

Intrinsic and Cyclin-dependent Kinase-dependent Control of Spindle Pole Body Duplication in Budding Yeast

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Centrosome duplication must be tightly controlled so that duplication occurs only once each cell cycle. Accumulation of multiple centrosomes can result in the assembly of a multipolar spindle and lead to chromosome mis-segregation and genomic instability. In metazoans, a centrosome-intrinsic mechanism prevents reduplication until centriole disengagement. Mitotic cyclin/cyclin-dependent kinases (CDKs) prevent reduplication of the budding yeast centrosome, called a spindle pole body (SPB), in late S-phase and G2/M, but the mechanism remains unclear. How SPB reduplication is prevented early in the cell cycle is also not understood. Here we show that, similar to metazoans, an SPB-intrinsic mechanism prevents reduplication early in the cell cycle. We also show that mitotic cyclins can inhibit SPB duplication when expressed before satellite assembly in early G1, but not later in G1, after the satellite had assembled. Moreover, electron microscopy revealed that SPBs do not assemble a satellite in cells expressing Clb2 in early G1. Finally, we demonstrate that Clb2 must localize to the cytoplasm in order to inhibit SPB duplication, suggesting the possibility for direct CDK inhibition of satellite components. These two mechanisms, intrinsic and extrinsic control by CDK, evoke two-step system that prevents SPB reduplication throughout the cell cycle.

INTRODUCTION

Maintenance of genome stability is dependent on faithful segregation of chromosomes at mitosis. The mitotic spindle mediates chromosome segregation through partitioning sister chromosomes to opposite poles. In most cell types, the mitotic spindle is organized by a pair of centrosomes present in the cell at mitosis. However, accumulation of additional centrosomes has been observed in a variety of human cancers (Lingle *et al.*, 1998; Pihan *et al.*, 1998). Supernumerary centrosomes have the potential to assemble a multipolar spindle, which can lead to genomic instability and aneuploidy through catastrophic errors in chromosome segregation. In fact, several studies have suggested that centrosome amplification can contribute to tumorigenesis (reviewed in D'Assoro *et al.*, 2002).

Several circumstances could lead to generation of supernumerary centrosomes, including failed mitosis, cell fusion, *de novo* centrosome formation, and centrosome reduplication during a single cell cycle (Nigg, 2002). Several studies demonstrate that centrosomes can reduplicate during an extended S-phase (Sluder and Lewis, 1987; Gard *et al.*, 1990; Balczon *et al.*, 1995), and this reduplication has been shown to be dependent on cell cycle regulatory proteins (Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999; Meraldi *et al.*, 1999). Additionally, mutations in a number of known cell cycle regulators are associated with centrosome amplification (re-

viewed in Nigg, 2002). These lines of evidence suggest that centrosome duplication is regulated during the cell cycle and that loss of regulation can lead to centrosome amplification.

There is evidence to suggest that the structure of the duplicating centrosome precludes reduplication. Cell fusion experiments patterned after the classic experiments of Rao and Johnson (1970) demonstrated that when cells with duplicated centrosomes were fused with cells that had yet to duplicate their centrosomes, the duplicated centrosomes did not reduplicate, even though they were placed in a cellular environment that promotes centrosome duplication (Wong and Stearns, 2003). This observation suggests that something about the constitution of the previously duplicated centrosome prevents duplication. Recently, this centrosome-intrinsic block to centrosome duplication was shown to be relieved by centriole disengagement (centriole disorientation; Tsou and Stearns, 2006). Disengagement normally occurs during late mitosis or early G1 (Kuriyama and Borisy, 1981) and appears to be driven by separase (Tsou and Stearns, 2006). These findings indicate that the structure of engaged centrioles is not permissive for centriole duplication and that disengagement exposes sites or structures necessary for centriole duplication after mitosis.

Several studies suggest a role for cyclin dependent kinases (CDKs) in regulating centrosome duplication during the cell cycle (reviewed in Delattre and Gonczy, 2004). In experimental systems that are permissive for centrosome reduplication, specific cyclin/CDK complexes promote (Cdk2, cyclins E and A; Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999; Meraldi *et al.*, 1999) and restrain (cyclin B; Lacey *et al.*, 1999) centrosome duplication. In *Drosophila* loss of Cdk1 leads to formation of additional centrioles, whereas expression of hyper-stable cyclin A and cyclin B may inhibit centriole duplication (Vidwans *et al.*, 1999, 2003). These findings suggest that specific cyclin/CDK complexes are important for both promoting centrosome duplication and inhibiting cen-

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Abbreviations used: SPB, spindle pole body; CDK, cyclin-dependent kinase.

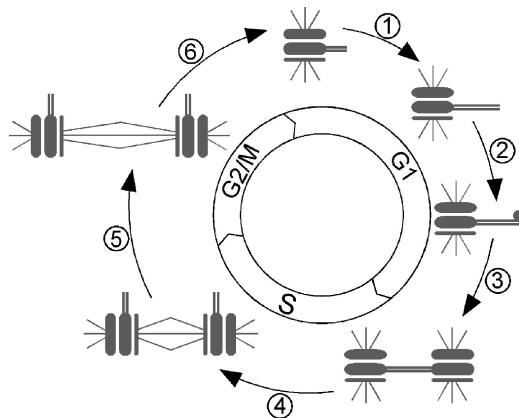


Figure 1. The SPB duplication cycle is coordinated with the cell cycle (reviewed in Chial and Winey, 1999). Steps in the SPB duplication cycle are indicated by number. Step 1, bridge elongation. The half-bridge doubles in length. Step 2, satellite assembly. An electron-dense mass of protein, thought to be a template for the new SPB, is deposited at the distal end of the half-bridge on the cytoplasmic face of the nuclear envelope. Step 3, SPB duplication. The SPB is assembled in the cytoplasm through an intermediate called the duplication plaque. The nascent SPB is then inserted into the nuclear envelope, resulting in two side-by-side SPBs connected by a full bridge. Step 4, SPB separation. The two SPBs move to opposite sides of the nucleus in a microtubule-dependent manner, splitting the full bridge into two half-bridges and creating a short spindle. Step 5, spindle elongation. At mitosis the spindle elongates, segregating chromosomes into the mother and daughter cells. Step 6, mitotic exit. At the completion of mitosis, the spindle disassembles, the cells separate, and each cell inherits a single SPB with a short half-bridge.

trosome reduplication during the normal cell cycle; however, it remains unclear whether CDKs control centrosome duplication directly or affect centrosome duplication indirectly by inhibiting cell cycle progression.

The centrosome analog in the budding yeast, *Saccharomyces cerevisiae*, called the spindle pole body (SPB), has been established as a model for many aspects of centrosome biology (Adams and Kilmartin, 2000). Electron microscopy has been used to describe the structure of the spindle pole body during the different stages of SPB duplication in great detail (Byers and Goetsch, 1974, 1975; Adams and Kilmartin, 1999), as summarized in Figure 1. Subsequent genetic screens identified several genes required for proper SPB duplication (reviewed in Chial and Winey, 1999; Jaspersen and Winey, 2004). By examining the structure of the unduplicated SPB in mutant cells, a detailed order of assembly for the SPB and the activities required for each step has been established (reviewed in Chial and Winey, 1999; Jaspersen and Winey, 2004). Nevertheless, relatively little is known about the regulation of SPB duplication.

Several studies have demonstrated a role for CDK activity in the SPB duplication cycle (reviewed in Jaspersen and Winey, 2004). Cln1,2/Cdk1 activity has been shown to be important for SPB duplication in G1 (Figure 1, step 3) by directly phosphorylating important proteins in SPB duplication (Byers and Goetsch, 1974; Jaspersen *et al.*, 2004). In addition, B-cyclins have been shown to be important for both promoting SPB duplication and preventing SPB reduplication (Haase *et al.*, 2001).

