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TORC1 kinase and the S-phase cyclin Clb5 collaborate to promote mitotic spindle assembly and DNA replication in *S. cerevisiae*

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Abstract

The Target of Rapamycin complex 1 (TORC1) is a central regulator of eukaryotic cell growth that is inhibited by the drug rapamycin. In the budding yeast *Saccharomyces cerevisiae*, translational defects associated with TORC1 inactivation inhibit cell cycle progression at an early stage in G1, but little is known about the possible roles for TORC1 later in the cell cycle. We investigated the rapamycin-hypersensitivity phenotype of cells lacking the S phase cyclin Clb5 (*clb5Δ*) as a basis for uncovering novel connections between TORC1 and the cell cycle regulatory machinery. Dosage suppression experiments suggested that the *clb5Δ* rapamycin hypersensitivity reflects a unique Clb5-associated cyclin-dependent kinase (CDK) function that cannot be performed by mitotic cyclins and that also involves motor proteins, particularly the kinesin-like protein Kip3. Synchronized cell experiments revealed rapamycin-induced defects in pre-anaphase spindle assembly and S phase progression that were more severe in *clb5Δ* than in wild type cells but no apparent activation of Rad53-dependent checkpoint pathways. Some rapamycin-treated cells had aberrant spindle morphologies, but rapamycin did not cause gross defects in the microtubule cytoskeleton. We propose a model in which TORC1 and Clb5/CDK act coordinately to promote both spindle assembly via a pathway involving Kip3 and S phase progression.

Keywords

rapamycin; Kip3; Clb5; cell cycle; S phase; microtubules

Introduction

The core cell division cycle of the budding yeast *Saccharomyces cerevisiae* is regulated by a single cyclin-dependent kinase (CDK), Cdc28, which associates with nine different cyclin partners at various times during the cycle (Mendenhall and Hodge 1998). Although the nine cyclins fall into three general categories—the G1, S phase, and mitotic cyclins—their activities are characterized both by subtle functional differences and robust functional overlap. For example, either of the S phase cyclins, Clb5 and Clb6, can activate timely entry into S phase, but only Clb5 can support efficient progression through S phase and completion of DNA replication (Epstein and Cross 1992; Kuhne and Linder 1993; Schwob

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and Nasmyth 1993). Given this partial redundancy in cyclin function, uncovering phenotypes that are specifically associated with the elimination of a particular cyclin is of special interest.

We observed that cells that lack the S phase cyclin Clb5 (*clb5Δ*) are hypersensitive to the drug rapamycin and that this phenotype is not associated with deletion of any other Cdc28-associated cyclin. This same result was also noted in a large-scale screen of the entire yeast gene deletion collection (Chan et al. 2000). Rapamycin is a bacterially derived compound that inhibits the growth of all eukaryotic cells by targeting a conserved, central cell growth regulatory protein, a serine/threonine protein kinase called TOR (target of rapamycin) (Crespo and Hall 2002). Rapamycin first binds to the immunophilin FKBP12 (Fpr1 in yeast), then the drug-immunophilin complex binds to and inhibits TOR. Budding yeast possess two TOR-encoding genes, *TOR1* and *TOR2*. The rapamycin-inhibited TOR functions are carried out redundantly by a complex referred to as TORC1, which consists of either Tor1 or Tor2 along with several other proteins. The functions of TORC1 include promotion of translation initiation and ribosome and tRNA biogenesis, as well as inhibition of processes that respond to nutrient limitation (Wullschlegel et al. 2006).

The translation defect that results from rapamycin-mediated inhibition of TORC1 arrests yeast cells in early G1, prior to the α -factor-mediated arrest point referred to as “Start” (Barbet et al. 1996). Two recent studies described unique post-Start functions for TORC1: 1) an S-phase cytoprotective role in response to chemical-induced DNA damage that also requires the Rad53-dependent DNA damage checkpoint machinery (Shen et al. 2007) and 2) an additional role in promoting timely mitotic entry via a pathway involving the protein kinase Cdc5 (Nakashima et al. 2008). The rapamycin hypersensitivity of *clb5Δ* cells could reflect a functional connection between Clb5 and one of these two pathways or, alternatively, a novel post-G1 TORC1 function that impinges upon the core cell cycle regulatory machinery.

In addition to its role in triggering late replication origin firing (Donaldson et al. 1998; Mendenhall and Hodge 1998), Clb5 is also important for mitotic spindle assembly and positioning (Segal et al. 2000; Segal et al. 1998). Deletion of *KIP2* or *CIN8*, genes that encode kinesin-like motor proteins involved in spindle assembly, also causes rapamycin hypersensitivity. Furthermore, rapamycin treatment of wild type cells was reported to cause defects in nuclear and mitotic spindle positioning that led to the accumulation of binucleate cells (Choi et al. 2000). Thus, the mitotic spindle seemed to be a plausible point of functional intersection between Clb5 and TORC1. We present results that implicate TORC1 in the process of pre-anaphase spindle assembly—an S phase event that follows the spindle pole body (SPB) duplication that occurs in early G1 (Lew et al. 1997). This function appears to be coordinately promoted by Clb5 and to involve the kinesin-like motor protein Kip3, likely through a mechanism that involves CDK-mediated Kip3 phosphorylation. Rapamycin-mediated TORC1 inhibition also exacerbates the slowed DNA replication associated with *CLB5* deletion but does not significantly affect replication timing in wild type cells and does not appear to activate the Rad53-dependent DNA replication or DNA damage checkpoints. Together, these results implicate TORC1 in novel post-Start cell cycle functions that are carried out in conjunction with Clb5/CDK.

Materials and methods

Chemical and biological reagents

Unless otherwise mentioned, all chemical and biological reagents were from Sigma-Aldrich (St. Louis, MO).

Yeast strains and growth conditions

The yeast strains used in this study are listed in Table 1. Single-gene deletion strains are from the complete *S. cerevisiae* gene knockout library (Giaever et al. 2002), which was purchased from Invitrogen Corp. (Carlsbad, CA). Yeast cells were grown according to standard procedures in yeast peptone dextrose (YPD) medium or in synthetic dextrose medium lacking uracil (SD-ura) (Sherman 1991). Plasmids were introduced using a standard lithium acetate transformation procedure (Gietz and Woods 2002). The JHY448 and JHY449 strains used for α -factor synchronization experiments (wild type and *clb5* Δ strains in which the *BAR1* gene is deleted) were generated by integrating *EcoRI/SalI*-digested pJGSST1 (Reneke et al. 1988) into BY4741 and JHY180, respectively, then selecting *ura3* auxotrophs on medium containing 5-fluoroorotic acid (Boeke et al. 1987). JHY437 was generated by replacing the *kanMX4* cassette of a *MATa clb5* Δ ::*kanMX4* strain (Giaever et al. 2002) with a *natMX4* cassette by homologous recombination (Tong et al. 2001); this strain was crossed to a *MATa tor1* Δ ::*kanMX4* strain (Giaever et al. 2002) and the diploid was sporulated to generate JHY444 (*clb5* Δ ::*natMX4 tor1* Δ ::*kanMX4*). *kanMX4*- and *natMX4*-positive strains were identified by their ability to grow on YPD plates containing genitcin (200 mg/L) or nourseothricin (100 mg/L; Werner BioAgents, Jena, Germany), respectively.

