

A cell sizer network involving Cln3 and Far1 controls entrance into S phase in the mitotic cycle of budding yeast

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S*accharomyces cerevisiae* must reach a carbon source-modulated critical cell size, protein content per cell at the onset of DNA replication (Ps), in order to enter S phase. Cells grown in glucose are larger than cells grown in ethanol. Here, we show that an increased level of the cyclin-dependent inhibitor Far1 increases cell size, whereas *far1*Δ cells start bud emergence and DNA replication at a smaller size than wild type. *Cln3*Δ, *far1*Δ, and strains overexpressing Far1 do

not delay budding during an ethanol glucose shift-up as wild type does. Together, these findings indicate that Cln3 has to overcome Far1 to trigger Cln–Cdc28 activation, which then turns on SBF- and MBF-dependent transcription. We show that a second threshold is required together with the Cln3/Far1 threshold for carbon source modulation of Ps. A new molecular network accounting for the setting of Ps is proposed.

Introduction

Cell viability requires coordination of cell growth and cell division (Tapon et al., 2001). In *Saccharomyces cerevisiae*, a model organism for cell cycle studies, such coordination mostly relies on the requirement of a nutritionally modulated critical cell size (protein content per cell at the onset of DNA replication [Ps]) to trigger budding and DNA replication and become committed to a new mitotic cell cycle (for reviews see Rupes, 2002; Wells, 2002). The *CLN3* gene encodes a cyclin that activates the Cdk Cdc28 in the events that commit a yeast cell to bud and to enter S phase (for review see Mendenhall and Hodge, 1998). Cln3 is a low abundance protein (Tyers et al., 1993; Ghaemmaghami et al., 2003). Cln3 level is modulated by nutrients (Hubler et al., 1993) and represents a constant fraction of cell mass during the G1 phase (Polymenis and Schmidt, 1997). In a given growth medium, deletion of the *CLN3* gene causes an increase in Ps, whereas *CLN3* overexpression, or mutations that increase Cln3 stability have the opposite effect (Tyers et al., 1993; Futcher, 1996). The above data indicate a key role of *CLN3* in the mechanism controlling the G1 to S transition and led to the suggestion that the increase of the

amount of Cln3 per cell might trigger entrance into S phase (Dirick et al., 1995; Futcher, 1996). A well-established role of Cln3–Cdc28 kinase is to activate SBF (Swi4–Swi6) and MBF (Mbp1–Swi6) transcriptional activators that drive the expression of a number of genes (~120), including those encoding the Cln1,2 and Clb5,6 cyclins (Koch et al., 1993; Cross et al., 1994), that in turn regulate many events required to enter S phase (Toone et al., 1997; Murray, 2004). Under physiological conditions, the timing of SBF/MBF activation is known to be determined by the onset of Cln3–Cdc28 activity (Dirick et al., 1995; Stuart and Wittenberg, 1995).

Cyclin–Cdc28 activity is negatively regulated by Cdk inhibitors (Ckis). Two Ckis are known in budding yeast: the first, Far1 specifically inhibits Cln1,2–Cdc28 and Cln3–Cdc28 complexes during the pheromone response (Peter et al., 1993; Tyers and Futcher, 1993), whereas the second, Sic1, specifically inhibits Clb–Cdc28 kinases (Mendenhall, 1993; Schwob et al., 1994). Clb5,6–Cdc28 inhibition is removed by Sic1 degradation that in turn is triggered by its Cln1,2–Cdc28-dependent phosphorylation, on six independent sites resulting in a steep, switchlike response that sets the onset of DNA replication (Nash et al., 2001).

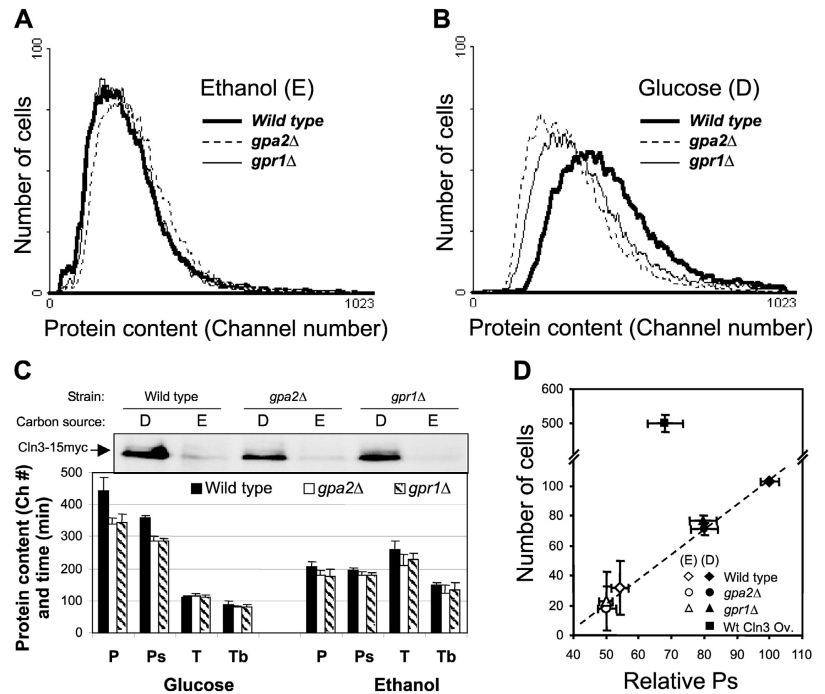
In *S. cerevisiae*, Ps increases in proportion with ploidy and is modulated by nutrients (Neufeld and Edgar, 1998; Rupes, 2002; Wells, 2002) and second messengers, such as cAMP (Baroni et al., 1989; Alberghina et al., 1998). In yeast cultures exponentially growing in shaken flasks the average protein

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Abbreviations used in this paper: Cki, Cdk inhibitors; P, protein content; Ps, protein content per cell at the onset of DNA replication; SC, synthetic complete; SCD, SC medium with glucose; SCE, SC medium with ethanol.

Figure 1. A functional extracellular glucose sensing system is required to properly set cell size (P) and protein content required for S phase initiation (Ps). (A) Size of wild-type cells (thick line), isogenic *gpa2Δ* cells (dashed line), and *gpr1Δ* cells (thin line) exponentially growing in ethanol (SCE medium) determined by FACS analysis of total cell protein after FITC staining. (B) Same as A for cells exponentially growing in glucose (SCD medium). (C) Immunoblot showing Cln3 protein level in wild-type and isogenic *gpa2Δ* and *gpr1Δ* cells grown in SCD and SCE (lanes labeled D and E, respectively). Extracts were loaded and bands quantified as detailed in Materials and methods. The values of average P, critical P at the beginning of DNA replication (Ps) determined by cytofluorimetric analysis (see Materials and methods for details), and duplication time (T) and length of budded phase (Tb) were determined for wild-type cells (black bars), *gpa2Δ* cells (white), and *gpr1Δ* cells (dashed) exponentially growing in SCE and SCD. Closed symbols indicate cells grown in SCD, open symbols indicate cells grown in SCE. Either average \pm SD of data derived from experiments repeated at least three times (C and D, bar chart) or representative results from one of such experiments (A, B, and Western blot in C) are shown.



content (P) is larger in conditions allowing fast growth rate, such as in glucose-supplemented media, than in populations growing at slow growth rate, for instance in the presence of ethanol as a carbon source (Lord and Wheals, 1980; Vanoni et al., 1983). Accordingly, data from glucose-limited continuous cultures indicate that cells are larger at dilution rates allowing fast growth than at dilution rates supporting slow growth (Porro et al., 2003). Thus, both in batch and continuous cultures the average cell P (and Ps) is fairly constant at low and intermediate growth rates and increases at high growth rates. The hyperactivation of cAMP signaling increases Ps (Baroni et al., 1992). Even if playing a key role in activating adenylyl cyclase (Broach, 1991), the Ras-cAMP pathway may not be directly involved in glucose signaling to adenylyl cyclase (Colombo et al., 1998). On the contrary, the G-protein coupled receptor Gpr1 and its cognate G α protein Gpa2 are required for glucose-induced cAMP signaling (Kraakman et al., 1999; Versele et al., 2001).

