The induction of the mating program in the phytopathogen *Ustilago maydis* is controlled by a G1 cyclin

Sonia Castillo-Lluva and José Pérez-Martín*

Department of Microbial Biotechnology, Centro Nacional de Biotecnología CSIC, Campus de Cantoblanco, UAM, 28049 Madrid, Spain

*corresponding autor

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is Dr. José Pérez-Martín, Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco-UAM, 28049 Madrid, Spain. Phone: +34-91-585-4704; FAX: +34-91-585-4506; e-mail: jperez@cnb.uam.es

Key Words: *Ustilago maydis*, Cell cycle, G1 cyclin, Fungal virulence

Running title: G1 cyclin controls mating in smuts

SUMMARY

Our understanding of how cell cycle regulation and virulence are coordinated during the induction of fungal pathogenesis is limited. In the corn smut fungus, *Ustilago maydis*, pathogenesis and sexual development are intricately interconnected. Furthermore, the first step in the infection process is mating and this is linked to cell cycle. In the present study, we have identified a new G1 cyclin gene from *U. maydis* that we have named *cln1*. We investigated the roles of Cln1 in growth and differentiation in *U. maydis* and found that although not essential for growth, its absence produces dramatic morphological defects. We provide results that are consistent with Cln1 having a conserved role in regulating the length of G1 and cell size, but also additional morphological functions. We also present experiments indicating that the cyclin Cln1 controls sexual development in *U. maydis*. Overexpression of *cln1* blocks sexual development, while its absence enables the cell to express sexual determinants in conditions where wild-type cells were unable to initiate this developmental program. We conclude that Cln1 contributes to negative regulation of the timing of sexual development, and we propose the existence of a negative cross-talk between mating program and vegetative growth, that may help explain why these two developmental options are incompatible in *U. maydis*.

INTRODUCTION

Developmental decisions leading to differentiation often require the cell cycle to be reset and the induction of new morphogenetic programs. However, understanding how growth and cell cycle progression are coordinately regulated during development still remains a challenge. Furthermore, there is little information available regarding the relationship of these processes with the induction of the virulence programs in pathogenic fungi. Hence, the role of cell cycle regulators as true virulence factors has still to be defined. Nevertheless, in the corn smut fungus *Ustilago maydis*, the different morphological changes that the fungal cells undergo during the pathogenic process indicates that cell cycle control is closely associated to these transitions (see Kahmann and Kamper, 2004 for a recent review).

The first step in the infection process is mating and this is linked to cell cycle. Indeed, when exposed to sexual pheromones, *U. maydis* cells undergo arrest in G2 phase, prior to the fusion of the cytoplasms, and then form the infective dikaryotic filament (García-Muse et al., 2003). Once this filament enters the plant, the fungal cells proliferate, forming filaments in which the septa partition the cell compartments so that each contain a pair of nuclei (Snetselaar and Mims, 1992; Banuett and Herskowitz, 1996). This morphological transition is clearly connected to cell cycle control and hence, *U. maydis* is perfectly suited to analyze the relationships between cell cycle, morphogenesis and pathogenicity (Basse and Steinberg, 2004). Two distinct Cdk-cyclin complexes are responsible for the different cell cycle transitions in *U. maydis*. While Cdk1-Clb1 is required for the G1/S and the G2/M transitions, the Cdk1-Clb2 complex is specific for the G2/M transition (García-Muse et al., 2004).

Whether cell cycle regulators may have a role in the pathogenicity of *U. maydis* has recently been addressed. When the transcription of mitotic cyclins is manipulated,

hyphal proliferation within the plant is affected and the fungal cells produced are unable to successfully infect the plant (García-Muse et al., 2004). Furthermore, we described that mutation of the Fizzy-related APC (Anaphase Promoting Factor) activator, Cru1, provoked defects at different stages of *U. maydis* plant infection (Castillo-Lluva et al., 2004). For instance, in Δ*cru1* cells, a low level of expression of the pheromone-encoding gene, *mfa1*, resulted in deficiency in mating and thereby a low frequency of dikaryotic infective filament formation. In addition, proliferation of the mutant fungus inside the plant was also affected, resulting in the inability to induce tumors in plants (Castillo-Lluva et al., 2004).

Like other Fizzy-related proteins in different organisms, Cru1 controls the length of G1 in *U. maydis*. Therefore, we proposed that an accurate control of the length of G1 could be a determinant of virulence in *U. maydis*. If this is the case, an exciting challenge would be to establish whether mutations in other regulators of G1 also affect the pathogenicity of *U. maydis* cells. Appealing candidates that should be studied include the genes encoding G1 cyclins.

In *Saccharomyces cerevisiae*, the length of G1 depends on the presence of G1 cyclins that induce the G1/S transition when coupled to their cyclin-dependent kinase (Cdk) partner (Murray and Hunt, 1993). One such G1 cyclin, Cln3, is strongly associated with the size threshold for division and hence, with G1 length. When the expression of *CLN3* is augmented, the length of G1 is shortened, and cells divide at a smaller than normal size. In contrast, when *CLN3* is deleted, the G1 phase is delayed, and cells reach a larger than normal size before dividing (Cross 1988; Nash et al., 1988). However, not all G1 cyclins are dedicated to G1 length control. The other two G1 cyclins from *S. cerevisiae*, Cln1 and Cln2, have a major morphogenetic role, inducing bud formation and cell polarization (Lew and Reed, 1993).

The human pathogen *Candida albicans* also contains homologues of G1 cyclins, including Cln1/Ccn1, Hgc1 and Cln3 (Loeb et al., 1999b; Zheng et al., 2004; Chapa y Lazo et al., 2005; Bachewich and Whiteway, 2005), but it seems that G1 cyclin homologues in this organism have evolved important roles in hyphal morphogenesis as opposed to cell cycle progression. Deletion of *CLN1* or *HGC1* results in defects to maintain hyphal growth (Loeb et al., 1999b; Zheng et al., 2004), while depletion of Cln3 causes yeast cells to arrest in G1, increase in size, and then develop into hyphae and pseudohyphae (Chapa y Lazo et al., 2005; Bachewich and Whiteway, 2005). Interestingly, repressing *CLN3* in environment-induced hyphae did not inhibit growth or cell cycle, suggesting that in *C. albicans* yeast and hyphal cell cycles may be regulated differently.

Finally in other organisms such as *Schizosaccharomyces pombe*, no clear role has been ascribed to the G1 cyclin Puc1, which is related to Cln3 at the sequence level, but it seems to have a minimal role in cell cycle, most likely because its activity is masked by that of the two mitotic cyclins, Cig1 and Cig2 (Martín-Castellanos et al., 2000).

In our attempts to clarify the connection between the cell cycle and virulence in smut fungi, we have identified a new G1 cyclin gene from *U. maydis* that we named *cln1*. We found that Cln1 is not essential but is required for normal morphogenesis and cell separation. Repression of *cln1* resulted in G1 delay and cell enlargement, while high levels of *cln1* expression induced hyphal-like growth. These results were consistent with Cln1 having a conserved role in regulating the length of G1 and cell size, but also additional morphological functions in influencing polar growth and cell separation. We also present experiments suggesting that the cyclin Cln1 controls sexual development in *U. maydis*. Our results indicate that *U. maydis* cells have

established a negative cross-talk between vegetative growth and mating to make these cell choices incompatible.

RESULTS

Identification of a G1 cyclin-related protein in *U. maydis*

To determine whether G1 cyclins exist in *U. maydis*, we used *S. cerevisiae* Cln1, Cln2 and Cln3 cyclin box sequences to search *U. maydis* genome database (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/index.html). From this approach, we obtained the sequence of a single gene that we named *cln1*. The conceptual translation of the *cln1* sequence produces a putative protein of 520 amino acids (accession no. DQ017758), that groups with members of G1-type cyclin family, including putative G1 cyclins from other basidiomycetes (Figure 1A). The predicted protein contained one cyclin box near the amino terminus, which shared 73-30% sequence similarity to that of other G1 cyclins characterized in fungi (Figure 1B and Supplemental Figure 1). We also found a PEST domain at the C terminus of *U. maydis* Cln1 (Figure 1B). The PEST domain, found in all three budding yeast G1 cyclins as well as in *S. pombe* Puc1 (Tyers et al., 1992; Forsburg and Nurse, 1994), was originally identified as a potential determinant of protein instability on the basis of its frequent occurrence in unstable proteins (Rechsteiner and Rogers, 1996).

Comparison of genomic and cDNA sequences indicated that *cln1* was intronless. Interestingly, the *cln1* mRNA 5' leader contains several salient features such as its unusual length (551 nucleotides) and the presence of two short upstream open reading frames (uORF, Figure 1C). A short uORF in the 5' leader of the *S. cerevisiae CLN3* mRNA has been shown to rate-limit Cln3 production at the translation initiation step by a "leaky scanning" mechanism (Polymenis and Schmidt, 1997), suggesting

that a similar mechanism could operate in the regulation of *U. maydis cln1* expression.

Cln1 is a G1-type cyclin

We first investigated whether the expression of *cln1* fluctuated through the cell cycle. Northern blot analysis was performed on total RNA isolated from cultures of G1 phase enriched wild-type cells, or cells arrested in S phase (in the presence of hydroxyurea) or in M phase (benomyl). We found high levels of *cln1* mRNA in G1-enriched cells (Figure 2A), which dramatically decreased in S phase-arrested cells. As a control we followed the expression levels of *clb1*, which encodes an essential mitotic cyclin that reached peak expression in S phase (García-Muse et al., 2004). This result was consistent with the preferential expression of *cln1* during G1. To analyze whether the levels of *cln1* mRNA expression mirror that of the protein, we exchanged the native *cln1* gene by a modified allele that encodes a C-terminal end myc-tagged Cln1 protein. The resulting strain showed no apparent defects with respect to a wild-type strain (not shown) indicating that the tagged protein was fully functional. However, we were unable to detect the Cln1 protein directly from crude extract (not shown). To circumvent this problem, we used an immunoprecipitation-based enrichment step, and we observed the presence of Cln1 protein in immunoprecipitates obtained from asynchronous and G1-enriched cultures, while only trace amounts were detected in immunoprecipitates obtained from S- and M-arrested cells (Figure 2B). From these results, we believe that the levels of Cln1 protein reproduce that of *cln1* mRNA expression and thus, it follows that the Cln1 protein could exert its main influence in G1 phase.

We also wished to prove that Cln1 might interact with Cdk1, the catalytic subunit of the mitotic cyclin-dependent kinase in *U. maydis* (García-Muse et al., 2004). For this,

proteins were precipitated from extracts of wild-type cells and cells expressing high levels of the functional myc-tagged version of Cln1. Initially, the protein extracts were incubated with agarose beads coupled to the *S. pombe* protein Suc1, which is known to bind specifically to Cdk1 with high affinity (Ducommun and Beach, 1990; García-Muse et al., 2004). The precipitates were then separated by SDS-PAGE and immunoblotted with anti-PSTAIRE (that detects Cdk1) and anti-MYC antibodies. Both Cdk1 and Cln1-myc were recovered in the fraction of proteins bound to Suc1-beads (Figure 2C left panel). The presence of Cdk1 was also analyzed in protein extracts precipitated with anti-MYC antibodies (Figure 2C, right panel). While these antibodies co-precipitated Cdk1 with Cln1-myc, neither Cln1 nor Cdk1 was detected in precipitates from wild-type cell extracts. In addition, the precipitate from tagged strains exhibited histone H1 kinase activity (not shown). These results demonstrate a physical association between the Cln1 protein and Cdk1, further evidence that Cln1 can be considered as a definitive cyclin.

Finally, we also investigated whether *U. maydis cln1* could complement the absence of G1 cyclins in *Saccharomyces cerevisiae*, as described for other G1-related cyclins (Forsburg and Nurse, 1991; Whiteway et al., 1992; Sherlock et al., 1994; Zheng et al., 2004). We tested this in an *S. cerevisiae* strain in which the *CLN1* and *CLN2* genes had been deleted, and where the *CLN3* gene was under the control of *GAL1* promoter. The viability of this strain is conditional and galactose-dependent (Tyers et al., 1993). We introduced into this strain the *U. maydis cln1* gene under the control of the constitutive *GPD1* promoter on a centromeric plasmid. As positive control, we used a centromeric plasmid carrying the *S. cerevisiae CLN3* locus. All the strains grew on solid medium carrying galactose, a condition that allows *GAL1-CLN3* expression, while when galactose was replaced by glucose, only the strains carrying

the *CLN3* or *cln1* plasmids grew, although the strain expressing *cln1* considerably more slowly (Figure 2D). The same test in liquid medium produce similar results. Interestingly, around 17% of the cells carrying the vector expressing the *U. maydis cln1* gene exhibited an elongated morphology (Figure 2E). The polarized morphology of the *S. cerevisiae* cells expressing *U. maydis cln1* might be a reflection of some intrinsic ability of this protein in promoting morphogenesis (see below). Although these results shown that *U. maydis cln1* may be only partially functional in *S. cerevisiae*, collectively these data support the notion that the *cln1* gene encodes a G1 cyclin in *U. maydis*.

Cln1 is not essential but is required for normal morphogenesis and cell separation

To analyze the function of Cln1, we inactivated one *cln1* allele in the diploid FBD11 strain replacing it with a carboxin resistance cassette (producing the Δ*cln1* null allele). When the meiotic offspring was analyzed after sporulation, half the resulting population was resistant to carboxin and carried the null allele, indicating that *cln1* is not essential for growth. However, in contrast to the characteristic cigar-shaped wild-type morphology, a *cln1* deficient strain generated very thick and irregular shaped cell aggregates (Figure 3A). In these cell aggregates most cells did not separate, and they form septa that could be stained with calcofluor (Figure 3B). Older cells often lost their polarity and became rounded, although the cells at the periphery of the aggregates remained elongated. We also found that some cells accumulated several nuclei (Figure 3A, arrowheads).

Repression of *cln1* results in G1 delay.

In *S. cerevisiae*, the absence of Cln3 causes a delay in G1, and an increase in cell size (Cross, 1998; Nash et al., 1988). The deletion of *cln1* in *U. maydis* resulted in enlarged cells. However, because of the cell separation defect producing cell aggregates, we were unable to correlate the absence of Cln1 with a delay in G1 (that could be measured as an increase in 1C DNA content in the cell population by FACS analysis). To circumvent this difficulty, we constructed a conditional allele, *cln1*^{nar}, in which the *cln1* gene was placed under the control of the *nar1* promoter (Brachmann et al., 2001). This enabled the down-regulation of *cln1* expression to be controlled by switching from nitrate- to ammonium-containing medium (Figure 4A). To avoid any putative negative regulation exerted by the long 5' UTR, in this chimeric construction we removed the native *cln1* mRNA leader region, leading to a shorter transcript when compared with the wild-type transcript. No defects in cell growth were apparent in the resulting strain under permissive conditions (not shown). However, when shifted to restrictive conditions, cells start to enlarge (compare with wild-type cells, Figure 4B inset) and to have cell separation defects (Figure 4B, 3 hours). After an extended period of time, cell aggregates started to form in which the older cells became rounded while the new ones remained elongated (Figure 4B, 6 hours). Prolonged incubations (more than 24 hours) lead to the appearance of large aggregates that were reminiscent of those seen in Δ*cln1* cells (not shown).

