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Dissection of mitotic functions of the yeast cyclin Clb2

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Abbreviations: APC/C, anaphase promoting complex/cyclosome; CDK, cyclin dependent kinase; GFP, green fluorescent protein; SPB, spindle pole body

Progression through mitosis requires the activity of cyclin-dependent kinases (CDKs) associated with regulatory cyclin subunits. In the yeast *Saccharomyces cerevisiae*, Clb2 has the most important role among the four mitotic cyclins, Clb1-4, manifested by data showing that simultaneous deletion of the *CLB1*, *CLB3* and *CLB4* genes has only minor effects on mitosis. Thus, Clb2 alone is sufficient for all essential CDK functions in mitosis, such as the assembly of bipolar spindles and spindle elongation. Here, we show that a modification of Clb2, by the C-terminal addition of a Myc12 epitope, causes the loss of one specific mitotic function of Clb2. Strains carrying *CLB2-MYC12* are nonviable in the absence of the *CLB3* and *CLB4* genes, because the modified Clb2 version fails to promote assembly of the mitotic spindle. In contrast, Clb2-Myc12 has no apparent defects in late mitotic functions and, furthermore, induces the switch from polarized to isotropic growth with similar efficiency as the endogenous Clb2. Thus, the presence of the Myc12 epitope selectively inactivates Clb2's capacity to promote spindle formation. Clb2-Myc12 represents therefore the first version of Clb2 impaired in one specific mitotic function. We conclude that the major mitotic functions of this cyclin can be unequivocally dissected.

Introduction

Cyclin-dependent kinases (Cdks) are key factors for the progression of the eukaryotic cell cycle. Their regulatory subunits, termed cyclins, are unstable proteins, whose abundance fluctuates during the cell cycle. They have fundamental roles for cell cycle regulation of Cdk activity.

Progression through mitosis requires activation of Cdks by mitotic cyclins.[1,2] The yeast *Saccharomyces cerevisiae* has four mitotic cyclins, termed Clb1-4, which have overlapping functions. Inactivation of all four cyclins results in a cell cycle arrest due to defects in the assembly of a mitotic spindle.[3-5] An additional characteristic of these cells is their highly elongated shape because they are impaired in the switch from polarized to isotropic growth.

Already the initial characterization of *CLB* genes in the early 90's revealed that Clb2 is the main mitotic cyclin in yeast.[3,4] A simultaneous deletion of the three *CLB1*, *CLB3* and *CLB4* genes did not cause distinct defects in mitosis. In contrast, a deletion of the *CLB2* gene in combination with one of the *CLB1* or *CLB3* genes resulted in drastic mitotic defects, and even the single deletion of *CLB2* caused delayed progression through mitosis and an elongated cell shape.[6]

The *CLB3* and *CLB4* genes are expressed about 20 minutes earlier than the *CLB1* and *CLB2* genes.[3] In fact, Clb3 and Clb4 have primarily roles in processes required for early mitosis, such as the separation of spindle pole bodies and the formation of bipolar mitotic spindles. Since *clb1*Δ *clb3*Δ *clb4*Δ and *clb3*Δ *clb4*Δ mutants are able to undergo mitosis, it is obvious that, despite its delayed expression, Clb2 can replace Clb3 and Clb4 in triggering spindle assembly.

It was also thought that the S-phase cyclin Clb5 participates in spindle assembly, because a deletion of *CLB5* combined with *clb3*Δ *clb4*Δ results in a G_2/M arrest, due to a failure to form bipolar spindles.[7] Later data however argued against a direct function of Clb5 in this process. Clb5 is rather involved in the inactivation of the anaphase promoting complex/cyclosome (APC/C), associated with its co-activator Cdh1.[8] Cdk1/Clb5 activity is thereby required to enable the efficient accumulation of Clb2.

In accordance with their delayed expression, Clb1 and Clb2 have important roles in later periods of mitosis, after the spindle has formed. It was recently shown that these functions include the activation of APC/C-Cdc20, thereby enabling the dissolution of sister chromatid cohesion by activating separase.[9] This study also demonstrated that Clb1 and Clb2 promote the elongation of the mitotic spindle during anaphase.

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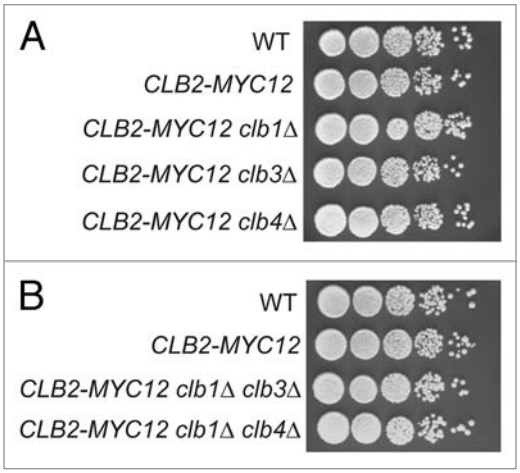

Figure 1. A *CLB2-MYC12* gene fusion combined with various *CLB* gene deletions has no apparent growth defect. Serial dilutions (1:10) spotted on YEPD agar plates. Strains were incubated at 30°C for 2, 5 days. Strains used are (from top): (A) S001/S519/S645/S646/S647; (B) S001/S519/S648/S649.

Here, we show that a modified version of Clb2, containing a large C-terminal epitope tag, is defective in promoting the assembly of the mitotic spindle. As a consequence, *CLB2-MYC12 clb3*Δ *clb4*Δ strains are nonviable. Intriguingly, *CLB2-MYC12 clb1*Δ strains are viable and have no discernible growth defects, suggesting that this version of Clb2 is functional with respect to functions in late mitosis. Thus, the C-terminal modification selectively inactivates one of the key functions of Clb2, the ability to trigger spindle formation.