The results presented in this paper indicate that Cln3, Far1, Clb5,6, Sic1, and Cdc28 are involved in the cell size network controlling entry into S phase. In fact, we show that an increased level of the cyclin-dependent inhibitor Far1 increases cell size, whereas *far1Δ* cells start bud emergence and DNA replication at a smaller size than wild type. Together, these findings indicate that Cln3 has to overcome Far1 in order to trigger Cln-Cdc28 activation, which is in turn required for SBF- and MBF-dependent transcription. Thus, the ratio between Cln3 and Far1 defines a threshold involved in the molecular mechanism sensing cell size. A second threshold given by degradation of the Clb5,6-specific cyclin-dependent inhibitor Sic1 is required for the onset of DNA replication. Both thresholds are targets of the modulation by glucose of Ps, because carbon source modulation of Ps is lost only when Sic1 is inactivated concurrently with either Far1 or Cln3.

Together, with a wide body of literature data, our results have been used to construct a model for the G1 to S transition that incorporates the Cln3/Far1 threshold as a first initial trigger of a unifying framework that includes all the mechanisms previously proposed to control S phase entry, that have alternatively stressed the role of Cln3 in the nucleus as a trigger (Futcher, 1996), the activation of the SBF and MBF transcription factors (Mendenhall and Hodge, 1998; Jorgensen et al., 2002) and the timing of degradation of Sic1 (Schwob et al., 1994; Nash et al., 2001).

Results

Modulation of Cln3 level and of cell size by the Gpr1-Gpa2 glucose-sensing pathway

Both carbon source and cAMP are known to modulate Ps (Broach, 1991; Baroni et al., 1992). Thus, it was of interest to investigate whether mutations in the glucose-sensing pathway that activates cAMP synthesis affect glucose modulation of cell size and Ps. An easy way to assess whether a cell has the ability to modulate cell size and Ps in response to carbon source is to measure the average P in glucose and ethanol media, for instance by analyzing protein distributions of exponentially growing populations (Alberghina and Porro, 1993). Mutants carrying a deletion in either the Gpr1 receptor or the cognate G α encoding gene (*gpr1Δ* or *gpa2Δ* strain, respectively) were analyzed during exponential growth in synthetic complete (SC) medium supplemented with either 2% ethanol (SCE) or 2% glucose (SCD). During exponential growth on SCE, *gpr1Δ* and *gpa2Δ* strains do not differ significantly from isogenic wild-type cells in any of the measured cell cycle parameters. These include duplication time (T), the length of the budded phase

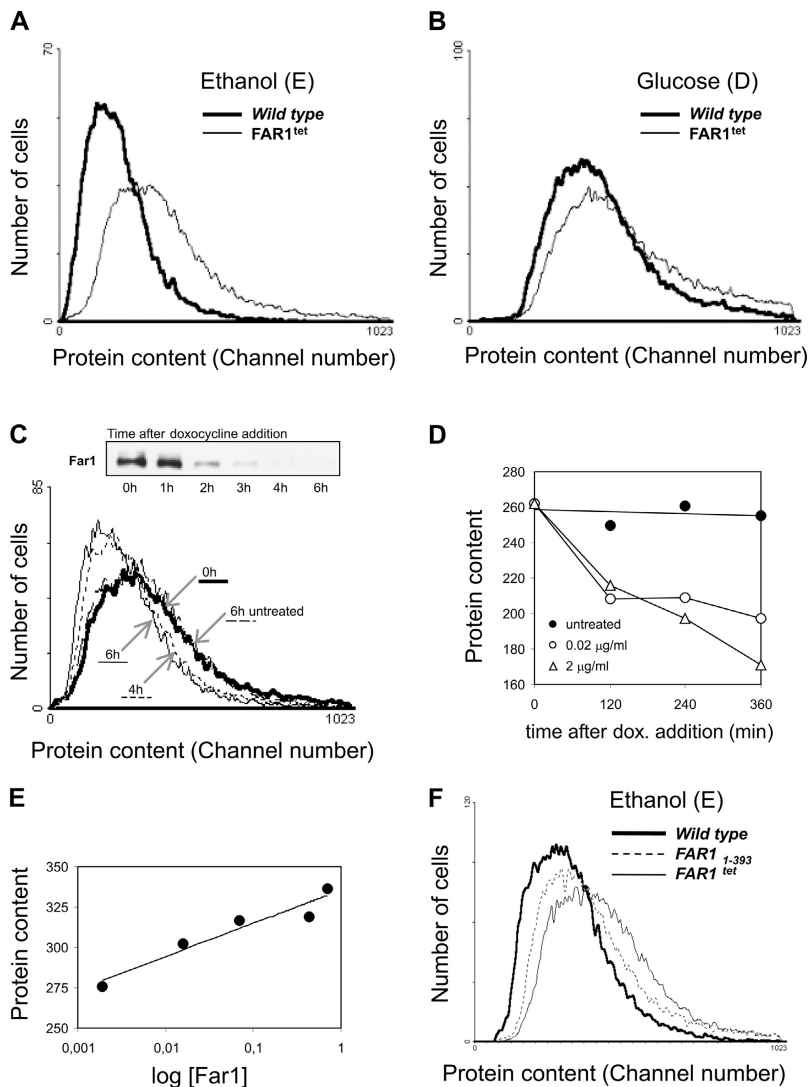


Figure 2. The *FAR1* gene is involved in cell size setting under both steady-state and transient growth conditions. (A) Size of wild-type cells (thick line) and *far1Δ* cells over-expressing *FAR1* under the control of a Tet-repressible promoter (*FAR1^{tet}* strain, thin line) during exponential growth in ethanol (SCE medium) determined by FACS analysis of total cell protein after FITC staining. (B). Same as A for cells exponentially growing in glucose (SCD medium). (C) *FAR1* expression in a *FAR1^{tet}* strain exponentially growing in SCE was switched off by addition of doxycycline at time 0. Far1 protein levels (top) and the average P (FACS analysis protein profile) were measured at the indicated time after doxycycline addition. (D) Average P from FACS analysis of untreated cells (closed circles), and after treatment with 0.02 µg/ml doxycycline (open circles) and 2 µg/ml doxycycline (open triangles). (E) Correlation between cellular P and intracellular Far1 level determined from quantification of immunoblot bands as detailed in Materials and methods. (F) Size of wild-type, *FAR1^{tet}*, and the *FAR1^{N393tet}* strains, the latter overexpressing a truncated form of Far1, during exponential growth in SCE determined by FACS analysis of total cell protein after FITC staining. Experiments were repeated twice with superimposable results. Representative results from one of these experiments are shown.

(Tb), the average P, and the average Ps (Fig. 1, A and C). The latter is operationally defined as the average P of cells with a DNA content just above 1c, as determined by two-dimensional flow cytometry (Cocchetti et al., 2004). Instead, *gpr1Δ* and *gpa2Δ* strains during exponential growth on SCD showed significantly altered protein distributions (Fig. 1 B), resulting in a reduction of both P and Ps (Fig. 1 C). The duplication time (T) and the length of the budded phase (Tb) were unaffected, consistent with the notion that signaling through the Gpr1–Gpa2 pathway specifically modulates Ps setting.