In cells lacking the mitotic B-cyclins, SPBs will reduplicate; however, in cells lacking all six of the B-cyclin genes, SPBs duplicate but do not reduplicate (Haase *et al.*, 2001). These observations indicate that B-cyclin/CDK activity is

required to promote SPB reduplication and is likely to trigger a step(s) in the SPB duplication cycle. The B-cyclin-dependent step appears to correlate with SPB separation, which is also known to require B-cyclin/CDK activity (Fitch *et al.*, 1992; Haase *et al.*, 2001; Crasta *et al.*, 2006). Although the mechanism by which mitotic cyclin/CDK complexes function to inhibit SPB reduplication is poorly understood, data suggest that mitotic cyclin/CDK acts directly, rather than through the suppression of *CLN2* transcription (Haase *et al.*, 2001). Taken together, these findings suggest a mechanism for preventing SPB reduplication reminiscent of the licensing model for the control of DNA replication (Haase *et al.*, 2001). Evidence points to CDK regulation at multiple levels throughout the SPB duplication cycle; however, it is not yet clear how cells prevent SPB reduplication early in the cell cycle before B-cyclins are expressed, or how mitotic cyclin/CDKs inhibit reduplication when expressed later in the cell cycle.

To address the mechanisms restraining SPB duplication to once per cell cycle, we examined both CDK-dependent and -independent mechanisms that prevent SPB reduplication. Our findings reveal a role for a SPB-intrinsic block to SPB reduplication, similar to the centrosome-intrinsic block to centrosome duplication, which prevents SPB reduplication early in the cell cycle. We also demonstrate that mitotic cyclin/CDKs but not S-phase or G1 cyclin/CDKs can block SPB duplication when expressed early in G1 and that mitotic cyclin/CDK activity can inhibit early steps in the SPB duplication cycle. Our results describe distinct mechanisms that coordinate to prevent SPB reduplication throughout the yeast cell cycle.

MATERIALS AND METHODS

Plasmid and Strain Construction

All strains used in this study are derivatives of BF264-15DU (*MATa*; *ade1*; *his2*; *leu2-3112*; *trp1-1*; *ura3Δns*; Richardson *et al.*, 1992). Relevant genotypes of strains are detailed in Table 1. SBY408 was constructed by cutting plasmid *pZSPC42-GFP* (Haase *et al.*, 2001) with BglIII and integrating at the *TRP1* locus. SBY844 was constructed by PCR amplification of the mRFP-KanMX6 tag from the genome of ATCC201389: *SPC42-mRFP-KanMX6* (Huh *et al.*, 2003) using oligonucleotides FW (5'ATGGCCTCCTCCGAGGACG3') and RV (5'GTTAGTATCGAATCGACAGC3') and cloning into pDrive (Qiagen, Valencia, CA). The sequence was reamplified using oligonucleotides FW (5'TATGTCAGAAAACATTCGCAACTCCCACTCCCAATAATCGAGGAATCGCTCTCCGAGGACG3') and RV (5'TATAAAAGGCCTTTACGTTTCCGCTTCTGTGGAAAATAGTATGATCGAATCGAATCGACAGC3') and integrated at the *SPC42* locus via homologous recombination. SBY533 was constructed by cutting plasmid YIpG2-*CLB2Δdb* (a gift from Steve Reed) with KpnI and integrating at the *LEU2* locus. SBY520 was made by cutting plasmid *pGAL1-SIC1Δ3P* (Verma *et al.*, 1997) with EcoRV and integrating into SBY408 at the *URA3* locus. SBY1353 was made by PCR amplification of *CIN8* from the genome followed by ligation into pDrive (Qiagen). The *cin8^{ts}* allele was made by site-directed mutagenesis using primers FW(5'GATAAAAGCGCCATATACCTGCACGTGAATCGAAATTGACC3') and RV (GGTCAATTCGATTCACGTGCAGGTATATGGCCGCTTTATC3') to generate the temperature-sensitive F429A mutation (Gheber *et al.*, 1999) and a unique PmlI site. A KpnI/XbaI fragment containing the *cin8^{ts}* allele was then subcloned into pRS306 (Sikorski and Hieter, 1989). The resulting plasmid was cut with BglIII and transformed into a version of SBY408 carrying *kip1Δ::KanMX4* at the *CIN8* locus. *CIN8* allele replacement was achieved by growing transformants in the presence of 5'-fluoroorotic acid (5'-FOA), and confirmed by PCR followed by PmlI digestion. The resulting strain was transformed with *pGAL1-Sic1Δ3P* (Verma *et al.*, 1997) cut with EcoRV for integration at the *URA3* locus. SBY1168 was made by amplification of the HA3 tag and Kan^R from plasmid *pKHA3* using primers FW (5'GGTTAGAAAAACCGCTATGATATAATGACCTTGCATGAACGCATCTTTACCCATACG3') and RV (5'CATACATTATATGGACATTTATCGATTATCGTTTTAGACATGCCATCCGTAAGATGC3') and integrating into SBY533 at both *CLB2Δdb* and *CLB2*. SBY1099 was made by cutting CWB194 (*pRS414-GAL1-CLN2-HA3*; Lanker *et al.*, 1996) with MfeI and integrating into SBY844 at the *TRP1* locus. SBY1157 was made by cutting plasmid DBRI (*pGAL1-HA3-CLB5Δdb*; Cross *et al.*, 1999) with EcoRV and integrating into SBY408 at the *URA3* locus.

The *pGAL1-CLB2ΔdbΔNES-GFP* and *pGAL1-CLB2ΔdbΔNLS-GFP* plasmids were made by swapping a BbvCI/ApaI fragment from the C-terminal half of

Table 1. Yeast strains used in this study

Strain	Relevant genotype
SBY408	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R</i>
SBY520	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; GAL-SIC1Δ3P-URA3</i>
SBY533	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; GAL-CLB2Δdb-LEU2</i>
SBY844	<i>MATa; bar1; SPC42-mRFP-KanMX6</i>
SBY916	<i>MATa; bar1; SPC42-mRFP-KanMX6; GAL-CLB2Δdb-GFP-TRP1</i>
SBY918	<i>MATa; bar1; SPC42-mRFP-KanMX6; GAL-CLB2ΔdbNES-GFP-TRP1</i>
SBY920	<i>MATa; bar1; SPC42-mRFP-KanMX6; GAL-CLB2ΔdbΔNLS-GFP-TRP1</i>
SBY1099	<i>MATa; bar1; SPC42-mRFP-KanMX6; GAL-CLN2-HA3-TRP1</i>
SBY1157	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; GAL-HA3-CLB5Δdb-URA3</i>
SBY1167	<i>MATa; bar1; CLB2-HA3-Kan^R</i>
SBY1168	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; GAL-CLB2Δdb-HA3-LEU2-Kan^R; CLB2-HA3-Kan^R</i>
SBY1171	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; GAL-HA3-CLB5Δdb-NES1-mRFP-URA3</i>
SBY1173	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; GAL-HA3-CLB5Δdb-NESA-mRFP-URA3</i>
SBY1353	<i>MATa; bar1; SPC42-GFP-TRP1-Zeo^R; cin8^{ts}; kip1Δ::KanMX4; GAL-SIC1Δ3P-URA3</i>

CLB2 in pPS2191 (2 μ *GAL1-CLB2Δdb-GFP*) with a BbvCI/ApaI fragment from either pPS2190 (2 μ *GAL1-CLB2(L303A)-GFP*) to make 2 μ *GAL1-CLB2ΔdbNES-GFP* or pPS2192 (2 μ *GAL1-CLB2(Δ183-200)-GFP*) to make 2 μ *GAL1-CLB2ΔdbΔNLS-GFP* (Hood *et al.*, 2001). pRS304 *GAL1-CLB2Δdb-GFP* (and Δ NES and Δ NLS derivatives) were made by cutting 2 μ plasmids containing the construct with EcoRI/NsiI and cloning fragments into pRS304 (Sikorski and Hieter, 1989) cut at EcoRI/PstI. SBY916, SBY918, and SBY920 were made by cutting pRS304 *GAL1-CLB2Δdb-GFP* constructs with Bsu36I and integrating at the *TRP1* locus of SBY844.

