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Cyclin-Specific Control of Ribosomal DNA Segregation▽

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Following chromosome duplication in S phase of the cell cycle, the sister chromatids are linked by cohesin. At the onset of anaphase, separase cleaves cohesin and thereby initiates sister chromatid separation. Separase activation results from the destruction of its inhibitor, securin, which is triggered by a ubiquitin ligase called the anaphase-promoting complex (APC). Here, we show in budding yeast that securin destruction and, thus, separase activation are not sufficient for the efficient segregation of the repetitive ribosomal DNA (rDNA). We find that rDNA segregation also requires the APC-mediated destruction of the S-phase cyclin Clb5, an activator of the protein kinase Cdk1. Mutations that prevent Clb5 destruction are lethal and cause defects in rDNA segregation and DNA synthesis. These defects are distinct from the mitotic-exit defects caused by stabilization of the mitotic cyclin Clb2, emphasizing the importance of cyclin specificity in the regulation of late-mitotic events. Efficient rDNA segregation, both in mitosis and meiosis, also requires APC-dependent destruction of Dbf4, an activator of the protein kinase Cdc7. We speculate that the dephosphorylation of Clb5-specific Cdk1 substrates and Dbf4-Cdc7 substrates drives the resolution of rDNA in early anaphase. The coincident destruction of securin, Clb5, and Dbf4 coordinates bulk chromosome segregation with segregation of rDNA.

Cyclin-dependent kinases (Cdks) determine the order of cell cycle events by phosphorylating different substrates at different points in the cell cycle. The temporal control of substrate phosphorylation is made possible by the periodic synthesis and destruction of multiple cyclin cofactors that bind and activate the Cdk (17). Different cyclins have different functional capacities, in part because they target the associated Cdk to specific substrates (2, 4). In the budding yeast *Saccharomyces cerevisiae*, for example, a complex of the S-phase cyclin Clb5 and Cdk1 displays high specificity for a small subset of Cdk1 substrates, due to a docking site on Clb5 that greatly increases its affinity for those substrates (16). Many Clb5-specific substrates are involved in DNA synthesis, helping to explain why Clb5-Cdk1 drives early cell cycle events.

The final events of M phase require dephosphorylation of Cdk1 targets, which results from a combination of cyclin destruction, induction of Cdk1 inhibitors, and activation of phosphatases, such as Cdc14 of budding yeast, that remove phosphates from Cdk1 sites (30). Clb5 and the major mitotic cyclin Clb2 are both targeted for destruction by the anaphase-promoting complex (APC), a ubiquitin ligase that is activated in metaphase by association with the cofactor Cdc20 (APC^{Cdc20}) (22, 32). APC^{Cdc20} triggers the complete destruction of Clb5 before anaphase but promotes only partial destruction of Clb2, which is completely destroyed later in mitosis when the APC is activated by Cdh1 (24, 39). These differences in the timing of cyclin destruction might provide a mechanism to allow the dephosphorylation of Clb5-specific substrates in early anaphase, while the general substrates of Clb2-Cdk1 remain phosphorylated until later (30). The Clb5-specific substrates Fin1 and Ase1, for example, are dephosphorylated in early anaphase, allowing them to help initiate important changes in mitotic spindle dynamics (12, 40).

If the order of cyclin destruction determines the timing of late-mitotic events, then the stabilization of different cyclins should have distinct effects on mitotic processes. Indeed, the stabilization of metazoan cyclin A (by mutation of sequences required for APC recognition) causes phenotypes very different than those caused by stabilized cyclin B (21, 25). In budding yeast, stabilization or overexpression of Clb2 causes defects in spindle breakdown and mitotic exit, whereas partially stabilized Clb5 has little apparent effect on late-mitotic events (39). Overexpression of Clb5 is highly toxic (11), but the cellular defects that result from excess Clb5-Cdk1 activity are not clear.

The initiating event of anaphase is sister chromatid separation, which results primarily from the proteolytic cleavage of cohesin by separase (19). In budding yeast, however, separase-mediated cohesin destruction is not sufficient for resolution of the highly repetitive ribosomal DNA (rDNA) locus on chromosome XII. Segregation of the rDNA also requires separase-dependent activation of the phosphatase Cdc14 (5, 28), suggesting that dephosphorylation of Cdk1 substrates is required.

It remains unclear if destruction of APC^{Cdc20} substrates other than securin is required for resolution of the rDNA in anaphase. Here, we investigate this question and find that the degradation of two additional APC^{Cdc20} substrates, Clb5 and Dbf4, contributes to rDNA segregation. Our results also provide evidence for cyclin specificity in late-mitotic control, as rDNA resolution depends on loss of Clb5 activity but not on loss of activity of the major mitotic cyclin Clb2. We also show that degradation of Clb5 is critical for origin relicensing and, hence, DNA replication. Finally, we demonstrate that the molecular mechanisms that govern loss of rDNA cohesion in mitosis are similar to those in anaphase I of the meiotic program.

MATERIALS AND METHODS

All mitotic experiments were performed in strain W303, and all meiotic experiments were performed in strain SK1. Epitope tagging of endogenous genes

