Compartmentalization of the functions and regulation of the mitotic cyclin Clb2 in *S. cerevisiae*

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Summary

Orderly progression through the eukaryotic cell cycle is a complex process involving both regulation of cyclin dependent kinase activity and control of specific substrate-Cdk interactions. In *Saccharomyces cerevisiae*, the mitotic cyclin Clb2 has a central role in regulating the onset of anaphase and in maintaining the cellular shape of the bud by inhibiting growth polarization induced in G1. However, how Clb2 and the partially redundant cyclin Clb1 confer specificity to Cdk1 in these processes still remains unclear. Here, we show that Clb2 mutants impaired in nuclear import or export are differentially affected for subsets of Clb2 functions while remaining fully functional for others. Our data support a direct role of the cytoplasmic pool of Clb1,2-Cdk1 in terminating cytoskeleton and growth polarization, independently of G1 cyclin transcriptional regulation. By contrast, the nuclear form of the cyclin is required for timely initiation of anaphase. Clb2 localization influences its stage-specific degradation as well. We report that Clb2 trapped in the cytoplasm is stabilized during anaphase but not at the time of mitotic exit. Altogether, our results demonstrate that the subcellular localization of the mitotic cyclin Clb2 is one of the key determinants of its biological function.

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Introduction

Most eukaryotic organisms use numerous cyclin-Cdk (cyclin dependent kinase) complexes to orchestrate cell cycle progression through phosphorylation of multiple substrates. One standing question is how a particular complex meets its specific substrates at the right place and the right moment. In addition to sequentially activating the Cdks through well-described differences in their timing and level of expression, the cyclins are thought to contribute to substrate recognition by direct binding. This is based on the identification of a conserved hydrophobic patch (referred to as the HP motif) that serves as a docking site for substrates containing a RXL sequence (Schulman et al., 1998). In the budding yeast *Saccharomyces cerevisiae*, the HP motif of the S-phase cyclin Clb5 is essential for enhancing the activity of Clb5-Cdk1 towards its preferred substrates (Loog and Morgan, 2005; Archambault et al., 2005). However, it remains unclear whether the role of the HP motif is conserved in the mitotic cyclin Clb2.

The apparent lack of Clb2 HP-dependent specificity is surprising if one considers the major role of Clb2 in promoting M phase (Fitch et al., 1992; Richardson et al., 1992). Indeed, inactivating Clb2 in combination with the closely related cyclins Clb1 or Clb3 is lethal whereas Clb2 alone confers enough Cdk1-dependent mitotic activity to sustain growth. Finally, Clb2 is the only mitotic cyclin whose inactivation shows a discernable phenotype with a delay in G2 and elongated cells. The role of Cdk1 in bud morphogenesis is particularly interesting in that the same kinase has opposite effects depending on the appended cyclin (Lew and Reed, 1993). During the vegetative cell cycle, cells polarize at the G1-S transition in order to orient cell growth for bud formation. Initiation of polarization requires the activity of Cdc28 complexed with the G1 cyclins, Cln1 or Cln2. Early bud growth is restricted to the bud tip (apical growth) through vectorial transport of secretory vesicles along actin cables. By contrast, activation of Cdc28 by Clb1 and Clb2 turns off the polarized growth in G2 and triggers a uniform growth on the bud surface through the reorganization of the actin cytoskeleton (isotropic growth). It has been shown that expression of Cln1 or Cln2 in G2 results in hyperpolarized growth whereas ectopic expression of stabilized Clb1 or Clb2 in G1 prevents polarization (Lew and Reed, 1993). One way to interpret this observation is that Cln1,2-Cdk1 complexes have a direct role in reorganizing the actin cytoskeleton, whereas Clb1 and Clb2 terminate polarized growth indirectly through repression of *CLN1,2* transcription (Amon et al., 1993). An alternative, but not exclusive, possibility is that Cln and Clb have a role in discriminating among Cdk1 targets with opposite effects on growth polarization. To date, this possibility has never been formally tested because of the lack of identified Cdk substrates involved in growth regulation and the lack of Clb2 mutants specifically affected in bud morphogenesis.

The role of the cyclins in directing Cdk towards distinct biological outcomes has been mainly addressed through

comparison of the two related B-type cyclins, Clb2 and Clb5 (Cross et al., 1999; Cross and Jacobson, 2000; Loog and Morgan, 2005). Loog and Morgan (Loog and Morgan, 2005) proposed that the specific functions of Clb2 stems from an enhanced capacity of Clb2 to activate the intrinsic kinase activity of Cdk1 as compared to Clb5. Subcellular localization of the cyclin constitutes an alternative mechanism by which cyclins may help discrimination between distinct substrates. This has been well demonstrated for example for the budding yeast G1 cyclins (Miller and Cross, 2000; Edgington and Futcher, 2001). Specific localization may also regulate cyclins degradation as well as Cdk activity through accessibility to specific inhibitors and activators.

We and others have previously shown that Clb2 localizes to several cellular compartments (Hood et al., 2001; Bailly et al., 2003). In order to further investigate the physiological relevance of Clb2 compartmentalization, we analyzed the functionality of Clb2 localization mutants in two processes: the G2-M progression and the switch from apical to isotropic growth. Our data show that these two functions can be largely assigned to specialized pools of the cyclin. The nuclear form of the protein is required for timely entry in anaphase while the cytoplasmic pool is required for bud morphogenesis. Our data support a model in which the switch from a polarized to an isotropic growth occurs as soon as the cytoplasmic concentration of Clb1 and Clb2 reaches an appropriate threshold independently of cell cycle progression. Furthermore, the morphogenetic Clb2 function occurs independently of its role in regulating *CLNs* expression, pointing towards a direct role of the mitotic Cdk in terminating growth polarization.

Results

Cellular distribution of Clb2 upon mutations in nuclear translocation signals

To assess the functional relevance of the Clb2 distribution pattern, we investigated the effect of altering its nucleo-cytoplasmic ratio. Clb2 contains a nuclear localization signal (NLS) located in the N-terminal part of the protein and two putative nuclear export signals (NES). Point mutation in NES2 and deletion of the NLS sequence have been shown to deeply affect the cellular distribution of the cyclin (Hood et al., 2001; Bailly et al., 2003). However, since the cellular localization of both mutant proteins had only been investigated upon overexpression, we re-examined their localization when expressed under more physiological conditions. Internal deletion of a fragment containing the NLS (an allele hereafter named *clb2*$^{\Delta NLS}$ for simplicity) and the *clb2*^{L303A} mutation, which inactivates NES2, were introduced at the chromosomal *CLB2* locus by homologous recombination and a single GFP tag was appended at the C terminus of the mutant proteins. Fig. 1 shows representative images of Clb2$^{\Delta NLS}$ and Clb2^{L303A} cellular distribution as compared to the wild-type protein. As previously described (Hood et al., 2001; Bailly et al., 2003), wild-type Clb2 localized primarily to the nucleus. It was detected also as a single nuclear dot in 50% of the cells before nuclear division. Co-labeling experiments with Spc29-CFP, a known spindle pole body (spb) component, showed that in most cells this dot did not co-localize with Spc29 but was located between or close to one of the two separated spb (see supplementary material Fig. S1). By contrast, the Clb2 foci