Table 1. Numbers of viable segregants resulting of a crossing of a *clb1*Δ *clb3*Δ *clb4*Δ strain (S13) with *a CLB2-MYC12* strain (S519), 68 tetrads were analyzed

Genotype	No. of viable segregants
WT	20
CLB2-MYC12	15
*clb1*Δ	15
*CLB2-MYC12 clb1*Δ	21
*clb3*Δ	12
*CLB2-MYC12 clb3*Δ	11
*clb4*Δ	20
*CLB2-MYC12 clb4*Δ	17
*clb1*Δ *clb3*Δ	14
*CLB2-MYC12 clb1*Δ *clb3*Δ	21
*clb1*Δ *clb4*Δ	8
*CLB2-MYC12 clb1*Δ *clb4*Δ	15
*clb3*Δ *clb4*Δ	15
*CLB2-MYC12 clb3*Δ *clb4*Δ	0
*clb1*Δ *clb3*Δ *clb4*Δ	23
*CLB2-MYC12 clb1*Δ *clb3*Δ *clb4*Δ	0

Table 2. Number of viable *clb3*Δ *clb4*Δ segregants resulting of a crossing of a *clb3*Δ *clb4*Δ *GAL-CLB3-HA3* strain (S541) with a *CLB2-MYC12* strain (S518) on YEP + Gal plates

Genotype	No. of viable segregants	Viability on YEPD
*clb3*Δ *clb4*Δ	7	7
*clb3*Δ *clb4*Δ *GAL-CLB3-HA3*	4	4
*CLB2-MYC12 clb3*Δ *clb4*Δ	0	0
*CLB2-MYC12 clb3*Δ *clb4*Δ *GAL-CLB3-HA3*	5	0

18 tetrads were analyzed.

Results

A *CLB2-MYC12* gene fusion fails to take over the functions of the *CLB3* and *CLB4* genes. We intended to construct a yeast strain, which contains a version of Clb2 with 12 Myc epitope tags protein as sole mitotic cyclin. For this purpose, a W303 yeast strain, in which the endogenous *CLB2* gene was replaced by a *CLB2-MYC12* gene fusion, was crossed with a yeast strain deleted for cyclin genes *CLB1*, *CLB3* and *CLB4* (*clb1*Δ *clb3*Δ *clb4*Δ). Upon mating, sporulation and tetrade dissection, we noticed that no viable *CLB2-MYC12 clb1*Δ *clb3*Δ *clb4*Δ segregants were obtained (**Table 1**). Similarly, we did not get segregants containing *CLB2-MYC12* in combination with *clb3*Δ *clb4*Δ double deletions. All other combinations were viable and produced colonies. Microscopic examination showed that spores, which did not produce colonies, were mostly germinated and that cells underwent at least one cell division. This observation provides evidence that *CLB2-MYC12* is synthetic lethal when combined with the *clb3*Δ *clb4*Δ double deletion. A possible lethality was further analyzed by crossing a *CLB2-MYC12* strain with a *clb3*Δ *clb4*Δ mutant. Tetrade analysis revealed no viable *CLB2-MYC12 clb3*Δ *clb4*Δ segregant (data not shown).

In a striking contrast to *CLB2-MYC12 clb3*Δ *clb4*Δ, each of segregants containing *CLB2-MYC12* in combination with single *clb1*Δ, *clb3*Δ or *clb4*Δ deletions as well as in combinations with *clb1*Δ *clb3*Δ or *clb1*Δ*clb4*Δ double deletions were viable and displayed no appreciable growth defects (**Fig. 1**). If the *CLB2-Myc12* gene fusion were an inactive version of Clb2, then one would expect that most of these strains would be nonviable, like e.g., *clb1*Δ *clb2*Δ or *clb2*Δ *clb3*Δ strains.[3,4] Viability and efficient growth of these strains implies that Clb2-Myc12 is not generally inactive, but only affected in a common process with Clb3 and Clb4.

To further confirm the lethality of *CLB2-MYC12* in the absence of *CLB3* and *CLB4,* we crossed a *CLB2-MYC12* strain with a *clb3*Δ *clb4*Δ strain carrying also *CLB3* (with an HA3-tag) expressed from the galactose inducible *GAL1* promoter. Upon sporulation, tetrads were dissected on plates containing galactose. Several *CLB2-MYC12 clb3*Δ *clb4*Δ segregants carrying *GAL-CLB3-HA3* were viable. However, upon streaking to glucose plates (YEPD), these strains were unable to grow (**Table 2**).

Lethality of these strains was analyzed in more detail by plating serial dilutions on plates containing either galactose or glucose. We found that virtually no colonies were produced by *CLB2-MYC12 clb3*Δ *clb4*Δ strains on glucose medium (**Fig.**

2A). Photographs of cells on glucose plates showed that most cells were unable to undergo more than 1–3 cell divisions (**Fig. 2B**).

The *CLB2-MYC12* gene fusion was verified by sequencing, in order to exclude that artificial mutations are responsible for the phenotype. Thereby, the presence of 12 Myc tags immediately before the stop codon of the *CLB2* gene was confirmed. To further rule out that this phenotype may be an artificial effect, we used an additional, independent strain containing *CLB2-MYC12*, described by Zachariae and Nasmyth.[10] Also this gene fusion was found to be lethal in combination with the *clb3*Δ *clb4*Δ double deletion (**Fig. 2A**).

These data suggest that the epitope tagging results in a modified version of Clb2, which fails to take over functions of Clb3 and Clb4.

The Myc12 epitope causes a loss of Clb2 function. To distinguish whether the tagging results in a loss of function of the Clb2 protein or whether a *CLB2-MYC12* fusion may have dominant effects in the *clb3*Δ *clb4*Δ strain background, we performed a complementation analysis with diploids strains. A *CLB2-MYC12 clb3*Δ *clb4*Δ *GAL-CLB3-HA3* strain was crossed with a *clb3*Δ *clb4*Δ strain and diploids were selected on galactose medium. This strain was fully viable on glucose medium, suggesting that the *CLB2-MYC12* gene fusion has a recessive effect (**Suppl. Fig. S1**). In a similar manner, the presence of a single copy of any of the *CLB3* or *CLB4* genes restored growth to *CLB2-MYC12 clb3*Δ *clb4*Δ strains (**Fig. S1**).

