S-Phase Cyclin-Dependent Kinases Promote Sister Chromatid Cohesion in Budding Yeast[▽]

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Genome stability depends on faithful chromosome segregation, which relies on maintenance of chromatid cohesion during S phase. In eukaryotes, Pds1/securin is the only known inhibitor that can prevent loss of cohesion. However, *pds1*Δ yeast cells and securin-null mice are viable. We sought to identify redundant mechanisms that promote cohesion within S phase in the absence of Pds1 and found that cells lacking the S-phase cyclins Clb5 and Clb6 have a cohesion defect under conditions of replication stress. Similar to the phenotype of *pds1*Δ cells, loss of cohesion in cells lacking Clb5 and Clb6 is dependent on Esp1. However, Pds1 phosphorylation by Cdk-cyclin is not required for cohesion. Moreover, cells lacking Clb5, Clb6, and Pds1 are inviable and lose cohesion during an unperturbed S phase, indicating that Pds1 and specific B-type cyclins promote cohesion independently of one another. Consistent with this, we find that Mcd1/Sccl is less abundant on chromosomes in cells lacking Clb5 and Clb6 during replication stress. However, *clb5*Δ *clb6*Δ cells do accumulate Mcd1/Sccl at centromeres upon mitotic arrest, suggesting that the cyclin-dependent mechanism is S phase specific. These data indicate that Clb5 and Clb6 promote cohesion which is then protected by Pds1 and that both mechanisms are required during replication stress.

A temporal coupling between S phase and mitosis is essential to prevent aberrant chromosome segregation that can result in the aneuploidy associated with many cancers. One crucial regulatory process in S phase ensures that loss of chromosome cohesion does not occur until after DNA replication has been completed. In budding yeast, Pds1 is the only known protein that prevents cohesion loss, and it functions by inhibiting the Esp1 protease that cleaves the cohesin subunit Mcd1/Sccl (9). Pds1 binds to Esp1, a yeast separase homolog, until anaphase, when Pds1 is removed by ubiquitin-dependent degradation (26). When this occurs, the Esp1 protease is able to cleave the cohesin subunit that holds sister chromatids together. This cleavage allows the separation of sister chromatids (44).

However, the role of Pds1 in cohesion maintenance appears to be limited to conditions of replication stress and has been observed only in late S phase (12). *pds1*-null mutants are viable and are able to prevent loss of cohesion when DNA replication is blocked in early S phase (47, 48). Mcd1/Sccl cleavage occurs in a cell cycle-regulated manner in the absence of Pds1 (2). Moreover, *pds1*-null cells can maintain cohesion throughout S phase when DNA replication proceeds in the absence of stress at replication forks (3). Only when DNA replication is slowed in the presence of the ribonucleotide reductase inhibitor hy-

droxyurea (HU), which depletes nucleotide pools, does premature loss of cohesion occur in *pds1*-null cells (12). Together, these data indicate that slow or stalled replication forks put stress on the mechanisms that lead to the establishment and maintenance of cohesion. In the absence of replication stress, there may be a redundant mechanism that renders Pds1 dispensable for cohesion.

In mammalian cells the Pds1 homolog, securin, is similarly dispensable for viability (24), and mice lacking securin are viable (29). Still, the gene encoding securin is often overexpressed in aggressive tumors (49). These facts indicate the central importance of Pds1/securin anaphase inhibitors but also indicate that other mechanisms must be sufficient for cohesion in mammals as well as in yeast.

In addition to securin, cyclin B1 has been shown to prevent the onset of anaphase and loss of cohesion during mitosis in vertebrates (7, 21, 38). A nondegradable cyclin B1 mutant inhibits anaphase by activating Cdk1 kinase. Based on biochemical studies, the mechanism by which Cdk1-cyclin B1 blocks anaphase may be related to its ability to inhibit separase, although direct proof of this mechanism from a biological perspective has been difficult to obtain (18).

In budding yeast, the cell cycle is controlled by the cyclin-dependent kinase Cdc28/Cdk1. B-type cyclins, Clb1 to -6, interact with Cdc28 as stage-specific activators at different points during the cell cycle. There is some redundancy in the functions of these B-type cyclins, and this has hampered dissection of their roles in the cell cycle. Clb5 and Clb6 promote the initiation of DNA replication (28). Although other Clbs can also initiate replication (30), S phase occurs more slowly in *clb5*-null cells (13). Cells lacking Clb3 and Clb4 have defects in spindle pole body separation and spindle formation (37), while either Clb1 or Clb2 is required for mitosis (28, 30).

Since, at least *in vitro*, vertebrate Cdk/B-type cyclin com-

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TABLE 1. Strains used in this work

Strain	Genotype
1671	<i>MATa bar1Δ wa3::HIS3:GFP-TUB1(URA3) ARG4</i>
2081	<i>MATa bar1Δ trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
1672	<i>MATa bar1Δ ura3::HIS3:GFP-TUB1(URA3) pds1-128</i>
2082	<i>MATa bar1Δ trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3) pds1-128</i>
2449	<i>MATa bar1Δ pds1-38-HA(URA3)</i>
4342	<i>MATa bar1Δ trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3) pds1-38-HA(URA3)</i>
1902	<i>MATα clb5::URA3</i>
2367	<i>MATa bar1Δ clb5::ARG4 ura::HIS3:GFP-TUB1</i>
2267	<i>MATα bar1Δ clb6::ADE1</i>
2560	<i>MATa clb6::KAN</i>
2457	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 GFP-TUB1(URA3)</i>
2574	<i>MATa bar1Δ clb5::URA3 clb6::KAN</i>
2532	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
2441	<i>MATa bar1Δ clb5::ARG4</i>
AGB1	<i>MATα clb3::TRP1 clb5::URA3</i>
SLE33	<i>MATa bar1Δ clb4::HIS2 clb5::ARG4</i>
SLE31	<i>clb3::TRP1 clb4::HIS2</i>
SLE35	<i>clb3::TRP1 clb6::ADE1</i>
AGB2	<i>MATa clb4::HIS2 clb6::KAN</i>
SLE13	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 pds1::KAN GAL1:PDS1(HA)3X(LEU2 KAN) trp1::lacO(TRP1 LEU2) ARG4</i>
SLE14	<i>MATα clb5::ARG4 clb6::ADE1 pds1::KAN GAL:PDS1(HA)3X(LEU2 KAN) trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
SLE15	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 pds1::KAN GAL:PDS1(HA)3X(LEU2 KAN) trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
SLE16	<i>MATα bar1Δ clb5::ARG4 clb6::ADE1 pds1::KAN GAL:PDS1(HA)3X(LEU2 KAN) trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
SLE18	<i>MATa bar1Δ pds1::KAN GAL:PDS1(HA)3X(LEU2 KAN) trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
SLE25	<i>MATα bar1Δ pds1::KAN GAL:PDS1(HA)3X(LEU2 KAN) trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3)</i>
AVY265	<i>MATa bar1Δ trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3) mcm7::MCM7-GFP(URA3)</i>
AVY266	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 trp1::lacO(TRP1 LEU2) his3::lacR-GFP(HIS3) mcm7::MCM7-GFP(URA3)</i>
4011	<i>MATa bar1Δ esp1-2^{td}(URA3) pGAL1-MYC-UBR1(HIS3) trp1::TetO(TRP1) CUP1::TetR-GFP(KAN)</i>
4292	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 esp1-2^{td}(URA3) ubr1::GAL1-MYC-UBR1(LEU2) trp1::TetO(TRP1) CUP1::TetR-GFP(KAN)</i>
2328	<i>MATa bar1Δ lys4::lacO(LEU2) his3::lacR-GFP(HIS3)</i>
4314	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 lys4::lacO(LEU2) his3::lacR-GFP(HIS3)</i>
2083	<i>MATa bar1Δ tel4::lacO(LEU2) his3::lacR-GFP(HIS3)</i>
4323	<i>MATa bar1Δ clb5::ARG4 clb6::ADE1 tel4::lacO(LEU2) his3::lacR-GFP(HIS3)</i>
2680	<i>MATa bar1Δ sec1::SCC1-MYC18(TRP1)</i>
AVY166	<i>MATa bar1Δ sec1::SCC1-MYC18(TRP1) clb5::ARG4 clb6::ADE1</i>