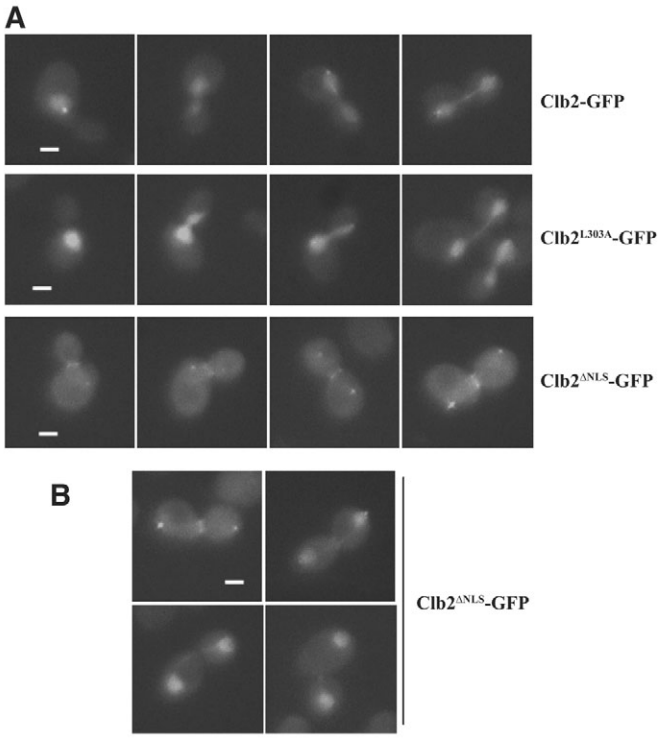

Fig. 1. (A,B) Cellular localization of the Clb2 mutants when expressed from the *CLB2* endogenous promoter. WT, *clb2*$^{\Delta NLS}$ and *clb2*^{L303} cells in which a GFP tag was introduced at the *CLB2* locus were grown to mid-log-phase on YPD plates supplemented with adenine. All images were taken with the same optical settings and processed with identical parameters into Adobe PhotoShop. (A) Cellular distribution of Clb2^{NLS} and Clb2^{L303A} compared with the wild-type cyclin. (B) Representative images of the pattern of distribution of Clb2$^{\Delta NLS}$-GFP in late anaphase. Bar, 2 μm.

detected from early to late anaphase colocalized with the spb (see supplementary material Fig. S1) (Bailly et al., 2003). Bud neck localization of Clb2 was more easily detected in late anaphase.

As expected, the nuclear signal was enhanced in *clb2*^{L303A} cells (Fig. 1A). Quantification of the fluorescence in the nucleus before nuclear division revealed that the amount of nuclear Clb2^{L303A} was about twofold that of the wild-type (WT) cyclin. The L303A mutation also prevented the localization of Clb2 to the bud neck, without affecting the nuclear dot-like localization in metaphase cells and the localization to the spb during anaphase (Fig. 1A and see supplementary material Fig. S1).

By contrast, the NLS deletion strongly prevented the nuclear localization of Clb2-GFP. The Clb2$^{\Delta NLS}$-GFP fusion was barely detected in the nucleus before nuclear division, a signal that dropped further during anaphase. Accordingly, the fluorescent signal in the cytoplasm, which was just over the background levels in WT cells, increased by a factor four in *clb2*$^{\Delta NLS}$ cells. Clb2$^{\Delta NLS}$-GFP also accumulated to the bud neck and the spb (Fig. 1A and see supplementary material Fig. S1A). Surprisingly, although the protein was hardly detected in the nucleus from metaphase to mid-anaphase, it relocalized to the nucleus in 45% of the cells with fully separated nuclei (Fig. 1B). This suggests that another mechanism of nuclear

translocation may operate in late anaphase independently of the NLS.

The nuclear form of Clb2 plays a prominent role in initiation of anaphase

Cell cycle progression was analyzed in *clb2*$^{\Delta NLS}$ and *clb2*^{L303A} cells after release from an α-factor-induced G1 arrest. Fig. 2A shows that DNA replication in the mutant and parental cells occurred at similar times. In WT cells, exit from mitosis started at 90 minutes whereas it was delayed by 15-30 minutes in *clb2Δ* cells, a delay that has been shown to stem mostly from a defect in initiation of anaphase (Richardson et al., 1992) (Fig. 2B). Cell cycle progression was also delayed by the *clb2*$^{\Delta NLS}$ mutation, but less dramatically than by the full deletion of *CLB2* (Fig. 2A,B). Analysis of the kinetics of nuclear division showed an accumulation of *clb2*$^{\Delta NLS}$ cells in a pre-anaphase state (i.e. with the nucleus localized to the bud neck) at 60 and 75 minutes after release and an enhanced percentage of binucleate cells at 90 and 105 minutes (Fig. 2B). This suggested that the cell cycle delay in *clb2*$^{\Delta NLS}$ cells results from additional defects in both anaphase initiation and mitotic exit. By contrast, the *clb2*^{L303A} allele appeared proficient for these two stages of cell cycle progression (Fig. 2A,B). The ΔNLS mutant is not affected in its capacity to activate Cdc28 in vitro (Bailly et al., 2003). Therefore, the delayed chromosome segregation in *clb2*$^{\Delta NLS}$ cells is probably linked to the mislocalization of the cyclin, as similar levels of Clb2$^{\Delta NLS}$ and Clb2^{L303A} were detected in metaphase-arrested cells (see Fig. 4A). Finally, the inactivation of the Cdc28 inhibitor Swe1 did not restore timely anaphase onset in *clb2*$^{\Delta NLS}$ cells (data not shown). This finding argued against the possibility that the G2-M delay in these cells could be the consequence of a misregulation of Swe1.

Together, these data point to the functional importance of the nuclear form of Clb2 in timely initiation of nuclear division. However, *clb2*$^{\Delta NLS}$ cells did not display the synthetic lethality with *clb1Δ* or *clb3Δ* exhibited by *clb2Δ* cells. This raised the possibility that the faint nuclear signal observed in *clb2*$^{\Delta NLS}$ cells was sufficient for the main function of the cyclin in triggering anaphase. To test this hypothesis, an ectopic nuclear export signal (NES) was fused at the N terminus of Clb2$^{\Delta NLS}$ in order to further force its cytoplasmic localization. A similar construct containing an inactive NES sequence was used as a control (mnes) (Miller and Cross, 2000). In these conditions, the NES fusion specifically prevented nuclear localization of Clb2$^{\Delta NLS}$ without affecting its bud neck and spb localizations (see supplementary material Fig. S2A). Both *NES-* and *mnes-clb2*$^{\Delta NLS}$ alleles were next introduced in a centromeric vector under the control of the *CLB2* promoter. Although the levels of expression of Clb2$^{\Delta NLS}$ and NES-Clb2$^{\Delta NLS}$ were equivalent (see supplementary material Fig. S2B), the latter was unable to sustain growth of *clb1,3,4Δ clb2ts* cells at the restrictive temperature (Fig. 2C). This negative effect was specific to the NES sequence since it was not observed with the inactive mnes signal. Analysis of cell cycle progression after release from a G1-arrest revealed that *NES-clb2*$^{\Delta NLS}$ cells are blocked before nuclear division (see supplementary material Fig. S2C). Altogether, these findings suggest that the nuclear function of Clb2 at anaphase initiation depends on two different nuclear localization signals.

To better evaluate the importance of the NLS for Clb2 functions, we next determined to which extent its deletion mimics the complete inactivation of the protein. For this, we tested the effect of the NLS deletion on the role of Clb2 in the FEAR (Cdc14 early anaphase release) network. Clb1,2-Cdk1 activate the FEAR network by phosphorylating Net1, which

