Candida albicans Cyclin Clb4 Carries S-Phase Cyclin Activity

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Cyclin-dependent kinases (CDKs) are key regulators of eukaryotic cell cycle progression. The cyclin subunit activates the CDK and also imparts to the complex, at least in some cases, substrate specificity. Saccharomyces cerevisiae, an organism in which the roles of individual cyclins are best studied, contains nine cyclins (three G₁ cyclins and six B-type cyclins) capable of activating the main cell cycle CDK, Cdc28. Analysis of the genome of the pathogenic yeast Candida albicans revealed only two sequences corresponding to B-type cyclins, C. albicans Clb2 (CaClb2) and CaClb4. Notably, no homolog of the S. cerevisiae S-phase-specific cyclins, Clb5/Clb6, could be detected. Here, we performed an in vitro analysis of the activity of CaClb2 and CaClb4 and of three G₁ cyclins, as well as an analysis of the phenotype of S. cerevisiae cells expressing CaClb2 or CaClb4 instead of Clb5. Remarkably, replacement of CLB5 by CaCLB4 caused rapid diploidization of S. cerevisiae. In addition, both in vitro and in vivo analyses indicate that, in spite of the higher sequence similarity of CaClb2 to Clb5/Clb6, CaClb4 is the functional homolog of Clb5/Clb6. The activity of a CaClb2/CaClb4 cyclin hybrid suggests that the cyclin box domain of CaClb4 carries the functional specificity of the protein. These results have implications for our understanding of the evolution of specificity of the cell cycle cyclins.

Cyclin-dependent kinases (CDKs) regulate many cellular processes but are best known for their role in the promotion of cell cycle progression. CDK activity depends on the binding of activatory subunits, the cyclins, which periodically appear during the cell cycle. Saccharomyces cerevisiae contains a single essential cell cycle CDK, S. cerevisiae Cdc28 (ScCdc28)/Cdk1, which in turn can be activated by nine cyclins: three G₁-type cyclins (Cln1, Cln2, and Cln3) and six B-type cyclins (S. cerevisiae Clb1 [ScClb1] to ScClb6) (34). Cln3 together with Cln1 and Cln2 (Cln1/2) induces a large class of cell cycle-regulated genes, including genes involved in S-phase initiation, such as the B-cyclins Clb5 and Clb6 (Clb5/6) (44, 47). Clb3 and Clb4 are expressed from early S phase to anaphase (22) and play a role in spindle orientation (Clb4) (31) and morphogenesis (Clb3 and Clb4) (25, 37), and Clb1 and Clb2 are expressed in G₂ (22) and play a role in entry into anaphase and spindle elongation (18). Genetic analysis suggests that the genes CLB1 to CLB4 have overlapping functions, as deletions of all four is lethal, but a mutant with deletion of all but CLB2 is still viable (18). Deletion of both CLB5 and CLB6 or of CLB5 alone is not lethal but results in a delay in S-phase initiation (41).

The diverged yeast Schizosaccharomyces pombe contains one G₁ cyclin and three B-type cyclins. Studies indicating that a single S. pombe B-type cyclin, Cdc13, is sufficient to promote cell cycle progression led to the suggestion that the cyclin’s function is solely to periodically activate the CDK (17, 32). It is now clear, however, that the cyclin subunit imparts specificity to the CDK in at least some cases. Notably, biochemical analysis suggests that the different cellular function of the S. cerevisiae B-type cyclins may be based upon different substrate specificities: comparative analysis by in vitro phosphorylation of CDK substrates by Clb2-Cdk1 versus Clb5-Cdk1 indicates that whereas Clb2-Cdk1 carries a higher kinase activity toward most substrates, Clb5-Cdk1 is differentially much more active on a subclass of CDK substrates, including many S-phase proteins (30). A specific region of the cyclin box domain of Clb5 was identified that is essential for interaction with S-phase-specific substrates such as Orc6 (46) and Cdc6 (1).

