Whi3 binds the mRNA of the G_1 cyclin *CLN3* to modulate cell fate in budding yeast

Eloi Garí,[1,3] Tom Volpe,[2,3] Hongyin Wang,[1] Carme Gallego,[1] Bruce Futcher,[2] and Martí Aldea[1,4]

[1]Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, 25198 Lleida, Catalunya, Spain; [2]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA

Eukaryotic cells commit in G_1 to a new mitotic cycle or to diverse differentiation processes. Here we show that Whi3 is a negative regulator of Cln3, a G_1 cyclin that promotes transcription of many genes to trigger the G_1/S transition in budding yeast. Whi3 contains an RNA-recognition motif that specifically binds the *CLN3* mRNA, with no obvious effects on Cln3 levels, and localizes the *CLN3* mRNA into discrete cytoplasmic foci. This is the first indication that G_1 events may be regulated by locally restricting the synthesis of a cyclin. Moreover, Whi3 is also required for restraining Cln3 function in meiosis, filamentation, and mating, thus playing a key role in cell fate determination in budding yeast.

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Like most other eukaryotes, *Saccharomyces cerevisiae* cells monitor internal and external signals to choose between developmental options in the G_1 phase of the cell cycle (Pringle and Hartwell 1981). Sexual pheromones arrest haploid cells in G_1 transiently, whereas nutrient limitation causes a G_1 arrest and a switch to either meiosis or pseudohyphal/invasive growth.

During mitotic proliferation, both external and internal signals are also integrated in G_1 to coordinate cell growth in mass with cell cycle entry. Once a critical cell volume is attained in late G_1, Cln3 bound to the cyclin-dependent kinase Cdc28 somehow activates the transcription factors SBF (Swi4/Swi6) and MBF (Mbp1/Swi6). These in turn induce the expression of a large set of genes in a process that leads to the G_1/S transition (Tyers et al. 1993; Dirick et al. 1995; Stuart and Wittenberg 1995; Spellman et al. 1998). Cln3 is already present in early G_1 cells, and Cln3 protein levels do not oscillate much throughout the cell cycle. However, a posttranslational mechanism that explains why SBF and MBF become active only in late G_1 (or at a critical cell size) is yet to be found.

Here we show that Whi3, a recently identified protein that regulates cell size in budding yeast (Nash et al. 2001), exerts a negative role on Cln3 function in the G_1 phase. Whi3 shows a C-terminal domain very similar to the RNA-recognition motif (RRM), and we show that it binds the *CLN3* mRNA specifically. However, Whi3 does not obviously affect the abundance or translation of the *CLN3* mRNA, or the overall Cln3 protein levels. Rather, Whi3 localizes the *CLN3* mRNA to distinct cytoplasmic foci that may locally restrict Cln3 synthesis to modulate its activity. Importantly, we have found that Whi3, also through its RNA-recognition motif, is necessary for repressing Cln3 function when cells are challenged in G_1 to decide among other developmental options such as meiosis or filamentation.

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[3]These authors contributed equally to this work.
[4]Corresponding author.
E-MAIL marti.aldea@cmb.udl.es; FAX 34-973-702426.
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Results and Discussion

Whi3 negatively regulates Cln3 function in G_1

The *WHI3* gene has been isolated as a regulator of cell size in budding yeast. Whi3-deficient cells show a 20% reduction in cell size with no changes in growth rate (Nash et al. 2001). To test a possible role of Whi3 in regulating the G_1 phase, we analyzed small (early G_1) cells obtained by elutriation from wild-type and *whi3* mutant strains. Whi3-deficient cells budded and entered S phase with a volume ~25% smaller than wild-type cells (Fig. 1A,B). These results suggest that Whi3 might be specifically involved in setting cell size at the G_1/S transition, the point where Cln3 plays a rate-limiting role (Cross 1988; Nash et al. 1988). Indeed, transcriptional activation of G_1/S genes such as *CLN2*, *CLB5*, and *SWI4* also took place at a smaller cell size in *whi3* cells (Fig. 1C). However, Cln3 protein and phosphorylation levels (Fig. 1C) were not affected.