The p*GAL1-HA3-CLB5Δdb-NES-mRFP* constructs were made as follows. *CUP1-CLB5* was amplified from p*KCUP1-CLB5-HA* (Haase *et al.*, 2001) using oligonucleotides FW (5'CTTGCATGCTGCAGGTC3') and RV (5'GTCGACCGCGCCGCACCTAAGATTAATAGATTTTGAAAGTGTATGCATTTC3'). The resulting product was cloned into pDrive (Qiagen). A Sall fragment of pDrive-*CUP1-CLB5* was cloned into Sall digested pDrive-mRFP-KanMX6 to make pDrive-*CUP1-CLB5-mRFP-KanMX6* of which a SacI/BglII fragment containing *CUP1-CLB5-mRFP* was subcloned into YCplac33 (Gietz and Sugino, 1988) digested with SacI/BamHI. The active (NES-A) and inactive (NES-I) cassettes were amplified from p306-NESA and p306-NESI plasmids, respectively (Edgington and Futcher, 2001), using oligonucleotides FW (5'CGAAATGCATAGCAACTTTCAAATCTATTTAAATCTTAAGGGTTTACGACTGCTAAATAGC3') and either NES-A RV (5'CGTCTCGGAGGAGGCCATAATCTGAATTCGTCGACAAGCACTACCGATATCTAAACCTG3') or NES-I RV (5'CGTCTCGGAGGAGGCCATAATCTGAATTCGTCGACAAGCACTACCGATATCAGCACCTG3') and cloned into SacI-digested YCplac33-*CUP1-CLB5-mRFP* via gap repair. *GAL1-HA3-CLB5Δdb* was subcloned from DBRI (Cross *et al.*, 1999) into pRS306 (Sikorski and Hieter, 1989) on EcoRI ends. A NotI/BspEI fragment containing the C-terminus of *CLB5* was replaced by NotI/BspEI fragments from YCplac33-*CUP1-CLB5-NESA-mRFP* or YCplac33-*CUP1-CLB5-NESI-mRFP* to make pRS306-*GAL1-HA3-CLB5Δdb-NESA-mRFP* and pRS306-*GAL1-HA3-CLB5Δdb-NESI-mRFP*. These constructs were digested with EcoRV and integrated into the *URA3* locus of SBY408 to make SBY1173 (NES-I) and SBY1171 (NES-A).

Cell Growth and Synchronization

Yeast cultures were grown in YEP medium (1% yeast extract, 2% peptone, 0.012% adenine, 0.006% uracil supplemented with 2% sugar: dextrose, sucrose, or galactose). Cells were grown at 30°C. Mating pheromone arrest was accomplished by adding 30–60 ng/ml alpha-factor (α -factor) to the growth medium. For synchronization experiments, cells were then released into growth medium without α -factor. For mitotic arrest, cells were treated with 15 μ g/ml nocodazole.

SPB reduplication experiments were performed as previously described (Haase *et al.*, 2001). For nocodazole experiments, hydroxyurea was used at 100 mM to slow progression through S-phase, and nocodazole was used at 15

μ g/ml to destabilize microtubules either at release from α -factor or at the time of induction of *GAL1-SIC1Δ3P*. For *cin8^{ts}kip1Δ* experiments, cells were incubated at either 25 or 38°C upon release from α -factor, and galactose was then added to both cultures to induce *GAL1-SIC1Δ3P* after the 25°C culture had become 80% budded.

Elutriation experiments were performed as follows. Cells were grown to 1.5×10^7 – 2.5×10^7 cells/ml in YEP-sucrose. The *GAL1* promoter was induced by addition of 2% galactose into the media and incubated for 45 min (for *CLN2* or *CLB5* constructs) or 60 min (for *CLB2* constructs). Cells were then put on ice, subjected to centrifugal elutriation, and small daughter cells were collected. Cells synchronized in early G1 were released into YEP-galactose at $t = 0$ min.

Microscopy

All samples analyzed by fluorescence microscopy were fixed in 2% paraformaldehyde for 15–30 min and then washed with PBS and stored in 30% glycerol. DNA staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics, Indianapolis, IN) was done at 1 μ g/ml to visualize nuclei. Cells were viewed using a Zeiss Axio Imager widefield fluorescence microscope with a 100 \times objective and standard filter sets (Thornwood, NY). Foci of Spc42-GFP or Spc42-mRFP were counted for a minimum of 200 cells per sample to determine the state of SPB duplication in the sample. Images were acquired with a Hamamatsu Orca ER monochrome cooled-CCD camera with IIEE (Bridgewater, NJ) and captured using Metamorph 7.1 (Universal Imaging, Downingtown, PA). Images were merged digitally using Photoshop 7.0 (Adobe Systems, Palo Alto, CA).

Samples were prepared for electron microscopy as previously described (Byers and Goetsch, 1991). Samples were viewed using Philips CM 12 transmission electron microscope (FEL, Hillsboro, OR), and images were captured using an AMT XR100 Digital Camera (Advanced Microscopy Techniques, Danvers, MA).

Immunoblotting

Cell lysates were subjected to SDS-PAGE and immunoblotting using the following antibodies: mouse anti-HA (Roche Diagnostics), mouse anti-green fluorescent protein (GFP; Covance, Berkeley, CA), mouse anti-PSTAIR (Abcam, Cambridge, MA), and HRP-conjugated goat anti-mouse (Pierce Biotechnology, Rockford, IL).

Flow Cytometry

Preparation of cells and flow cytometric analysis of DNA content was carried out as described previously (Haase and Reed, 2002).

RESULTS

SPB Separation Is Required for SPB Reduplication

Mitotic cyclin/CDKs inhibit SPB reduplication during the cell cycle (Haase *et al.*, 2001), but mitotic cyclins are not expressed until S-phase and G2. Thus, SPB reduplication cannot be inhibited by mitotic cyclins in G1 and early S-phase, a period when SPB components are expressed and proteins are known to be important for SPB duplication are active (Jaspersen *et al.*, 2004; Orlando *et al.*, 2008). Previous studies indicate that B-cyclin/CDK activity is required after SPB duplication in order for SPBs to duplicate in the next cycle (Haase *et al.*, 2001). We investigated whether B-cyclin/CDKs might function to relieve an inhibitory mechanism that normally prevents SPB reduplication in late G1 and early S-phase.

During the normal cell cycle, the SPBs separate to form a short spindle in late S-phase/G2 (Figure 1, step 4), whereas cells lacking mitotic B-cyclins, cells go on to reduplicate SPBs after separation (Haase *et al.*, 2001). Because cells lacking all the B-cyclins neither separate, nor reduplicate their SPBs (Haase *et al.*, 2001) and separation has been shown to be dependent on B-cyclin/CDK activity (Figure 1, step 3; Fitch *et al.*, 1992; Crasta *et al.*, 2006), we hypothesized that SPB separation may be required to relieve a block to SPB reduplication. To test this hypothesis, we took advantage of the finding that SPB separation is dependent on the presence of microtubules (Jacobs *et al.*, 1988).

SPB separation was blocked by treating cells with the microtubule depolymerizing drug, nocodazole, and the ability of cells to reduplicate SPBs under conditions previously

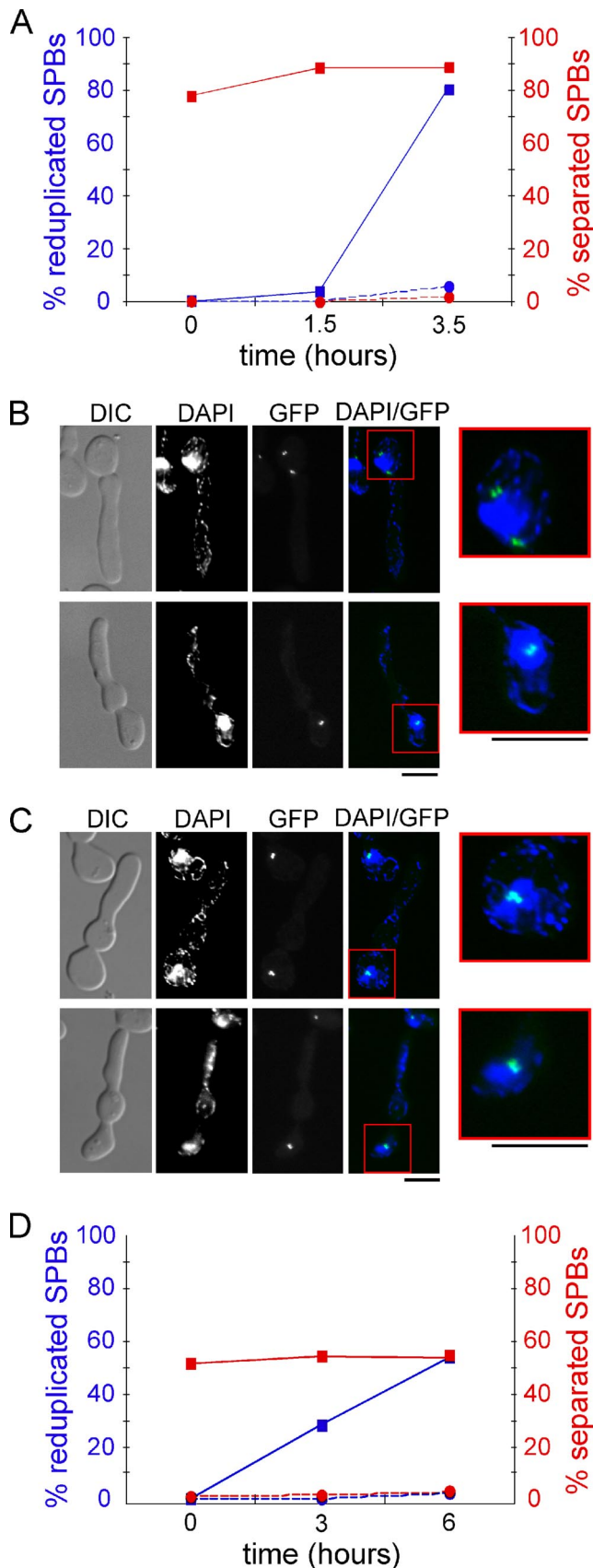


Figure 2. SPB separation is required for SPB duplication. Cells were synchronized in G1 and cell aliquots were either allowed to