plexes can inhibit separase activity, we asked if one mechanism of S-phase cohesion is mediated by Cdc28/cyclin. To investigate possible roles of B-type cyclins in S-phase control of cohesion, we surveyed the sensitivity of *clb* deletion strains to replication stress induced by HU. We observed a marked HU sensitivity specifically in *clb5Δclb6Δ* strains and present evidence that these particular cyclins are required in S phase for sister chromatid cohesion. The yeast Cdk, Cdc28, must phosphorylate Pds1 to allow efficient binding of Pds1 to Esp1. But we find that these phosphorylations are not necessary for cohesion. Therefore, the role of cyclin in promoting cohesion is independent of Pds1 phosphorylation. In agreement, there is a synthetic interaction between *pds1Δ* and *clb5Δ clb6Δ*. The triple mutant lacked the ability to maintain cohesion in S phase even in the absence of exogenous replication stress. Moreover, loss of cohesion in *clb5Δ clb6Δ* cells required Esp1, and similarly to vertebrates, we were able to detect Cdc28/Esp1 complexes in yeast cell extracts. However, intriguingly we found that the cohesin subunit Mcd1/Sccl does not become enriched on chromatin in early-S-phase *clb5Δ clb6Δ* cells during replication stress. *clb5Δ clb6Δ* cells were not defective in enhancing the abundance of Mcd1/Sccl near centromeres upon mitotic arrest. Together, the data indicate that parallel mechanisms using both Pds1 and B-type cyclins ensure cohesion between the sister chromatids in S phase.

MATERIALS AND METHODS

Yeast strain construction. Strains described herein were derived from BF264-15 *15DU:MATa ura3Δns ade1 his2 leu2-3,112 trp1-1^a* (35) and were grown at 30°C unless temperature sensitive in medium containing yeast extract and peptone (YEP) plus dextrose (YEPD) or galactose (YEPG) as a carbon source. Strain genotypes are shown in Table 1.

Cell cycle analysis. Overnight cultures were grown with extra adenine to decrease background green fluorescence. The temperature-sensitive *clb5Δ clb6Δ pds1Δ* triple deletion strain was grown overnight at 25°C in YEPG to maintain *PDS1* expression until cells were synchronized in YEPD. The *Esp1-2^{td}* protein was inactivated as previously described (33). All other strains were grown at 30°C overnight in YEPD. For synchrony, cells were diluted to an optical density (OD) of 0.15 in YEPD with mating pheromone (concentrations varying from 1:1,000 to 1:5,000 of a 1-mg/ml stock). After 2 to 3 h, cells were monitored microscopically until G₁ synchrony (85 to 100%) was reached. Mating pheromone was washed off with water, and cells were released under experimental conditions. For each strain, 100 to 200 cells were counted and samples were taken for FACS analysis as described previously (11, 20) using Sytox green DNA stain (Molecular Probes, Inc., Eugene, OR).

Microscopy. Spindle morphologies were visualized using *TUB1-GFP* (40) and sister chromatid segregation with the LacO/green fluorescent protein (GFP)-LacI system (39). Fluorescence and differential interference contrast (DIC) microscopy with Plan Apo 63×/1.4 and Alpha Plan Fluor 100×/1.45 objectives and a Zeiss Axio Plan II microscope were used for scoring and capturing images of live cells with a Zeiss AxioCam camera and AxioVision software.

Western blots. Cell cultures were handled as described above except with no extra adenine addition. Collected samples were washed with ice water, and pellets were kept at −80°C until protein was extracted using glass beads in NP-40 buffer (50 mM Tris-HCl at pH 7.5, 250 mM NaCl, 0.1% NP-40, 10 mM sodium pyrophosphate, 5 mM EDTA, 0.1 mM orthovanadate, 1 mM phenylmethylsul-

fonyl fluoride [PMSF], 2 μ g/ml aprotinin, 2 μ g/ml pepstatin, and 1 μ g/ml leupeptin) (similar to what was described in reference 25). At 4°C, cells were vortexed in 1.5-ml tubes for 10 min and centrifuged at 14,000 rpm for 10 min and again for 5 min to remove sediment. Equal levels of protein, as determined by 280-nm absorption, were used for SDS-PAGE. Immunostaining was performed with a 1:1,000 dilution of anti-Myc (9E10; Santa Cruz) and a 1:10,000 dilution of anti-PSTAIRE. Secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce, Rockford, IL), was used at 1:5,000. Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) was used per the manufacturer's instructions. For the Suc1 precipitations, extracts were made in lysis buffer with 1% Triton. Cdc28 was precipitated using Suc1 beads (Upstate Biotechnology) at 4°C, washed three times with lysis buffer, and resuspended in 2 $\times$ loading dye.