Because the G1 cyclin Cln3 has been suggested to play a pivotal role in Ps setting (Futcher, 1996), we analyzed Cln3 level in the above-mentioned *gpr1Δ* and *gpa2Δ* mutants. Fig. 1 C shows that during growth in glucose both *gpr1Δ* and *gpa2Δ* mutations cause a reduction in the Cln3 level. Such a decrease correlates well with the decrease in Ps (Fig. 1 D). On the contrary, ectopic Cln3 overexpression in SCD medium brings about a decrease in population cell size, as reported previously (Nash et al., 1988), as well as a decrease in Ps (Fig. 1 D).

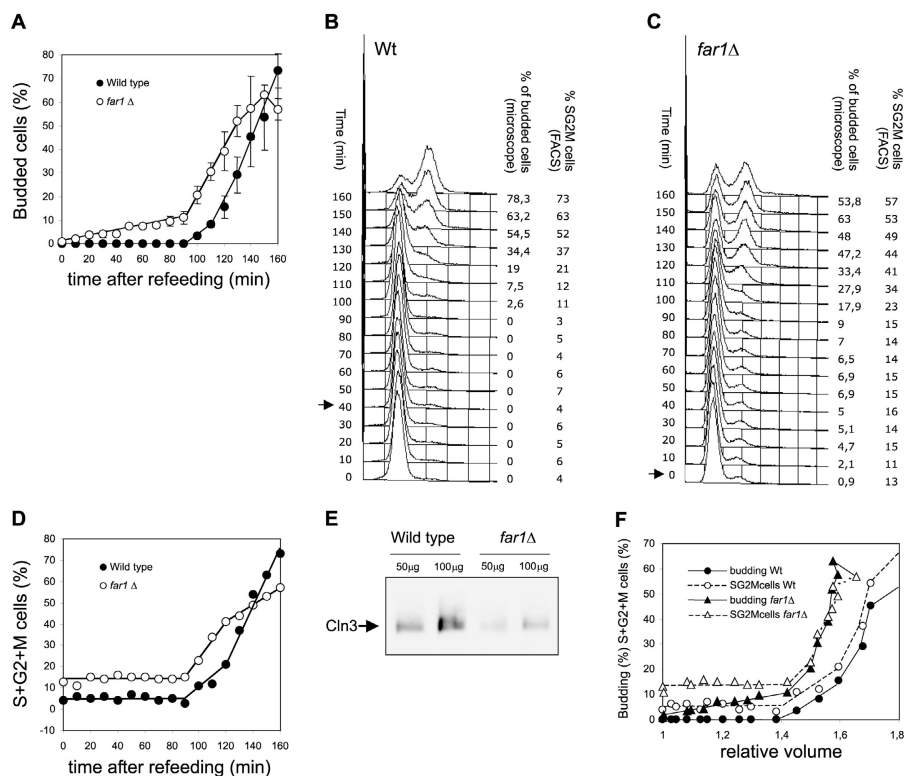
In any previously tested growth condition, increasing the level of Cln3 alone, by overexpression (Tyers et al., 1993) or

mutational stabilization of the protein (Cross and Blake, 1993), was shown to decrease cell size and Ps, which are instead increased in *cln3Δ* cells (Cross, 1988). On the contrary, when the Cln3 level is modulated by a change in the carbon source (Fig. 1; Hall et al., 1998) or by a defect in glucose sensing (Fig. 1), both cell size and Ps are directly proportional to Cln3 level. At a first glance these data may appear in conflict with one another (Hall et al., 1998), but they can be easily explained if Cln3 is not the only determinant of the cell sizer mechanism regulating entrance into S phase.

***FAR1* overexpression leads to a carbon source-dependent increase of cell size**

Based on a number of published data (Cross and Blake, 1993; Tyers and Futcher, 1993; Oehlen et al., 1996; Henchoz et al., 1997; Jeoung et al., 1998), we proposed previously (Alberghina et al., 2001) that the Cki Far1, that has long been recognized to inhibit the G1 to S transition in response to mating pheromones by inhibiting Cln–Cdc28 functions (Elion, 2000), may cooperate with Cln3 in establishing a nutritionally modulated threshold controlling S phase entry also in the mitotic cycle.

Figure 3. **FAR1 deletion partially uncouples budding and DNA synthesis and alters timing and cell size of S phase entry.** Wild-type and *far1Δ* cells were grown in SCD, elutriated as reported in Materials and methods and small cells refed with fresh prewarmed SCD at time 0. (A) Percentage of budded cells determined by microscopic observation (a minimum of 300 cells were counted for each time point) for wild-type cells (closed circles) and *far1Δ* cells (open circles). (B) DNA FACS distributions of wild-type cells synchronized by centrifugal elutriation and released in fresh SCD medium were determined every 10 min after refeeding. The percentage of budded cells and of cells with DNA content greater than 1c (S+G2+M) is indicated on the right side. (C) Same as in B for *far1Δ* cells. The single black arrow in the left side of B and C indicates the time point in which wild-type and *far1Δ* cells had the same volume (26 fl) as determined by coulter counter analysis. (D) Percentage of S+G2+M cells determined FACS analysis for wild-type cells (closed circles) and *far1Δ* cells (open circles). (E) Cln3 protein level were evaluated by Western blot analysis in wild-type (W303-p strain) and in *far1Δ* cells (*far1Δ*-p strain) exponentially growing in glucose (SCD medium); quantities of crude extracts loaded are indicated. (F) The percentage of budded cells and of S+G2+M cells from A and D are plotted as function of relative cell volume, where the initial volume of both cell types at time 0 was made equal to 1. Experiments were repeated twice with superimposable results. Representative results from one of these experiments are shown.



Our hypothesis is that the cell sizer mechanism governing the G1 to S transition is based on the molecular ratio between Cln3 and Far1, rather than based only on the increase in the concentration of Cln3 (Futcher, 1996). The presence of Far1 should not allow the formation of active Cln3–Cdc28 until the increase in Cln3 amount, driven by cell mass accumulation, overcomes the action of the Far1 inhibitor and hence permits the activation of SBF/MBF transcription by Cln3–Cdc28. This mechanism is thus a threshold driven by the increase in Cln3 and set by the amount of Far1 endowed to the cell at the beginning of the cycle.

The Cln3/Far1 threshold hypothesis, predicts a direct correlation between *FAR1* expression and population cell size. Data reported in Fig. 2 in fact show that overexpression of Far1 driven by a tetracycline-repressible promoter resulted in a shift of the protein distribution leading to increased average P in ethanol-grown cells (Fig. 2 A) and, to a much smaller extent, in glucose-grown cell (Fig. 2 B). Moreover, when *FAR1* expression was shut off by addition of tetracycline to SCE-growing cells, a time-dependent reduction of Far1 level and of the total P was observed (Fig. 2, C and D). Fig. 2 E shows that a linear relation between cellular P and the log of intracellular Far1 concentration can be observed. Finally, the ability to increase cell size upon overexpression is retained, at least partially, by the Far1₁₋₃₉₃ truncated protein (Fig. 2 F), which still contains the minimal Far1 domain able to elicit G1 arrest after α factor treatment (Valtz et al., 1995; Gartner et al., 1998).