Candida albicans is a pathogenic yeast in the order Saccharomycetales, distantly related to S. cerevisiae. Given the cumbersome genetics of C. albicans, a diploid organism lacking a traditional sexual cycle, assignment of gene function in C. albicans has often been informed by sequence comparison with S. cerevisiae. However, the complete genome sequence of C. albicans, while including a Cdk1/Cdc28 homolog as well as sequence homologs of the cyclins Cln1/2, Cln3, Clb2, and Clb4—5 predicted Cdk1/Cdc28 cyclins in total—lacks an obvious homolog of Clb5/6. Here, we show by biochemical analysis and functional complementation that the homologous function of ScClb5 is carried by C. albicans Clb4 (CaClb4).

MATERIALS AND METHODS

Media and strains. Medium included either yeast extract-peptone-dextrose (YPD) or synthetic complete medium, both described in Sherman et al. (43). Selection for uridine prototrophy was performed on synthetic complete plates lacking uridine and uracil (SC-URA). The C. albicans strains used were all derived from CAI4 (20).

S. cerevisiae strains are listed in Table 1. The S. cerevisiae strains Scclb5Δ:CaClb2 (KY1286) and Scclb5Δ:CaClb4 (KY1292 and KY1318) were constructed by homologous gene replacement into BY4741 or BY4743, using PCR products obtained with primers 963, 964, and 965 (see Table S1 in the supplemental material) and plasmids KB2099 and KB31927 as templates. Scclb5Δ:CaClb2/CaClb4 (KY1346) was constructed by homologous gene replacement into BY4741 using a PCR product obtained with primers 963 and 965 (see Table S1) and plasmid KB2053 as a template. The substitution was confirmed by PCR. The KY1330 and KY1331 strains were derived by sporulation
sulfonyle fluoride, 1:100 of aprotinin solution (Sigma), 50 μg/ml tosylsulfonyle phenylalanyl chloromethyl ketone (TPCK), 50 μg/ml Na-p-tosyl-L-lysine chloromethyl ketone (TLCK), and 1:500 of an antiprotease cocktail containing leupeptin, pepstatin, and chymostatin, each 10 mg/ml in dimethyl sulfoxide). A 0.2-mg protein extract (5 to 20 μl) was incubated with the 9E10 monoclonal antibody for 30 min on ice, and then 20 μl of a 50% slurry of protein A-agarose beads (Pharmacia) and 200 μl of extract buffer were added, and the tubes were incubated with tumbling for 1 h at 4°C. The agarose beads were then washed three times with extract buffer lacking aprotinin, TPCK, TLCK, and the antiprotease cocktail and two times with kinase buffer. For each phosphorylation reaction, the agarose bead pellet was incubated for 30 min at 30°C with 1 μg of the generic substrates histone H1 and myelin basic protein (MBP) or with about 50 ng of the recombinant proteins in 10 μl of kinase buffer with 1 μCi of [γ-32P]ATP and 10 μM unlabeled ATP. All the kinase reactions were terminated by the addition of 10 μl of protein loading buffer and separated by SDS-PAGE. Bands were quantitated using a Fuji BAS phosphorimager.

Fluorescence-activated cell sorting (FACS) analysis. A log-phase culture (1.5 ml) was collected and fixed with 70% ethanol for at least 1 h at 4°C. All washing steps were performed with 1 ml of 50 mM Tris-HCl, pH 8. The cells were washed twice and resuspended in 300 μl of 1 mg/ml RNase A (Sigma R-8475) in 50 mM Tris-HCl, pH 8. The cells were incubated for 1 h at 37°C, then washed twice, resuspended in 50 μl of 10 mg/ml proteinase K (Sigma, p-2308), and incubated for 1 h at 37°C. The cells were then washed once, resuspended in 200 μl of SYBR green (diluted 1:1,000 in 10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8), incubated in the dark for 1 h at room temperature, sonicated for 30 s (10 s on/10 s off), diluted with 800 μl of 3% Tritos-HCI, pH 8, and analyzed with a FACS-Calibur cell sorter (BD Biosciences).