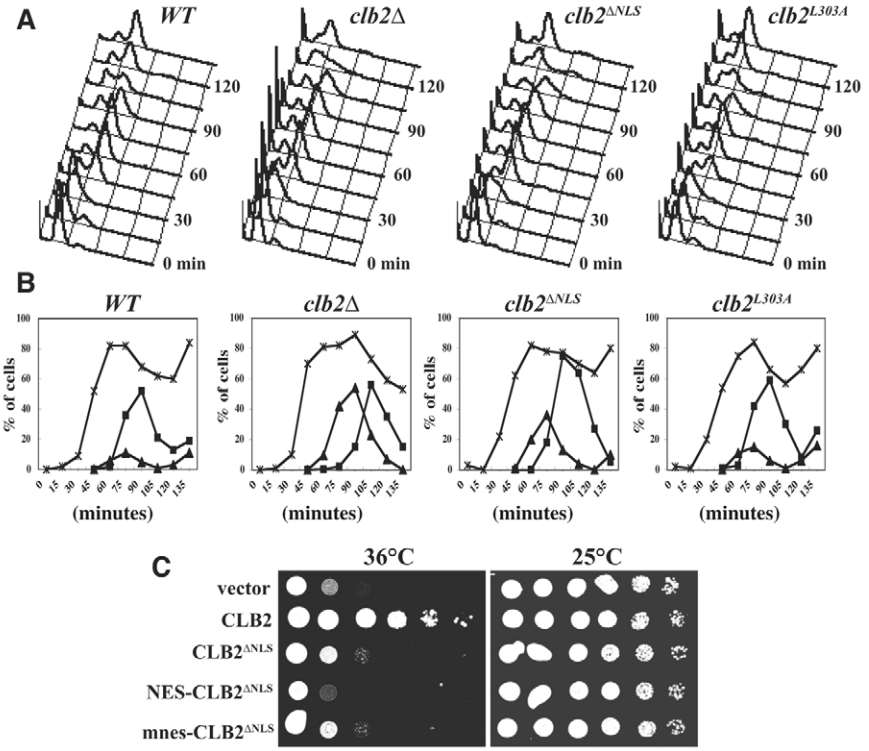

Fig. 2. (A,B) Cell cycle progression of cells expressing mutant forms of *CLB2*. The indicated cells were synchronously released from an α-factor-induced G1 arrest at 30°C. Aliquots were taken every 15 minutes. (A) DNA content was analyzed by flow cytometry and (B) the percentage of budded (crosses), pre-anaphase (triangles) and binucleate (squares) cells determined microscopically after a brief sonication. Pre-anaphase cells were defined as having the nucleus located at the bud neck. Note that in our strain background, about 20% of *clb2Δ* cells had the nucleus at the bud side of the neck just before anaphase and were classified as pre-anaphase cells. Each panel is representative of at least two independent experiments. More than 200 cells were counted for each time point. (C) Cells from the strain W303 *clb1,3,4Δ clb2ts* (Amon et al., 1993) were transformed with the indicated vectors and assayed for growth at 36°C. Serial 5x dilutions of saturated cultures were spotted on YPD plates and incubated at the indicated temperatures for 36 hours.

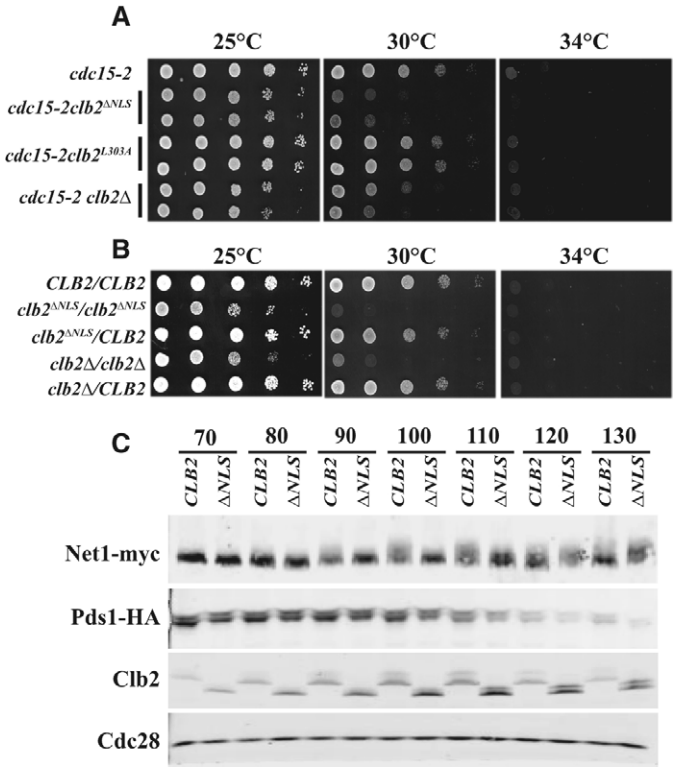

Fig. 3. Delayed phosphorylation of Net1 in *clb2*$^{\Delta NLS}$-expressing cells. (A) *clb2*$^{\Delta NLS}$ but not *clb2*^{L303A} exacerbates the temperature-sensitive growth phenotype of *cdc15-2*. Serial 10× dilutions of saturated cultures were spotted on YPD plates and incubated at the indicated temperatures for 3-4 days. (B) The synthetic effect of the *clb2*$^{\Delta NLS}$ allele is recessive. The same experiment as in A was repeated with *cdc15-2/cdc15-2* diploid cells expressing the indicated *CLB2* alleles. (C) The kinetics of Net1 phosphorylation was analyzed in extracts from *clb2*$^{\Delta NLS}$ *clb1Δ* and *clb1Δ* cells expressing *NET1-Myc* and *PDS1-HA* alleles. Cells were released from an α-factor-induced G1 arrest at 25°C and aliquots taken every 10 minutes. Total cellular extracts were separated by a long migration on 8% SDS-polyacrylamide gels to detect Net1-Myc, and by 10% SDS-PAGE for Clb2, Pds1 and Cdc28. To better compare the Net1 migration profiles, *clb2*$^{\Delta NLS}$ *clb1Δ* and *clb1Δ* extracts were run on the same gel. Clb2 and Pds1 were used as markers of cell cycle progression.

maintains Cdc14 trapped in the nucleolus until early anaphase (Azzam et al., 2004; Queralt et al., 2006). As a consequence, the *CLB2* deletion enhances the temperature-sensitive phenotype of *cdc15-2*, a mutant of the mitotic exit network (MEN) (Azzam et al., 2004). *clb2*$^{\Delta NLS}$ but not *clb2*^{L303A}, displayed a synthetic phenotype with *cdc15-2,* similar to the full deletion of *CLB2* (Fig. 3A). This phenotype is recessive (Fig. 3B) indicating that it truly stemmed from a lack of function of the mutant protein rather than from a dominant negative effect due to the accumulation of the protein in the cytoplasm.

Net1 phosphorylation can be monitored by the appearance of slower migrating forms of the protein that occur at the time of Cdc14 release from the nucleolus (Visintin et al., 2003). The extent and timing of Net1 phosphorylation were analyzed in cells deleted for *CLB1* and expressing the *clb2*$^{\Delta NLS}$ allele. In *clb1Δ*, *clb2*$^{\Delta NLS}$ cells synchronously released from a G1 arrest, appearance of slow migrating forms of Net1 was delayed by 20 minutes when compared to parental *clb1Δ* (Fig. 3C) or *clb1Δ*, *clb2*^{L303A} cells (see supplementary material Fig. S3A). To confirm that the delayed phosphorylation of Net1 was not due to a failure upstream of FEAR activation, we analyzed the securin Pds1 that was degraded with similar timing in *clb1Δ* and *clb1Δ*, *clb2*$^{\Delta NLS}$ cells (Fig. 3C and see supplementary material Fig. S3B).

The NLS deletion affects Clb2 degradation at anaphase but not during mitotic exit

Spatial regulation of proteolysis is also crucial for the coordination of many cell cycle transitions (Clute and Pines, 1999; Jaquenoud et al., 2002). Clb2 degradation is dependent on the anaphase-promoting complex/cyclosome (APC/C). Although APC/C activity is believed to localize to the nucleus (Jaquenoud et al., 2002; Melloy and Holloway, 2004), it is still unknown whether destruction of the mitotic cyclins is spatially regulated in *S. cerevisiae*. The mitotic exit defect exhibited by the *clb2*$^{\Delta NLS}$ cells raised the possibility that the cyclin trapped in the cytoplasm was not degraded as efficiently as the WT protein. Clb2 proteolysis occurs in two sequential waves mediated by Cdc20 and Hct1, two substrate-specific adaptors of APC/C (Baumer et al., 2000; Yeong et al., 2000; Wasch and Cross, 2002). The first wave of Clb2 destruction takes place at the metaphase-anaphase transition and is dependent on Cdc20, whereas the second wave, in telophase-G1, requires activation of Hct1 and possibly also Cdc20. To gain insight into the spatial regulation of Clb2 degradation, we compared Clb2 levels in WT and mutant cells blocked in metaphase by the spindle depolymerizing drug nocodazole, to levels in cells arrested in late anaphase-telophase by the ts allele *cdc15-2*. As expected, the amount of the WT protein was lower in telophase than in metaphase cells (Fig. 4A). Interestingly, although a similar pattern was observed with Clb2^{L303A}, similar levels of Clb2$^{\Delta NLS}$ were found at both stages of the cell cycle.