FAR1 deletion partially uncouples budding and DNA synthesis and alters timing and cell size of entry into S phase

If a Cln3/Far1 threshold is operative, also a deletion of *FAR1* should affect timing and cell size for entry into S phase. To address this question, wild-type and *far1Δ* cells were grown in SCD, size-selected by centrifugal elutriation, and the smallest cells were refed in fresh prewarmed medium. In exponential growth in glucose, wild-type and *far1Δ* strains showed similar protein and DNA distributions (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200405102/DC1>). Biparametric flow cytometry for DNA/protein showed that the P at the beginning of the cell cycle was reproducibly larger in *far1Δ* cells than in the wild-type strain (Fig. S1, A–C). In agreement with this observation, the smallest elutriable *far1Δ* cells were consistently larger than their wild-type counterparts.

As shown in Fig. 3 A, during the first 90 min after fresh medium refeeding, no bud emergence is observed in wild-type cells, whereas mutant *far1Δ* cells started to bud as soon as fresh medium was refed. Only small buds were produced in this period (not depicted). During the same time window, no net DNA synthesis was observed in either wild-type (Fig. 3 B) or *far1Δ* cells (Fig. 3 C), although in the latter strain a constant low level of cells with DNA content higher than 1c was observed. Because DNA synthesis requires overcoming of a second, Sic1-dependent, threshold (Nash et al., 2001), these results may be due to partial uncoupling of budding and DNA synthesis, al-

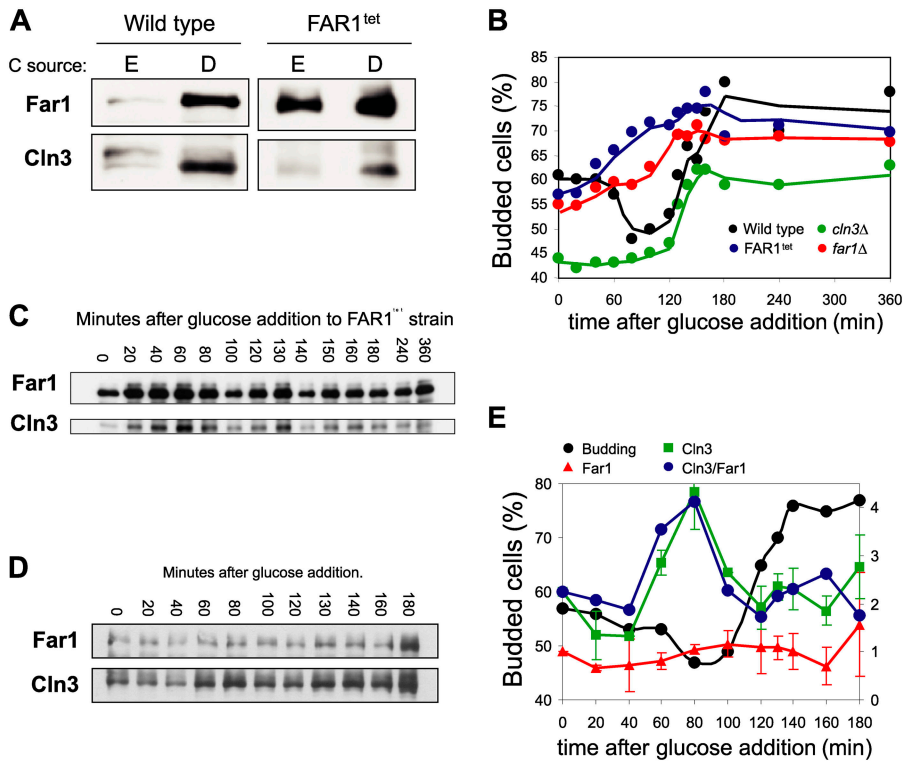


Figure 4. Far1-dependent delay of budding in an ethanol to glucose nutritional shift up. (A) Far1 and Cln3 protein levels in wild-type and FAR1 overexpressing strain (FAR1^{tet}): both strains were grown in SCE (E) and SCD (D) and mid log phase cells were collected for protein extraction followed by Western blot analysis with anti-Myc antibodies as detailed in Materials and methods. (B) At time 0 2% glucose was added to cells exponentially growing in SCE medium and samples were collected to evaluate the percentage of budded cells in wild-type (black), *far1Δ* (red), *cln3Δ* (green), and FAR1 overexpressing (FAR1^{tet}, blue) strains. (C) Western blots of Far1 and Cln3 proteins from cells overexpressing FAR1 (FAR1^{tet} strain) during an ethanol/glucose shift up. (D) Western blot of Far1 and Cln3 proteins from wild-type cells (W303-CF strain) during an ethanol/glucose shift-up. (E) Cln3 (green) and Far1 (red) protein levels and their ratio (blue) were determined during ethanol/glucose shift-up by quantification of Western blot bands of wild-type cells (W303-CF strain) as detailed in Materials and methods. Percentage of budded cells (black) and Cln3/Far1 mean values ratio (blue) are also plotted as a function of time. Protein levels shown are average \pm SD of data derived from independent experiments repeated at least twice. Blots shown are representative of experiments repeated at least twice with similar results.

though limited sensitivity of flow cytometry may hamper detection with sufficient resolution of cells entering just the earliest stage of S phase. After 90 min, budding and DNA synthesis were activated in both wild-type and *far1Δ* cells. The kinetics of budding and DNA synthesis completion are directly compared in Fig. 3 (A and D), respectively. The initial rate of budding in *far1Δ* cells (time 0–90 min) was significantly lower than that observed in the same cells after 90 min, that paralleled that observed in the wild type. Right after elutriation, *far1Δ* cells showed a low level of cells with DNA content higher than 1c, but only after 90 min DNA synthesis started, with a rate very similar to that observed in wild type (Fig. 3 D). Some delay in the completion of S (or in the entry of all cells into S) was also apparent. The above data clearly indicate that budding is anticipated in the absence of Far1, as expected. In *far1Δ* cells bud emergence proceeds steadily, although with an initial slow kinetics that may depend at least in part upon the lower steady-state level of Cln3 in *far1Δ* cells (Fig. 3 E).

The black arrow on the left side of Fig. 3 (B and C) indicates the time point in which wild-type and *far1Δ* cells had the same volume (26 fl) as determined by Coulter counter analysis, i.e., wild-type cells reach the same size of the smallest *far1Δ* elutriated cells only 40 min after fresh medium has been refed. Nevertheless, they will start budding only after an additional 60 min.

The percentage of budded cells and the percentage of cells with a DNA content higher than 1c were thus replotted as a function of relative cell size, taking as 1 the cell size of the smallest elutriated cells (Fig. 3 F). Both budding and net DNA synthesis can be shown to start at a smaller relative cell size in *far1Δ* cells than in wild type. Therefore, the lack of Far1 affects both the timing and cell size at which bud emergence and