Plasmid construction

Plasmids used in this study are listed in Table 2. Plasmid pJH272 contains a 2.8 kb genomic *CLB2 EcoRI/XhoI* fragment cloned into the corresponding sites of pRS426 (Sikorski and Hieter 1989). Plasmids pJH299 and pJH298 contain a 2.4 kb genomic *CLB6 BamHI/EcoRI* fragment cloned into the corresponding sites of pRS313 and pRS423 (Sikorski and Hieter 1989), respectively. pJH426 contains *CLB5::CLB2* from *pRS314-CLB5::CLB2* (a gift from Fred Cross, Rockefeller University) swapped into pRS426 (Sikorski and Hieter 1989) via homologous recombination in yeast. The untagged *KIP3(T254A)* allele (pJH379) was created by site-directed mutagenesis of pEH389 using the QuikChange kit (Stratagene/Agilent Technologies, Cedar Creek, TX) with primers JHD140 (5'-CCTTTTGAAACCAGAGGCACCATCTAAAAGATTGG-3') and JHD141 (5'-CCAATCTTTTAGATGGTGCCTCTGGTTTCAAAAAGG-3') (nucleotide changes from the wild type sequence are underlined). The 6-myc-tagged *KIP3(T254A)* allele was created in a similar manner by site-directed mutagenesis of pB956. All mutant constructs were confirmed by DNA sequencing.

Cell dilution assays

Yeast strains were grown overnight in YPD or appropriate synthetic dropout media to select for plasmid retention. Five 1:10 serial dilutions of the cells in sterile water were performed in 96-well microplates, starting with suspensions of 5×10^5 cells/ μ l. Two-microliter volumes of the cell suspensions were spotted onto YPD or YPD supplemented with various concentrations of rapamycin (A.G. Scientific, San Diego, CA). Plates were incubated at 30°C for 3–6 days and photographed using a Kodak Gel Logic 200 imaging system.

Yeast lysate preparation and western blotting

For Clb5-HA and Kip3-myc detection, yeast lysates were prepared as previously described (Hood and Silver 1998). For Rad53 detection, cells harvested from 10 ml of early log-phase culture were resuspended in 2 ml 20% trichloroacetic acid (TCA) and pelleted by centrifugation. The pellets were resuspended in 50 μ l of 20% TCA; approximately 100 μ l of glass beads were added, and cells were lysed by vortexing for 2 min at room temperature. Lysed samples were centrifuged 10 min at 3000 rpm in a microcentrifuge, and the supernatants were removed. Precipitated proteins were resuspended in 100 μ l 1X protein

sample buffer (1% sodium dodecyl sulfate; 5% glycerol; 50 mM Tris, pH 6.8; 100 mM dithiothreitol; 0.1% bromophenol blue), and 35 μ l 1M Tris base was added to return the pH to neutral and the dye color to blue; if additional base was needed to cause the color change, an appropriate amount of 5X sample buffer was also added to maintain the starting concentration. Tris-HCl Criterion gels (BioRad, Hercules, CA) were used for SDS-PAGE. Mouse monoclonal anti-HA and anti-myc primary antibodies were from Roche Diagnostics Corp. (Indianapolis, IN) and Sigma, respectively; rabbit polyclonal anti-PSTAIRE, goat polyclonal anti-Rad53, and rabbit polyclonal anti-Clb2 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Rad53 antibodies were used at 1:250 dilution; all other primary antibodies were used at 1:1000 dilution. HRP-conjugated anti-mouse, anti-rabbit, and anti-goat secondary antibodies were from Jackson ImmunoResearch (West Grove, PA) and were diluted 1:5000. Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ) were used for detection. ImageJ software (<http://rsbweb.nih.gov/ij/>) was used to quantify band intensities.

Assessing cell viability after short exposure to rapamycin

To assess the cytotoxic vs. cytostatic nature of short-term rapamycin exposure, early-log-phase cultures grown at 30°C in either YPD (untransformed BY4741 and JHY180 strains) or SD-ura medium (JHY180 transformed with pRS426, pFC706, pEH389, or pJH379) were treated with 50 ng/ml rapamycin or left untreated for 4 h. After treatment, cells were pelleted and washed once with drug-free medium. The washed cells were diluted to an OD₆₀₀ of 5×10^{-4} , and equal volumes of each diluted sample were plated on each of three YPD (for untransformed strains) or SD-ura (for transformed strains) plates. In all cases, colonies appeared more slowly for the rapamycin-treated samples than for the controls, so colonies were counted after 3 days of incubation at 30°C for the controls and 6 days for the drug-treated samples. Colony formation for the rapamycin-treated samples relative to the untreated controls was calculated as the ratio of the average number of colonies on the three plates (treated/untreated) and expressed as a percentage. Statistical analyses were performed using JMP 7 software (SAS Institute, Inc., Cary, NC).

Synchronized cell assays

Alpha-factor from a 1×10^{-3} M stock in 0.1 N HCl was added to early-log-phase JHY448 and JHY449 cells to a final concentration of 1.5×10^{-8} M, and the cultures were incubated 2.5 h at 30°C to allow cell cycle arrest in G1. Arrested cells were harvested and washed twice in YPD containing 0.1 mg/ml Pronase E (protease from *S. griseus*) to eliminate the pheromone. Washed cells were resuspended in YPD $-/+$ rapamycin and incubated at 30°C for the duration of all time-course assays.

Anti-tubulin immunostaining and microscopy

For anti-tubulin immunostaining, cells from 1-ml culture aliquots were fixed in 3.7% formaldehyde/0.1 M potassium phosphate buffer (KPi), pH 6.4, at 4°C overnight or 1 h at room temperature. Fixed cells were washed three times in 1 ml KPi, resuspended in 1 ml sorbitol-citrate (1.2 M sorbitol/36 mM citric acid/10 mM K₂HPO₄), and stored at -20°C . Cells were prepared for immunostaining by 1 h treatment in digestion mix (200 μ l sorbitol-citrate/20 μ l glucylase (Perkin Elmer Life Sciences, Boston, MA)/10 μ l 10 mg/ml zymolyase (U.S. Biological, Swampscott, MA)) at 30°C with rocking. After digestion, cells were pelleted at 3,000 rpm in a microcentrifuge, washed with 1 ml sorbitol-citrate, then resuspended in an appropriate volume of sorbitol-citrate. Cells were applied to microscope slide wells pre-treated with 0.1% polylysine; excess cells were aspirated after 10 min. Cells were permeabilized by placing slides in ice-cold methanol for 3 min followed by ice-cold acetone for 10 sec. Immunostaining was performed at room temperature in a humid chamber using rat anti-tubulin antibodies (AbD Serotec, Raleigh, NC) followed by FITC-conjugated

goat anti-rat secondary antibodies (Jackson Immunoresearch, West Grove, PA), both diluted 1:100 from manufacturer stocks. Both antibody incubations were 1 h in duration and were followed by five washes in PBS/BSA (1% BSA/40 mM K₂HPO₄/10 mM KH₂PO₄/150 mM NaCl/0.1% NaN₃). To stain nuclei, cells were treated with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min, then washed three times with PBS/BSA. Mounting medium (9 parts glycerol/1 part 1% p-phenylenediamine in PBS with pH adjusted to > 8.0 with 0.5 M sodium carbonate, pH 9.0) was added to all wells before sealing the slides. Stained cells were examined on a Nikon Eclipse 80i fluorescence microscope, and images were acquired with a QImaging Retiga 2000R digital camera and Nikon NIS-Elements AR 2.3 software. For analysis of cell cycle stages, approximately 100 DAPI and anti-tubulin-stained cells were scored for each sample at each time-point using the following categories: unbudded, budded with undivided nucleus, and budded with dividing or divided nucleus; unduplicated spindle pole body, duplicated spindle pole bodies (side-by-side); short spindle (pre-anaphase), and long spindle (anaphase).