To address whether *cln1*-deficient cells have an elongated G1 phase, we grew wild-type and *cln1*^{nar} cells in permissive conditions (nitrate-containing minimal medium) until early exponential phase and then cells were arrested at M phase with benomyl and transferred to repressive conditions for *cln1* expression (complete medium, CMD) for one hour. After being released in benomyl-free CMD medium, the DNA content was followed as these cells proceed through mitosis and enter in G1 and S

phase (Figure 4C). We observed that upon release, mutant cells were delayed in G1 phase with respect to wild-type cells (compare the 1C population on wild-type and *cln1*^{nar} cells at 120 minutes). These results were consistent with a role of Cln1 promoting the transition through G1 phase.

High levels of *cln1* expression induces hyphal-like growth

G1 cyclins are thought to be involved during G1 in coordinating cell growth and division. For instance, an increase in the amount of *CLN3* in *S. cerevisiae* results in cells that divide when they are smaller than normal (Cross, 1988; Nash et al., 1988). We introduced an extra copy of *cln1* into *U. maydis* cells under the control of the *crg1* promoter, which can be induced by arabinose (Bottin et al. 1996). In this construction we removed the long 5' mRNA leader region, producing a shorter transcript, and the resulting protein was myc-tagged at its C-terminal end. In cells carrying this construct, mRNA transcribed from the *crg1* promoter (Figure 5A, *crg:cln1*), was detected after less than 1 hour of induction and the accumulation of this RNA reached a peak of expression around 25 times the levels of native *cln1* mRNA expression. The increase in mRNA expression was followed by protein accumulation (Figure 5B). Strikingly, these high levels of *cln1* expression from the *crg1* promoter resulted in the down-regulation of the mRNA transcribed from the native promoter (Figure 5A, *cln1*). This is suggestive of the existence of autoregulatory feedback-inhibition as reported for other cyclins (Ayte et al., 2001).

Unexpectedly, cells overexpressing *cln1* displayed hyperpolarized growth after the shift to inducing conditions (Figure 5C). The cells started to grew apically and after 3 hours, they clearly evaginated outgrowths carrying a single nucleus each, with a 2C DNA content (Figure 5D). Subsequently, the filaments began to undergo mitosis, and

by 8 hours, around half of the cell population contained four nuclei. Cells not overexpressing *cln1* and growing in arabinose-containing medium would have gone through approximately four cell divisions. Thus, *cln1*-induced filaments appear to be elongated buds that can progress through the nuclear cell cycle, albeit with an apparent delay. Prolonged incubation (24 hours) resulted in the development of highly elongated cells that resembled hyphae, with several nuclei evenly distributed along the entire length of the filament. Staining of the cells with calcofluor showed the presence of septal cross walls between hyphal compartments (Figure 5E).

Cln1 is a nuclear protein that also localizes to hyphal tip

The previous data suggesting a morphological role of Cln1 prompted us to analyze the sub-cellular localization of Cln1. For this, we constructed a strain that contained the GFP fused to the C terminus of the endogenous *cln1* gene. This strain had a wild-type appearance, although we were unable to detect Cln1-GFP by epifluorescence. Therefore, for in vivo localization we expressed this protein fusion under control of the *crg1* promoter, and analyzed the cells during the first two hours. The Cln1-GFP fusion protein localized to nucleus (Figure 6A), as it was expected for a cyclin protein. Strikingly, we also found fluorescent single spots that were associated to the region just behind the hyphal tip (Figure 6B). These spots were motile, showing random short-distance mobility (unpublished observations). Currently, we do not have a satisfactory explanation for this localization, but it was compatible with a morphogenetic role of Cln1 in *U. maydis*.

High level of Cln1 represses the induction of the *mfa1* gene

The above results were consistent with Cln1 having a conserved role in regulating the length of G1 and cell size, but also additional morphological functions. Because our previous suggestion that induction of the sexual program -as measured by the expression of *mfa1* gene, encoding the pheromone precursor- was related to G1 length (Castillo-Lluva et al., 2004), we wished to examine the relationships between Cln1 levels and *mfa1* expression. Since the culture medium has a strong influence in the *mfa1* expression (it is expressed in complete- and minimal-based media but not in YEP-based medium; Spellig et al., 1994), we constructed a new *cln1* conditional allele, $cln1^{crg}$, in which the endogenous *cln1* gene was placed under the control of the *crg1* promoter. This enabled us to down-regulate or to overexpress the *cln1* gene in any medium by switching the carbon source from glucose to arabinose, respectively (Figure 7A). In glucose-containing media, these cells displayed the altered morphology observed in a *cln1* deficient strain, while in arabinose-containing media they had the hyphal-like growth observed when *cln1* was overexpressed ectopically (not shown). We found that depletion of Cln1 enables the cells to express *mfa1* even on media like YEP-based medium, for which respective wild-type strain was unable to express this gene (Spellig et al., 1994; Castillo-Lluva et al., 2004). In contrast, overexpression of *cln1* abrogates the *mfa1* expression in media like complete medium (CM, Figure 7A), where *mfa1* is efficiently expressed in wild-type strains (Spellig et al., 1994). To study in detail this correlation, we grew $cln1^{crg}$ cells in CMD, conditions in which no expression of *cln1* was observed and a strong signal in *mfa1* was apparent (Figure 7B) and transferred to arabinose-containing complete medium (CMA). Consistently, we found strict inverse correlation between the *cln1* and *mfa1* mRNA levels (Figure 7B). Transfer of wild-type cells from CMD to CMA has no influence on the levels of *mfa1* expression (not shown).

The negative role of Cln1 in the expression of *mfa1* contrasts with the positive role that the Fizzy-related protein Cru1 has in pheromone gene expression (Castillo-Lluva et al., 2004). Since Cru1 is also implicated in G1 regulation, we decided to examine the relationships between Cln1 and Cru1 with respect to *mfa1* expression. Therefore we deleted *cln1* in cells already lacking the *cru1* gene. While cells lacking *cln1* showed higher levels of mRNA than wild-type cells, Δ*cru1* cells were unable to express *mfa1*. Strikingly, cells lacking both regulators were unable to express *mfa1* (Figure 7C), indicating a dominant effect of Δ*cru1* mutation over of Δ*cln1*.

cln1 expression is regulated by the cAMP/PKA and MAPK pathways

The cAMP/PKA and MAPK pathways regulate the expression of the pheromone gene (Kaffarnik et al., 2003). The relationship between Cln1 levels and pheromone gene expression brought us to assess whether these pathways also control *cln1* expression. For this, we examined its mRNA levels in Δ*adr1* and Δ*fuz7* mutants, in which the cAMP/PKA and the pheromone MAP kinase pathways are blocked respectively (Dürrenberger et al., 1998; Banuett and Herskowitz, 1994; Andrews et al., 2000). We also analyzed the *cln1* expression in cells carrying the Δ*ubc1* mutation, in which the PKA pathway is constitutively activated (Gold et al., 1994), and in cells expressing an activated allele of the Fuz7 MAPKK, $fuz7^{DD}$ (Müller et al., 2003) under a regulatable promoter.

When the regulatory subunit of PKA, *ubc1*, was deleted, the resulting constitutive activation of the cAMP/PKA pathway down-regulated the *cln1* expression (Figure 8A). Conversely, a deletion of the PKA catalytic subunit that interrupts the pathway produced an increase in the *cln1* mRNA levels (Figure 8A). With respect to the pheromone MAP kinase pathway, we found that deletion of the *fuz7* gene increases

cln1 expression around three-fold (Figure 8A), while that the expression of the constitutive active *fuz7*^{DD} allele strongly repressed the *cln1* expression (figure 8B). In wild-type cells, the expression of *cln1* was not affected by the transfer from glucose- to arabinose-containing media (not shown).

Cln1 and Cru1 control the expression of *prf1*, encoding the master regulator of sexual development.

The transcription factor Prf1 receives the information from both cAMP/PKA and MAPK pathways and activates the expression of genes involved in sexual development, including the *mfa1* gene (Hartmann et al., 1996; Kaffarnik et al., 2003). Since Cln1 affects *mfa1* expression and it is regulated by the same pathways as Prf1 is, we decided to analyze putative relationships between *prf1* and *cln1*.

Firstly, we wondered whether Prf1 negatively regulates *cln1* expression. We expressed the constitutive active *fuz7*^{DD} allele in Δ*prf1* cells, and we found that the absence of Prf1 did not suppress the negative regulation over *cln1* of an activated MAPK pathway (Figure 8B).

We also analyzed the level of expression of the *prf1* gene in cells carrying the conditional *cln1*^{crg} allele in induction (CMA) and non-induction (CMD) conditions, as well as in Δ*cru1* cells. We observed (Figure 9A) that *prf1* mRNA was not detectable in the absence of *cru1* or in conditions of high *cln1* expression, mirroring the *mfa1* levels previously observed (Figures 7A and 7C). This result suggests that the absence of *prf1* transcription in these genetic backgrounds might be the responsible for the absence of *mfa1* expression in these conditions. To support this interpretation, we put in these strains the *prf1* gene under the control of the regulatable *nar1* promoter. We observed *mfa1* expression in Δ*cru1* cells when transcription of the *prf1*

gene was induced (Figure 9B). We also were able to bypass the *cln1*-mediated repression of *mfa1* expression by induced expression of *prf1* under P*nar1* control (Figure 9C). These results support the notion that Cru1 and Cln1 control the induction of sexual development in *U. maydis* via the master regulator Prf1.

Absence of Cln1 affects cell-cell fusion during mating

Given the connections between cell cycle control and pathogenesis in *U. maydis* we were interested in determining whether cells deficient in *cln1* were able to infect plants. For this, we infected corn plants with a mixture of compatible (i.e. with different *a* and *b* loci) Δ*cln1* strains. While in control experiments with mixtures of wild-type compatible strains 95% of infected plants showed tumor formation, compatible Δ*cln1* strains were completely impaired in pathogenic development. No one of the 74 plants inoculated with the compatible mutant cells, showed tumors (Table 1).

Such a severe defect in virulence led us to investigate the ability of Δ*cln1* cells to mate, a prerequisite to initiate the pathogenic development. The mating reaction in *U. maydis* can be easily scored by co-spotting compatible strains on solid media containing charcoal. In these plates, cell fusion and development of the infective dikaryotic filament resulted in the formation of a white layer of aerial hyphae on the surface of the growing colony. While control mating reactions between compatible wild-type strains produce a clear formation of aerial filaments, when the strain carrying the Δ*cln1* allele was co-spotted with a wild-type compatible strain, the formation of the aerial filaments was attenuated, and additional deficiency was observed when two compatible Δ*cln1* strains were mixed together (Figure 10A). Furthermore, in spite of the fuzzy aspect of small areas in the mutant mixture, we

were unable to detect any dikaryotic filament in these mixtures (not shown). The formation of dikaryotic hyphae requires the fusion of conjugation tubes, and therefore it is possible that the inability to produce a successful mating in Δ*cln1* cells is a consequence of defects in cell-cell fusion. To circumvent the need for cell fusion we deleted the *cln1* gene in the solopathogenic strain SG200, which carries the genetic information required to form hyphae without a mating partner (Bölker et al., 1995). Strikingly, a solopathogenic Δ*cln1* strain was able to produce infective hyphae as well as the SG200 strain (Figure 10A). We also observed formation of normal appressoria in the plant surface (Supplemental Figure 2) and finally, in virulence studies the mutant Δ*cln1* strain was fully pathogenic (Table 1).

Since these results indicated that the defects associated to the absence of Cln1 were prior to cell-cell fusion, we analyzed the ability of mutant cells to secrete pheromone. For this we took advantage of the strain FBD12-17 -a tester strain used to check for pheromone production. If this strain is co-spotted together to cells secreting a1 pheromone, a strong formation of hyphal growth is induced (Spellig et al., 1994). We spotted together with a constant amount of tester cells increasing amounts of wild-type and Δ*cln1* cells, and we found that although mutants cells were able to secrete pheromone, they were clearly impaired in this process, in comparison to wild-type cells (Figure 10B).

We also analyzed the ability of Δ*cln1* cells to produce conjugative tubes in response to the presence of compatible pheromone. We treated wild-type and Δ*cln1* cells with synthetic a2-mating pheromone, and we found that mutant cells formed significantly less tubes (Figure 10C), that in general were thicker and more irregular than those produced by wild-type cells (Figure 10D), indicating that deletion of *cln1* affects induction and morphology of conjugation tubes.

In summary, haploid Δ*cln1* cells were non-virulent mainly because a defect in cell-cell fusion that we believe arises at least from impaired pheromone secretion and conjugative tube formation.

DISCUSSION

Fungal cells take different developmental options in response to external signals, and frequently these options are incompatible. In the phytopathogenic fungus *Ustilago maydis*, pathogenicity and sexual development are intimately intertwined, and both are part of the same developmental program. Entering the mating program involves cell cycle arrest, suggesting that to have an active mitotic cycle could be incompatible with the induction of the sexual program. This work concerns the relationships between key molecules involved either in the regulation of G1 phase or in the activation of the sexual development in *U. maydis*, and provides some clues as to how smut fungus make these choices incompatible.

Role of Cln1 in G1 progression and morphogenesis

U. maydis cln1 gene encodes a protein with high sequence similarities to G1 cyclins from other fungi. This protein can interact with the catalytic subunit of the mitotic cyclin-dependent kinase and it is able to complement the lack of G1 cyclins when expressed in *S. cerevisiae*. We also showed that the *cln1* gene is preferentially expressed in G1 phase. In agreement with this view, cell cycle arrest/release experiments indicated that cells lacking Cln1 had a delayed G1 phase with respect to wild-type cells. G1 cyclins in fungi and D-type cyclins in mammalian are though to be essential regulators that mediated G1/S transition. In *S. cerevisiae* there are three major G1 cyclins, Cln1, Cln2 and Cln3. Although they have different roles, G1 cyclins

in budding yeast are functionally redundant, since viability is not compromised by the deletion of any two of the three; however, deletion of all three is lethal (Richardson et al., 1989). In *U. maydis,* Cln1 is dispensable to growth. However, we found no additional sequences in the available genome sequence with capacity to encode another G1-like cyclin. It could be possible that additional elements, like Bck2 in *S. cerevisiae,* share function with Cln1 in activating G1/S transition (Epstein and Cross, 1994; Di Como et al., 1995), or that its activity is masked by other Cdk-cyclin complexes, as it happens in *S. pombe*, where the solely G1 cyclin Puc1 is masked by the activity of the two mitotic cyclins, Cig1 and Cig2 (Martín-Castellanos et al., 2000). In any case, our data indicated that Cln1 has a conserved role in regulating the length of G1.