ChIP and real-time qPCR. Yeast cells were collected at each designated time point after release from G_1 arrest and into medium containing hydroxyurea and/or nocodazole. The cells were fixed with 1% formaldehyde. Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (16) using anti-Myc antibodies (9E10; Santa Cruz Biotechnology). DNA was analyzed by real-time quantitative PCR (qPCR) using the Roche LightCycler 480 system, and the primer pairs were designed to detect Mcd1/Sccl1-Myc occupancy at *CEN16*, *TRP1*, and *LYS4* loci according to the genome-wide mapping of Mcd1/Sccl1 (17). The primer pairs used were as follows: *TRP1*, 5' TGACGAAGAGG ATCTTCCTG, 3' CCAGCTCCACCACTCGT; *LYS4*, 5' AATGGAATTTT GCTTGTGGA, 3' TGCCGTGCTGGGTAAGAT; *CEN16*, 5' TGAGCAAAC AATTGAACAGAAA, 3' CGCTTTAGAACCCTACCAT.

RESULTS

Sensitivity to replication stress upon deletion of B-type cyclins. Pds1-null yeast are viable and can maintain cohesion during S phase, indicating the existence of a second mechanism that promotes cohesion. Budding yeast synthetic genetic arrays with *pds1*-null mutants have revealed important information about Pds1 functions but have not identified a pathway that acts redundantly with Pds1 (36). Cyclin B1 has been implicated in inhibition of anaphase onset in mammals, but it is not known whether B-type cyclins promote cohesion in S phase. Although viable, yeast *pds1*-null mutants are sensitive to replication inhibitors such as hydroxyurea (HU) and lose cohesion in S phase under conditions of replication stress. To ask if B-type cyclins might participate in cohesion, we surveyed HU sensitivity in single and double deletion mutants. None of the single B-type cyclin deletions were HU sensitive, with the exception of mildly sensitive *clb5 Δ* cells (Fig. 1A) (10). Double deletions, including the *clb5 Δ* mutants, had similar mild sensitivities, but *clb5 Δ clb6 Δ* mutants alone were markedly sensitive to HU (Fig. 1B). This indicated a specific S-phase function shared by Clb5 and Clb6, needed for a faithful S phase under the condition of replication stress.

Some checkpoint mutants are sensitive to HU because of a failure to sustain nucleotide pool levels, a defect that can be corrected by upregulation of *RNR1* (15, 45). However, the HU sensitivity of *clb5 Δ clb6 Δ* mutants was not suppressed by *RNR1* overexpression (Fig. 1C), indicating that an alternative deficiency must account for the lethality during perturbed replication.

Premature loss of cohesion in *clb5 Δ clb6 Δ* mutants in HU. A cohesion defect in S phase could explain the HU sensitivity of *clb5 Δ clb6 Δ* cells. To determine if *clb5 Δ clb6 Δ* mutants have a cohesion defect, strains containing a LacO(*TRP1*)/GFP-LacI combination were used to assay cohesion (39). Its proximity to *ARS1* ensures that the *TRP1* locus is replicated in early S phase, but cohesion keeps these loci close together (seen as a single green fluorescent dot; Fig. 2A, left) until shortly before

anaphase (when the dots separate and then rapidly segregate; Fig. 2A, middle and right). Premature loss of cohesion is indicated if cells possess separated *TRP1* loci before S phase is complete. To ask if *clb5 Δ clb6 Δ* mutants can maintain *TRP1* cohesion in S phase, the cells were arrested in G_1 using mating pheromone and then released into the cell cycle in the presence of 75 mM HU. In the strain background used, doses of HU below 0.3 M slow replication but do not cause an S-phase arrest. Wild-type cells completed DNA replication after $\sim$ 2.5 h, at which time no cells were seen with two green fluorescent dots (Fig. 2B and C). Thirty minutes later, *TRP1* separation was observed in some cells, followed shortly thereafter by anaphase. In contrast, loss of cohesion at *TRP1* in *clb5 Δ clb6 Δ* cells was observed before the first cells reached G_2 . Therefore, some cells must have failed to maintain cohesion in S phase. This can account for the sensitivity of *clb5 Δ clb6 Δ* mutant cells to HU and implicates these cyclins, presumably in association with Cdc28, in sister chromatid cohesion.

We compared loss of cohesion in the *clb5 Δ clb6 Δ* mutant and a *pds1* mutant that had been previously studied (Fig. 2D and E) (11, 12). Similarly to the *pds1-128* mutant, loss of cohesion in *clb5 Δ clb6 Δ* cells did not occur until mid- to late S phase. We also asked if loss of cohesion was due to the lower rate of DNA replication in *clb5 Δ clb6 Δ* cells. DNA replication in wild-type cells was decreased to a similar rate by using higher doses of HU. Under these conditions, wild-type cells were capable of maintaining cohesion even when replication was as slow as in *clb5 Δ clb6 Δ* cells (Fig. 2D and E). Therefore, the reduced rate of replication *per se* is not responsible for premature loss of cohesion in *clb5 Δ clb6 Δ* cells.

Loss of cohesion in *clb5 Δ clb6 Δ* cells occurs before nuclear export of Mcm7 in HU. In the above experiments, *clb5 Δ clb6 Δ* mutants lost cohesion before the cells entered G_2 as judged by fluorescence-activated cell sorting (FACS) analysis of DNA content. To examine replication and cohesion simultaneously, we developed a single-cell assay to identify S-phase cells using a GFP-tagged Mcm7. The minichromosome maintenance (MCM) complex is imported into the nucleus at the end of mitosis and remains nuclear until the completion of S phase, after which it is exported into the cytoplasm (32). G_1 -arrested wild-type cells expressing *MCM7-GFP* and possessing the LacO(*TRP1*)/GFP-LacI combination were washed and released into the cell cycle (Fig. 3). In wild type, all of the cells in which Mcm7-GFP remained nuclear had a single fluorescent signal at the *TRP1* locus. Loss of cohesion at *TRP1* was observed only in cells without nuclear Mcm7-GFP fluorescence, and this category of cells was observed concomitantly with the appearance of anaphase cells (Fig. 3B). Therefore, as expected, loss of cohesion occurs only after S phase, based on observation of Mcm7-GFP nuclear export. To ask if *clb5 Δ clb6 Δ* mutants lost cohesion during S phase in HU, we repeated the above experiment and released G_1 -arrested wild-type and *clb5 Δ clb6 Δ* strains into the cell cycle in the presence of 75 mM HU (Fig. 3C). Cells with separated *TRP1* loci were categorized into those with nuclear Mcm7-GFP or diffusely localized Mcm7-GFP. We observed that a substantial fraction of *clb5 Δ clb6 Δ* cells lost cohesion with nuclear Mcm7-GFP, indicating that these cells had not completed S phase. Thus, the data indicate that premature loss of cohesion in *clb5 Δ clb6 Δ* mutants is a consequence of failed cohesion during S phase.

