Mol Gen Genomics (2006) 276:211–229
DOI 10.1007/s00438-006-0152-6

REVIEW

Pathocycles: *Ustilago maydis* as a model to study the relationships between cell cycle and virulence in pathogenic fungi

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Received: 17 May 2006 / Accepted: 5 July 2006 / Published online: 29 July 2006

Abstract Activation of virulence in pathogenic fungi often involves differentiation processes that need the reset of the cell cycle and induction of a new morphogenetic program. Therefore, the fungal capability to modify its cell cycle constitutes an important determinant in carrying out a successful infection. The dimorphic fungus *Ustilago maydis* is the causative agent of corn smut disease and has lately become a highly attractive model in addressing fundamental questions about development in pathogenic fungi. The different morphological and genetic changes of *U. maydis* cells during the pathogenic process advocate an accurate control of the cell cycle in these transitions. This is why this model pathogen deserves attention as a powerful tool in analyzing the relationships between cell cycle, morphogenesis, and pathogenicity. The aim of this review is to summarize recent advances in the unveiling of cell cycle regulation in *U. maydis*. We also discuss the connection between cell cycle and virulence and how cell cycle control is an important downstream target in the fungus-plant interaction.

Introduction

Pathogenic fungi are characterized by a great variability in their lifestyles and the symptoms they cause. This is an important aspect for the search of common targets in antifungal research because it is likely that disease is caused by different attributes in almost each fungus. However, all of them have in common the requirement of accurate developmental decisions for the induction of the pathogenic program (Gow 1995). Developmental decisions often involve differentiation processes that need the reset of the cell cycle and the induction of a morphogenetic program. Therefore, the fungal capability to modify its cell cycle must be an important determinant in carrying out a successful infection. Until now, the main experimental approaches taken to define and to study regulation of the pathogenic developmental programs in fungi have been focused on studying signal transduction and transcriptional changes. However, during the last years, novel opportunities have become available to investigate the molecular basis of fungal pathogenicity under a novel point of view that is complementary to previous approaches in the field. The main idea of these studies was to assume novel roles for cell cycle regulators in pathogenic fungi; roles that may help adapting the cell cycle to the virulence program. How growth and cell cycle progression are regulated coordinately during development in eukaryotic cells is an active area of research. However, little knowledge has been added on how these processes are related to induction of the virulence program in pathogenic fungi. To address this, different pathogenic fungi are currently being used. One of these model systems is the phytopathogenic fungus *Ustilago maydis*. This dimorphic fungus, which belongs to the basidiomycete group, is the causative agent of corn smut disease and the culprit of considerable losses of grain yields (Agrios 1997). In recent years, *U. maydis* has become a highly attractive model

Communicated by T. Nyström

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in addressing fundamental questions of pathogenic development in fungi (Bölker 2001; Basse and Steinberg 2004). *U. maydis* provides an ideal experimental system due to its facile sexual cycle, its easiness to grow under laboratory conditions, and the availability of molecular genetic tools. An excellent transformation system makes molecular manipulations in this fungus very efficient (Tsukuda et al. 1988). Homologous recombination events are frequent and transformants are easily obtained by means of introductions or deletions of genes (Fotheringham and Holloman 1990). The availability of vectors carrying autonomously replicating sequences (ARS) allows non-integrative transformations and simplifies gene complementation studies, as well as plasmid rescue in bacteria (Kojic and Holloman 2000). A plethora of selectable markers, reporter genes, and both constitutive and regulatable promoters has been developed (Bottin et al. 1996; Spellig et al. 1996; Brachmann et al. 2001, 2004). In addition, the *U. maydis* sequence has recently been determined (J. Kämper et al. 2006, submitted) and is currently manually annotated (MUMDB database, http://www.mips.gsf.de/genre/proj/ustilago/).