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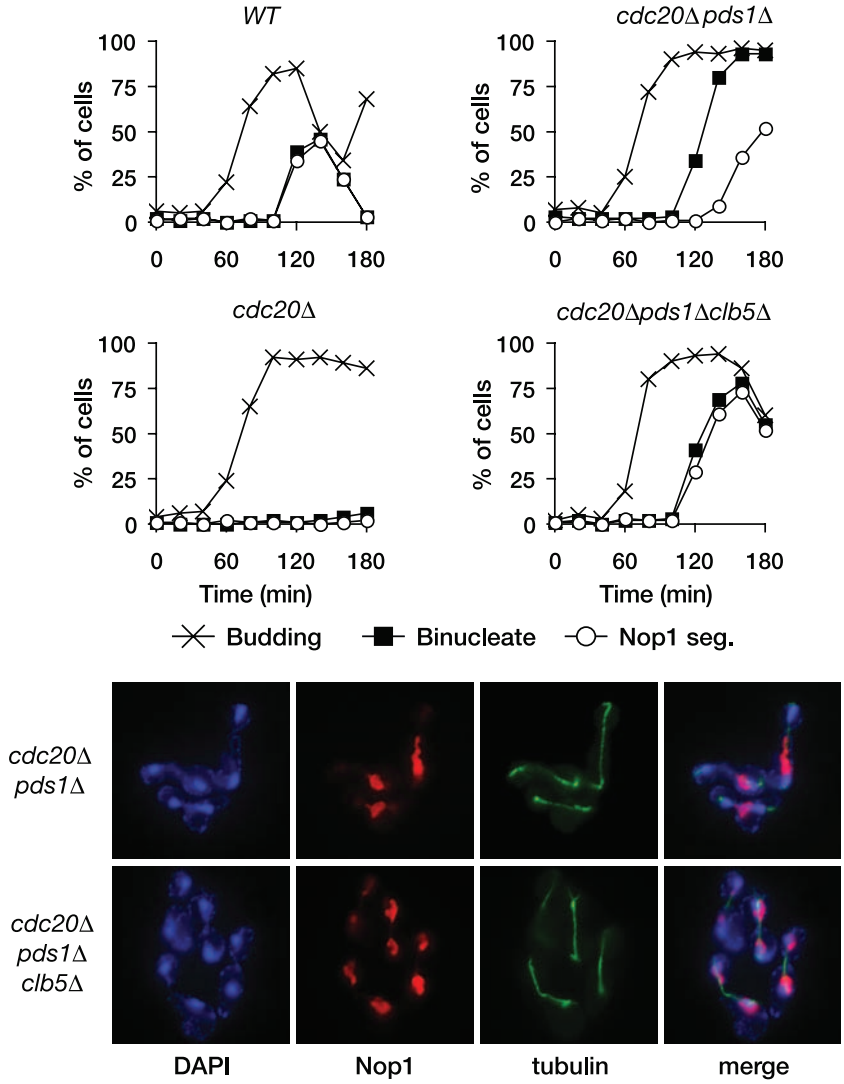

FIG. 1. APC^{Cdc20}-dependent degradation of securin and Clb5 allows rDNA segregation. Wild-type (WT), *cdc20*Δ, *cdc20*Δ *pds1*Δ, and *cdc20*Δ *pds1*Δ *clb5*Δ yeast expressing *GAL-CDC20* were arrested in G_1 with α-factor and then released into fresh dextrose media to repress *CDC20* transcription. Samples were collected every 20 min for analysis of budding, binucleate formation (bulk chromosome segregation, assessed by DAPI [4′,6-diamidino-2-phenylindole] staining), and Nop1 segregation (a marker of nucleolar rDNA segregation) (top panel). The bottom panel shows representative images of *cdc20*Δ *pds1*Δ and *cdc20*Δ *pds1*Δ *clb5*Δ yeast 140 min after release from G_1.

and gene deletions was performed by gene targeting using PCR products. The *CLB5* gene, along with three C-terminal hemagglutinin epitopes and 451 bp of the *CLB5* promoter, was amplified from budding yeast and cloned to allow integration of an extra copy of *CLB5-HA3* at the *URA3* locus. This plasmid was modified to generate the N-terminal deletion, *CLB5-ΔN*, by removal of the coding sequence for amino acids 2 to 95. For expression in meiosis, the coding sequence of *CLB5-ΔN-TAP* was placed in front of 341 bp of the *DMC1* promoter to allow integration of *pDMC1-CLB5-ΔN-TAP* at the *HIS3* locus. To express high levels of cyclins in mitosis, *CLB5-ΔN-TAP* or *CLB2-ΔN-HA3* (encoding Clb2 lacking amino acids 2 to 176) was cloned in front of the *GAL1-10* promoter. To make stabilized Dbf4, a plasmid that contained 523 bp of the *DBF4* promoter and a portion of the *DBF4* gene with the coding sequence for amino acids 2 to 65 removed was created. This plasmid was linearized to integrate at the *DBF4* locus and to switch the endogenous gene to *DBF4-ΔN* and was confirmed by PCR.

Conditions for growth and release of synchronous cultures from arrest in G_1 by α-factor were performed as described previously (16). In the *GAL-CDC20* shutoff experiment (Fig. 1), cells were grown in raffinose/galactose media for α-factor arrest and released into glucose media. In the *GAL-CLB* experiment (Fig. 2), cells were grown in raffinose media for α-factor arrest and released into raffinose/galactose media. In experiments involving *GAL-SIC1*, cells were grown in raffinose/galactose media for α-factor arrest (Fig. 3, 4, and 5). Once arrested, glucose was added for 30 min, and cells were released from the arrest into fresh glucose media. Synchronous meiotic experiments were performed as described previously (29). Western blotting, budding counts, and immunofluorescence analysis were performed as described previously (28). The antibodies used were α-Nop1 clone MCA28F2 (Encor), α-tubulin YOL1/34 (Serotec), α-hemagglutinin clone 16B12 (Babco), and α-myc clone 9E10 (Babco).

RESULTS

Resolution of the rDNA requires APC^{Cdc20}-mediated degradation of Clb5 and securin. To test whether the destruction of APC^{Cdc20} targets other than securin governs rDNA resolution, we examined chromosome segregation in budding-yeast cells lacking Cdc20 and securin (Pds1 in yeast). Wild-type, *cdc20*Δ, and *cdc20*Δ *pds1*Δ strains, expressing Cdc20 from the repressible *GAL1-10* promoter, were arrested in G_1 by α-factor treatment

