The Cln3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast

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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 G1 phase of the cycle (Pringle and Hartwell, 1981). Haploid cells arrest temporarily in G1 in response to sexual pheromones and, after conjugation, enter a diploid cell cycle. Both haploid and diploid cells also arrest in G1 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 G0 state. The molecular pathway by which sexual pheromones cause a G1 arrest has been characterized in great detail (reviewed by Herskowitz, 1995). In contrast, little is known about the mechanisms that arrest yeast cells in G1 when they are deprived of nutrients.

Commitment to divide takes place in late G1, 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 G1 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, RNRI 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; Cvrcıková 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 G1–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 G1 under starvation conditions. Although cells deficient in Ras-cAMP activity arrest in G1, they also stop growing, thus making it difficult to
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 G1 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 G1 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 G1 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 G1 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 G1 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 G1-S transition.

As Cln cyclins play a fundamental role in the G1-S transition, we first asked whether the G1 arrest caused by nitrogen deprivation requires a down-regulation of the activity of these G1 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 G1 arrest under nitrogen deprivation conditions.

To characterize further the apparent uncoupling of cell growth and cell cycle kinetics observed in starved cultures, small early G1 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 G1 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 G1 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 G1 arrest, we asked whether nitrogen deprivation causes a transcriptional down-regulation of those genes known to be induced during the G1-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 G1. 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. G1 cells arrested by nitrogen deprivation are able to grow past the critical volume for budding. (A) Small early G1 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 (VMC). (B) DNA content distributions.

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 G1 (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 G1. 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 G1 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 G1 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 G1–S transition unless Sic1 is removed (Schneider et al., 1996), the absence of Cln proteins and the sustained presence of Sic1 could explain the G1 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
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Fig. 3. SBF- and MBF-driven gene expression is down-regulated by nitrogen deprivation by regular cell cycle mechanisms as cells arrest in G1. (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 G1–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-
regulation of Cln3 levels during nitrogen deprivation could be due to an increased turnover of the protein.

We recently have adapted to yeast cells (Gari 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-based protein turnover rate in the presence of the absence of the nitrogen source by either total protein decay after promoter repression or pulse–chase analysis. As shown 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

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**Fig. 4.** G1 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) Western blot to detect Swi6, Swi4, Mbp1 and Cdc28 with α-Swi6, α-Swi4, α-HA and α-PSTAIRE antibodies, respectively. CML141 (Δswi6::TRP1), K1939 (Δswi4::LEU2; K.Nasmyth), CML128 (wt) and CML225 (Δpho85::kan′) cell extracts were used as controls.
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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 G1 phase. We have found that nitrogen-deprived G1-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 G1 arrest when cells are nutritionally deprived, even under condi-

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Fig. 6. Cln3 becomes more unstable upon nitrogen deprivation. CML133 (wild-type), CML200 (cdc28-13) and HY600 (cdc34-2) cells carrying the CLN3-3HA construct under the control of the tetracycline-repressible tetO2 promoter (pCM166), were transferred to media with (+N, ○) or without (−N, □) the nitrogen source at time 0. (A) Repression kinetics of the tetO2 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).

One of the landmarks of the G1–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 G1 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 G1 when deprived of the nitrogen source, with many cells remaining in a budded state (Hadwiger et al., 1989; Lankar 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 G1 by nutrient deprivation.

Repression of SBF- and MBF-dependent transcription occurs during G2 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 G1. If the G1 arrest takes place before activation of SBF and MBF, cells should accumulate in G1, 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 G1–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 G1 before SBF and MBF activation takes place in late G1.

Cln3 is a potent G1 cyclin regarding the timely activation of SBF- and MBF-dependent promoters in late G1 (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 G1–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 G1 could be the intracellular parameter by which cells assess growth conditions before traversing the G1–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 G1 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.

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 G1 progression (Barbet et al., 1996) to regulation of translation initiation. Although rapamycin causes a G1 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 invariant 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 G1 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 G1 arrest (Johnston et al., 1977; our unpublished observations), progression through G1 seems to be the phase of the cell cycle most sensitive to unspecific protein synthesis inhibition. Thus, the rapamycin-mediated G1 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 G1 arrest under nutrient deprivation conditions.

Although loss of Cln3 in starved cells may be an important mechanism to achieve a G1 arrest, the fact that strains lacking CLN3 are still able to perform the G1–S transition indicates that some other mechanisms must exist to explain the G1 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 G1–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 G1, thus lowering Clb5 levels by degradation. Finally, the sustained presence of Sic1 in starved cells could explain in molecular terms why cells arrest in G1 when deprived of the nitrogen source.

The G1 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 G1-arrested cells occurs by both translational regulation and decreased degradation (Hengst and Reed, 1996). Translation and stability of other key regulators of the G1–S transition such as D-type cyclins may also contribute to link trophic signals to G1 arrest and differentiation in higher eukaryotic cells.

### Materials and methods

#### Strains, plasmids and growth conditions

Our parental strain CML128 (MATa, leu2-3,112, ura3-52, trp1-1, his4, can-1) is a spore of 1788 (Lee et al., 1993), a homogenous diploid strain obtained from EG123 (I.Herskowitz). The following CML128 derivatives were obtained: CML133 (LEU2::tryA), CML232 (LEU2::tryA, TRP1, HIS4), CML198 (cdc28-13), CML200 (LEU2::tryA, cdc28-13), CML233 (LEU2::tryA, TRP1, HIS4, cdc28-13), CML141 (MATa, TRP1, CML210 (CLN1-3HA), CML204 (CLN2-3HA), CML203 (CLN3-3HA), CML206 (CLB5-3HA) and CML208 (SIC1-3HA). Strain CML213 (MATa, ura3-52) is a spore of FY1679 (B.Dujon), a S288C derivative. Strain HTY600 (cdc34-2; L.Johnston) is a derivative of W303-1A (MATa, leu2-3,112, ura3-52, trp1-1, his4-11,75, ade2-1, can1-100; L.Johnston). The tetracycline-repressible transactivator tTA was introduced into yeast cells by integration of pCM87 (Gari et al., 1997) at the LEU2 locus. Chromosomal C-terminal fusions to the HA epitope were performed by gene transplacement with Pif1-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 BamHI and AscI 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 CLN2 ORF under the control of the tetO2, promoter (Gari et al., 1997) in YCplac33. pMBP1-1A (L.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 Pif1-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 OD600 = 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 Na2HPO4 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 Na2HPO4 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-DUT 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 Trin35S-label (ICN), 0.1 μM unlabeled methionine and 0.1 μM 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 (Gari 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-PSTAIRRE 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 μl of Tran3’-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 N2.

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 EN3HANCE (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. Binning 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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