shown to promote SPB reduplication (Haase *et al.*, 2001) was determined. Cells bearing a hyper-stabilized allele of *SIC1* (*SIC1Δ3P*) controlled by the *GAL1* promoter were arrested in G1 with α -factor and then released in the presence or absence of nocodazole in non-inducing medium. When cells growing in medium lacking nocodazole had separated SPBs, galactose was added to both cell cultures to induce the expression of *SIC1Δ3P*, thereby inhibiting B-cyclin/CDK activity and allowing SPB reduplication. SPB separation and reduplication were monitored by fluorescence microscopy of Spc42-GFP.

In cells treated with nocodazole upon release from an α -factor arrest, SPBs duplicated, but neither separated nor reduplicated, whereas untreated cells separated and reduplicated SPBs (Figure 2, A and B). To control for unanticipated effects of nocodazole treatment on SPB duplication, a third aliquot of cells was allowed to separate SPBs and form a short spindle before the addition of nocodazole, which was added concurrent with addition of galactose to stimulate expression of Sic1Δ3P. In these cells, the short spindle collapses (Jacobs *et al.*, 1988), but some of the SPBs go on to reduplicate despite their close proximity (Figure 2C).

We also investigated the necessity of SPB separation before SPB reduplication using mutations in the motor proteins Cin8 and Kip1. SPB separation is defective in *cin8 kip1* double mutants, and cells arrest with duplicated SPBs still attached by a full bridge structure (Hoyt *et al.*, 1992; Roof *et al.*, 1992). *cin8^{ts}kip1Δ* cells were released from α -factor arrest at either restrictive or non-restrictive temperature. Galactose was added to induce transcription of *SIC1Δ3P* from the *GAL1* promoter in both cultures after the cells had been given sufficient time to separate their SPBs at non-restrictive temperature (25°C). SPB separation and reduplication were monitored by fluorescent microscopy of Spc42-GFP. Many of the *cin8^{ts}kip1Δ* cells released at 25°C separated their SPBs and by 6 h after induction of *SIC1Δ3P* most of the cells with separated SPBs had reduplicated SPBs (Figure 2D). As expected, few *cin8^{ts}kip1Δ* cells separated SPBs at 38°C, and only a small fraction of cells released at restrictive temperature (38°C) reduplicated SPBs after 6 h (Figure 2D). The few cells at 38°C that went on to reduplicate SPBs had also separated their SPBs.

Taken together, these results demonstrate that SPB separation is essential for SPB reduplication and suggest that duplicated side-by-side SPBs are unable to reduplicate until they have separated into two individual SPBs, each with its own short half-bridge (Figure 1, step 4).

separate SPBs or were prevented from separating SPBs by addition of nocodazole or shifting *cin8^{ts}kip1Δ* cells to 38°C. Sic1Δ3P expression was induced in all aliquots to promote SPB reduplication. SPBs were examined by fluorescent microscopy of Spc42-GFP. (A) Percentages of separated and reduplicated SPBs in cells either treated with nocodazole upon release from α -factor (●; broken line) or untreated (■; solid line) over time. (B) Top, images showing separated and reduplicated SPBs in untreated cells. Bottom, images showing unseparated and duplicated (but not reduplicated) SPBs in cells treated with nocodazole upon release from α -factor. Bar, 50 μ m. (C) Images showing collapsed and reduplicated SPBs in cells released from α -factor in the absence of nocodazole to allow SPB separation and then treated with nocodazole to collapse short spindles upon expression of Sic1Δ3P. Bar, 50 μ m. (D) Percentages of separated and reduplicated SPB in *cin8^{ts}kip1Δ* cells released at either 38°C (●; broken line) or 25°C (■; solid line) over time.

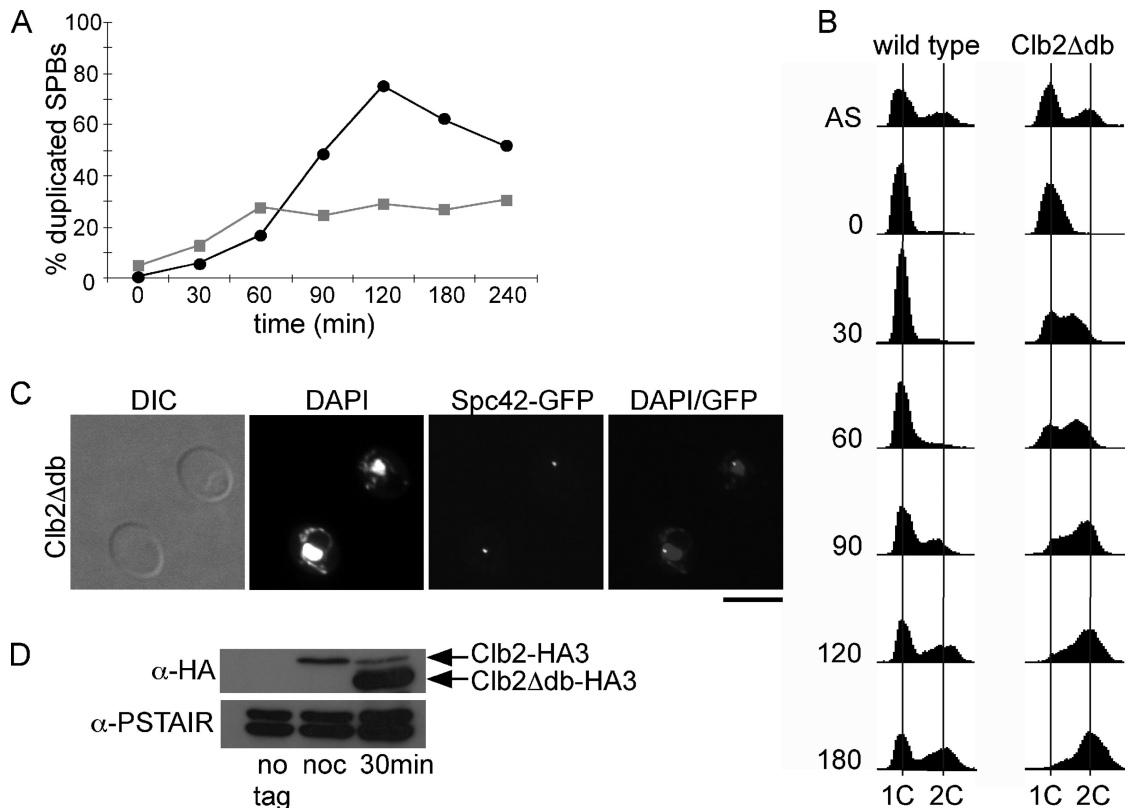


Figure 3. Ectopic expression of Clb2 in early G1 inhibits SPB duplication. Clb2 Δ db expression was induced from the *GAL1* promoter in asynchronous populations of cells, and then small daughter cells were collected by elutriation. SPB duplication (A) and DNA content (B) were monitored over time by fluorescence microscopy and flow cytometry in cells expressing (■, gray) or not expressing (●, black) Clb2 Δ db. (C) Images of representative cells expressing Clb2 Δ db 120 min after elutriation. Bar, 50 μ m. (D) Western blot of Clb2 protein levels in untagged cells, cells synchronized in α -factor and released into nocodazole for 1.5 h, and in elutriated cells 30 min after elutriation. Cdk1 levels (α -PSTAIR) are shown as a loading control.