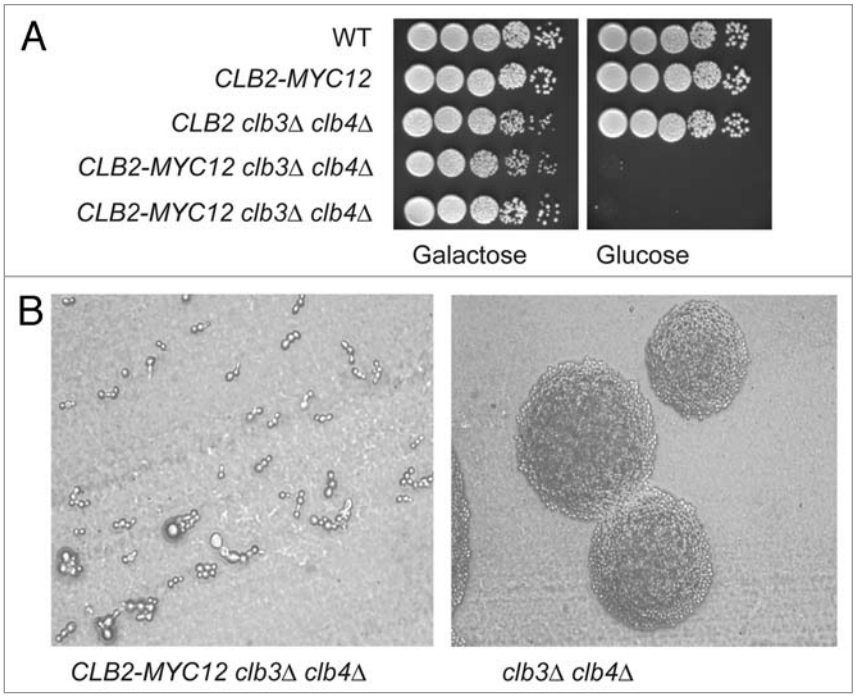

Figure 2. *CLB2-MYC12* is synthetic lethal with *clb3*Δ *clb4*Δ mutations. (A) Wild-type (S001), *CLB2-MYC12* (S519) and *clb3*Δ *clb4*Δ (S541) strains as well as two independent *CLB2-MYC12 clb3*Δ *clb4*Δ strains (upper: S539, lower: S575), both kept alive by a *GAL-CLB3-HA3* construct, were pregrown on galactose plates, transferred to liquid YEP medium and spotted in serial dilutions on agar plates containing either glucose or galactose. Plates were incubated at 30°C for 2, 5 days. (B) Photographs of strains S539 (left) and S541 (right) on agar plates containing glucose, after an incubation period of 24 hours.

It could be argued that the lethality of the *CLB2-MYC12 clb3*Δ *clb4*Δ strain is not due to a loss of Clb2 function, but rather because the large epitope tag may interfere with expression of the *CLB5* gene, located adjacent to the *CLB2* gene. Indeed, *clb3*Δ *clb4*Δ *clb5*Δ strains are nonviable.[7] To address this possibility, we crossed the *CLB2-MYC12 clb3*Δ *clb4*Δ strain with a *clb3*Δ *clb4*Δ *clb5*Δ strain to produce diploids. If Clb5 function were impaired by the *CLB2-MYC12* gene fusion, then diploids would be unable to grow. However, diploids were fully viable, suggesting that Clb5 is functional in *CLB2-MYC12* strains and that the tagging did not affect Clb5, but solely Clb2 function (**Fig. S2**). Indeed, crossing of *CLB2-MYC12 clb3*Δ *clb4*Δ with a *clb2*Δ *clb3*Δ *clb4*Δ strains resulted in non-viable diploids.

In summary, this genetic analysis demonstrates that the *CLB2-MYC12* gene fusion results in a loss of a Clb2 function.

***CLB2-MYC12 clb3*Δ *clb4*Δ strains are impaired in spindle formation.** To examine the cell division defect of *CLB2-MYC12 clb3*Δ *clb4*Δ strains, cells kept alive by a *GAL-CLB3-HA3* fusion were pre-grown in galactose medium and then shifted to glucose medium. 5 hours after the shift, the majority of cells were large-budded. Visualization of nuclei and spindles revealed that virtually all these cells displayed failures to assemble a bipolar spindle (**Fig. 3A**), thereby resembling the previously described phenotype of strains lacking all four mitotic cyclins.[3,4] As a consequence, cells arrested with undivided nuclei.

To examine this cell cycle arrest in synchronized yeast cultures, this strain and, as control, a *clb3*Δ *clb4*Δ strain, were pregrown in galactose medium and then arrested in G_1-phase with the pheromone α-factor. Glucose was added and cells were subsequently released into glucose medium. Budding was initiated in both strains with a similar kinetics. Microscopic examination showed that the *clb3*Δ *clb4*Δ strain was able to undergo mitosis and G_1-phase, manifested by the accumulation of a substantial fraction of unbudded cells (**Fig. 3B**). In contrast, more than 90% of cells of the *CLB2-MYC12 clb3*Δ *clb4*Δ strain accumulated as large-budded cells. Immunofluorescence microscopy showed that these cells failed to form bipolar spindles (**Fig. 3C**).

We conclude that, in contrast to Clb2, Clb2-Myc12 is unable to promote spindle formation.

Prior to the assembly of the mitotic spindle, spindle pole bodies (SPB) initially connected by a bridge-like structure need to be separated.[11] The structure of SPBs in cells containing Clb2-Myc12 is at present unknown. However, it is likely that these cells have non-separated SPBs, because astral microtubules in virtually all cells emanate from one single dot suggesting that the two SPBs are at a common location (**Fig. 3A and C**).

Microscopic examination further showed that *CLB2-MYC12 clb3*Δ *clb4*Δ strains had a mostly round or oval shape on glucose