Flow cytometry

One-milliliter yeast culture aliquots were harvested by centrifugation. Cells were resuspended in 1 ml 70% ice-cold ethanol and fixed overnight at 4°C. Fixed cells were washed in 1 ml 50 mM Tris, pH 7.5, incubated overnight in 0.5 ml 20 mg/ml RNaseA at 37°C with rocking, incubated 30 min in 0.5 ml 5 mg/ml pepsin/55 mM HCl, washed in 1 ml FACS buffer (200 mM Tris, pH 7.5/211 mM NaCl/78 mM MgCl₂), and resuspended in 0.5 ml 1 mg/ml propidium iodide. Stained cells were analyzed for DNA content using a FACS Calibur flow cytometer (BD Biosciences), and data were processed using FlowJo software (Tree Star, Inc., Ashland, OR).

Results

Genetic results implicate TORC1 and Clb5 in a common process

A high-throughput screen of the entire yeast deletion collection identified *clb5Δ* cells as rapamycin hypersensitive (Chan et al. 2000). We also discovered this phenotype through an independent line of investigation. Using a cell dilution assay, we confirmed that the *clb5Δ* rapamycin hypersensitivity was unique within the set of strains bearing deletions in the nine *CLB* and *CLN* cyclin genes (Fig. 1A). We also verified that expression of dominant, rapamycin-insensitive mutant TOR (*TOR2(S1975I)* or *TOR2(S1975R)*) (Lorenz and Heitman 1995) or deletion of *FPR1* eliminated the rapamycin hypersensitivity of *clb5Δ* cells, thus confirming that this phenotype was not due to some unexpected, off-pathway effect of the drug (data not shown).

The rapamycin hypersensitivity of *clb5Δ* cells suggested that Clb5 and the rapamycin-insensitive TOR complex, TORC1, might impinge on a shared cellular function. As an initial test of this hypothesis, we constructed a *clb5Δtor1Δ* double mutant strain and assessed its rapamycin sensitivity. The *clb5Δtor1Δ* cells were significantly more sensitive to the drug than were cells bearing either single deletion (Fig. 1B), further supporting the idea that TORC1 and Clb5 influence a common cellular process.

High-copy *CLB6* but not *CLB2* can suppress *clb5Δ* rapamycin hypersensitivity

To learn more about the specific nature of the *clb5Δ* rapamycin hypersensitivity, we asked whether over-expression of other B-type cyclins might suppress this phenotype. We first considered the other S phase cyclin, Clb6. The amino acid sequence of Clb6 is approximately 50% identical to that of Clb5, and both proteins accumulate with similar kinetics in late G1 (Jackson et al. 2006; Schwob and Nasmyth 1993). Both Clb5 and Clb6 can activate entry into S phase, but only Clb5 can support timely progression and

completion of DNA replication (Epstein and Cross 1992; Schwob and Nasmyth 1993). This functional distinction is due to differences in the timing of degradation of these cyclins: whereas Clb6 is degraded at the G1/S transition, Clb5 persists until cells enter mitosis (Jackson et al. 2006). Slightly increasing the dosage of *CLB6* by transforming *clb5Δ* cells with a centromeric *CLB6* plasmid barely improved the growth of *clb5Δ* cells on rapamycin-containing medium, but *clb5Δ* cells overexpressing Clb6 from a high-copy (2μ) plasmid grew quite well—in fact better than those in which *CLB5* was restored on a centromeric plasmid (Fig. 2A). This suggests that Clb6/Cdc28 can perform the TOR-related function responsible for *clb5Δ* rapamycin hypersensitivity, but that it is not normally present in sufficient quantities or at the right time to do so.

In contrast to Clb6, the mitotic cyclin Clb2 had little effect on the rapamycin hypersensitivity of *clb5Δ* cells, even when expressed from a high-copy plasmid (Fig. 2A). Clb2 begins to accumulate during late S phase and peaks in abundance at the G2/M transition (Grandin and Reed 1993). In addition, the CDK inhibitory kinase Swe1 specifically targets Clb2-associated Cdc28 for inhibitory phosphorylation with high affinity prior to the G2/M transition (Hu and Aparicio 2005). Clb2 expressed under the control of the *CLB5* promoter (*CLB5::CLB2*) accumulates with timing that mirrors that of Clb5 (Cross et al. 1999), but this early-expressed Clb2 can only trigger timely DNA replication when *SWE1* is deleted (Hu and Aparicio 2005). Therefore, we tested the ability of low- or high-copy *CLB5::CLB2* to suppress the *clb5Δ* rapamycin hypersensitivity either in *SWE1* or *swe1Δ* cells (Fig. 2B and C). Surprisingly, unlike what was observed for DNA replication (Hu and Aparicio 2005), neither the *CEN* nor the 2μ *CLB5::CLB2* plasmid was able to suppress the *clb5Δ* rapamycin hypersensitivity in either the *SWE1* or the *swe1Δ* cells (Fig. 2B and C). In addition, neither *CLB3* nor *CLB4* expressed from 2μ plasmids suppressed the *clb5Δ* rapamycin hypersensitivity to any significant degree (data not shown). Clb3 and Clb4 are mitotic cyclins that accumulate earlier in S phase than Clb2 and form complexes with Cdc28 that are only slightly inhibited by Swe1 (Grandin and Reed 1993; Hu and Aparicio 2005). These results imply that the inability of the mitotic cyclins to perform the rapamycin-sensitive Clb5 function is not merely due to differences in their expression timing but rather to inherent functional differences.

The Clb5 hydrophobic patch is important for the rapamycin-sensitive Clb5 function

B-type cyclins contain a “hydrophobic patch” that has been implicated in binding directly to substrates to confer phosphorylation specificity (Archambault et al. 2005; Schulman et al. 1998). The hydrophobic patch has been shown to be particularly important for targeting Clb5 to its specific substrate proteins (Loog and Morgan 2005; Wilmes et al. 2004). A version of Clb5 in which this hydrophobic patch is mutated (*clb5^{hpm}*) (Cross et al. 1999) was unable to restore the growth of *clb5Δ* cells on rapamycin-containing medium to the level achieved by plasmid-based expression of wild type Clb5, even though the two proteins were expressed to equal abundance (Fig. 2D and E). These results suggested the possibility that the rapamycin hypersensitivity of *clb5Δ* cells was caused by diminished phosphorylation of some substrate(s) preferentially phosphorylated by Clb5/Cdc28 in a hydrophobic patch-dependent manner. Increasing the expression of such proteins might allow them to be phosphorylated by a cyclin-CDK complex with lower inherent affinity for those substrates—e.g. Clb6/Cdc28. Following this line of reasoning, we sought to identify additional genes that could suppress *clb5Δ* rapamycin hypersensitivity when present in high copy number.

