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The Cln3 cyclin is down-regulated by translational repression and degradation during the G_1 arrest caused by nitrogen deprivation in budding yeast

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Nutrients are among the most important trophic factors in all organisms. When deprived of essential nutrients, yeast cells use accumulated reserves to complete the current cycle and arrest in the following G_1 phase. We show here that the Cln3 cyclin, which has a key role in the timely activation of SBF (Swi4–Swi6)- and MBF (Mbp1–Swi6)-dependent promoters in late G_1, is down-regulated rapidly at a post-transcriptional level in cells deprived of the nitrogen source. In addition to the fact that Cln3 is degraded faster by ubiquitin-dependent mechanisms, we have found that translation of the *CLN3* mRNA is repressed ~8-fold under nitrogen deprivation conditions. As a consequence, both SBF- and MBF-dependent expression is strongly down-regulated. Mainly because of their transcriptional dependence on SBF, and perhaps with the contribution of similar post-transcriptional mechanisms to those found for Cln3, the G_1 cyclins Cln1 and 2 become undetectable in starved cells. The complete loss of Cln cyclins and the sustained presence of the Clb–cyclin kinase inhibitor Sic1 in starved cells may provide the molecular basis for the G_1 arrest caused by nitrogen deprivation.

Keywords: cell cycle regulation/G_1 cyclins/nutrients/yeast

Introduction

The budding yeast *Saccharomyces cerevisiae* is a model system to study the regulatory networks that link cell cycle progression to cell growth. Like most other eukaryotes, budding yeast cells take developmental decisions during the G_1 phase of the cycle (Pringle and Hartwell, 1981). Haploid cells arrest temporarily in G_1 in response to sexual pheromones and, after conjugation, enter a diploid cell cycle. Both haploid and diploid cells also arrest in G_1 under nutrient starvation conditions. While diploid cells proceed into a meiotic cycle and finally sporulate, haploid cells suffer multiple metabolic adaptations for survival and become quiescent by entering a G_0 state. The molecular pathway by which sexual pheromones cause a G_1 arrest has been characterized in great detail (reviewed by Herskowitz, 1995). In contrast, little is known about the mechanisms that arrest yeast cells in G_1 when they are deprived of nutrients.

Commitment to divide takes place in late G_1, at a point called Start, where three essential processes of the cell cycle are initiated: budding, spindle-pole body duplication and DNA replication. The budding yeast cyclin-dependent kinase, Cdc28, associated with the Cln3 cyclin seems to be the most upstream activator of Start, and acts as a potent inducer of two distinct transcriptional complexes, SBF (Swi4–Swi6) and MBF (Mbp1–Swi6) (Tyers *et al.*, 1993; Dirick *et al.*, 1995; Stuart and Wittenberg, 1995; Levine *et al.*, 1996). While Swi6 plays a regulatory role in both complexes, Swi4 and Mbp1 constitute the DNA-binding proteins of SBF and MBF, respectively (Andrews and Herskowitz, 1989; Lowndes *et al.*, 1991, 1992; Primig *et al.*, 1992; Koch *et al.*, 1993). Transcription of four G_1 cyclin genes, *CLN1* and *2* and *PCL1* and *2*, and the *HO* endonuclease, which is required for mating-type interconversion, is activated by SBF while, on the other hand, two B-type cyclins, *CLB5* and *6*, the *SWI4* gene and several genes involved in DNA synthesis such as *POL1*, *RNR1* and *TMP1* are under the transcriptional control of MBF (reviewed by Koch and Nasmyth, 1994; Cross, 1995). While Cln1 and 2 are involved mainly in initiating budding (Benton *et al.*, 1993; Cvrcková and Nasmyth, 1993), the Clb5 and 6 cyclins are specially required for initiation of DNA replication (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). Clb5,6–Cdc28 active complexes appear during the G_1–S transition as a result of Cln cyclin accumulation by two different mechanisms, thus providing a way to coordinate S-phase entry and budding initiation. First, Clb degradation initiated during exit from the previous mitosis is inhibited by Cln cyclins (Amon *et al.*, 1994). Second, degradation of Sic1, which inhibits Clb5,6–Cdc28 complexes, is triggered by Cln–Cdc28 kinase activity (Schwob *et al.*, 1994; Schneider *et al.*, 1996).

The Ras-cAMP pathway has been implicated in coordinating cell cycle progression and cell growth, although with apparently conflicting roles. When starved cells are re-fed, transcriptional activation of *CLN1* and *2* during the first Start event depends on cAMP, which suggests that the Ras-cAMP pathway has a positive role in cell cycle progression (Huble *et al.*, 1993). On the contrary, cAMP exerts a repressor effect on *CLN1* and *2* transcription, implicating this pathway in regulating cell size under different growth conditions (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994). Perhaps cAMP-dependent mechanisms operate with opposite effects at distinct molecular steps of transcriptional activation of *CLN1* and *2*, i.e. attainment of a threshold Cln3 level and, on the other hand, SBF activation. Nevertheless, these reports do not address the question as to whether the Ras-cAMP pathway is involved in arresting cells in G_1 under starvation conditions. Although cells deficient in Ras-cAMP activity arrest in G_1, they also stop growing, thus making it difficult to

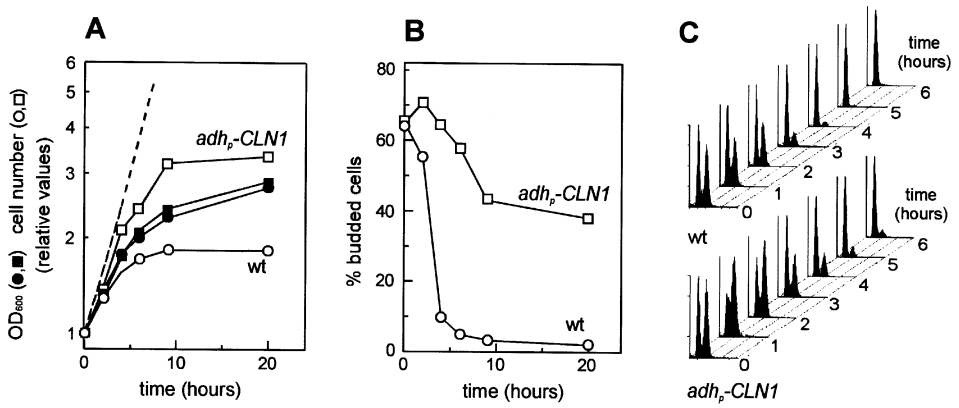

Fig. 1. Unregulated *CLN1* expression perturbs the G_1 arrest caused by nitrogen deprivation. CML128 [YCplac22] (wt, circles) and CML128 [pCM64] (*adh$_p$-CLN1*, squares) cells were deprived of nitrogen at time 0. (**A**) Cell growth (closed symbols) and cell number (open symbols) kinetics. The broken line indicates the growth rate in the presence of the nitrogen source. (**B**) Budding indexes. (**C**) DNA content distributions.

establish a direct role for this pathway in cell cycle arrest. Moreover, activation of the Ras-cAMP pathway does not prevent repression of *CLN2* and *HO* transcription in cells deprived of nitrogen (Markwardt *et al.*, 1995). A rapamycin-sensitive pathway mediated by the Tor1 and 2 phosphatidylinositol kinases has been involved in regulating early G_1 progression by nutrient limitation through control of translation initiation (Barbet *et al.*, 1996; Di Como and Arndt, 1996). Inhibition of Tor function by rapamycin causes a strong reduction in translation initiation and a G_1 arrest that can be suppressed by *CLN3* overexpression (Barbet *et al.*, 1996). However, as cell growth is seriously impaired, it is again difficult to establish a direct relationship between Tor activity and cell cycle progression.

Here we show that the G_1 arrest caused by nitrogen deprivation is mediated by down-regulation of *CLN3* at a post-transcriptional level. In addition to the fact that the Cln3 protein becomes more unstable, we have found that translation of the *CLN3* mRNA is repressed rapidly upon nitrogen deprivation and, as a consequence, SBF- and MBF-dependent transcription is also down-regulated.

Results

The G_1 arrest caused by nitrogen deprivation may involve active mechanisms to down-regulate Cln activity

When exponentially growing yeast cells were transferred to minimal media without the nitrogen source, both growth and division rates slowly declined, while cells arrested in G_1 as determined by DNA contents and budding index (Figure 1). We have obtained totally comparable results in three different strain backgrounds (EG123, W303 and S288C), being also independent of the presence of amino acid auxotrophic markers (data not shown). The relative increase in cell mass was always higher than that corresponding to the cell number, suggesting that the cell cycle arrest was not merely a consequence of an inability to meet the growth requirements of the G_1–S transition.