Western blotting. Protein levels were assessed by Western blotting by using the monoclonal antibody 9E10 to detect the Myc epitope. Proteins were extracted by the quantitative NaOH–2-mercaptoethanol method (28). To compare steady-state protein levels, equal protein amounts were loaded; to monitor protein disappearance after promoter shutoff, equal culture volume equivalents were loaded. Loading and transfer were monitored by Ponceau staining of the membrane or by the mouse anti-beta-actin antibody (Abcam ab8224).

Sequence comparison. Predicted cyclin sequences were extracted from the GenBank and other databases by sequence homology with known S. cerevisiae and C. albicans sequences. Sequence alignment was performed using the MAFFT (multiple alignment using fast Fourier transform) L-INS-I algorithm (27). A sequence phylogeny maximum-likelihood tree was obtained by feeding the alignment into PhyML (14).

RESULTS

Sequence analysis of fungal cyclins. In an attempt to identify the regulator of S-phase initiation that fulfills the role of ScCld5/6 in S. cerevisiae and after the whole-genome duplication (WGD) event. Align-
clade (Candida spp. excluding Candida glabrata, and Pichia spp., and Lodderomyces elongisporus) and from the Saccharomyces clade (post-WGD species and Ashbya gossypii, Kluyveromyces lactis, and Kluyveromyces waltii), and each also contains an outlying sequence from Yarrowia lipolytica, in good correspondence with the suggested phylogeny of the Saccharomycotina subphylum (19). Two sequence clusters corresponding to Clb2 and Clb4 are detectable, with a difference, however: the distance between the Candida clade and the Saccharomyces clade is larger within the Clb4 cluster than within the Clb2 cluster. Significantly, all the Saccharomyces clade species but not a single Candida clade species are represented in the Clb5/6 cluster sequences, indicating that the absence of Clb5 from C. albicans does, in fact, result from evolutionary gene gain or loss. The short distance separating the Clb2 and Clb5 clusters, as well as the adjacent location of Clb2 and Clb5 on the chromosomes of many species, supports the suggestion that Clb5 is a product from a Clb2 gene duplication event that took place after the divergence between the Candida and Saccharomyces clades (1). The situation with regard to the G1 cyclins is as follows: whereas Cln1/Cln2/Hgc1 and Cln3 homologs are detectable in all species, a Ccn1 cluster emerges that is found only in the Candida clade, but K. waltii and K. lactis each also exhibits a sequence most closely related to Ccn1.

**Biochemical analysis of C. albicans cyclin specificity.** The sequence comparisons shown above, while establishing each C. albicans cyclin as a member of a distinct Cdc28 cyclin family, did not allow a prediction of the identity of the cyclin carrying the Clb5 function. CaClb2 by its homology to ScClb5, Ccn1 by its occurrence mainly in species lacking Clb5, and CaClb4 by the divergence between the Candida and Saccharomyces sequences, all made equally plausible candidates for the S-phase cyclin. We therefore turned to biochemistry to try to identify the C. albicans S-phase cyclin. The five cyclins were epitope tagged and expressed from the inducible MAL2 promoter. Immunoprecipitation of cyclins under native conditions usually maintains their interaction with the CDK. Kinase activity associated with each cyclin was therefore tested by immunoprecipitating the tagged cyclin using protein A-Sepharose beads and mixing the beads with recombinant substrates and [32P]ATP. Generic substrates such as histone H1 (for CaClb2 and CaClb4) and myelin basic protein (MBP) (for G1 cyclins) were used throughout to determine the cyclin-associated kinase activity of each immunoprecipitate.