Over-expression of the kinesin-like protein Kip3 suppresses *clb5Δ* rapamycin hypersensitivity

Inhibition of TORC1 by rapamycin has previously been reported to affect nuclear positioning; furthermore, deletion of genes that encode some of the motor proteins involved in mitotic spindle positioning causes rapamycin hypersensitivity (Choi et al. 2000). Because Clb5 is also known to affect nuclear positioning (Segal et al. 2000; Segal et al. 1998), we hypothesized that the rapamycin hypersensitivity of *clb5Δ* cells might be related to a mitotic spindle defect. Thus, we asked whether over-expression of motor protein genes from high-copy plasmids might suppress *clb5Δ* rapamycin hypersensitivity. We focused on the five spindle-related yeast kinesin-like motor protein genes: *KIP1-3*, *CIN8*, and *KAR3* (Hildebrandt and Hoyt 2000). *KIP3* stood out in its ability to confer improved growth on medium containing up to 50 ng/ml rapamycin when expressed from a 2 μ plasmid (Fig. 3A). *KIP2* and *KAR3* also slightly improved the growth of *clb5Δ* cells in the presence of lower concentrations of rapamycin (e.g. 10 ng/ml) in some experiments, but the effect was weaker than for *KIP3* and less reproducible (Fig. 3A and data not shown). *KIP1* and *CIN8* had no apparent effect on *clb5Δ* rapamycin hypersensitivity, as was also the case for other spindle-related genes we tested, including *ASE1*, *BIK1*, and *SLK19* (Pellman et al. 1995; Zeng et al. 1999) (Fig. 3A and data not shown).

All CDKs are proline-directed serine and threonine kinases, and they typically phosphorylate residues contained within the consensus motif S/T-P-X-K/R, where X is any amino acid (Brown et al. 1999; Nigg 1993). Kip3 contains a single such motif composed of amino acids 254–257 of the protein (TPSK), which falls within its motor domain, (*Saccharomyces* Genome Database, <http://www.yeastgenome.org/>, accessed March 1, 2009; Hildebrandt and Hoyt 2000). If Kip3 is normally phosphorylated most efficiently by Clb5/Cdc28, increasing its expression level might allow it to be more efficiently phosphorylated by another Clb/Cdc28 complex; as described above, such a scenario might suppress the rapamycin hypersensitivity of *clb5Δ* cells. To test whether phosphorylation of the threonine in the Kip3 CDK phosphorylation motif might be required for the observed dosage suppression, we mutated it to alanine and tested the mutant allele, *KIP3(T254A)*, in the cell dilution assay. *KIP3(T254A)* expressed from a 2 μ plasmid was not able to suppress the *clb5Δ* rapamycin hypersensitivity; in fact it caused the cells to grow even more poorly than the vector control on rapamycin-containing medium (Fig. 3B). However, over-expression of *KIP3(T254A)* did not affect the growth of *clb5Δ* cells in the absence of rapamycin (Fig. 3B). In addition, neither *KIP3* nor *KIP3(T254A)* affected the rapamycin sensitivity of wild type cells when over-expressed, even when the concentration of rapamycin was increased to 200 ng/ml (Fig. 3B and data not shown).

We considered the possibility that the different behavior of *KIP3* and *KIP3(T254A)* in the *clb5Δ* rapamycin hypersensitivity suppression assay might be due to different expression levels of the two proteins. Western blotting of lysates from cycling *clb5Δ* cells expressing 6x-myc epitope-tagged versions of Kip3 and Kip3(T254A) indicated that the mutant protein was approximately two-to-three-fold more abundant than the wild type protein as determined by quantification of band intensities (Fig. 3C). It is therefore possible that the T254A mutation affects Kip3 stability. In addition, the Kip3(T254A) protein consistently appeared to migrate slightly faster in the gel than the wild type protein (Fig. 3C). This is consistent with the hypothesis that the T254A mutation eliminates a phosphorylation site.

Transient rapamycin exposure reduces viability

Rapamycin typically has a cytostatic rather than a cytotoxic effect on yeast, but certain rapamycin-sensitive mutants do exhibit rapamycin cytotoxicity (Zinzalla et al. 2007). To assess the cytotoxic vs. cytostatic nature of *clb5Δ* rapamycin hypersensitivity, we treated

log-phase wild type or *clb5Δ* cells with 50 ng/ml rapamycin for four hours then washed the cells in drug-free medium and plated equal numbers of treated and untreated cells on YPD to assess their ability to form colonies. During the four-hour incubation, both wild type and *clb5Δ* cultures exhibited a modest accumulation of cells with G1 DNA content, but significant G2 populations remained at the end of the treatment (Fig. 4A). This is consistent with the results of Nakashima et al. (2008), who found that the hallmark rapamycin-induced G1 arrest was incomplete until some time between six and 20 hours of treatment with the drug. In contrast to previously published results (Zinzalla et al. 2007), we observed decreased colony formation for the rapamycin-treated wild type cells to about 55% of that of the untreated control (Fig. 4B, WT). This effect was more severe for *clb5Δ* cells, with the drug-treated *clb5Δ* culture exhibiting only about 25% of the colony forming potential of the untreated control (Fig. 4B, *clb5Δ*). Thus, over a period corresponding to between two and three cell doubling times, rapamycin had a cytotoxic effect on some cells in both wild type and *clb5Δ* populations, but approximately twice as many *clb5Δ* cells were susceptible to the cytotoxicity.

***KIP3(T254A)* diminishes the viability of rapamycin-treated *clb5Δ* cells**

To further document the opposing effects of *KIP3* and *KIP3(T254)* observed in the cell dilution assay (Fig. 3A and B), we subjected *clb5Δ* cells transformed with *KIP3* or *KIP3(T254A)* over-expression plasmids to the post-rapamycin colony formation assay, comparing them to *clb5Δ* transformed with either an empty vector or a *CEN CLB5* plasmid. Importantly, the vector- and *CLB5* plasmid-transformed cells behaved similarly their *clb5Δ* and wild type counterparts in assay, despite the fact that the plasmid-transformed cells were grown in synthetic media and the untransformed cells were grown in YPD (Fig. 4B; compare *clb5Δ* and WT with *clb5Δ*+vec and *clb5Δ*+*CLB5*, respectively). Over-expression of *KIP3(T254A)* caused a significant reduction in the colony forming potential of rapamycin-treated *clb5Δ* cells compared to both the *CLB5* and *KIP3*-transformed samples (Fig. 4B, right side). Due to large standard deviations for the vector, *CLB5*, and *KIP3*-transformed strains in data from four independent trials, the results for these strains were not significantly different from each other as determined by the Tukey-Kramer HSD test; the same trends were observed in all trials, however: the *CLB5*-transformed cells formed more colonies than the vector-transformed cells, and the *KIP3*-transformed cells formed an intermediate number of colonies, indicating a partial rescue of viability.

