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G_1 cyclins block the Ime1 pathway to make mitosis and meiosis incompatible in budding yeast

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Diploid yeast cells switch from mitosis to meiosis when starved of essential nutrients. While G_1 cyclins play a key role in initiating the mitotic cell cycle, entry into meiosis depends on Ime1, a transcriptional activator regulated by both nutritional and cell-type signals. We show here that G_1 cyclins downregulate *IME1* transcription and prevent the accumulation of the Ime1 protein within the nucleus, which results in repression of early-meiotic gene expression. As G_1-cyclin deficient cells do not require nutrient starvation to undergo meiosis, G_1 cyclin would exert its role by transmitting essential nutritional signals to Ime1 function. The existence of a negative cross-talk mechanism between mitosis and meiosis may help explain why these two developmental options are incompatible in budding yeast.

Keywords: cell cycle/G_1 cyclins/Ime1/meiosis/nutrients

Introduction

Nutrients are among the most important trophic factors for yeast and, like most other eukaryotes, *Saccharomyces cerevisiae* takes different developmental options depending on environmental conditions during the G_1 phase of the cell cycle. Depending on the nutrient limitation conditions, haploid cells either arrest in G_1 or initiate invasive growth. Diploid cells also arrest in G_1 or produce pseudo-hyphae, but they have an additional option: entry into meiosis.

Ime1 is a transcriptional activator that routes both nutritional and cell-type signals to the expression of meiotic genes, and has a central role in triggering meiosis (for review see Kupiec *et al*., 1997). Only diploid cells are able to enter meiosis as they possess both components of the Mat**a**1–Matα2 complex, which allows expression of the *IME1* gene by two separate pathways. In addition, nutrient starvation signals regulate *IME1* at both transcriptional and post-transcriptional levels. The *IME1* promoter is repressed by glucose and nitrogen and is induced in the presence of acetate (Kassir *et al*., 1988; Sagee *et al*., 1998). In addition, Ime1's function as a transcriptional activator depends on its ability to interact with Ume6, a protein that binds at the promoters of early meiotic genes to inhibit or activate their expression depending on the interacting proteins (Strich *et al*., 1994; Bowdish *et al*., 1995; Kadosh and Struhl, 1997). Interaction between Ime1 and Ume6, which is elicited by the Rim11 and Rim15 kinases (Rubin-Bejerano *et al*., 1996; Vidan and Mitchell, 1997), has been shown to be a key target for glucose-mediated inhibition of Ime1 activity (Malathi *et al*., 1997). S-phase entry during meiosis is completely dependent on Ime1 (Kassir *et al*., 1988), partly through the Ime2 protein kinase (Foiani *et al*., 1996). However, the mechanisms by which this transcriptional activator is able to trigger initiation of DNA replication have not been characterized. It has been proposed that Cdc28, the central cyclin-dependent kinase that regulates the mitotic cell cycle, may not have a role since *cdc28* thermosensitive cells arrest meiosis after DNA replication (Shuster and Byers, 1989), but as yet no additional evidence is available to confirm this idea. Although Cln1, Cln2 and Cln3, the three yeast G_1 cyclins, show clear functional redundancy, they perform different roles during the G_1–S transition in the mitotic cell cycle. Cln3 is the most potent activator regarding SBF- and MBF-dependent transcription of a set of genes including *CLN1* and *CLN2* (Tyers *et al*., 1993; Dirick *et al*., 1995; Stuart and Wittenberg, 1995; Levine *et al*., 1996). On the other hand, Cln1 and Cln2 have more specialized roles in budding initiation (Benton *et al*., 1993; Cvrcková and Nasmyth, 1993) and also in DNA synthesis initiation through degradation of the Clb-Cdc28 inhibitor Sic1 (Schwob *et al*., 1994; Schneider *et al*., 1996; Feldman *et al*., 1997; Skowyra *et al*., 1997). The possible role of G_1 cyclins in regulating entry into pre-meiotic S phase has not been characterized.

This work deals with the relationships between key molecules involved in initiating either mitosis or meiosis. Here we show that, although mitosis and meiosis share some important similarities during S-phase entry, G_1 cyclins are not required to trigger pre-meiotic DNA replication. In fact, we have found that G_1 cyclins block the Ime1 pathway to inhibit meiosis by two different mechanisms: (i) downregulating *IME1* transcription and (ii) preventing Ime1 accumulation within the nucleus. Our results indicate that yeast cells have established a negative cross-talk mechanism between mitosis and meiosis to make these cell cycle choices incompatible.

Results

G_1 cyclins are rapidly lost during entry into meiosis

In accordance with their essential role in the G_1–S transition, we have shown previously that G_1-cyclin levels are downregulated very rapidly in haploid yeast cells deprived of an essential nutrient such as nitrogen (Gallego *et al*., 1997). Contrary to haploid cells, which arrest in G_1, diploid yeast cells switch from the mitotic to the meiotic cell cycle under nitrogen starvation conditions in the

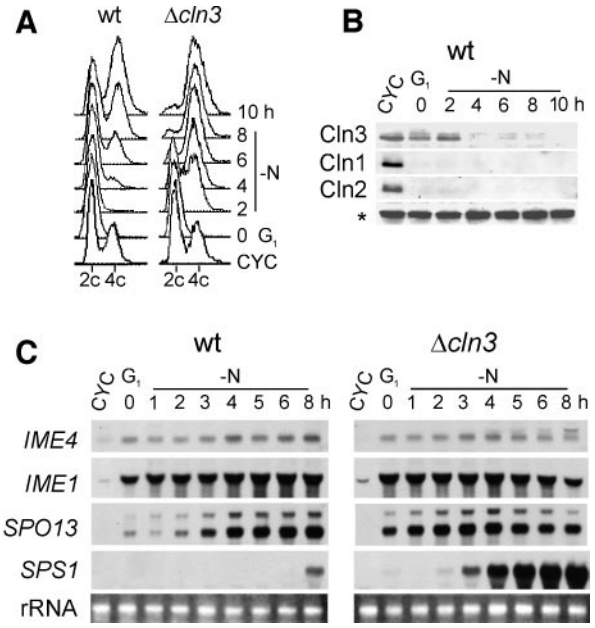

Fig. 1. G_1 cyclins are lost early during entry into meiosis. (**A**) DNA content distributions of wild-type 1788 (wt) and Cln3-deficient CML254 (Δ*cln3*) cells during entry into meiosis. Samples were obtained during mitotic growth in YPA medium (CYC), after carbon-source limitation (G_1, 0 h), and at different times under sporulation conditions (–N). (**B**) Protein levels for G_1-cyclins Cln1, Cln2 and Cln3 as determined by Western blot of samples taken as in (A). The 12CA5 cross-reactive band (*) serves as a loading control. (**C**) mRNA levels for meiotic genes *IME4* and *IME1*, as well as *SPO13* (an Ime1-dependent early gene) and *SPS1* (a middle gene) were determined by Northern blot analysis from samples taken as in (A). The 25S rRNA serves as a loading control.

presence of a non-fermentable carbon source. To understand the basis of these different cell fates, we first focused on the key molecules of the mitotic G_1–S transition during entry into meiosis.