We first tested the activity of the CaClb2 and CaClb4 cyclin immunoprecipitates on a known S. cerevisiae S-phase-specific substrate. ScCdc6 is an essential regulator of initiation of replication that is preferentially phosphorylated by ScClb5-Cdc28 (30). The N-terminal domain of ScCdc6, shown to be specifically targeted by ScClb5 (1), was produced in bacteria. Whereas both C. albicans B-type cyclins were able to phosphorylate ScCdc6, the reaction was stronger with CaClb4 (Fig. 2A). Taking into account that the generic kinase activity associated with CaClb4, as determined by its activity on histone H1, was actually lower than that associated with CaClb2 (Fig. 2A), the relative activity of CaClb4 on ScCdc6 appears even higher. It has been shown before that substrate specificity of cyclins can coevolve with their substrates, leading to a loss of specificity between the cyclin and the homolog of its substrate in a diverged species (21). Thus, phosphorylation of an S. cerevisiae protein may not be indicative of specificity of a C. albicans cyclin. In order to identify potential C. albicans S-phase substrates, we used sequence comparison and identified the presumed C. albicans homolog of ScCdc6 as orf19.5242 (CaCdc6) and that of ScMcm4, a member of the prereplication complex which is phosphorylated by CDK in the N-terminal domain (15), as orf19.3761 (CaMcm4). Activity of the C. albicans cyclins was then tested on these two recombinant proteins (Fig. 2B). The three G1 cyclins, Ccn1, Hgc1, and CaCln3, did not display any activity toward CaCdc6 or CaMcm4. In contrast,
both B-type cyclins phosphorylated the two substrates, but CaClb4 exhibited higher activity than CaClb2.

To more accurately compare the relative activities of CaClb2 and CaClb4 toward CaCdc6, phosphorylation of CaCdc6 and of histone H1 was carried out side by side using the same starting extracts, similar to the experiment shown in Fig. 2A. Quantitation of the resulting phosphorylations (Fig. 2C) indicated that the activity of CaClb4 toward CaCdc6, normalized to activity on histone H1, was some 20-fold higher than that of CaClb2.

Complementation of ScClb5 S-phase function by CaClb4. The higher activity of CaClb4 on S-phase substrates of both C. albicans and S. cerevisiae suggests that this is the cyclin that fulfills the role of ScClb5 in C. albicans. As an additional test of this notion, we replaced the open reading frame of CLB5 in S. cerevisiae with that of either CaCLB2 or CaCLB4. Neither strain showed any growth defect compared to the wild-type starting strain or to the Scclb5Δ mutant. Since the activity of the C. albicans cyclins in S. cerevisiae might

Effect of the CDK inhibitors ScSic1 and CaSol1 on CaClb2 and CaClb4 activity. A possible confounding factor in assessing C. albicans cyclin activity in S. cerevisiae is the presence of the CDK inhibitor ScSic1, which is specific for CDK-B-type cyclin complexes (36). C. albicans possesses an Sic1 homolog, CaSol1, which may have a somewhat distinct function; notably, it is less able to inhibit the G1/S transition than ScSic1 (2). Since the activity of the C. albicans cyclins in S. cerevisiae might
be affected by their sensitivity to ScSic1 versus CaSol1, we tested the effect of these inhibitors on kinase activity in vitro. As shown in Fig. 4, CaClb2/Cdc28 and CaClb4/Cdc28, when tested on either the generic substrate histone H1 (Fig. 4A) or the specific substrate CaMcm4 (Fig. 4B), were similarly sensitive to ScSic1. Therefore, the differential activity of CaClb2 and CaClb4 cannot be explained by different sensitivities to ScSic1. This experiment also shows that the sensitivities of both C. albicans cyclins to their cognate inhibitor CaSol1 may be slightly higher than to ScSic1. Note also that CaSol1 at high concentration is itself a substrate of both CaClb2 and CaClb4.

**Diploidization of the S. cerevisiae clb5Δ::CaCLB4 strain.** Examination of the FACS pattern of the Scclb5Δ::CaCLB4 strain (Fig. 3) revealed another surprising feature: in addition to the 1N DNA and 2N DNA peaks expected, a third, smaller peak corresponding to 4N DNA was consistently detectable. This peak could have resulted from diploidization of part of the cell population or from a defect in cytokinesis elicited by CaCLB4, causing G1 cells to enter a new round of replication before separation of the bud from the mother cell. However, the cytokinesis defect should have resulted in a very-large-budded cell. Instead, microscopic observation of the 4N cells isolated by FACS showed the expected distribution of G2/M phase cells, ranging from small-budded to large-budded cells (data not shown). Furthermore, further subculturing of the Scclb5Δ::CaCLB4 strain led to a rapid increase in the 4N subpopulation and a decrease in the 1N population, to the extent that after subculturing the population for two additional days, the whole Scclb5Δ::CaCLB4 cell population had shifted to a diploid pattern of one G1 peak containing 2N DNA and one G2/M peak containing 4N DNA (Fig. 5A).