Because rapamycin binds to TOR/Fpr1 with nanomolar affinity (Schreiber 1991), its effects can persist even after the drug has been removed from the medium. Caffeine is a lower-affinity TORC1 inhibitor that can be washed out more easily (Reinke et al. 2006). In an attempt to obtain viability assay data with lower standard deviations, we repeated the colony formation experiment substituting a four-hour treatment with 20 mM caffeine for the rapamycin treatment. Unfortunately, the standard deviations were not improved under this regimen, and no additional statistically significant differences emerged from it. However, the data followed the same trends observed in the rapamycin experiment (data not shown). Taken together, the results from the cell dilution assays and the post-drug rapamycin and caffeine colony formation assays generally support the conclusion that wild type *KIP3* counters the enhanced rapamycin cytotoxicity of *clb5Δ* cells whereas *KIP3(T254A)* actually seems to promote the cytotoxic effects of these drugs in *clb5Δ* cells.

Suppression of rapamycin hypersensitivity by *KIP3* is specific to *clb5Δ*

DNA replication is slowed in *clb5Δ* cells due to a delay in late origin firing (Donaldson et al. 1998). We sought to determine whether other strains that experience a prolonged S phase might also be hypersensitive to rapamycin and, if so, whether high-copy *KIP3* might also suppress such phenotypes. *RNR4* encodes a small subunit of the ribonucleotide reductase,

which is required for synthesis of deoxyribonucleotides (Huang and Elledge 1997; Wang et al. 1997). Loss-of-function mutations in *RNR4* significantly prolong S phase (Huang and Elledge 1997; Wang et al. 1997), and deletion of the gene was reported to cause rapamycin hypersensitivity in a genomic-scale study (Xie et al. 2005). We confirmed that *rnr4Δ* cells were slightly hypersensitive to rapamycin, but found that this hypersensitivity was much milder than that of *clb5Δ* cells (Fig. 5; compare to Fig. 1). In contrast to the result for the *clb5Δ* rapamycin hypersensitivity (Fig. 3A), high-copy *KIP3* had no effect on the *rnr4Δ* phenotype, nor did high-copy *CLB5* (Fig. 5). We also examined yeast slowed in S phase due to the temperature-sensitive *cdc6-1* mutation (Li and Herskowitz 1993); these cells did not exhibit rapamycin hypersensitivity at either permissive or semi-permissive temperatures (data not shown). Therefore, the rapamycin hypersensitive phenotype is not common to all strains that are slowed in DNA replication, and the phenotypic suppression by high-copy *KIP3* is specific to *clb5Δ* cells.

Rapamycin does not cause significant changes in microtubule morphology

Choi et al. (2000) reported rapid changes in microtubule morphology in wild type cells treated with a high dose of rapamycin. Specifically, they described the disappearance of cytoplasmic microtubules and the thinning and shortening of pre-anaphase spindles after 20 minutes of treatment with 200 nM rapamycin. Since the Kip3 over-expression data supported a link between *clb5Δ* rapamycin hypersensitivity and mitotic spindle function, we examined the microtubule morphology of rapamycin-treated *clb5Δ* cells and compared it to that of wild type cells. In contrast to the results of Choi et al., we did not observe any significant changes in microtubule morphology in either wild type or *clb5Δ* cells, even after two hours of treatment with 200 nM rapamycin (Fig. 6). Since we used an immunolocalization method similar to that of Choi et al., the reason for our different results is unclear; however, we also obtained the same results using wild type and *clb5Δ* cells expressing green fluorescent protein-tagged tubulin (Straight et al. 1997) (data not shown), bolstering the conclusion that rapamycin does not have a dramatic effect on the overall integrity of the microtubule cytoskeleton.

Rapamycin causes inefficient short spindle assembly and aberrant spindle morphology

Because we did not detect any gross changes in the microtubule cytoskeleton of rapamycin-treated *clb5Δ* cells, we looked for the possible occurrence of more subtle defects in cell cycle progression by observing the nuclear division and spindle morphologies of wild type and *clb5Δ* cells synchronized in G1 with α -factor and released into the cell cycle either in the absence or presence of 200 ng/ml rapamycin. We assessed cell cycle-related features of the synchronized cell populations at 20-min intervals up to three hours following the α -factor release by brightfield and fluorescence microscopy of DAPI and anti-tubulin immunostained cells (Fig. 7). Bud emergence occurred between 20 and 40 min post-release in the control and rapamycin-treated populations for both wild type and *clb5Δ* cells (Fig. 7A). Nuclear division (assessed by DAPI staining of the DNA) was delayed in both rapamycin-treated samples, peaking at 120 min for the drug-treated cells as compared to 80 min for the untreated control cells (Fig. 7B).

The anti-tubulin immunostaining of the synchronized cells provided more details regarding the nature of the delayed nuclear division in the rapamycin-treated samples. The appearance of short, pre-anaphase spindles (see Fig. 7E, panel iii) was delayed relative to the untreated controls in both rapamycin-treated wild type and *clb5Δ* cells (Fig. 7C). The rapamycin-treated wild type cells still exhibited a clear peak in this population, but at 100 min post-release rather than at 60 min as seen for the untreated cells. In contrast, there was no clear peak in the population of cells with pre-anaphase spindles for the rapamycin-treated *clb5Δ* cells; rather, this population was broadly distributed over the 60–180 min post-release period

(Fig. 7C). We examined the cell cycle kinetics in the phase prior to spindle assembly by scoring for the presence of single or side-by-side duplicated spindle pole bodies (SPBs; see Fig. 7E, panels i and ii). The onset of SPB duplication was coincident with bud emergence in all cell populations, but the cells with side-by-side SPBs persisted for a longer period of time in both rapamycin-treated samples, and this period was further prolonged in *clb5Δ* cells compared to wild type (Fig. 7D). These results suggest that TORC1 inhibition causes a delay in the conversion of duplicated SPBs to short spindles that is amplified by elimination of Clb5/Cdc28 kinase activity.

In scoring the synchronized cell populations depicted in Fig. 7A–D, we noted what appeared to be an altered interpolar spindle morphology in some cells in the rapamycin-treated populations. The spindles in these cells exhibited a punctate appearance (Fig. 7E, panel iv) rather than the continuous fluorescence seen in normal spindles (Fig. 7E, panel iii). We also observed small numbers of binucleate cells (unbudded or two nuclei in the mother cell) and anucleate cells. These observations support the conclusion that TORC1 inhibition affects the fidelity of nuclear division, as previously reported (Choi et al. 2000).