To obtain synchronous entry into pre-meiotic S phase, the non-standard pre-growth regimen developed by Padmore *et al*. (1991) was used (see Materials and methods). Diploid wild-type 1788 cells growing exponentially in acetate-based rich medium were allowed to reach a high cell density and accumulate in G_1 as their growth became limited by the carbon source. Upon transfer to sporulation medium with carbon source added but lacking the nitrogen source, these G_1 cells readily initiated a pre-meiotic S phase in ~4 h (Figure 1A) and proceeded into the meiotic nuclear divisions to produce spores in 24 h. Figure 1B shows that Cln1 and Cln2 are already absent in G_1 cells limited by the carbon source, while Cln3 is lost early after transfer to sporulation conditions, suggesting that G_1 cyclins might not be required at all in this specialized version of the yeast cell cycle. As Cln3 is the only detectable G_1 cyclin during entry into meiosis, we used Cln3-deficient cells to analyze Ime1-dependent gene expression and pre-meiotic S-phase entry kinetics. Unexpectedly, not only did Cln3-deficient cells sporulate with high efficiency (84% compared with 75% for the wild type) but they underwent pre-meiotic S phase earlier and more efficiently. More than 80% of Cln3-deficient cells had completed DNA replication 4 h after being transferred to sporulation medium, while wild-type cells were just initiating pre-meiotic S phase at that time (Figure 1A). Induction of *SPO13* (an Ime1-dependent early meiotic gene) and *SPS1* (a middle meiotic gene) also occurred earlier in Cln3-deficient cells (Figure 1C). Thus, G_1 cyclins do not seem to be required to initiate meiosis. The experiments shown in Figure 4 with a Δ*cln1* Δ*cln2 GAL1p–CLN3* strain (see below) provide further support to this idea. Rather, the fact that Cln3-deficient cells are able to enter pre-meiotic S phase more efficiently suggests that G_1 cyclins might have a negative role in meiosis initiation.

G_1-cyclin overexpression inhibits meiosis and blocks Ime1 function

If Cln3 exerts a negative effect on Ime1 function, entry into meiosis should be inhibited by overexpression of G_1 cyclins from a constitutive promoter. We used the tetracycline-regulatable system that we had adapted to yeast (Garí *et al*., 1997) to drive expression of *CLN1*, *CLN2* or *CLN3* from a centromeric vector in cells growing exponentially in acetate-based rich medium without tetracycline to induce the $tetO_2$ promoter. Figure 2A shows the percentages of asci and budded cells 24 h after nitrogen deprivation in cells overexpressing *CLN1*, *CLN2* or *CLN3*. As expected from the negative role of Cln3 during entry into meiosis, G_1-cyclin overexpression inhibited sporulation and forced cells to enter mitosis as deduced from the final budding indexes. Similar results were obtained in W303 diploid cells (data not shown). Constitutive overexpression of *CLN3* from the $tetO_2$ promoter did not prevent the G_1 arrest produced by carbon-source limitation in acetate-based rich media as deduced from the DNA content distributions (data not shown). Wild-type cells arrested in G_1 by carbon-source limitation did not increase their number significantly during the 24 h upon transfer to sporulation conditions. On the contrary, *CLN3*-overexpressing cells doubled their number and arrested with a high percentage of cells with a 4c DNA content, which is in agreement with the high final budding index attained by these cells. Thus, G_1-cyclin overexpression not only inhibits sporulation but also drives cells into mitosis under conditions where wild-type cells enter meiosis very efficiently.

Figure 2B shows that expression of both *SPO13*, which depends directly on Ime1 as an early gene, and *SPS1*, which is induced further downstream in the Ime1 pathway as a middle gene, is strongly repressed by *CLN3* overexpression under sporulation conditions. As *IME1* mRNA levels showed only a moderate decrease, these results suggest that *CLN3* overexpression may block Ime1 function at a post-transcriptional level. It has been proposed that Ime1 may retro-activate its own transcription during the earliest steps of meiosis (Shefer-Vaida *et al*., 1995). To avoid possible effects on *IME1* transcription due to Ime1 inhibition at a post-transcriptional level, Ime1-deficient cells were subject to *CLN3* overexpression during entry into meiosis. *IME1* expression was evaluated from a plasmid construct that lacks a functional *IME1* open reading frame (ORF) (*ime1-2*), and from the homozygous *kanMX4*-disrupted chromosomal copies (*ime1-1*) that retain the *IME1* promoter and transcription termination sequences (Figure 3). In both constructs, *CLN3* overexpression was able to repress the *IME1* promoter. Accordingly, *CLN3*-overexpression repression effects on *IME1* transcription were also observed in *rim11* null mutants (data not

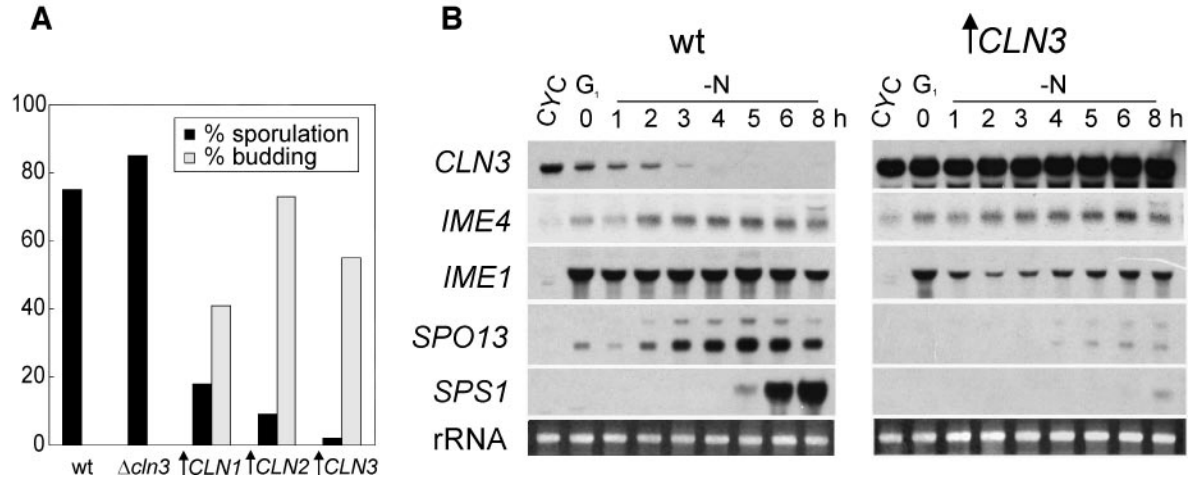

Fig. 2. G_1-cyclin overexpression inhibits meiosis by blocking Ime1 function. (**A**) Sporulation and budding indexes were determined after 24 h under sporulation conditions in wild-type CML256 (wt) and Cln3-deficient CML254 (Δ*cln3*) cells, as well as in CML256 cells overexpressing either *CLN1* from pCM207 (↑*CLN1*), *CLN2* from pCM214 (↑*CLN2*) or *CLN3* from pCM166 (↑*CLN3*). (**B**) Wild-type CML256 cells transformed with the empty vector (wt) or pCM166 to overexpress *CLN3* (↑*CLN3*) were used to determine mRNA levels for cyclin *CLN3*, the meiotic genes *IME4* and *IME1*, as well as *SPO13* (an Ime1-dependent early gene) and *SPS1* (a middle gene). Cells were grown in YPA medium in the absence of tetracycline to allow for expression of the $tetO_2$–*CLN3* construct. Lanes correspond to samples obtained during mitotic growth in YPA medium (CYC), after carbon-source limitation (G_1, 0 h), and at different times in sporulation medium (–N). The 25S rRNA serves as a loading control.