As Cln cyclins play a fundamental role in the G_1–S transition, we first asked whether the G_1 arrest caused by nitrogen deprivation requires a down-regulation of the activity of these G_1 cyclins. Figure 1 shows the cell cycle arrest kinetics of cells expressing *CLN1* from a mild constitutive promoter. While the relative increase in cell mass was unaffected, the cell number increase was much higher compared with the wild-type, indicating that most cells were able to complete one additional cell cycle under these conditions. Moreover, the high final budding index and the clear delay observed in the accumulation of cells with 1N DNA content indicate that *CLN1* transcriptional down-regulation may be essential in order to achieve a proper G_1 arrest under nitrogen deprivation conditions.

To characterize further the apparent uncoupling of cell growth and cell cycle kinetics observed in starved cultures, small early G_1 cells were released into minimal media with or without the nitrogen source, and cell volume, budding indexes and DNA content distributions were monitored (Figure 2). Under starvation conditions, these cells showed a first cycle G_1 arrest while cell volume increased clearly above 51 fl, the average budding cell volume (i.e. the small-budded mother cell volume) in the non-starved culture. Taken together, these results strongly suggest that the G_1 arrest caused by nitrogen deprivation is mediated by specific mechanisms, some of them involving the down-regulation of Cln proteins, and cannot simply be explained as a passive consequence of a growth arrest that would prevent cells from reaching the critical cell volume to bud and initiate S phase.

SBF- and MBF-driven expression is repressed owing to intrinsic cell cycle mechanisms

Since constitutive expression of *CLN1* prevented a proper G_1 arrest, we asked whether nitrogen deprivation causes a transcriptional down-regulation of those genes known to be induced during the G_1–S transition. Figure 3A shows that transcript levels of SBF- and MBF-driven genes decreased between 2 and 4 h after nitrogen deprivation, at a time when most cells accumulated in G_1. In contrast, transcript levels of *CLN3*, *SWI6* and *MBP1*, which do not oscillate much through the cell cycle, remained constant. Finally, *SIC1* mRNA levels slightly increased during the first hours after nitrogen deprivation. Comparable results were obtained in W303-1A, an independent background strain (data not shown).

The observed repression of SBF- and MBF-driven genes initially could be explained by two different mechanisms: (i) a specific repression of SBF- and MBF-dependent promoters by a *trans*-acting factor modulated by a nutrient

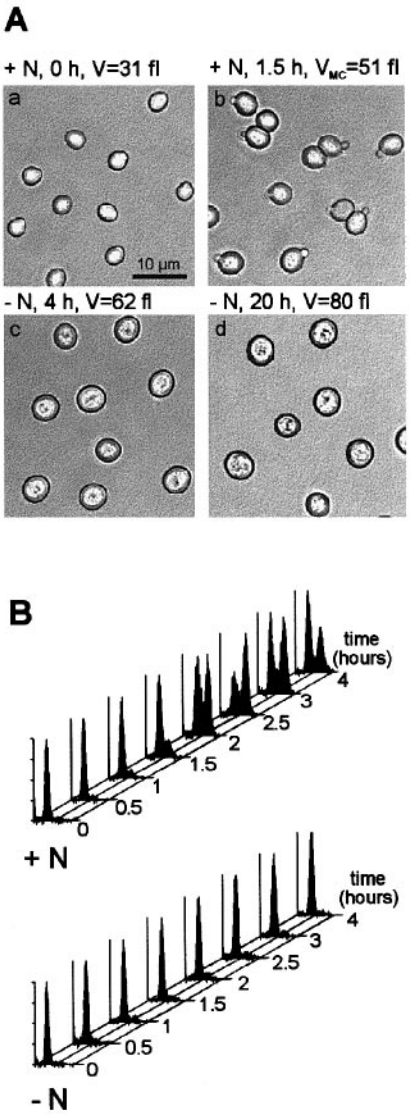

Fig. 2. G_1 cells arrested by nitrogen deprivation are able to grow past the critical volume for budding. (**A**) Small early G_1 CML128 (wt) cells were inoculated into minimal medium with (+N) or without (–N) the nitrogen source and samples were taken at the indicated time points to obtain the average total cell volume (V) or the small-budded mother cell volume (V_{MC}). (**B**) DNA content distributions.

signaling pathway; or (ii) as a direct consequence of a G_1 arrest prior to the induction of those promoters. In preliminary experiments with strains lacking Swi6, where *CLB5* is constitutively expressed during the cell cycle (Schwob and Nasmyth, 1993), we observed that this gene was not repressed at all during nitrogen deprivation (data not shown), thus favoring the latter hypothesis. However, this result does not rule out the possibility of a specific repressor linked to a nutrient signaling pathway, since it still could require or involve the Swi6 protein in order to exert its repressor function. To test these two different possibilities further, we used cells arrested with moderate or high *CLN1*, *CLN2* and *CLB5* transcript levels and asked whether nitrogen deprivation could still repress their expression. Figure 3B shows that *cdc28-13* cells arrested at the restrictive temperature for 2 h prior to starvation were not able to down-regulate *CLN1*, *CLN2* and *CLB5* transcription further when deprived of nitrogen. Very similar results were obtained with a thermosensitive mutation in the *CDC34* gene (data not shown), which is not involved in the transcriptional activation of those genes during the cell cycle. To avoid the heat shock produced when testing thermosensitive strains, we used hydroxyurea-arrested cells and obtained comparable results (Figure 3C). These data strongly argue against the existence of a specific repressor mechanism and favor the idea that SBF- and MBF-dependent promoters are repressed in starved cells by the mechanisms that operate during G_2 and M phases, as these cells complete their cycle and accumulate in the next G_1 before they can activate SBF- and MBF-dependent promoters again.

The Cln3 protein is lost rapidly in starved cells

Cln3 plays a key role in transcriptional activation of SBF- and MBF-driven genes in late G_1 (Tyers *et al.*, 1993; Dirick *et al.*, 1995; Stuart and Wittenberg, 1995). To test the possibility that nitrogen deprivation might prevent activation of SBF- and MBF-dependent promoters by down-regulating this protein, we used a triple hemagglutinin (HA)-tagged construct to determine Cln3 levels by immunoblotting techniques in extracts obtained from starved cells. Figure 4B shows that Cln3 protein levels decreased sharply during the first 2 h after nitrogen deprivation. As cells maintained invariable levels of the *CLN3* transcript for a much longer period of time (Figure 3A), this result suggested that *CLN3* is down-regulated by post-transcriptional mechanisms when cells are deprived of nitrogen.

None of the components of SBF and MBF was clearly down-regulated at the protein level during the first 6 h after nitrogen deprivation (Figure 4C). In addition, the amount of Cdc28 also did not change significantly. These results support the idea that nitrogen deprivation down-regulates SBF and MBF activity as a result of the loss of Cln3, which would prevent their activation in late G_1. Accordingly, expression of *CLN3* from a tetracycline-regulatable promoter (see below) was able to suppress *CLN1* repression in starved cells (data not shown).

Levels of other G_1 cyclins, Cln1, Cln2 and Clb5, decreased as expected from their transcript kinetics (Figure 4B). Although the respective mRNAs always showed easily detectable residual levels at 4 h after nitrogen deprivation, these proteins could not be detected even in highly exposed films. Those results suggest that these G_1 cyclins are also down-regulated by post-transcriptional mechanisms in starved cells. Sic1 protein levels did not change significantly during the starvation period analyzed. As a triple *cln* mutant is not able to traverse the G_1–S transition unless Sic1 is removed (Schneider *et al.*, 1996), the absence of Cln proteins and the sustained presence of Sic1 could explain the G_1 arrest produced by nitrogen deprivation in terms of molecular requirements.