The effects of *WHI3* on cell volume are dose-dependent, and *WHI3* overexpression causes a lethal arrest in G_1 (Fig. 2A,B; Nash et al. 2001). Moderate *WHI3* overexpression also repressed transcription of *CLN1*, *CLN2*, and *CLB5* (Fig. 2C) by preventing their activation in late G_1 as deduced from elutriated cultures (Fig. 2E,F). Loss of Whi3 therefore accelerates the expression of genes controlled by SBF and MBF, and overproduction of Whi3 delays or represses expression of the same genes. The G_1 arrest (but not the lethality) caused by *WHI3* overexpression was suppressed by constitutive expression of *CLN2* (data not shown), a condition able to compensate the major cell cycle deficiencies of a *cln3* mutant (Stuart and Wittenberg 1995). Furthermore, a C-terminal truncated form of Swi4 that is largely independent of Swi6 or Cln3 (Baetz and Andrews 1999) was able to activate G_1/S gene expression during *WHI3* overexpression (data not shown). Again, however, lethality was not suppressed, suggesting that overexpressed *WHI3* may interfere with additional targets. These observations are consistent with the idea that Whi3 inhibits the activation of SBF and MBF by Cln3. To cause a G_1 arrest Whi3 should also down-regulate other activators of Start, perhaps Cln1 and Cln2 (and see below), for we have found that Cdc24, a protein that is retained in the nucleus by Far1 in haploid cells and is exported to the cytoplasm depending on Cdc28–Cln1,2 activity (Shimada et al. 2000), remains nuclear in the G_1 arrest caused by Whi3 overexpression

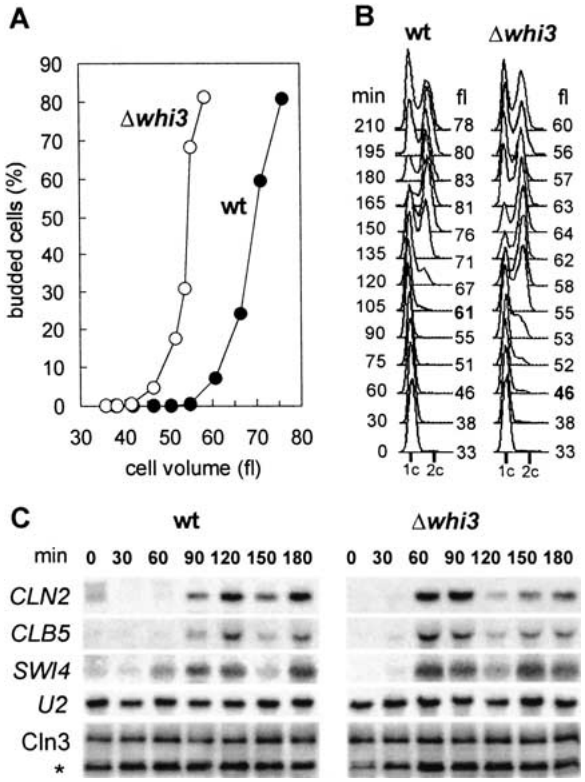

Figure 1. Whi3-deficient cells enter the cell cycle at a smaller cell size. Small G_1 wild-type (wt) or mutant (Δ*whi3*) cells were grown in YPD at 30°C, and samples were taken at different time points to plot (*A*) budding indexes versus cell volume mean values, and (*B*) DNA content distributions. (*C*) Northern analysis of G_1/S genes, and Western analysis of Cln3 protein levels from samples taken at the specified time points. The *U2* RNA and the αHA cross-reacting band (*) served as loading controls.

(data not shown). In any event, neither the G_1 arrest nor the lethal effects caused by high doses of Whi3 can be solely explained by its effects on Cln3 function through SCB- and MCB-driven transcription, pointing to the existence of additional essential targets of Whi3.

To ask whether Whi3 regulates G_1/S gene expression via Cln3 we compared *whi3*, *cln3*, and double *whi3 cln3* mutants. Deletion of *WHI3* had no effect in a *cln3* mutant strain (see Fig. 5A, below); that is, *cln3* is epistatic to *whi3* for cell size (and this is also true for other phenotypes of *whi3*; see below), suggesting that Whi3 acts via Cln3. In contrast, *bck2*, another mutation causing large cell size at Start (Di Como et al. 1995; Wijnen and Futcher 1999), was not epistatic to *whi3* (data not shown). These genetic results support the idea that Whi3 is a negative regulator of Cln3 function.