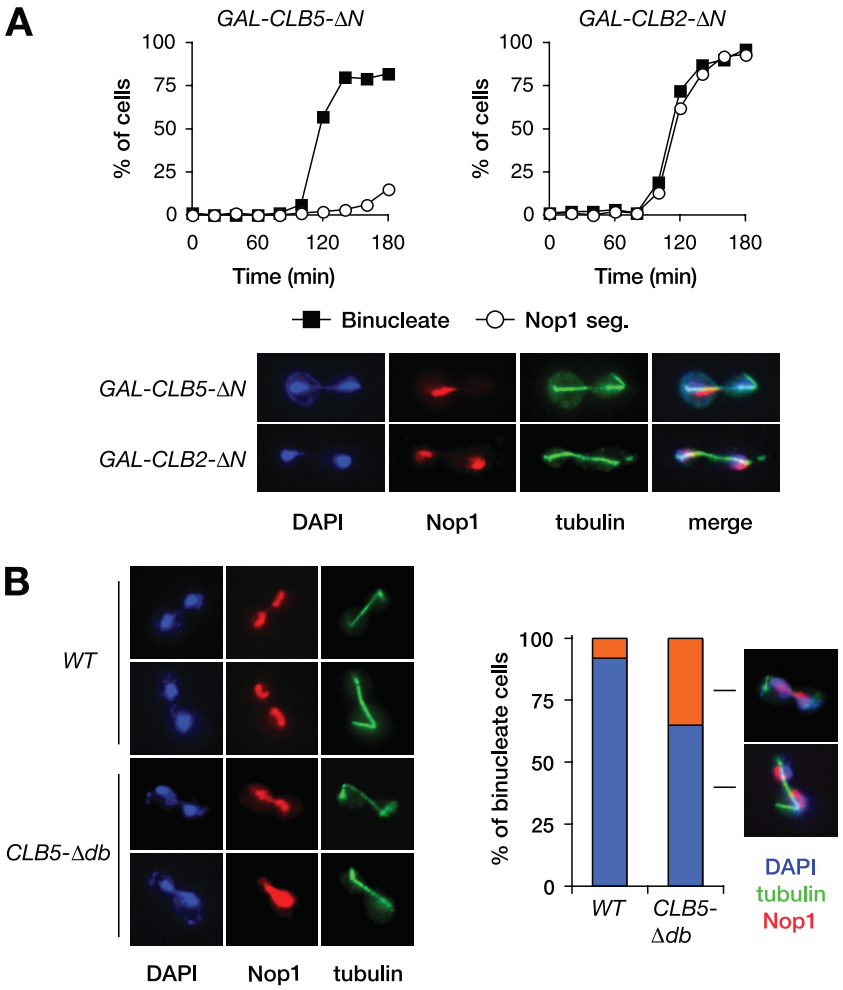

FIG. 2. Clb5 inhibits rDNA segregation. (A) Cells carrying either *GAL-CLB5-ΔN* or *GAL-CLB2-ΔN* were arrested in G_1 with α-factor and then released into fresh galactose media to induce cyclin expression. Samples were collected every 15 min for analysis of binucleate formation and Nop1 segregation. Representative images are shown of *GAL-CLB5-ΔN* yeast and *GAL-CLB2-ΔN* yeast 120 min after release from G_1. (B) Asynchronous *CLB5* (WT) and *CLB5-Δdb* cells were collected and binucleates analyzed for the number of segregated and unsegregated Nop1 masses. Representative images are shown (left).

and then released into dextrose media to repress transcription of *CDC20*. Budding and binucleate formation were scored. To examine rDNA segregation, we used immunofluorescence analysis of Nop1, a nucleolar protein that is involved in rDNA processing. Nop1 associates with the rDNA throughout the budding-yeast cell cycle, and its division into two distinct foci at anaphase is a useful marker of rDNA segregation. Wild-type cells segregated bulk DNA and rDNA with roughly equivalent timing, while *cdc20*Δ cells failed to segregate either (Fig. 1). In contrast, bulk chromosome segregation in *cdc20*Δ *pds1*Δ cells occurred with normal timing, but rDNA segregation was delayed. Destruction of APC^{Cdc20} targets other than securin might therefore be necessary for efficient rDNA segregation.

Deletion of *CLB5* is known to restore viability to *cdc20*Δ *pds1*Δ mutants (24). As Clb5 is an APC^{Cdc20} substrate, this suggests that the failure of *cdc20*Δ *pds1*Δ yeast to efficiently segregate their rDNA might be due to the continued presence of Clb5. Consistent with this possibility, we found that deletion of *CLB5* almost completely abolished the rDNA segregation delay in the *cdc20*Δ *pds1*Δ strain (Fig. 1). Thus, APC^{Cdc20} promotes rDNA segregation at anaphase onset by targeting both securin and Clb5 for destruction.

rDNA cohesion is controlled specifically by Clb5-Cdk1 activity. The rDNA segregation defect caused by Clb5 could be due to either a specific role for Clb5-Cdk1 or a general increase in Cdk1 activity. To distinguish between these possibilities, we compared the effects of different cyclins on rDNA segregation. Cells were released from G_1 in the presence of galactose-inducible Clb5 or Clb2 protein (each stabilized by removal of amino-terminal APC-targeting domains, as discussed below). Cells overexpressing either cyclin arrested as large-budded cells with segregated DNA masses (Fig. 2A), and examination of chromosome III segregation revealed that bulk chromatin segregated accurately (data not shown). Strikingly, however, rDNA segregation was almost completely blocked in cells overexpressing Clb5 but was unperturbed in cells overexpressing Clb2. This result indicates that rDNA cohesion is mediated specifically by Clb5-Cdk1 activity.

Stabilized Clb5 mutants cause rDNA segregation defects. If efficient rDNA resolution requires Clb5 destruction, then seg-