FIG. 1. Genetic interactions among B-type cyclins result in hydroxyurea sensitivity. Spotting assays indicating yeast growth on rich medium or medium containing hydroxyurea at 30°C. From top to bottom of each panel, 5,000, 500, or 50 cells were plated per spot for each strain. (A) From left to right, comparison of wild-type (WT), *pds1*-128, *clb5*::*URA3*, *clb5*::*ARG4*, *clb6*::*ADE2*, *clb6*::*KAN*, *clb5*::*ARG4* *clb6*::*ADE2*, and *clb5*::*URA3* *clb6*::*KAN* strains. (B) From left to right, comparison of WT, *pds1*-128, *clb5*::*ARG4* *clb6*::*ADE2*, *clb5*::*ARG4*, *clb5*::*ARG4* *clb3*::*TRP1*, *clb5*::*ARG4* *clb4*::*HIS2*, *clb3*::*TRP1* *clb4*::*HIS2*, *clb3*::*TRP1* *clb6*::*ADE2*, and *clb4*::*HIS2* *clb6*::*ADE2* strains. (C) Comparison of *clb5*::*ARG4* *clb6*::*ADE2* strain and three independent isolates of the same strain but containing YEp-*RNR1*(*URA3*). This high-copy-number plasmid did rescue inviability of a *rad53*-null strain (reference 11 and data not shown).

clb5 *clb6* Δ deletion strains had previously been shown to have compromised G₂ DNA damage checkpoint controls when grown in the presence of the alkylating agent methyl methane-sulfonate (MMS) (31). Using nuclear Mcm7-GFP as an S-

phase marker, we found that loss of cohesion at *TRP1* occurred after the completion of S phase (data not shown), unlike the HU-treated *clb5* *clb6* Δ cells, which lost cohesion while the cells were still in S phase. This suggests that depleted nucleo-

FIG. 2. Premature loss of cohesion in *clb5Δ clb6Δ* mutants in hydroxyurea. Analysis of synchronous cell cycles in the presence of hydroxyurea in wild-type, *clb5::ARG4 clb6::ADE1*, *clb5::URA3 clb6::KAN*, and *pds1-128* strains. (A) Micrographs of wild-type cells carrying *LacO(TRP1)/GFP-LacI*. A single GFP dot indicates cohered sister chromatids (left). Two adjacent GFP dots indicate loss of cohesion (middle). A GFP dot in each daughter cell indicates anaphase (right). (B to E) After release from G_1 synchrony, samples were taken for scoring loss of cohesion at *TRP1* (B and D) and for FACS analysis of DNA content (C and E). Approximate S-phase periods are indicated on the graphs (shaded regions), and in the corresponding DNA content histogram plots, overlaid vertical lines indicate approximate 1C and 2C DNA contents, while the experimental data for each time point are shown in gray.

tide pools (HU treatment) place a distinct type of stress on replication forks, compromising cohesion in the absence of Clb5/Clb6.

Pds1 phosphorylation by Cdk is not needed for viability upon replication stress. Cdk activity is required for Pds1 phosphorylation that occurs on three sites during S phase, although the cyclin partner(s) of Cdk needed for these phosphorylations

is not known (1). These phosphorylations stabilize the interaction of Pds1 with Esp1, an interaction that promotes maintenance of cohesion (1). Esp1/Pds1 complexes enter the nucleus around the time of late S phase (25), consistent with the timing of loss of cohesion in *clb5Δ clb6Δ* mutants in HU. A possible role of Clb5 and Clb6 in promoting cohesion is therefore the phosphorylation of Pds1. In this case, a lack of Pds1

FIG. 3. A single cell assay demonstrates that *clb5Δ clb6Δ* cells lose cohesion during DNA replication in the presence of hydroxyurea. (A) Micrographs of wild-type cells expressing Mcm7-GFP and carrying the LacO(*TRP1*)/GFP-LacI combination. The top row shows the bright GFP-LacI signal in a low-exposure image, and the middle row shows a longer exposure to reveal the relatively less bright Mcm7-GFP signal. (i to iii) G₁ and S-phase cells with a single *TRP1* locus dot and nuclear Mcm7-GFP. (iv) G₂ cell with single *TRP1* locus and diffuse Mcm7-GFP. (B) Wild-type cells from panel A released from G₁ synchrony after mating pheromone-induced arrest. Cells were scored into categories according to whether the *TRP1* loci were cohered or separated (LOC) and whether Mcm7-GFP was confined to the nucleus or diffused throughout the cell. (C) Cell cycle analysis of wild-type and *clb5Δ clb6Δ* strains expressing Mcm7-GFP and harboring LacO(*TRP1*)/GFP-LacI as described in panel A. Synchrony and release were performed as in panel B except in the presence of 75 mM hydroxyurea. Approximate S-phase periods are indicated on the graphs by shaded regions, estimated from FACS analysis of DNA content as shown in Fig. 2. The right panel shows an example of a *clb5Δ clb6Δ* cell that has lost cohesion at *TRP1* and has nuclear Mcm7-GFP.

modification at these sites should render cells HU sensitive and result in premature loss of cohesion in S phase. We therefore asked if *pds1-38* cells in which all three of the Cdc28 phosphorylation sites are converted to alanines (S277A, S292A, and T304A), thus perturbing the Pds1-Esp1 interaction (1), are sensitive to HU. Using HU-sensitive *pds1-128* cells as a positive control, the *pds1-38* cells surprisingly had a viability indistinguishable from wild-type cells when grown on HU-containing solid medium (Fig. 4A). Thus, the HU sensitivity of *clb5Δ clb6Δ* cells cannot be due to a failure of Pds1 phosphorylation on these three Cdk sites. In agreement with these data, we did not observe premature loss of cohesion in *pds1-38* cells grown in HU (data not shown).

***clb5Δ clb6Δ pds1Δ*-null cells lose cohesion in S phase and become aneuploid.** That Clb5 and Clb6 might promote cohesion through a different mechanism, independent of Pds1 phosphorylation, predicts that a combined lack of Pds1 and these cyclins would enhance the cohesion defect. We therefore crossed strains containing *clb5Δ clb6Δ* and *pds1Δ* to obtain a

triple mutant. Tetrad dissection revealed a synthetic interaction among these mutant combinations—viable triple mutant spores were not recovered (Fig. 4B and data not shown). In-viable spores germinated and then divided once or twice, indicating a possible cell division catastrophe (Fig. 4B, lower panel).