Diploidization of S. cerevisiae normally results from mating between strains of opposite mating types, MATα and MATα. Mating within a homogenous haploid population can potentially occur when a fraction of the population switches its mating type. The resulting diploids are then expected to be of MATα/MATα mating type, nonmating and nonresponding to mating pheromone. However, the diploids that arose from the MATα clb5Δ::CaCLB4 strain were able to mate and responded to α-pheromone (data not shown), indicating that they were...
of MATa/MATa genotype, and most probably resulted from an endomitotic event. We also constructed a MATa Scclb5Δ::CaCLB4 strain and found that it diploidized at the same frequency as the MATa strain, and we obtained tetraploids from matings between the MATa and MATa Scclb5Δ::CaCLB4 strains (Fig. 5B). The tetraploids could be sporulated and gave rise to diploid spores. Viability of the spores was lower than normal, but the phenotype distribution of the tetrads with four viable spores suggested an independent assortment of four segregating alleles of MET13 and of LYS2, as expected (data not shown).

Effect on cell cycle progression of CaClb4 overexpression in S. cerevisiae. The data shown above suggest that the diploidization of the Scclb5Δ::CaCLB4 strains occurred as the result of endoreduplication or endomitotic events. Microscopic observation of the haploid Scclb5Δ::CaCLB4 cells did not reveal any obvious cell cycle defects or delays. Therefore, to exacerbate any potential effects, CaClb4 was cloned and overexpressed under the strong GAL1 promoter. Plating on galactose showed that CaClb4 overexpression inhibits cell proliferation (Fig. 6A). In order to further characterize potential cell cycle defects, CaClb4 was similarly overexpressed in a TUB1::GFP (where GFP is green fluorescent protein) strain and in an NUF2::GFP strain, allowing visualization of the mitotic spindle and the spindle pole body (SPB), respectively, in living cells. Microscopic examination of liquid-grown cells after galactose induction showed that CaClb4 overexpression caused various anomalies. Many cells appeared in chains of three or more (30% of cells after 6 h in galactose; \( n = 160 \)), with abnormally broad bud necks and an extended spindle spanning the length of the cell chain (Fig. 6B). Another anomaly was that CaClb4-expressing unbudded cells frequently exhibited duplicated SPBs, either adjacent to each other (21% versus 3% in control cells; \( n = 100 \)) or well separated (26% versus 0%) (Fig. 6B). These observations suggest a loss of coordination between cytokinesis and the nuclear cycle.

If these anomalies, which may account for an increased frequency of endomitosis, were to occur even at a low frequency in the cells expressing CaClb2 under the CLB5 promoter, this might explain the diploidization of the Scclb5Δ::CaCLB4 strain. One possibility for the occurrence of these anomalies is that CaClb4 is misregulated in S. cerevisiae. Cell cycle cyclins are mainly regulated at the level of degradation apparatus. We therefore asked whether degradation of CaClb4 varies between C. albicans and S. cerevisiae. The cyclin was epitope tagged and expressed under the glucose-repressible promoters CaMAL2 in C. albicans and GAL1 in S. cerevisiae, and its levels were monitored following promoter shutdown. As shown in Fig. 6C, whereas CaClb4 was unstable in C. albicans, it was totally stable in S. cerevisiae, indicating that CaClb4 is not recognized by the S. cerevisiae degradation apparatus.