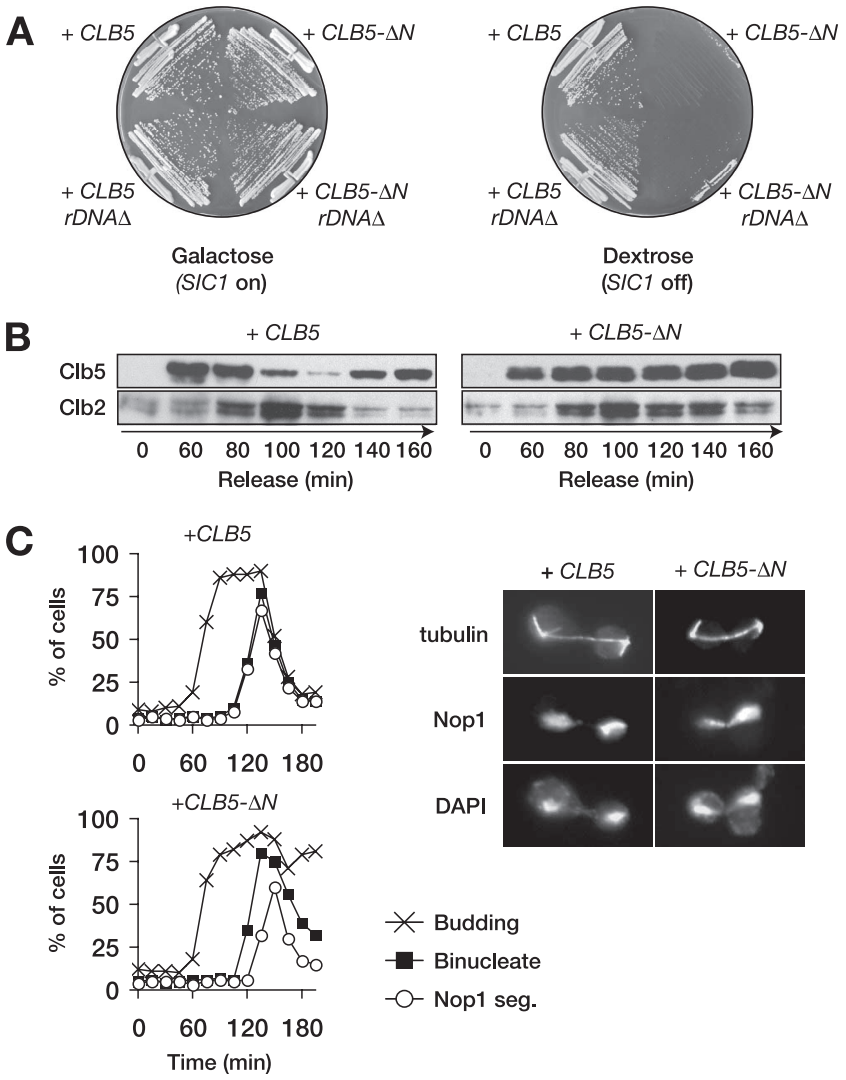

FIG. 3. *CLB5-ΔN* is lethal and causes an rDNA segregation delay. (A) Wild-type or *rDNA*Δ cells, carrying *GAL-SIC1* and an extra copy of the *CLB5* or *CLB5-ΔN* gene (expressed from the *CLB5* promoter), were plated on either galactose or dextrose media. (B) *GAL-SIC1* cells carrying an extra copy of *CLB5* or *CLB5-ΔN* were arrested in G_1 by α-factor treatment and released into fresh dextrose media to repress *SIC1* transcription. Samples were taken every 20 min, and the levels of Clb5 and Clb2 were analyzed by Western blotting. (C) Procedure was as in panel B, but α-factor was added 90 min after release to arrest cells in the following G_1. Samples were collected every 15 min for analysis of budding, binucleate formation, and Nop1 segregation. Images on the right show representative cells 140 min after release from G_1.

regation defects should result when stabilized mutant forms of Clb5 are expressed at physiological levels. APC^{Cdc20} recognizes its substrates through interactions with specific sequence motifs such as the D-box (22). The amino-terminal region of Clb5 contains a D-box, the removal of which partly stabilizes it in anaphase (the Clb5-Δdb mutant) (39). Clb5-Δdb is not lethal and has no clear effect on mitotic progression. We examined rDNA segregation in asynchronous populations of wild-type and *CLB5-Δdb* cells. 92% of wild-type cells that had segregated their chromosomes had also segregated their rDNA, whereas this was the case for only 65% of *CLB5-Δdb* cells (Fig. 2B). Thus, partly stabilized Clb5 slows rDNA segregation.

Many APC substrates contain multiple destruction determinants in addition to the D-box. Full stabilization of cyclins, for example, often requires the deletion of their entire N-terminal regions. To produce fully stabilized Clb5, we removed its 95 amino-terminal residues, leaving its Cdk1-binding domain intact (the Clb5-ΔN mutant). Transformation of wild-type cells with a plasmid carrying *CLB5-ΔN* under the control of its own promoter was lethal. This lethality was suppressed by overexpression of *SIC1* from the *GAL1-10* promoter (Fig. 3A). When expression of *GAL-SIC1* was repressed by plating on glucose media, cells carrying the *CLB5-ΔN* formed inviable microcolonies. To confirm that Clb5 was stabilized by the removal of its N terminus, cells containing an extra copy of either *CLB5* or *CLB5-ΔN* were released from G_1 arrest in the absence of *SIC1* expression. Levels of wild-type Clb5 fell at anaphase onset, while the amount of Clb5-ΔN was unchanged (Fig. 3B). Thus, removal of the N terminus of Clb5 stabilizes it in anaphase and causes cell lethality.

We analyzed anaphase progression in *CLB5* and *CLB5-ΔN* cells, again shutting off *SIC1* expression after release from G_1.

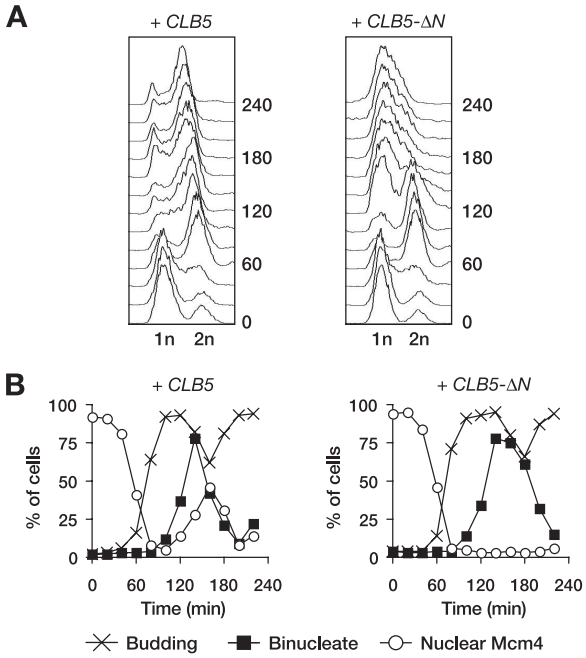

FIG. 4. *CLB5*-Δ*N* causes DNA replication defects. (A) *CLB5* and *CLB5*-Δ*N* cells were released from G_1 arrest in the absence of *SIC1* as in Fig. 3B, and flow cytometry was used to analyze cellular DNA content. (B) Procedure was as in panel A but with *MCM4-GFP* cells, which were analyzed for budding, binucleates, and the localization of the Mcm4-GFP protein.