Rapamycin induces an S phase delay that is enhanced in *clb5Δ* cells

The synchronized cell data shown in Fig. 7 identified defects in spindle dynamics as a likely contributing factor to the overall slowed mitotic entry of cells in which TORC1 function is inhibited by rapamycin. Delayed completion of DNA replication induces a checkpoint that inhibits anaphase but does not prevent assembly of short spindles (Lew et al. 1997). To determine whether slowed DNA replication might also be impacting the cell cycle progression of rapamycin-treated cells, we performed flow cytometry to quantify the DNA content of cells treated similarly to those represented in Fig. 7A–D. Rapamycin-treated wild type cells showed only a minor delay in DNA replication, with 40 and 60-min G2 DNA content peaks that were not as sharply defined as in untreated cells (Fig. 8). This S phase delay was much more significant in rapamycin-treated *clb5Δ* cells, with a substantial population of cells with G1 DNA content remaining at 60 min post-release (Fig. 8). Thus, inhibition of TORC1 function and elimination of Clb5/Cdc28 activity synergistically slow S phase progression in addition to suppressing short spindle assembly.

Rapamycin does not induce Rad53 phosphorylation in wild type or *clb5Δ* cells

The S phase delay noted in rapamycin-treated *clb5Δ* cells could be due to induction of an S phase cell cycle checkpoint. The DNA damage and DNA replication checkpoints both require the kinase Rad53 to inhibit mitotic progression (reviewed by Putnam et al. 2009). Rad53 activation involves its hyperphosphorylation (Sanchez et al. 1996). To determine whether TORC1 inhibition and elimination of Clb5/Cdc28 activity might synergistically activate a Rad53-dependent checkpoint, we performed immunoblotting to assess the Rad53 phosphorylation status in wild type and *clb5Δ* cells released from an α -factor arrest into rapamycin-containing medium or drug-free medium. We saw no evidence of time-dependent Rad53 hyperphosphorylation in any of the strains (Supplementary Fig. 1). Therefore, our results do not support the hypothesis that rapamycin induces a Rad53-dependent checkpoint in wild type or *clb5Δ* cells.

Discussion

Cell growth and cell division (or proliferation) are distinct processes that are controlled by different regulatory pathways, but it is fundamentally clear that they must be coordinated to maintain cell viability (Schmelzle and Hall 2000). The best understood link between the cellular machineries that regulate these two processes is in the G1 phase of the cell cycle, where the central cell growth regulator TOR promotes translation of key cell cycle

regulators, such as G1 cyclins (Bjornsti and Houghton 2004; Inoki and Guan 2006; Schmelzle and Hall 2000). Here, we investigated the unique rapamycin hypersensitivity of *clb5Δ* yeast cells as a means to uncovering other possible connections between the TORC1 pathway and the cell cycle machinery outside of G1. Taken together, our data suggest that TORC1 and Clb5 act coordinately to promote both mitotic spindle assembly and efficient S phase progression.

The cyclin-specific nature of *clb5Δ* rapamycin hypersensitivity

The synergistic rapamycin hypersensitivity of the *tor1Δclb5Δ* double mutant (Fig. 1B) is consistent with Clb5 and TORC1 acting in parallel pathways to promote the same cellular process. Clb5 and Clb6 are functionally redundant with regard to initiation of S phase, but Clb5 remains important beyond the G1/S transition (Epstein and Cross 1992; Schwob and Nasmyth 1993) after Clb6 has been targeted for degradation by the SCF^{Cdc4} ubiquitin ligase (Jackson et al. 2006). The fact that *clb5Δ* cells are hypersensitive to rapamycin but *Δclb6* cells are not (Fig. 1A) may indicate that the functional overlap between TORC1 and Clb5 occurs after Clb6 is normally degraded. Clb6 was able to suppress the *clb5Δ* rapamycin hypersensitivity quite effectively when over-expressed (Fig. 2A). If the post-G1/S model for the Clb5 rapamycin-sensitive function is correct, an increased amount of Clb6 might overwhelm its degradation targeting machinery such that the protein is less efficiently degraded and persists later into the cell cycle, where it can perform the TORC1-related function. The suppression of *clb5Δ* rapamycin hypersensitivity could also have two other explanations. First, it could be that Clb5 and Clb6 are completely redundant with regard to the rapamycin-sensitive function, but that Clb6 is expressed at a lower level than Clb5, providing insufficient S phase cyclin activity to compensate for lack of Clb5 unless present in high copy. This is unlikely, however, since Jackson et al. (2006) saw no difference in the abundance of Clb5 and Clb6 at the point when they first accumulate. Alternatively, whatever substrates need to be phosphorylated by Clb5/Cdc28 to accomplish the TORC1-related function may be inherently less efficiently targeted by Clb6/Cdc28, but high levels of Clb6 may compensate for this decreased substrate affinity.

The results with the *CLB2*, *CLB5::CLB2*, and *clb5^{hpm}* constructs (Fig. 2B–D) support the idea that the basis for the cyclin specificity of the TORC1-related Clb5 function relates to its ability to target Cdc28 to specific phosphorylation substrates via hydrophobic patch-mediated protein-protein interactions. The mitotic cyclin Clb2 was unable to suppress *clb5Δ* rapamycin hypersensitivity even when expressed with the same timing as Clb5 and with the Clb2/Cdc28-specific Swe1 inhibitory activity removed (i.e. from the *CLB5::CLB2* construct in *clb5Δswe1Δ* cells, Fig. 2C). This suggests that Clb2/Cdc28 cannot efficiently phosphorylate the relevant TORC1-related Clb5 substrate(s). In contrast, *CLB5::CLB2* was largely able to correct the slowed DNA replication in *clb5Δ* cells when *SWE1* was deleted (Hu and Aparicio 2005). The failure of the *clb5^{hpm}* hydrophobic patch mutant to rescue *clb5Δ* rapamycin hypersensitivity (Fig. 2D) is consistent with the results of Loog and Morgan (2005), who demonstrated the importance of the Clb5 hydrophobic patch in conferring Clb5/Cdc28 phosphorylation substrate specificity. All three amino acid residues that are mutated in the *clb5^{hpm}* protein are conserved in Clb6 (Bazan 1996; Cross et al. 1999); additional flanking elements have been proposed to contribute specificity to the hydrophobic patch substrate targeting function, however (Cross et al. 1999). Therefore, Clb5 and Clb6 could still differ in this regard.

Connections between *clb5Δ* rapamycin hypersensitivity, motor proteins, and spindle assembly

Kip3 over-expression partially suppressed *clb5Δ* rapamycin hypersensitivity (Fig. 3A). Rapamycin treatment also delayed formation of pre-anaphase spindles, with cells persisting

at the side-by-side duplicated SPB stage, and caused a punctate spindle morphology in some cells (Fig. 7). Together, these results point to coordinate involvement of Clb5 and TORC1 in spindle assembly and possibly also maintenance of spindle integrity. Cottingham et al. (1999) found that either Kip3 or Kar3 is required for normal spindle assembly and integrity. Kip3 is a plus-end motor, whereas Kar3 moves toward microtubule minus-ends; both proteins possess plus-end-specific microtubule depolymerizing activity, however (Gupta et al. 2006; Sproul et al. 2005; Varga et al. 2006). Cottingham et al. (1999) observed partial rescue of the temperature sensitivity of the $\Delta kip3 kar3-64$ strain by the microtubule depolymerizing drug benomyl, leading them to conclude that it is the depolymerizing activity of Kip3 and Kar3 that is relevant in spindle assembly. In addition, these researchers noted fewer short spindles in $\Delta kip3 kar3-64$ cells at the non-permissive temperature, similar to the phenotype we observed in rapamycin-treated cells. Thus, it is likely the Kip3 microtubule depolymerizing activity that is important for suppression of *clb5 Δ* rapamycin hypersensitivity.