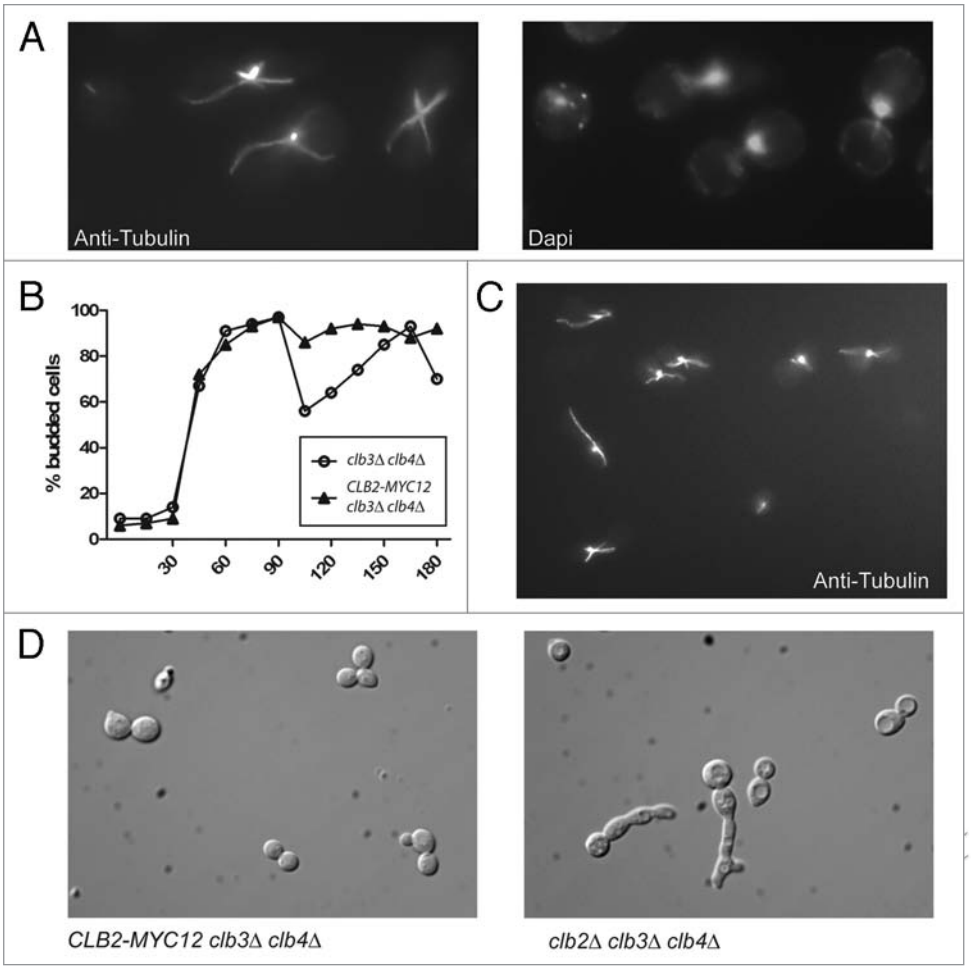

Figure 3. *CLB2-MYC12 clb3Δ clb4Δ* strains accumulate as large budded cells containing undivided nuclei and lacking bipolar spindles. (A) A *CLB2-MYC12 clb3Δ clb4Δ* strain containing *GAL-CLB3-HA3* (S575) was pre-grown in YEP + Gal medium to exponential growth phase, centrifuged and then transferred to YEPD medium. After a 5 hours incubation period in YEPD, cells were analyzed by immunofluorescence microscopy, using DAPI and anti-tubulin antibodies to visualize nuclei or spindles, respectively. (B and C) A *clb3Δ clb4Δ* strain containing *GAL-CLB3-HA3* (S541) and S575 were pregrown in YEP + Gal and then treated with the pheromone α-factor to synchronize cells in G_1 phase. After 2 hours, glucose was added and cells were incubated for additional 45 minutes in the presence of pheromone. Then cells were washed to remove the pheromone and transferred to fresh YEPD medium. Release from the G_1 arrest and progression through the cell cycle was examined by counting the percentage of budded cells (B). (D) Morphology of strain S575 compared to a *clb2Δ clb3Δ clb4Δ GAL-CLB3-HA3* strain (S652) five hours after transfer to YEPD medium.

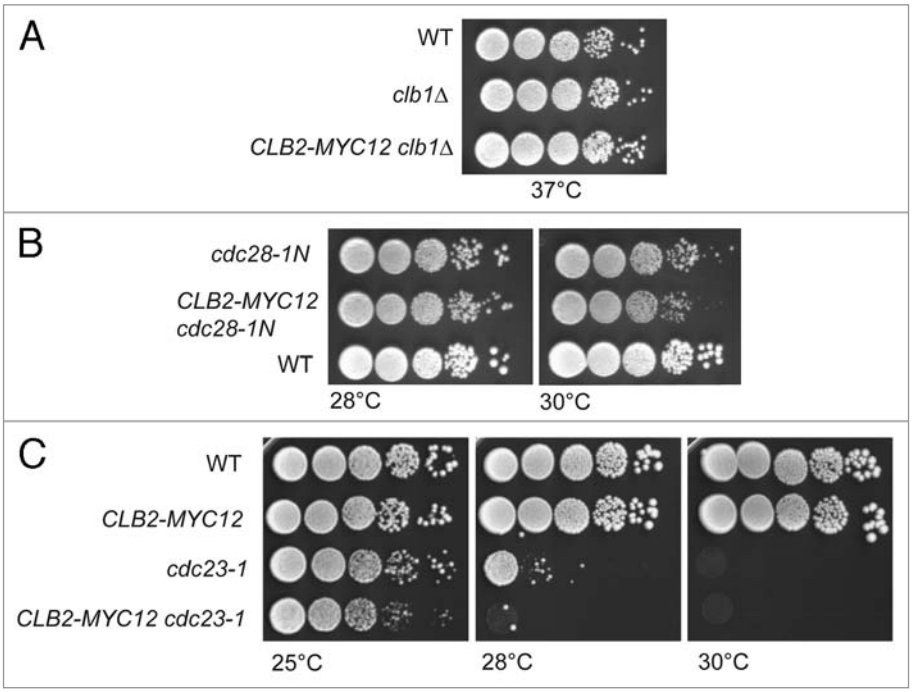

Figure 4. Viability of strains containing *CLB2-MYC12* and either *clb1Δ cdc28-1N* or *cdc23-1* mutations. (A) Serial dilutions were incubated at 37°C. Strains used are (from top): S001/S702/S644. (B and C) Serial dilutions of strains incubated at indicated temperatures. Prior to dilutions, strains were pre-grown at 25°C. Strains used are (from top): S247/S715/S001 (B) and S001/S086/S265/S703 (C).

plates, which is in contrast to *clb2*Δ *clb3*Δ *clb4*Δ strains containing frequently elongated cells (**Fig. 3D**). Thus, it appears that the presence of *CLB2-MYC12* results in isotropic growth of cells.

Clb2-Myc12 efficiently executes Clb2 functions in later periods of mitosis. The finding that strains containing *CLB2-MYC12* are viable in the absence of the *CLB1* gene (**Fig. 1**) argues that the tagged protein is active with respect to the shared functions of Clb1 and Clb2. These two cyclins are important for processes following spindle assembly, such as sister chromatid separation and spindle elongation.[9] *CLB2-MYC12 clb1*Δ strains have no apparent growth defects even at 37°C (**Fig. 4A**). This clearly indicates that Clb2-Myc12 has retained its ability to bind and activate Cdk1.

To further assess the functionality of Clb2-Myc12, we crossed strains containing the *CLB2-MYC12* allele with temperature-sensitive *cdc28-1N* and *cdc23-1* mutants, which are defective in the metaphase/anaphase transition. *cdc28-1N* mutants arrest at the restrictive temperature with short spindles due to an impaired function of Cdk1.[6] *cdc23-1* mutants, which are defective in APC/C function, arrest in metaphase because of a failure to separate sister chromatids.[12] Combinations of either of these alleles with a *clb2* deletion are known to be synthetic lethal at permissive temperatures, 25°C.[6,12,13] In contrast, we found that both *CLB2-MYC12 cdc28-1N* and *CLB2-MYC12 cdc23-1* segregants were viable (**Fig. 4B and C**). We analyzed growth of these strains at elevated, semi-permissive temperatures. *cdc28-1N* and *CLB2-MYC12 cdc28-1N* had a similar viability at 28°C and 30°C. A modest growth reduction of *CLB2-MYC12* was observed in the *cdc23-1* background at 28°C (**Fig. 4C**).