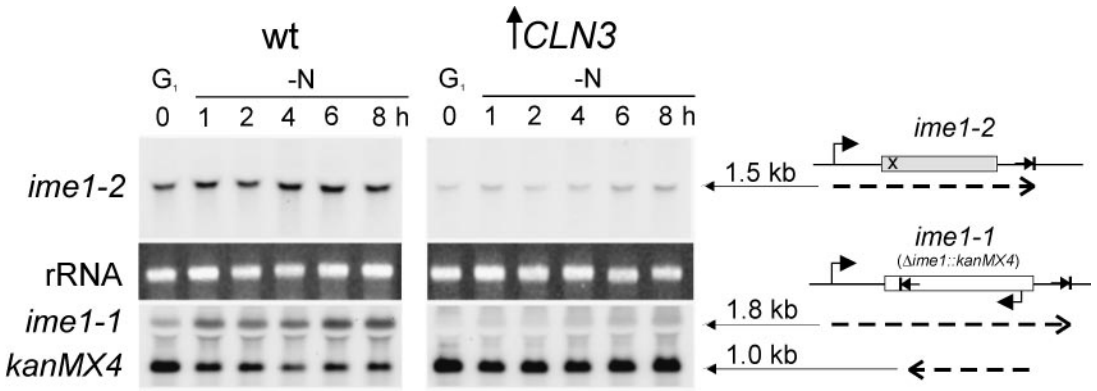

Fig. 3. CLN3 overexpression inhibits IME1 transcription. Ime1-deficient CML342 cells carrying a homozygous *ime1-1* deletion (Δ*ime1::kanMX4)* and a truncated *ime1-2* allele on pCM268, were transformed with empty vector (wt) or pCM166 to overexpress *CLN3* (↑*CLN3*). mRNA levels produced by the *IME1* promoter from the chromosomal *ime1-1* allele were determined with a *kanMX4* probe, which also detects the *kanMX4* transcript. The plasmid-borne *ime1-2* mRNA was detected with an *IME1* probe that does not cover any of the *IME1* sequences left in *ime1-1*. Cells were grown in YPA medium in the absence of tetracycline to allow for expression of the $tetO_2$–*CLN3* construct. Lanes correspond to samples taken after carbon-source limitation (G_1, 0 h), and at different times under sporulation conditions (–N). The 25S rRNA is shown as a loading control. The origin of the transcripts detected is outlined on the right.

shown), where Ime1-dependent transcription is completely repressed (Bowdish *et al*., 1994). Thus, despite the likely existence of post-transcriptional mechanisms (see below), our results indicate that high Cln-cyclin levels have a role in repressing *IME1* transcription.

Cells deprived of G_1 cyclins switch from mitosis to meiosis in rich media

Nitrogen starvation has been the most efficient environmental condition used to induce meiosis in diploid yeast cells (Freese *et al*., 1982). On the other hand, nitrogen starvation causes a rapid decrease of G_1-cyclin levels in haploid cells (Gallego *et al*., 1997). We have shown here that (i) G_1 cyclins are lost early during entry into meiosis; (ii) Cln3-deficient cells undergo meiosis more efficiently than wild-type cells; and (iii) G_1-cyclin overexpression inhibits meiosis and drives cells into mitosis even under nitrogen starvation conditions. Thus, nitrogen starvation could exert its essential role in inducing meiosis through downregulation of G_1-cyclin levels.

To test whether G_1-cyclin downregulation is not only a necessary but also a sufficient condition to allow entry into pre-meiotic S phase independently of the nutritional status of the cell, we used a homozygous Δ*cln1* Δ*cln2 GAL1p–CLN3* strain that depends on the presence of galactose to execute the mitotic G_1–S transition. In order to determine first the essential role of G_1 cyclins on Ime1 function inhibition at a post-transcriptional level, *IME1* was consitutively expressed in a centromeric vector from the *Schizosaccharomyces pombe adh* promoter, which attains expression levels similar to those produced by the natural *IME1* promoter under sporulation conditions (data not shown). Figure 4A shows that when these cells were transferred to acetate-based rich medium to shut off *CLN3* expression (Figure 4B), they arrested temporarily in G_1, proceeded into an S phase with no signs of budding and

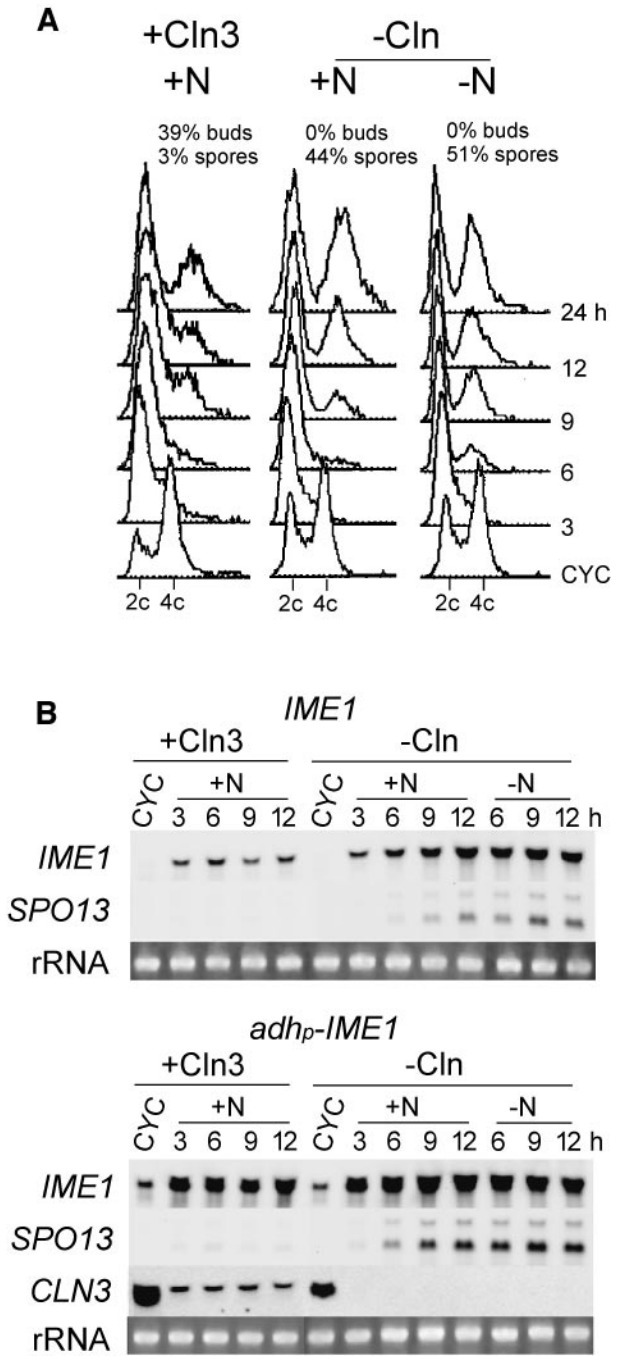

Fig. 4. G_1-cyclin-deficient cells switch from mitosis to meiosis in rich media. (**A**) DNA content distributions of CML353, a homozygous Δ*cln1* Δ*cln2 GAL1p–CLN3* strain that expresses *IME1* constitutively from pCM284, transformed either with an empty vector (–Cln) or pCM194 (+Cln3), which contains the *CLN3* gene under its own promoter sequences. Samples were obtained during mitotic growth in YPGal medium (CYC) and at different times after transfer to YPA medium (+N) to repress *CLN3* expression from the *GAL1* promoter. After 3 h in YPA, a portion of G_1-cyclin-deficient cells (–Cln) were transferred to sporulation medium (–N) and samples were taken thereafter. Budding and sporulation indexes obtained at 24 h are indicated. (**B**) CML353 cells constitutively expressing *IME1 (adhp–IME1*) or not (*IME1*) from plasmid pCM284, and transformed with either an empty vector (–Cln) or pCM194 (+Cln3), were used to determine mRNA levels of *IME1*, *SPO13* and *CLN3* by Northern blot analysis from samples taken as in (A). The 25S rRNA is shown as a loading control.