Whi3 binds the CLN3 *mRNA specifically*

The Whi3 protein contains a domain similar to the RNA-recognition motif. Deletions and point mutations destroying the putative RRM yield null alleles of *WHI3* with respect to both cell size (Nash et al. 2001) and developmental defects (see below), which suggests that the putative RRM is critical for the function of Whi3. To test whether Whi3 is in fact an RNA-binding protein and whether it binds the *CLN3* mRNA specifically, we performed pull-down assays with a Whi3–TAP fusion expressed from the *WHI3* promoter in yeast cells (Fig. 3A). The *CLN3* mRNA was enriched more than 10-fold in Whi3–TAP beads compared to untagged controls as measured by dot-blot analysis, but neither *URA3* nor *ACT1* mRNA ratios differed significantly from 1 (Fig. 3C). In addition, despite the fact that the *CLN3* mRNA is extremely unstable (Schneider et al. 1998), most *CLN3* mRNA in the Whi3–TAP pull down was full length (Fig. 3B). Fusing the Whi3 RRM to the calmodulin-binding peptide (Fig. 3D) allowed us to perform in vitro binding assays and test a direct involvement of Whi3 in binding

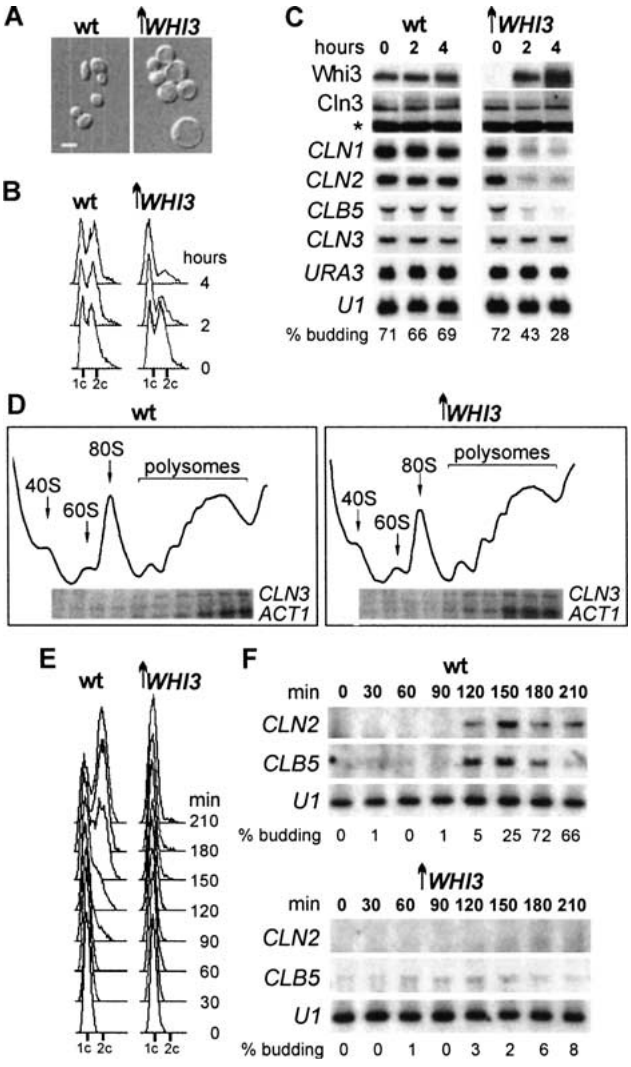

Figure 2. *WHI3* overexpression prevents Cln3 from inducing G_1/S gene expression and arrests cells in G_1. Wild-type (wt) or *GAL1p–WHI3* (↑*WHI3*) cells were added to 2% galactose to induce expression of *WHI3*, and samples were analyzed at 4 h or otherwise-specified time points. (*A*) Phase-contrast micrographs. (*B*) DNA content distributions. (*C*) Northern analysis of G_1/S genes and Western analysis of Whi3 and Cln3 protein levels. (*D*) *CLN3* mRNA distribution on polysomal profiles. Small G_1 cells were elutriated 60 min after galactose addition and grown under inducing conditions to obtain (*E*) DNA content distributions and perform (*F*) Northern analysis of G_1/S genes.