All these data suggest that Clb2-Myc12 has retained most of its activity for processes following spindle formation.

CLB2-Myc12 promotes the switch to isotropic growth with similar efficiency as Clb2. A further collective function of Clb1 and Clb2 is the switch from polarized to isotropic growth. *clb2*Δ mutants are characterized by a moderately elongated cell shape and *clb1*Δ *clb2*Δ cells are highly elongated.[6]

A closer examination of the *CLB2-MYC12* strains revealed that cells were mostly roundish (**Fig. 5**). Remarkably, even a *CLB2-MYC12 clb1*Δ does not display morphological characteristics distinctly different from wild-type strains. This is a striking contrast to *clb1*Δ *clb2*Δ strains, kept alive by a *GAL-CLB2* fusion, after transfer to glucose medium.

These findings show that Clb2-Myc12 can promote the shift

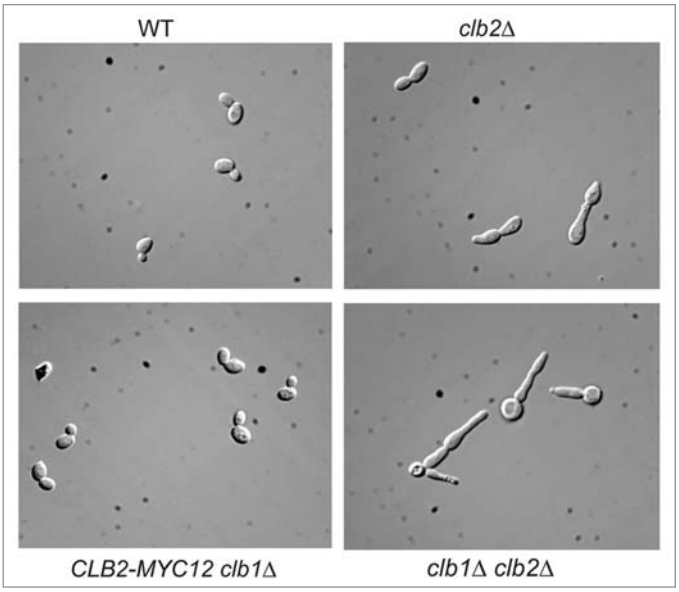

Figure 5. *CLB2-MYC12* is efficiently functional in promoting isotropic growth even in the absence of the *CLB1* gene. Photographs of cells of logarithmic cultures in liquid YEPD medium, except strains S018, which is nonviable on glucose medium. Strain S018 was pregrown in YEP + Gal medium and subsequently incubated in YEPD medium for 5 hours. Strains used are S001 and S008 (top) and S644 and S018 (bottom).

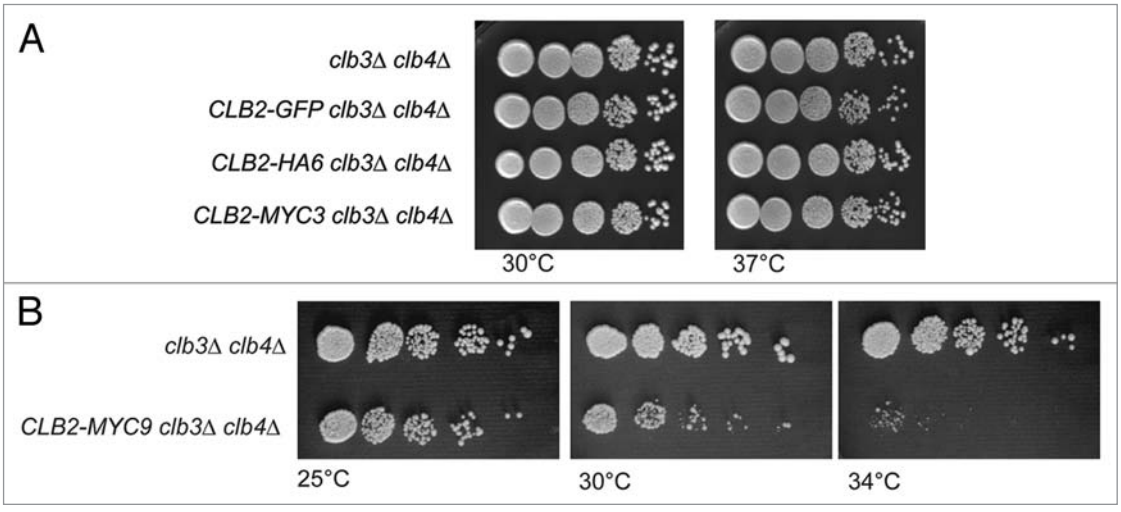

Figure 6. Short epitopes and GFP do not interfere with Clb2 function in the *clb3*Δ *clb4*Δ background, but a Myc9 epitope causes a temperature-dependent phenotype. (A and B) Serial dilutions of strains pre-grown on YEPD plates were incubated at indicated temperatures. Strains used are (from top): S014/S697/S698/S768 (A) and S014/S687 (B).