Narrowing the region of cyclin specificity with a cyclin hybrid. Alignment of the fungal B-type cyclins shows a region of relative conservation in the C-terminal half of the sequence, corresponding to the cyclin box domains, and a region of lower conservation in the N-terminal half of the sequence (see Fig. S1 in the supplemental material). In order to identify which part of the CaClb4 sequence is responsible for its functional specificity toward S-phase substrates, we constructed a series of hybrids between CaClb2 and CaClb4. Both hybrids were over-expressed under the GAL1 promoter and integrated to replace ScCLB5. Of several hybrids tested, a single hybrid, which spliced together the N-terminal half of CaClb2, extending to position 221, and the C-terminal half of CaClb4, extending from position 248, was toxic when overexpressed (data not shown). By sequence comparison with a solved human B-cyclin crystal structure (5), this hybrid is predicted to include the cyclin box domain of CaClb4 and 10 residues preceding it and the N-terminal domain of CaClb2, including the predicted \( \alpha \) N-terminal helix. In addition, when integrated to replace ScCLB5, this CaCLB2/4 hybrid caused diploidization of S. cerevisiae at a higher rate even than CaClb4 because even without subculturing, the cell population was diploid by FACS analysis. Finally, comparison of the Scclb5Δ::CaCLB2/4 diploid FACS pattern to either wild-type or Scclb5Δ diploid S. cerevisiae...
siae indicated, on one hand, suppression of the S-phase delay seen in the ScblΔ mutant and, on the other hand, an increase in the Gi cell population (Fig. 7).

**DISCUSSION**

**Role of CaClb4 in S-phase initiation.** Homology analysis performed here and elsewhere (1) suggested that the *S. cerevisiae* S-phase cyclins Clb5 and Clb6 represent a relatively recent addition to the fungal cyclin complement. This raised the question of which, if any, of the Cdc28 cyclins in the other fungal clades such as *Candida* carries the S-phase cyclin function or whether, indeed, there is a function homologous to ScClb5/6 in *Candida*, the alternative possibility being that S-phase initiation in *Candida* is driven entirely by other kinases such as, e.g., the Dbf4-dependent kinase. We show here that a *C. albicans* B-type cyclin, CaClb4, is the ScClb5 functional homolog because (i) it has higher specificity for the S-phase substrates tested, both from *C. albicans* and from *S. cerevisiae*, and (ii) CaClb4 is able to functionally replace ScClb5 in *S. cerevisiae*.

One possible explanation for the *in vivo* complementation of ScclbΔ by CaCLB4 expressed under the promoter of ScCLB5 was that the timing of expression is what matters for cyclin function, rather than a distinct substrate specificity of the cyclin. This possibility is, however, contradicted by two observations: first, CaCLB2 expressed under the promoter of ScCLB5 is unable to complement the ScclbΔ S-phase defect (Fig. 3). Second, neither ScCLB2 nor the closest yeast homolog of CaCLB4, namely, ScCLB4, is able to replace ScCLB5 in S-phase initiation when placed under the ScCLB5 promoter (13, 16). Thus, the simplest conclusion about the *in vivo* phenotypes is that the ability of CaClb4 to replace the ScClb5 S-phase cyclin is due to the intrinsic activity of the CaClb4-associated complex.

The ability of CaClb4, but not CaClb2, ScClb2, or ScClb4, to replace ScClb5 *in vivo* could be due to cyclin-associated substrate specificity; alternatively, it could be due to differential sensitivity of different cyclin-CDK complexes to inhibitors of Cdk1 activity. One such inhibitor is the CDK inhibitor Sic1 (in *S. cerevisiae*) (40) and its *C. albicans* homolog Ssl1 (2). We find, however, that CaClb4/Cdc28 is equally as sensitive to Sic1 inhibition as CaClb2/Cdc28 (Fig. 4). Another mechanism of inhibition is via inhibitory phosphorylation of Cdc28 on Tyr19 by the tyrosine kinase Sww1; different Clb-Cdk-associated CDK complexes are differentially sensitive to this type of inhibition, with Clb2-Cdc28 complexes being most sensitive, Clb4-Cdc28 being moderately sensitive, and Clb5-Cdc28 being insensitive (24). Thus, the *in vivo* complementation data could not rule out that the ability of CaClb4 to replace ScClb5 in *S. cerevisiae* is due to reduced sensitivity to Sww1 inhibition compared to CaClb2. The *in vitro* kinase activity data showing equal or higher relative activity of the CaClb2-associated complex on the generic substrate histone H1, are not consistent, however, with a differential sensitivity to inhibitory phosphorylations. Rather, the *in vitro* kinase assays suggest that CaClb4 is able to replace ScClb5 thanks to its similar affinity for S-phase substrates.