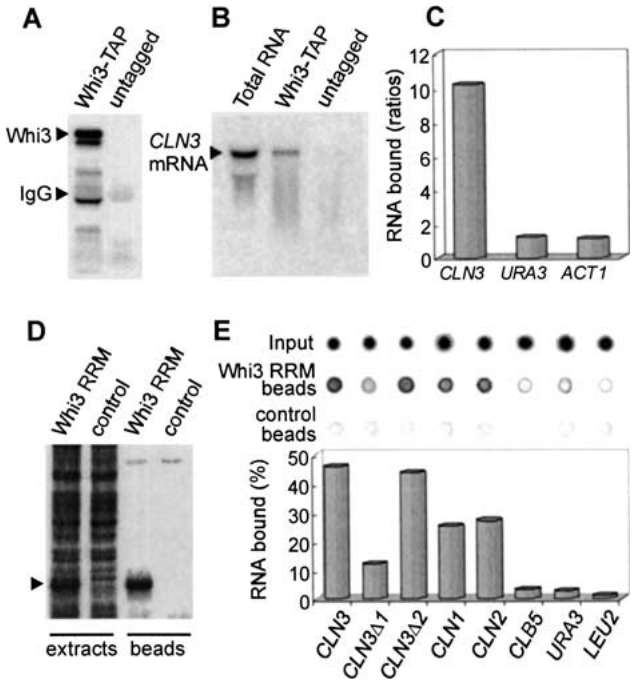

Figure 3. Whi3 binds the *CLN3* mRNA. (*A*) For in vivo analysis, *WHI3*–TAP and untagged yeast strains were used in pull-down assays with IgG beads. Bound proteins detected with a peroxidase anti-peroxidase antibody are shown. (*B*) Northern detection of bound *CLN3* mRNA. Total RNA is shown as control. (*C*) Dot-blot detection of bound *CLN3*, *URA3*, and *ACT1* mRNAs. Plotted are the values obtained from Whi3–TAP cells relative to untagged cells. (*D*) For in vitro analysis, the Whi3 RRM fused to the calmodulin-binding peptide (arrowhead) was bound to calmodulin beads to perform RNA binding assays. Shown are Coomassie-stained proteins in Whi3 RRM or control cell extracts and beads. (*E*) Dot-blot detection of input and bound RNAs to Whi3 RRM and control beads. Percentages of bound to input values are plotted.

the *CLN3* mRNA. Similarly to the in vivo assays, the Whi3 RRM was able to bind very efficiently and specifically to the *CLN3* mRNA in vitro (Fig. 3E). Although the binding of *CLN3* mRNA remained unaffected by the addition of cold yeast total RNA in a 10-fold excess, the binding of *URA3* and *LEU2* mRNAs showed a fivefold decrease (data not shown). Interestingly, the Whi3 RRM was also able to bind the mRNAs of the two other G_1 cyclins, *CLN1* and *CLN2*, albeit with reduced efficiency, whereas there was no binding to the mRNA of the S-phase cyclin *CLB5*. These results reinforce the idea that the G_1 arrest caused by Whi3 overexpression may involve the functional inhibition of all Cln cyclins. A preliminary deletion analysis showed that the Whi3 RRM may bind the *CLN3* mRNA at more than one region, although the 3′ region of the *CLN3* open reading frame seems to contain the most important determinants for binding to Whi3 (Fig. 3E; see below). These observations support the idea that Whi3 acts as an RNA-binding protein, and that the *CLN3* mRNA may be a direct target.

We therefore examined the effects of Whi3 on *CLN3* mRNA and protein. Neither overexpression nor deletion of *WHI3* had any apparent effect on *CLN3* mRNA levels, or on Cln3 protein levels (Figs. 1C, 2C). The polysome profile of *CLN3* mRNA did not change during *WHI3* overexpression (Fig. 2D), suggesting that translation of Cln3 was not affected. We could see no effect of Whi3 on levels of Cln3–Cdc28 histone H1 kinase activity (data not shown) or Cln3 protein phosphorylation levels (Figs. 1C, 2C), which suggest that Whi3 does not directly inhibit Cln3–Cdc28 complexes.