finally sporulated with high efficiency, indicating that G_1-cyclin downregulation is sufficient for entry into meiosis in rich media as long as *IME1* constitutive expression is provided. S-phase entry and sporulation efficiencies were as high as those obtained when cells were starved for nitrogen by transfer to sporulation medium. Thus, nitrogen starvation may exert its essential role in meiosis induction by downregulating G_1 cyclins. As expected, when *CLN3* expression was provided from its natural promoter sequences in a centromeric vector, those cells also arrested temporarily in G_1 but resumed cell proliferation by mitosis as deduced from budding indexes (Figure 4A) and cell number increase (data not shown). It has been proposed that cell size may be an important requirement for entry into meiosis (Calvert and Dawes, 1984). G_1-deficient cells entered pre-meiotic S phase in rich media with a mean cell volume of 184 fl, while *CLN3*-containing cells entered mitotic S phase under the same conditions with a mean cell volume of 187 fl. Both mean cell volumes are very similar and, on the other hand, larger than that shown by the two strains when they entered pre-meiotic S phase in sporulation media (125 and 128 fl). Thus, the fact that cells either lacking or containing Cln3 enter pre-meiotic or mitotic S phase, respectively, cannot simply be explained as a consequence of having smaller or larger cell volumes than that required for sporulation.

Although the *IME1* promoter senses a variety of nutritional signals to become fully active (Sagee *et al*., 1998), when we performed the same cell cycle analysis in acetate-based rich medium with a Δ*cln1* Δ*cln2 GAL1p–CLN3* strain with *IME1* under its own promoter, cells did indeed enter S phase with no signs of budding (data not shown) and sporulated, albeit at lower frequencies (15% compared with 40% in sporulation medium). These results suggest that *IME1* expression from its own promoter in acetate-based rich medium is sufficient for entry into meiosis as long as G_1 cyclins are not present in the cell, which agrees with the fact that *CLN3* overexpression represses *IME1* transcription (Figure 2; see above). Figure 4B shows that after an initial increase in *IME1* transcription due to the transfer from galactose to acetate-based rich media, Cln-deficient cells induced *IME1* transcription at levels much higher than those attained by cells containing Cln3. In addition, Ime1-dependent induction of *SPO13* expression only took place in Cln-deficient cells. This transcriptional activation was not merely due to higher Ime1 levels as *IME1* transcription increased. Cln3-containing cells that expressed constitutive levels of *IME1* mRNA were not able to activate *SPO13* transcription (Figure 4B). The same inhibitory effects on Ime1 were observed with a strain where progression through the mitotic cycle depended on Cln1 (data not shown), which agrees with the fact that constitutive overexpression of *CLN1* and *CLN2* inhibits meiosis under optimal nutritional conditions for sporulation (see above). These results indicate that G_1 cyclins are able to downregulate Ime1 function at both transcriptional and post-transcriptional levels.

Figure 4B shows that both *IME1* and *SPO13* expression levels were similar in Cln-deficient cells independently of the presence of the nitrogen source, supporting the idea that the essential effect of nitrogen starvation to induce meiosis is the downregulation of G_1 cyclins, which will in turn allow for full activation of Ime1 function.

Table I. Two-hybrid system for Ime1–Ume6 interaction analysis

Protein fusions	+C (glucose)	8 h –N (acetate)	
	wt	wt	Δ*rim11*
TetR–VP16	221	301	273
TetR–Ime1id	3	5	4
TetR–Ime1id + Ume6id–VP16	9	233	7

β-galactosidase activities as Miller units were determined in wild-type 1788 (wt) and Rim11-deficient CML359 (Δ*rim11*) cells expressing different hybrid proteins from pCM293 (TetR–VP16), pCM295 (TetR–Ime1id) and pCM298 (TetR–Ime1id + Ume6id–VP16). TetR-driven expression of the *lacZ* gene was monitored with plasmid pCM286. Samples were taken from cells growing exponentially in glucose-based minimal media (+C), or 8 h after transfer to sporulation conditions (8 h –N).

Table II. Ume6–Ime1 interaction under different nutritional conditions

Strain	glucose		acetate			
	CYC	G_1	CYC	G_1	8 h –N	24 h –N
wt	0.05	0.10	0.75	0.75	0.79	0.79
Δ*cln3*	0.05	0.12	0.69	0.63	0.65	0.74

β-galactosidase activities expressed as relative values were determined in wild-type 1788 (wt) and Cln3-deficient CML254 (Δ*cln3*) cells containing either pCM293 (TetR–VP16) or pCM298 (TetR–Ime1id + Ume6id–VP16). TetR-driven expression of the *lacZ* gene was monitored with plasmid pCM286. Samples were taken from cells either growing exponentially (CYC), under carbon-source limitation (G_1) or nitrogen starvation conditions (–N) in the presence of a fermentable (glucose) or a nonfermentable (acetate) carbon source. β-galactosidase activities determined from pCM298 (TetR–Ime1id + Ume6id–VP16) were made relative to those obtained from pCM293 (TetR–VP16) for each condition.

Not all meiotic nutritional requirements could be mimicked by G_1-cyclin deprivation. Upon transfer of Δ*cln1* Δ*cln2 GAL1p–CLN3* cells from galactose to glucose-based rich medium, which also represses *CLN3* expression, they rapidly arrested in G_1 but did not undergo meiosis as deduced from DNA content distributions, lack of *SPO13* expression and absence of asci (data not shown). Transcription of *IME1* was not induced under these conditions, which is in agreement with the fact that the *IME1* promoter is repressed by glucose (Sagee *et al*., 1998). In addition, and possibly through Rim15 (Vidan and Mitchell, 1997), glucose inhibits the physical interaction between Ume6 and Ime1 proteins (Malathi *et al*., 1997), which is essential to activate early-gene promoters during meiosis. Accordingly, although constitutive expression of *IME1* resulted also in increased Ime1 protein levels in Cln-deprived cells by glucose, it did not allow for any detectable *SPO13* induction (data not shown).

G_1-cyclin deficient cells are able to complete meiosis in the presence of nitrogen, which agrees with the fact that glucose but not the presence of a nitrogen source inhibits late steps during meiosis (Lee and Honigberg, 1996).