Our results also indicated that Cln1 has additional roles in morphogenesis. The cell aggregates produced by the absence of Cln1 are composed of cells that lost its ability to divide by budding, remain attached after cytokinesis and often lost their polarity. In contrast, high levels of Cln1 provokes a strong polar growth that results in filaments composed of cells separated by septa. The involvement of G1 cyclins in fungal morphogenesis is well known. In *S. cerevisiae*, the G1 cyclins Cln1 and Cln2 play a role in the establishment of growth polarization and budding (Benton et al., 1993; Cvrckova and Nasmyth, 1993; Lew and Reed, 1993), and mutants that enhance or prolong the activities of Cln1,2-Cdc28 complexes sometimes cause significant bud elongation (Lew and Reed, 1993; Loeb et al., 1999a). The different G1-like cyclins from *C. albicans* have distinct morphogenetic roles. Deletion of *CLN1* results in the inability to maintain hyphal growth under certain conditions (Loeb et al.,1999b), while deletion of *HCG1* prevents hyphal growth under all hypha-inducing conditions (Zheng et al., 2004). In contrast deletion of *CLN3* produces hyphal growth

in the absence of environmental inducing conditions (Chapa y Lazo et al., 2005; Bachewich and Whiteway, 2005). From our data we infer that in *U. maydis* Cln1 could be related with cell polarity and budding, recapitulating some of the roles of the three different G1 cyclins from *S. cerevisiae*. The mechanism by which Cln1 may affect morphogenesis in *U. maydis* is unknown. In addition to a nuclear distribution, we were able to locate Cln1-GFP, when overexpressed, as small unstable dots in the tip of the filament (Suplemental Video 1). However, in spite of the dramatic morphological effect observed when overexpressed, we believe that Cln1 is not directly involved in hyphal-like promotion in *U. maydis*. We found that conditions producing hyphal-like growth in *U. maydis*, such as the induced expression of an activated form of the Fuz7 MAPK kinase or the expression of an active bE/bW heterodimer, were not affected by the absence of Cln1 (Supplemental Figure 3). Moreover, we believe that the hyphal-like growth resulting from a continuous supply of Cln1 through the cell cycle could be the consequence of the interference with a correct morphogenesis, and this could be producing a cell cycle delay that leads to hyperfilamentation.

Mitosis or mating: a role for G1 cyclins

In *U. maydis*, entering the sexual program seems to be incompatible with an active mitotic cell cycle. The response to pheromone and the activation of the mating program induces a cell cycle arrest in *U. maydis* (Garcia-Muse et al., 2003), whereas Cdk1-cyclin complexes seem to be repressive for the induction of sexual determinants (Figure 11). High levels of *cln1* expression impairs the expression of *mfa1*, encoding the precursor of sexual pheromone a1, while down-regulation of *cln1* enables the expression of *mfa1* even in nutritional conditions that represses the

expression of the *a* mating types. Similarly, we previously reported that the requirement of the APC activator Cru1 for *mfa1* expression could be bypassed by down-regulation of *clb1* expression (Castillo-Lluva et al., 2004), suggesting that the requirement of Cru1 for pheromone expression is just to remove Clb1. Several results support that Cru1 and Cln1 are acting in the same pathway, or at least in the same target. We showed that the deletion of *cln1* failed to rescue the *mfa1* expression in Δ*cru1* cells, suggesting that either the inhibitory function of Cln1 is upstream of Cru1 activating function or that the Cru1 functions "in parallel" to the Cln1 cyclin in regulating pheromone expression but Cru1 requirement has a stronger effect on the process. The inability to express *mfa1* seems to be related with the absence of *prf1* expression when Cru1 is absent or *cln1* is overexpressed. Moreover, the effect on *mfa1* expression of the absence of *cru1* and the overexpression of *cln1* are suppressed by heterologous expression of *prf1*. These results suggest that the expression of *prf1* seems to be the target of the negative cell cycle regulation. The *prf1* promoter has previously been shown to underlie a complex regulation, with at least three different positive regulators acting on distinct regulatory regions: Prf1 itself, which binds pheromone response elements (PREs, Hartmann et al., 1996), Crk1, a kinase that acts through the UAS (Garrido et al., 2004) and Rop1, which binds RRS sites (Brefort et al., 2005). Whether the Cdk1-cyclin complexes act inhibiting any of these regulatory inputs or some new regulatory player is currently unknown, and future experiments will address these issues.

Why in *U. maydis* are mating and mitosis incompatible options? We can imagine one possible explanation. *U. maydis* is a saprophytic organism that in Nature enters the sexual cycle only in particular conditions (the surface of its host plant), that most likely cursed with poor nutritional environment. Under favorable nutritional conditions,

U. maydis cells would prefer to reproduce asexually by means of the mitotic cell cycle. An active mitotic cycle correlates with high Cdk1-cyclin activity. In contrast, when they experience starvation, for instance in the plant surface, the cell cycle is turn down and therefore the window for mating process begins by the induction of sexual development. Therefore, it seems logical that smut cells need a system to carefully time the start of sexual development, when nutrients become limiting and the possibilities to proliferate by fast mitotic divisions are uncertain. The presence of a control system involving positive (*cln1*, *clb1*) and negative (*cru1*-APC) regulators of cell cycle progression may constitute a sophisticated mechanism by which the optimal time for mating, and therefore virulence is determined. This kind of dichotomy could be more general than expected in fungi. In *S. cerevisiae*, where mitosis and sporulation are incompatible options, a negative cross-talk takes place by the inhibitory effect of G1 cyclins on *IME1* transcription, the master regulator of sporulation (Colomina et al., 1999). Also, in *S. pombe*, Puc1 contributes to negative regulation of the timing of sexual development and functions at the transition between cycling and non-cycling cells, although the putative target of this inhibition is currently unknown (Forsburg and Nurse, 1994).

Cln1 and virulence

One of the aims of this work was to define the role of G1 regulators in *U. maydis* virulence. Previous studies (García-Muse et al., 2004; Castillo-Lluva et al., 2004) indicated that in *U. maydis*, an accurate control of the cell cycle is predicted to be necessary not only for cells growing in axenic conditions but also during the infection process. However, we found that a solopathogenic strain lacking the *cln1* gene was fully virulent, suggesting a minor role, if any, of this regulator during the pathogenic

phase. It is possible that in spite of the dramatic effect during the yeast phase, Cln1 plays no role during the hyphal phase of life cycle in *U. maydis*, and therefore it suggest that the cell cycle may not be regulated in the same manner in these two cell types. This situation is reminiscent of *C. albicans*, where there is a differential requirement for the G1 cyclin Cln3 in yeast versus hyphal cells (Chapa y Lazo et al., 2005; Bachewich and Whiteway, 2005).

Strikingly, we found that haploid cells were unable to infect plants. However, since mutant solopathogenic strains were fully virulent, we traced the deficiency to steps previous to the cell-cell fusion process. Although we cannot discard a direct role of Cln1 in this process, we believe that the observed defect is a consequence of the severe morphogenetic deficit of cells lacking Cln1. Haploid Δ*cln1* cells secreted less pheromone than wild-type cells and had an impaired response to exogenous pheromone. Conditions that affect a correct morphogenesis like treatment of cells with the actin inhibitor latrunculin A (Fuchs et al., 2005) or mutations in the *myo5* gene, encoding a class-V myosin (Weber et al., 2003), also results in defects during early steps of mating, strengthening the importance of a correct morphogenesis during the mating process.

METHODS

Strains and growth conditions

The yeast strains used in this study are listed in Table 2. *S. cerevisiae* cells were grown in defined minimal (SC) media supplemented with the appropriate nutrients for plasmid selection (Sherman et al., 1986) and the *U. maydis* cells were grown in YEP-based, complete (CM) or minimal medium (MM) (Holliday, 1974). The expression of genes under the control of the *crg1* and *nar1* promoters, FACS analysis and cell

cycle arrest were all performed as described previously (Brachmann et al., 2001; García-Muse et al., 2003; García-Muse et al., 2004).

DNA, RNA, and protein analysis

The procedures used here for *U. maydis* RNA isolation and Northern analysis, DNA isolation and transformation, and for obtaining protein extracts, for Suc1-purification of Cdk complexes, for immunoprecipitation and Western analysis have all been described previously (Tsukuda et al., 1988; Garrido and Pérez-Martín, 2003; García-Muse et al., 2004). The anti-PSTAIRE (Santa Cruz Biotechnology) and anti-myc 9E10 (Roche Diagnostics Gmb) antibodies were diluted 1:10,000 in phosphate-buffered saline + 0.1% Tween + 10% dry milk for use. Anti-mouse-Ig-horseradish peroxidase and anti-rabbit-Ig-horseradish peroxidase (Roche Diagnostics Gmb) were used as secondary antibodies at a dilution of 1:10,000. All western blots were visualized using enhanced chemiluminescence (Renaissance ®, Perkin Elmer).

Plasmid and strain constructions

The plasmids utilized in this study are described below and the oligonucleotides are shown in Table 3. The PCR fragments amplified were sequenced with an automated sequencer (ABI 373A) and analyzed with standard bioinformatic tools. To generate the different strains, the constructs indicated were used to transform protoplasts as previously described (Tsukuda et al., 1988). The integration of the plasmids into the corresponding loci was verified by diagnostic PCR and subsequently by Southern blotting.

The *cln1* gene was deleted using the pKOCLN1plasmid. This plasmid was produced by ligating a pair of DNA fragments flanking the *cln1* ORF into pNEB-Cbx (+), a *U.*

maydis integration vector containing a carboxine resistance cassette (Brachmann et al., 2001). The 5' fragment spans from nucleotide -2250 to nucleotide -321 (considering the adenine in the ATG as nucleotide +1) and was produced by PCR of *U. maydis* genomic DNA using the primers CLN1-KO1 and CLN1-KO2. The 3' fragment spans from nucleotide +1314 to nucleotide +1666 and was produced by PCR amplification using the primers CLN1-KO3 and CLN1-KO4. After digestion with *Kpn*I, the pKOCLN1 plasmid was integrated by homologous recombination into the *cln1* locus.

To overexpress the *cln1* gene, the *cln1* open reading frame was first tagged with three copies of the *myc* epitope. A fragment *Nde*I-*Eco*RI carrying the entire *cln1* ORF sequence without the stop codon was obtained by PCR amplification of *U. maydis* genomic DNA with the primers CLN1-N5 and TGCLN1-3 and subsequent digestion with the appropriated enzymes. This fragment was cloned into the plasmid pGNB-myc, a pGEX-2T derivative (Pharmacia Biosciences) that carries three copies of the *myc* epitope (Garrido et al., 2004), permitting the expression of the tagged fusion protein in bacteria. The *cln1-myc* fusion was excised from the resulting plasmid (pGNB-cln1-myc) as a *Nde*I-*Afl*II fragment and sub-cloned into the pRU11 plasmid (Brachmann et al., 2001). The pRU11 plasmid is an integrative *U. maydis* vector that contains the *crg1* promoter (Brachmann et al., 2001) and the resulting plasmid (pRU11-cln1-myc) was linearized and integrated into the *cbx1* locus by homologous recombination.

To overexpress the *cln1-GFP* fusion, the plasmid pRU11-cln1-myc was digested with *Eco*RI and a 1 Kb *Eco*RI fragment from p123 (Spelling et al., 1996) carrying the *gfp* open reading frame was cloned in phase to produce pRU11-cln1-GFP, which was linearized and integrated into the *cbx1* locus by homologous recombination.

To produce the conditional *cln1*^{nar} and *cln1*^{crg} alleles, we ligated two fragments into pRU2 and pRU11, these being *U. maydis* integration vectors containing the promoter of the *nar1* gene (Brachmann et al., 2001) and the promoter of the *crg1* gene (Bottin et al., 1996) respectively. The 5' fragment was produced by PCR using the primers CLN1-KO1 and CLN1-KO2, while the 3' fragment was isolated from pGNB-cln1-myc. The resulting pCLN1nar and pCLN1crg plasmids were linearized and integrated into the *cln1* locus by homologous recombination.

To tag the endogenous *cln1* locus, the plasmid pRU11-cln1-myc was digested with *Afl*II-*Sex*AI to remove the P_{crg1} promoter as well as the 5´half of *cln1* gene. The resulting plasmid, pCLN1-MYC was linearized with *Bst*XI and and integrated into the *cln1* locus by homologous recombination.

To express the *cln1* gene in *S. cerevisiae*, we PCR amplified the *cln1* ORF from *U. maydis* genomic DNA with the primers SCG1-1 and SCG1-2. This PCR fragment was cloned into p426GPD, a centromeric plasmid carrying the *URA3* marker and the promoter of the *GPD1* gene (Mumberg et al., 1995). The plasmid pCM194 contains the *CLN3* gene under its own promoter (Gallego et al., 1997).

Microscopy

A Leica DMLB microscope with phase contrast was used to analyze the material, coupled to a Leica 100 camera. Epifluorescence was observed using standard FITC and DAPI filter sets and images were processed with Photoshop (Adobe). Analysis of GFP fusions was done using a Nikon Eclipse 600 FN microscope coupled to a Hamamatsu ORCA-100 cooled CCD camera, that was controlled by the MetaMorph (Universal Imaging) software. Nuclear staining was carried out with DAPI as

described previously (Garcia-Muse et al., 2003) and WGA staining was performed as described in Castillo-Lluva et al. (2004).

Mating and plant infection

To test for mating, compatible strains were co-spotted on charcoal-containing plates (Holliday, 1974), which were sealed with parafilm and incubated at 21ºC for 48 hours. Pheromone stimulation was performed as described previously (Weber et al., 2003). Synthetic a2 pheromone (a gift of Dr. G. Steinberg) was used at a final concentration of 2,5 ng/ml.

Plant infection was performed as described in Gillissen et al. (1992) using the maize cultivar Early Golden Bantam (Old Seeds, Madison, WI, USA).

Sequence analyses

Protein sequences of fungal G1 cyclins were downloaded from PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). The alignment and phylogenetic dendrograms were constructed using the ClustalW and NJPlot programs (Thompson et al., 1997).

ACKNOWLEDGEMENTS

We thank Prof. M. A. Peñalva (CIB, Madrid) for essential help with fluorescence microscopy; Dr. Gero Steinberg (MPI, Marburg) for the valued gift of synthetic a2 pheromone; Prof. Martí Aldea (Universidad de Lerida) for *S. cerevisiae* strains. We wish to thank the anonymous referees for excellent suggestions, that clearly improved the work. This work was supported by a Grant from the MCyT (BIO2002-

03503) and a Grant from the CAM (07B/0040/2002). Sonia Castillo-Lluva was a recipient of a FPI fellowship from the MCyT.