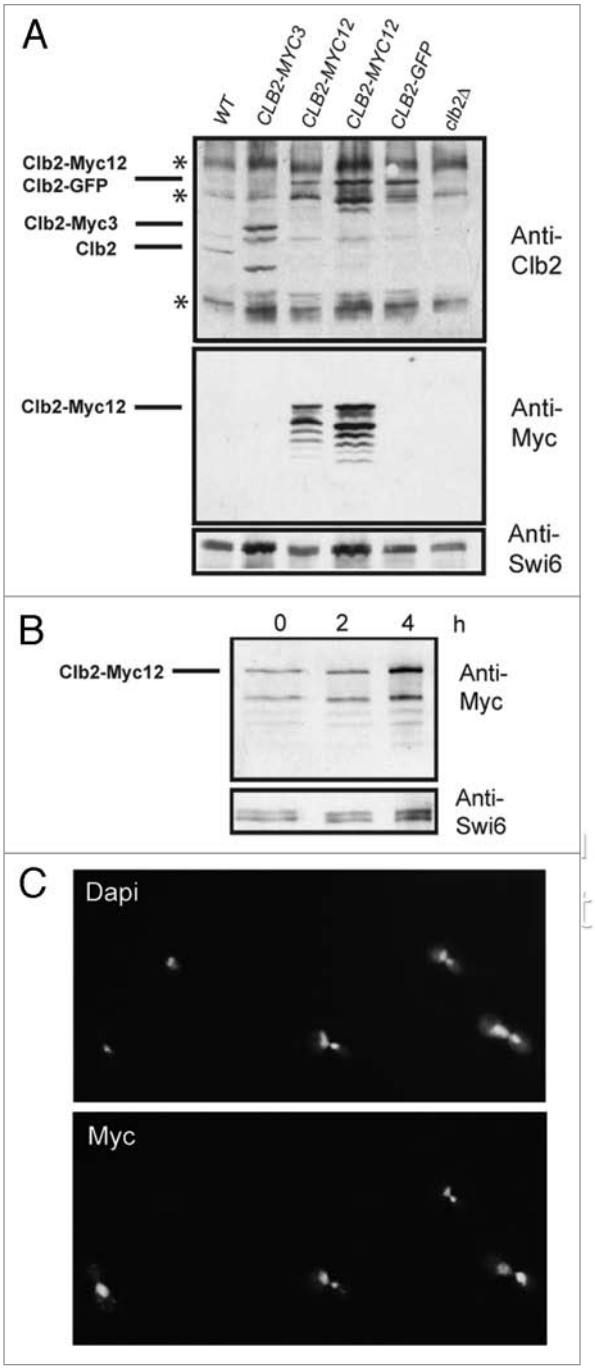

Figure 7. Protein levels and nuclear localization of Clb2-Myc12. (A) Immunoblotting of cell extracts of strains carrying different C-terminally tagged versions of Clb2. A wild-type strain (S001), a *CLB2-MYC3* strain (S695), two independent *CLB2-MYC12* strains (S519 and S86; lines 3 and 4, respectively), a *CLB2-GFP* strain (S699) and a *clb2*Δ strain (S008) were grown to logarithmic phase before breaking cells and preparation of cell extracts. An anti-Clb2 antibody was used to detect tagged proteins as well as endogenous Clb2 from a wild-type strain. Note that Clb2-GFP has approximately the same size as Clb2-Myc12. Unspecific background signals obtained with the Clb2 antibody are marked with asterisks. An anti-Myc antibody was used to visualize specifically Myc-tagged proteins. Clb2-Myc3 was not detected, probably because of the small size of the tag. Swi6 was used as loading control. (B and C) A *CLB2-MYC12 clb3*Δ *clb4*Δ strain, containing a *GAL-CLB3-HA3* fusion (S575) was pre-grown in YEP + Gal medium and then transferred to YEPD medium. Samples were collected at 0, 2 and 4 hours after the transfer. For immunoblotting, anti-Myc and anti-Swi6 (loading control) antibodies were used (B). Immunofluorescence microscopy of representative cells of S575 using DAPI and anti-Myc antibody to visualize nuclei and Clb2-Myc12, respectively (C).

from polarized to isotropic growth with a similar efficiency as endogenous Clb2 implying that the epitope tag does not affect this specific function of Clb2.

The Myc epitope affects Clb2 in a size-dependent manner. We next addressed the question whether the loss of a specific function of Clb2-Myc12 is due to the presence of a long epitope tag or whether modifications at the C-terminal region generally interfere with Clb2 function.

To test this, Clb2 was fused with various tags of different sizes: Myc3, HA6 and GFP. GFP is a relatively large tag comparable to Myc12. We crossed strains containing these gene fusions with a *clb3*Δ *clb4*Δ strain and found that all segregants containing tagged Clb2 were viable in the *clb3*Δ *clb4*Δ background (**Fig. 6A**). Serial dilutions showed that neither small epitopes nor the GFP had any detectable effects on Clb2 activity, even at 37°C. Therefore the loss of function is not generally caused by C-terminal modifications, but seems to be specific for large Myc tags.

Since a short Myc3 epitope had no effect on Clb2 activity, we further examined the dependence of the length of the Myc epitope on Clb2 function. To this end, nine repeats of the Myc epitope were added to the C-terminal end of Clb2. Viable segregants carrying *CLB2-MYC9* combined with the *clb3*Δ *clb4*Δ deletion were obtained. Growth of this strain was modestly affected at 25°C. Serial dilutions incubated at different temperatures revealed that *CLB2-MYC9* caused a temperature-dependent phenotype (**Fig. 6B**). *CLB2-MYC9 clb3*Δ *clb4*Δ were retarded in growth at 30°C and virtually nonviable at 34°C.

We conclude that the Myc epitope eliminates Clb2 function in a size-dependent manner, suggesting large tags may act as sterical hindrances, which may interfere with the interaction of Clb2 with substrates or with its proper intracellular localization.

Clb2-Myc12 protein is abundant and predominantly nuclear. It may be argued that large Myc epitope tags results in reduced protein levels, perhaps by causing protein instability or inefficient translation. Therefore, we examined whether the defect of Clb2-Myc12 may be due to low protein levels. Clb2 levels in strains containing various tagged versions of Clb2 were compared by immunoblotting. We found that Clb2-Myc12 is present in similar amounts as Clb2, Clb2-Myc3 and Clb2-GFP proteins (**Fig. 7A**). Importantly, Clb2-Myc12 levels are not reduced compared to Clb2-GFP, which has a similar molecular weight and is fully functional (**Fig. 6A**).

We also addressed the question whether the cell cycle arrest of *CLB2-MYC12 clb3*Δ *clb4*Δ strains may be caused by low Clb2-Myc12 protein level. By shifting a *CLB2-MYC12 clb3*Δ *clb4*Δ strain containing *GAL-CLB3-HA3* to glucose medium, we found that Clb2-Myc12 remained on high levels during a 4 hours incubation time (**Fig. 7B**).

Clb2 was shown to be localized mostly to the nucleus.[14,15] To test, whether the Myc12 epitope tag may interfere with the import of the fusion protein into the nucleus, we performed

immunofluorescence microscopy with anti-Myc antibody. Localization studies showed that the fusion protein is predominantly nuclear (**Fig. 7C**).

Thus, we conclude that the mitotic defect of strains containing *CLB2-MYC12* is neither caused by reduced protein levels nor by impaired nuclear import.

Discussion

Selective inactivation of a specific mitotic function of Clb2. It is known for almost two decades that Clb2 has the most prominent function among the four mitotic cyclins in *Saccharomyces cerevisiae*. This was manifested by the analysis of single deletions, and by combining all combinations of double and triple deletions of *CLB* cyclin genes.[3,4,6] The conclusion from these data was, that Clb2 alone is sufficient to trigger all essential functions of cyclin-dependent kinases in mitosis, including assembly of a bipolar mitotic spindle, activation of the ubiquitin ligase APC/C to induce sister chromatid separation and elongation of the spindle.