G_1 cyclins prevent Ime1 accumulation in the nucleus

To activate transcription, Ime1 must interact with Ume6, a DNA-binding protein that plays a dual role in regulating meiotic early-gene expression. While Ume6 represses early-gene promoters in mitotically active cells, its interaction with Ime1 converts the complex into a transcriptional activator under sporulation conditions (Rubin-Bejerano *et al*., 1996). This interaction depends on two protein kinases, Rim11 and Rim15 (Rubin-Bejerano *et al*., 1996; Vidan and Mitchell, 1997). Although Rim15 may transmit some nutritional signals to Ime1 function (absence of fermentable carbon sources such as glucose), no physiological role has yet been established for Rim11. Since we have shown that G_1 cyclins inhibit Ime1 function at a post-transcriptional level, we wished to determine whether this effect was exerted upon the interaction between Ume6 and Ime1. To test this possibility we used the tetracycline-regulatable expression system (Garí *et al*., 1997) and built a two-hybrid analysis model by fusing the *tetO*-binding domain of the *Escherichia coli* Tet repressor (TetR) to the interaction domain of Ime1 (Ime1id) and, on the other hand, the VP16 transactivator to the interaction domain of Ume6 (Ume6id). Similarly to previous work where the Ime1–Ume6 interaction was first shown by two-hybrid analysis (Rubin-Bejerano *et al*., 1996), the presence of both TetR–Ime1id and Ume6id–VP16 constructs in diploid cells gave rise to high β-galactosidase levels (comparable to those obtained with TetR–VP16), but only under sporulation conditions, while these high expression levels were completely dependent on Rim11 (Table I). We then used wild-type and homozygous *cln3* null mutant strains to determine β-galactosidase activity in cycling and G_1-arrested cells in glucose-based or acetate-based media and under the nitrogen starvation conditions used to induce entry into meiosis. Table II shows that the main nutritional requirement for the Ume6–Ime1 interaction is the presence of a non-fermentable carbon source such as acetate, independently of cell cycle position (cycling versus G_1-arrested cells) or the presence or absence of the nitrogen source. Cln3-deficient cells showed a very similar behavior, which indicates that the Ume6–Ime1 interaction is not modulated by the presence of Cln3. Similar negative results were obtained when using Δ*cln1* Δ*cln2 GAL1p–CLN3* cells to analyze the Ume6–Ime1 interaction by two-hybrid analysis in the experimental approach shown in Figure 4 (data not shown). Thus we concluded that G_1 cyclins block Ime1 function by mechanisms that seem not to involve its interaction with Ume6.

Ime1 is a nuclear protein under sporulation conditions (Smith *et al*., 1993) and its localization does not depend on Rim11 (Rubin-Bejerano *et al*., 1996). By using a constitutively expressed hemagglutinin (HA)-tagged *IME1* gene that fully complements homozygous *ime1* null mutants, we determined its cellular localization by immunofluorescence in the Δ*cln1* Δ*cln2 GAL1p–CLN3* strain after transfer to acetate-rich media (Figure 5A). We have shown that under these conditions, Cln-deficient cells induce Ime1-dependent transcription and enter into premeiotic S phase, while mitotically cycling cells do not. Figure 5C shows that the overall Ime1 protein levels were similar as measured in immunoblots. However, the Ime1 protein did only accumulate in the nuclei of Cln-deficient cells. Mitotically cycling cells could prevent Ime1 accumulation in the nucleus either by G_1-cyclin activity or more indirectly by the action of other molecules only present in mitotically active cells. To test these two possibilities

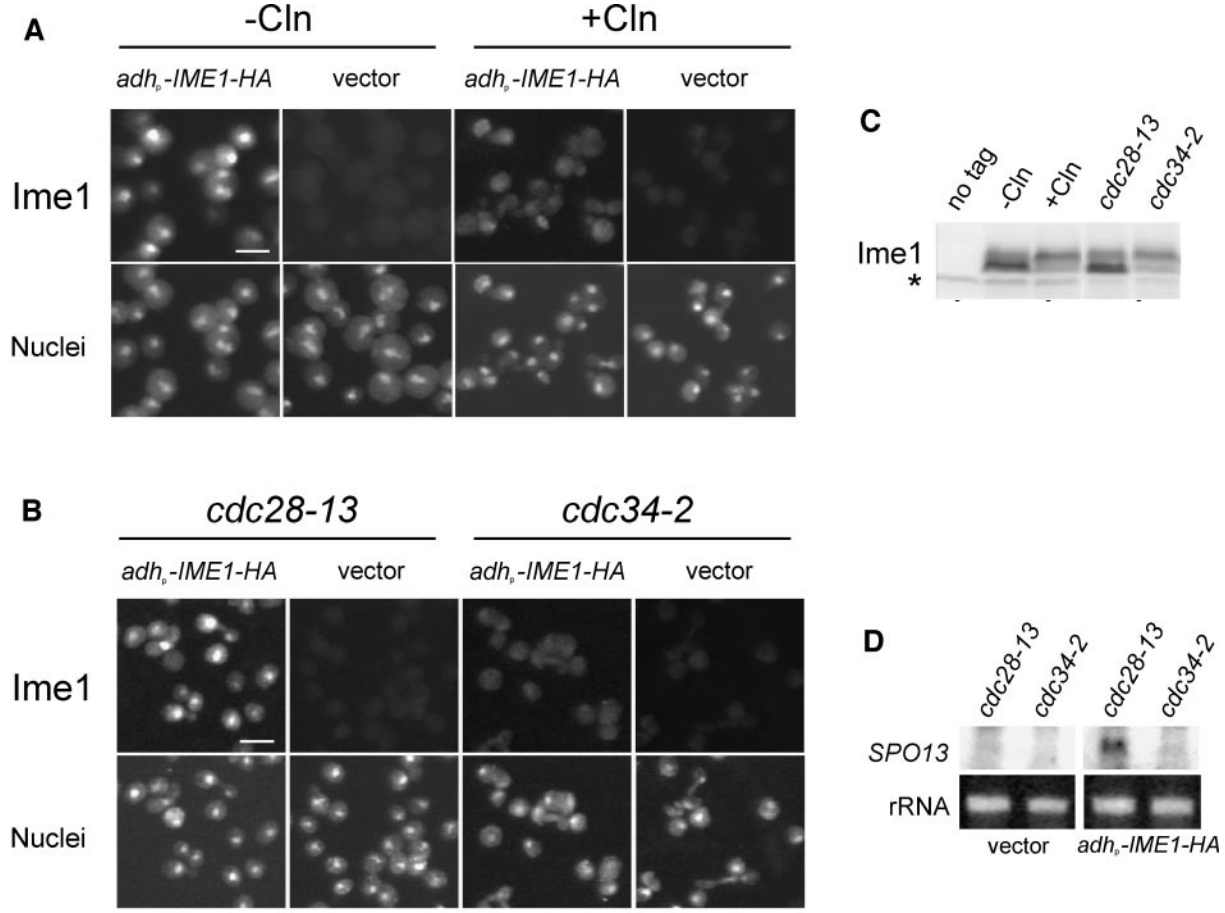

Fig. 5. G_1 cyclins prevent Ime1 accumulation within the nucleus. (**A**) Diploid CML353 (–Cln) and CML348 (+Cln) cells were transformed with either an empty vector or pCM284 (*adh$_p$–IME1-HA*). Samples were taken 9 h after transfer to YPA medium as described under Figure 4. The Ime1 protein was visualized by immunofluorescence (Ime1) and nuclei were counterstained with propidium iodide. Bar represents 10 μm. (**B**) Haploid *cdc28*-thermosensitive CML200 (*cdc28-13*) and *cdc34*-thermosensitive CML344 (*cdc34-2*) cells were transformed with an empty vector or plasmid pCM279 (*adh$_p$-IME1-HA*). Cells were grown in YPA at 25°C and samples were taken 4 h after transfer to 37°C (*cdc28-13*) or 34°C (*cdc34-2*). The Ime1 protein was localized by immunofluorescence (Ime1) and nuclei were counterstained with propidium iodide. Bar represents 10 μm. (**C**) Ime1 levels were determined by Western blot in samples taken as described in (A) and (B). CML353 cells without pCM284 were used as negative control (no tag). A 12CA5 cross-reactive band (*) serves as a control for loading. (**D**) *SPO13* mRNA levels were determined by Northern blot in samples taken as described in (B). The 25S rRNA is shown as a loading control.

