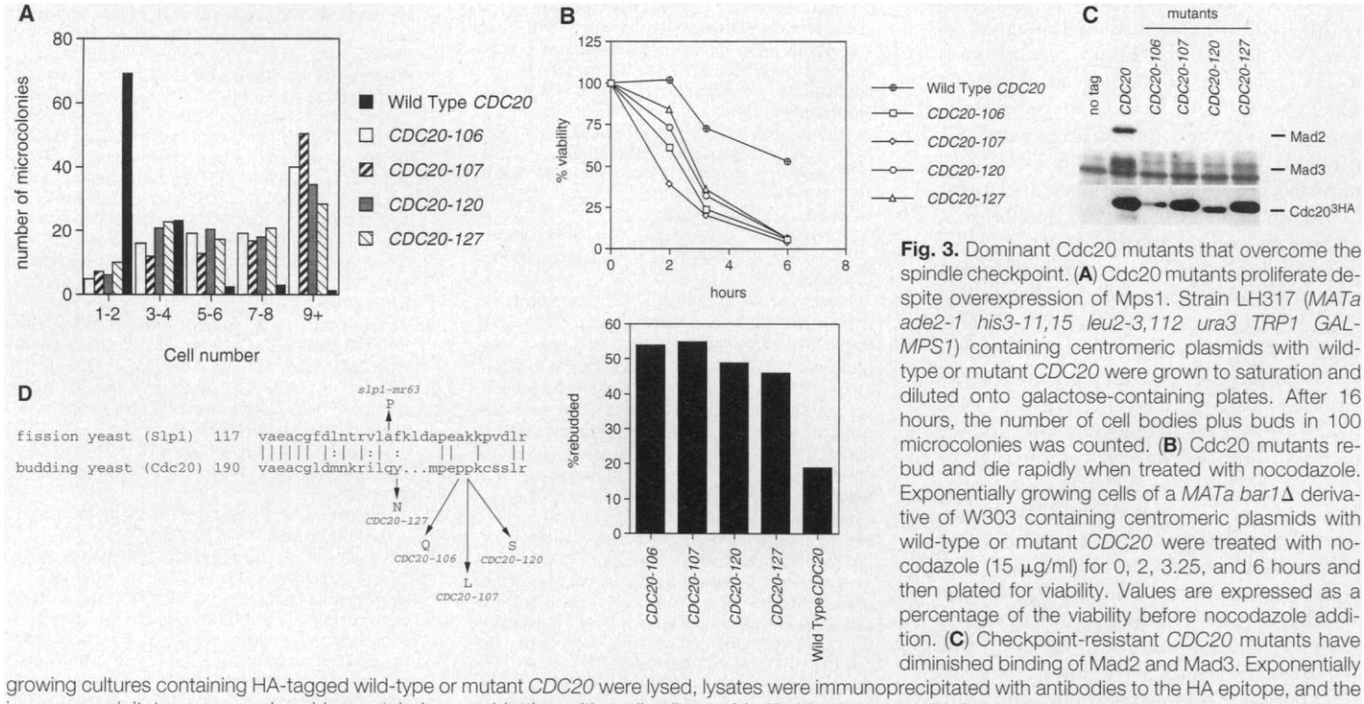


Fig. 3. Dominant Cdc20 mutants that overcome the spindle checkpoint. (**A**) Cdc20 mutants proliferate despite overexpression of Mps1. Strain LH317 (*MATa ade2-1 his3-11,15 leu2-3,112 ura3 TRP1 GAL-MPS1*) containing centromeric plasmids with wild-type or mutant *CDC20* were grown to saturation and diluted onto galactose-containing plates. After 16 hours, the number of cell bodies plus buds in 100 microcolonies was counted. (**B**) Cdc20 mutants rebud and die rapidly when treated with nocodazole. Exponentially growing cells of a *MATa bar1*Δ derivative of W303 containing centromeric plasmids with wild-type or mutant *CDC20* were treated with nocodazole (15 μg/ml) for 0, 2, 3.25, and 6 hours and then plated for viability. Values are expressed as a percentage of the viability before nocodazole addition. (**C**) Checkpoint-resistant *CDC20* mutants have diminished binding of Mad2 and Mad3. Exponentially growing cultures containing HA-tagged wild-type or mutant *CDC20* were lysed, lysates were immunoprecipitated with antibodies to the HA epitope, and the immunoprecipitates were analyzed by protein immunoblotting with antibodies to Mad2, Mad3, or HA. (**D**) Sequence changes in dominant, checkpoint-resistant *CDC20* alleles. Shown are changes from the wild-type sequence in four mutants and the comparison with the sequence of Slp1 (the fission yeast homolog of Cdc20) and the checkpoint-resistant Slp1 mutant described by Kim *et al.* (*7*). The *CDC20-120* allele includes two changes outside this region, Trp^{226}→Ser and Trp^{247}→Ile; we do not know if these changes are required to confer the mutant phenotype. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

modification of one of the Mad proteins converts it into a form that can inhibit as well as bind to Cdc20. Although we have not detected modification of Mad2 and Mad3, Mad1 becomes hyperphosphorylated on activation of the checkpoint (8). An alternative possibility is that other, undiscovered proteins show checkpoint-dependent binding to Mad2 or Mad3 and inhibit Cdc20 activity.

Overexpression of Cdc20 and dominant Cdc20 mutants interferes with both the DNA damage and spindle checkpoints. The role of Cdc20 in the DNA damage checkpoint is independent of the Mad proteins because *mad* mutants have an intact DNA damage checkpoint (*27*). We speculate that in all eukaryotes the spindle checkpoint prevents the onset of anaphase by inhibiting Cdc20. In contrast, inhibition of mitotic exit by the DNA damage checkpoint is likely to be confined to organisms like