To analyze this phenotype further, we produced triple deletion strains in which viability was restored using a *GAL1*-driven *PDS1* gene (*GAL1-PDS1*{3xHA} *clb5Δ clb6Δ pds1Δ*). Such strains were viable on medium containing galactose but not on glucose-containing medium, which represses *GAL1*-driven transcription (although after several days some colonies grew out on the glucose plates, most likely from single cells that had achieved *GAL1-PDS1* derepression) (Fig. 4C and data not shown). Since Pds1 is degraded in G₁ phase, we could achieve a mating pheromone-arrested population lacking Pds1 protein. After release from G₁ in rich medium containing glucose, *GAL1-PDS1 clb5Δ clb6Δ pds1Δ* cells initiated DNA replication with a delay compared to wild type (Fig. 4D). While replication

FIG. 4. *clb5Δ clb6Δ pds1Δ*-null cells lose cohesion in S phase and become aneuploid. (A) Spotting assay indicating yeast growth at either 26°C or 30°C on rich medium or medium containing hydroxyurea. From left to right, 5,000, 500, or 50 cells were plated per spot for each strain: WT, *pds1-128*, and *pds1-38* (lacking the Cdk phosphorylation sites; see text) strains. (B) Tetrad dissection showing spore viability on rich medium from a *clb5Δ clb6Δ* × *pds1Δ* cross (upper panel) and the cell morphology of the inviable spores (lower panel). (C) Growth of *GAL1-PDS1(3XHA) clb5Δ clb6Δ pds1Δ* cells and *GAL1-PDS1(3XHA) pds1Δ* cells spotted onto rich medium with either galactose or glucose as a carbon source. (D) Analysis of a synchronous cell cycle after *G*₁ arrest in rich medium in wild-type or *GAL1-PDS1(3XHA) clb5Δ clb6Δ pds1Δ* cells. Cells were synchronized in *G*₁ with mating pheromone for 2.5 h in medium with glucose to repress *GAL1-PDS1(3XHA)* synthesis. After release from *G*₁ into rich medium with glucose, samples were taken for scoring loss of cohesion at *TRP1* and for FACScan analysis of DNA content. Approximate S-phase periods are indicated on the graphs (shaded regions), and in the corresponding DNA content histogram plots, overlaid vertical lines indicate approximate 1C and 2C DNA contents, while the experimental data for each time point are shown in gray. Note that although the triple mutant cells began replication with a delay compared to wild-type cells, bulk DNA replication was completed within about 20 min, similar to wild-type cells. (E) Dot plots of DNA content against forward scatter obtained from FACScan samples (from the experiment in panel D) 3 h or 5 h after release from *G*₁ in glucose. Open arrows indicate approximate positions of 1C and 2C DNA contents (left to right). Closed arrows indicate presumed cell population with less than a 1C DNA content in *PDS1(3XHA) clb5Δ clb6Δ pds1Δ* strain or lack thereof in wild type. Dex, dextrose.

was complete in wild type cells by ~40 min, it took until ~70 min for the *GAL1-PDS1(3XHA) clb5Δ clb6Δ pds1Δ* cells to finish bulk replication. Loss of cohesion and anaphase onset began after 50 min in wild-type cells, but in the triple mutant, loss of cohesion was observed before cells reached *G*₂. Thus, these mutant cells were unable to maintain cohesion during S phase even in the absence of replication stress. After several cell cycles in the presence of glucose, *GAL1-PDS1(3xHA)*

clb5Δ clb6Δ pds1Δ cells became aneuploid (Fig. 4E), consistent with loss of cohesion in S phase, resulting in missegregation of chromosomes during anaphase. This synthetic interaction between *pds1Δ* and *clb5Δ clb6Δ* indicates that independent mechanisms enforce cohesion, one relying on Pds1 function and the other on an S-phase cyclin-dependent function.

HU sensitivity of *cks1* and *cdc28* mutants and synthetic lethality with *pds1* mutants. We next investigated if the *Clb5/*

FIG. 5. Cdk and Pds1 maintain cohesion in S phase. (A) Spotting assays indicating yeast growth on rich (YPD) medium or medium containing 100 mM hydroxyurea. From left to right, 5,000, 500, or 50 cells were plated per spot for each strain. (B and C) Analysis of synchronous cell cycles in *cdc28-1N cdc28-as1 pds1Δ* cells with and without ATP analog 1-NM-PP1. After release from G₁, ATP analog was added to half the culture to inactivate the *cdc28-as1* kinase, leaving Cdc28-1N as the only active Cdk present. Samples were taken for scoring loss of cohesion at *TRP1* (B) and for FACS analysis of DNA content (C). Approximate S-phase periods are indicated on the graphs (shaded regions), and in the corresponding DNA content histogram plots, overlaid vertical lines indicate approximate 1C and 2C DNA contents, while the experimental data for each time point are shown in gray. (D) Western blots of cell extracts from wild-type and *clb5Δ clb6Δ* cells (left) and precipitations from those extracts (right) using protein G-Sepharose (PGS) or Suc1-PGS beads. Both strains expressed Myc epitope-tagged Esp1. The PSTAIRE antibody was used to detect Cdc28.

Clb6 function in promoting cohesion depends on Cdc28 kinase. We observed that *cdc28-1N* mutant cells are sensitive to HU (Fig. 5A). This allele is a temperature-sensitive allele of *CDC28* that retains kinase activity at the restrictive temperature but fails to bind to Cks1 and therefore fails to act upon a subset of its substrates (42). We found that like *cdc28-1N* and

cks1Δ mutants were as sensitive to HU as *clb5Δ clb6Δ* mutants (Fig. 5A). If complexes of Cks1-Cdc28-Clb5 or Cks1-Cdc28-Clb6 contribute to the Pds1-independent function that promotes cohesion in S phase, we would expect that *cdc28-1N* and *cks1* mutants would be synthetically lethal with *pds1* mutants. We therefore crossed *cdc28-1N* and two temperature-sensitive

TABLE 2. Results of crosses between mutant strains

Mutant	Result of cross with <i>pds1</i> mutant	
	<i>pds1Δ</i>	<i>pds1-128</i>
<i>cks1-35</i>	Dead	Sick
<i>cks1-38</i>	Dead	Sick
<i>cdc28-1N</i>	Dead	Dead

cks1 mutants to the *pds1Δ* and *pds1-128* strains. All of the mutants either were inviable or showed synthetic sickness in combination with the *pds1* mutants (Table 2). These data support the hypothesis that Cks1-bound Cdc28/Clb5 or Cdc28/Clb6 complexes promote cohesion independently of Pds1.