CLN3 is down-regulated at a post-transcriptional level

Cln3 is a very unstable protein (Cross and Blake, 1993; Yaglom *et al.*, 1995) and, for this reason, its cellular contents should be extremely sensitive to the overall protein synthesis rate, as opposed to very stable proteins that adjust their levels very slowly to new steady-state conditions. To determine whether the observed decrease

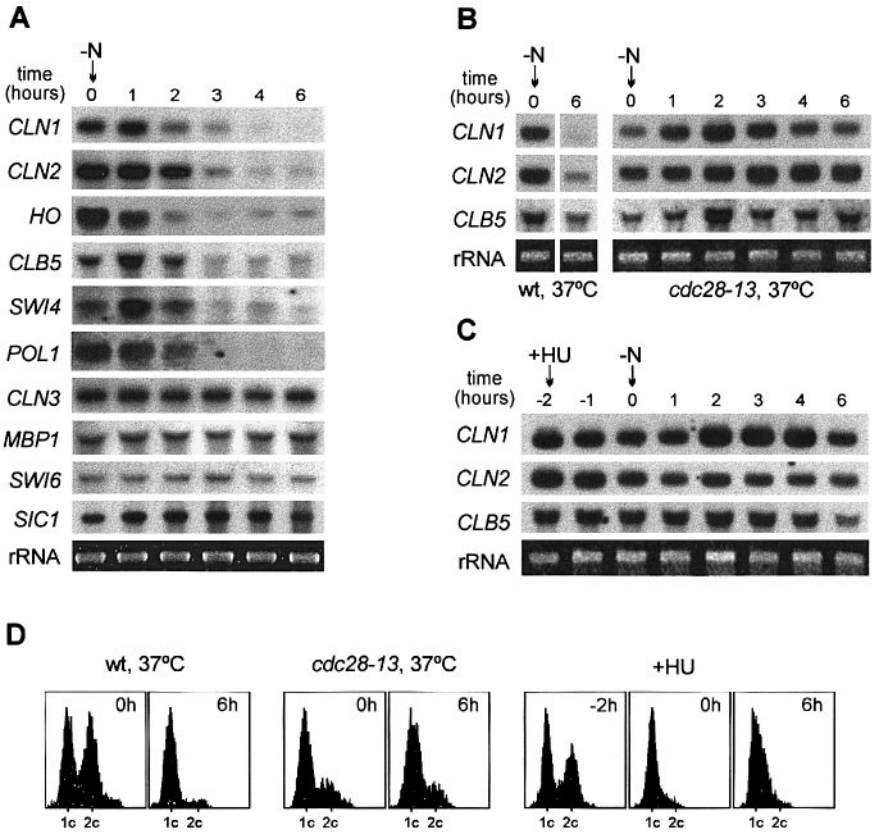

Fig. 3. SBF- and MBF-driven gene expression is down-regulated by nitrogen deprivation by regular cell cycle mechanisms as cells arrest in G_1. (**A**) CML128 (wt) cells were deprived of the nitrogen source at time 0 and samples were taken at different time points to analyze transcript levels by Northern blot with the indicated probes. (**B**) CML128 (wt) and CML198 (*cdc28-13*) cells previously transferred at 37°C during 2 h were deprived of nitrogen at time 0 and kept at 37°C during the course of the experiment. (**C**) CML128 (wt) cells were arrested in early S-phase with hydroxyurea for 2 h and then deprived of the nitrogen source in the continued presence of hydroxyurea. (**D**) DNA content distributions of samples corresponding to (B) and (C) for the indicated time points.

in Cln3 protein levels in starved cells could be explained by an equivalent decrease in the translational efficiency of the cell, we measured the overall protein synthesis rate by pulse-labeling analysis during nitrogen deprivation conditions. Figure 5A shows the protein synthesis patterns on SDS–PAGE from cells pulse-labeled under semi-quantitative conditions during nitrogen deprivation. Although the overall protein synthesis rate decreased during the first 2 h of starvation, this decrease could not explain the complete loss of Cln3 protein observed under these conditions (Figure 4B). On the other hand, the synthesis of some proteins was clearly induced 2 h after nitrogen deprivation, indicating that yeast cells sense starvation and adjust their expression requirements well before cell growth becomes seriously impaired.

As a very careful measurement of the overall translation rate by pulse-labeling was required, we first determined the minimal total extracellular concentration of sulfur-containing amino acids required to override the effects produced by changes in amino acid intracellular pools and uptake rates during the starvation period (see Materials and methods). Independently of the amino acid requirements of the strain used, the overall protein synthesis rate decayed slowly during the first 4 h after nitrogen deprivation (Figure 5B), which agrees with the slow decrease in growth rate observed under these conditions (Figure 1A). In contrast, Cln3 levels decreased much faster, becoming almost undetectable 2 h after nitrogen deprivation (Figure 5D), when translation efficiency had only decreased ~2-fold under the three conditions analyzed. In addition, while the decay in protein synthesis rate did not change much, the rate of Cln3 loss was faster when the strain used had no amino acid requirements. Totally comparable results were obtained in CML213 cells, a S288C-related strain (data not shown). These observations rule out the possibility that Cln3 down-regulation could be influenced by the presence of amino acid auxotrophic markers in some of the strains used. Moreover, amino acid depletion as measured by translational induction of *GCN4* was not detectable until 3 h after nitrogen starvation (Figure 5C), while Cln3 levels had dropped to undetectable levels much earlier. Thus, the observed loss of Cln3 in starved cells cannot be explained simply as a result of the decay in the overall protein synthesis rate, implying the existence of post-transcriptional mechanisms that down-regulate Cln3 levels when cells are deprived of the nitrogen source.

Cln3 becomes more unstable under nitrogen deprivation conditions

Programmed cyclin destruction occurs in both the G_1–S transition (Blondel and Mann, 1996) and exit from mitosis (Surana *et al.*, 1993). In addition, turnover of both Cln1 and Cln2 depends on *GRR1*, a gene that is involved in nutrient uptake pathways (Barral *et al.*, 1995). Thus, we decided to test whether the post-transcriptional down-

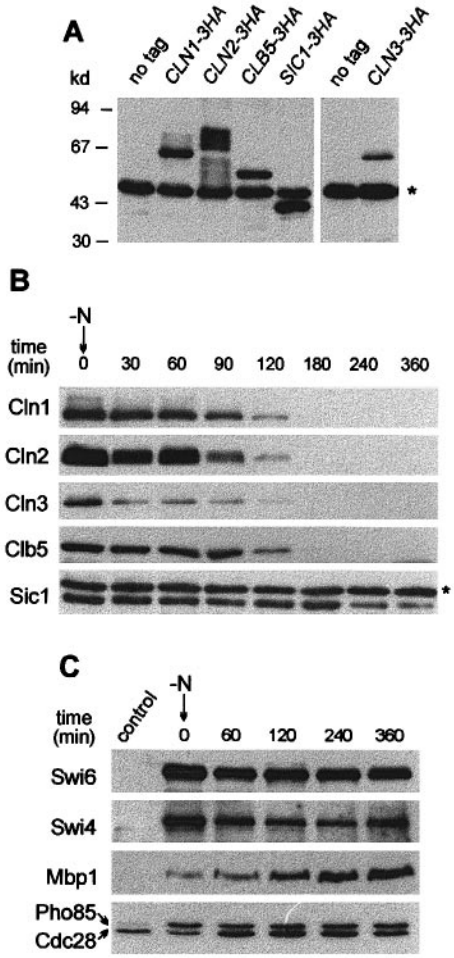

Fig. 4. G_1 cyclin proteins are lost rapidly in starved cells. (**A**) Western blot of HA-tagged proteins in cell extracts from CML128 (no tag), CML210 (*CLN1-3HA*), CML204 (*CLN2-3HA*), CML203 (*CLN3-3HA*), CML206 (*CLB5-3HA*) and CML208 (*SIC1-3HA*). A non-specific band that cross-reacts with the 12CA5 antibody is indicated with an asterisk. (**B**) Cells carrying the different HA-tagged proteins were deprived of nitrogen at time 0 and samples were taken at the indicated time points for Western blot analysis with the α-HA 12CA5 monoclonal antibody. (**C**) Cell extracts of CML128 [pMBP1-HA] during nitrogen deprivation conditions were analyzed by Western blot to detect Swi6, Swi4, Mbp1 and Cdc28 with α-Swi6, α-Swi4, α-HA and α-PSTAIRE antibodies, respectively. CML141 (Δ*swi6::TRP1*), K1939 (Δ*swi4::LEU2;* K.Nasmyth), CML128 (wt) and CML225 (Δ*pho85::kan*^{r}) cell extracts were used as controls.

regulation of Cln3 levels during nitrogen deprivation could be due to an increased turnover of the protein.

We recently have adapted to yeast cells (Garí *et al.*, 1997) a regulatable promoter system, initially developed for higher eukaryotic cells, that is mediated by a tetracycline-repressible transactivator protein (Gossen and Bujard, 1992). Repression of this promoter system by tetracycline does not involve any metabolic changes to yeast cells and does not affect their growth rate, making it especially suitable for manipulating gene expression to study nutrient-dependent regulatory mechanisms. After fusing the HA-tagged Cln3 to the *tet* promoter, we first determined that Cln3 levels responded perfectly to the tetracycline concentration under steady-state conditions, which resulted in ~20-fold the wild-type levels with no tertracycline added to the medium, and almost undetectable levels in the presence of 2 μg/ml tetracycline (data not shown). According to previously described results (Yaglom *et al.*, 1995), although cell size was clearly reduced, yeast cells could tolerate high levels of Cln3 protein with no effect on their growth rate. Figure 6A shows that the *tet* promoter can be turned off quite rapidly, the transcript levels becoming almost undetectable 30 min after tetracycline addition.