We were unable to directly analyze the effect of Caclb4 mutations in *C. albicans* because these mutants are not amenable to cell cycle analysis. *C. albicans* often switches to a hyphal morphology upon disturbance of normal cell cycle progression. In particular, the Caclb4−/− mutant, as well as the Caclb4+/− MET3-CaCLB4 and Caclb2−/− MET3-CaCLB2 strains grown under MET3-repressing conditions, rapidly forms pseudohyphae, as previously reported (4), to an extent that prohibits cell cycle distribution measurements. We do note, however, that in a recent global cell cycle expression study, CaCLB4 was shown to be periodically expressed, with a peak of expression around the Gi/S transition (10). Analysis of the expression data indicates that the peak of CaCLB4 expression is 18% earlier (expressed as percentage of the cell cycle length) than the median histone expression peak, a hallmark of S phase. This is comparable to the peak of ScCLB5 expression in *S. cerevisiae*, which precedes histone expression by 25%. These data are thus consistent with a role for CaClb4 in S-phase initiation in *C. albicans* that is similar to the role of ScClb5 in *S. cerevisiae*.

**Effect of CaClb4 on *S. cerevisiae* ploidy.** An unexpected consequence of expressing CaCLB4 in *S. cerevisiae* was a relatively rapid diploidization of the strain. The diploids that were obtained in the ScclbΔ::CaCLB4 strains were of the *MATa*/*MATa* and *MATa/*MATa types, suggesting that they did not arise as a consequence of mating-type switching but, rather, as a consequence of either an endoreplication or an endomitosis event. Several yeast mutants were previously reported to cause increases in ploidy in the absence of mating-type switching. Many of the mutant genes (*CDC31, ESP1, SPA1, KARI, NDC1, MPS1,* and *MPS2*) encode components or interacting
proteins of the SPB (reference 7 and references within). Therefore, it seems likely that defects in SPB dynamics constitute one mechanism by which *S. cerevisiae* can diploidize, presumably as a consequence of endomitosis. SPBs undergo a duplication and segregation cycle that is coordinated with the cell cycle: the SPB is duplicated during the G$_1$ phase; the two SPBs separate during S phase and move further apart during anaphase, at the end of which they are segregated between the mother and the daughter cell (11). The activity of several cyclin/CDK complexes is crucial to the SPB cycle: SPB duplication requires Cln/Cdk1, and SPB separation requires Cln5/6 although other B-type cyclins can carry out this function in the absence of Clb5/6 (23). In addition, the presence of Clb5/6 alone causes reduplication of the SPB, an activity that is apparently counteracted by Clb1 to Clb4 (23). Thus, it is possible that the unexpected diploidization of the *S. cerevisiae* CLB4 strain is due to interference in SPB dynamics. This assumption is supported by the high level of premature SPB separation in the *clb5* null strain. This function was taken over by Clb5/6 in the *clb5* null strain.

Evolution of B-cyclin substrate specificity. The simplest scenario for the evolution of substrate specificity by B-type cyclins is that the ancestral Clb4 did carry specificity for S-phase substrates; this function was taken over by Clb1 to Clb4 (23). Thus, it is possible that the unexpected diploidization of the *S. cerevisiae* CLB4 strain is due to interference in SPB dynamics. This assumption is supported by the high level of premature SPB separation in the *clb5* null strain. This function was taken over by Clb5/6 in the *clb5* null strain.

486 of CaClb4 (CaClb21–221/CaClb4248–486) and carrying the conserved in CaClb4-overexpressing cells. This observation could reflect a role of CaClb4 in SPB dynamics, similar to that of ScClb5. The stability of CaClb4 in *S. cerevisiae*, in contrast to its normal rapid degradation in *C. albicans* (Fig. 6C), could be the cause of interference with SPB dynamics in the Scclb5Δ::CaCLB4 strain.

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