***cdc28-1N pds1Δ* strains show premature loss of cohesion.** To ask if *cdc28-1N* is synthetically lethal with *pds1Δ* due to failed cohesion during S phase, we constructed a *cdc28-1N pds1Δ* strain kept alive with an analog-sensitive *CDC28* allele, *cdc28-as1* (43). Inhibition of Cdc28-as1 kinase by the ATP analog 1-NM-PP1 has been previously characterized (43). We monitored cohesion at *TRP1* in the presence or absence of 1-NM-PP1 after synchronizing cells in G₁ (Fig. 5B and C). After releasing the cells from the G₁ arrest, the strains were shifted to 30°C, at which temperature Cdc28-1N is severely defective for binding Cks1 (6). We found that *cdc28-1N cdc28-as1 pds1Δ* cells lose cohesion during S phase in the presence of 1-NM-PP1 but not in the absence of inhibitor, thereby suggesting that Cdc28 functions to promote cohesion during S phase and that the interaction with Cks1 is required for this function.

Clb5/Clb6 are required for the association of Esp1 with Cdc28/cyclin complexes. In human cells, Cdk1/cyclin B1/hsCks1 complexes bind and inhibit Esp1/separase (18). To determine if Cks1/Cdc28/cyclin complexes bind to Esp1, we precipitated Cdc28 using Suc1 Sepharose beads. (Suc1 is the highly conserved *Schizosaccharomyces pombe* homolog of Cks1.) Precipitations were performed with extracts from wild-type and *clb5Δ clb6Δ* strains that expressed a Myc epitope-tagged allele of *ESP1*. The reaction mixtures were then run on SDS-PAGE gels and Western blotted. The blots were probed with anti-Myc antibodies to detect Esp1 and PSTAIRE antibodies to detect Cdc28. We found that in wild-type strains, Esp1 and Cdc28 coprecipitated with Suc1 beads but did not precipitate with protein G-Sepharose (PGS) beads (Fig. 5D). This result suggests that Esp1 can bind to Cdc28 complexes. We were unable to precipitate Esp1 using extract from the *clb5Δ clb6Δ* strain, indicating that Esp1 may not bind directly to Suc1 beads or Cdc28 associated with the beads and that the interaction is likely to depend directly on Clb5 or Clb6. In human cells, less than 5% of separase protein was estimated to be associated with Cdk1/cyclin B1/hsCks1 (18). Similarly, the corresponding complexes in yeast were not efficiently copurified. Nevertheless, these data leave open the possibility that Esp1 may be directly inhibited by Cdk-cyclin complexes in yeast.

Esp1 is required for loss of cohesion in HU-treated *clb5Δ clb6Δ* cells. The data described above indicate that B-type cyclins Clb5 and Clb6 together with Cdc28 promote cohesion during S phase in parallel with Pds1. If the cohesion defect in *clb5Δ clb6Δ* cells is due to misregulation of cohesin complexes,

the defect ought to be suppressed in the absence of Esp1. We constructed *clb5Δ clb6Δ* mutant strains with a heat-inducible degron allele of the *esp1-2* temperature-sensitive allele of *ESP1* (*esp1-2^{td}*). Additionally, the strain carried a galactose-inducible *UBR1* gene to enhance degradation of Esp1-2^{td} at the restrictive temperature (37°C). To determine if loss of Esp1 prevented the premature loss of cohesion at the *TRP1* locus, wild-type, *esp1-2^{td}*, *clb5Δ clb6Δ*, and *esp1-2^{td} clb5Δ clb6Δ* strains were synchronized in G₁ with mating pheromone. G₁-arrested cells were washed and released into medium containing 75 mM HU at 37°C (Fig. 6A and B). As previously observed, *clb5Δ clb6Δ* strains displayed premature loss of cohesion in S phase. *esp1-2^{td}* strains maintained cohesion in S phase, as would be predicted. Furthermore, *esp1-2^{td} clb5Δ clb6Δ* strains maintained cohesion during S phase. Therefore, the data indicate that the cohesion function of Clb5 and Clb6 is mechanistically directed at regulation of the cohesin complex.

Because Esp1 cleaves the Mcd1/Sccl subunit of cohesin to allow sister chromatid separation at anaphase, we predicted that premature Mcd1/Sccl cleavage would occur in *clb5Δ clb6Δ* cells. Surprisingly, under normal growth conditions, the cleaved forms of Mcd1/Sccl were less abundant than in wild-type cells (Fig. 6C). Furthermore, we were not able to detect the cleaved forms in *clb5Δ clb6Δ* cells under conditions of replication stress (Fig. 6D). One possible explanation of these data is a more rapid degradation of the cleavage products. Alternatively, Clb5 and Clb6 might not regulate cohesin via its cleavage.

The cohesion defect in *clb5Δ clb6Δ* cells treated with HU is observed cytologically only in the presence of spindle tension. The suppression of loss of cohesion in *esp1-2^{td} clb5Δ clb6Δ* indicates that *TRP1* locus separation is mechanistically due to misregulation of the cohesin complex. However, an additional factor that could promote premature loss of cohesion is prolonged spindle forces that might pull the *TRP1* locus apart. A recent study by Renshaw et al. used the LacO/GFP-LacI reporter system to observe the centromere, the *HIS3* locus, and a telomere on the same chromosome, and their data showed that unlike centromeric regions, which separate at the onset of anaphase, chromosome arms do not separate until midanaphase upon further spindle extension (34). They showed that complete cohesin removal requires sufficient spindle pulling force in addition to Esp1 activity. We therefore asked if attachment of chromosomes to the spindle is required for *TRP1* separation. Wild-type and *clb5Δ clb6Δ* strains were arrested in G₁, washed, and then released into rich medium with 50 mM HU and in the presence or absence of nocodazole. Separation of the *TRP1* locus was monitored through the cell cycle as described for Fig. 2. In the presence of nocodazole, wild-type and *clb5Δ clb6Δ* cells did not show any loss of cohesion at *TRP1* (Fig. 7A). This result suggested that spindle tension is required for loss of cohesion to be observed in *clb5Δ clb6Δ* cells.