We next wanted to determine whether the NLS deletion also interferes with Clb2 degradation during mitotic exit. We sought to create conditions where the WT and the Clb2$^{\Delta NLS}$ proteins were subjected to the same APC activity to compare their patterns of degradation. For this, we took advantage of the fact that Clb2$^{\Delta NLS}$ migrated faster on SDS-PAGE and could be easily distinguished from the WT protein in western blots. The levels of both cyclins were analyzed in heterozygous *CLB2/clb2*$^{\Delta NLS}$ diploid cells released from a *cdc15-2*-induced telophase block. As shown in Fig. 4B, the profiles of the two proteins differed only during the first minutes after release at 25°C, probably before *cdc15-2* cells fully recovered from the arrest. For the rest of the time course, Clb2$^{\Delta NLS}$ degradation perfectly paralleled that of the WT cyclin, including an abrupt reduction at 30 minutes after release as previously reported (Schwab et al., 1997). This finding strongly suggests that Clb2 degradation is not affected by the ΔNLS mutation during mitotic exit. Consistently, we did not observe an accumulation of the mutant cyclin in G1 and early S phase in synchronized *clb2*$^{\Delta NLS}$ cultures (see supplementary material Fig. S3 and Fig. S4). Furthermore, as predicted for an increased level of Clb2 (Cross et al., 2005), the *clb2*$^{\Delta NLS}$ mutation is colethal with the deletion of *CDH1*. Taken together, our data support the view that Clb2$^{\Delta NLS}$ stability is specifically affected during anaphase but not during mitotic exit.

The Clb2$^{\Delta NLS}$ mutant induces a premature inhibition of growth polarization

CLB2-deleted cells form tubular buds as a consequence of a delay in the induction of the switch from apical to isotropic growth (Lew and Reed, 1993). We thus wondered whether the apical to isotropic switch also required nuclear localization of Clb2. To evaluate the timing of the isotropic switch, we monitored the polarization of the protein Bem1 in Clb2 mutant cells. Bem1 is an adaptor protein required for efficient actin polarization (Butty et al., 2002). Like most of the proteins involved in polarized growth, Bem1 shows a highly cell-cycle-regulated localization (Ayscough et al., 1997). First detected at the pre-bud site in late G1, the protein is maintained as a cap at the tip of the enlarging buds. In large-budded cells, the Bem1-GFP signal becomes more diffuse, appearing as small dots dispersed through the entire surface of the bud, before repolarizing to the mother bud neck during cytokinesis (Fig. 5A). As a consequence of this localization pattern, in a WT log-phase culture, 72% of medium budded cells showed a bud tip polarization of Bem1 compared to only 30% in large budded cells (Fig. 5B). By contrast, *clb2Δ* cells showed a high percentage of large-budded cells with polarized Bem1, consistent with the observation that Bem1 remains at the site of polarization when a polarized growth is artificially maintained (Butty et al., 2002). Nevertheless, *clb2Δ* cells still retained some capacity to inhibit polarized growth as shown by the fact that Bem1-GFP signed was diffuse in 80% of cells with a very large (>5 μm) bud. By contrast, *clb2*$^{\Delta NLS}$ cells showed a premature inhibition of Bem1 polarization. This was already noticeable in cells with medium sized buds (37% of Bem1 polarization, as compared with 72% in WT cells), this defect becoming even more conspicuous in cells with larger buds. The polarization of Bem1 to the pre-bud site, to the tip of the small buds and to the site of cytokinesis was not affected by the ΔNLS mutation (data not shown), showing that *clb2*$^{\Delta NLS}$ cells were not affected in their global capacity to polarize growth. Moreover, the defect of *clb2*$^{\Delta NLS}$ cells in maintaining polarization was not restricted to Bem1, as similar results were obtained with Spa2, a component of the polarisome (not shown). Together, these data suggest that accumulation of Clb2 in the cytoplasm may induce a premature inhibition of the polarized growth.

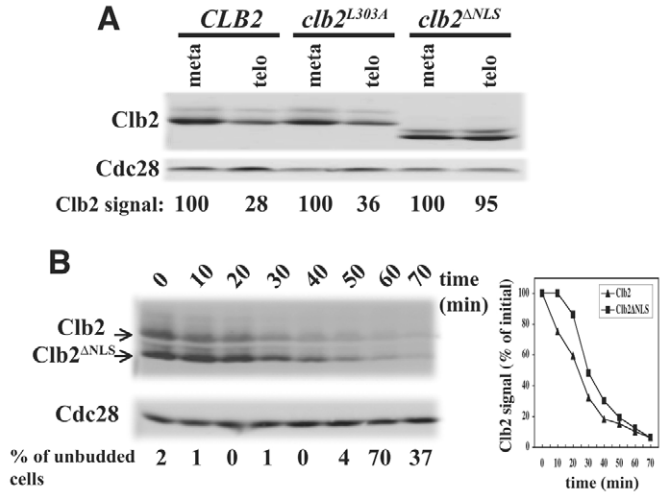

Fig. 4. Degradation profile of the Clb2$^{\Delta NLS}$ mutant protein. (A) Comparison of Clb2 levels in metaphase and telophase-arrested cells. *WT* and *cdc15-2* cells expressing the indicated *CLB2* alleles were exponentially grown at 25°C and transferred at 37°C with (WT background, metaphase arrest) or without (*cdc15-2* background, telophase arrest) 15 μg/ml nocodazole for 2 hours. Metaphase arrests were performed at 37°C to normalize the temperature shift required to block cells in telophase. Cellular extracts were analyzed by western blot with the indicated antibodies. Signals from Alexa Fluor-coupled secondary antibodies were quantified with an Odyssey scan. Clb2 signals were normalized using Cdc28 as a loading control. Signals from metaphase-arrested cells were considered as 100%. (B) Kinetics of Clb2 and Clb2$^{\Delta NLS}$ degradation during mitotic exit. *cdc15-2/cdc15-2 CLB2/clb2*$^{\Delta NLS}$ diploid cells were released from a temperature shift-induced telophase arrest as described in Materials and Methods. Clb2 signals were normalized using Cdc28 as a loading control and are expressed as the percentage of the signals measured at t0 (100%).

If Clb2 is required in the cytoplasm to induce the isotropic switch, this activity should be limiting in cells expressing the *clb2*^{L303A} allele. These cells, however, did not display an elongated phenotype and showed only a slight increase of Bem1 polarization in large-budded cells (Fig. 5B). Because the L303A substitution may not completely abolish nuclear export of Clb2, we hypothesized that the threshold of cytoplasmic mitotic cyclins required for the apical/isotropic switch in these cells could be achieved by the presence of Clb1. Indeed, the deletion of *CLB1* in *clb2*^{L303A} cells, led to a mildly elongated phenotype (not shown). Accordingly, *clb1Δ clb2*^{L303A} cells failed to correctly delocalize Bem1, as demonstrated by a high percentage of large-budded cells that preserved a polarized localization of Bem1-GFP (76%; Fig. 5B). Remarkably, when analyzed in synchronized cells blocked in metaphase, the defect of *clb1Δ clb2*^{L303A} cells in ending polarized growth was more dramatic than for *clb1Δ* or *clb2Δ* single mutants. As shown in Fig. 5C, at a time when the actin cytoskeleton is depolarized in all WT cells, 78% of the double mutant cells had actin patches concentrated at the bud tip, whereas most of *clb2Δ* and *clb1Δ* cells showed actin patches uniformly distributed over the surface of the buds (88 and 92%, respectively). Importantly, the elongated phenotype of the *clb1Δ clb2*^{L303A} cells was not the consequence of a lower capacity of the mutant cyclin to activate Cdc28 since similar levels of H1 kinase activities co-immunoprecipitated with WT and Clb2^{L303A} cyclins from metaphase arrested cells (see supplementary material Fig. S4A). Hyperpolarization of *clb1Δ clb2*^{L303A} cells did not stem from some illegitimate feedback loop interactions with Swe1, since a similar elongated phenotype was observed in *clb1Δ clb2*^{L303A} *swe1Δ* cells (Fig. 5C).