α-Factor was added back to the cultures after the initiation of budding. In *CLB5*-Δ*N* cells, budding and chromosome segregation occurred with normal timing (Fig. 3C). However, there was a significant delay in rDNA segregation in these cells, demonstrating that Clb5 degradation is necessary for timely resolution of the rDNA. Unlike wild-type cells, *CLB5*-Δ*N* cells failed to arrest in the following G_1, suggesting that Clb5-ΔN prevented the G_1 arrest mediated by mating pheromone.

We considered the possibility that Clb5-ΔN prevents rDNA segregation by blocking activation of the phosphatase Cdc14, which is required for rDNA segregation (5, 28). However, the phenotypes of the *CLB5*-Δ*N* mutant are clearly distinct from those of temperature-sensitive *cdc14* mutants. *cdc14-1* cells, in addition to delaying rDNA segregation, fail to break down the anaphase spindle, fail to undergo cytokinesis and do not enter the next cell cycle (26). In contrast, *CLB5*-Δ*N* cells, like *CLB5*-Δ*db* cells (39), showed no significant defects in mitotic exit, as judged by spindle disassembly, cytokinesis, or new cycle entry, although Clb2 destruction was slightly delayed (Fig. 3B). Furthermore, previous studies of *cdc20*Δ *pds1*Δ cells showed that Cdc14 is released efficiently from the nucleolus at anaphase onset even though Clb5 levels remain high (24). Together, these lines of evidence suggest that the delayed rDNA segregation of *CLB5*-Δ*N* cells is not due to problems with Cdc14 activation but instead that Cdk1-Clb5 promotes rDNA cohesion through another mechanism.

One possibility is that Clb5 opposes the effects of Cdc14 on the phosphorylation state of Cdk1 targets involved in rDNA cohesion. Consistent with this possibility, we found that deletion of *CLB5* partly suppressed rDNA segregation defects in *cdc14-1* cells. When arrested for 2.5 h at 37°C, rDNA segregation occurred in 44% of *cdc14-1* cells and 68% of *cdc14-1* Δ*clb5* cells in three separate experiments.

We next tested if the lethality of the *CLB5*-Δ*N* mutation results from a failure to efficiently segregate chromosome XII, which contains the rDNA. We used a yeast strain from which the entire rDNA locus is deleted and viability is supported by multiple copies of a plasmid carrying an rDNA repeat (36). A *GAL-SIC1* plasmid was introduced into this strain, along with a plasmid carrying either *CLB5* or *CLB5*-Δ*N*. Deletion of the rDNA locus did not restore the viability of *CLB5*-Δ*N* cells when *SIC1* expression was repressed on dextrose media (Fig. 3A). We conclude that rDNA segregation defects are not the sole cause of *CLB5*-Δ*N* lethality.

DNA replication fails in *CLB5*-Δ*N* cells. Clb5-Cdk1 is a critical regulator of DNA replication. To investigate whether DNA replication is aberrant in cells expressing stabilized Clb5, we measured the DNA content of cells expressing *CLB5* or *CLB5*-Δ*N* after release from G_1 in the absence of Sic1. Both strains completed the first round of DNA replication and progressed into mitosis with similar timing, revealing no significant defects in the first cycle of DNA replication (Fig. 4A). The *CLB5* strain then entered the next cell cycle and completed another S phase and progressed through the second and subsequent cell cycles. In contrast, *CLB5*-Δ*N* cells failed to undergo a second round of DNA replication and arrested as large-budded cells with a 1n DNA content (Fig. 4A). Thus, the continued presence of Clb5 after anaphase prevents DNA replication and thereby causes cell death.

We suspected that Clb5-ΔN blocked DNA replication by inhibiting the formation of prereplicative complexes (pre-RCs) at replication origins. From early S to late M phases, Cdk1 blocks pre-RC assembly by phosphorylating and thereby inhibiting several pre-RC subunits; Cdk1 inactivation in late mitosis then triggers pre-RC assembly (1, 20). A key pre-RC component is the Mcm complex, the phosphorylation of which prevents its nuclear import (14, 15). We examined the localization of a GFP-tagged Mcm complex in cells released from a G_1 arrest. In cells expressing wild-type *CLB5*, the Mcm complex moved from the nucleus to the cytoplasm during S phase and reentered the nucleus in late mitosis (Fig. 4B). In contrast, late-mitotic nuclear reentry of the Mcm complex did not occur in *CLB5*-Δ*N* cells, and it remained cytoplasmic. Thus, the continued presence of Clb5 after anaphase blocks the nuclear import of the Mcm complex, presumably preventing efficient pre-RC formation. It seems likely that this problem leads to defects in the initiation of DNA replication, and the resulting replication stress triggers a DNA damage response that blocks cell cycle progression.