Considering these data, then we asked if *clb5Δ clb6Δ* mutants prematurely elongate their spindles in HU, coordinately with loss of cohesion. We synchronized *clb5Δ clb6Δ* and wild-type cells, expressing *GFP-TUB1*, in G₁ and then released them into the cell cycle in the presence of 100 mM HU. Wild-type cells reached G₂ ~3.5 h after release from G₁, and spindle

FIG. 6. *Esp1* is required for loss of cohesion in *clb5Δ clb6Δ* cells. (A and B) Analysis of synchronous cell cycles in the presence of 75 mM hydroxyurea in wild-type, *clb5Δ clb6Δ*, *esp1-2^{td}*, and *clb5Δ clb6Δ esp1-2^{td}* strains. After release from G₁, samples were taken for scoring loss of cohesion at *TRP1* (A) as described in Fig. 2 and for FACS analysis of DNA content (B). The *esp1-2^{td}*-encoded protein was inactivated by the exogenous *UBR1* induction at 37°C, as previously described (33). Approximate S-phase periods are indicated on the graphs (shaded regions), and in the corresponding DNA content histogram plots, overlaid vertical lines indicate approximate 1C and 2C DNA contents. (C and D) Mcd1/Scc1 cleavage in synchronous *clb5Δ clb6Δ* cells. Western blots from synchronous cultures released from G₁ arrest and grown in rich medium (top panel) or in the presence of 75 mM hydroxyurea (bottom panel). Wild-type and *clb5Δ clb6Δ* strains expressed epitope-tagged Scc1/Mcd1 that allows cleaved products to be detected.

elongation took place ~30 min later (Fig. 7B). In *clb5Δ clb6Δ* mutants, like wild-type cells, spindle elongation was delayed until bulk DNA replication was complete (Fig. 7B). Therefore, we found no evidence that the cohesion defect in *clb5Δ clb6Δ* cells is due to premature spindle elongation.

We next examined cohesion at loci unaffected by spindle tension: *LYS4* and a locus adjacent to *TEL4*. Using the conditions in Fig. 2, we observed no loss of cohesion in wild-type cells or in *clb5Δ clb6Δ* cells during replication in the presence of HU (Fig. 8). We also noted that unlike *TRP1*, these loci separated coincident with anaphase in wild-type cells. These data are therefore consistent with the findings of Renshaw et al. that anaphase spindle forces are required to fully remove cohesin and separate chromosome arm loci (34). Our failure to observe premature loss of cohesion at *LYS4* and *TEL4* loci, as well as at the *TRP1* locus in the presence of nocodazole, in *clb5Δ clb6Δ* cells is presumably due to the lack of preanaphase spindle-pulling forces.

Reduced abundance of chromosomal Mcd1/Scc1-Myc in *clb5Δ clb6Δ* cells treated with HU. The above experiments

showed that preanaphase spindle tension is required for premature *TRP1* locus separation in *clb5Δ clb6Δ* cells. Importantly, however, wild-type cells were able to endure prolonged spindle tension under the same conditions of replication stress (Fig. 2D): the *TRP1* locus did not separate prematurely and the cells were resistant to HU, indicating faithful chromosome segregation. Together these data indicated that a *bona fide* cohesion defect in *clb5Δ clb6Δ* cells is compounded by prolonged spindle tension to result in premature *TRP1* locus separation. To test this hypothesis directly, we sought to compare the abundance of cohesin on chromatin in the wild-type and *clb5Δ clb6Δ* cells using chromatin immunoprecipitates of Mcd1/Scc1-Myc and real-time quantitative PCR (qPCR).

First, we determined that Mcd1/Scc1-Myc was present at equivalent levels in wild-type and *clb5Δ clb6Δ* cells and could be immunoprecipitated with similar efficiencies (Fig. 9A). In order to establish the efficiency with which we could detect chromatin-associated cohesin, we used extracts from cells arrested in G₁ with mating pheromone or in mitosis with nocodazole. The rationale for this was that cohesin had been

FIG. 7. *TRP1* locus separation in *clb5Δ clb6Δ* mutant requires spindle tension. (A) Separation of the *TRP1* locus depends on spindle tension in *clb5Δ clb6Δ* mutants. Analysis of synchronous cell cycles in the presence of 50 mM hydroxyurea with or without nocodazole. Cell cycle parameters were determined in cells released from G₁ arrest by scoring loss of cohesion at *TRP1* as described in Fig. 2. (B) Delayed spindle elongation in *clb5Δ clb6Δ* mutants in the presence of 100 mM hydroxyurea. Cell cycle parameters were determined by scoring % unbudded cells (G₁), spindle morphology, and FACS analysis of DNA content (data not shown). Approximate S-phase periods are indicated on the graphs (shaded regions).

previously shown to become enriched at pericentric regions by *de novo* loading when cells are arrested in mitosis with microtubule-depolymerizing drugs (14). The mechanism of *de novo* loading appears to be different from that which establishes cohesion concurrent with DNA replication (14, 41, 46). Therefore, we predicted that *clb5Δ clb6Δ* cells would be competent to load cohesin at pericentric regions during mitotic arrest. After arrest in G₁, or upon arrest in the presence of nocodazole, samples were taken to compare wild-type and *clb5Δ clb6Δ* cells, using ChIP and real-time qPCR. The abundance of Mcd1/Sccl-Myc near a centromere locus (*CEN16*) was comparable in the two strains, and as described previously (46), cohesin was enriched in the mitosis-arrested cells (Fig. 9B). These data therefore establish that the *de novo* loading of cohesin upon mitotic arrest occurs in both wild-type and *clb5Δ clb6Δ* cells.

We then prepared extracts from cells progressing synchronously through S phase in the presence of HU (using the same

conditions as for the cytological assays in Fig. 2). We compared samples in which wild-type and *clb5Δ clb6Δ* cells were in early S, mid-S, and late S, as well as postmitotic cells, to ask how abundant Mcd1/Sccl-Myc was at *TRP1* and *LYS4* (Fig. 9C). In wild-type cells, as previously described (4, 5), the abundance of Mcd1/Sccl-Myc at *TRP1* and *LYS4* peaked in early/mid-S phase and dropped as the cell population completed bulk replication. However, in *clb5Δ clb6Δ* cells, Mcd1/Sccl-Myc did not peak in abundance at either locus. Therefore, regardless of the presence (*TRP1*) or absence (*LYS4*) of spindle tension, *clb5Δ clb6Δ* cells have reduced levels of cohesin on chromatin under the conditions of replication stress that result from reduced nucleotide pool levels. Together with the cytological assays for locus separation, these data indicate that *clb5Δ clb6Δ* cells have a cohesion defect at *TRP1* and *LYS4* and that premature separation of the replicated loci requires spindle tension applied at that locus. The combination of spindle tension during a prolonged S phase and reduced cohesin abundance on chromatin presumably leads to *TRP1* separation and the aneuploidy observed in *clb5Δ clb6Δ* cells following replication stress.

DISCUSSION

Identification of the anaphase inhibitor Pds1/securin led to a greater understanding of how chromosome cohesion is maintained until anaphase in eukaryotes (47, 48). However, the viability of *pds1*-null yeast and securin-null mammals (23, 24, 47, 48) indicated that cohesion is maintained in S phase independently of Pds1. Pds1 inhibits the protease Esp1, which cleaves the Mcd1/Sccl component of cohesin. Maintenance of cohesion in S-phase *pds1Δ* cells might be a fortuitous result of the reduced Esp1 activity (8) and compromised nuclear import of Esp1 (25) when Pds1 is not present. However, loss of cohesion occurs during S phase in *pds1*-null cells under conditions of replication stress. Therefore, Pds1-independent mechanisms are required for cohesion when replication is perturbed.