As Clb1 and Clb2 have been shown to be required for timely repression of *CLN1* and *CLN2* transcription (Amon et al., 1993), we next asked whether the hyperpolarized phenotype of *clb1Δ clb2*^{L303A} cells was due to a misregulation of the G1 cyclins. This did not appear to be the case. Indeed, the level of Cln2 dropped 60 minutes after release from a G1 arrest in *clb1Δ clb2*^{L303A} cells as it did in *clb1Δ* cells (Fig. 6A). Similarly, we did not observe an early repression of *CLN2* expression in *clb2*$^{\Delta NLS}$ cells that could explain the premature switch of the polarized growth (see supplementary material Fig. S4B). This approach, however, did not rule out the

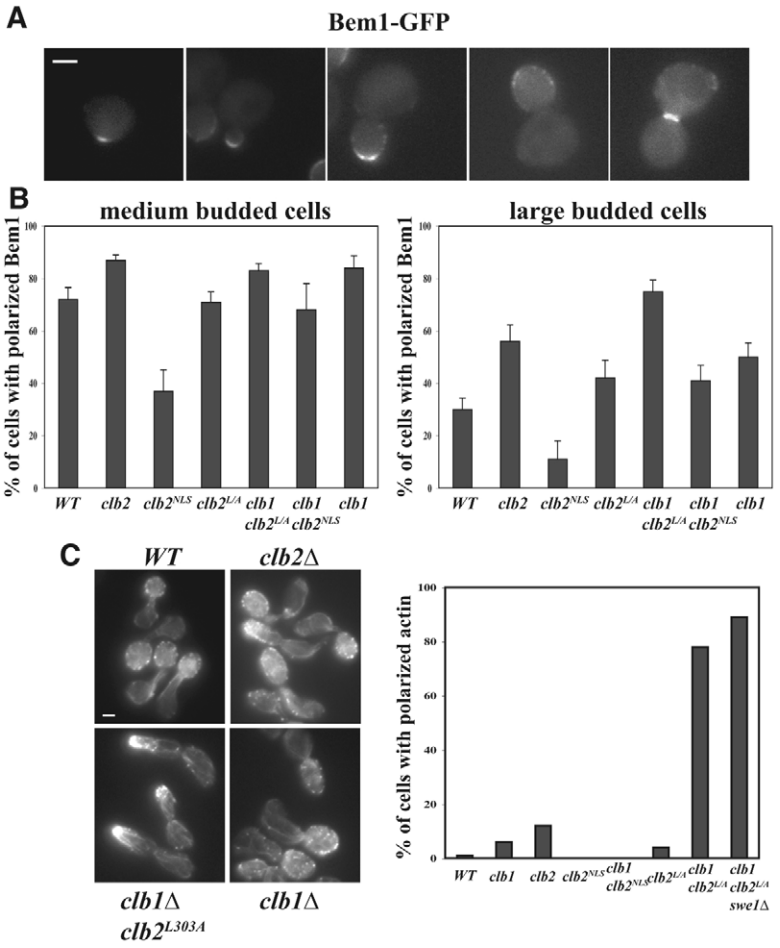

Fig. 5. *clb2$^{\Delta NLS}$* cells display a premature switch of growth polarization. (A) Cell cycle regulation of Bem1-GFP localization. (B) The percentage of cells with Bem1-GFP polarized at the bud tip was monitored in the indicated strains in log-phase cultures at 25°C. Medium and large buds were 2,5-3,5 μm and 3,5-5 μm in length respectively. Large budded cells with Bem1-GFP localized at the mother-bud neck were not scored. Bars indicate the standard deviation of at least three independent experiments with more than 500 cells counted for each strain. (C) Cells from the indicated strains were arrested in G1 with 5 μg/ml α-factor, washed and synchronously released in fresh YPD medium containing 15 μg/ml nocodazole. The cells were fixed after 75 minutes at 30°C and processed for actin staining with Rhodamine-phalloidin. The percentage of cells displaying a polarized distribution of cortical actin patches was quantified for each strain by counting at least 150 cells. Bars, 2 μm.

possibility of subtle variations of Clns levels that would account for the phenotype of the mutant cells. To investigate this possibility, we monitored whether the hyperpolarized phenotype of *clb1Δ clb2^{L303A}* cells could be reversed by decreasing the levels of Cln1 and Cln2. *cln1,2Δ* cells are viable because of the presence of the partially redundant cyclins Pcl1 and Pcl2, which activate the Cdc28-related kinase Pho85 in G1 (Moffat and Andrews, 2004). *clb1Δ, clb2^{L303A}* mutations were combined with the deletion of both *CLN1* and *CLN2* and buds morphology and actin distribution analyzed in metaphase-arrested cells. As shown in Fig. 6B, *clb1Δ*, *clb2^{L303A}*, *cln1Δ*, *cln2Δ* cells blocked in metaphase exhibited an elongated bud phenotype and showed an accumulation of actin patches at the bud tip (80% of the cells). These findings demonstrate that the *clb2^{L303A}* allele has a *CLN1,2*-independent defect in bud morphogenesis.

clb2Δ and *clb2^{L303A}* cells are sensitive to high dosage of Cdc42-GTP

The key event of cell polarization is the local activation of the Rho-family GTPase Cdc42. The cycling of Cdc42 between inactive GDP- and active GTP-bound states, is catalyzed by the guanine nucleotide exchange factor Cdc24 and the GTPase activating proteins (GAPs) Rga1, Rga2, Bem2 and Bem3 (for a review, see Chang and Peter, 2003). Apical growth requires a continuous local production of Cdc42-GTP (Gladfelter et al., 2002). In addition, the elongated bud morphology of *bem3Δ rga1Δ* cells suggests that inactivation of Cdc42 is required for cessation of polar bud growth (Smith et al., 2002; Caviston et al., 2003). This observation prompted us to investigate the effect of combining the inactivation of Cdc42 GAPs with the deletion of *CLB2*. Fig. 7A shows that *clb2Δ bem3Δ* and *clb2Δ rga1Δ* cells display a more elongated phenotype than any of the single mutants. By contrast, no genetic interactions could be detected between *CLB2* and *BEM2* (data not shown). Bem3 and Rga1 are known to be involved in the organization of septins (Caviston et al., 2003), a process monitored by the morphogenetic checkpoint (Barral et al., 1999; Longtine et al., 2000). However, the elongated phenotype of *clb2Δ bem3Δ* and *clb2Δ rga1Δ* cells appeared independent of Swe1 and hence of MCP activation (Fig. 7A). Next, we examined the occurrence of genetic interactions between the GAPs and the localization mutants of Clb2. As shown in the Fig. 7B, the deletion of *RGA1* slightly increased the length of *clb2^{L303A}* cells but had no morphological effect on *clb2$^{\Delta NLS}$* cells. Similar results were obtained with the deletion of *BEM3* (not shown). These data further support the idea that the level of Clb2-Cdc28 competent for the isotropic switch was limiting in *clb2^{L303A}* cells.