One explanation for the suppression of *clb5 Δ* rapamycin hypersensitivity by high-copy *KIP3* is that Kip3 is normally a preferential (but not exclusive) Clb5/Cdc28 phosphorylation substrate and that its phosphorylation is important for cell survival under conditions of TORC1 inhibition. Increased Kip3 expression could allow phosphorylation of a sufficient population of the protein by another cyclin/CDK pair that has lower inherent affinity for it—most likely Clb6/Cdc28, given the data we present in Fig. 2. The proteomic study of budding yeast Cdc28 substrates performed by Ubersax et al. (Ubersax et al. 2003) identified both Kip3 and Kar3 as in vitro targets. The follow-up study by Loog and Morgan (2005) showed that Clb5/Cdc28 phosphorylated both of these proteins more efficiently than Clb2/Cdc28 did. Despite the similarities between Kip3 and Kar3 mentioned here and above, over-expressed *KAR3* did not show the same ability to suppress *clb5 Δ* rapamycin hypersensitivity as *KIP3* did. Unraveling the precise contributions of these two motor proteins to the Clb5- and TORC1-dependent spindle function will require further investigations.

Neither the Ubersax et al. (2003) nor the Loog and Morgan (2005) study mapped phosphorylation sites, so we do not know whether the threonine residue within the full CDK consensus motif that we mutated (T254) is indeed the Kip3 site that is phosphorylated by Clb5/Cdc28, but this is most reasonable interpretation. Although preliminary, the slight difference in mobility of the Kip3(T254A) protein relative to wild type Kip3 observed in Fig. 3C is consistent with T254 being a bona fide in vivo phosphorylation site. Over-expressed Kip3(T254A) together with rapamycin had an additive inhibitory effect on the growth of *clb5 Δ* cells in the cell dilution assay (Fig. 3B). The results for the vector- and *KIP3(T254A)*-transformed *clb5 Δ* cells did not show a statistically significant difference in the post-rapamycin colony viability assay due to the large standard deviations for the vector control strain (Fig. 4B); however, the additive growth inhibition was also evident in this assay. The fact that over-expressed Kip3(T254A) did not affect the growth of *clb5 Δ* cells in the absence of rapamycin (Fig. 3B) indicates that TORC1 inhibition creates some particular condition within the cell that is negatively impacted by the mutant Kip3 protein. Furthermore, the fact that wild type cells were not similarly affected suggests that Clb5/Cdc28 may have additional targets that counteract the negative impact of Kip3(T254A) in the presence of rapamycin. Kar3 could be such a target. To advance understanding of Kip3 function, it will be important to verify the phosphorylation of T254 and to determine whether the T254A mutation influences Kip3 stability or otherwise alters Kip3 function— affecting its microtubule depolymerizing activity, for example. Considering all of the Kip3 results together, we favor a model in which Kip3 is a preferential Clb5/Cdc28 substrate and therefore contributes to the shared function of Clb5 and TORC1 as part of the Clb5 branch of the pathway rather than acting in another parallel branch. Deletion of *KIP3* on its own does not cause rapamycin hypersensitivity, and it also does not enhance the rapamycin

sensitivity of *clb5Δ* cells (J. Hood-DeGrenier, unpublished data). These results strongly support our interpretation that Kip3 acts in the same pathway as Clb5 and also suggests that additional Clb5/Cdc28 substrates are relevant to the *clb5Δ* rapamycin hypersensitivity phenotype.

Coordinate involvement of TORC1 and Clb5 in S phase progression

Our flow cytometry analysis of synchronized cell populations showed that normal S phase delay of *clb5Δ* cells (Epstein and Cross 1992; Kuhne and Linder 1993; Schwob and Nasmyth 1993) was significantly extended in the presence of rapamycin (Fig. 8). Our experiments with *rnr4Δ* and *cdc6-1* cells indicate that strong rapamycin hypersensitivity is not a general feature of all cells with an S phase delay, but rather is specific to *clb5Δ* (Fig. 5 and data not shown). We favor the interpretation that the strong rapamycin hypersensitivity of *clb5Δ* cells is caused by the combined effect of abrogating two different functions that coordinately involve Clb5 and TORC1: one related to spindle assembly and the other to S phase progression. It is also possible that these two functions are related in some as yet undefined way. The exact nature of the S phase defect in rapamycin-treated *clb5Δ* cells is still unclear, but our data provide no evidence to support the idea that Rad53-activating DNA structures are accumulating in these cells (Supplementary Fig. 1). Our results contrast with those of Gibson et al. (2004), who reported low levels of hyperphosphorylated Rad53 in otherwise unperturbed, synchronized *clb5Δ* cells. It is possible that we are not detecting some low level of hyperphosphorylated Rad53 in our experiment; however, TORC1 inhibition does not appear to activate a Rad53 checkpoint response that is sufficiently robust to fully account for the observed S phase delay in rapamycin-treated *clb5Δ* cells.

In summary, our results indicate that TOR1C and Clb5 act in parallel to promote both completion of DNA replication and mitotic spindle assembly. Together with the previously described Cdc5-dependent role for TORC1 in mitotic entry (Nakashima et al. 2008), a general model is emerging in which the TORC1 signaling pathway is not only important for G1 progression but interfaces with many different regulatory components through all phases of the cell cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *clb5*Δ cells exhibit rapamycin hypersensitivity that is additive with *tor1*Δ. **A** Ten-fold serial dilutions (decreasing in concentration from left to right) of wild type (WT, BY4741) and otherwise isogenic yeast strains bearing the indicated gene deletions were spotted onto YPD plates containing the indicated concentrations of rapamycin (rap) or control plates without rapamycin and grown for five days at 30°C. **B** BY4741 (WT), *clb5*Δ (JHY437), *tor1*Δ, and *clb5*Δ *tor1*Δ (JHY444) cells were treated as in **A**.

Fig. 2. Cyclin specificity in the reversal of *clb5Δ* rapamycin hypersensitivity. **A–D** *clb5Δ* (JHY180) or *clb5Δswe1Δ* (JHY639) cells transformed with an empty vector (vec) or the indicated centromeric (*CEN*) or high-copy (*2μ*) expression plasmids were grown in SD-ura medium, then equal volumes of ten-fold serial dilutions (decreasing in concentration from left to right) were spotted onto YPD + 50 ng/ml rapamycin plates and grown for five days at 30°C. *clb5^{hpm}* is the triple hydrophobic patch mutant (M197A, L201A, W204A; Cross and Jacobson 2000). **E** Anti-HA Western blot of lysates prepared from JHY180 cells bearing a *CEN CLB5-HA* or *clb5^{hpm}-HA* plasmid or an empty vector. Anti-PSTAIRE panel shows equal loading of total protein for each sample.

Fig. 3.