We next used the *tetp–CLN3-HA* construct to estimate the protein turnover rate in the presence or the absence of the nitrogen source by either total protein decay after promoter repression or pulse–chase analysis. As seen in Figure 6, Cln3 protein levels decayed faster in starved cells after tetracycline addition. The same difference was observed when the *tet* promoter was turned off by tetracycline addition 15 min before transferring cells to media with or without the nitrogen source (data not shown). In addition, this difference was completely suppressed in *cdc34-2* and *cdc28-13* mutants that had been transferred to the restrictive temperature 45 min before tetracycline addition. In agreement with previous work by Yaglom *et al.* (1995), Cln3 was largely stabilized in both mutants at the restrictive temperature, with the phosphorylated and unphosphorylated forms of Cln3 accumulated in the *cdc34-2* and *cdc28-13* mutants, respectively. To avoid unwanted effects due to the high levels of Cln3 protein attained during the preceding incubation at the restrictive temperature, we performed the temperature shift at the same time that cells were transferred to media with tetracycline in the presence or absence of the nitrogen source, and comparable results were obtained (data not shown). Thus, the faster turnover of Cln3 observed under nitrogen deprivation would be mediated by the same ubiquitin-dependent mechanisms that operate under regular growth conditions. However, assuming that Cln3 turnover follows either first- or second-order reaction kinetics, the moderate differences in protein stability could not explain the total loss of Cln3 observed in starved cells as *CLN3* is constantly expressed from its natural promoter sequences, suggesting the existence of additional post-transcriptional mechanisms.

CLN3 mRNA translation is repressed by nitrogen deprivation

Due to the differences observed in protein turnover and to the fact that Cln3 protein levels decrease very rapidly under nitrogen deprivation conditions, an unequivocal assessment of translational repression mechanisms by pulse-labeling and immunoprecipitation would not be possible in wild-type cells. For this reason, we decided to take advantage of (i) the high Cln3 stability that we had observed in both starved and non-starved *cdc28-11* cells at the restrictive temperature; and (ii) the prediction that these cells should accumulate high Cln3 protein levels very rapidly after transfer to the restrictive temperature under otherwise normal growth conditions.

As expected, *cdc28-13* cells accumulated very high levels of Cln3 protein over a period of 3 h after the transfer to the restrictive temperature in the presence of the nitrogen source (Figure 7A), and this accumulation was not due to a concomitant increase in the mRNA levels. On the contrary, while *CLN3* mRNA levels behaved similarly, these cells were not able to accumulate Cln3 at all under nitrogen deprivation conditions. Comparable

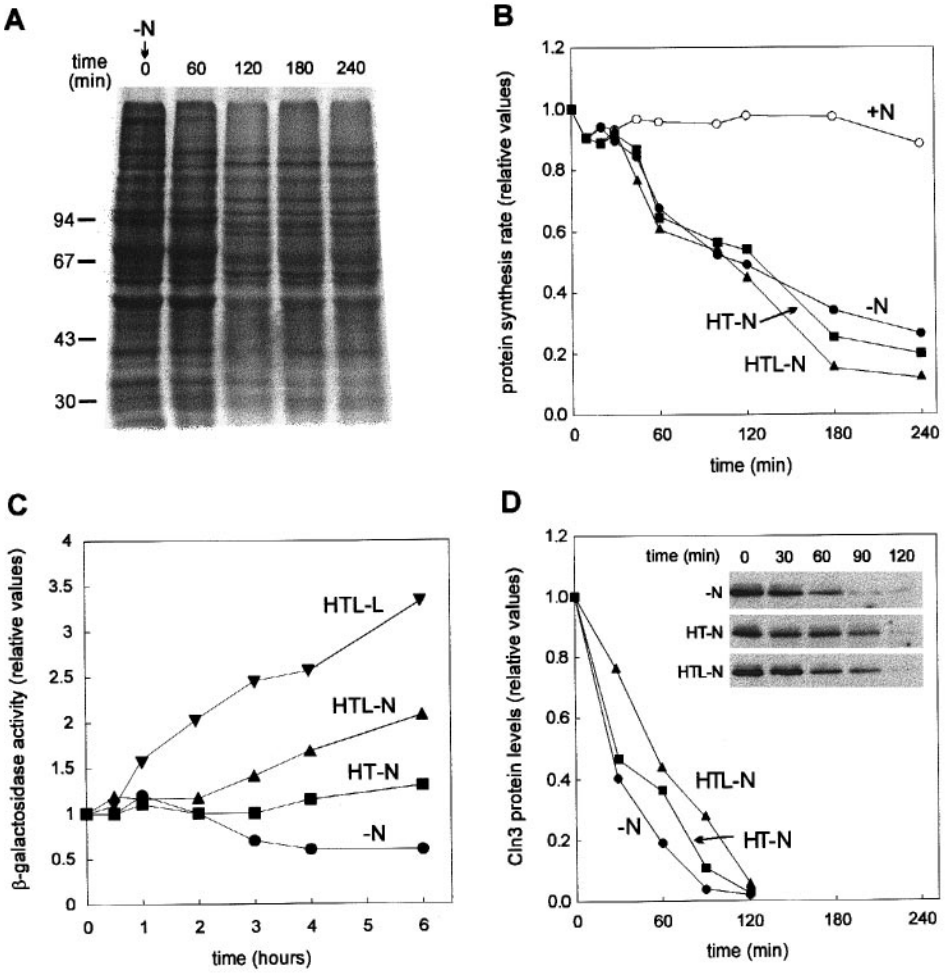

Fig. 5. Down-regulation of *CLN3* occurs at a post-transcriptional level. *CLN3-3HA* cells having different amino acid requirements, CML203 (histidine, tryptophan and leucine, HTL, triangles), CML133 [pCM194] (histidine and tryptophan, HT, squares) and CML232 [pCM194] (none, circles), were transferred to media with (+N, open symbols) or without the nitrogen source (–N, closed symbols) at time 0. (**A**) Autoradiography of an SDS gel loaded with total protein extracts of pulse-labeled CML203 cells. (**B**) Overall protein synthesis rate measured by pulse labeling. (**C**) *GCN4* induction kinetics. Strains mentioned above were transformed with p180 (*GCN4::lacZ*), and β-galactosidase units were determined. CML203 [p180] cells were deprived of leucine at time 0 as a control (–L, ▼). (**D**) Cln3 protein levels obtained by Western blot analysis (insert) were quantified as values relative to time 0.

results were obtained in our prototrophic strain CML234 (data not shown). Protein stability was determined in *cdc28-13* cells at the restrictive temperature for a period of 3 h and, in agreement with the results shown in Figure 6, we found no differences in the protein half-life obtained in the presence or absence of the nitrogen source (data not shown). Moreover, the relative overall protein synthesis rate decreased slowly during nitrogen deprivation (Figure 7C), in a way very similar to that shown in Figure 5B for wild-type cells at 30°C. Thus, the inability of starved *cdc28-13* cells to accumulate Cln3 could only be explained as a result of a translational repression of the *CLN3* mRNA. Comparable relative differences in Cln3 accumulation were also obtained in a *cdc34*-2 mutant derived from W303 (data not shown), excluding the unlikely possibility that the observed translational repression effect could be exerted in an indirect way by the *cdc28-13* mutation. To evaluate the extent of this translational repression, we measured Cln3 protein synthesis rate by pulse-labeling analysis 2 h after transfer of *cdc28-13* cells to the restrictive temperature, in the presence or absence of the nitrogen source. As shown in Figure 7D, nitrogen deprivation caused a net 8-fold reduction in the Cln3 protein synthesis rate. Thus, although nitrogen deprivation causes a moderate increase in its turnover rate, Cln3 loss in starved cells is due mainly to a specific translational repression mechanism.