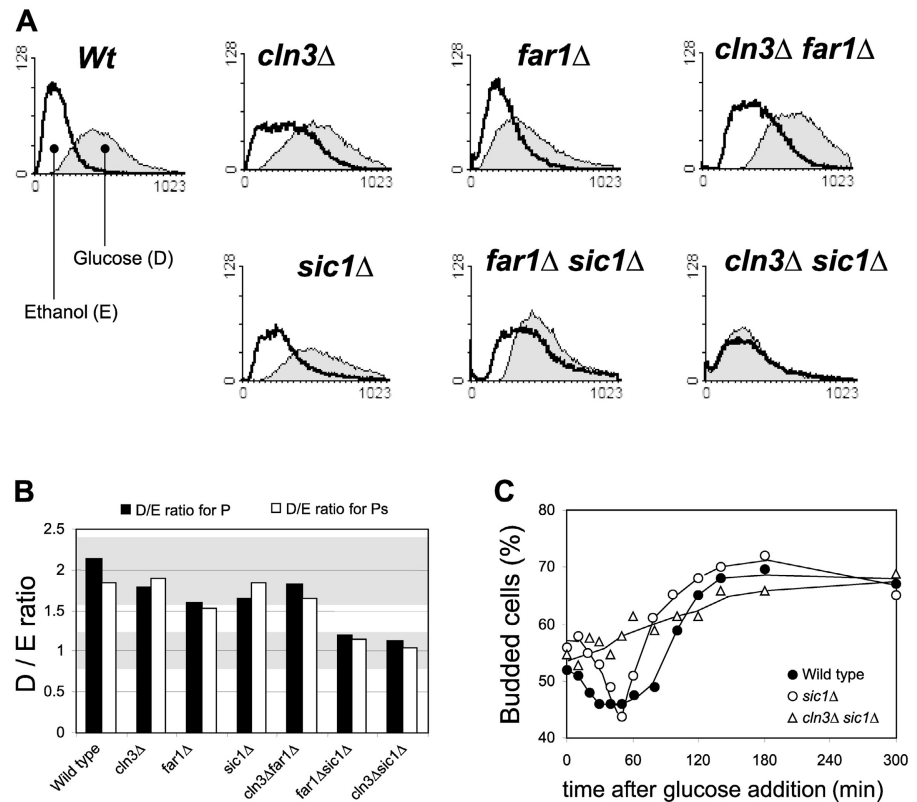
DNA replication start. Together, these data strongly support our hypothesis of a role for Far1 in setting the control of entrance into S phase during the mitotic cycle.

On the other hand, although the data of Fig. 3 (A and C) indicate that cell lacking Far1 preferentially anticipate budding in elutriated G1 cells compared with wild type, they also show that full-fledged budding and DNA synthesis take place at a similar period after refeeding (90 min) in both strains, thereby suggesting that a second control step has to be overcome to allow completion of budding and DNA synthesis. The degradation of Sic1, the Cki that blocks Clb5,6–Cdc28 activity appears well suited as this second control point (Nash et al., 2001).

Resetting of the Cln3/Far1 threshold regulates the G1 to S transition during a nutritional shift-up

We showed previously that cells undergoing a nutritional shift-up delay execution of the G1 to S transition as indicated by a transient drop in the fraction of budded cells, while at the same time cell size increases, consistent with a resetting of Ps to a new, higher value that is typical of the new medium (Alberghina et al., 1998). If the above described Cln3/Far1 threshold is acting as a regulatory device during the ethanol glucose shift-up, we expect that the signature drop in budding should be lost in cells where the balance between the two components of the threshold is altered. In fact, the absence of the Far1 inhibitor in *far1Δ* cells should make cells respond to the change in carbon source as soon as Cln3 increases. Accordingly, in *cln3Δ* cells the target for inhibition by Far1 is absent, so that no drop in budding should be expected. Finally, because overexpression of Far1 results in a large excess of Far1 over Cln3 (Fig. 4 A,

Figure 5. Carbon source modulation of cell size is totally lost when both Far1- and Sic1-dependent thresholds are disrupted. (A) Size of wild-type cells and isogenic single or double mutants exponentially growing in medium supplemented with ethanol (SCE, open distributions) or glucose (SCD, gray distributions) by FACS analysis of total cell protein after FITC staining. (B) Mean values of P distributions of wild-type cells and deletion mutant cells in A exponentially growing in glucose and in ethanol were determined by FACS analysis (the complete set of mean values and SDs from three independent experiments is reported in Fig. S2); the ratio between mean value in glucose, P(D), and mean value in ethanol, P(E), is indicated as D/E ratio and is shown in the histogram for wild-type and each deletion mutant (black bars). In the same way and for the same cells, Ps were determined by FACS analysis (see Fig. S2 for values and Materials and methods for details). The corresponding D/E ratios for Ps are shown in the histogram as white bars. Shaded areas cover a 20% variation of D/E ratio around ordinate values of 1 (absence of carbon source-dependent modulation of size) and 2 (full wild-type modulation). (C) At time 0 2% glucose was added to cells exponentially growing in SCE medium and samples were collected to evaluate the percentage of budded cells. Wild type (closed circles), *sic1*Δ (open circles), and *cln3*Δ*sic1*Δ (open triangles).



where protein levels can be directly compared because both Far1 and Cln3 are fused to the same 15-myc tag), we expect that this strain should display a phenotype similar to that of *cln3*Δ cells. Data reported in Fig. 4 B show budding kinetics during a shift-up for wild-type (black), *far1*Δ (red), *cln3*Δ (green), and Far1 overexpressing (Far1^{tet}, blue) cells. In keeping with our predictions, the drop in budding is present only in wild-type cells, and is lost in strains where the normal interplay between Cln3 and Far1 is disrupted.

In Far1 overexpressing cells, Far1 remains constantly well above Cln3 within the examined time window even though Cln3 starts to increase as soon as glucose is added (Fig. 4 C). Besides, in wild-type cells exponentially growing in media supplemented with either ethanol or glucose, the ratio between Cln3 and Far1 remains similar, although the level of both proteins changes substantially (Fig. 4 A, left). During a shift-up an excess of free Cln3 builds up during the first 90 min of the shift-up and therefore an increase in the Cln3/Far1 ratio is observed (Fig. 4, D and E, blue line). After a maximum in the Cln3/Far1 ratio has been reached, budding increases. At later times Cln3 and Far1 reach the steady-state level typical of the new carbon source. Together, the above-mentioned results are consistent with the hypothesis that a Cln3/Far1 cell size control entry into S phase.

Two sequential Far1 and Sic1 mediated thresholds cooperate in modulating cell size in different carbon sources

It has long been known that *cln3*Δ cells are larger than their wild-type isogenic counterpart (Nash et al., 1988; Futcher,

1996). Protein distributions from wild-type, *cln3*Δ, *far1*Δ, and *cln3*Δ*far1*Δ double mutants are reported in the top row of Fig. 5 A for cells exponentially growing in ethanol and glucose supplemented media. Average P and average Ps were computed from these distributions and are reported in Fig. S2 (A and B; available at <http://www.jcb.org/cgi/content/full/jcb.200405102/DC1>). Fig. 5 B shows the ratios of P and Ps measured in glucose over that measured in ethanol (black and white bars, respectively). Both P and Ps in wild-type cells growing in glucose are about twice as much as those found in cells growing in ethanol. We consider that carbon source modulation is present for values of the ratio glucose/ethanol above 1.6 (Fig. 5 B, top shaded area) and lost for values of the ratio around 1.0 (Fig. 5 B, bottom shaded area). *cln3*Δ, *far1*Δ and *cln3*Δ*far1*Δ cells only marginally lose their ability to modulate P and Ps in response to carbon source (Fig. 5, A and B), thereby indicating that the Cln3/Far1-dependent cell size mechanism is not the only determinant in modulation of DNA synthesis initiation by carbon source.

Therefore, we asked whether the Sic1 Cki, whose degradation is known to be critical for the onset of DNA replication, might play a role in the network regulating carbon source modulation of cell size-dependent G1 to S transition in yeast. As shown in Fig. 5 (A and B), *SIC1* deletion has only a minor effect on glucose modulation of cell size and Ps in exponentially growing cells, similar to what observed for the *cln3*Δ and *far1*Δ deletions. Strikingly, concurrent deletion of *SIC1* and either *CLN3* or *FAR1*, almost completely abolished glucose modulation of both P and Ps (Fig. 5, A and B). The ratio for both parameters is in fact close to one in both *cln3*Δ *sic1*Δ and *far1*Δ *sic1*Δ cells, indicating that in exponentially growing

cells, the glucose-dependent setting of cell size lies entirely on these two cyclin–Cdc28/Cki-mediated thresholds.