Over-expression of the kinesin-like motor protein Kip3 rescues *clb5Δ* rapamycin hypersensitivity, likely in a phosphorylation-dependent manner. **A, B** *clb5Δ* (JHY180) cells transformed with an empty vector (vec) or the indicated centromeric (*CEN*) or high-copy (*2μ*) expression plasmids were grown in SD-ura medium, then equal volumes of ten-fold serial dilutions (decreasing in concentration from left to right) were spotted onto YPD plates supplemented with the indicated concentrations of rapamycin and grown for six days at 30°C. *KIP3^m* denotes the *KIP3*(T254A) mutation, which eliminates a consensus CDK phosphorylation site within the encoded Kip3 protein. **C** Anti-myc Western blot of lysates from vector-transformed control cells or JHY180 cells bearing plasmids expressing 6x-myc-tagged *KIP3* or *KIP3^m*. Amounts of lysates containing 10, 5, or 2.5 μg of total protein were loaded in the three sets of lanes. Equal loading of total protein was verified by Ponceau S staining prior to antibody detection.

Fig. 4.

Colony formation after a short rapamycin treatment. Early log-phase wild type (WT, BY4741) and *clb5Δ* (JHY180) cells grown in YPD or JHY180 cells transformed with an empty vector, *CEN CLB5*, 2μ *KIP3*, or 2μ *KIP3(T254A)* and grown in SD-ura were left untreated or treated with 50 ng/ml rapamycin for four hours. After treatment, the cells were washed once with drug-free medium and resuspended to a constant OD₆₀₀ value. **A** Flow cytometry analysis of the DNA content of wild type and *clb5Δ* cells at the end of the 4-hour rapamycin treatment and untreated control cells. **B** Washed cells diluted to a constant OD₆₀₀ were plated on YPD (WT and *clb5Δ*) or SD-ura (plasmid-transformed strains) to assess colony formation. Colonies were counted after three (untreated controls) or six (rapamycin-treated samples) days of growth at 30°C. The graph represents the relative colony formation of rapamycin-treated samples as percentages of the colonies obtained for the untreated, paired controls. Error bars indicate standard deviations for at least three independent trials (four trials for *clb5Δ* + vec, *CLB5*, and *KIP3* samples). Brackets and asterisks indicate pairs of samples for which differences in colony formation were statistically significant within the experimental groups separated by the vertical line. Statistical significance between WT and *clb5Δ* samples was determined by the Student's t-test; differences between the vector transformed strains were assessed by ANOVA and the Tukey-Kramer HSD test.

Fig. 5.

rrr4Δ cells exhibit a mild rapamycin hypersensitivity that is not rescued by high-copy *KIP3* or *CLB5*. Wild type (WT, BY4741) or *rrr4Δ* cells transformed with an empty vector (vec) or the indicated 2 μ expression plasmids were grown in SD-ura medium, then equal volumes of ten-fold serial dilutions (decreasing in concentration from left to right) were spotted onto YPD plates with or without 200 ng/ml rapamycin and grown for six days at 30°C.

Fig. 6. Anti-tubulin immunostaining of rapamycin-treated wild type and *clb5Δ* cells. Wild type (WT, BY4741) and *clb5Δ* (JHY180) cells were either untreated (control) or treated with 200 nM rapamycin for 20 or 120 min, formaldehyde fixed, and subjected to anti-tubulin immunostaining and fluorescence microscopy as described in Materials and Methods.

Fig 7. Effect of rapamycin on cell cycle progression in synchronized wild type and $\Delta clb5$ cells. Alpha-factor-synchronized YPD cultures of wild type (WT, JHY448) or $\Delta clb5$ (JHY449) cells were released from the G1 arrest at time zero into YPD or YPD supplemented with 200 ng/ml rapamycin. At 20-min intervals up to three hours, cells were formaldehyde fixed and processed for anti-tubulin immunostaining and DAPI DNA staining followed by fluorescence microscopy. Approximately 100 cells from each sample were scored for various cell cycle progression markers: **A** all budded cells as a percentage of the total scored for each timepoint; **B** budded cells with divided or dividing nuclei; **C** cells with pre-anaphase (short) spindles; **D** cells with side-by-side duplicated spindle pole bodies (SPBs) but no assembled spindle. Symbols used in the graphs are explained in the legend (lower left). **E** Examples of scored as having a single SPB (i), side-by-side duplicated SPBs (ii), a normal short spindle (iii), or a “punctate” spindle (iv). The arrow in “iv” points to a region of diminished fluorescence signal within the spindle.

Fig. 8.

Effect of rapamycin treatment on S phase progression in synchronized wild type and *clb5Δ* cells. Alpha-factor-synchronized YPD cultures of wild type (WT, JHY448) or *clb5Δ* (JHY449) cells were released from the G1 arrest at time zero into YPD (control) or YPD supplemented with 200 ng/ml rapamycin (+ rap). At 20-min intervals up to three hours, cells were ethanol fixed and processed for flow cytometry to measure DNA content as described in Materials and Methods.

Table 1Yeast strains used in this study^a

Strain name	Relevant genotype	Reference/Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(Brachmann et al. 1998)
JHY180	<i>MATa clb5Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(Giaever et al. 2002)
JHY448	<i>BY4741 bar1Δ</i>	This study
JHY449	<i>JHY180 bar1Δ</i>	This study
JHY437	<i>MATa clb5Δ::natMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	This study
JHY444	<i>MATa tor1Δ::kanMX4 clb5Δ::natMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	This study
JHY639	<i>clb5Δ::natMX4 swe1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	This study

^a Single gene deletion strains used in the study but not listed in the table are all from the yeast deletion collection described by Giaever *et al.* (2002) and have the same genetic background as BY4741 with individual genes replaced by *kanMX4*.

Table 2

Plasmids used in this study

Plasmid name	Description	Reference/Source
pRS426	<i>2μ URA3</i>	Sikorski, 1989
pFC706	<i>CEN URA3 CLB5</i>	Fred Cross (Rockefeller U.)
pB1224	<i>CEN URA3 CLB2</i>	David Pellman (Harvard U.)
pJH272	<i>2μ URA3 CLB2</i>	This study
pJH299	<i>CEN HIS3 CLB6</i>	This study
pJH298	<i>2μ HIS3 CLB6</i>	This study
FC443	<i>CEN URA3 CLB5::CLB2</i>	Cross et al. 1999
pJH426	<i>2μ URA3 CLB5::CLB2</i>	This study
pFC432	<i>CEN URA3 CLB5-HA</i>	(Cross and Jacobson 2000)
pFC433	<i>CEN URA3 clb5-hpm-HA</i>	(Cross and Jacobson 2000)
pJH355	<i>2μ URA3 KIP1</i>	Andrew Hoyt (Johns Hopkins U.)
pEH410	<i>2μ URA3 KIP2</i>	Andrew Hoyt (Johns Hopkins U.)
pEH389	<i>2μ URA3 KIP3</i>	Andrew Hoyt (Johns Hopkins U.)
pMA1199	<i>2μ URA3 CIN8</i>	Andrew Hoyt (Johns Hopkins U.)
pJH379	<i>2μ URA3 KIP3(T254A)</i>	This study
pB956	<i>CEN LEU2 KIP3-6myc</i>	David Pellman (Harvard U.)
pJH417	<i>CEN LEU2 KIP3(T254A)-6myc</i>	This study
pMR794	<i>2μ URA3 KAR3</i>	(Meluh and Rose 1990)