A Mitotic Cyclin Expressed in G1 Can Inhibit SPB Duplication

After separation, it has been demonstrated that mitotic cyclin/CDKs prevent SPB reduplication (Haase *et al.*, 2001). The structure of the SPB does not detectably change after separation until the next G1 (Figure 1, steps 5, 6, and 1; Byers and Goetsch, 1974, 1975). We posited that in the absence of mitotic cyclins, the cell cycle arrests at metaphase, but the SPB duplication cycle continues by progressing directly from the separated phase (Figure 1, after step 4) through the early steps of the duplication cycle (Figure 1, steps 1–3). The orientation of reduplicated SPBs (two sets of side-by-side SPBs separated by a short spindle; Figure 2A; Haase *et al.*, 2001) is consistent with this hypothesis. To determine whether mitotic cyclin/CDKs normally prevent SPB reduplication by preventing the premature passage through these early steps in the SPB duplication cycle, we ectopically expressed a destruction box mutant allele of the mitotic B-cyclin Clb2, Clb2 Δ db, which is stable in G1, from the *GAL1* promoter in early G1 cells.

Daughter cells in early G1 were collected from an asynchronous population of cells by centrifugal elutriation 60 min after the induction of Clb2 Δ db expression. SPB duplication was then monitored over time by fluorescence microscopy (Figure 3, A and C). Two hours after elutriation, cells not expressing Clb2 Δ db had duplicated their SPBs and initiated mitosis; however, \sim 70% of cells expressing Clb2 Δ db failed to duplicate their SPBs (Figure 3A). Although early G1 cells expressing Clb2 Δ db fail to bud (data

not shown; Lew and Reed, 1993), they do rapidly replicate DNA and arrest with 2C DNA content (Figure 3B; Amon *et al.*, 1994), indicating that they progress through the G1/S transition.

These results demonstrate that ectopic expression of Clb2 in early G1 can inhibit SPB duplication; however, there is normally very little CDK activity during early G1 (Miller and Cross, 2001). To ensure that the inhibition of SPB duplication is specific to mitotic B-cyclins and not a simply a function of excess CDK activity in early G1, we also assayed the effects of expressing the G1 cyclin, Cln2, and a destruction box mutant of the S-phase cyclin, Clb5 (Clb5 Δ db; Cross *et al.*, 1999), from the *GAL1* promoter in early G1. Although Cln2 and Clb5 promote SPB duplication later in G1, it is possible that a high level of Cln2/Cdk1 or Clb5/Cdk1 activity before START would have an inhibitory role similar to a high level of Clb2/Cdk1 activity. However, expression of either Cln2 or Clb5 in early G1 did not inhibit SPB duplication, but rather accelerated the duplication process either by direct phosphorylation or by indirectly driving the cells more rapidly through G1 (as evidenced by early bud emergence in cells expressing either Cln2 or Clb5 Δ db; Figure 4). Additionally, cells expressing Clb5 Δ db failed to exit mitosis (Figure 4, A and B) as previously described (Jacobson *et al.*, 2000). Our findings indicate that the inhibition of SPB duplication in early G1 is specific to mitotic cyclins and is not due to a general increase in CDK activity during this time period.

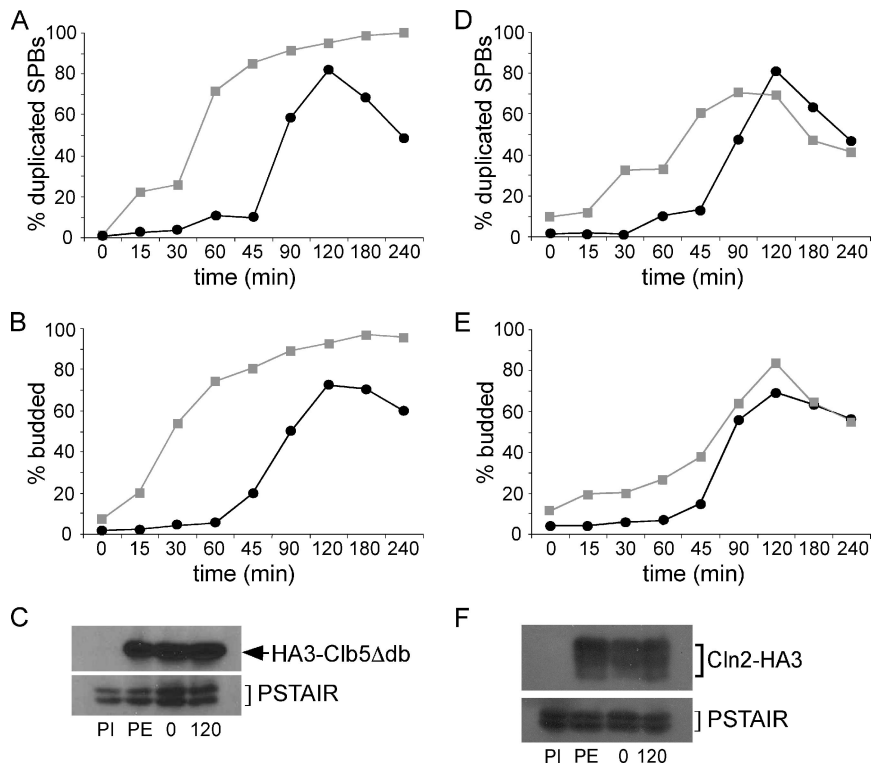


Figure 4. Ectopic expression of G1 or S-phase cyclins in early G1 cannot inhibit SPB duplication. HA3-Clb5 Δ db (A–C) or Cln2-HA3 (D–F) was expressed from the *GAL1* promoter in asynchronous populations of cells, and then small daughter cells were collected by centrifugal elutriation. SPB duplication (A and D) and bud emergence (B and E) were monitored over time by fluorescence microscopy in cells expressing (■, gray) or not expressing (●, black) cyclin. (C and F) Western blot of cyclin levels before cyclin induction (PI), before elutriation (PE), after elutriation (0), and 120 min after elutriation (120). Cdk1 levels (α -PSTAIR) are shown as a loading control.

Clb2 Expression in Early G1 Inhibits Satellite Assembly

Mitotic cyclin/CDK activity could block a number of early steps in the SPB duplication cycle including bridge elongation, satellite assembly, or the assembly of a new SPB (Figure 1, steps 1–3). To determine whether Clb2 inhibits SPB duplication after satellite assembly, we asked whether Clb2 could inhibit SPB duplication in cells arrested in α -factor, when SPBs are known to have assembled a satellite (Byers and Goetsch, 1974, 1975). Cells were arrested in α -factor and then released into media containing galactose to induce expression of Clb2 Δ db. Cells expressing Clb2 Δ db and control cells not expressing Clb2 Δ db duplicated their SPBs at a similar rate (Figure 5A). Furthermore, SPB duplication was driven by the expression of Clb2 Δ db in cells that were maintained in α -factor (Figure 5B), consistent with previous observations (Amon *et al.*, 1994). These findings suggest that Clb2 inhibits early steps in the SPB duplication cycle; however, once a satellite has formed, Clb2 no longer inhibits SPB duplication but can actually drive later steps in the SPB duplication cycle.

To investigate which early steps in the SPB duplication cycle might be inhibited by Clb2, we examined SPBs by electron microscopy in cells where SPB duplication was blocked by the expression of Clb2. We analyzed 50 SPBs from cells expressing Clb2 Δ db 2 h after elutriated cells were inoculated into fresh medium (Figure 5D). Half-bridges were observed in 33 of 50 SPBs; however, we did not observe satellite structures in any cells expressing Clb2. This result confirms that Clb2 inhibits SPB duplication before satellite assembly, although we were unable to discern whether Clb2 inhibits bridge elongation, satellite assembly, or both. Interestingly, about one-third of the SPBs were observed to be larger than 100 nm in diameter. Large SPB size has been previously observed in cells with mutations that affect SPB duplication (Byers, 1981; Winey *et al.*, 1991; Adams and Kilmartin, 1999).

To control for our ability to identify satellite structures, we examined an isogenic strain (not expressing Clb2 Δ db) that was released into medium containing α -factor to arrest the cells at START after elutriation. Cells arrested at START should contain satellite bearing SPBs as previously reported (Byers and Goetsch, 1974, 1975; Adams and Kilmartin, 1999). In the 29 control cells examined, all SPBs observed were between 75 and 100 nm in diameter, a normal size for haploid SPBs (Byers and Goetsch, 1974). Eleven SPBs had observable half-bridges and nine of those SPBs had satellite structures associated with the half-bridge (Figure 5E).