In *U. maydis*, virulence and sexual development are intricately interconnected (Kahmann and Kämper 2004). Haploid cells are saprophytic and grow in a yeast-like unicellular form (so-called sporidium) that divides by budding. The pathogenic form, the filamentous dikaryon, is established after mating of two sporidia that have to harbor different alleles of *a* and *b* mating types. The *a* locus controls the cell fusion via a pheromone-receptor based system. Upon pheromone stimulation, cells arrest budding growth and start the formation of conjugation tubes (Spellig et al. 1994). These mating filaments undergo directed tip growth toward the pheromone source (Snetselaar et al. 1996), followed by cell fusion and the formation of dikaryotic hyphae. The subsequent steps in filament formation and pathogenic development are controlled by the multiallelic *b* locus, which encodes two distinct homeodomain transcription factors, bE and bW. A heterodimeric complex of these two proteins is formed when they are derived from different alleles, and the presence of this complex is sufficient to initiate pathogenic development (Feldbrügge et al. 2004). The dikaryon formed after the fusion of compatible sporidia is arrested in cell cycle; to further develop, the dikaryon requires plant signals that have not yet been identified. On the plant surface, the filaments differentiate appressoria and penetrate the leaf (Snetselaar and Mims 1992, 1993). In contrast to appressoria from other phytopathogenic fungi, such as *Magnaporthe grisea* or *Colletotrichum* species (Bechinger et al. 1999; Talbot 2003), appressoria of *U. maydis* are not melanized and only slightly swollen relative to hyphae and form penetration structures that are less constricted (Snetselaar and Mims 1993; Snetselaar et al. 2001). Since it is unlikely that entry of *U. maydis* occurs by mechanical force, it is believed that appressoria simply mark the point at which growth direction changes. Once the filament enters the plant, the cell cycle is reactivated, and the fungal cells proliferate forming a network of filaments in which septa divide cell compartments, each containing a pair of nuclei (Snetselaar and Mims 1992; Banuett and Herskowitz 1996). These dikaryotic filaments induce the formation of plant tumors, which consist of an increased number of enlarged host plant cells. Later on, inside the tumor, massive fungal proliferation is followed by nuclear fusion and fragmentation of the hyphae, a process that releases individual cells that will produce the diploid spore (teliospore). Once dispersed by the action of wind, eventual germination of the teliospores is associated to meiosis, which leads to the production of four haploid cells, thus closing the life circle of *U. maydis* (Fig. 1a).

In summary, the virulence process in *U. maydis* implies not only strong morphological changes (bud to hypha, and hypha to teliospore transition) but also genetic changes (haploid to dikaryotic, then to diploid transition). Therefore, we soundly believe that this model pathogen deserves attention as a powerful tool in analyzing the relationships between cell cycle, morphogenesis, and pathogenicity (Fig. 1b). The aim of this review is to summarize recent advances in the untangling of how cell cycle is regulated in *U. maydis*, in particular in comparison to other well-known yeast systems, highlighting striking similarities and key differences. Defining central players of conserved pathways in cell cycle regulation in pathogenic fungi should enable us, in the next step, to investigate at the molecular level which unique cell cycle features are relevant to cause pathogenicity, and finally to address the proposed role of cell cycle regulators as true virulence factors.

Overview of cell cycle control

The cell cycle encompasses all events required for replication and segregation of the genome. In eukaryotic organisms, major cell cycle controls regulate G1/S and G2/M transitions, and ensure that these events occur in the correct sequence. Central to these controls are the cyclin-dependent kinases (CDKs), which are composed of a catalytic subunit and a regulatory subunit called