Whi3 colocalizes with the CLN3 *mRNA into cytoplasmic foci*

We next asked whether Whi3 could affect the localization of *CLN3* mRNA. Analysis by FISH showed that the *CLN3* mRNA forms discrete cytoplasmic foci in G_2/M cells (Fig. 4A), a property found in mRNAs that are localized to direct the encoded protein to specific cytoplasmic compartments (Bashirullah et al. 1998). The brightness of these foci was greatly diminished in *whi3* cells (Fig. 4A), whereas unrelated mRNAs such as *URA3* or *kanMX4* were unaffected (data not shown). In addition, a *CLN3* mRNA deletion construct (*CLN3Δ1*) that shows reduced binding to the Whi3 RRM in vitro (see above) also produced much dimmer particles (Fig. 4A). Levels of *CLN3* and *CLN3Δ1* mRNAs were totally comparable when detected by Northern analysis in otherwise wild-type cells (Fig. 4B). Neither a *CLN3* mRNA construct that codes for an identically C-terminal truncated product but retains the 3′ mRNA sequences (*CLN3-1*), nor a mutant that lacks the Start codon (*cln3nt*) affected particle appearance (Fig. 4A). Therefore, the localization of *CLN3* mRNA into these foci requires Whi3 and certain *CLN3* mRNA sequences, but does not require Cln3 protein or full translation of the *CLN3* mRNA. Immunofluorescence showed that Whi3 was also present in cytoplasmic foci (Fig. 4C). A combination of FISH and immunofluorescence showed that the *CLN3* mRNA foci colocalized with a large fraction of the Whi3 foci (Fig. 4D). The fact that we could only clearly detect the *CLN3* mRNA foci in G_2/M cells could be attributed to the transcriptional up-regulation of *WHI3* during mitosis (Spellman et al. 1998; Gari et al., unpubl.). In addition, Whi3 overexpression increased the brightness of *CLN3* mRNA foci in G_1 cells (data not shown), suggesting that the Whi3/*CLN3* mRNA ratio may also be crucial for foci formation and Cln3 function.

Localization of *ASH1* mRNA particles into daughter cells depends on efficient transport via the actin cytoskeleton (Jansen et al. 1996; Long et al. 1997; Takizawa et al. 1997), whereas localization of other mRNAs in higher eukaryotes depends on their movement via microtubules (Arn and Macdonald 1998; Bashirullah et al. 1998). Latrunculin or nocodazole treatment of cells, which disrupts the actin or microtubule cytoskeletons, respectively, did not affect *CLN3* mRNA foci appearance or number (data not shown), suggesting that transport may not be a key functional aspect of the *CLN3* mRNA foci. Rather, as Cln3 is an extremely short-lived protein (Schneider et al. 1998), binding of Whi3 to the *CLN3* mRNA could restrict both synthesis and presence of the Cln3 protein to a narrow molecular environment. If this environment were unfavorable for the activation of SBF and MBF, then Cln3 function would be inhibited, despite wild-type levels of Cln3 protein. Although Miller and Cross (2000) did not detect endogenous Cln3 protein in G_1 cells or small-budded cells by immunofluoresence, they used several genetic approaches to propose that