Cytoplasmic Localization of Clb2 Is Required to Inhibit SPB Duplication

Satellite assembly is spatially confined to the cytoplasmic face of the half-bridge (Adams and Kilmartin, 1999). Clb2 has been shown to shuttle between the nucleus and cytoplasm (Hood *et al.*, 2001) and to localize to spindle poles (Bailey *et al.*, 2003). Thus, it is possible that Clb2 may inhibit satellite assembly by phosphorylating targets on the cytoplasmic face of the SPB. Therefore, we examined whether Clb2 localization was important for its ability to block SPB duplication in early G1 using *CLB2* alleles that have mutations in either the nuclear localization sequence (NLS, Δ 183–200) or the nuclear export sequence (NES, L303A; Hood *et al.*, 2001). Clb2, Clb2 Δ NES, and Clb2 Δ NLS alleles with mutations in the destruction box were fused to GFP, expressed under control of the *GAL1* promoter, and integrated in cells with monomeric red fluorescent protein (mRFP)-tagged Spc42 so that both Clb2 localization and SPB duplication could be monitored. We ectopically expressed these Clb2 mutants in cells synchronized in early G1 by elutriation as described above. As expected, Clb2 localized to both the nucleus and the cytoplasm, Clb2 Δ NES localized to the nucleus and Clb2 Δ NLS exhibited increased cytoplasmic local-

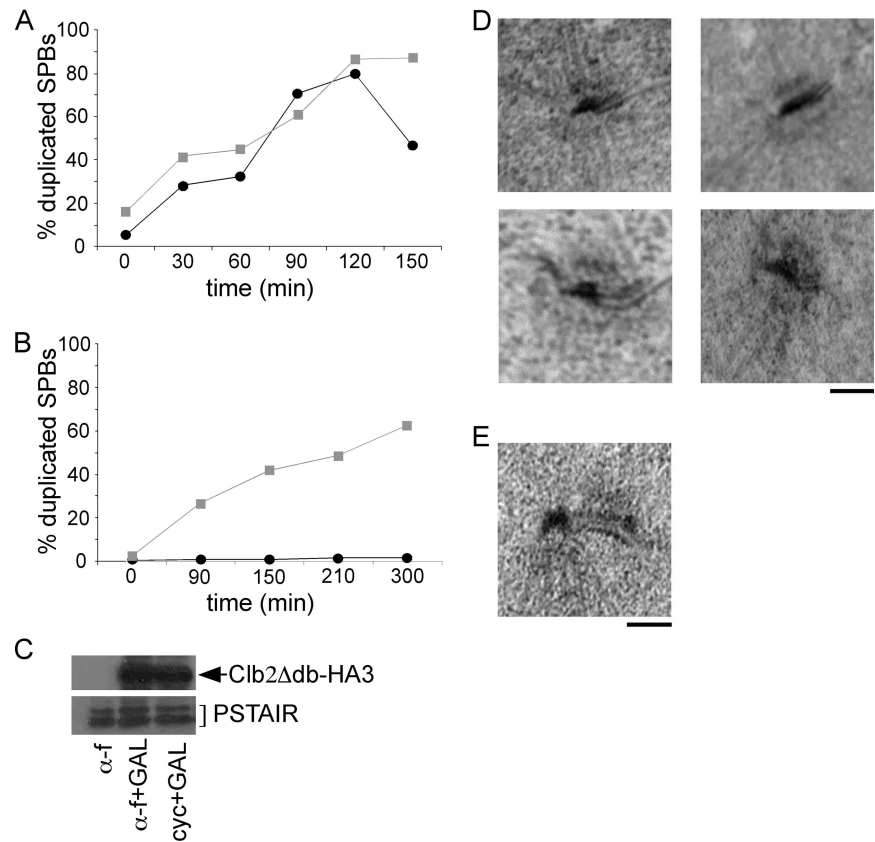


Figure 5. Clb2 inhibits early steps in SPB duplication. (A) Asynchronous populations of cells were arrested in α -factor and released into media inducing expression of Clb2 Δ db. (B) Cells were held in α -factor arrest as Clb2 Δ db expression was induced. For both experiments, SPB duplication was monitored by fluorescence microscopy of Spc42-GFP over time for cells expressing (■, gray) and not expressing (●, black) Clb2 Δ db. (C) Western blot showing Clb2 Δ db-HA3 levels in arrested cells (α -f), arrested cells 30 min after Clb2 Δ db induction (α -f + GAL), and in cells released from α -factor and induced to express Clb2 Δ db for 30 min (cyc + GAL). Cdk1 levels (α -PSTAIR) are shown as a loading control. (D) SPB structure was analyzed by electron microscopy in elutriated cells expressing Clb2 Δ db 2 h after elutriation. (E) SPB structure was analyzed by electron microscopy in cells arrested in α -factor after elutriation. Bar, 100 nm.

ization but was not excluded from the nucleus (Figure 6C; Hood *et al.*, 2001). SPB duplication was inhibited in cells expressing either Clb2 or Clb2 Δ NLS, but not in cells expressing Clb2 Δ NES (Figure 6A). All three constructs were expressed at comparable levels (Figure 6D). Additionally, although cells expressing Clb2 or Clb Δ NLS failed to bud, some, but not all cells expressing Clb2 Δ NES budded over time (Figure 6B). These findings indicate that Clb2 must localize to the cytoplasm to inhibit SPB duplication.

Cytoplasmic Localization Cannot Confer the Ability to Inhibit SPB Duplication to Clb5

Previous studies demonstrate that the mitotic B-cyclins (Clb1, Clb2, Clb3, and Clb4) can inhibit SPB reduplication, whereas the S-phase B-cyclins, Clb5 and Clb6, cannot (Haase *et al.*, 2001). As we have shown that Clb2 must be cytoplasmic in order to inhibit SPB duplication, it is possible that Clb5, a nuclear protein (Jacobson *et al.*, 2000), cannot block SPB duplication because it does not localize to the cytoplasm. Thus, we asked if Clb5 could block SPB duplication if it was forced to localize to the cytoplasm. Two active leucine-rich NES sequences (NESA) or two inactive NES sequences that have key leucines mutated to alanine (NESI; Edgington and Futcher, 2001) were fused to mRFP-tagged Clb5 Δ db (Cross *et al.*, 1999) and expressed from the *GAL1* promoter in early G1 cells. As expected, Clb5-NESI localized to the nucleus (Figure 7D). Clb5-NESA was observed in the nucleus and cytoplasm and was observed to colocalize with Spc42-GFP (Figure 7C), as has been shown previously for both Clb2 and Clb4 (Hood *et al.*, 2001; Bailly *et al.*, 2003; Maekawa and Schiebel, 2004). However, localization to the cytoplasm (and the spindle pole) did not confer the ability to inhibit SPB duplication on Clb5 (Figure 7A). Thus, Clb5/

Cdk1 cannot phosphorylate the cytoplasmic Clb2/Cdk1 targets important to inhibit SPB reduplication.

DISCUSSION

Ensuring that centrosomes are duplicated only once during each cell cycle is critical for assembling a proper bipolar spindle and for preventing mis-segregation events during mitosis. In budding yeast, we have now characterized both SPB-intrinsic and extrinsic control mechanisms that prevent reduplication of the SPB during distinct cell cycle intervals.