budding yeast that lack a clearly defined transition between G_2 and mitosis. In animals and fission yeast, DNA damage can arrest cells in G_2, thus keeping them from entering mitosis and condensing their chromosomes. In budding yeast, the mitotic spindle assembles normally in cells with damaged or unreplicated DNA. Thus, the checkpoints that detect these defects must be able to prevent the exit from rather than the entry into metaphase. The budding yeast Pds1 protein may be involved in this evolutionary shift in the target of the DNA damage checkpoint. Destruction of Pds1 and its fission yeast homolog (Cut2) are required to separate sister chromatids (*9*, *11*), and in budding yeast Pds1 is required for the DNA damage checkpoint in G_2 (*28*). One explanation for this dual requirement is that Pds1 acts both as a substrate and an inhibitor of the APC, with inhibitory function being independent of the destruction box. In undamaged cells, Pds1 binding to the activated APC would partially inhibit APC activity, but because the APC can target Pds1 for destruction, cells could rapidly escape this inhibition. DNA damage would induce modifications of Pds1 that increased its ability to inhibit the APC; consequently, Pds1 and mitotic cyclins would be stable, sister chromatids would not separate, and cells would remain in metaphase. This model explains why an indestructible form of Pds1 arrests budding yeast in metaphase. A similar mutation in Cut2, the fission yeast homolog of Pds1, prevents sister chromatid separation but not cyclin B destruction and the exit from mitosis. This difference correlates with the different organization of the cell cycle in the two yeasts.

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17. All experiments in this study were done in strains isogenic to W303 with standard media (*29*). Wild-type or *cdc26Δ* strains contained the centromeric plasmid pLH68, which contains the *CDC20* gene with a triple hemagglutinin (HA) tag and a six-histidine tag at its COOH-terminus and the *URA3* gene. Cultures were grown in medium without uracil until mid-log phase, transferred to rich medium without further treatment, and then treated with α-factor (1 μg/ml) to induce G_1 arrest or with nocodazole (15 μg/ml) to activate the spindle checkpoint; in *cdc26Δ* strains, the cells were shifted to 37°C to inactivate the APC. Cells were harvested and lysed, and lysates were immunoprecipitated and protein immunoblotted as described (*19*), with the exception that the lysis buffer contained 50 mM β-glycerophosphate instead of 1 mM sodium vanadate.