APC^{Cdc20}-mediated destruction of Dbf4 contributes to timely rDNA disjunction. We noted earlier that *cdc20*Δ *pds1*Δ cells exhibited a 30-min delay in rDNA segregation (Fig. 1). However, expression of Clb5-ΔN in otherwise wild-type cells caused only a 15-min delay in rDNA segregation (Fig. 3C). One explanation for this difference is that Clb5-ΔN is not fully stabilized, but we could find no evidence for that (Fig. 3B). An alternate possibility is that degradation of an additional APC^{Cdc20} substrate contributes to rDNA segregation. One appealing candidate was Dbf4, the activating subunit for the protein kinase Cdc7. Dbf4 is known to be an APC substrate

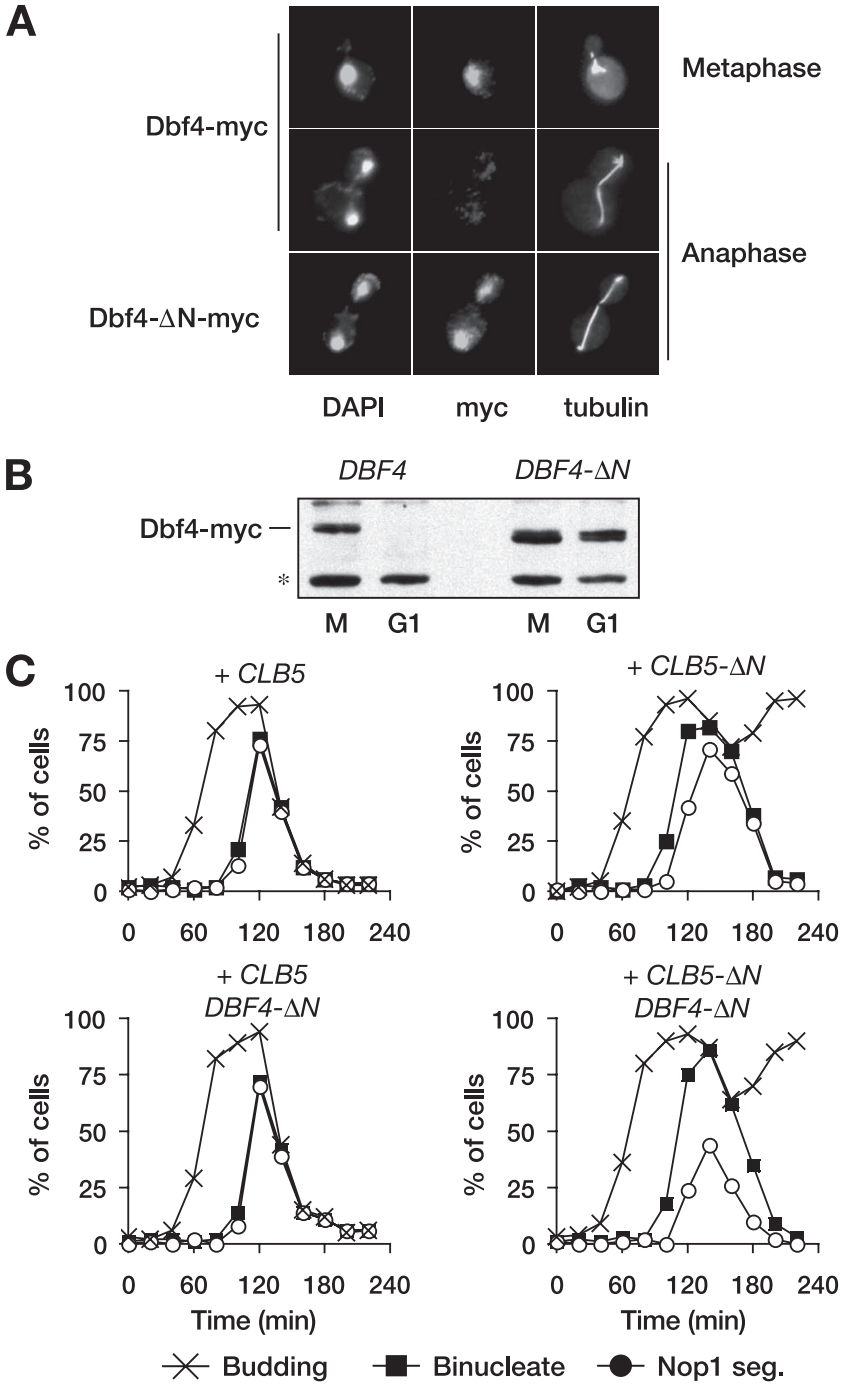

FIG. 5. Stabilization of Dbf4 contributes to the rDNA segregation delay. (A) Representative images of Dbf4-myc in metaphase or anaphase from wild-type or *DBF4-ΔN* cells. (B) *DBF4* and *DBF4-ΔN* cells were arrested in M phase by nocodazole treatment or G_1 by α-factor treatment, and the levels of Dbf4 were analyzed. The asterisk indicates a nonspecific background band recognized by the anti-myc antibody. (C) *DBF4* and *DBF4-ΔN* cells, carrying an extra copy of either *CLB5* or *CLB5-ΔN*, were released from a G_1 arrest as in Fig. 3C, and α-factor was re-added 80 min after release to arrest cells in the following G_1. Samples were collected every 20 min for analysis of budding, binucleate formation, and Nop1 segregation.

whose levels drop in mitosis, although the precise timing of its destruction and the APC cofactor involved are not known (7). We found that APC^{Cdc20} ubiquitinates Dbf4 in vitro and that Dbf4 is destroyed at anaphase onset, independently of *CDH1* (Fig. 5A and data not shown), suggesting that Dbf4 is an APC^{Cdc20} substrate. Removal of its amino-terminal destruction sequences stabilized Dbf4 in mitosis and G_1 (Fig. 5A and B).

To assess the contribution of Dbf4 to rDNA segregation, we constructed a strain in which the endogenous *DBF4* gene was