Discussion

Cell cycle control by trophic factors has a key role in regulation of cell proliferation in all organisms. Nutrients are among the most important trophic factors for yeast cells and, hence, mechanisms must exist that couple nutrient availability to cell cycle crucial transitions. When deprived of essential nutrients, *Saccharomyces cerevisiae* haploid cells use nutritional stores to complete the current cycle and arrest in the following G_1 phase. We have found that nitrogen-deprived G_1-arrested cells are able to grow, reaching a volume much larger than the average budding volume of non-starved cells. This observation suggests the existence of specific mechanisms causing a G_1 arrest when cells are nutritionally deprived, even under condi-

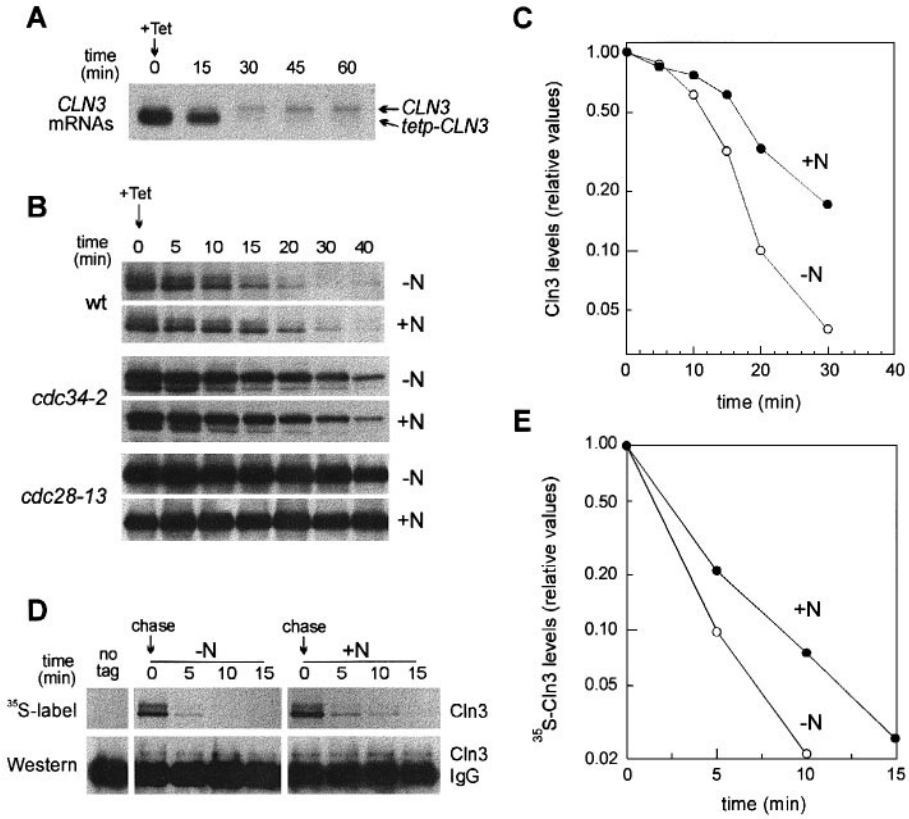

Fig. 6. Cln3 becomes more unstable upon nitrogen deprivation. CML133 (wild-type), CML200 (*cdc28-13*) and HTY600 (*cdc34-2*) cells carrying the *CLN3-3HA* construct under the control of the tetracycline-repressible $tetO_2$ promoter (pCM166), were transferred to media with (+N, ●) or without (–N, ○) the nitrogen source at time 0. (**A**) Repression kinetics of the $tetO_2$ promoter in CML133 [pCM166] after tetracycline (Tet) addition as determined by Northern blot. (**B**) Western blot analysis of Cln3 total protein decay after tetracycline addition. (**C**) Quantification of Cln3 bands shown in (B) for wild-type CML133 [pCM166] cells. (**D**) Pulse–chase analysis of Cln3 stability in wild-type CML133 [pCM166] cells as described in Materials and methods. CML133 [YCplac33] cells were labeled and used as the negative control for immunoprecipitation (no tag). (**E**) Quantification of radioactivity in Cln3 bands shown in (D).

tions where the critical cell mass for budding seems to be largely surpassed.

One of the landmarks of the G_1–S transition is the transcriptional activation of a set of genes regulated by two transcriptional factors with distinct DNA-binding moieties, SBF and MBF (Nasmyth, 1996). As expected from a G_1 arrest, we show here that SBF- and MBF-dependent gene expression is repressed under nitrogen deprivation conditions. Cells expressing hyperstable alleles of *CLN2* do not arrest properly in G_1 when deprived of the nitrogen source, with many cells remaining in a budded state (Hadwiger *et al.*, 1989; Lanker *et al.*, 1996). Consistent with these results, we have found that constitutive expression of *CLN1* causes very similar effects, implying that proper regulation of *CLN* activity is required to arrest cells in G_1 by nutrient deprivation.

Repression of SBF- and MBF-dependent transcription occurs during G_2 and M phases in actively growing cells (Amon *et al.*, 1993; Koch *et al.*, 1996). Starved cells use their nutrient stores to complete the current cycle and arrest in the next G_1. If the G_1 arrest takes place before activation of SBF and MBF, cells should accumulate in G_1, with very low levels of transcripts expressed by these transcriptional factors. Alternatively, nitrogen deprivation could cause a direct repression of SBF and MBF, thus blocking cells before entering S-phase. We have shown that cells blocked at the G_1–S transition (*cdc28-13* and *cdc34*-2 mutants at the restrictive temperature) or in early S-phase (after hydroxyurea addition) with moderate or high levels of *CLN1*, *CLN2* and *CLB5* transcripts are not able to down-regulate these transcripts when deprived of nitrogen, implying that nitrogen deprivation does not cause a direct repression of SBF and MBF. Rather, nitrogen-deprived cells arrest in G_1 before SBF and MBF activation takes place in late G_1.

Cln3 is a potent G_1 cyclin regarding the timely activation of SBF- and MBF-dependent promoters in late G_1 (Tyers *et al.*, 1993; Dirick *et al.*, 1995; Stuart and Wittenberg, 1995), and raising its cellular contents by either increased transcription or altered protein turnover has profound effects on cell size (Nash *et al.*, 1988; Yaglom *et al.*, 1995). Although neither mRNA nor protein levels oscillate much during the G_1–S transition (Wittenberg *et al.*, 1990; Cross and Blake, 1993; Tyers *et al.*, 1993; McInerny *et al.*, 1997), those properties suggest that subtle differences in the Cln3 accumulation rate during G_1 could be the intracellular parameter by which cells assess growth conditions before traversing the G_1–S transition. Consistent with this role, Cln3 is an extremely short-lived protein (Cross and Blake, 1993; Yaglom *et al.*, 1995) and, hence, its levels may respond rapidly to changes in the overall protein synthesis rate. Thus, a simple model to explain the G_1 arrest caused by nutrient deprivation would imply that, due to the reduction in protein synthesis rate, starved