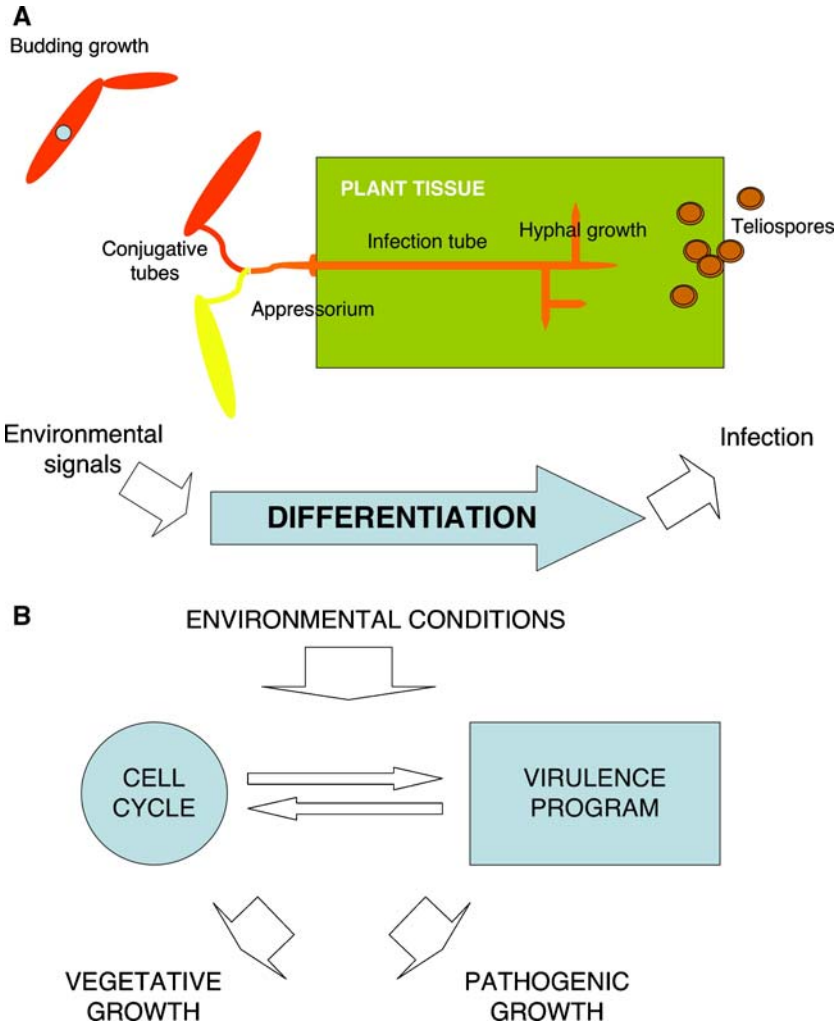

Fig. 1 Life cycle of *Ustilago maydis*. **a** Scheme depicting the different morphological transitions in *Ustilago maydis* cells during the infection process. Haploid cells grow by budding. On the plant surface, two sexually compatible cells (schematized by different color) produce conjugative tubes that allow cell fusion to generate the pathogenic filamentous dikaryon. After formation of the appressorium, the infective filament penetrates the plant tissue and proliferates as a dikaryotic filament that eventually will render diploid teliospores, where meiosis takes place producing again haploid budding cells. **b** The decision to enter into pathogenic development or vegetative growth will depend on the interplay between cell cycle regulation and the induction of the virulence program, which in the last instance depends on environmental conditions

cyclin. In multicellular eukaryotes, more than one catalytic subunit is present, forming a variety of complexes with different cyclins that regulate progression through the cell cycle (Pines 1995). In fungi, however, a single catalytic subunit is implicated in cell cycle control. It associates with different cyclins that function during G1 for the onset of S-phase and, later in the cell cycle, during G2 for the onset of mitosis. Therefore, this catalytic subunit is required for all cell cycle transitions (Krylov et al. 2003). CDK activity is controlled by a variety of mechanisms, both positive and negative (Fig. 2). Activation steps include the binding to cyclins (Murray 2004) and phosphorylation of residue Thr-161 (or an equivalent) by the CDK-activating kinases (CAK; Kaldis 1999). Active cyclin/CDK complexes can be inhibited in different ways. The phosphorylation of Thr-14 and Tyr-15 residues, mediated by kinases of the Wee1 family, interferes with correct binding of the cofactor ATP, and therefore inhibites CDK activity (Dunphy 1994). Indirectly, kinase activity is also inhibited by controlled ubiquitin-mediated proteolysis of cyclins. Ubiquitination requires the generation of polyubiquitin chains on substrate protein through combined action of ubiquitin-carrying enzymes (UBCs or E2s) and ubiquitin-protein ligases (or E3s) that bring substrates and UBCs together. Two related E3 complexes are most intimately dedicated to basic cell cycle control, namely the anaphase-promoting complex or cyclosome (APC/C) (Peters 2002) and the Skp1/cullin/F-box protein (SCF) complex (Deshaies 1999). These complexes gain specificity of substrate through the interaction with specific proteins called activators or adaptors (Zachariae and Nasmyth 1999).