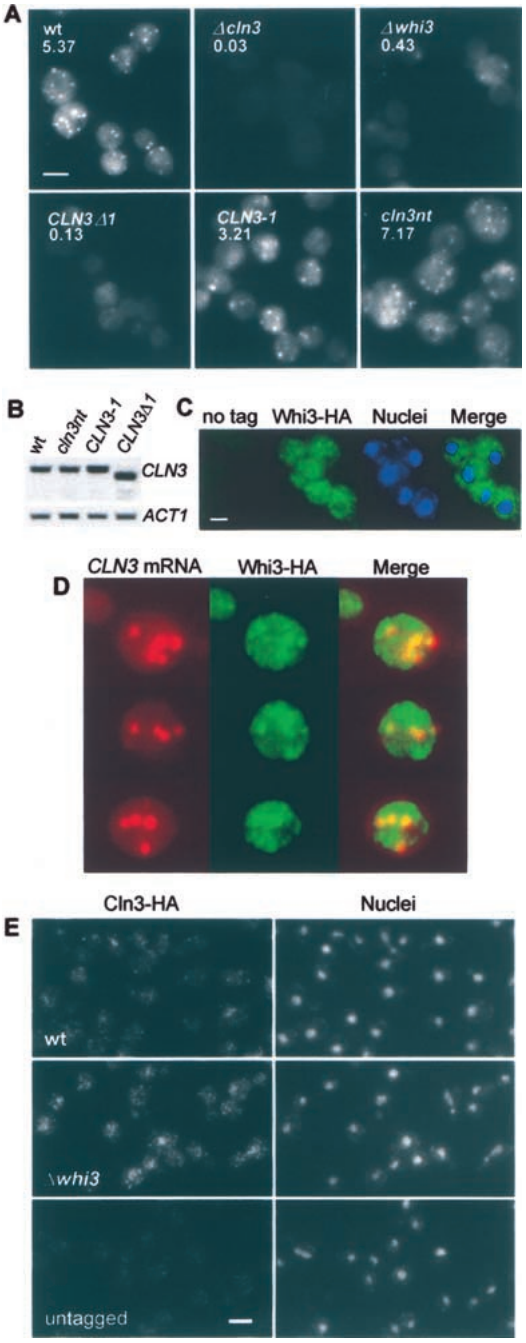

Figure 4. The *CLN3* mRNA is localized to cytoplasmic foci that depend on and colocalize with Whi3. (*A*) *CLN3* mRNA FISH analysis of wild-type (wt), Δ*cln3* as negative control, and Δ*whi3* strains, as well as wild-type cells carrying a deletion in the 3′ region of the ORF (*CLN3-Δ1*), the ORF-equivalent point mutation (*CLN3-1*), or a nontranslatable *CLN3* mRNA (*cln3nt*). (*B*) mRNA levels of the above *CLN3* constructs detected by Northern using the *ACT1* mRNA as loading control. (*C*) Immunofluorescence of Whi3-HA cells (untagged cells as negative control), showing also nuclei and merged images. (*D*) Combined *CLN3* mRNA FISH and Whi3-HA immunofluorescence, showing also merged images. (*E*) Signal-amplification immunofluorescence of Cln3-HA in wild-type (wt), *whi3* mutant (Δ*whi3*), and untagged cells as negative control, showing also nuclei stained with DAPI. Bars, 5 μm.

Cln3 should exert a rate-limiting function in the nucleus. By using a powerful signal-amplification method for immunofluorescence we have been able to detect endogenously expressed Cln3 in the cytoplasm of all wild-type cells (Fig. 4E), with some cells also showing a distinctive nuclear signal. In contrast, Whi3-deficient cells showed a more general and brighter nuclear pattern, including unbudded cells, suggesting that Whi3 regulates a cytoplasmic event that, directly or indirectly, affects the ability of Cln3 to accumulate in the nucleus.

Whi3 is required to repress Cln3 function during meiosis and filamentous growth

Down-regulation of Cln3 function seems to be a key condition for yeast cells to take other developmental options in G_1 such as meiosis, filamentous growth, and mating. For example, Cln3 blocks the function of the meiotic inducer Ime1, thus making mitosis and meiosis incompatible (Colomina et al. 1999). Whi3 may be important for down-regulation of Cln3 in meiosis, because a homozygous *whi3* strain entered meiotic S phase and sporulated very poorly, but a *whi3 cln3* double mutant was as proficient as the parental strain (Fig. 5B). Similarly, Cln3 has an inhibitory effect on filamentous growth (Loeb et al. 1999), and *whi3* has been independently identified in a search for mutants unable to undergo pseudohyphal differentiation (Mosch and Fink 1997). We therefore asked whether the failure of *whi3* cells to filament could be alleviated by deletion of *CLN3*. Although deletion of *whi3* blocked pseudohyphal differentiation in diploid strains and invasive growth in haploid strains, the corresponding double *whi3 cln3* mutants showed similar pseudohyphal and invasive growth ability as parental strains (Fig. 5C,D). The behavior of a *whi3ΔRRM* mutant lacking the RNA-binding domain was indistinguishable from a complete deletion of the *WHI3* gene in all these epistatic analyses (data not shown), which reinforces the notion that the observed direct interaction of Whi3 with the *CLN3* mRNA plays an important role in regulating G_1 events. Finally, *whi3* cells mate with low efficiencies, and this defect shows strong genetic interactions with a hyperactive allele of *CLN3* (Nash et al. 2001). In summary, the observed G_1 cell cycle and developmental deficiencies of *whi3* mutant cells can be all alleviated by loss of *CLN3*, which confirms both the negative effect of Whi3 on Cln3 function and the specificity of their functional relationship.