LEGENDS TO THE FIGURES

Figure 1. Cln1 is a G1-type cyclin.

(A) Dendrogram of G1 cyclins. The tree was reconstructed using the ClustalW method (http://www.ebi.ac.uk/clustalw/). Bar: 0,05 substitutions per aa. *S. cerevisiae* (Sc) cyclins were Cln1 (AAA65724), Cln2 (AAA65725) and Cln3 (CAA32143); *S. pombe* (Sp) Puc1 (CAA18649); *C. albicans* (Ca) Cln1 (CAA56336), Cln3 (EAK96708) and Hgc1 (EAK99984). We also include putative G1 cyclins from two basidiomycetes: *Cryptococcus neoformans* (Cn, Cnd06233) and *Coprinus cinerea* (Cc, ccd00715). *U. maydis* Cln1 (UmCln1) is showed in boldface.

(B) Scheme of the Cln1 protein in relation to the described G1 cyclins from other fungi. The cyclin boxes are shown in black and the percentage values inside each box represent the sequence identity to *U. maydis* Cln1 cyclin box. The putative PEST domains are indicated as hatched boxes. The cyclin boxes and PEST domains were identified using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

(C) Schematic representation of *cln1* gene structure, showing the *cln1* ORF and the upstream open reading frames (uORF). Relative positions of nucleotide sites including the 5´end of the mRNA and of the predicted uORF are indicated and numbered with respect to the initiation codob of in *cln1* coding sequences.

Figure 2. Characterization of the Cln1 protein.

(A) Levels of *cln1* expression at different stages of the cell cycle. RNA extracted from wild-type FB1 cells arrested at S phase, arrested at M phase, enriched in G1 phase, or growing asynchronously (As) was analyzed by Northern blotting. The filters were hybridized with probes for *cln1*, *clb1* and 18s *rRNA* as a loading control.

(B) Cln1 protein levels at different stages of the cell cycle. Protein extracts from SONU59 (*a1 b1 cln1-myc*) cultures arrested at S phase, arrested at M phase, enriched in G1 phase, or growing asynchronously (As) were immunoprecipitated with anti-MYC antibodies and the precipitates were separated by SDS-PAGE and immunoblotted with anti-MYC to detect Cln1-myc.

(C) Cln1 associates with Cdk1. Lysates prepared from wild-type FB1 cells (*cln1*) and SONU58 cells expressing a myc-tagged version of Cln1 (*cln1-myc*) were incubated with Suc1 beads to pull down Cdk1 (left panel), or the lysates were immunoprecipitated with anti-MYC antibodies to pull down Cln1-myc (right panel). The whole cell lysates, as well as the precipitates, were separated by SDS-PAGE and immunoblotted with anti-PSTAIRE and anti-MYC to detect Cdk1 and Cln1-myc respectively.

(D) *U. maydis cln1* supports the growth of an *S. cerevisiae* strain without endogenous G1 cyclins. The yeast strain BF305-15#21 (*cln1*Δ, *cln2*Δ, and *GAL1-CLN3*) was transformed with a control plasmid (vector), a plasmid expressing *S. cerevisiae CLN3* (*CLN3*) or a plasmid expressing *U. maydis cln1* (*cln1*). The resulting strains were spotted in solid medium containing 2% galactose or glucose as a carbon source, and they were incubated at 30ºC for 2 days.

(E) Liquid cultures of the above strains were grown in minimal medium with 2% galactose to mid-exponential phase and then were transferred to minimal medium with 2% glucose and incubated for 6 hours. Note that some of the *cln1*-expressing cells exhibited an elongated morphology.

Figure 3. Cln1 is required for normal morphogenesis and cell separation.

(A) Morphology of Δ*cln1* cells. Wild-type (FB1) and Δ*cln1* (SONU76) cells grown in CMD medium until the exponential phase were observed by phase contrast and by epifluoresence to visualize the DAPI staining (Bar 10 μm). Arrowheads indicated cells carrying more than one nucleus.

(B) Chitin distribution in Δ*cln1* cells. Calcofluor (CFW) detects chitin at cell tips in wild-type FB1 cells while in SONU76 cells, it stains the septa (Bar 10 μm).

Figure 4. Cln1 regulates G1 length

(A) Levels of *cln1* mRNA in the *cln1*^{nar} conditional strain. Cultures of wild-type FB1 (WT) and conditional strain SONU64 (*cln1*^{nar}) were grown under permissive conditions (minimal medium with nitrate as nitrogen source, MM-NO_3) to an OD_{600} of 0.2 and shifted to permissive (MM-NO_3) or restrictive (complete medium, CMD) conditions for 4 hours. The RNA was then extracted and analysed by Northern blotting using a probe for *cln1* and for 18s *rRNA* as a loading control.

(B) Morphology of conditional *cln1* mutant cells at different times after transfer to restrictive conditions (CMD; Bar 10 μm). Inset shows a wild-type cell at same magnification.

(C) Analysis of G1 length in the absence of Cln1. Wild-type and *cln1*^{nar}cells were grown under permissive conditions (MM-NO_3) to an OD_{600} of 0.2 and then shifted to CMD medium amended with benomyl for 90 minutes. Cells arrested at G2/M transition were released in benomyl-free CMD medium, and samples were taken at the indicated times. FACS analysis was obtained from these samples.

Figure 5. Ectopic expression of *cln1* induces polar growth.

(A) Northern analysis of wild-type FB1 cells and SONU58 cells expressing an ectopic copy of *cln1-myc* under the control of the *crg1* promoter. Cells were grown under repressing conditions (complete medium with glucose, CMD) to an OD_{600} of 0.2 and then shifted to inducing conditions (complete medium with arabinose, CMA). Samples were taken at the times indicated. Note the different migration of the mRNA species generated from the native *cln1* promoter (*cln1* arrowhead) and the *crg1-cln1* fusion (*crg:cln1* arrowhead) as a result of the different transcriptional start point.

(B) Cln1 protein levels in SONU59 cells growing under inducing conditions (complete medium with arabinose, CMA). Protein extracts obtained at the time indicated after shift to inducing conditions were separated by SDS-PAGE and immunoblotted with anti-MYC and anti-PSTAIRE to detect Cln1-myc and Cdk1 as loading control. Cdk2 is the homolog of *S. cerevisiae* Pho85 that is also recognized with anti-PSTAIRE.

(C) Time course of the morphological changes in SONU58 cells grown in inducing conditions. Note that after 24 hours, the cells form filaments carrying several nuclei (arrow; Bar 20 μm).

(D) Flow cytometry analysis of SONU58 cells (P*crg:cln1*) expressing high levels of *cln1* using wild-type FB1 cells as controls. Samples were taken for FACS analysis at the times indicated after transfer to induction conditions (complete medium with arabinose).

(E) Calcofluor staining of SONU58 cells after 24 hours of growth in inducing conditions. Bottom image is an Photoshop inverted image to show more clear the septum positions (asterisks). Bar 40 μm.

Figure 6. Sub-cellular location of Cln1-GFP.

(A) SONU84 cells expressing a Cln1-GFP fusion were grown for 2 hours in inducing conditions (CMA). Cln1-GFP localizes in the nucleus (Bar 20 μm)

(B) Location of Cln1-GFP dots around the hyphal tip. Single dots are marked with arrowheads. Because the different fluorescence intensity threshold between the nucleus and the rest of the cell, only the tip is showed. Bar 10 μm.

Figure 7. Cln1 represses sexual development.

(A) Effects of the *cln1*^{crg} allele on the expression of the pheromone gene *mfa1* are analyzed by Northern blot. SONU73 cells were grown on YEP-based and complete media amended with glucose (YPD, CMD) or arabinose (YPA, CMA) as carbon source until mid exponential phase. The filter was hybridized in succession with probes for *cln1*, *mfa1* and 18s *rRNA*, as indicate at left.

(B) Time course induction of *cln1* gene and its effect on *mfa1* expression. SONU73 cells growing in glucose-containing complete medium were washed three times, transferred to arabinose-containing complete medium, and samples were obtained at the indicated times (in hours). RNA was isolated and submitted to Northern blot analysis as above.

(C) Relationships between *cru1* and *cln1* in pheromone gene expression. Wild-type cells (FB1), the single Δ*cru1* (UMP7) and Δ*cln1* (SONU76) mutants and the double Δ*cru1* Δ*cln1* mutant (SONU79) were grown in CMD to mid-exponential phase. The RNA was then extracted and analysed by Northern blotting using a probe for *mfa1*, *cru1*, *cln1* and for 18s *rRNA* as a loading control.

Figure 8. The mRNA levels of *cln1* are regulated by cAMP/PKA and MAPK pathways.

(A) Levels of *cln1* mRNA in different signaling mutants. Total RNA from the strains indicated on the top (WT, FB1; Δ*adr1*, SONU24; Δ*ubc1*, UME18; Δ*fuz7*, FB1Δfuz7) growing in CMD until mid-exponential phase was isolated and subjected to Northern analysis with the probes indicated on the left.

(B) *cln1* is repressed by the MAPK cascade. SONU8 cells carrying an ectopic copy of $fuz7^{DD}$, a constitutive allele of the Fuz7 MAPK kinase under the control of P*crg1* promoter were grown to an OD_{600} of 0.2 in non-inducing conditions (CMD) and then shifted to non-inducing conditions (CMD) and inducing conditions (CMA) for six hours. A derivative strain (SONU34) without the *prf1* gene was used to assess the putative implication of this transcriptional regulator in the down-regulation of *cln1* expression.

Figure 9. Induced expression of *prf1* bypasses the cyclin-mediated repression.

(A) Expression of *prf1* is under Cln1 and Cru1 control. Wild-type, SONU73 ($cln1^{crg}$) and UMP7 (Δ*cru1*) cells were grown until mid-exponential phase in the indicated media and submitted to Northern analysis with a *prf1* probe and 18s *rRNA* as a loading control.

(B) Induced expression of *prf1* bypasses the Cru1 requirement for *mfa1* expression. Cells carrying a $prf1^{nar}$ conditional allele, with (SONU27) or without a functional *cru1* gene (SONU38), were grown in inducing ($MMNO_3$) and non-inducing conditions ($MMNH_4$) for $prf1^{nar}$ expression until mid-exponential phase. The RNA was then extracted and analysed by Northern blotting using a probe for *mfa1* and for 18s *rRNA* as a loading control.

(C) Effects of the $prf1^{nar}$ allele on the expression of the pheromone gene *mfa1* in strains overexpressing *cln1*. Strains SONU27 ($prf1^{nar}$) and SONU115 ($prf1^{nar}$ $cln1^{crg}$)

were grown in the conditions indicated until mid-exponential phase, and isolated RNA was submitted to Northern blot to detect *mfa1* transcripts.

Figure 10. Cells lacking Cln1 are impaired in cell-cell fusion

(A) Effects of absence of *cln1* in formation of infective filaments. Mixtures of compatible wild-type (FB1 and FB2), wild-type and *cln1* mutant (SONU76 and FB2), and mutant (SONU76 and SONU75) strains (top row) or the solopathogenic strain SG200 and its Δ*cln1* derivate (SONU130) were spotted in CM-charcoal plates. The appearance of white infective filaments was assayed after 24 hours incubation at room temperature.

(B) Assay for a1 pheromone production in mutant cells. 10^4 cells from tester strain FBD12-17 were co-spotted with the indicated amount of cells of wild-type (FB1) and Δ*cln1* mutant (SONU 76) strains in CM-charcoal plates. Formation of infective filaments was scored after 24 hours at room temperature.

(C) Formation of conjugative tubes in wild-type (FB1) and Δ*cln1* mutant (SONU 76) cells after incubation in CMD amended with 2,5 ng/ml synthetic a2 pheromone. A total of 100 cells were counted in each assay.

(D) Morphology of conjugative tubes in FB1 wild-type and Δ*cln1* mutant (SONU 76) cells. Cells were incubated as above during 8 hours. Note that Δ*cln1* mutant cells are actually composed for more than one cell compartment, explaining the presence of more than one tube per cell.

Figure 11. Model of the negative relationships between the cell cycle and the virulence program in *Ustilago maydis*.

An active cell cycle represses the induction of the mating program, while the induction of the mating program arrests the cell cycle. Cell cycle transmits the negative information to the sexual program most likely via Cdk1-cyclin complexes that negatively regulate the transcription of *prf1*, the master sexual regulator. Environmental information is transmitted via cAMP/PKA and MAPK pathways to the different elements. Dashed arrows indicate putative interactions that remain to be identified (see discussion).

Table 1. Pathogenicity assays

Inoculum	Genotype	Anthocyanin formation		Tumor formation	
		Total	Percentage	Total	Percentage
FB1 x FB2	*a1 b1* x *a2 b2*	57/60	95	57/60	95
SONU76 x SONU75	*a1 b1*Δ*cln1*x *a2 b2* Δ*cln1*	0/74	0	0/74	0
SG200	*a1mfa2 bW2bE1*	46/52	88	43/52	82
SONU130	*a1mfa2 bW2bE1* Δ*cln1*	72/77	93	70/77	90

Table 2. Yeast strains used in this study

Strain	Relevant genotype	Reference
***U. maydis* strains**		
FB1	*a1 b1*	Banuett and Herskowitz,1989
FB2	*a2 b2*	Banuett and Herskowitz,1989
FBD11	*a1a2 b1b2*	Banuett and Herskowitz,1989
FBD12-17	*a2a2 b1b2*	Banuett and Herskowitz,1989
FB1Δfuz7	*a1 b1 Δfuz7*	Müller et al., 2003
UME18	*a1 b1 Δubc1*	Garrido and Pérez-Martín, 2003
UMP7	*a1 b1 Δcru1*	Castillo-Lluva et al., 2004
SG200	*a1mfa2 bW2bE1*	Bölker et al., 1995
SONU8	*a1 b1* P_{crg}*:fuz7*^{DD}	This work
SONU24	*a1 b1 Δadr1*	Garrido and Pérez-Martín, 2003
SONU27	*a1 b1 prf1*^{nar}	Garrido et al., 2004
SONU34	*a1 b1* P_{crg}*:fuz7*^{DD} *Δprf1*	This work
SONU38	*a1 b1 prf1*^{nar} *Δcru1*	This work
SONU58	*a1 b1* P_{crg}*:cln1-myc*	This work
SONU59	*a1 b1 cln1-myc*	This work
SONU64	*a1 b1 cln1*^{nar}	This work
SONU68	*a1a2 b1b2 cln1/Δcln1*	This work
SONU73	*a1 b1 cln1*^{crg}	This work
SONU75	*a2 b2 Δcln1*	This work
SONU76	*a1 b1 Δcln1*	This work
SONU79	*a1 b1 Δcln1 Δcru1*	This work
SONU84	*a1 b1* P_{crg}*:cln1-GFP*	This work
SONU115	*a1 b1 prf1*^{nar} *cln1*^{crg}	This work
SONU130	*a1mfa2 bW2bE1 Δcln1*	This work
***S. cerevisiae* strains**		
BF305-15#21	*cln1*::*HIS3 cln2*::*TRP1 GAL1-CLN3*	Tyers et al. 1993
SCS1	*cln1*::*HIS3 cln2*::*TRP1 GAL1-CLN3/* p426-GPD	This work
SCS2	*cln1*::*HIS3 cln2*::*TRP1 GAL1-CLN3/* pCM194	This work
SCS3	*cln1*::*HIS3 cln2*::*TRP1 GAL1-CLN3/* pCLN1	This work

Table 3. Oligonucleotides used in this study

Primer	Sequence
CLN1-KO1	5'CCCGGTACCGACACCATCGCTGCATGA3'
CLN1-KO2	5'CCGAATTCTTGAGATCGATGAGGGCCTTCCA3'
CLN1-KO3	5'CCCGAATTCTCTCTTTATTCATTGCAC3'
CLN1-KO4	5'CCCGGTACCGCCAGAATGCTAATCGAG3'
CLN1-N5	5'TCGCCATATGACCGCCCTCTGTCAGCTCGA3'
TGCLN1-3	5'GCGAATTCGAACTGCTGGACATTAACG3'
SCG1-1	5'GGACTAGTTTTCCACCCCATCTCAACG3'
SCG1-2	5'CGGAATTCGAGAGAGAAAGATGCTTGC3'

REFERENCES

Andrews, D.L., Egan, J.D., Mayorga, M.E., and Gold, S.E. (2000). The *Ustilago maydis ubc4* and *ubc5* genes encode members of a MAP kinase cascade required for filamentous growth. Mol. Plant-Microbe Interact. **13,** 781-786.