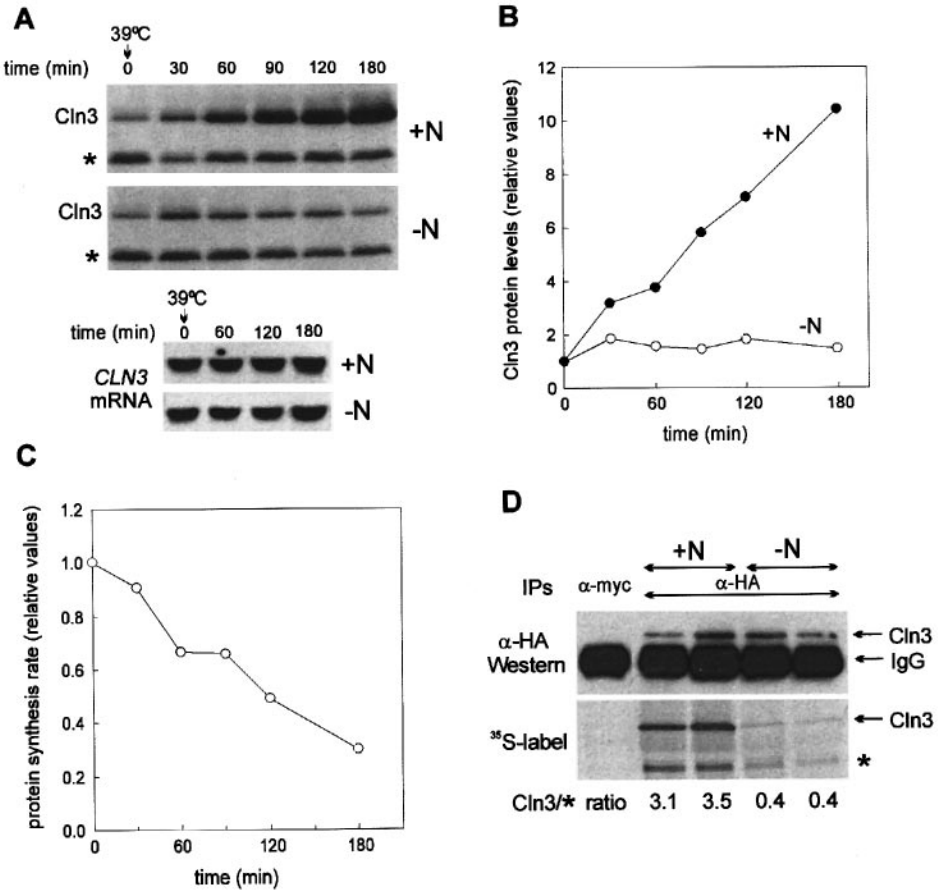

Fig. 7. Cln3 is subject to translational repression in cells deprived of nitrogen. CML200 (*cdc28-13*) cells carrying the *CLN3-3HA* gene under its own promoter sequences in pCM194 were grown at 25°C and transferred to 39°C in media with (+N, ●) or without (–N, ○) the nitrogen source at time 0. (**A**) Cln3 protein and *CLN3* mRNA levels at the indicated time points as measured by Western and Northern blot analysis. A non-specific band that cross-reacts with the 12CA5 antibody is marked with an asterisk. (**B**) Quantification of Cln3 protein levels shown in (A). (**C**) Overall protein synthesis rate as measured by pulse labeling analysis during nitrogen deprivation. (**D**) Pulse analysis of Cln3 was performed for 10 min as described in Materials and methods in duplicate samples 2 h after transferring the cells to media with (+N) or without (–N) the nitrogen source. In order to have similar total Cln3 protein levels in all immunoprecipitations (IPs), unlabeled extracts were used as described in Materials and methods. An additional labeled extract from cells grown with the nitrogen source was immunoprecipitated with an α-myc monoclonal antibody as negative control. Relative Cln3 synthesis rates (Cln3/* ratios) were evaluated by using the 45 kDa band (indicated with an asterisk) that cross-reacts with the 12CA5 antibody as an internal control for incorporation and immunoprecipitation efficiencies.

cells could not reach the Cln3 threshold level required to execute the G_1–S transition (Richardson *et al.*, 1989). However, cells carrying a hyperstable *CLN3* allele, which show a reduced critical mass for budding in exponentially growing cultures, still arrest mainly in an unbudded state under starvation conditions (Sudbery *et al.*, 1980), albeit at later times than wild-type cells (our unpublished results), suggesting the existence of specific mechanisms that could overcome the higher stability of Cln3. We have shown that Cln3 is down-regulated mainly at the translation level by nitrogen deprivation, while the faster turnover observed under these conditions would help to decrease the Cln3 levels more quickly. Thus, our findings would explain why an altered turnover of Cln3 does not *per se* cause a major effect in the G_1 arrest kinetics.

The precise mechanism that causes an 8-fold reduction in *CLN3* mRNA translation efficiency under nitrogen deprivation conditions remains to be elucidated. Preliminary work using the *tetp–CLN3* construct, where the *CLN3* open reading frame (ORF) is fused to the 5′-untranslated region (UTR) from *CYC1*, suggested that the observed translational repression requires the 5′-UTR of *CLN3*. However, we have not been able to suppress this repression by analyzing overlapping deletions of the *CLN3* 5′-UTR as described in Figures 5 and 7 (our unpublished results), indicating that translational repression does not seem to be mediated by binding of a specific *trans*-acting factor to the *CLN3* 5′-UTR. A rapamycin-sensitive pathway mediated by the Tor1 and 2 phosphatidylinositol kinases has been involved recently in linking nutrient availability (Di Como and Arndt, 1996) and early G_1 progression (Barbet *et al.*, 1996) to regulation of translation initiation. Although rapamycin causes a G_1 arrest similar to nitrogen deprivation, there are some important differences: (i) rapamycin causes a strong down-regulation of *CLN3* transcript levels at times where they are kept essentially invariable by nitrogen deprivation (Barbet *et al.*, 1996; our unpublished results); (ii) rapamycin addition over a wide range of concentrations causes a 10-fold reduction in the overall protein synthesis rate (Barbet *et al.*, 1996), indicating that rapamycin effectively blocks most of translation initiation events in yeast cells, while nitrogen deprivation imposes a specific translational control on the *CLN3* mRNA when the overall protein synthesis rate is

only reduced by 2-fold; and (iii) both Cln3 protein levels and the overall protein synthesis rate decrease with the same kinetics after rapamycin addition, even at the minimal concentrations able to cause a G_1 arrest (our unpublished results), indicating that, in contrast to nitrogen deprivation, translational repression mediated by rapamycin does not act in an specific manner on the *CLN3* mRNA. Overall protein synthesis requirements have been established in multiple points of the cell cycle (Burke and Church, 1991). However, as low concentrations of cycloheximide cause a G_1 arrest (Johnston *et al*., 1977; our unpublished observations), progression through G_1 seems to be the phase of the cell cycle most sensitive to unspecific protein synthesis inhibition. Thus, the rapamycin-mediated G_1 arrest could be explained in very similar terms. Taken together, these observations suggest that translational repression of the *CLN3* mRNA very early during nitrogen deprivation does not involve the Tor pathway. Perhaps the Tor pathway plays its essential role in repressing translation initiation of most mRNAs at later times during starvation, when nutrients are almost exhausted, thus preventing accumulation of truncated proteins in the cell. Alternatively, yeast cells could have redundant mechanisms to ensure a G_1 arrest under nutrient deprivation conditions.

Although loss of Cln3 in starved cells may be an important mechanism to achieve a G_1 arrest, the fact that strains lacking *CLN3* are still able to perform the G_1–S transition indicates that some other mechanisms must exist to explain the G_1 arrest caused by nitrogen deprivation. Cln1, Cln2 and Clb5 residual levels are down-regulated much more severely than those corresponding to their respective transcripts in starved cells, suggesting that these proteins may also be subject to post-transcriptional mechanisms. Similarly to the faster decay of Cln3 observed in starved cells, we have found that Cln1 and Cln2 show an increased turnover under these conditions (E.Garí, N.Colomina, C.Gallego, E.Herrero and M.Aldea, in preparation). This additional mechanism could reduce their residual levels down to a level such that no sufficient Cln activity would be present in the cell to execute the G_1–S transition. Moreover, since Cln protein levels remain very low, the activated Clb degradation during exit from the previous mitosis would still be active as cells accumulate in G_1, thus lowering Clb5 levels by degradation. Finally, the sustained presence of Sic1 in starved cells could explain in molecular terms why cells arrest in G_1 when deprived of the nitrogen source.

The G_1 arrest caused by trophic factors in higher eukaryotes seems to involve mechanisms similar to those operating in yeast cells. Thus, translational control has been demonstrated in transforming growth factor (TGF)β-dependent down-regulation of Cdk4 (Ewen *et al.*, 1993). In addition, up-regulation of the cdk–cyclin inhibitor p27 in G_1-arrested cells occurs by both translational regulation and decreased degradation (Hengst and Reed, 1996). Translation and stability of other key regulators of the G_1–S transition such as D-type cyclins may also contribute to link trophic signals to G_1 arrest and differentiation in higher eukaryotic cells.

Materials and methods

Strains, plasmids and growth conditions

Our parental strain CML128 (*MAT***a**, *leu2-3,112, ura3-52, trp1-1, his4, can-1*^{r}) is a spore of 1788 (Lee *et al.*, 1993), a homogeneous diploid strain obtained from EG123 (I.Herskowitz). The following CML128 derivatives were obtained: CML133 (*LEU2*::*tTA*), CML232 (*LEU2*::*tTA, TRP1, HIS4*), CML198 (*cdc28-13*), CML200 (*LEU2*::*tTA, cdc28-13*), CML234 (*LEU2*::*tTA, TRP1, HIS4, cdc28-13*), CML141 (Δ*swi6::TRP1*), CML210 (*CLN1-3HA*), CML204 (*CLN2-3HA*), CML203 (*CLN3-3HA*), CML206 (*CLB5-3HA*) and CML208 (*SIC1-3HA*). Strain CML213 (*MAT***a**, *ura3-52*) is a spore of FY1679 (B.Dujon), a S288C derivative. Strain HTY600 (*cdc34-2*; L.Johnston) is a derivative of W303-1A (*MAT***a**, *leu2-3,112, ura3-52, trp1-1, his3-11,75, ade2-1, can1-100*; L.Johnston). The tetracycline-repressible transactivator tTA was introduced into yeast cells by integration of pCM87 (Garí *et al*., 1997) at the *LEU2* locus. Chromosomal C-terminal fusions to the HA epitope were performed by gene transplacement with *Pfu*-PCR products obtained from pCM133 (see below) essentially as described (Wach *et al*., 1994). Transformation procedures and gene transplacement methods were done as described (Ausubel *et al.*, 1987).