In this study, we showed that a modified version of Clb2, carrying a Myc12 epitope, is specifically defective in one of these processes, in spindle formation. We found that *CLB2-MYC12 clb3*Δ *clb4*Δ strains, which contain the tagged *CLB2* version in place of the endogenous *CLB2* gene, are nonviable. In contrast to wild-type Clb2, Clb2-Myc12 obviously fails to take over the functions of Clb3 and Clb4. As a consequence cells are blocked in mitosis because they fail to form a spindle, in analogy to the cell cycle arrest of a strain deleted of all four mitotic cyclins.[3,4] Thus, the *CLB2-MYC12* allele causes a similar phenotype as a *clb2* deletion. The distinct cell cycle defect indicates that the presence of the Myc12 epitope tag causes a complete loss of Clb2's capacity to promote spindle formation.

In a striking contrast, Clb2-Myc12 appears to be potently active with respect to other mitotic processes. *CLB2-MYC12 clb1*Δ strains have no apparent growth defects, implying that the fusion protein can take over the common functions of Clb1/Clb2. In a recent report by the Amon lab, it was demonstrated that Clb1 and Clb2 have pivotal roles in late periods of mitosis, after the bipolar spindle is assembled.[9] By using a temperature-sensitive *clb1*Δ *clb2-ts* strain, the authors showed that Cdk1-Clb1/Clb2 is required for APC/C activation and elongation of the anaphase spindle. The effective growth of *CLB2-MYC12 clb1*Δ strains implies that Clb2-Myc12 can efficiently trigger these processes.

Consistent with this model are the data with temperature-sensitive *cdc28-1N* and *cdc23-1* mutants. At the restrictive temperatures, both *cdc28-1N* and *cdc23-1* mutants are able to assemble a bipolar spindle, but fail to undergo the metaphase/anaphase transition. It is known that a *clb2* deletion is lethal for both *cdc28-1N* and *cdc23-1* mutants at 25°C.[6,12] In contrast, both of these mutants carrying *CLB2-MYC12* instead of the endogenous *CLB2* gene are viable, displaying at most minor growth defects at semipermissive temperatures (**Fig. 4B and C**). The viability of each of the *clb1*Δ, *cdc28-1N* and *cdc23-1* mutants carrying *CLB2-MYC12* indicates that the epitope tagged protein has largely retained its activity for functions following spindle assembly.

Clb1 and Clb2 are also required for the switch from polarized to isotropic growth. In a strain lacking both cyclins, growth is highly polarized and even a *clb2*Δ strain is characterized by an elongated cell shape.[6] Clb2-Myc12 retained the function in promoting isotropic growth, which is most pronounced when comparing the cell shape of *CLB2-MYC12 clb1*Δ strains with the highly elongated cells produced by *clb1*Δ *clb2*Δ strains (**Fig. 5**).

Taken together, Clb2-Myc12 can efficiently perform all of the collective functions of Clb1 and Clb2, but is nonfunctional with respect to the joint role of cyclins Clb2, Clb3 and Clb4 in promoting spindle formation. The data presented here show for the first time that functions of Clb2 can be unequivocally separated.

A C-terminal epitope affecting Clb2 function in a size-dependent manner. The characterization of the *CLB2-MYC12* gene fusion demonstrates that the tagging of a protein can severely affect its activity, but that a loss of function may not be recognized in wild-type strains, due to redundancies with other proteins.

How may this epitope tag affect Clb2 function? By constructing Clb2 proteins containing different repeats of the Myc epitope, we found that this epitope interferes with Clb2 function in a size-dependent manner. A version with nine epitopes resulted in partial growth defect at high temperatures, whereas three epitopes had no effect.

However, the size of a C-terminal tag is evidently not the only cause for an impaired Clb2 function, but possibly also its structure. GFP, a polypeptide of similar size as Myc12, has no appreciable effect on Clb2 activity. Clb2-GFP has been applied for earlier localization studies, implying that GFP properly folds and adopts a defined 3D structure.[14,15] In contrast to GFP, it is unlikely that a large Myc tag, 12 repeats of a short amino acid motif, has a defined structure, but may rather be a long tail extruding from the protein. Each Myc repeat, EQKLISEEDL, has three acidic amino acids. It is tempting to speculate that an unstructured tail and/or the presence of many negative charges could prevent either the access of Clb2 to certain subcellular locations or its interaction with specific substrates.

Like endogenous Clb2,[14] Clb2-Myc12 is mostly localized in the nucleus, arguing against an impaired nuclear import of this fusion protein (**Fig. 7C**). We cannot rule out that import of Clb2-Myc12 is less effective than of Clb2. However, it is known that reducing Clb2 in the nucleus by eliminating nuclear localization signals does not cause growth defects.[14] A fraction of Clb2 is known to be localized in the cytoplasm, where the protein is found at the bud neck.[14,15] We did not detect a bud neck localization of Clb2-Myc12 by indirect immunofluorescence. It was shown that impaired bud neck localization is known to cause enhanced polarized growth, a phenotype not seen with strains carrying Clb2-Myc12. Furthermore, a Clb2 version lacking a nuclear export signal has no distinct growth defects.[14] We conclude that it is unlikely that the Myc12 epitope primarily affects nucleocytoplasmic transport of Clb2.

Possibly, the large epitope interferes with the interaction of Clb2 with other proteins. A crucial interaction partner of Clb2 is Cdk1. Our data clearly argue against a weakening of this interaction, because we found that Clb2-Myc12 is effective in executing