Ayte, J., Schweitzer, C., Zarzov, P., Nurse, P. and deCaprio, J.A. (2001). Feedback regulation of the MBF transcription factor by cyclin Cig2. Nat. Cell Biol. **3,** 1043-1050.

Bachewich, C., and Whiteway M. (2005). Cyclin Cln3p links G1 progression to hyphal and pseudohyphal development in *Candida albicans*. Eukaryot. Cell **4,** 95-102.

Banuett, F. and Herskowitz, I. (1989). Different *a* alleles are necessary for maintenance of filamentous growth but not for meiosis. *Proc. Natl. Acad. Sci. USA* **86,** 5878-5882.

Banuett, F. and Herskowitz, I. (1994). Identification of *fuz7*, a *Ustilago maydis* MEK/MAPKK homolog required for *a*-locus-dependent and –independent steps in the fungal life cycle. Genes Dev. **8,** 1367-1378.

Banuett, F. and Herskowitz, I. (1996). Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*. Development **122,** 2965-2976.

Basse, C.W., and Steinberg, G. (2004). *Ustilago maydis*, model system for analysis of the molecular basis of fungal pathogenicity. Mol. Plant Pathol. **5,** 83-92.

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

Bölker, M., Genin, S., Lehmler, C., and Kahmann, R. (1995). Genetic regulation of mating and dimorphisms in *Ustilago maydis*. Can. J. Bot. **73,** 320-325.

Bottin, A., Kämper, J. and Kahmann, R. (1996). Isolation of a carbon source-regulated gene from *Ustilago maydis*. Mol. Gen. Genet. **253,** 342-352.

Brachmann, A., Weinzierl, G, Kämper, J. and Kahmann, R. (2001). Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis*. Mol. Microbiol. **42,** 1047-1063.

Brefort, T., **Mülller, P. and Kahmann, R.** (2005). The High-Mobility-Group domain transcription factor Rop1 is adirect regulator of *prf1* in *Ustilago maydis*. Eukaryot. Cell 4, 379-391.

Castillo-Lluva, S., García-Muse, T., and Pérez-Martín, J. (2004). A member of the Fizzy-related family of APC activators is required at different stages of plant infection by *Ustilago maydis*. J. Cell. Sci. **117,** 4143-4156.

Chapa y Lazo, B., Bates, S., and Sudbery, P. (2005). The G1 cyclin Cln3 regulates morphogenesis in *Candida albicans*. Eukaryot. Cell **4,**90-94.

Colomina, N., Garí, E., Gallego, C., Herrero, E. and Aldea, M. (1999). G1 cyclins block the Ime1 pathway to make mitosis and meiosis incompatible in budding yeast. EMBO J. **18,** 320-329.

Cross, F. R. (1988). *DAF1*, a mutant gene affecting size control, pheromone arrest and cell cycle kinetics of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8,** 4675-4684.

Cvrkova, F., and Nasmyth, K. (1993). Yeast G1 cyclins CLN1 and CLN2 and a GAP-like protein have a role in bud formation. EMBO J. **12,** 5277-5286.

Ducommun, B. and Beach, D. (1990). A versatile microtiter assay for the universal cdc2 cell cycle regulator. Anal. Biochem. **187,** 94-97.

Dürrenberger, F., Wong, K., and Kronstad, J.W. (1998). Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis.* Proc. Natl. Acad. Sci. USA **95,** 5684-5689.

Di Como, C.J., Chang, H., and Arndt, K.T. (1995). Activation of *CLN1* and *CLN2* cyclin gene expression by BCK2. Mol. Cell. Biol. **15,** 1835-1846.

Epstein, C.B., and Cross, F.R. (1994). Genes that can bypass the *CLN* requirement for *Saccharomyces cerevisiae* cell cycle START. Mol. Cell. Biol. **14,** 2041-2047.

Forsburg, S. L. and Nurse, P. (1991). Identification of a G1-type cyclin *puc1*+ in the fission yeast *Schizosaccharomyces pombe*. Nature **351,** 245-248.

Forsburg, S. L. and Nurse, P. (1994). Analysis of the *Schizosaccharomyces pombe* cyclin puc1: evidence for a role in cell cycle exit. J. Cell Sci. **107,** 601-613.

Fuchs, U., Manns, I., and Steinberg, G. (2005). Microtubules are dispensable for the initial pathogenic development but required for long-distance hyphal growth in the corn smut fungus *Ustilago maydis*. Mol. Biol. Cell **16,** 2746-2758.

Gallego, C., Garí, E., Colomina, N., Herrero, E. and Aldea, M. (1997). The Cln3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast. EMBO J. **16,** 7196-7206.

García-Muse, T., Steinberg, G. and Pérez-Martín, J. (2003). Pheromone-induced G2 arrest in the phytopathogenic fungus *Ustilago maydis*. Eukaryot. Cell **2,** 494-500.

García-Muse, T., Steinberg, G. and Pérez-Martín, J. (2004). Characterization of B-type cyclins in the smut fungus *Ustilago maydis*: roles in morphogenesis and pathogenicity. J. Cell Sci. **117,** 487-506.

Garrido, E. and Pérez-Martín, J. (2003). The *crk1* gene encodes an Ime2-related protein that is required for morphogenesis in the plant pathogen *Ustilago maydis*. Mol. Microbiol. **47,** 729-743.

Garrido, E., Voß, U., Müller, P., Castillo-Lluva, S., Kahmann, R. and Pérez-Martín, J. (2004). The induction of sexual development and virulence in the smut fungus *Ustilago maydis* depends on Crk1, a novel MAPK protein. Genes Dev. **18,** 3117-3130.

Gillissen, B., Bergemann, J., Sandmann, C., Schrör, B., Bölker, M. and Kahmann, R. (1992). A two-component regulatory system for self/non-self recognition in *Ustilago maydis*. Cell **68,** 647-657.

Gold, S.E., Duncan, G., Barrett, K., and Kronstad, J.W. (1994). cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis.* Genes Dev. **8,** 2805-2816.

Hartmann, H.A., Kahmann, R., and Bölker, M. (1996). The pheromone response factor coordinates filamentous growth and pathogenic development in *Ustilago maydis*. EMBO J. **15,** 1632-1641.

Holliday, R. (1974). *Ustilago maydis*. In *Handbook of Genetics*, (ed. R. C. King) Plenum Press, New York, USA pp. 575-595.

Kaffarnik, F., Müller, P., Leibundgut, M., Kahmann, R., and Feldbrügge, M. (2003). PKA and MAPK phosphorylation of Prf1 allows promoter discrimination in *Ustilago maydis*. EMBO J. **22,** 5817-5826.

Kahmann, R. and Kämper, J. (2004). *Ustilago maydis*: how its biology relates to pathogenic development. New Phytologist **164,** 31-42.

Lew, D. J. and Reed S.I. (1993). Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. J. Cell Biol. **120,** 1305-1320.

Loeb, J.D.J., Kerensteva, T.A., Pan, T., Sepulveda-Becerra, M. and Liu, H. (1999a). *Saccharomyces cerevisiae* G1 cyclins are differentially involved in invasive and pseudohyphal growth independent of the filamentation mitogen-activated protein kinase pathway. Genetics **153,** 1535-1546.

Loeb, J.D.J., Sepulveda-Becerra, M., Hazan, I., and Liu, H. (1999b). A G1 cyclin is necessary for maintenance of filamentous growth in *Candida albicans*. Mol. Cell. Biol. **19,** 4019-4027.

Martín-Castellanos, C., Blanco, M.A., de Prada, J.M. and Moreno, S. (2000). The puc1 cyclin regulates the G1 phase of the fission yeast cell cycle in response to cell size. Mol. Biol. Cell **11,** 543-554.

Mumberg, D., Müller, R. and Funk, M. (1995). Yeast vectors for the controlled expression of heterologous protein in different genetic backgrounds. Gene **156,** 119-122.

Müller, P., Weinzierl, G., Brachmann, A., Feldbrugge, M., and Kahmann, R. (2003). Mating and pathogenic development of the smut fungus *Ustilago maydis* are regulated by one mitogen-activated protein kinase cascade. Eukaryot. Cell **2,** 1187-1199.

Murray, A. and Hunt, T. (1993). The cell cycle. (New York: Oxford University Press).

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

Polymenis, M. and Schmidt, E.V. (1997). Coupling of cell division to cell growth by translational control of the G1 cyclin *CLN3* in yeast. Genes and Dev. **11,** 2522-2531.

Rechsteiner, M. and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem. Sci. **21,** 267-271.

Richardson, H.E., Wittenberg, C., Cross, F., and Reed, S.I. (1989). An essential G1 function for cyclin-like proteins in yeast. Cell **59,** 1127-1133.

Sherlock, G., Bahman, A.M., Mahal, A., Shieh, J.C., Ferreira, M. and Rosamond, J. (1994). Molecular cloning and analysis of CDC28 and cyclin homologues from the human fungal pathogen *Candida albicans*. Mol. Gen. Genet. **245,** 716-723.

Sherman, F., Fink, G.R., and Hicks, J.B. (1986). Laboratory course manual for methods in yeast genetics (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Snetselaar, K.M. and Mims, C.W. (1992). Sporidial fusion and infection of maize seedlings by the smut fungus *Ustilago maydis*. Mycologia **84,** 193-203.

Spellig, T., Bölker, M., Lottspeich, F., Frank, R.W., and Kahmann, R. (1994). Pheromone trigger filamentous growth in *Ustilago maydis*. EMBO J **13,** 1620-1627.

Spellig, T., Bottin, A., and Kahmann, R. (1996). Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. Mol. Gen. Genet. **252,** 503-509.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple alignment aided by quality analysis tools. Nucleic Acid Res. **25,** 4876-4882.

Tsukuda, T., Carleton, S., Fotheringham, S. and Holloman, W.K. (1988). Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. Mol. Cell. Biol. **8,** 3703-3709.

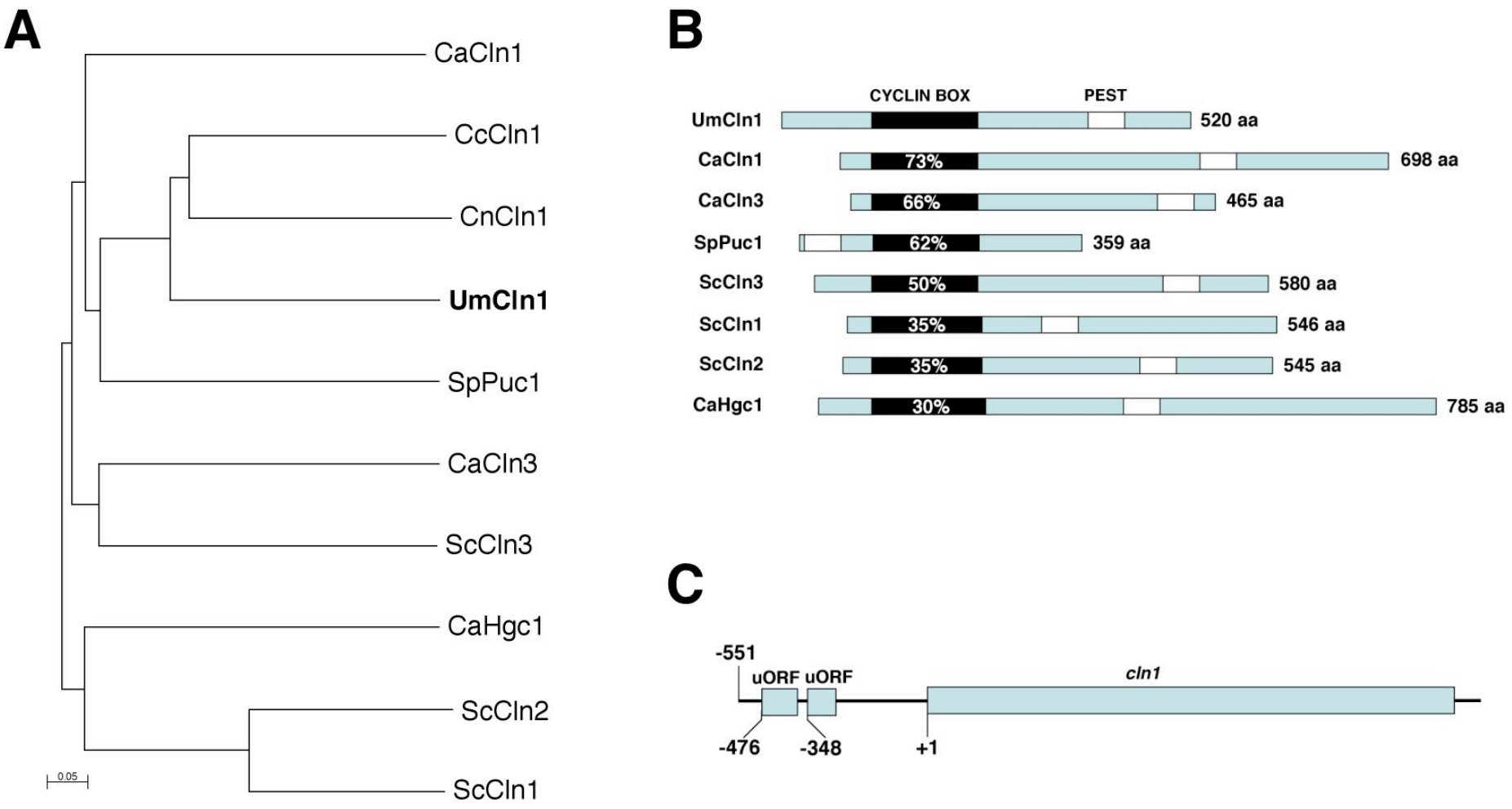
Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992). The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. **11,** 1773-1784.