Our results show that Whi3 inhibits Cln3 function, and suggest that the direct binding of Whi3 to *CLN3* mRNA in cytoplasmic foci may be important for this inhibition. As Cln3 is an extremely short-lived protein, localizing its translation to particular cytoplasmic regions, or molecular environments, could modulate the ability of Cln3 to reach its presumptive nuclear targets and, hence, to induce G_1/S gene expression at a proper cell size during the budding mitotic cycle. As a possibility, the ratio of unstable activators of Start (e.g., *CLN3* mRNA and/or Cln3) to stable inhibitors (e.g., Whi3) may promptly reflect changes in growth rate versus mass or volume, which could determine cell size at Start for a particular set of external conditions. Whatever the precise molecular mechanism of inhibition, Whi3 is also very important for repressing Cln3 function in other developmental options that yeast cells take in G_1: meiosis, filamentation, and mating (Fig. 5E). D-type cyclin hyper-

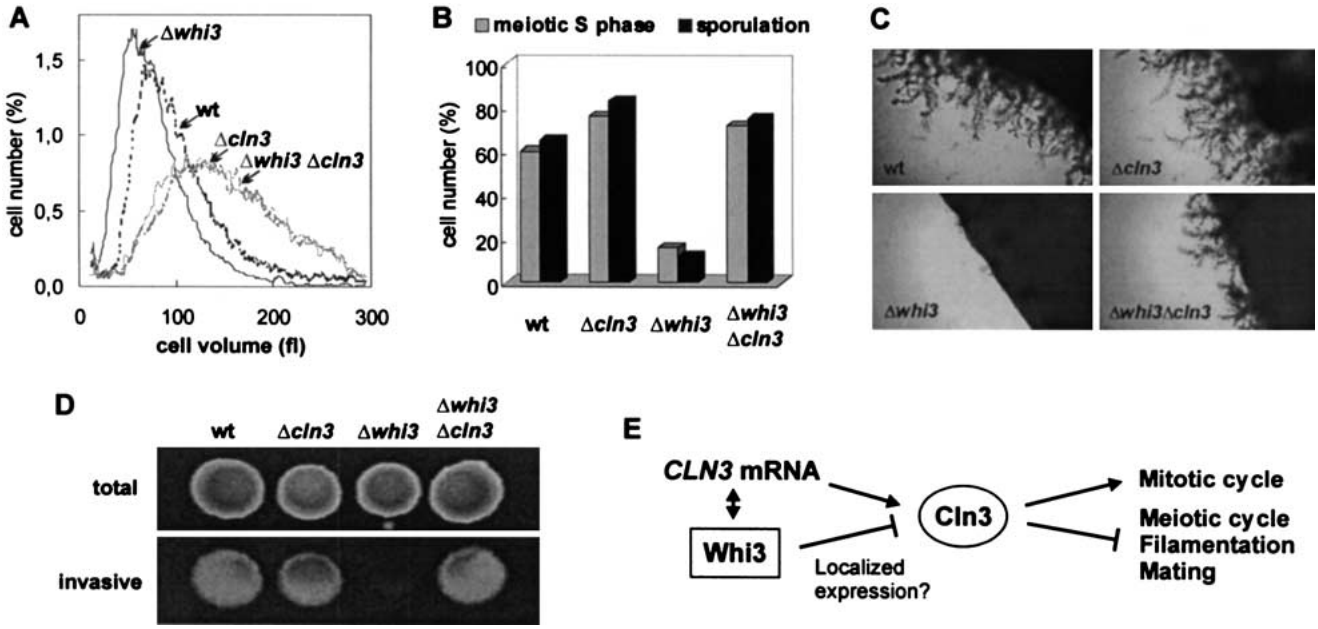

Figure 5. Cell size and developmental defects of Whi3-deficient cells depend on Cln3. Wild-type (wt), Δ*cln3*, Δ*whi3*, and double Δ*cln3* Δ*whi3* mutant strains were used to analyze (*A*) cell volume distributions of haploid 1788 strains, (*B*) meiotic S-phase entry at 12 h and sporulation efficiencies at 24 h of diploid 1788 strains, (*C*) pseudohyphal growth of diploid Σ1278 strains in SLAD plates, and (*D*) invasive growth of haploid Σ1278 strains in YEPD plates. (*E*) Scheme to depict Whi3 and Cln3 functional interactions.

activity also perturbs cell size control in G_1 (Ohtsubo and Roberts 1993; Quelle et al. 1993) and inhibits differentiation (Skapek et al. 1995; Zhang et al. 1999) in mammalian cells. Although an specific RNA-binding protein has not yet been identified, localization of cyclin B1 mRNA by CPEB and maskin to the spindle and centrosomes in *Xenopus* oocytes is important for spindle dynamics (Groisman et al. 2000). The further functional and molecular characterization of the Whi3 device may therefore help us understand how eukaryotic cells integrate and centralize molecular information to regulate their cycle and take different cell fate options.