During transitory states, such as an ethanol/glucose shift-up, the role of the Cln3/Far1 threshold may be more relevant. In fact a drop in budding after shift-up was observed both in wild-type and *sic1Δ* strains, although both the drop and the subsequent rise in budding were somehow steeper in *sic1Δ* cells (Fig. 5 E). Moreover, double *sic1Δ cln3Δ* mutants under the same conditions, as previously observed for *cln3Δ* or *far1Δ* mutants (Fig. 4 C) did not show the transient drop in budding. Thus, resetting of the cell size-dependent threshold during a shift-up takes place mostly through an alteration of the Cln3/Far1 ratio. As expected, because of the role of the Gpr1–Gpa2 pathway in glucose sensing, a similar lack of the transient drop in budding is observed in the *gpr1Δ* mutants (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200405102/DC1>).

Discussion

The main novel contribution of this paper is to provide experimental evidence for the involvement of Far1 in the cell sizer mechanism that controls entry into S phase in the mitotic cell cycle and to show that Sic1 has a previously unrecognized role in the modulation by carbon source of the critical size Ps for entry into S phase.

A molecular mechanism for the Cln3/Far1 threshold

The Cln3/Far1 threshold allows to rationalize why overexpression of Cln3 in a given medium results in a reduction in cell size, whereas the opposite holds when Cln3 level is physiologically regulated by a change of carbon source (i.e., ethanol vs. glucose). Far1 is a large protein that is present mainly during late G2, M, and early G1 phases (Oehlen et al., 1996). Phosphorylation by Cln–Cdc28 primes it for degradation (McKinney and Cross, 1995). It shuttles between the cytoplasm and the nucleus, where it is enriched during G1. Overexpression of a nonphosphorylatable, stable Far1 mutant is able to induce cell cycle arrest in the absence of α factor, cells arresting as large unbudded cells with a G1 DNA content and low Cln–Cdc28 activity (Henchoz et al., 1997). Mating pheromones, in contrast, enhance Far1 synthesis, accumulation and nuclear export (Nern and Arkowitz, 2000). Far1 has long been recognized to inhibit the G1 to S transition in response to mating pheromones by inhibiting Cln–Cdc28 functions required for budding (Elion, 2000). The mechanism through which Far1 inhibits Cln–Cdc28 function is not yet completely understood: it may involve direct inhibition of Cln–Cdc28 kinase activity, as well as shielding of substrates from the active site of the complex, and/or restriction of the Cln–Cdc28 complex to subcellular compartments where the kinase complex cannot complete its cell cycle specific function (for review see Elion, 2000). Notably, Far1 has been reported to bind to (Tyers and Futcher, 1993), and inhibit the kinase activity of (Jeoung et al., 1998), Cln3–Cdc28 kinase. More recently, a report suggesting the involvement of Far1 in the control of the G1 to S transition in mitotic cells on the basis of the ability of a *far1* deletion to res-

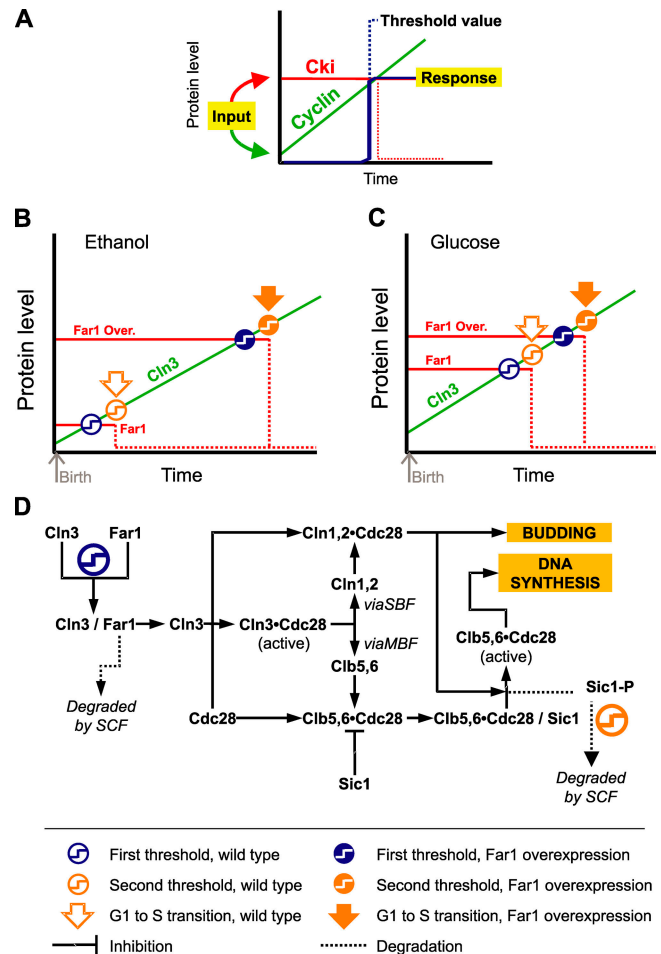


Figure 6. Molecular blueprint of the network controlling the G1 to S transition in budding yeast. (A) Schematic representation of a molecular threshold: the antagonistic effect of an activator (cyclin, green) and an inhibitor (Cki, red) sets the threshold for the response (blue). (B) Effect of Far1 overexpression on the G1 to S transition execution (large arrows) in cells growing in ethanol, and (C) in cells growing in glucose. The Cln3/Far1 threshold (blue) is set by the level of Far1 (red line) received by newborn cells. The threshold is executed when Cln3 (green line), exceeds Far1 and is made irreversible by Far1 degradation (red dotted line). The second threshold (orange) is set by Sic1 degradation. (D) Model of a two threshold network for the control of the G1 to S transition.

cue the G1 arrest of a *cdc48*-td mutant was published previously (Fu et al., 2003). The results presented in this paper directly confirm the involvement of Far1 in the control of the mitotic cell cycle at the G1 to S transition.

The threshold mechanism is outlined in Fig. 6 A. During G1, Cln3 amount per cell increases proportionally to cell mass (Cross and Blake, 1993). The Cln3/Far1 threshold (blue) is set by the level of Far1 (red line) that is mostly endowed to the cell at the previous cell division and remains approximately constant in G1 cells. When the amount of Cln3 (green line), very likely localized in the nucleus (Edgington and Futcher, 2001; Miller and Cross, 2001), overcomes that of Far1 (red solid line), a first threshold is overcome. The threshold is made irreversible by Cln–Cdc28-primed Far1 degradation (red dotted line). The S-shaped, thick blue line indicates the response of the threshold.

Nutrients modulate the accumulation of both Cln3 and Far1, because exponentially growing cells have higher average levels (~10-fold) of both Cln3 and Far1 when grown on glucose supplemented media than when the carbon source is ethanol, whereas the Cln3/Far1 ratio remains similar under both conditions (Fig. 4 A, left). A further layer of complexity is given by the observation that the Cln3 level may also depend on the presence of Far1 as suggested by the reduced levels of Cln3 in *far1Δ* strains (Fig. 3 E).