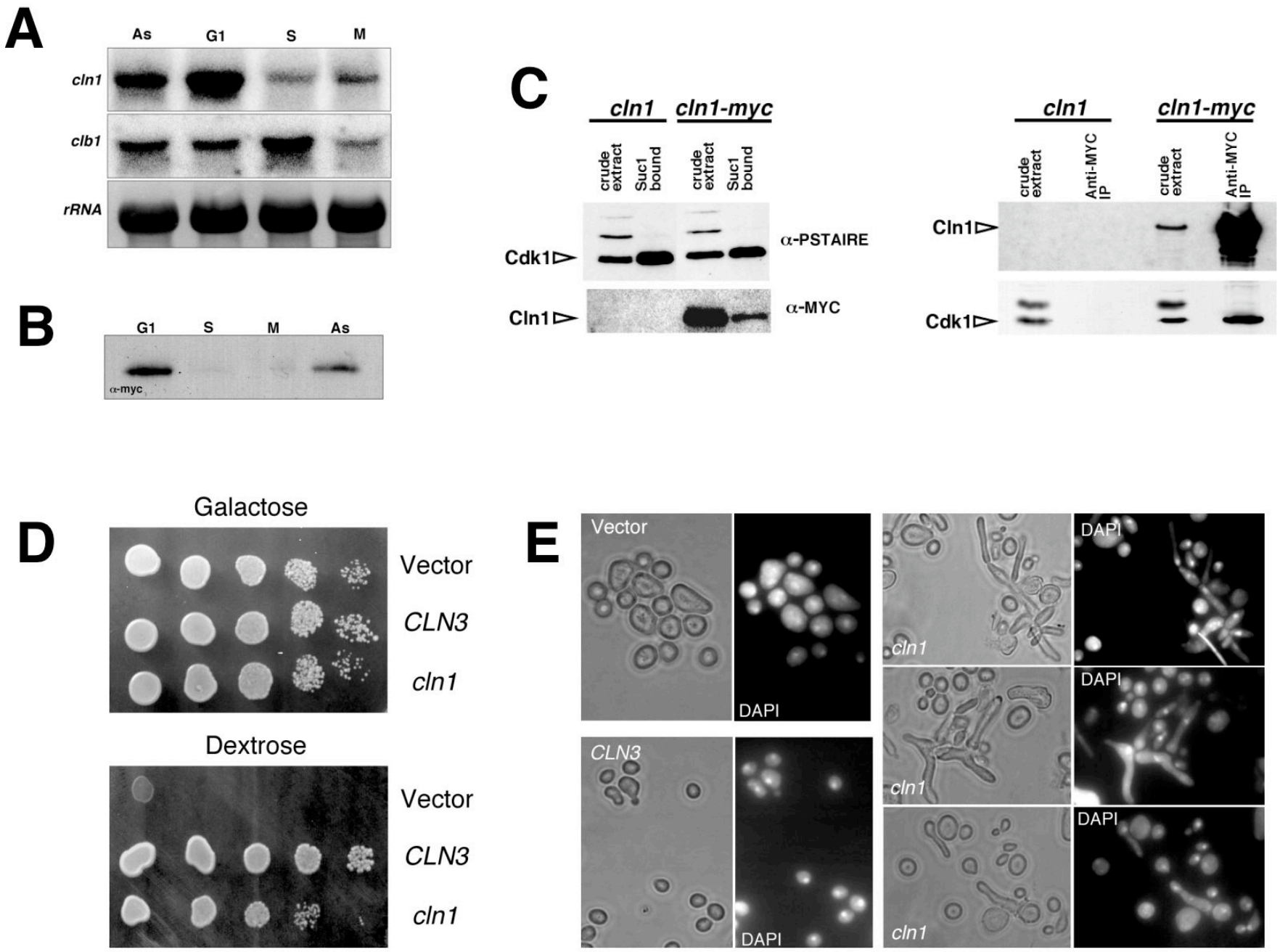
Tyers, M., Tokiwa, G. and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. **12,** 1955-1968.

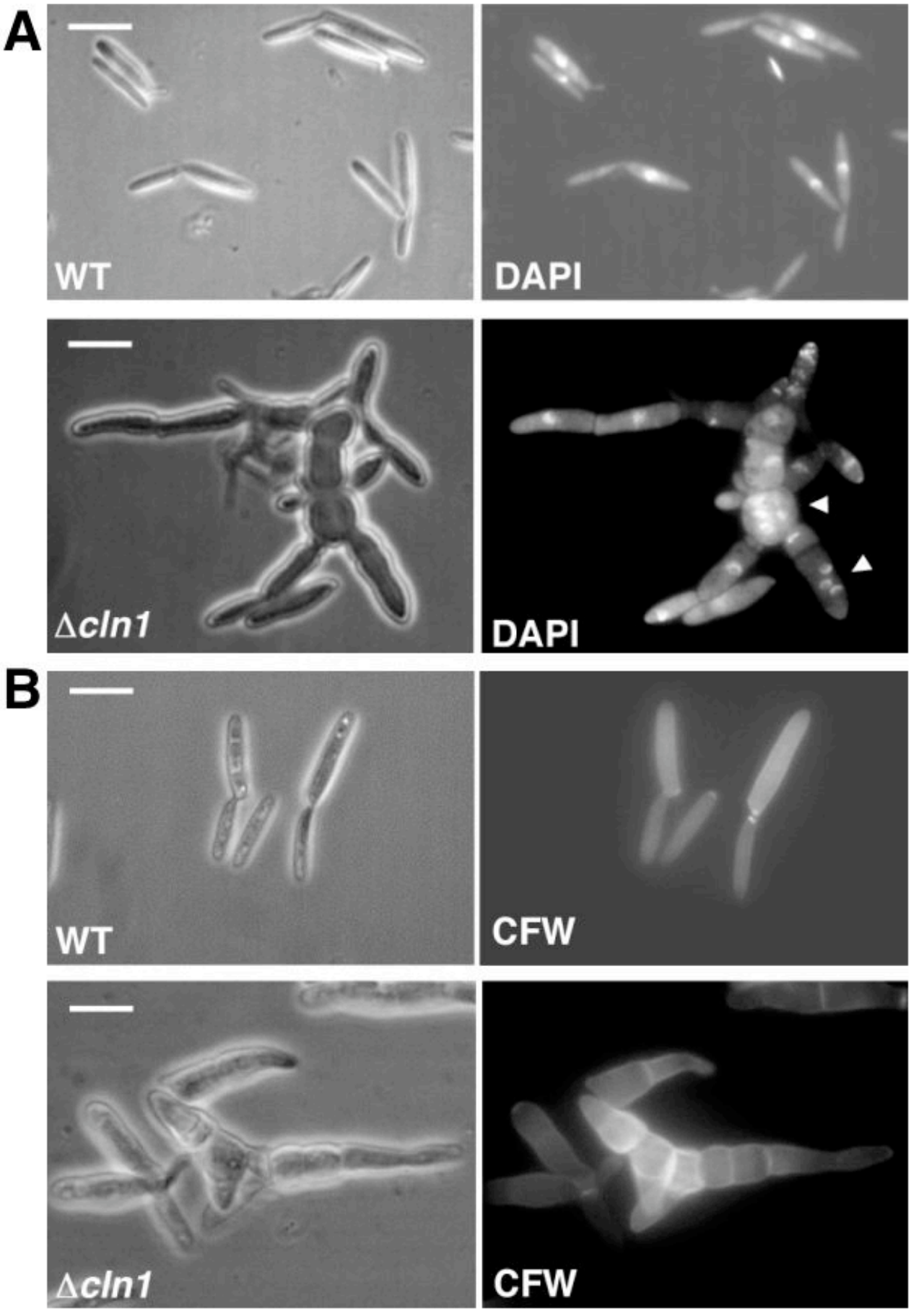
Weber, I., Gruber, C., and Steinberg, G. (2003). A class-V myosin required for mating, hyphal growth, and pathogenicity in the dimorphic plant pathogen *Ustilago maydis*. Plant Cell **15,** 2826-2842.

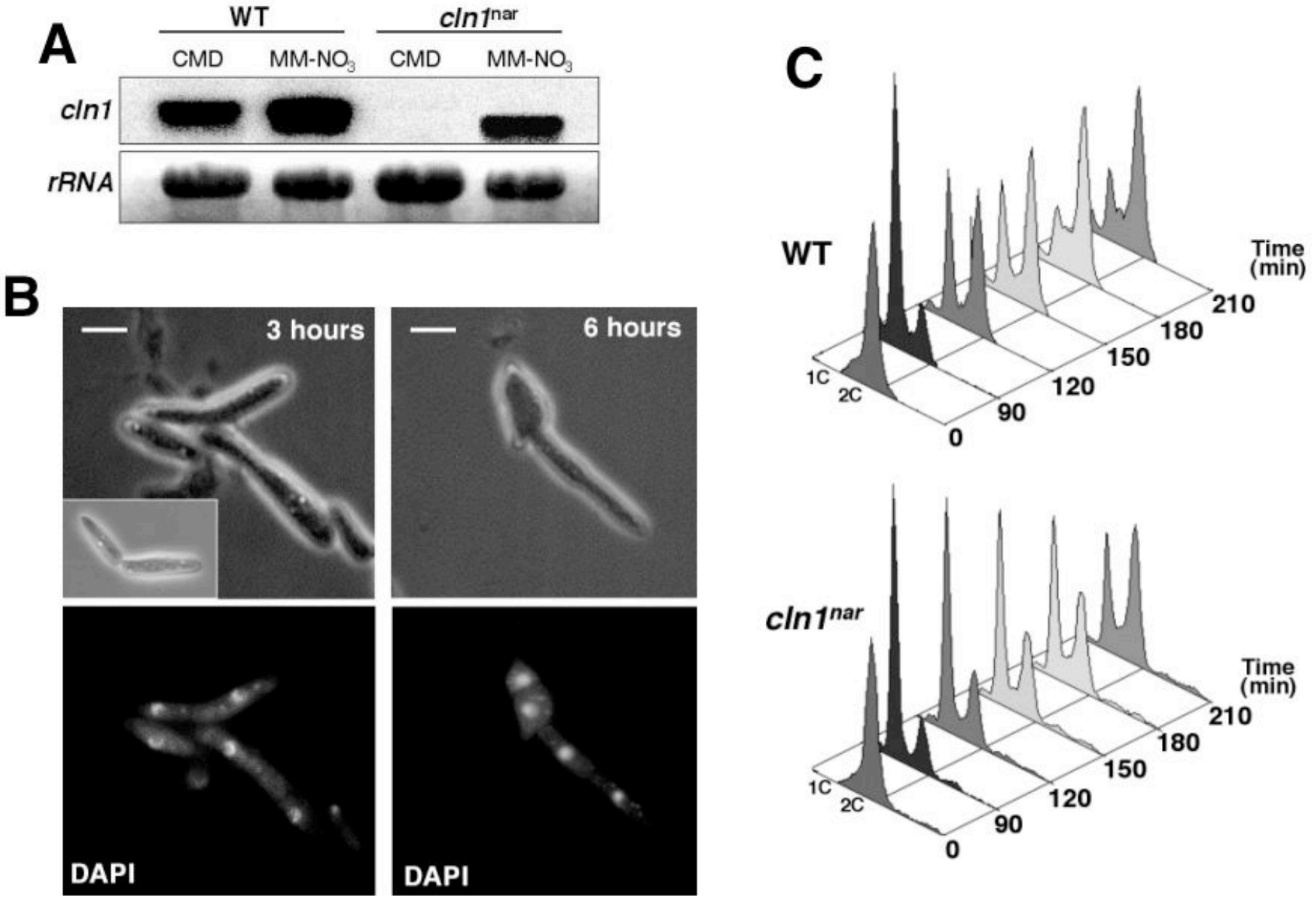
Whiteway, M. D., Dignard, D. and Thomas, D. Y. (1992). Dominant negative selection of heterologous genes: isolation of *Candida albicans* genes that interfere with *Saccharomyces cerevisiae* mating factor-induced cell cycle arrest. Proc. Natl. Acad. Sci. USA **89,** 9410-9414.

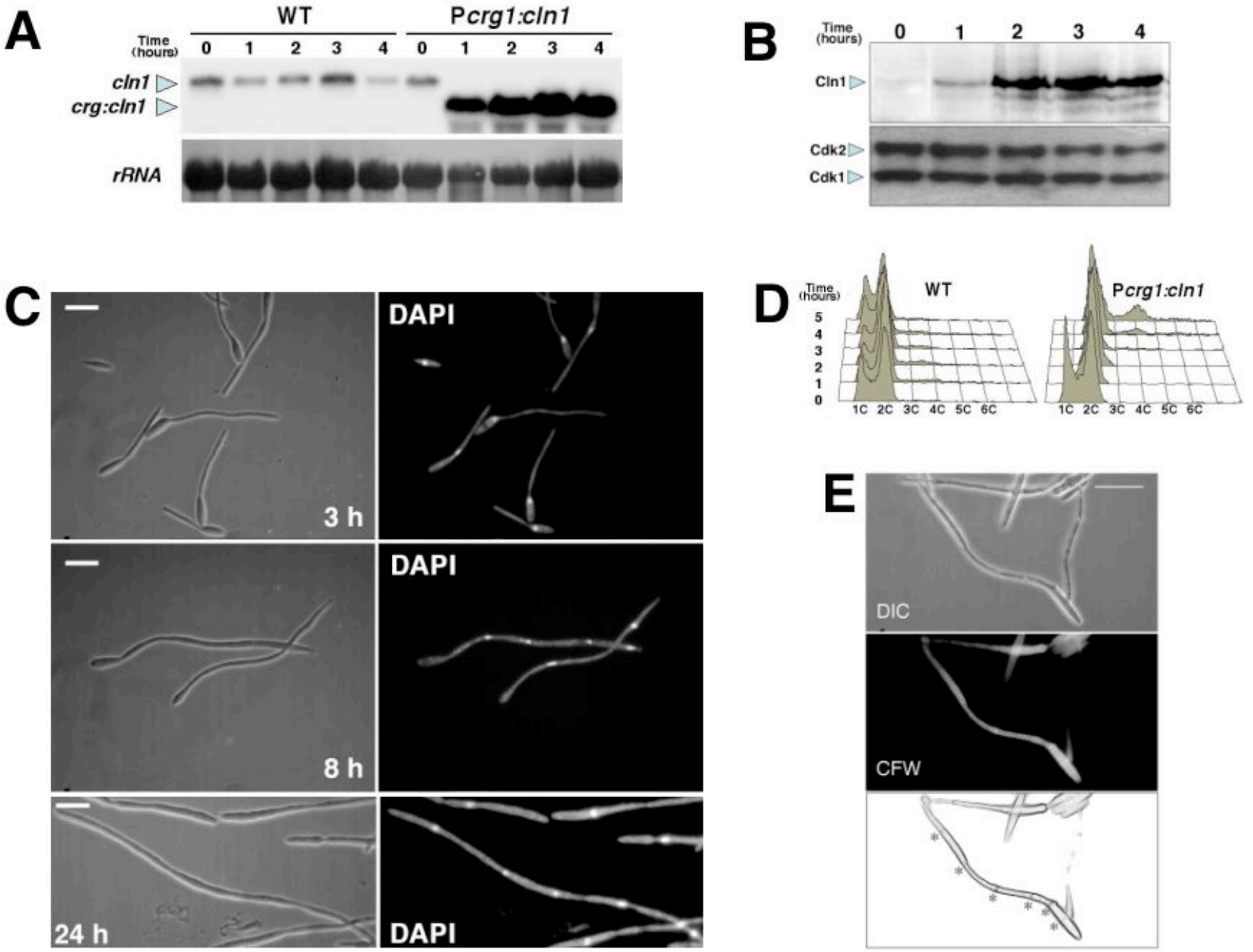
Zheng, X., Wang, Y. and Wang, Y. (2004). Hcg1, a novel hypgha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. EMBO J. **23,** 1845-1856