Plasmid pCM133 was built from pFA6a-kanMX3 (Wach *et al*., 1994) by replacing the β-galactosidase gene by three copies of the HA epitope (Tyers *et al.*, 1993) between the *Bam*HI and *Asc*I sites. pCM194 is a YCplac33 (*URA3*; Gietz and Sugino, 1988) derivative that carries the HA-tagged *CLN3* gene (Tyers *et al*., 1993) under its own transcriptional regulatory sequences. Plasmid pCM166 was built by placing the HA-tagged *CLN3* ORF under the control of the *tetO*$_2$ promoter (Garí *et al*., 1997) in YCplac33. pMBP1-HA (N.Lowndes) contains the 3HA-tagged *MBP1* gene in YCplac33. The *CLN1* ORF was placed under the control of the *Schizosaccharomyces pombe adh1* promoter (A.Bueno) in YCplac22 (*TRP1*; Gietz and Sugino, 1988), resulting in plasmid pCM64. Finally, p180 carries a translational fusion of the *GCN4* gene to the β-galactosidase coding sequences (Hinnebusch, 1985) in a centromeric *URA3* vector. DNA manipulations, either by regular subcloning or *Pfu*-PCR techniques, were done as described in Ausubel *et al.* (1987) and Weiner *et al*. (1994).

Yeast cells were grown in SD minimal media at 30°C unless stated otherwise, and supplements were added as required (Ausubel *et al*., 1987). Tetracycline was added to 2 μg/ml when repression of the tTA transactivator was needed. DNA replication was inhibited by addition of 20 mg/ml hydroxyurea. Yeast nitrogen base without ammonium sulfate was used as recommended by the manufacturer (Difco) to prepare SD media without the nitrogen source, and the required amino acids were added to the following final concentrations: 15 μg/ml leucine, 5 μg/ml histidine and 10 μg/ml tryptophan. Nitrogen deprivation experiments were done with cells growing exponentially for 14–16 h until OD_{600} = 0.3–0.4, which were then collected by filtration and, after a quick wash, resuspended at the same cellular concentration in pre-warmed medium lacking the nitrogen source. Small newly born cells were isolated from Ficoll gradients as described (Mitchison, 1988).

Northern blot analysis

Total RNA samples were obtained from yeast cell pellets, electrophoresed in formaldehyde–agarose gels and transferred to positively charged nylon membranes as described (Ausubel *et al.*, 1987). After UV cross-linking, membranes were washed twice in washing buffer (1% SDS, 20 mM Na_2HPO_4 pH 7.2, 1 mM EDTA) at 65°C, pre-hybridized for 1 h at 65°C in 20% SDS, 0.5% blocking reagent (Boehringer Mannheim), 250 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, and hybridized overnight at 65°C with digoxigenin-labeled probes at 2 ng/ml. After three washes at 65°C in washing buffer, immunological detection steps were performed as described by the manufacturer (Boehringer Mannheim) using CDP* (Tropix) as the chemiluminescent substrate. Signal quantification was performed with a CCD camera-based system (Lumi-Imager) from Boehringer Mannheim. DNA fragments containing only ORF sequences, obtained by either PCR or restriction digestion, were used to synthesize probes by random-PCR with digoxigenin-dUTP labeling mixture as directed by Boehringer Mannheim.

Protein synthesis rate and β-galactosidase activity measurements

The overall protein synthesis rate was determined by pulse analysis, which was performed by incubating 100 μl of culture for 5 min at the specified temperature with 10 μl of a labeling mixture containing 1 μCi/μl Tran^{35}S-label (ICN), 0.1 mM unlabeled methionine and 0.1 mM unlabeled cysteine in growth medium. After addition of 1 ml of cold 10% trichloroacetic acid (TCA), samples were incubated at 0°C for at least 1 h and TCA-precipitable counts were recovered by filtration on GF/C filters with the help of a Bio-Dot (Bio-Rad), which were then washed four times with 5% cold TCA and twice with cold ethanol, and

finally air-dried. Quantification was performed with a BAS-1000 (Fuji). β-Galactosidase activity was measured as described (Garí *et al.*, 1997).

Western blot analysis, labeling conditions and immunoprecipitations

Samples for Western blot analysis were prepared from frozen cell pellets with 5 OD_{600} equivalents, which were added to 15 µl of 5 M urea and immediately boiled for 2 min. After addition of an equal volume of glass beads, cell suspensions were vortexed for 7 min at room temperature, supplemented with 50 µl of 2% SDS, 0.125 M Tris–HCl pH 6.8, vortexed for 1 min, boiled for 2 min and microfuged. The protein concentration in the supernatants was determined by a Micro DC protein assay (Bio-Rad). Equal amounts of total protein were resolved by SDS–PAGE (Ausubel *et al.*, 1987), and ECL immunoblot analysis was carried out as directed by the manufacturer (Amersham). HA-tagged proteins were detected with a 1:2500 dilution of the 12CA5 monoclonal antibody (ascites fluid). The α-Swi4 and α-Swi6 polyclonal antibodies (N.Lowndes), as well as the α-PSTAIRE antibody (Santa Cruz), were used at a 1:10 000 dilution. Chemiluminescent signal quantification was performed with a CCD camera-based system (Lumi-Imager) from Boehringer Mannheim.

The Cln3 degradation rate was measured by pulse–chase analysis as follows. CML133 (pCM166) cells growing exponentially in SD minimal medium were transferred to SD with or without the nitrogen source, and 120 ml of culture were added to 240 µCi of Tran^{35}S-label (ICN) and incubated for 10 min at 30°C. Samples of 30 ml were removed at 0, 5, 10 and 15 min after addition of unlabeled methionine and cysteine to a final concentration of 10 µM. Cells were then rapidly filtered, washed with cold water, resuspended in 0.5 ml cold water and microfuged, and cell pellets were quickly frozen in liquid N_2.

The Cln3 synthesis rate was evaluated by pulse analysis in duplicated samples with the same labeling conditions as described above. In order to achieve similar total Cln3 protein levels in all extracts and easily comparable immunoprecipitation efficiencies, labeled proteins from starved and unstarved cells were mixed with the same amount of unlabeled proteins from unstarved and starved cells, respectively.

Double immunoprecipitation of cell extracts with the 12CA5 mouse monoclonal antibody against the HA epitope was essentially as described (Blondel and Mann, 1996). One-fifth of the total immunoprecipitate was used to quantitate the total HA-tagged Cln3 protein by Western blot as described above. The remainder was used to detect the labeled HA-tagged Cln3 in SDS gels treated for fluorography with EN^{3}HANCE (DuPont), and quantitated with a BAS-1000 (Fuji).

Flow cytometry and microscopy

DNA content distributions were obtained by propidium iodide staining as described (Nash *et al.*, 1988) with an Epics XL flow cytometer (Coulter), which was also used to quantitate relative cell concentrations from samples that had been fixed in 1% formaldehyde, 1× SSC, and sonicated for 5 s. Budding indexes were obtained by inspecting a minimum of 200 fixed cells under a phase-contrast microscope. Both long and short axes of a minimum of 100 cells were measured under a Zeiss LSM microscope, and average cell volumes were determined by considering yeast cells as regular obloids.

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References

Amon,A., Tyers,M., Futcher,B. and Nasmyth,K. (1993) Mechanisms that help the yeast cell cycle clock tick: G_2 cyclins transcriptionally activate G2 cyclins and repress G_1 cyclins. *Cell*, **74**, 993–1007.

Amon,A., Irniger,S. and Nasmyth,K. (1994) Closing the cell cycle circle in yeast: G_2 cyclin proteolysis initiated at mitosis persists until the activation of G_1 cyclins in the next cycle. *Cell*, **77**, 1037–1050.

Andrews,B.J. and Herskowitz,I. (1989) The yeast Swi4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. *Nature*, **342**, 830–833.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) *Current Protocols in Molecular Biology*. Wiley Interscience, New York.

Barbet,N.C., Schneider,U., Helliwell,S.B., Stansfield,I., Tuite,M.F. and Hall,M.N. (1996) *TOR* controls translation initiation and early G_1 progression in yeast. *Mol. Biol. Cell*, **7**, 25–42.

Baroni,M.D., Monti,P. and Alberghina,L. (1994) Repression of growth-regulated G_1 cyclin expression by cyclic AMP in budding yeast. *Nature*, **371**, 339–342.

Barral,Y., Jentsch,S. and Mann,C. (1995) G_1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. *Genes Dev.*, **9**, 399–409.