we determined the cellular localization of Ime1 in *cdc28-13* cells arrested in G_1 with very low Cln-Cdc28 kinase activity (Wittenberg and Reed, 1988; Wittenberg *et al*., 1990), and *cdc34-2* cells arrested at the G_1–S transition with high Cln-Cdc28 levels (Deshaies *et al*., 1995; Yaglom *et al*., 1995). As seen in Figure 5B, Ime1 was clearly detected in the nuclei of *cdc28-13* cells, while *cdc34-2* cells showed a non-localized signal. Equivalent results were obtained in the W303 background. Similar lower-mobility forms of Ime1 can be detected in extracts obtained from cycling and *cdc34*-arrested cells but to a much lesser extent in extracts from G_1-cyclin deficient or *cdc28*-arrested cells (Figure 5C), which suggests that Ime1 is subject to similar post-translational modifications when not localized to the nucleus. *SPO13* induction took only place in *cdc28-13* cells, indicating that Ime1 accumulation in the nucleus may be a key target for G_1-cyclin inhibition of meiotic gene expression (Figure 5D).

Discussion

Almost all cells take different developmental options in response to external signals, and frequently these options are incompatible. Thus, diploid yeast cells initiate either mitosis or meiosis, depending on environmental signals. While the presence of nutrients exerts an inducing role for mitosis, their absence is an essential condition for entry into meiosis. This work concerns the relationships between key molecules involved in initiating either mitosis or meiosis, and provides some clues as to how yeast cells make these cell cycle choices incompatible.

Different mechanisms for entry into mitotic and pre-meiotic S phases

We have shown that G_1 cyclins are downregulated rapidly in haploid yeast cells under nitrogen starvation conditions (Gallego *et al*., 1997), and we show here that diploid cells behave very similarly when deprived of nitrogen. However, while haploid cells arrest in G_1 and enter a quiescent state termed G_0, diploid cells proceed into a pre-meiotic S phase and finally sporulate. Here we show that G_1 cyclins are not required for entry into meiosis, confirming the findings published recently by Dirick *et al*. (1998). Although the absence of G_1 cyclins may explain why the meiotic cycle proceeds without budding, entry into pre-meiotic S phase must be exerted by mechanisms different than those

present in mitosis, where Cln3 has a key role in MBF-dependent expression of S-phase cyclins *CLB5* and *CLB6* (Schwob and Nasmyth, 1993), and on the other hand, Cln1 and Cln2 trigger degradation of Sic1, the yeast Clb-Cdc28 kinase inhibitor (Schwob *et al*., 1994). Dirick *et al*. (1998) have shown that pre-meiotic S phase also requires *CLB5/6* cyclins, while degradation of the Clb-Cdc28 inhibitor Sic1 during meiosis depends on *IME2*, an early-meiotic gene induced by Ime1. In addition, *CLB5* expression during entry into meiosis depends on Ime1 (our unpublished results). Taken together, these results would explain why *ime1* mutants arrest before pre-meiotic S phase (Kassir *et al*., 1988; Foiani *et al*., 1996).

Whether the Cdc28 kinase is required for pre-meiotic S phase entry remains to be elucidated. Analysis of the arrest phenotype of *cdc28* mutants has led to the proposition that pre-meiotic S phase would not require Cdc28-kinase activity (Shuster and Byers, 1989). However, the different leakiness of the *cdc28* mutants used regarding their arrest at G_1–S or G_2–M in the mitotic cycle could mask a putative function for Cdc28 in pre-meiotic S phase. In addition, as *CLB5* expression and Sic1 loss do not depend on Cln-Cdc28 kinase in meiosis, even those *cdc28* mutations that produce a G_1 arrest in the mitotic cycle might be suppressed by elevated Ime1-dependent Clb5 levels during meiosis, mainly when the Clb-Cdc28 inhibitor, Sic1, is being downregulated by Cln-Cdc28-independent mechanisms.

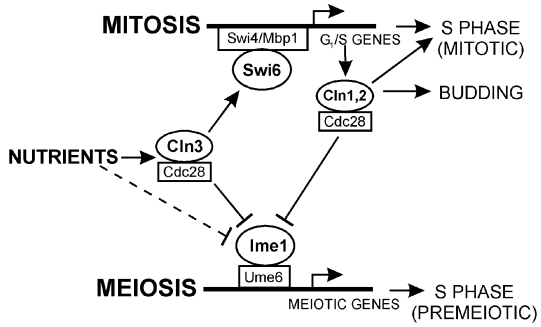

Fig. 6. G_1 cyclins, the key activators of the mitotic G_1–S transition, block the Ime1 pathway to inhibit meiosis. G_1 cyclins transmit essential nutritional signals to the Ime1 pathway. In the presence of nutrients, G_1-cyclin levels are high and cells are driven to the mitotic G_1–S transition. G_1 cyclins have a key role in activating SBF(Swi4/Swi6)- and MBF(Mbp1/Swi6)-dependent gene expression and in the degradation of Sic1, the Clb-Cdc28 kinase inibitor. In addition, high G_1-cyclin levels downregulate *IME1* transcription and prevent the accumulation of Ime1 in the nucleus, thus inhibiting meiotic gene expression. As G_1-cyclin levels fall rapidly under nitrogen starvation conditions, cells arrest mitotic proliferation in G_1 and, if in the presence of a nonfermentable carbon source, full activation of Ime1 at both transcriptional and post-translational levels takes place, thus allowing cells to enter pre-meiotic S phase. The broken line indicates G_1-cyclin independent mechanisms that downregulate Ime1 function also at both transcriptional and post-transcriptional levels by the carbon source (see Discussion).

G_1 cyclins transmit essential nutritional signals to the Ime1 pathway to inhibit meiosis

The essential nutritional requirements for entry into meiosis in budding yeast are: (i) nitrogen starvation conditions; and (ii) presence of only a nonfermentable carbon source. Ime1 function has been shown to be regulated by these nutritional signals through different transcriptional and post-transcriptional mechanisms.

Our results indicate that high G_1-cyclin levels downregulate *IME1* expression under optimal nutritional conditions for sporulation. Moreover, G_1-cyclin-deficient cells enter meiosis in acetate-based rich media by increasing *IME1* expression levels regardless of the presence of the nitrogen source, while these cells show no alterations in the induction caused by acetate or in the repression exerted by glucose. As a consequence, G_1-cyclin deficiency seems to mimick most of the nitrogen-starvation signals for *IME1* expression. The *IME1* promoter is repressed by nitrogen at the UCS1 and IREu regions (Sagee *et al*., 1998). *IME1* expression is de-repressed 3- to 4-fold in *swi4* and *swi6* mutants, this effect being attributed to the IREu sequence, which contains an SCB-like motif (Sagee *et al*., 1998). Similar mechanisms could operate at the UCS1 region, since it also contains SCB-like sequences. Thus, in addition to its role as an activator during the mitotic G_1–S transition, SBF could act as a repressor through G_1-cyclin-dependent mechanisms to inhibit *IME1* expression. Alternatively, other transcriptional factors involved in activating expression from the IREu and UCS1 sequences could be subject to Cln-Cdc28 mediated inhibition.