A C-terminal motif in Clb2 required for its bud neck localization

The L303A substitution not only affects the nuclear export of Clb2 but also its localization to the mother-bud neck (Hood et al., 2001). Two recent studies support a direct role of the septins in determining the pattern of bud growth (Caviston et al., 2003; Gladfelter et al., 2005). Since Clb2 localizes to the bud neck in a septin-dependent manner (Bailly et al., 2003), we wanted to check whether the premature switch observed in *clb2$^{\Delta NLS}$* cells was related to the fraction of the protein that concentrates to the neck. For this, we analyzed a new allele of *CLB2* carrying a double substitution of two conserved residues (K459, Y460) in the C-terminal part of the protein. These mutations were designed based on the occurrence in sea urchin cyclin B of natural splice variants affecting the C terminus of the protein (Lozano et al., 1998). Sequence comparison indicated that one structurally important consequence of these splicing events is the loss of an evolutionarily conserved KY

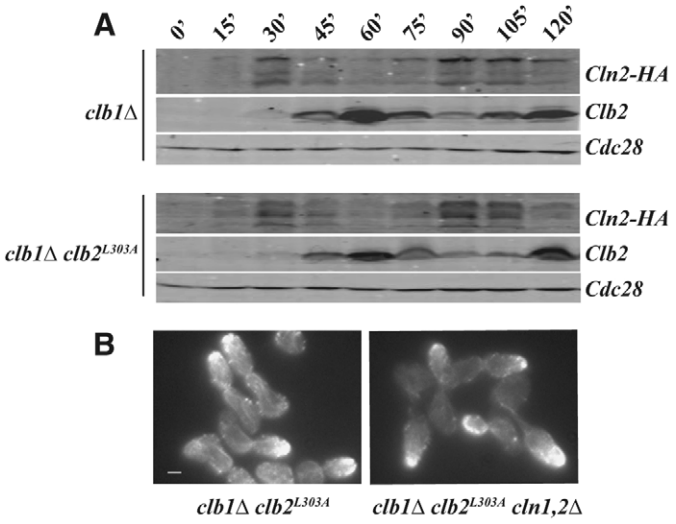

Fig. 6. The hyperpolarized phenotype of *clb1Δ clb2*^{L303A} cells is independent of *CLN1,2*. (A) *clb1Δ* and *clb1Δ clb2*^{L303A} cells expressing a *CLN2-HA3* allele were released from an α-factor arrest at 30°C and analyzed at the indicated times for the amount of Cln2-HA, Clb2 and Cdc28 by western blot analysis. (B) *clb1Δ clb2*^{L303A} and *clb1Δ clb2*^{L303A} *cln1Δ cln2Δ* cells were processed as described in the legend of Fig. 5C before staining for actin with Rhodamine-phalloidin. Bar, 2 μm.

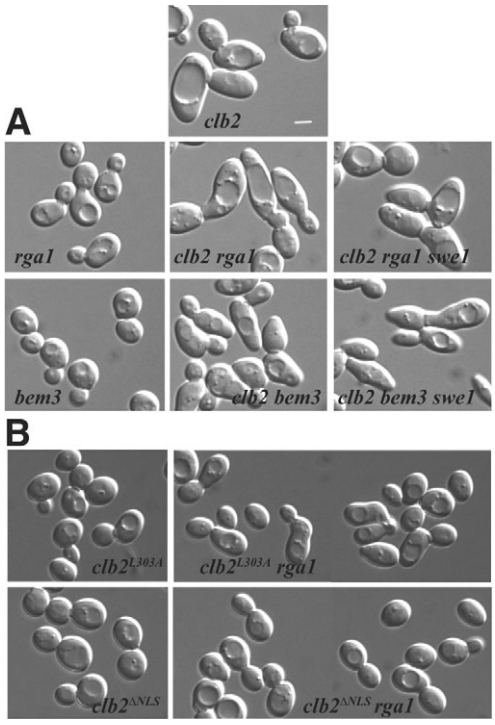

Fig. 7. Additive effects of GAP and *CLB2* deletions on cell elongation. (A,B) The indicated strains were grown to exponential phase on YPD plates at 25°C and examined by DIC microscopy. Bar, 3 μm.

motif (Lozano et al., 1998) (see supplementary material Fig. S5A). When expressed as a GFP fusion protein from the *CLB2* locus, Clb2^{K459A,Y460C} was not detected at the bud neck (Fig. 8A). In addition, although localized to the spb during anaphase (Fig. 8A and see supplementary material Fig. S5B), the signal was fainter in these cells (see supplementary material Fig. S1), suggesting that the K459A,Y460C double mutation also partially impaired the localization to the spb. Clb2^{K459A,Y460C} was not observed at the bud neck even when overproduced from the GAL1 promoter (Fig. 8B), indicating that the mutation not only decreased but completely abolished bud neck targeting of Clb2. Upon overexpression, Clb2^{K459A,Y460C}-GFP was no longer detectable at the spb, probably because the low signal was masked by the increased fluorescence in the nucleus. This observation further confirmed that the K459A,Y460C mutation weakened the localization of Clb2 to the spb in addition to its deleterious effect on bud neck localization. Finally, we tested whether Clb2^{K459A,Y460C} could be targeted to the neck when forced to reside in the cytoplasm by deletion of its NLS. As expected, the Clb2$^{K459A,Y460C,\Delta NLS}$ protein was trapped in the cytoplasm, yet it did not localize to the neck. Similarly to the yeast Clb2^{K459A,Y460C} mutant protein, one major effect of the KY deletion in the sea urchin cyclin B splice variant is the modification of the cellular distribution of the cyclin (Lozano et al., 1998). The role of the KY motif in mediating specific cellular distribution is therefore likely to be conserved among B-type cyclins.

The *clb2*^{K459A,Y460C} mutation did not affect the interaction of the cyclin with its Cdk counterpart as measured by the capacity of p13suc1 affinity-purified Cdc28 to retain WT or K459A,Y460C substituted Clb2 (see supplementary material Fig. S5C). In addition, *clb2*^{K459A,Y460C} cells did not show the G2-M delay characteristic of an inactivated cyclin. Instead, they entered mitosis 60 minutes after release from an α-factor-induced G1 arrest as WT cells did (see supplementary material Fig. S5D).

clb2^{K459A,Y460C} cells showed a Bem1 profile similar to that of WT cells (compare Fig. 8C with Fig. 5B). Trapping the Clb2^{K459A,Y460C} mutant in the cytoplasm by the additional deletion of the NLS resulted in a premature depolarization of Bem1. As the Clb2$^{K459A,Y460C,\Delta NLS}$ mutant still lacks bud neck localization, we conclude that the cytoplasmic rather than the bud neck localization of Clb2 is responsible for Bem1 delocalization from the bud tip in G2.

Discussion

A nuclear function of Clb2 in anaphase initiation

In the present study, we describe a cellular cartography of Clb2-Cdc28 where nuclear Clb2 emerges as a key factor for timely entry in anaphase, while the cytoplasmic form of the cyclin plays a prominent role in regulating bud shape. The critical role of the nuclear fraction of Clb2 is not unexpected if we consider the known substrates of the Clb1,2-Cdk1 complexes, for instance Net1, whose phosphorylation at the metaphase-anaphase transition, releases active Cdc14 from the nucleolus (Shou et al., 1999; Visintin et al., 1999; Azzam et al., 2004; Queralt et al., 2006). This first wave of Cdc14 release not only stimulates the MEN activity, but also regulates the change in microtubule dynamics observed at anaphase onset (Higuchi and Uhlmann, 2005). Consequently, the delayed Net1 phosphorylation observed in *clb2*$^{\Delta NLS}$ cells could be part of the anaphase defect of these cells. Another function of nuclear Clb2 might be the transcriptional control of key cell cycle