Previous work established that SPBs reduplicate in the absence of mitotic cyclin/CDKs (Haase *et al.*, 2001). However, we have now demonstrated that in the absence of B-cyclin/CDK activities, inhibiting SPB separation also prevents reduplication (Figure 2) before mitotic cyclin expression. As SPBs in both nocodazole-treated and *cin8 kip1* cells have been shown to arrest with duplicated SPBs with an intact bridge (Jacobs *et al.*, 1988; Hoyt *et al.*, 1992; Roof *et al.*, 1992), these findings suggest that duplicated, side-by-side SPBs lack the proper structure to initiate a new round of SPB duplication. The SPB duplication cycle normally begins with the elongation of the half-bridge structure and the assembly of a satellite at the distal end of the half-bridge (Figure 1, steps 1 and 2; Byers and Goetsch, 1974, 1975). It is likely that the proper cues for satellite assembly are contained in the elongated half-bridge, but not the full bridge that connects side-by-side SPBs. Thus, SPB reduplication is inhibited by an SPB-intrinsic mechanism until SPB separation exposes new half-bridge structures that can support satellite assembly. This mechanism would prevent multiple rounds of SPB duplication during G1 when B-cyclin/CDK activity is low

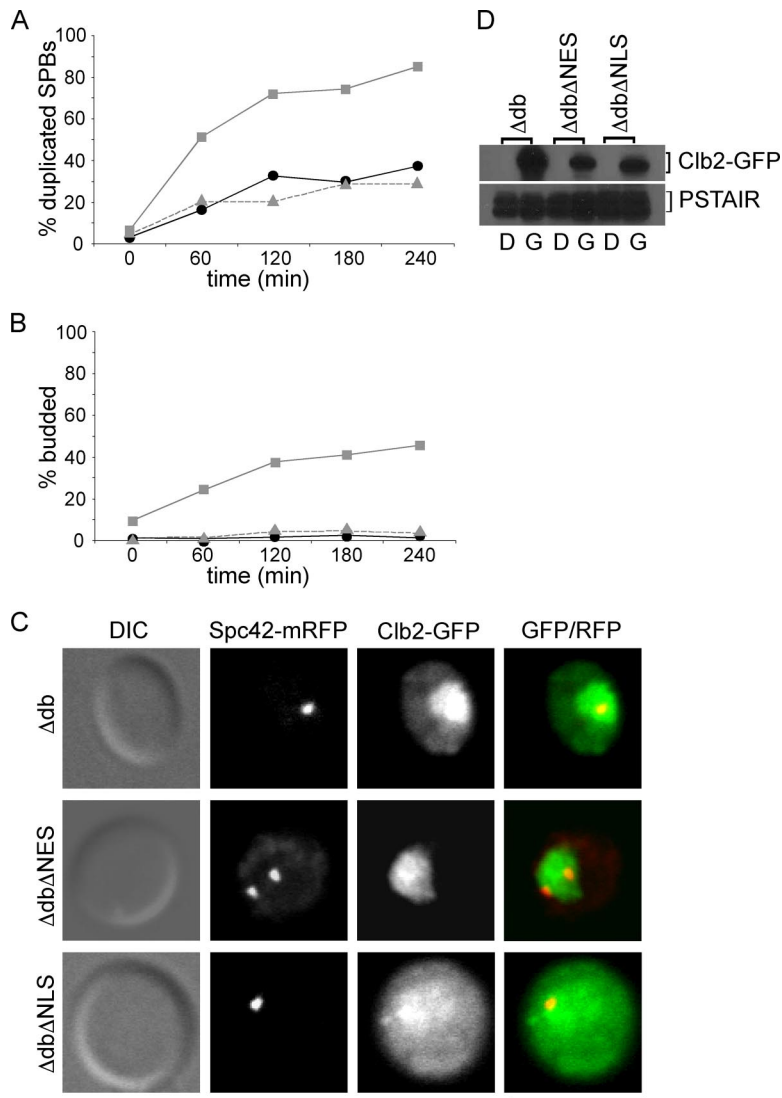


Figure 6. Clb2 must be cytoplasmic in order to inhibit SPB duplication. (A–C) Asynchronous populations of cells were induced to express GFP tagged Clb2Δdb (●, black), Clb2ΔdbΔNES (■, gray), or Clb2ΔdbΔNLS (▲, gray) from the *GAL1* promoter, and small daughter cells were then collected by centrifugal elutriation. SPB duplication (A) and bud emergence (B) were monitored over time by fluorescence microscopy. (C) Representative images of cells at 120 min after elutriation. In merged image, GFP is green, mRFP is red, and colocalization is yellow. Bar, 50 μ m. (D) Western blot showing levels of Clb2 constructs in asynchronous cells grown in either dextrose “D” or galactose “G”. Cdk1 levels (α -PSTAIR) are shown as a loading control.

and SPB components are expressed (Jaspersen and Winey, 2004; Orlando *et al.*, 2008).

Similar centrosome-intrinsic mechanisms have been proposed for preventing centrosome reduplication in metazoans. Like side-by-side SPBs that are connected by a full bridge, engaged centrioles cannot initiate a new round of centriole duplication (Tsou and Stearns, 2006). Centriole disengagement is essential for a new round of centriole duplication and appears to be regulated by separase (Tsou and Stearns, 2006). Despite the similarity in mechanism, it is unlikely that separase plays a role in SPB separation, as separase mutants (*esp1*) do not exhibit a defect in SPB separation (Baum *et al.*, 1988). Our work and the work of others (Fitch *et al.*, 1992; Crasta *et al.*, 2006) suggest that B-cyclin/CDKs trigger SPB separation, although the mechanisms have yet to be elucidated.

Mutations in the bridge protein Sfi1 have been found that block SPB separation (Anderson *et al.*, 2007). Both of these mutations fall in a consensus CDK phosphorylation site and Sfi1 has been shown to be highly phosphorylated by Clb2/Cdk1 (Ubersax *et al.*, 2003; Anderson *et al.*, 2007). Additionally, these mutations are located in the C-terminus of Sfi1, where Sfi1 is proposed to bind the C-termini of other Sfi1

molecules forming the structure of the bridge (Li *et al.*, 2006). Thus, it is possible that phosphorylation of Sfi1 by mitotic cyclin/CDKs may contribute to SPB separation by destabilization of the bridge.

A previous study demonstrated that extrinsic control by mitotic cyclin/CDK also prevents SPB reduplication (Haase *et al.*, 2001); however, the mechanisms by which mitotic cyclins prevent SPB reduplication remained unexplored. By expressing a stabilized version of the mitotic B-cyclin (Clb2Δdb) in at different points in G1, we established that mitotic cyclin/CDKs are likely to prevent reduplication by inhibiting early events of the SPB duplication cycle (Figures 1, 3, and 5). The fact that Clb2 cannot inhibit SPB duplication after α -factor arrest suggests that SPBs are already “licensed” to duplicate at START. Previous studies dissecting SPB structure during the duplication cycle have shown that as cells transit from early G1 to START, the SPB half-bridge elongates, and a satellite is assembled at the distal end of the bridge (Byers and Goetsch, 1974, 1975). Thus mitotic cyclin/CDKs may inhibit bridge elongation or satellite assembly. Indeed, when we examined the SPB structure by EM in cells that expressed Clb2Δdb in early G1, we did not observe satellites in any of the cells analyzed, although we could not