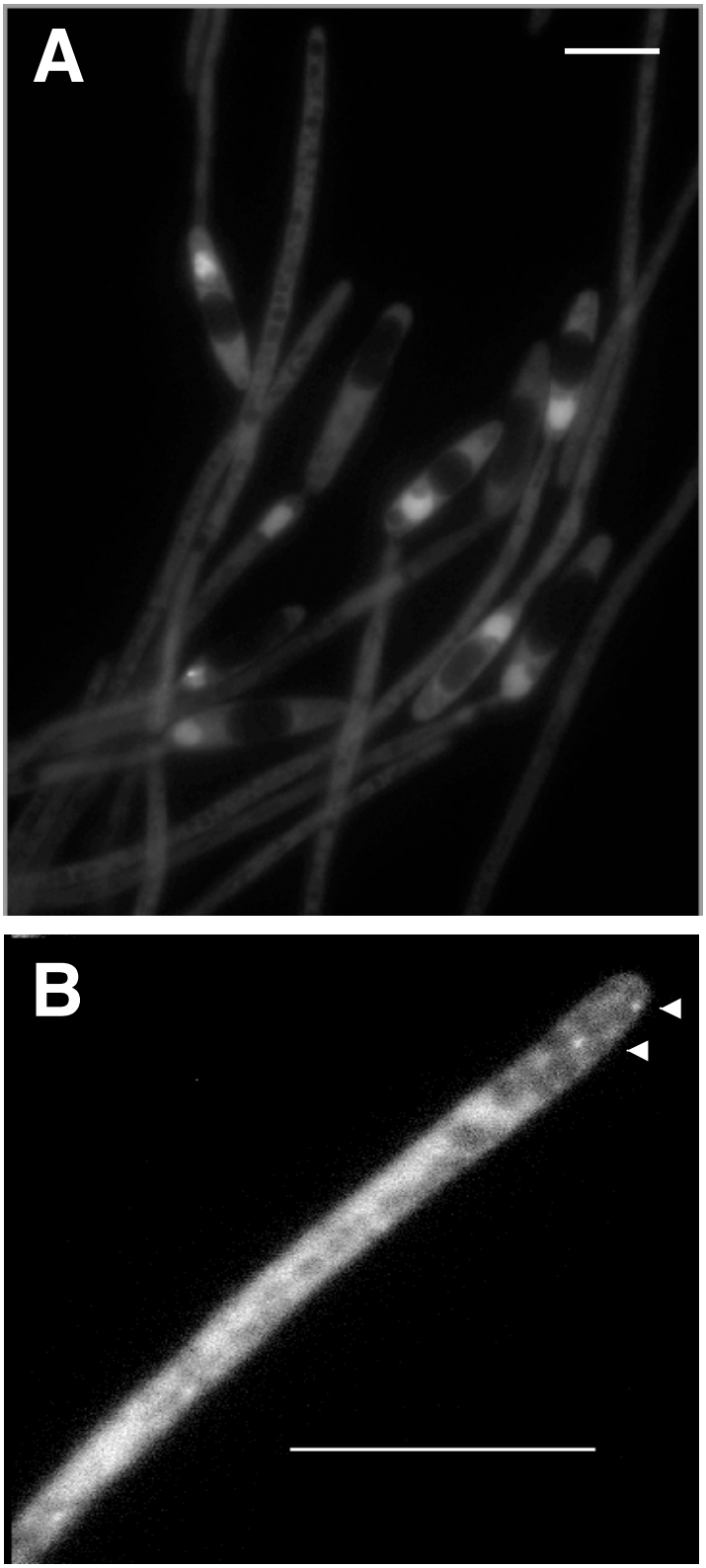

A
CaCln1
CcCln1
CnCln1
UmCln1
SpPuc1
CaCln3
ScCln3
CaHgc1
ScCln2
ScCln1
0.05
B
CYCLIN BOX
PEST
UmCln1
520 aa
CaCln1
73%
698 aa
CaCln3
66%
465 aa
SpPuc1
62%
359 aa
ScCln3
50%
580 aa
ScCln1
35%
546 aa
ScCln2
35%
545 aa
CaHgc1
30%
785 aa
C
-551
uORF uORF
cln1
-476
-348
+1

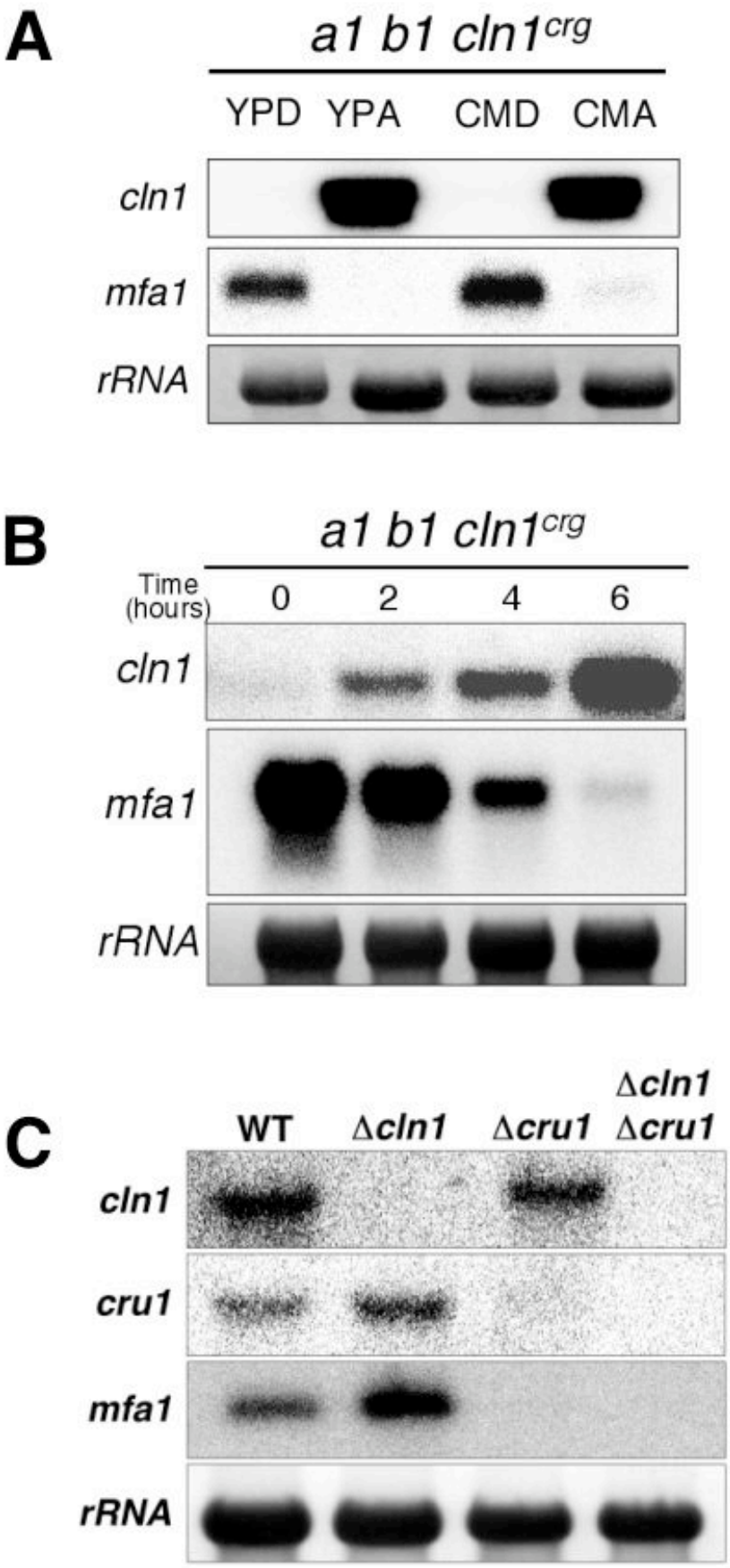

A
As
G1
S
M
cln1
clb1
rRNA
B
G1
S
M
As
α-myc
C
cln1
cln1-myc
crude extract
Suc1 bound
crude extract
Suc1 bound
Cdk1
α-PSTAIRE
Cln1
α-MYC
cln1
cln1-myc
crude extract
Anti-MYC IP
crude extract
Anti-MYC IP
Cln1
Cdk1
D
Galactose
Vector
CLN3
cln1
Dextrose
Vector
CLN3
cln1
E
Vector
DAPI
CLN3
DAPI
cln1
DAPI
cln1
DAPI
cln1
DAPI