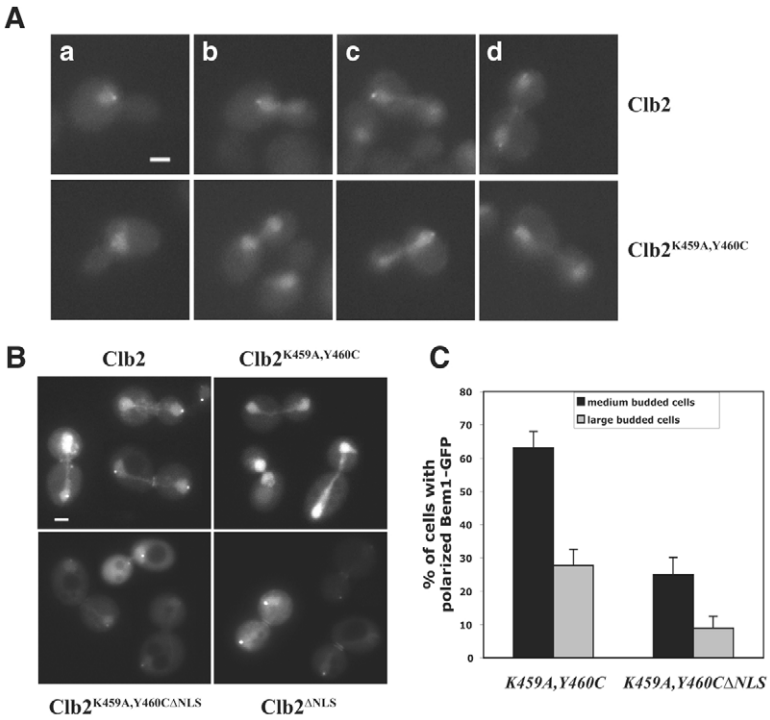

Fig. 8. Mutation of a C-terminal KY motif affects bud neck localization of Clb2. (A) A GFP tag was appended to the *clb2*^{K459A,Y460C} allele at the *CLB2* locus. Cells were processed for microscopic observations as described in Fig. 1 and are shown in comparison with *CLB2-GFP* cells before (a) and during (b-d) nuclear division. (B) The indicated mitotic cyclins were overexpressed as GFP fusion under the control of the *GAL1* promoter. Cells grown in SC-raffinose were collected on nitrocellulose filters and induced for 2 hours on YEP-galactose plates before microscopic observation. (C) The percentage of cells with Bem1-GFP polarized at the bud tip was monitored in *clb2*^{K459A,Y460C} and *clb2*$^{K459A,Y460C,\Delta NLS}$ cells as described in Fig. 5. Bar, 2 μm.

regulators. Indeed, Clb2-Cdk1 complexes play an important role in the expression of the *CLB2* cluster of genes through phosphorylation of the nuclear protein Ndd1, a co-activator of the transcription factor Mcm1-Fkh2 (Darieva et al., 2003).

Considering the strong reduction of the nuclear Clb2 levels upon deletion of its NLS, one intriguing result of this study is the moderate G2-M delay of *clb2*$^{\Delta NLS}$ cells, as compared to *clb2Δ* cells. Furthermore, the *clb2*$^{\Delta NLS}$ allele is not lethal in the absence of the other mitotic cyclins as it is the case for *clb2Δ* (Fig. 2) (Hood et al., 2001). This phenotype raises the possibility that another, non nuclear, pool of Clb2 might play of a prominent role at anaphase onset. This does not seem to be the case, because adding an extra NES that forces the translocation of Clb2$^{\Delta NLS}$ out of the nucleus, results in further impairment of its capacity to initiate anaphase. This result suggests the implication of an additional, unknown, nuclear translocation pathway independent of the NLS. Furthermore, it indicates that a low level of Clb2 nucleo-cytoplasmic shuttling is sufficient to reach the threshold of kinase activity necessary for anaphase entry. This finding raises the question about the role of the NLS sequence and about the requirement to accumulate high levels of nuclear mitotic cyclins in metaphase. Two, non exclusive, hypotheses may be considered. (1) The NLS-dependent accumulation of nuclear Clb2 might strength the robustness of mitotic entry. This function, that would be almost dispensable during a normal cell cycle, could become instrumental in assuring cell cycle progression when the cells are exposed to internal or external stresses or in the absence of other regulators of mitotic progression. In favor of this hypothesis, we found that the NLS deletion, as the inactivation of Clb2, decreases cells viability after DNA damage (E.B., unpublished results). (2) Nuclear Clb2 accumulation might be required for preparing mitotic exit. The latter already starts at the metaphase-anaphase transition via Cdk-dependent phosphorylation of Net1 and further requires APC^{Cdc20}-regulated destruction of the mitotic cyclins during anaphase (Azzam et al., 2004; Queralt et al., 2006; Wasch and Cross, 2002). In agreement with a role of the NLS in mitotic exit, we show that the *clb2*$^{\Delta NLS}$ allele delays Net1 phosphorylation and has a synthetic effect with *cdc15-2* similar to that observed with the full deletion of *CLB2*. In addition, the NLS deletion affects Clb2 degradation during anaphase (see below).

Spatial regulation of Clb2 proteolysis

We have investigated whether altering the nucleo-cytoplasmic ratio of Clb2 affects its degradation. Analysis of Clb2$^{\Delta NLS}$ degradation showed a complex pattern where the mutant cyclin accumulates specifically in anaphase but not during mitotic exit. Clb2 degradation at the metaphase-anaphase transition has been reported to mainly depend on APC^{Cdc20} (Baumer et al., 2000; Yeong et al., 2000; Wasch and Cross, 2002). In this context, stabilization of Clb2$^{\Delta NLS}$ might stem either from a lower accessibility of the mutant protein to the degradation machinery or from an altered APC^{Cdc20} activity. Indeed Cdk1 activates APC^{Cdc20} in at least two ways: the phosphorylation of several components of APC/C that is required for Cdc20-dependent APC activity (Rudner and Murray, 2000) and the transcriptional regulation of the *CLB2* cluster of genes, which includes *CDC20* (Spellman et al., 1998; Darieva et al., 2003). However, Pds1 was degraded with similar timing in *clb1Δ clb2*$^{\Delta NLS}$ and *clb1Δ* cells indicating that APC^{Cdc20} activity is not strongly affected in *clb2*$^{\Delta NLS}$ cells during a normal cell cycle. Therefore, our data suggest that the cytoplasmic form of the cyclin is not easily accessible to APC^{Cdc20}. This is consistent with the fact that Cdc20 is nuclear at all stages of the cell cycle (Jaquenoud et al., 2002). The restriction of Clb2 degradation to the nucleus opens the possibility of modulating Clb2 levels via the regulation of its nucleo-cytoplasmic shuttling. Protecting part of Clb2 from degradation could also be important to preserve some of the cyclin functions in the cytoplasm.

In contrast to Cdc20, Cdh1 localization is cell cycle regulated. During anaphase, Cdh1 is found only in the cytoplasm, whereas it dynamically shuttles between the nucleus and the cytoplasm at late mitosis (Jaquenoud et al., 2002). Further work will be necessary to establish whether the efficient Clb2$^{\Delta NLS}$ degradation at mitotic exit is because APC^{Hct1} is more potent than APC^{Cdc20} in recognizing cytoplasmic substrates or because

Clb2$^{\Delta NLS}$ translocates to the nucleus at mitotic exit by an NLS-independent mechanism. The finding that Clb2$^{\Delta NLS}$ localizes to the nucleus in about 50% of clb2$^{\Delta NLS}$ cells in late anaphase is compatible with the hypothesis, first proposed by Jaquenoud et al. (Jaquenoud et al., 2002), that Clb2 translocates to the nucleus as a complex with Cdh1. This idea is further supported by the observation that Cdh1 binds to its substrates independently of APC/C and thus may target proteins to the ubiquitin ligase (Burton and Solomon, 2001; Pfleger et al., 2001; Schwab et al., 2001). Alternatively, the second pathway of nuclear translocation that seems to operate in metaphase cells could be upregulated during mitotic exit.