Induction of the *IME1* promoter depends on binding of Msn2,4 at the IREu sequence (Sagee *et al*., 1998), and as Msn2,4 import to the nucleus is prevented by protein kinase A (PKA) activity (Görner *et al*., 1998), it may explain the repressor effects that glucose exerts through the cAMP–PKA pathway on *IME1* expression. However, this mechanism cannot explain the fact that PKA-deficient cells are able to induce *IME1* expression not only in the presence of glucose, but also in the presence of nitrogen, a nutrient that does not modulate the cAMP–PKA pathway (discussed in Kupiec *et al*., 1997), and seems to repress the *IME1* promoter at sequences different than those bound by Msn2,4 (Sagee *et al*., 1998). Our results indicate that G_1-cyclin loss may mimick most of the nitrogen starvation signals for induction of *IME1* expression. On the other hand, G_1-cyclin gene expression has been shown to depend on the cAMP–PKA pathway (Huble *et al*., 1993; Hall *et al*., 1998). Taken together, these observations could explain why PKA-deficient cells are able to bypass both glucose and nitrogen repressor effects on *IME1* expression.

Overexpression of *IME1* overcomes *MAT*-mediated requirements for entry into meiosis, but only when growth becomes limited in acetate-based media (Kassir *et al*., 1988; Smith *et al*., 1990), which indicates that nutrients exert important repressor effects upon Ime1 activity. We show here that G_1-cyclin loss activates Ime1 at a post-transcriptional level to increase expression of *SPO13* in acetate-based media, independently of the presence of the nitrogen source. As for transcriptional control of *IME1*, G_1-cyclin deficiency also seems to mimic most of the nitrogen starvation signals required for post-transcriptional activation of Ime1.

The Ime1 protein interacts with Ume6 to transactivate early-gene expression; this interaction is mediated by the Rim11 and Rim15 kinases. Many observations indicate that this interaction is a key target for regulation of Ime1 function by the carbon source present, but not by nitrogen starvation signals (Bowdish *et al*., 1994; Rubin-Bejerano

Table III. Yeast strains

Strain	Genotype	Source or reference
CML128 derivatives		
CML133	*MAT***a**, *LEU2::tTA*	Gallego *et al*. (1997)
CML200	*MAT***a**, *cdc28-13, LEU2::tTA*	Gallego *et al*. (1997)
CML344	*MAT***a**, *cdc34-2, LEU2::tTA*	this work
CML257	*MAT***a**/α, *CLN3-3HA/CLN3*	this work
CML262	*MAT***a**/α, *CLN1-3HA/CLN1*	this work
CML362	*MAT***a**/α, *CLN2-3HA/CLN2*	this work
CML256	*MAT***a**/α, *LEU2::tTA/leu2-3,112*	this work
CML337	*MAT***a**/α, *IME1-3HA/IME1-3HA*	this work
CML315	*MAT***a**/α, *IME1-3HA/IME1-3HA, LEU2::tTA/leu2-3,112*	this work
CML268	*MAT***a**/α, *Δime1::kanMX4/Δime1::kanMX4*	this work
CML342	*MAT***a**/α, *Δime1::kanMX4/Δime1::kanMX4, LEU2::tTA/leu2-3,112*	this work
CML359	*MAT***a**/α, *Δrim11::kanMX4/Δrim11::kanMX4*	this work
CML331	*MAT***a**/α, *Δrim11::kanMX4/Δrim11::kanMX4, LEU2::tTA/leu2-3,112*	this work
CML254	*MAT***a**/α, *Δcln3::LEU2/Δcln3::LEU2*	this work
CML319	*MAT***a**/α, *Δcln3::LEU2/Δcln3::LEU2, IME1-3HA/IME1-3HA*	this work
CML346	*MAT***a**/α, *Δcln3::LEU2/Δcln3::LEU2, Δime1::kanMX4/Δime1::kanMX4*	this work
CML348	*MAT***a**/α, *Δcln1::HIS3/CLN1, Δcln2::TRP1/CLN2, GAL1p-CLN3/CLN3*	this work
CML353	*MAT***a**/α, *Δcln1::HIS3/Δcln1::HIS3, Δcln2::TRP1/Δcln2::TRP1, GAL1p-CLN3/GAL1p-CLN3*	this work
W303 derivatives		
CMY1036	*MAT***a**, *cdc34-2*	gift from C.Mann
CML363	*MAT***a**/α, *LEU2::tTA/leu2-3,112*	this work

et al., 1996; Malathi *et al*., 1997; Vidan and Mitchell, 1997). Using a different two-hybrid approach, our results confirm that the Ume6–Ime1 interaction is mainly regulated by the carbon source regardless of nitrogen starvation conditions and cell cycle position, i.e. G_1-arrested versus cycling cells. In addition, we have found that G_1-cyclin deficiency does not increase transcription levels driven by the Ume6–Ime1 interaction in acetate-based rich media, whereas glucose-mediated inhibition of Ime1–Ume6 interaction is not relieved by G_1-cyclin deficiency. Thus, *IME1* post-transcriptional regulation by G_1 cyclins does not seem to be exerted at the level of Ime1 protein interaction with Ume6.

Ime1 is a nuclear protein during sporulation (Smith *et al*., 1993) and its localization does not depend on the Rim11 kinase (Rubin-Bejerano *et al*., 1996). We have found that Ime1 accumulates in the nucleus of G_1-cyclin-deficient cells but not in cycling cells growing in acetate-based rich media. As *SPO13* expression is only induced in G_1-cyclin-deficient cells, Ime1 accumulation in the nucleus may be an important mechanism for entry into meiosis by regulating Ime1 function at a post-translational level. That Cln cyclins have a role in regulating Ime1 localization to the nucleus is supported by the fact that *cdc28*-arrested cells, which contain very low Cln-Cdc28 kinase activity, accumulate Ime1 in their nuclei and express *SPO13* in an Ime1-dependent manner, while *cdc34*-arrested cells, which contain high Cln-Cdc28 levels, do not accumulate Ime1 in their nuclei and show no *SPO13* mRNA at detectable levels. Whether Cln-Cdc28 activity prevents Ime1 accumulation in the nucleus by direct mechanisms remains to be elucidated. However, some data suggest that this may be the case since (i) the lower-mobility forms of Ime1 associated with the presence of Cln-Cdc28 activity can be collapsed into a higher-mobility band by lambda protein phosphatase treatment; and (ii) Ime1 is phosphorylated *in vitro* by both Cln2 and Cln3 immunoprecipitates with equal efficiency (our unpublished results). Phosphorylation is a widespread mechanism used to link environmental and internal signals to the activity of transcriptional factors, often regulating their import to the nucleus. Thus, nuclear import of the Swi5 transcriptional activator is restricted to the G_1 phase of the cell cycle in budding yeast (Nasmyth *et al*., 1990), and the Cdc28 kinase phosphorylates Swi5 at its nuclear localization signal (NLS) to prevent its import to the nucleus (Moll *et al*., 1991). Further work will be required to determine whether Ime1 import to the nucleus is regulated by Cdc28-dependent mechanisms.