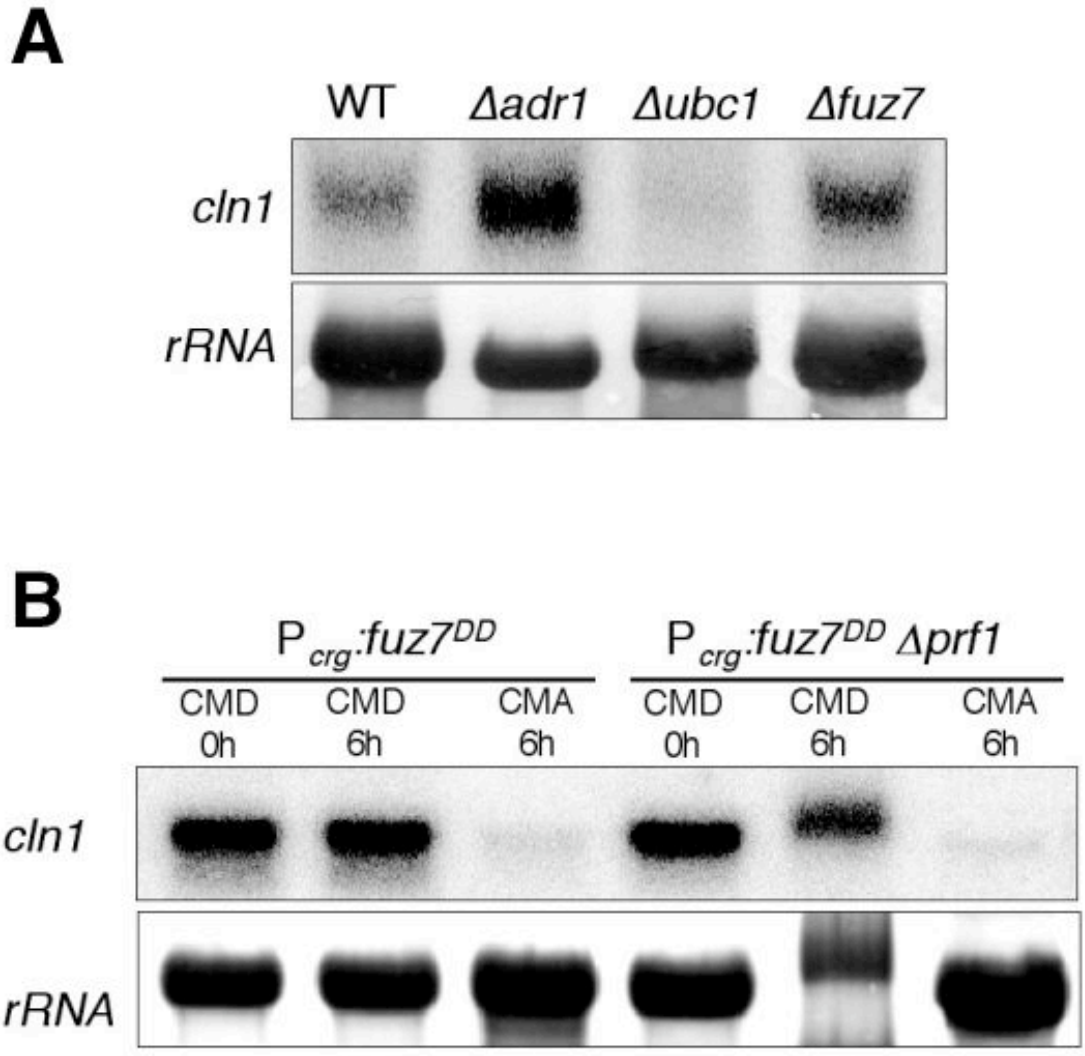

A
WT
DAPI
Δcln1
DAPI
B
WT
CFW
Δcln1
CFW

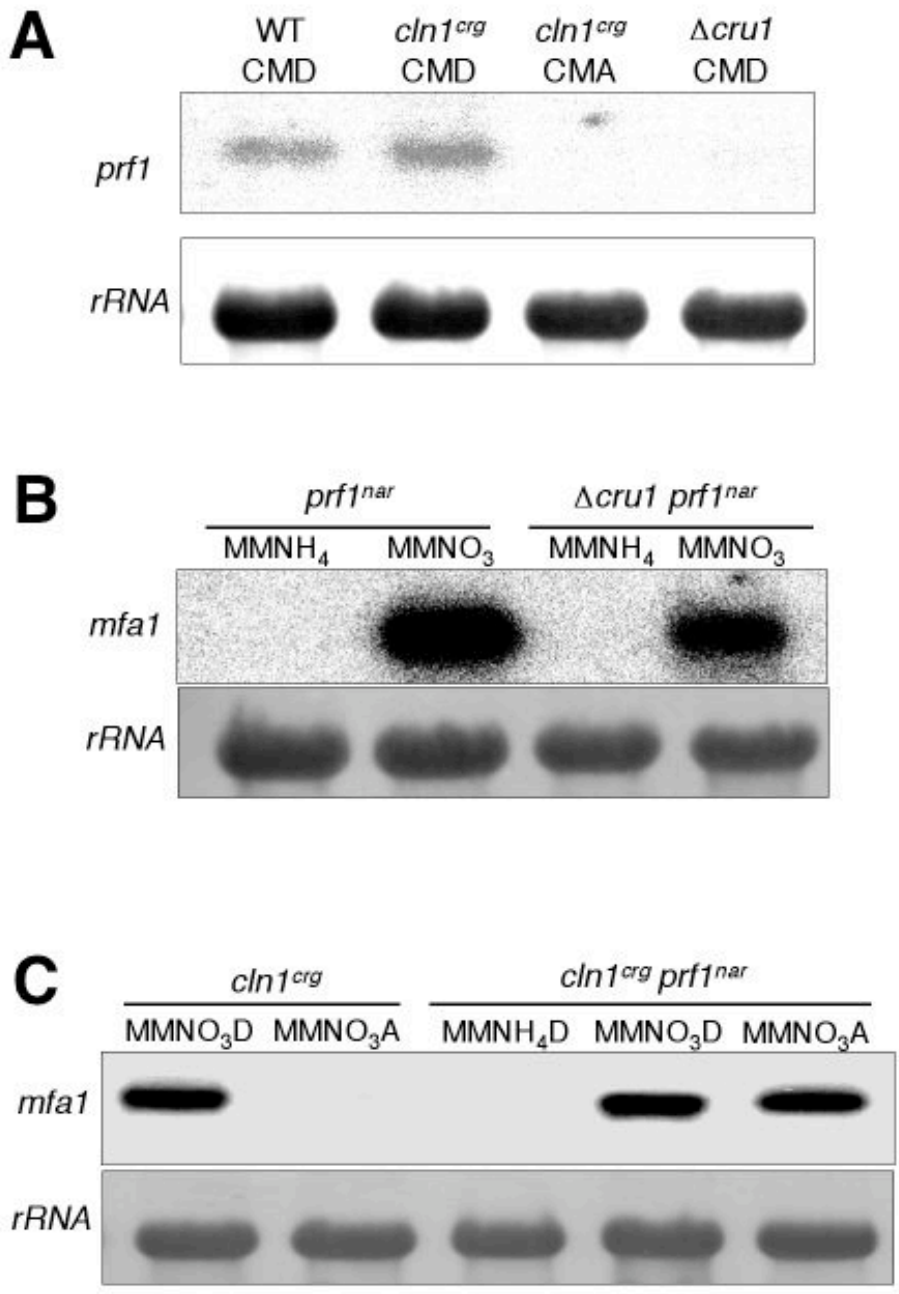

A
WT
cln1nar
CMD
MM-NO3
CMD
MM-NO3
cln1
rRNA
B
3 hours
6 hours
DAPI
DAPI
C
WT
Time (min)
1C
2C
0
90
120
150
180
210
cln1nar
Time (min)
1C
2C
0
90
120
150
180
210

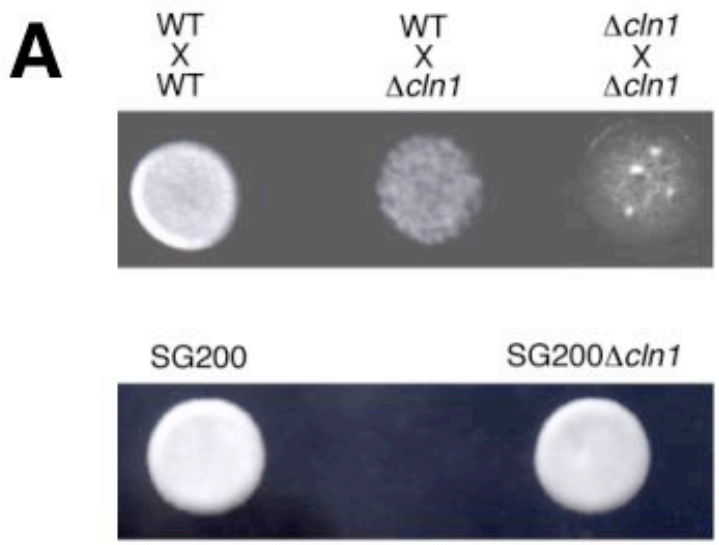

A
WT
Pcrg1:cln1
Time (hours)
0 1 2 3 4 0 1 2 3 4
cln1
crg:cln1
rRNA
B
Time (hours)
0 1 2 3 4
Cln1
Cdk2
Cdk1
C
DAPI
3 h
8 h
24 h
D
Time (hours)
WT
Pcrg1:cln1
5
4
3
2
1
0
1C 2C 3C 4C 5C 6C
E
DIC
CFW

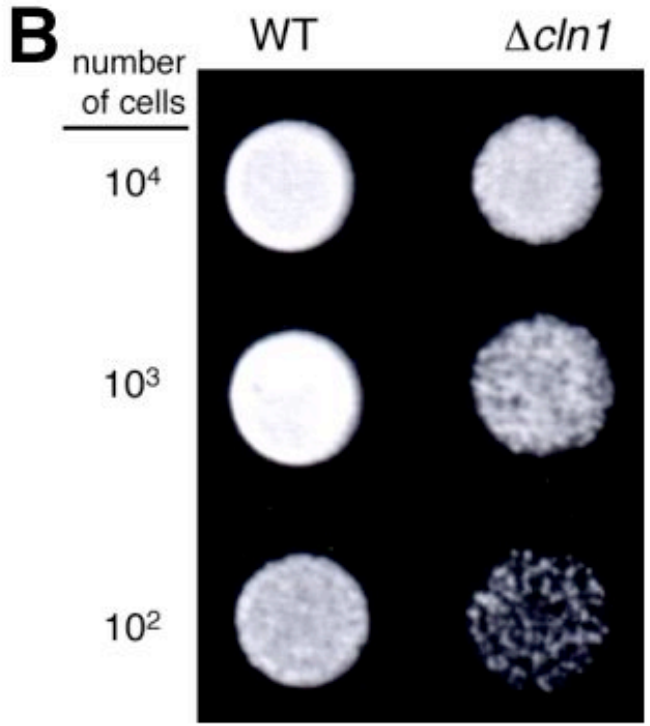

A
B

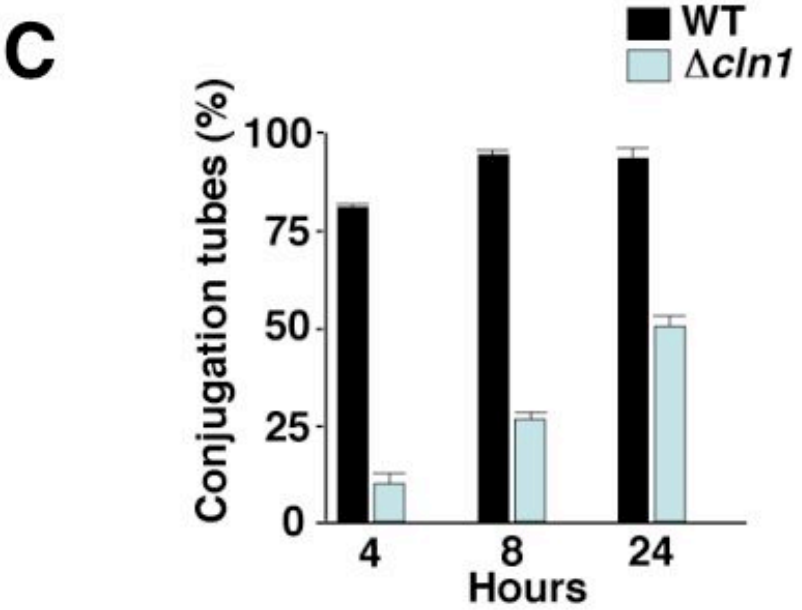

A
a1 b1 cln1crg
YPD
YPA
CMD
CMA
cln1
mfa1
rRNA
B
a1 b1 cln1crg
Time (hours)
0
2
4
6
cln1
mfa1
rRNA
C
WT
Δcln1
Δcru1
Δcln1 Δcru1
cln1
cru1
mfa1
rRNA

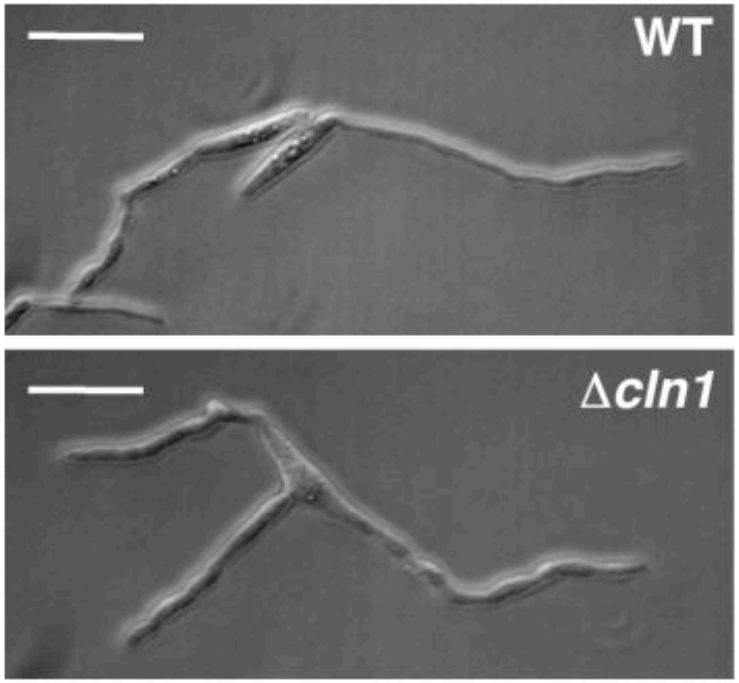

A
WT
Δadr1
Δubc1
Δfuz7
cln1
rRNA
B
P crg:fuz7DD
P crg:fuz7DD Δprf1
CMD 0h
CMD 6h
CMA 6h
CMD 0h
CMD 6h
CMA 6h
cln1
rRNA

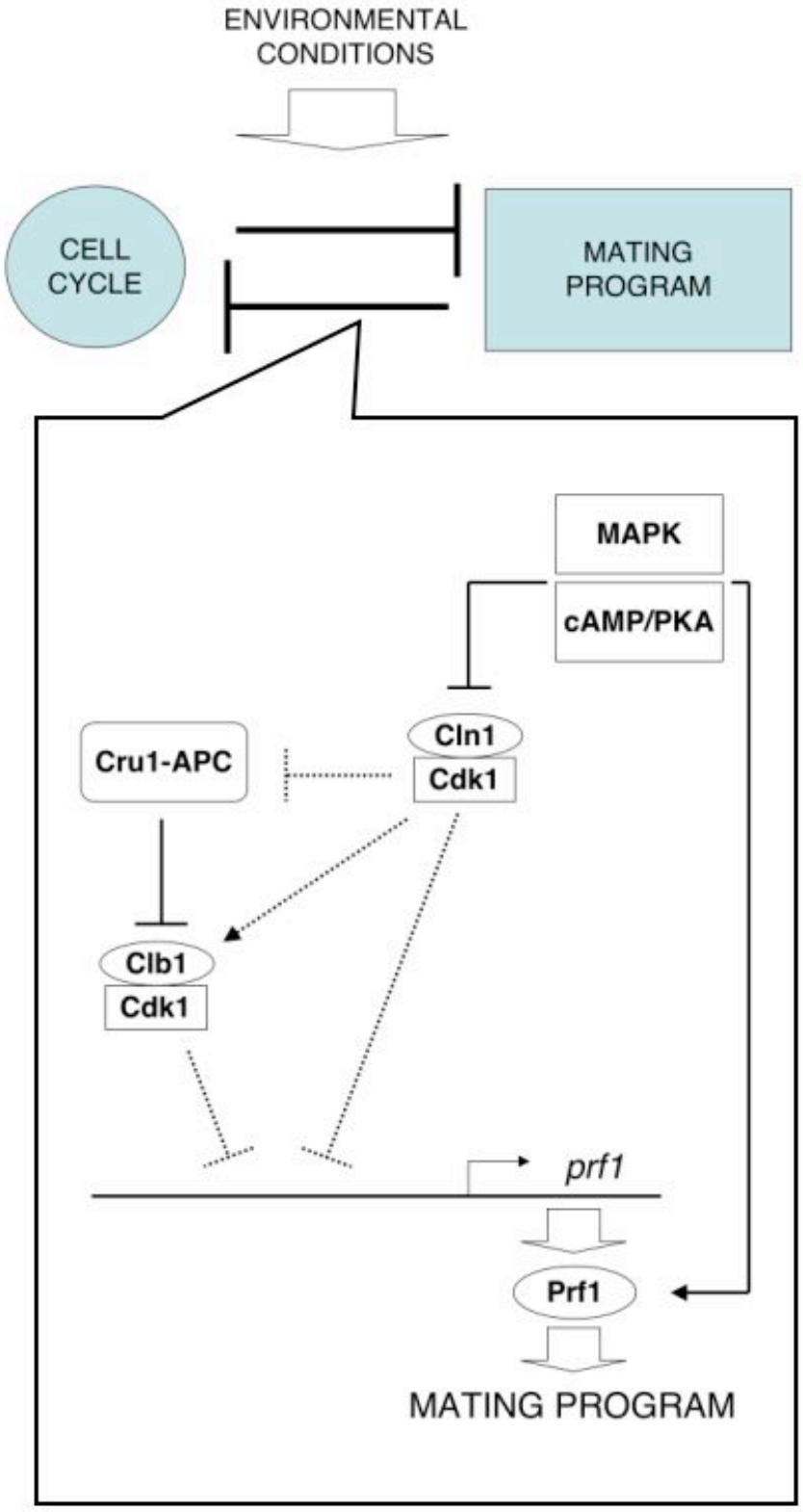

A
WT
CMD
cln1crg
CMD
cln1crg
CMA
Δcru1
CMD
prf1
rRNA
B
prf1nar
Δcru1 prf1nar
MMNH4
MMNO3
MMNH4
MMNO3
mfa1
rRNA
C
cln1crg
cln1crg prf1nar
MMNO3D
MMNO3A
MMNH4D
MMNO3D
MMNO3A
mfa1
rRNA


A
WT X WT
WT X Δcln1
Δcln1 X Δcln1
SG200
SG200Δcln1


B
WT
Δcln1
number of cells
10⁴
10³
10²


C
WT
Δcln1
Conjugation tubes (%)
100
75
50
25
0
4
8
24
Hours


D
WT
Δcln1


ENVIRONMENTAL CONDITIONS
CELL CYCLE
MATING PROGRAM
MAPK
cAMP/PKA
Cln1
Cdk1
Cru1-APC
Clb1
Cdk1
prf1
Prf1
MATING PROGRAM