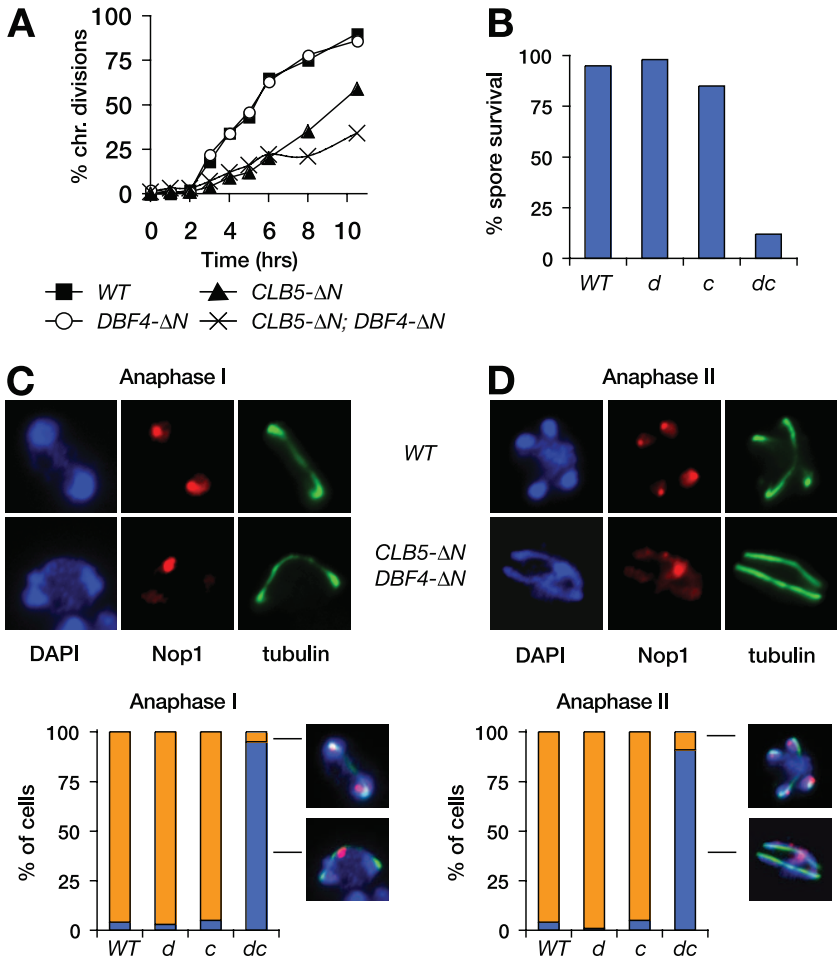

FIG. 6. Meiotic rDNA segregation fails in the presence of stabilized Dbf4 and Clb5. Diploid *DBF4* and *DBF4-ΔN* SK1 cells, with or without *pDMC1-CLB5-ΔN*, were followed through synchronous meiosis. Chromosome divisions were scored (A), as were the survival rates of resulting spores (B) and the segregation patterns of Nop1 through both anaphase I (C) and anaphase II (D). Histograms are labeled *WT* (wild type), *d* (*DBF4-ΔN*), *c* (*pDMC1-CLB5-ΔN*), and *dc* (*DBF4-ΔN* with *pDMC1-CLB5-ΔN*).

replaced with *DBF4-ΔN*. This strain displayed no apparent defects in cell viability, mitotic progression, or rDNA segregation (Fig. 5C). We then introduced either *CLB5* or *CLB5-ΔN* in combination with *GAL-SIC1* and examined cell cycle progression. Notably, cells carrying both *CLB5-ΔN* and *DBF4-ΔN* exhibited an rDNA segregation defect that was more severe than that seen in *CLB5-ΔN* cells. The average delay in rDNA segregation relative to bulk chromatin in the *CLB5-ΔN DBF4-ΔN* cells was similar to that in *cdc20Δ pds1Δ* cells, and many cells failed to separate their nucleolus before cytokinesis. Thus, Clb5 and Dbf4 are APC^{Cdc20} targets that must both be destroyed for efficient rDNA segregation.

Meiotic rDNA segregation fails in the continued presence of Clb5 and Dbf4. In anaphase I of meiosis, homologs are resolved by loss of cohesin on the chromosome arms. However, rDNA cohesion must also be resolved to allow segregation of the chromosome XII homologs. We investigated whether meiotic rDNA segregation involves the same mechanisms as those we identified in mitosis. Immunofluorescence analysis revealed that both Clb5 and Dbf4 were present at high levels in metaphase I and absent in anaphase I, consistent with degradation via APC^{Cdc20} (data not shown). We then expressed stabilized Clb5 and/or Dbf4 in the SK1 budding-yeast strain, which allows analysis of synchronous meiosis. Replacement of endogenous *DBF4* with *DBF4-ΔN* had no apparent impact on meiosis (Fig. 6A and B). Expression of *CLB5-ΔN* from the meiosis-specific *DMC1* promoter caused a delay in meiotic progression and a small decrease in spore viability (Fig. 6A and B) but no apparent defect in rDNA segregation in anaphase I. However, cells expressing both Clb5-ΔN and Dbf4-ΔN exhibited defects in meiotic progression and particularly in spore viability. Strikingly, rDNA segregation failed completely in the double mutant cells, as can be seen clearly in both anaphase I and II cells, where only a single, unsegregated nucleolar mass was visible (Fig. 6C and D). Thus, APC^{Cdc20}-mediated destruction of both Clb5 and Dbf4 contributes to rDNA segregation in meiosis, and a failure to destroy both in anaphase I completely prevents rDNA disjunction.

DISCUSSION

Sister chromatid cohesion depends primarily on cohesin linkages (19). In budding yeast, an additional mechanism op-

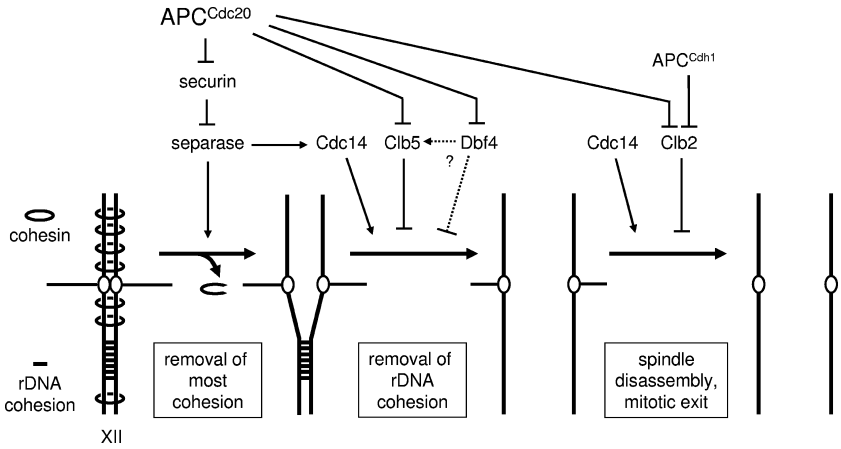

FIG. 7. Model of regulatory mechanisms governing chromosome segregation in budding yeast. Bulk chromosome separation occurs when cohesin is cleaved by separase, the activation of which results from the APC^{Cdc20}-dependent destruction of securin. Efficient removal of rDNA cohesion also requires the dephosphorylation of Clb5-specific Cdk1 targets, which results from the activation of Cdc14 by separase and the destruction of Clb5 via APC^{Cdc20}. By uncertain mechanisms, APC^{Cdc20}-dependent Dbf4 destruction also contributes. Destruction of Clb2 allows dephosphorylation of its targets after anaphase, leading to spindle disassembly and mitotic exit.