Mitosis or meiosis: a role for G_1 cyclins

It has long been known that diploid yeast cells switch from mitosis to meiosis under nutrient starvation conditions, and since *IME1* was first isolated (Kassir *et al*., 1988) a pathway has emerged where different transcriptional and post-transcriptional regulatory mechanisms have been identified that link its activity to nutrient starvation. On the other hand, work done with haploid cells, where nutrient limitation causes a G_1 arrest, has demonstrated that G_1 cyclins are a key target for cell cycle regulation by nutrient availability (Huble *et al*., 1993; Gallego *et al*., 1997; Hall *et al*., 1998; Parviz *et al*., 1998). Here we show that G_1 cyclins block the Ime1 pathway by downregulating *IME1* transcription and preventing the accumulation of Ime1 within the nucleus, thus repressing early-meiotic gene expression. These observations, which are outlined in Figure 6, indicate that yeast cells have developed a negative cross-talk mechanism between the key initiator molecules of mitosis and meiosis. If nutrient availability were to regulate these two processes independently, intermediate nutrient limiting situations would exist where no option or, much more detrimental for cell survival, both options could be taken. The existence of a negative cross-talk between G_1 cyclins and Ime1, although unidirectional, may help explain why mitosis and meiosis are two incompatible choices for yeast cells.

Materials and methods

Strains and plasmids

Table III lists the strains used in this work. Our parental diploid 1788 and haploid CML128 (*MAT***a**, *leu2-3,112, ura3-52, trp1-1, his4, can1*^{r}) strains have been described (Gallego *et al*., 1997). Some strains used derive from W303-1A (*MAT***a**, *leu2-3,112, ura3-52, trp1-1, his3-11,75, ade2-1, can1-100*) (gift from N.Lowndes).

The tetracycline-repressible transactivator tTA was introduced in yeast cells by integration of pCM87 (Garí *et al*., 1997) at the *LEU2* locus. Chromosomal gene disruptions and C-terminal fusions to the 3HA epitope were obtained by gene transplacement as described previously (Gallego *et al*., 1997).

Plasmids pCM207, pCM214 and pCM166 carry the 3HA-tagged *CLN1*, *CLN2* and *CLN3* ORFs, respectively, under the control of the *tetO*$_2$ promoter (Garí *et al*., 1997) in YCplac33 (*URA3*; Gietz and Sugino, 1988). The 3HA-tagged *IME1* ORF under the control of the *S.pombe adh* promoter (gift from A.Bueno) was inserted in YCplac22 (*TRP1*; Gietz and Sugino, 1988) and YCplac111 (*LEU2*; Gietz and Sugino, 1988) resulting in plasmids pCM279 and pCM284, respectively. The 3HA-tagged *IME1* gene under its own promoter sequences was placed in YCplac22 (*TRP1*; Gietz and Sugino, 1988), resulting in plasmid pCM267. The *ime1-2* allele in pCM268 codes for a stop codon at amino acid 13, and was obtained from pCM267 by site-directed mutagenesis (Weiner *et al*., 1994). Plasmid pCM194 contains the *CLN3* gene under its own promoter (Gallego *et al*., 1997). The TetR–VP16 (tTA) protein fusion used has been described previously (Garí *et al*., 1997). The TetR–Ime1id hybrid was obtained by fusing the TetR domain from tTA to amino acids 270–360 of Ime1. On the other hand, the Ume6id–VP16 hybrid was constructed by fusing amino acids 1–232 from Ume6 to the VP16 domain of tTA. Expression of genes coding for the TetR–VP16 and TetR–Ime1id protein fusions is driven by the cytomegalovirus (CMV) promoter (Garí *et al*., 1997) in plasmids pCM293 and pCM295, respectively, which are both YCplac22 derivatives. Plasmid pCM298 is a derivative of pCM295 which also contains the gene coding for Ume6id–VP16 under the control of the *S.pombe adh* promoter. The *lacZ*-reporter plasmid used to monitor TetR-driven transcription, pCM286, is a YCplac33-based version of pCM159 (Garí *et al*., 1997). Measurement of β-galactosidase activity in permeabilized cells has been described previously (Garí *et al*., 1997). Details of strain and plasmid constructions are available upon request.

Growth and sporulation conditions

In addition to 2% peptone and 1% yeast extract, YPD, YPGal and YPA contained 2% glucose, 2% galactose and 1% potassium acetate, respectively. Sporulation medium was 0.3% potassium acetate, to which the required amino acids were added to the following final concentrations: 15 μg/ml leucine, 5 μg/ml histidine and 10 μg/ml tryptophan. To obtain acceptable levels of synchrony in pre-meiotic S-phase entry, the non-standard pre-growth regimen described by Padmore *et al*. (1991) was used with only slight modifications. Briefly, cells grown in YPD (with 50 μg/ml uracil for *ura3* strains) for 36–48 h to reach OD_{600} values of 25–30 were washed, resuspended in YPA at an OD_{600} of 0.3, and incubated at 30°C for 20 h with vigorous agitation to reach an OD_{600} of 2.5–3. At this point cells were uniformly arrested in G_1 by carbon-source limitation, as deduced from both budding indexes and DNA content distributions. To initiate meiosis, cells were then washed, resuspended in sporulation medium at an OD_{600} of 1, and incubated at 30°C. Minimal media with 2% glucose or 2% galactose with the required amino acids (Gallego *et al*., 1997) was substituted for YPD when using strains containing centromeric plasmids. In this case, most cells (>95%) had retained the plasmid after growth to saturation in YPA as deduced from plating efficiencies under selective and non-selective conditions for the plasmid marker used. To obtain samples from cells growing exponentially in YPA medium, cells grown in YPD as above were washed, resuspended at an OD_{600} of 0.025 in YPA and incubated 16–20 h at 30°C, unless otherwise indicated. Tetracycline was added to 2 μg/ml when repression of the tTA transactivator was needed (Garí *et al*., 1997).

Northern and Western blot analyses

Total RNA samples were analyzed by Northern blot as described previously (Gallego *et al*., 1997). DNA fragments containing only ORF sequences, obtained by either PCR or restriction digestion, were used to synthesize probes by random-PCR with a digoxigenin-dUTP labeling mixture as directed by Boehringer Mannheim. RNA samples were blotted and probed onto the same membrane when accurate comparison was required. Western blot analysis of whole cell extracts with the mouse anti-HA antibody (clone 12CA5) from Boehringer Mannheim were performed as described previously (Gallego *et al*., 1997).

Immunofluorescence

The intracellular localization of the 3HA-tagged Ime1 protein was determined by indirect immunofluorescence techniques essentially as described by Rose *et al*. (1990). The rat anti-HA antibody (clone 3F10, Boehringer Mannheim) was used at 1 μg/ml, and the FITC-conjugated goat anti-rat antibody (Southern Biotechnology Associates) was used at 10 μg/ml in the presence of 100 μg/ml RNase. Nuclei were stained with 50 μg/ml propidium iodide and fluorescent images were obtained in a Zeiss LSM410 confocal microscope equipped with a 488 nm laser, a 63×/1.4 objective and the required band-pass filters (515–545 nm for FITC and 575–640 nm for propidium iodide).

Flow cytometry and morphological determinations

DNA content distributions were obtained by propidium iodide staining as described (Nash *et al*., 1988) with an Epics XL flow cytometer (Coulter). Budding and sporulation percentages were obtained under a phase-contrast microscope by inspecting a minimum of 200 cells that had been fixed in 1% formaldehyde, 1× SSC, and sonicated for 5 s. Mean cell volumes were determined in a Coulter Z2 counter.

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