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23. The plasmid YIplac211GAL-CDC20 contains the wild-type *CDC20* open reading frame (ORF) fused to the *GAL1* promoter and was directed to integrate into the *URA3* gene by digestion with Nco I. To examine the spindle and DNA replication checkpoints, we grew wild-type cells (A1015), cells carrying two copies of a *GAL-CDC20* fusion (A1016), or *GAL-CDC20 cdc23-1* (A1022) cells to exponential phase and treated them with nocodazole (15 μg/ml) or hydroxyurea (10 mg/ml) in YEP (yeast extract, peptone) raffinose at 23°C for 165 min to induce cell cycle arrest before adding galactose to 2% to induce production of excess CDC20. Samples were taken at the indicated times after galactose addition to determine DNA content and the percentage of cells that had rebudded. To examine the DNA damage checkpoint, we grew the *cdc13-1* (A1017), *cdc13-1 GAL-CDC20* (A1018), and *cdc13-1 cdc23-1 GAL-CDC20* (A1023) strains to exponential phase at 23°C and shifted them to 33°C for 165 min in YEP raffinose medium before adding galactose. Samples were taken at the indicated times after galactose addition to determine DNA content and the fraction of cells that had rebudded. Viability was measured by adding nocodazole (15 μg/ml) or hydroxyurea (10 mg/ml), or shifting the temperature to 33°C (for *cdc13-1*) for wild-type, *mad1::URA3* (A928), *mec1-1* (K2888) *cdc13-1*, or *cdc13-1 rad9::URA3* (K2554) strains. For corresponding strains that also contained *GAL-CDC20*, galactose was added to 2% at the time of the other addition or temperature shift. Samples were withdrawn at the indicated times and plated on YEP glucose plates. Colonies were counted after 3 days. Induction of *GAL-CDC20* leads to spindle elongation in cells arrested by the *cdc13-1* mutation but not in cells arrested by hydroxyurea treatment (*20*, *24*).
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26. The plasmid pCM4 is derived from the *URA3* centromeric plasmid YCPlac33 (*30*) and contains the wild-type *CDC20* gene under the control of its own promoter. The ORF, 848 bases upstream, and 232 bases downstream were amplified by mutagenic polymerase chain reaction. The product DNA was mixed with a Bst EII–Sac I fragment of pCM4, which lacks all but 487 bases of the *CDC20* ORF and transformed into the diploid strain DA2050A (*a/a bar1/bar1 mec1-1/mec1-1 CDC28-VF::LEU2/CDC28-VF::LEU2 GAL1-MPS1/GAL1-MPS1*). The *CDC28-VF* mutation blocks adaptation to the spindle assembly checkpoint (*31*), whereas homozygosity at *MAT* and the *mec1-1* mutation have no effect on the behavior of checkpoint-resistant mutants. The transformation mix was plated on URA$^-$ plates containing 2% galactose. Plasmid DNA was recovered from colonies that grew on these plates, then was purified and amplified by transformation into *Escherichia coli* and retransformed into DA2050A; transformants were selected on URA$^-$ glucose plates. pCM4 derivatives that carried the checkpoint-resistant *CDC20* mutants were transformed into strains LH317 (*MATa ade2-1 his3-11,15 leu2-3,112 ura3 TRP1 GAL-MPS1*) and a *MATa bar1Δ* derivative of W303. For microcolony assays and rebudding, cultures were grown to saturation in URA$^-$ glucose medium, diluted 1:1000, and sonicated to break up cell clumps, and 5 μl was spotted on YEP 2% galactose plates (for transformants in LH317) or YEP 2% glucose plus benomyl plates (10 μg/ml) (for transformants in *MATa bar1Δ*). At the indicated times, the number of cells plus buds was counted in at least 100 microcolonies. To monitor nocodazole induced death, we diluted cultures grown to saturation in URA$^-$ glucose medium into YEP glucose medium, then incubated them at 30°C for 16 hours before adding nocodazole to 15 μg/ml. Cells were plated for viability on URA$^-$ glucose plates at the time of nocodazole addition and 2, 3.25, and 6 hours later.
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