Indications of the dynamics of the Cln3/Far1 threshold come from the following observations. In ethanol-growing wild-type cells we detect a linear relationship between the log of the average level of Far1 in the cell and the average cell size (Fig. 2, C–E). Under the same conditions, ectopic expression of *FAR1* results in a level of Far1 that largely exceeds that of Cln3 (Fig. 4 A, compare lanes marked E showing extracts from wild-type with Far1 overexpressing strains). This event severely delays overcoming of the Far1 level by Cln3, i.e., threshold overcoming, so that cell size in the population is significantly increased. As a result, the second, Sic1-mediated threshold is executed at a size much larger than in wild type (Fig. 6 B). On the contrary, in glucose-grown cells, ectopic Far1 expression only marginally increases Far1 level as compared with the wild-type situation (Fig. 4 A, compare lanes marked D in cell prepared from wild-type with Far1 overexpressing strains), so that the Ps cell sizer is set to a value quite similar to that observed in wild-type cells (Fig. 6 C). As a result, the Cln3/Far1 threshold is only marginally up-regulated. Moreover, the larger amount of Cln3–Cdc28 that remains free after the threshold overcoming (due to the shielding role of the more abundant Far1 present in glucose-grown cells) should allow a faster build-up of Cln1,2 and Clb5,6. As a result execution of the Sic1 threshold is accelerated, so that very marginal effects on the population cell size and Ps are expected, as observed.

The increase in cell size and Ps brought about by Far1 overexpression in ethanol growing cells is largely due to the NH₂-terminal domain, which has been shown to be responsible for cell cycle arrest of α factor treated cells. In fact, cells overexpressing Far1₁₋₃₉₃ are significantly larger than wild-type cells, though not as large as cells overexpressing full-length Far1. Far1₁₋₃₉₃ retains cell cycle inhibitory and localization signals (Gartner et al., 1998), whereas it lacks the Cdc24 binding site (Cross and Blake, 1993; Butty et al., 1998). Therefore, because Far1₁₋₃₉₃ is expressed in a *far1Δ* background, Cdc24 is likely to be mostly cytoplasmic.

The effects of a *FAR1* deletion are subtler. In wild-type cells, SBF and MBF activation is delayed until Cln–Cdc28 phosphorylates Far1 (Henchoz et al., 1997), that can then be degraded, giving rise to a burst of free Cln3–Cdc28 complexes that irreversibly and efficiently complete the overcoming of the Cln3/Far1 threshold. In the absence of Far1, free Cln3–Cdc28 complex is formed earlier in the cycle, but its level is much lower than the level released in wild-type after Far1 degradation. In fact, a small amount of Cln3 is present at the beginning of the cycle, and most cellular Cln3 will accumulate throughout the cell cycle as mass increases. Moreover, the absence of Far1 decreases the overall steady-state Cln3 level (Fig. 3 E). The

low level of active Cln3Cdc28 complex in *far1Δ* elutriated cells cause premature budding initiation at a low but consistent rate (Fig. 3 A). Indeed, when the larger size of newborn cells is taken into account, *far1Δ* cells are shown to start both budding and DNA synthesis at a size smaller than the isogenic wild-type cells. Thus, strains either overexpressing or lacking Far1 show a cell cycle behavior consistent with a role of Far1 in setting a threshold required for triggering budding and DNA replication.

A two-threshold network for the control of the G1 to S transition

Integration of the results presented in this paper with a wide body of literature data led to the model of the network controlling the G1 to S transition reported in Fig. 6 D that allows to unify in the same framework all the mechanisms previously proposed to control S phase entry, namely a triggering role for nuclear Cln3 accumulation (Futcher, 1996), the activation of the SBF and MBF transcription factors (Mendenhall and Hodge, 1998) and Sic1 degradation (Schwob et al., 1994; Nash et al., 2001).

At the core of the network lies the above-described Cln3/Far1 threshold. Once the threshold has been overcome, free Cln3 is ready to form active Cln3–Cdc28 complexes that in turn trigger the SBF- and MBF-dependent transcription of the *CLN1,2* and *CLB5,6* genes and the synthesis of the encoded proteins. Whether Far1 binds Cln3 directly (Fig. 6 D) or only after the Cln3–Cdc28 complex has been formed, is not critical at the present stage to our model, as long as it is the excess of Cln3 over Far1 that brings to active Cln3–Cdc28 complex and therefore triggers entrance into S phase. Far1 phosphorylation by nuclear Cln–Cdc28 directs Far1 to degradation (Henchoz et al., 1997); hence it frees more Cln3 molecules that give a sizeable burst to the Cln3–Cdc28 activity in the cell. The newly formed Clb5,6–Cdc28 complexes are not immediately active, due to the presence of their Sic1 inhibitor. Only after Sic1 removal by SCF-mediated degradation, the onset of DNA replication occurs. Sic1 degradation requires phosphorylation by Cln1,2–Cdc28 on at least six of its nine Cdk-phosphorylatable serines. This requirement originates a switch response (Nash et al., 2001) and may be an example of a more general mechanism to set thresholds in regulated protein–protein interactions (for review see Deshaies and Ferrell, 2001). Therefore, the cell sizer network controlling S phase entry requires the overcoming of at least two sequential thresholds that modulate Cdc28 activity, the first involving Cln3 and Far1, the second one Cln1,2, Clb5,6, and Sic1.

Cell size modulation by carbon source involves both a Cln3–Far1 and Clb5,6–Sic1 threshold

The Gpr1–Gpa2 glucose-sensing pathway that leads to adenyl cyclase activation is required to modulate the critical cell size Ps in response to glucose (Fig. 1). In the meantime, the inactivation of the glucose sensing system does not affect the growth rate of the cells. This finding indicates that yeast cells are able to modify their cell cycle machinery in response to an external signal, showing a sort of growth factor/hormone-like

control on cell cycle entry as observed in mammalian cells (Wells, 2002). Cyclic AMP hyperactivation has been shown to inhibit the expression of Cln1 and Cln2, quite likely by inhibiting transcription of the corresponding genes (Baroni et al., 1994; Tokiwa et al., 1994). However, inactivation of the Gpr1–Gpa2 pathway does not completely abrogate glucose modulation of cell size, because *gpr1Δ* or *gpa2Δ* cells growing in the presence of glucose are still larger than those growing in ethanol-containing media. On the contrary, protein distributions of *cln3Δ sic1Δ* cells growing in either ethanol or glucose-containing media are superimposable, indicating that concurrent inactivation of both the Cln3–Far1 and Clb5,6–Sic1-mediated thresholds is required to make a yeast cell totally unable to modulate its Ps in response to glucose (Fig. 5).

The partial effect of mutations in the Gpr1–Gpa2 pathway indicates that both intracellular and extracellular glucose cooperate to set Ps. A requirement for both intracellular and intracellular glucose signaling has previously shown for glucose-induced cAMP signaling (Rolland et al., 2000). Although dysfunction of the signal transduction pathway monitoring extracellular glucose only partially affects glucose-modulation of Ps, disruption of components of both thresholds, which are likely to respond to intracellular glucose as well, completely abolishes glucose control over Ps.

In conclusion, the presence of two sequential thresholds, each one able to integrate cell signaling information coming from external and internal conditions should provide the cell with a very relevant safety control device over the commitment to enter S phase. Because the second threshold is dependent on the first one and both are modulated by the carbon source, one or the other may appear predominant in a certain condition (defined as the combination of carbon source and genetic make up). The distribution of control over several sequential steps is a general property of metabolic pathways, and has been previously reported in signal transduction pathways as well (Thomas and Fell, 1998).