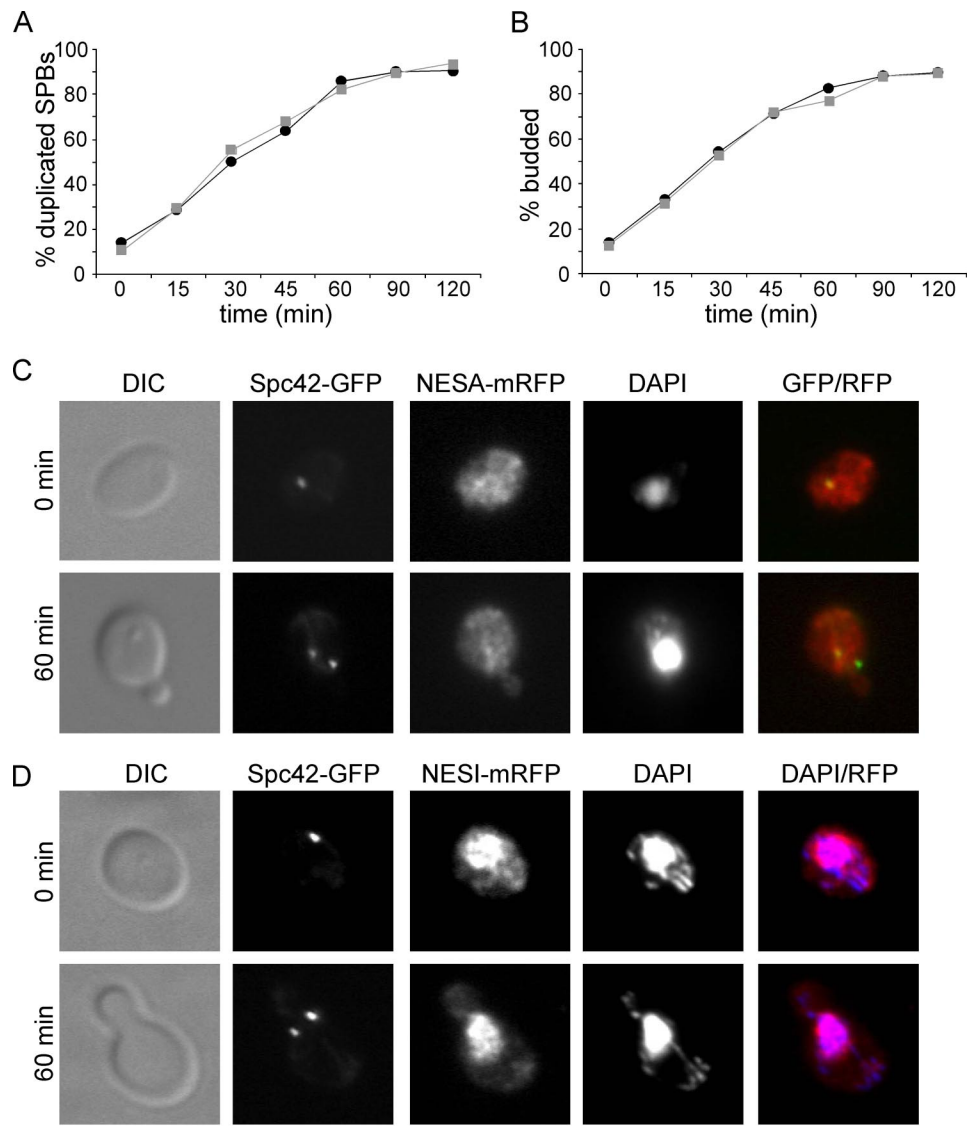


Figure 7. Cytoplasmic Clb5 Δ db cannot inhibit SPB duplication. Cells were induced to express Clb5 Δ db-NESA-mRFP (active NES; ■, gray) or Clb5 Δ db-NESI-mRFP (inactive NES; ●, black) from the *GAL1* promoter and small daughter cells were collected by centrifugal elutriation. SPB (A) duplication and bud emergence (B) were monitored over time by fluorescence microscopy. (C) Representative images showing Clb5 Δ db-NESA-mRFP colocalization with Spc42-GFP as well as SPB duplication at 0 and 60 min after elutriation. In merged image GFP is red, mRFP is green, and colocalization is yellow. (D) Representative images showing Clb5 Δ db-NESI-mRFP colocalization with DNA as well as SPB duplication at 0 and 60 min after elutriation. In merged image GFP is green, DAPI is blue and colocalization is magenta. Bar, 50 μ m.

determine unambiguously if bridge elongation was inhibited. Taken together, these findings argue that the satellite-bearing SPB may be analogous to the preduplicative complex in DNA licensing models as previously hypothesized (Adams and Kilmartin, 1999; Haase *et al.*, 2001) and that one or more steps leading up to satellite assembly are inhibited by mitotic cyclin/CDKs.

Mitotic cyclin/CDKs could inhibit satellite assembly indirectly or by direct phosphorylation of satellite or half-bridge proteins. Interestingly, previous studies have shown that mitotic cyclins Clb2 (Hood *et al.*, 2001; Bailly *et al.*, 2003) and Clb4 (Maekawa and Schiebel, 2004) colocalize with the SPB, suggesting that direct phosphorylation of satellite or half-bridge proteins could occur. The satellite assembles on the cytoplasmic face of the SPB (Adams and Kilmartin, 1999), suggesting that the subcellular localization of Clb2 may be important for its ability to inhibit SPB licensing. Our finding that Clb2 must be localized to the cytoplasm in order to inhibit licensing suggests that the inhibitory targets of Clb2 may indeed be associated with the half-bridge and/or satellite.

Although Clb2 can inhibit SPB duplication if expressed in early G1, neither Clb5 nor Cln2 can inhibit SPB duplication

when expressed similarly, indicating that inhibition of SPB duplication is specific to Clb2 and not a function of elevated CDK activity in early G1 (Figures 3 and 4). Furthermore, we have shown that cytoplasmic localization of Clb5 is not sufficient for inhibiting SPB duplication (Figure 7), suggesting that the targets of Clb2/Cdk1 involved in inhibition of SPB duplication are not efficiently phosphorylated by Clb5/Cdk1. This finding is consistent with *in vitro* studies indicating that Clb5/Cdk1 may have preferences for specific substrates, whereas Clb2/Cdk1 can phosphorylate a wider range of targets (Loog and Morgan, 2005).

The results presented here suggest that at least two distinct mechanisms work together to prevent the reduplication of SPBs during the cell cycle. In early G1, when there is low mitotic cyclin/CDK activity, the preduplicative structure, the satellite bearing SPB, is allowed to assemble. After START, the activity of cyclin/CDK complexes (G1 cyclin/CDK under normal circumstances, but mitotic cyclin/CDK will suffice; Figure 5B) triggers the assembly of a new SPB, resulting in a structure with two side-by-side SPBs connected by a full bridge. This structure establishes a SPB-intrinsic block to SPB reduplication during late G1 and early S-phase when the components of the SPB are expressed

(Orlando *et al.*, 2008) and the proper activating kinases are present (Jaspersen *et al.*, 2004). SPB separation is then triggered by the activation of mitotic B-cyclins (Fitch *et al.*, 1992; Crasta *et al.*, 2006) or inefficiently by S-phase cyclins (Haase *et al.*, 2001), releasing the SPB-intrinsic block to SPB reduplication. After promoting SPB separation, mitotic cyclin/CDKs inhibit SPB reduplication for the remainder of the cycle by preventing the premature assembly of the pre-duplicative SPB complex. At the end of mitosis, the inhibition and destruction of mitotic cyclins releases this inhibition, and cells are able to reassemble the preduplicative SPB complex in the early G1.

As has been shown for DNA replication, we suspect that mitotic cyclin/CDKs phosphorylate multiple protein targets and that any of those phosphorylations could block SPB reduplication. However, our findings here suggest that the important targets are localized to the cytoplasm and at least a subset of these proteins may be structural components of the satellite or bridge structure. In a large-scale screen for CDK targets, three of the four known components of the bridge or satellite tested were found to be phosphorylated by Clb2/Cdk1 (Ubersax *et al.*, 2003), suggesting that many bridge and satellite proteins may, in fact, be regulated by CDK activity. One of these proteins, Spc42, a protein that has been shown to localize to the satellite (Adams and Kilmartin, 1999), is known to be phosphorylated by CDK (Donaldson and Kilmartin, 1996; Ubersax *et al.*, 2003; Jaspersen *et al.*, 2004). Cln1,2/Cdk1 phosphorylations have been mapped to two of the eight minimal CDK consensus sites within the Spc42. These phosphorylations likely contribute to SPB assembly (Jaspersen *et al.*, 2004); however, it remains possible that phosphorylation of additional residues in Spc42 by B-cyclin/CDK could be important in the inhibition of SPB reduplication later in the cell cycle.

It remains unclear whether centrosome duplication in metazoans is governed by the same mechanisms we have identified in yeast, but remarkable similarities between the duplication cycles (reviewed in Adams and Kilmartin, 2000) suggest that analogous regulatory themes may exist. Centrosome-intrinsic mechanisms clearly prevent centrosome reduplication, and centriole disengagement may be an analogous process to SPB separation. However, centriole disengagement in metazoans appears to be regulated by separase (Tsou and Stearns, 2006), whereas in budding yeast, separation is regulated by B-cyclin/CDKs (Fitch *et al.*, 1992; Crasta *et al.*, 2006). Furthermore, centriole disengagement does not occur until mitosis, so the centrosome-intrinsic mechanism prevents reduplication for the bulk of the cell cycle. The question then arises, are additional mechanisms required to prevent centrosome reduplication during the interval between centriole disengagement in mitosis and cytokinesis, and if so, do CDKs inhibit the assembly of a preduplicative centrosome complex, perhaps by inhibiting procentriole assembly? Although several studies have suggested that cyclin B/Cdk1 inhibits centrosome duplication (Hinchcliffe *et al.*, 1998; Lacey *et al.*, 1999; Vidwans *et al.*, 1999, 2003), it is not clear whether cyclin B/Cdk1 inhibits centrosome duplication directly, if it inhibits separase activation (Stemmann *et al.*, 2001), or if it inhibits progression into cell cycle phases permissive for centrosome duplication.

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