Materials and methods

Strains, plasmids, and growth conditions

Yeast parental strains (1788, W303, and Σ1278), standard growing conditions, and special media to induce sporulation, filamentation, or invasive growth were as described (Roberts and Fink 1994; Gallego et al. 1997; Colomina et al. 1999). Chromosomal gene disruptions, C-terminal fusions to a triple HA epitope or the TAP tag, as well as gene fusions to the *GAL1* promoter were performed by gene transplacement methods as described (Gallego et al. 1997). *CLN3-Δ1* carries a deletion from nucleotide +1213 to the end of the *CLN3* ORF. *CLN3-Δ2* lacks the first 1212 nucleotides of the *CLN3* ORF. *CLN3-1* has a nonsense mutation at +1210 (Nash et al. 1988), and *cln3nt* carries a point mutation that eliminates the Start codon. Details of strain and plasmid constructions are available upon request.

RNA-binding assays

Yeast cells expressing the Whi3–TAP protein were used in pull-down assays (Rigaut et al. 1999). Washed IgG beads were eluted with 0.5% SDS, 10 mM EDTA, 10 mM HCl-Tris at pH 8 for 5 min at RT, and RNA was isolated (Ausubel et al. 1999) to detect various mRNAs (*CLN3*, *URA3*, and *LEU2*) by dot or Northern blot analysis (see below). As deduced from Northern analysis, *CLN3* mRNA stability was not particularly affected during extract preparation when compared to *URA3* and *ACT1* mRNAs used as internal controls. For in vitro binding assays, the Whi3RRM was fused to the calmodulin-binding peptide (CBP) in pCAL-n and affinity-purified on calmodulin beads (Stratagene), which were then used to test binding of ^{32}P-labeled RNAs synthesized from T7/T3-expression vectors (Ausubel et al. 1999). Binding reactions contained 0.3 µg of ^{32}P-labeled RNA and 1 µg of either CBP–Whi3RRM or CBP bound to calmodulin beads in cold CBB buffer (Stratagene). Binding was performed at 4°C for 1 h, beads were washed four times with cold CBB buffer, and bound RNAs were eluted as described above and spotted on Nylon$^+$ membranes to quantify radioactivity with a Fuji BAS-1000 reader.

FISH and immunofluorescence

Cells were treated for FISH with a digoxigenin-labeled *CLN3* riboprobe essentially as described (Takizawa et al. 1997), using a detection method based on development of a HNPP-fluorescent precipitate (Roche). As the *CLN3* mRNA signal was significantly stronger during mitosis, cells were treated with 10 µg/mL nocodazole for 90 min before fixation. Foci per cell were counted in images all treated in such a way that negative control cells (Δ*cln3*) were already not distinguishable against the field background, and mean values were obtained by counting a minimum of 300 cells per sample. The localization of Whi3-HA was done by immunofluoresence (Colomina et al. 1999) with a rat anti-HA antibody (clone 3F10, Roche), followed by incubation with Alexa488-labeled anti-rat antibody (Molecular Probes). To combine FISH and immunofluoresence, rat anti-HA and sheep alkaline-phosphatase anti-digoxigenin (Roche) antibodies were added together after washing the probe, followed by incubation with Alexa488-labeled anti-rat antibody, and HNPP precipitate development as above. Cln3-HA was detected by a signal-amplification method based on regular immunofluorescence procedures (Colomina et al. 1999). Briefly, after incubation with rat anti-HA antibody, slides were sequentially incubated with goat anti-rat, rabbit anti-goat, and goat anti-rabbit antibodies labeled with Alexa488 (Molecular Probes).

Miscellaneous

Northern methods and probes used, as well as Western conditions to detect specific tagged proteins, were done as described previously (Gallego et al. 1997). TAP-tagged proteins were detected with a peroxidase-labeled rabbit anti-peroxidase antibody soluble complex (Sigma) as described (Rigaut et al. 1999). Cln3 immunoprecipitation and associated-kinase assays were also as described (Tyers et al. 1993). The distribution of the *CLN3* mRNA in polysomes was analyzed in sucrose gradients

(Cigan et al. 1991). Small G_1 cells were obtained by elutriation (Tyers et al. 1993). Cell volume distributions were obtained in a Z2 Coulter Counter, and DNA content distributions and budding indexes were determined as described (Gallego et al. 1997).

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