erates specifically at the rDNA locus of chromosome XII. Removal of both cohesive mechanisms requires APC^{Cdc20}-dependent degradation of securin and the liberation of separase, which cleaves a subunit of cohesin and also promotes dissolution of rDNA cohesion by activating the phosphatase Cdc14 (5, 27, 28, 31). Although it is well established that the only critical role of APC^{Cdc20} in cohesin removal is the promotion of securin degradation, it was not known if the destruction of additional targets is necessary for the resolution of rDNA. Our results demonstrate that efficient rDNA resolution also depends on APC^{Cdc20}-dependent destruction of Clb5 and Dbf4.

Although delayed, rDNA segregation does eventually occur in cells expressing stabilized forms of Clb5 and Dbf4, indicating that the destruction of these proteins is not essential for rDNA segregation in mitosis. In meiosis, however, rDNA segregation fails completely in the presence of stabilized Clb5 and Dbf4, revealing a critical function for the inactivation of these kinases in meiotic anaphase.

Previous evidence that the phosphatase Cdc14 promotes rDNA segregation (5, 28) suggested that rDNA cohesion depends on the phosphorylation of Cdk1 substrates. Our results are consistent with this possibility and also suggest that rDNA resolution depends specifically on the dephosphorylation of Clb5-Cdk1 targets, as rDNA segregation was not significantly affected by Clb2 overexpression. Given the known timing of Clb5 and Clb2 destruction, this cyclin specificity supports a model (Fig. 7) in which Clb5 destruction and Cdc14 activation trigger the dephosphorylation in early anaphase of specific Cdk1 targets involved in rDNA segregation. The significant levels of Clb2 that remain in the anaphase cell are apparently insufficient to maintain the phosphorylation of these targets in the face of separase-dependent Cdc14 activation. However, we speculate that Clb2-Cdk1 activity in anaphase does maintain the phosphorylation state of other Cdk1 targets involved in spindle disassembly and mitotic exit; the dephosphorylation of these targets occurs after anaphase as a result of Clb2 destruction, perhaps coupled with the increased Cdc14 activity that results from activation of the mitotic-exit network (30). Stabilization of Clb2 therefore blocks mitotic exit but not rDNA segregation or other anaphase processes.

What are the key targets of Clb5-Cdk1 in rDNA cohesion? The molecular basis of rDNA cohesion is not well understood but seems to depend on complex mechanisms involving topoisomerase II, sumoylation, condensin, and RNA polymerase I (5, 10, 18, 28, 33, 34, 38). Clb5-Cdk1 may act through one or multiple substrates to govern these mechanisms. We identified several Clb5-specific Cdk1 substrates in our previous work (16), but none of these appear to be a clear candidate for the control of rDNA cohesion. A significant future challenge will be the systematic identification of all Clb5-specific Cdk1 substrates, which should allow discovery of the critical Clb5 substrates involved in rDNA resolution and other anaphase processes.

We also identified the Cdc7-binding partner Dbf4 as a potential regulator of rDNA cohesion in budding yeast. Dbf4-Cdc7 is known primarily as a regulator of the initiation of DNA synthesis, and no mitotic roles have been described for this kinase. How might Dbf4-Cdc7 regulate rDNA cohesion? The fact that the *DBF4-ΔN* mutant has no apparent cell cycle defect alone but enhances the segregation delay from the *CLB5-ΔN* mutant suggests that one function of Dbf4-Cdc7 might be to enhance Clb5-Cdk1 function, either by increasing its intrinsic kinase activity or by enhancing its activity toward some Clb5-Cdk1 target. Alternatively, Dbf4-Cdc7 could promote cohesion by directly phosphorylating some component of the rDNA machinery. Notably, recent work suggests that Dbf4-Cdc7 helps promote DNA recombination in meiotic prophase and monopolar chromosome attachment in meiosis I (23, 35, 37). Dbf4-Cdc7 might therefore be involved in the control of multiple processes other than DNA replication.

In fission yeast and higher eukaryotes, it is not clear if there are cohesin-independent chromosome linkages like those operating at budding-yeast rDNA. The fission yeast ortholog of Cdc14, Clp1, is not required for rDNA segregation (3), but it remains possible that other phosphatases contribute to the

dephosphorylation of Cdk1 substrates involved in rDNA cohesion. In mammalian cells, noncohesin rDNA linkages, if they exist, may be lost early in mitosis as a result of the "prophase" pathway that drives chromosome arm resolution and separation before metaphase (8, 13). Alternatively, rDNA resolution in animal cells might depend on the degradation of cyclin A in prometaphase. In some cell types, expression of APC-resistant cyclin A leads to a metaphase-like arrest with unseparated sister chromatids (6, 9, 21, 25), and cohesin-independent linkages may contribute to this separation defect.

Future models of mitosis should take into account the qualitative contributions of individual cyclin-Cdk1 complexes, in terms of both phosphorylation events early in the cell cycle and dephosphorylation events as cyclins are sequentially destroyed in late mitosis. Early destruction of cyclins that display strong substrate specificity, such as Clb5 and cyclin A, may be required for the correct timing of anaphase events. The substrate specificity of phosphatases such as Cdc14 provides another layer of complexity in the control of Cdk substrate dephosphorylation. These mechanisms collaborate to ensure that key Cdk substrates involved in processes such as chromosome segregation and spindle elongation are dephosphorylated at anaphase onset, while dephosphorylation of other Cdk substrates is delayed until later in mitosis.

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