Table 3. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source
S001	W303-1A wild-type strain *MATa*, *ade2-1, trp1-1. leu2-3,112, his3-11,15, ura3, can1-100, GAL, psi*$^{+}$	R. Rothstein
S008	*MATa, clb2::LEU2*	K. Nasmyth
S013	*MATa, clb1::URA3, clb3::TRP1, clb4::HIS3*	K. Nasmyth
S014	*MATa, clb3::TRP1, clb4::HIS3*	K. Nasmyth
S018	*MATa, clb1::URA3, clb2::LEU2, GAL-CLB2/TRP1*	K. Nasmyth
S086	*MATa, CLB2-MYC12/URA3*	K. Nasmyth
S247	*MATa, cdc28-1N*	K. Nasmyth
S265	*MATa, cdc23-1*	K. Nasmyth
S518	*MATα, CLB2-MYC12/Kan-R*	S. Kron
S519	*MATa, CLB2-MYC12/Kan-R*	S. Kron
S539	*MATα, CLB2-MYC12/Kan-R, clb3::TRP1, clb4::HIS3 GAL-CLB3-HA3::URA3*	this work
S541	*MATa, clb3::TRP1, clb4::HIS3 GAL-CLB3-HA3::URA3*	this work
S575	*MATa, CLB2-MYC12/URA3 clb3::TRP1, clb4::HIS3 GAL-CLB3-HA3:URA3*	this work
S644	*MATα, CLB2-MYC12/Kan-R, clb1::URA3*	this work
S646	*MATα, CLB2-MYC12/Kan-R, clb3::TRP1*	this work
S647	*MATa, CLB2-MYC12/Kan-R, clb4::HIS3*	this work
S648	*MATα, CLB2-MYC12/Kan-R, clb1::URA3, clb3::TRP1*	this work
S649	*MATα, CLB2-MYC12/Kan-R, clb1::URA3, clb4::HIS3*	this work
S652	*MATa, clb2::LEU2, clb3::TRP1, clb4::HIS3, GAL-CLB3-HA3 (URA3)*	this work
S675	*MATa, clb3::TRP1, clb4::HIS3, clb5::URA3, GAL-CLB3-HA3 (URA3)*	this work
S687	*MATa, CLB2-MYC9/TRP1, clb3::TRP1, clb4::HIS3*	this work
S695	*MATα, CLB2-MYC3/Kan-R*	this work
S696	*MATα, CLB2-MYC9/TRP1*	this work
S697	*MATa, CLB2-GFP/Kan-R, clb3::TRP1, clb4::HIS3*	this work
S698	MATa, *CLB2-HA6/TRP1, clb3::TRP1, clb4::HIS3*	this work
S699	*MATα, CLB2-GFP/Kan-R*	this work
S702	*MATα, clb1::URA3*	this work
S703	*MATa, CLB2-MYC12/URA3, cdc23-1*	this work
S709	*MATa, CLB2-MYC12/URA3, clb1::URA3*	this work
S715	*MATa, CLB2-MYC12/URA3, cdc28-1N*	this work
S768	*MATa, CLB2-MYC3/Kan-R, clb3::TRP1, clb4::HIS3*	this work

functions of Clb2 in late mitosis, even in the absence of *CLB1*. Thus, it is evident that Clb2-Myc12 properly binds and activates Cdk1.

It is more likely, that an impaired interaction of Clb2-Myc12 with a specific substrate or with a subset of substrates may be responsible for the defect in spindle formation. Previously, 181 budding yeast Cdk1 substrates were identified,[16] of which 110 were found to be phosphorylated by Cdk1/Clb2.[17] One of these is Cdh1, the co-activator of APC/C. This protein contains eleven Cdk1 phosphorylation sites[18,19] and was reported to be an important substrate with respect to spindle assembly.[20-22] Phosphorylation of Cdh1 is a prerequisite for efficient APC/C inactivation and the accumulation of microtubule associated proteins Ase1, Kip1 and Cin8. All these proteins are substrates of APC/C-Cdh1 and they all are thought to directly trigger SPB separation by mechanical forces. To test the possibility that the Myc12 tag may prevent the interaction of Clb2 with Cdh1 and/or other Cdk1 substrates will be a focus of future experimental approaches.

In summary, we have identified here Clb2-Myc12 as the first version of Clb2, which is affected in one specific mitotic function. We propose that this Clb2 variant with a defined cell cycle defect should be a suitable tool for the future to get more detailed insights into the various functions of this cyclin in mitosis. It may also help to further unravel the outstanding importance of Clb2 among the four yeast mitotic cyclins.

Materials and Methods

Yeast strains. All strains used in this study are derivatives of W303 wild-type strains. Strains are listed in **Table 3**. Two independent yeast strains containing *CLB2-MYC12*, S086 and S519, were received from K. Nasmyth and S. Kron, respectively. Both gene fusions were verified by sequencing, they contained the 12 repeats of the Myc epitope motif EQKLISEEDL inserted after the last codon of the *CLB2* open reading frame. S086 and S519 were crossed with S541 to receive two independent *CLB2-MYC12 clb3*Δ *clb4*Δ strains, S575 and S539, respectively, both kept alive by a *GAL-CLB3-HA3* construct.[23] C-terminal epitope tagging of Clb2 to obtain *CLB2-MYC3*, *CLB2-MYC9*, *CLB2-HA6* and *CLB2-MYC9* was performed as previously described using plasmids pYM4, PYM6, pYM3 and pYM12, respectively.[24] Primers Clb2-S2 (5'GGA CAT TTA TCG ATT ATC GTT TTA GAT ATT TTA AGC ATC TGC CCC TCT TCA TCG ATG AAT TCG AGC TCG-3') and Clb2-S3 (5'-GGC TTT AAA GGT TAG AAA AAA CGG CTA TGA TAT AAT GAC CTT GCA TGA ACG TAC GCT GCA GGT CGA C-3') were used for PCR amplification of each of the cassettes.

Manipulation of yeast strains. Standard genetic procedures were used for yeast strain constructions by transformation or crossings. Diploids strains were constructed by either selection of diploids on selective media or, if a selection was not possible, by pulling zygotes with a micromanipulator from a mating suspension 6–8 hours after mixing *MATa* and *MAT*α strains. All diploid used in this study (see **Fig. S1 and S2**) were verified by confirming asci formation on sporulation media.

Growth conditions. Yeast strains were grown in YEP medium containing 2% of either glucose (YEPD), raffinose (YEP + Raf) or galactose (YEP + Gal). YEP + Gal also contained 1% raffinose as additional carbon source. Prior to induction of the *GAL1* promoter, strains were pre-grown on YEP + Raf medium. 2% galactose (end concentration) was used to induce the *GAL1* promoter. To shut off the *GAL1* promoter, cells were centrifuged, washed and transferred to YEPD medium.

For serial dilutions, strains freshly grown over night on agar plates were transferred with a toothpick into 100 µl liquid YEP medium (w/o carbon source), followed by successive 1:10

dilutions in YEP. Then 10 µl of each dilution were spotted on agar plates.

Cell cycle synchronization. For a pheromone induced G_1 arrest, cells were treated with α-factor (1:1,000 dilution from a 5 mg/ml stock solution). Cells were washed with several volumes medium to remove the pheromone and were then transferred to fresh medium. G_1 arrest and release were monitored by counting the numbers of budded cells.

Immunoblotting and immunofluorescence microscopy. For immunoblotting, cells used were lysed with glass beads and extracts were then used for SDS gel electrophoresis. Clb2 (1:1,000), Swi6 (1:10,000) and Myc (1:1,000) antibodies were used in the indicated dilutions.

Immunofluorescence microscopy was performed as described.[25] Anti-tubulin antibodies (YOL1/34) and anti-myc antibodies were used to analyze spindles and Clb2-Myc12 protein, respectively. 4',6-Diamidino-2-phenylindol (DAPI) was used to visualize nuclei.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/KuczeraCC9-13-Sup.pdf

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