Table 1. Strains used in this work

Strain	Relevant genotype
W303-CF	<i>cln3::KAN1</i> , pCLN3-15Myc, <i>FAR1-15Myc-URA3</i>
W303-p	<i>cln3::KAN1</i> , pCLN3-15Myc
<i>cln3Δ</i>	<i>cln3::KAN1</i>
<i>far1Δ</i>	<i>far1::HIS3</i>
<i>far1Δ-p</i>	<i>cln3::KAN1</i> , pCLN3-15Myc, <i>far1::HIS3</i>
<i>sic1Δ</i>	<i>sic1::HIS3</i>
<i>cln3Δ sic1Δ</i>	<i>cln3::KAN1</i> , <i>sic1::HIS3</i>
<i>cln3Δ far1Δ</i>	<i>cln3::KAN1</i> , <i>sic1::HIS3</i>
<i>far1Δ sic1Δ</i>	<i>far1::HIS3</i> , <i>sic1::KAN1</i>
<i>gpa2Δ</i>	<i>cln3::KAN1</i> , pCLN3-15Myc, <i>gpa2::LEU2</i>
<i>gpr1Δ</i>	<i>cln3::KAN1</i> , pCLN3-15Myc, <i>gpr1::LEU2</i>
CLN3 ^{tet}	<i>cln3::KAN1</i> , pTet-CLN3-3HA
FAR1 ^{tet}	<i>cln3::KAN1</i> , pCLN3-15Myc, <i>far1::HIS3</i> , pTet-FAR1-15Myc
FAR1 ^{N393-tet}	<i>cln3::KAN1</i> , pCLN3-15Myc, <i>far1::HIS3</i> , pTet-FAR1 ₁₋₃₉₃ -15Myc

All the strains are obtained from, and are isogenic to, W303-1a (*MAT-α*, *ade2-1*, *can1-100*, *his3-11*, *leu2-3*, *trp1-1*, *ura3-1*) with the exception of *far1Δ sic1Δ* strain obtained by crossing W303-1a *far1::HIS3* with W303-1b *sic1::KAN1* isogenic to W303-1b (*MAT-α*, *ade2-1*, *can1-100*, *his3-11*, *leu2-3*, *trp1-1*, *ura3-1*).

Materials and methods

Yeast strains

Strains used in this work are listed in Table 1.

Molecular and microbiological techniques

DNA manipulations and yeast transformations were performed according to standard techniques. The 3HA sequence of plasmid pCM194 (a YCplac33 derivative) was supplied by M. Aldea; Universitat de Lleida, Lleida, Spain) was excised as a BamHI–AscI cassette and replaced with a 15Myc sequence. Plasmid pCLN3-15Myc was obtained, carrying a *CLN3* gene under the control of its own promoter and followed by an in-frame 15Myc sequence.

Far1 protein was tagged with a COOH-terminal 15Myc epitope, by in-locus 3' in-frame insertion. We replaced a 15Myc encoding sequence as a BamHI–AscI fragment in the pFA6a-GFP(S65T)-HIS3MX6 plasmid (Wach et al., 1998) obtaining the pFA6a-15Myc-HIS3MX6 plasmid, subsequently used as short flanking homology PCR template to amplify the 15Myc-HIS3 region flanked by Far1 COOH-terminal replacement sequences as described in Wach et al. (1998). Oligonucleotides used were the following: *FAR1-W*: 5'-TGGTAAAGCAGCAAGAATTCATCAGACCCTGGAAGTCCCAACCCCGTACGCTGCAGGTCGAC-3'. *FAR1-C*: 5'-AAAGGAAAAACAAAAGCCTCGAAATACGGGCTCGATTCCCGAACTAATCGATGATTTCGAGCTCG-3'. Tagged strains were phenotypically indistinguishable from the parent strain in a variety of growth conditions; duplication times, fraction of budded cells, P, and overall morphology were assayed in glucose and ethanol containing media.

To obtain the Far1 overexpression, *FAR1* gene has been amplified by PCR, omitting start codon, and inserted in plasmid pCM189 (Gari et al., 1997) as a NotI–PstI cassette; a PmeI–NotI cassette carrying the 15Myc sequence has been inserted obtaining plasmid pTet-FAR1-15Myc used to transform a *far1Δ* strain. To obtain the Cln3 overexpression, *CLN3* gene with a 3'-3HA sequence has been amplified by PCR from pCM194 and inserted in plasmid pCM184 (Gari et al., 1997) as a ClaI–ApaI cassette. Plasmid pTet-CLN3-3HA has been obtained and used to transform a *cln3Δ* strain.

The NH₂-terminal truncated form of Far1 has been obtained via PCR mutagenesis. The 394th codon of *FAR1* sequence on plasmid pTet-FAR1-15Myc was replaced with an amber stop codon via PCR mutagenesis, using the following primers: *MUT394W*: 5'-CATGTTATGGTCAGTGTAGATTTCAAGAAATGCGG-3'; *MUT394C*: 5'-CCGCATTTCTGAAAC-TACACTGACCATAACATG-3'.

Western blot

Crude protein extracts were obtained by standard glass beads methods. 50 μg of extract were loaded on each lane of 10% SDS-PAGE, unless otherwise indicated. As a loading control, all the blotted membranes have been stained for total protein with Ponceau red (Sigma-Aldrich) before immunodecoration. Myc-tagged proteins were detected with monoclonal anti-Myc antibodies (9E10; Santa Cruz Biotechnology, Inc.). ECL system (Amersham Biosciences) was used for immunoblot detection according to manufacturer's instructions.

Image acquisition

Images were scanned at a minimum resolution of 300 dpi. Cln3 and Far1 protein levels were quantified by densitometry of raw TIFF images using the NIH Image-based software Scion Image (Scion Corporation). TIFF files were resized and eventually brightness adjusted (process applied to the whole image) with Corel Photo-Paint 7.0 for figures preparation.

Growth condition and elutriations

SC media was prepared assembling 2% carbon source (glucose or ethanol, SCD or SCE, respectively), 6.7 g/liter yeast nitrogen base, 0.6 g/liter complete supplemented mixtures (Bio101) omitting amino acids as necessary for selection. Cells were grown in SC media unless otherwise stated. Budding index was determined by direct microscopic count of at least 300 cells and cells were counted with a counter (model Z2; Beckman Coulter). Duplication time (T) and length of the budded phase (T_b) were determined as described previously (Cocchetti et al., 2004). Elutriation was performed starting from 4 liters of cells grown to mid-exponential phase, (≤10⁷ cells/ml), using a 40-ml chamber elutriator (Beckman Coulter). Elutriated cells were released in fresh, prewarmed medium, and scored for growth parameters and samples collected for Western analysis of Myc-tagged proteins as described above.

Flow cytofluorimetric analysis

A total of 2 × 10⁷ cells exponentially growing has been collected by filtration for each sample, fixed in 70% ethanol, eventually stocked (over-

night to 1 wk) at 4°C and subsequently processed for FACS analysis using a BD FACStarPlus equipped with a Coherent Innova 70 Ion-Argon laser with a 488-nm laser emission. Cell size was operationally defined as the average P determined by flow cytometry in the channel of FITC fluorescence of FITC-stained cells. DNA, protein and double parameter analysis (DNA and proteins on the same sample), determination of the critical size at the beginning of DNA replication (Ps) or phase-specific average protein content (Po, for new born cells) were performed as described previously [Cocchetti et al., 2004].

Online supplemental material

Fig. S1 shows that wild-type and *far1Δ* cells have the same overall size but newborn cells are larger in the mutant. Fig. S2 illustrates P and duplication times for wild-type cells and deletion mutants. Fig. S3 shows that *gpr1Δ* cells do not show the drop of budding after an ethanol/glucose shift up. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200405102/DC1>.

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