Here we have described evidence that, in budding yeast, the S-phase cyclins Clb5 and Clb6 are important regulators of cohesion. A cohesion defect in *clb5Δ clb6Δ* cells was observed cytologically (separation of the newly replicated *TRP1* loci) and biochemically (reduced abundance of Mcd1/Sccl-Myc at *TRP1* and *LYS4*). Under conditions of replication stress (in HU), either a lack of Pds1 or a lack of Clb5 and Clb6 results in a cohesion defect. Thus, neither of these factors is sufficient when replication fork progression is perturbed. When both Pds1 and the cyclins are absent, cells lose cohesion in S phase even in the absence of the replication stress induced by HU. The consequences are aneuploidy and cell death. We also find that Cdc28-dependent phosphorylation of Pds1 is not required for cohesion in S phase. These data indicate that the function of Clb5 and Clb6 in promoting cohesion does not depend on Pds1 but rather constitutes a distinct mechanism.

Interestingly, we did not observe premature loss of cohesion at the *TRP1* locus in *clb5Δ clb6Δ* cells treated with HU and nocodazole. We also failed to observe premature loss of cohesion at the *LYS4* locus and near the telomere of chromosome IV in *clb5Δ clb6Δ* cells treated with HU alone. These experiments raised the possibility that prolonged spindle forces during S phase might pull the centromeric regions further apart, giving rise to the observed

FIG. 8. Timing of loss of cohesion at the *LYS4* and *TEL4* loci in hydroxyurea. Analysis of synchronous cell cycles in the presence of 75 mM hydroxyurea in wild-type and *clb5Δ clb6Δ* strains. After release from G₁, samples were taken for scoring loss of cohesion at *LYS4* (A) and *TEL4* (C) and for FACS analysis of DNA content (B and D). Approximate S-phase periods are indicated on the graphs (shaded regions), and in the corresponding DNA content histogram plots, overlaid vertical lines indicate approximate 1C and 2C DNA contents.

separation of the *TRP1* loci. Counter to this possibility, in *clb5Δ clb6Δ* mutants treated with HU, the mitotic spindle did not elongate until after S phase was completed, indicating that anaphase spindle forces are not prematurely initiated. More importantly, we observed comparably reduced abundances of chromosomal Mcd1/Sccl-Myc at *TRP1* and *LYS4* in *clb5Δ clb6Δ* cells treated with HU. Spindle forces are therefore not required for the cohesin deficiency in *clb5Δ clb6Δ* cells because *LYS4* is not subject to tension during S phase. Presumably, the lack of *TRP1* separation in *clb5Δ clb6Δ* cells treated with nocodazole and HU demonstrates that this cytological assay requires spindle force in order for the cohesion defect to be observed. The reduced abundance of Mcd1/Sccl-Myc on chromosomes in S-phase *clb5Δ clb6Δ* cells is intriguing given a recent study which demonstrated that reduced levels of cohesin on replicating DNA slow down the replication forks (19). This suggests that the prolonged DNA repli-

cation observed in *clb5Δ clb6Δ* mutants might be a consequence of reduced chromosomal cohesin loading.

We also find that loss of cohesion does not occur in *clb5Δ clb6Δ esp1-2^{td}* cells. However, we did not observe premature Mcd1/Sccl cleavage in *clb5Δ clb6Δ* cells, indicating that the cohesion defect is not due to premature Esp1 activation. Indeed, the Mcd1/Sccl cleavage products were less abundant in *clb5Δ clb6Δ* cells compared to wild-type cells. Mcd1/Sccl cleavage occurs preferentially on the chromatin-associated pool of cohesin complexes (22, 27). The data are therefore consistent with our observation that Mcd1/Sccl is less abundant on chromatin during S phase in *clb5Δ clb6Δ* cells than in wild-type cells. Together the experiments suggest that the cohesin defect in *clb5Δ clb6Δ* cells, and the reduced level of cleavage products, is due to a cohesin loading defect in early S phase. The cytological assay, observation of *TRP1* locus separation, indicated a cohesion defect in the latter half of S phase.

FIG. 9. Relative abundance of Mcd1/Sccl-Myc at *CEN16*, *TRP1*, and *LYS4* during S phase in hydroxyurea or after mitotic arrest in nocodazole. (A) Western blotting of Mcd1/Sccl-Myc in wild-type and *clb5Δ clb6Δ* cells in whole-cell extracts (WCE) or after immunoprecipitation (IP). PSTAIR is a loading control. (B) Real-time quantitative PCR of the *CEN16* locus in chromatin immunoprecipitates from extracts prepared after cells were arrested in G₁ with mating pheromone or after mitotic arrest following G₁ synchrony and release in the presence of nocodazole and hydroxyurea. (C) Analysis of synchronous cell cycles of wild-type and *clb5Δ clb6Δ* cells following G₁ arrest and release into medium containing 75 mM hydroxyurea. (Left) Real-time quantitative PCR of the *TRP1* and *LYS4* loci in chromatin immunoprecipitates from samples at the indicated time points (see Materials and Methods for details). (Right) FACS analysis of DNA content at each time point shown on the left. (D) A model describing how Pds1 may act redundantly with Clb5- and Clb6-Cdc28 to ensure cohesion is maintained during an unperturbed S phase. In the absence of replication stress, either efficient cohesin loading (Clb5 and Clb6 dependent) or robust protection of cohesin from cleavage (Pds1 dependent) is sufficient for cohesion. When replication is perturbed by reduced nucleotide pools, both mechanisms are required to ensure cohesion in S phase; otherwise, spindle tension results in loss of cohesion under these conditions.

Since loss of cohesion required Esp1 and spindle tension, a model to explain these data is that the low level of chromosomal Mcd1/Sccl-Myc in *clb5Δ clb6Δ* cells is sufficient for cohesion until later in S phase when Esp1 activity is increased and spindle forces contribute to pulling the locus apart. However, we cannot rule out the possibility that S-phase cyclins also act directly on Esp1, given that we were able to coprecipitate Esp1 in complexes with Cdc28 and Cks1.

In summary, we describe evidence that specific yeast S-phase cyclins promote loading of cohesin complexes onto chromatin in early S phase (Fig. 9D). In an unperturbed S phase, this mechanism acts in parallel with Pds1 to ensure cohesion is sufficient to hold sister chromatids together. When replication fork progression is stressed, both efficient cohesin loading (Clb5 and Clb6

function) and protection (Pds1 function) are required for cohesion.

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