Cytoplasmic Clb2 has a direct role in regulating bud morphogenesis

In spite of being delayed in the nuclear cycle, Clb2$^{\Delta NLS}$ induces a premature switch of the polarized growth. This is reminiscent of the accelerated transition observed upon overproduction of Clb2 (Lew and Reed, 1993), suggesting that inhibition of polarized growth occurs as soon as the cytoplasmic Clb1,2/Cdc28 activity reaches an appropriate threshold. This hypothesis is consistent with the slight defect of *clb2*^{L303A} cells to depolarize growth, a defect further enhanced by the deletion of *CLB1*. Moreover, the synergistic effect observed upon combination of *clb2*^{L303A} with deletions of Cdc42 GAPs further supports the idea that the depolarizing activity is limiting in these cells. Importantly, we showed that the hyperpolarized phenotype of the *clb1Δ, clb2*^{L303A} cells was not caused by an altered regulation of *CLN1* and *CLN2* or by upregulation of Swe1. Our data suggest a direct role of Clb1 and Clb2 in regulating cytoskeleton polarization. Several proteins involved in growth polarization have been identified as in vitro substrates of Cdc28 (Ubersax et al., 2003). Although this screen was performed with Clb2-Cdk1 complexes, it was probably not discriminating in terms of cyclin specificity. Accordingly, some of the identified proteins turned out to specifically interact with Cln1,2 using another experimental approach (Archambault et al., 2004). This is for example the case of Rga1 and Bem3, whose phosphorylation matches Cln1,2 expression (our unpublished data). The development of cell polarity is critical for a wide range of cellular processes including morphogenesis, cell motility and adhesion. All these processes require dynamic assembly and rearrangements of the actin cytoskeleton controlled by local activation and inactivation of Rho GTPase (Ridley et al., 2001), and their deregulation is probably one key determinant of tumors cells motility (Fritz and Kaina, 2006). The direct role of the mitotic kinase Clb2-Cdk1 in terminating growth polarization adds to the increasing number of examples that link cell cycle machinery and cytoskeleton organization (Besson et al., 2004). This raises the possibility that deregulation of cyclin-Cdk activity in cancer cells affects not only proliferation but also invasiveness.

Materials and Methods

Yeast strains and plasmids

All strains were isogenic with BF264-15DU (*MATa leu2 ura3 trp1 his2 ade1*) (Richardson et al., 1989) unless otherwise stated and are listed in supplementary material Table S1. Strains were constructed and analyzed by standard genetic methods. Deletions of *BEM3*, *RGA1* and *CLB1* were generated by in vivo recombination of appropriate PCR-amplified cassettes (Wach et al., 1994). Deletion of SWE1 was performed with a p*swe1*::LEU2 vector (Booher et al., 1993) and checked by PCR analysis with appropriate oligonucleotides. Cln2 has been HA-tagged using a Yiplac211-CLN2-HA plasmid kindly provided by Curt Wittenberg. The *clb2*^{K459A,Y460C} allele was constructed by in vitro PCR mutagenesis with the following oligonucleotide GAATTTCATAGAGCAGCTCAATCTAGAAGA. The underlined nucleotides introduced the mutations and an extra *Sph*I site. The *clb2*$^{\Delta NLS}$ allele encodes a truncated form of Clb2, Clb2$^{176-213}$ (Bailly et al., 2003). The *clb2*^{L303A} allele was a generous gift from Pamela Silver (Hood et al., 2001). These mutations were introduced at the genomic *CLB2* locus by a two step procedure. The *CLB2* gene was first deleted with a *clb2::URA3* cassette. *CLB2* mutations were introduced with the appropriate enzymes into the pB-*CLB2* vector, which contains a 2823 bp *Xho*I-*Eco*RI *CLB2* fragment. *Xho*I-*Eco*RI fragments containing the desired mutations were transformed in *clb2::URA3* cells and selected on 5-FOA. Correct integrations of the mutations were identified by PCR and restriction analysis. Net1-Myc and Clb2-GFP fusions were constructed by using the PCR reaction-based method (Wach et al., 1994).

NES-CLB2$^{\Delta NLS}$*-HA* and *mnes-CLB2*$^{\Delta NLS}$*-HA* were constructed by PCR amplification of the *CLB2*$^{\Delta NLS}$ sequence with a 5′ primer that contains an *Eco*RI restriction site followed by the NES or the mnes localization signals (Miller and Cross, 2000) in frame with *CLB2*: NES-CLB2: 5′-GGGAATTCATCTTATAG-ATGGAATTAGCCTTGAAATTAGCAGGTCTTGATATCAACAAGACATCCAA-CCCAATAGAAAACAC-3′; *mnesCLB2:* 5′-GGGAATTCATCTTATAGATGGA-ATTAGCCTTGAAATTAGCAGGTgcTGATATCAACAAGACATCCAACCCAAT-AGAAAACAC-3′ (with the localization signal underlined and the bases changed to inactivate the NES in lower cases). The 3′ primer, 5′-CGCGGATCCTCAG-CGGCCGCCATGCAAGGTCATTATATCAT-3′ contains a *Not*I restriction site. The PCR products were subcloned as *Eco*RI-*Not*I fragment in a pRS316 derivative (pPC2) containing a 800 bp fragment of the *CLB2* promoter and a HA3 tag in frame with the *Not*I site (this study). The entire sequences of the fusion ORFs were checked by sequence analysis.

Microscopy

Fluorescence and DIC microscopy were performed using a Zeiss Axiovert microscope with 100 and 63× objectives, respectively. Cell images were captured with an ORCA-ER Camera (Hamamatsu) and subsequently exported into Adobe PhotoShop CS2 for image analysis. Induction of Clb2-GFP fusion proteins from the GAL1 promoter was performed as described previously (Bailly et al., 2003). For monitoring Bem1 polarization, a GFP tag was introduced at the *BEM1* locus using a pk-*BEM1*-GFP (KAN^{R}) vector (this study). Cells were grown at low density overnight at 25°C on YPD plates supplemented with adenine and suspended in minimal medium before microscopic observation. Images were analyzed for Bem1-GFP localization and for the size of the buds using the Imaris software. Bud sizes, measured as the length between the bud neck and the bud tip, were classified as medium (2.5-3.5 μm) or large (3.5-5 μm). *clb2Δ* cells were unique in forming a significant percentage of very large buds >5 μm, which were not included in the figures. F-actin was labeled with Rhodamine-phalloidin according to Lew and Reed (Lew and Reed, 1993). Quantification of the nuclear and cytoplasmic fluorescence from Clb2-GFP fusions was performed with the ImageJ software. For each strain, fluorescence levels in the nucleus and the cytoplasm were measured in a defined square for at least 50 pre-anaphase cells. The background fluorescence was measured as the level of fluorescence in an unbudded G1 cell in the same field using the same square and deducted from the nuclear or cytoplasmic values.

Cell growth and synchronization

Synchronization of cells in G1 was achieved with 60 ng/ml α-factor for *bar1Δ* cells and 3 μg/ml for *BAR1* cells for 2 hours at 30°C. Cells were released from the G1 arrest and resuspended at 6×10^{6} cells/ml in fresh medium. The *cdc15-2* mutation was used to impose a cell cycle arrest in late anaphase-telophase. Cells were presynchronized in S phase by 300 mM hydroxy-urea for 3 hours at 25°C, washed and released in fresh medium at 37°C for 90 minutes. Cells were judged to be arrested when more than 90% of cells had a large bud. Cells were released from the telophase arrest in fresh medium at 25°C. Pre-synchronization in S phase avoided long incubations at 37°C and improved the synchrony upon release from the telophase arrest.

Immunoblots

Yeast extracts were prepared as described in Bailly et al. (Bailly et al., 2003) except for analysis of Net1-Myc phosphorylation (Fig. 3 and supplementary material Fig. S3) and quantification of Clb2 degradation (Fig. 4). For the latter, crude extracts were prepared according to Azzam et al. (Azzam et al., 2004). Anti-Clb2 and anti-Cdc28 polyclonal antibodies were generous gifts from Carl Mann. Secondary antibodies were either coupled to alkaline phosphatase (see supplementary material Fig. S4) or Alexa Fluor 680 and IRDye 800 (Invitrogen). Alexa Fluor- and IRDye-coupled secondary antibodies allowed linear quantification of the fluorescent signal with the Odyssey^{R} infrared imaging systems (Li-Cor, Biosciences, Lincoln, NE).

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