Benton,B.K., Tinkelenberg,A.H., Jean,D., Plump,S.D. and Cross,F.R. (1993) Genetic analysis of Cln/Cdc28 regulation of cell morphogenesis in budding yeast. *EMBO J.*, **12**, 5267–5275.

Blondel,M. and Mann,C. (1996) G_2 cyclins are required for the degradation of G_1 cyclins in yeast. *Nature*, **384**, 279–282.

Burke,D.J. and Church,D. (1991) Protein synthesis requirements for nuclear division, cytokinesis, and cell separation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 3691–3698.

Cross,F.R. (1995) Starting the cell cycle: what's the point? *Curr. Opin. Cell. Biol.*, **7**, 790–797.

Cross,F.R. and Blake,C.M. (1993) The yeast Cln3 protein is an unstable activator of Cdc28. *Mol. Cell. Biol.*, **13**, 3266–3271.

Cvrcková,F. and Nasmyth,K. (1993) Yeast G_1 cyclins *CLN1* and *CLN2* and a GAP-like protein have a role in bud formation. *EMBO J.*, **12**, 5277–5286.

Di Como,C.J. and Arndt,K.T. (1996) Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev.*, **10**, 1904–1916.

Dirick,L., Böhm,T. and Nasmyth,K. (1995) Roles and regulation of Cln–Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. *EMBO J.*, **14**, 4803–4813.

Epstein,C.B. and Cross,F.R. (1992) *CLB5*: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev.*, **6**, 1695–1706.

Ewen,M.E., Sluss,H.K., Whitehouse,L.L. and Livingston,D.M. (1993) TGFβ inhibition of Cdk4 synthesis is linked to cell cycle arrest. *Cell*, **74**, 1009–1020.

Garí,E., Piedrafita,L., Aldea,M. and Herrero,E. (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast*, **13**, 837–848.

Gietz,R.D. and Sugino,A. (1988) New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 3065–3073.

Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.

Hadwiger,J.A., Wittenberg,C., Richardson,H.E., De Barros Lopes,M. and Reed,S.I. (1989) A family of cyclin homologs that control the G_1 phase in yeast. *Proc. Natl Acad. Sci. USA*, **86**, 6255–6259.

Hengst,L. and Reed,S.I. (1996) Translational control of $p27^{Kip1}$ accumulation during the cell cycle. *Science*, **271**, 1861–1864.

Herskowitz,I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell*, **80**, 187–197.

Hinnebusch,A.G. (1985) A hierarchy of *trans*-acting factors modulate translation of an activator of amino acid biosynthetic genes in yeast. *Mol. Cell. Biol.*, **5**, 2349–2360.

Huble,L., Bradshaw-Rouse,J. and Heideman,W. (1993) Connections between the Ras-cyclic AMP pathway and G_1 cyclin expression in the budding yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **13**, 6274–6282.

Johnston,G.C., Pringle,J.R. and Hartwell,L.H. (1977) Coordination of growth and cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.*, **205**, 79–98.

Koch,C. and Nasmyth,K. (1994) Cell cycle regulated transcription in yeast. *Curr. Opin. Cell Biol.*, **6**, 451–459.

Koch,C., Moll,T., Neuberg,M., Ahorn,H. and Nasmyth,K. (1993) A role for the transcription factors Mbp1 and Swi4 in progression from G_1 to S phase. *Science*, **261**, 1551–1557.

Koch,C., Schleiffer,A., Ammerer,G. and Nasmyth,K. (1996) Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G_1. *Genes Dev.*, **10**, 129–141.

Lanker,S., Valdivieso,M.H. and Wittenberg,C. (1996) Rapid degradation of the G_2 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science*, **271**, 1597–1601.

Lee,K.S., Hines,L.K. and Levin,D.E. (1993) A pair of functionally redundant yeast genes (*PPZ1* and *PPZ2*) encoding type 1-related protein phosphatases function within the *PKC1*-mediated pathway. *Mol. Cell. Biol.*, **13**, 5843–5853.

Levine,K., Huang,K. and Cross,F.R. (1996) *Saccharomyces cerevisiae* G_1 cyclins differ in their intrinsic functional properties. *Mol. Cell. Biol.*, **16**, 6794–6803.

Lowndes,N.F., Johnson,A.L. and Johnston,L.H. (1991) Coordination of expression of DNA synthesis genes in budding yeast by a cell-cycle regulated trans-factor. *Nature*, **350**, 247–250.

Lowndes,N.F., Johnson,A.L., Breeden,L. and Johnston,L.H. (1992) Swi6 protein is required for transcription of the periodically expressed DNA synthesis genes in budding yeast. *Nature*, **357**, 505–508.

Markwardt,D.D., Garret,J.M., Eberhardy,S. and Heideman,W. (1995) Activation of the Ras/cyclic AMP pathway in the yeast *Saccharomyces cerevisiae* does not prevent G_1 arrest in response to nitrogen starvation. *J. Bacteriol.*, **177**, 6761–6765.

McInerny,C.J., Partridge,J.F., Mikesell,G.E., Creemer,D.P. and Breeden,L.L. (1997) A novel Mcm1-dependent element in the *SWI4*, *CLN3*, *CDC6*, and *CDC47* promoters activates M/G_1-specific transcription. *Genes Dev.*, **11**, 1277–1288.

Mitchison,J. (1988) Synchronous cultures and age fractionation. In Campbell,I. and Duffus,J.H. (eds), *Yeast*: *A Practical Approach*. IRL Press, Oxford, UK, pp. 51–64.

Nash,R., Tokiwa,G., Anand,S., Erickson,K. and Futcher,B. (1988) The *WHI1*$^+$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.*, **7**, 4335–4346.

Nasmyth,K. (1996) At the heart of the budding yeast cell cycle. *Trends Genet.*, **12**, 405–412.

Primig,M., Sockanathan,S., Auer,H. and Nasmyth,K. (1992) Anatomy of a transcription factor important for the cell cycle of *Saccharomyces cerevisiae*. *Nature*, **358**, 593–597.

Pringle,J.R. and Hartwell,L.H. (1981) The *Saccharomyces cerevisiae* cell cycle. In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces cerevisiae*: *Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. I, pp. 97–142.

Richardson,H.E., Wittenberg,C., Cross,F.R. and Reed,S.I. (1989) An essential G_1 function for cyclin-like proteins in yeast. *Cell*, **56**, 1127–1133.

Schneider,B.L., Yang,Q.-H. and Futcher,B. (1996) Linkage of replication to Start by the Cdk inhibitor Sic1. *Science*, **272**, 880–882.

Schwob,E. and Nasmyth,K. (1993) *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes Dev.*, **7**, 1160–1175.

Schwob,E., Böhm,T., Mendenhall,M.D. and Nasmyth,K. (1994) The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G_1 to S transition in *S.cerevisiae*. *Cell*, **79**, 233–244.

Stuart,D. and Wittenberg,C. (1995) *CLN3*, not positive feedback, determines the timing of *CLN2* transcription in cycling cells. *Genes Dev.*, **9**, 2780–2794.

Sudbery,P.E., Goodey,A.R. and Carter,B.L.A. (1980) Genes which control cell proliferation in the yeast *Saccharomyces cerevisiae*. *Nature*, **288**, 401–404.

Surana,U., Amon,A., Dowzer,C., Mcgrew,J., Byers,B. and Nasmyth,K. (1993) Destruction of the Cdc28/Clb kinase is not required for metaphase/anaphase transition in yeast. *EMBO J.*, **12**, 1969–1978.

Tokiwa,G., Tyers,M., Volpe,T. and Futcher,B. (1994) Inhibition of G_1 cyclin activity by the Ras/cAMP pathway in yeast. *Nature*, **371**, 342–345.

Tyers,M., Tokiwa,G. and Futcher,B. (1993) Comparison of the *Saccharomyces cerevisiae* G_1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.*, **12**, 1955–1968.

Wach,A., Brachat,A., Pöhlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.

Weiner,M.P., Costa,G.L., Shoettlin,W., Cline,J., Mathur,E. and Bauer,J.C. (1994) Site-directed mutagenesis of double strand DNA by the polymerase chain reaction. *Gene*, **151**, 119–123.

Wittenberg,C., Sugimoto,K. and Reed,S.I. (1990) G_1-specific cyclins of *S.cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with $p34^{CDC28}$ protein kinase. *Cell*, **62**, 225–237.

Yaglom,J., Linskens,M.H.K., Sadis,S., Rubin,D.M., Futcher,B. and Finley,D. (1995) $p34^{Cdc28}$-mediated control of Cln3 degradation. *Mol. Cell. Biol.*, **15**, 731–741.

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