Another mechanism of negative regulation of CDK activity is binding to the CDK complex by a family of low molecular weight proteins, named CDK inhibitors (CKI), that block the ability of CDK to phosphorylate its substrates (Sherr and Roberts 1999).

For a long time, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have been excellent genetic models in studying cell cycle control in eukaryotic cells for long time. In

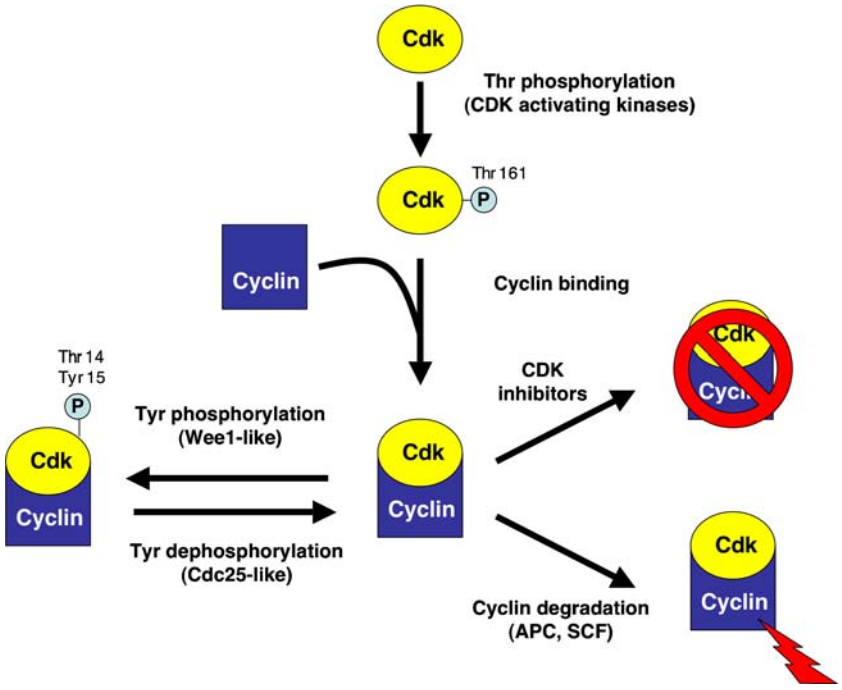

Fig. 2 Principles of CDK regulation. A simplified model of the major mechanisms used to regulate CDK activity both negatively and positively. The phosphorylation states of CDK conserved residues Tyr-15 (within the active site) and Thr-161 (within a regulatory loop) dictate catalytic activity. In addition, CDK can be directly controlled by the interaction with CKI and indirectly via degradation of cyclins performed by the APC/C and SCF complexes

spite of their simplicity, both yeasts share cell cycle characteristics with higher eukaryotes, such as G1, S, G2, and M phases, similar CDK regulatory systems, and checkpoint controls. However, below these general principles and common elements, each system has particularities. The evolutionary divergence of these yeasts is about the same as that between each of them and that of the human (Sipiczki 2000). The distinct growth mode and division of both organisms preclude different ways of regulating the two main transitions: G1/S and G2/M. We will summarize these differences since they could be illustrative about the way *U. maydis* controls cell cycle. Readers may know of an extensive published literature on yeast cell cycle regulation, including meriting reviews in their own right, to which we cannot do justice here (see for instance Rupes 2002; Bähler 2005). Instead, we will focus on a few points germane to G1/S and G2/M transition in our system model.

G1/S and G2/M transitions in budding yeast

In budding yeast, cell division is asymmetrical and produces cells of unequal size, the daughter cell being smaller than the mother cell. Since growth and cell division are coordinated in fungi, daughter cells must attain a sufficient growth rate to enter a new division cycle. In budding yeasts, cell growth is measured mainly during G1 phase (Fig. 3a). The G1 cyclin Cln3 is a primary indicator by which the cell cycle apparatus measures cellular growth rate. Cln3 levels appear to respond to the cellular growth rate via several inputs, including transcription and translation ratios (Polyeminis and Schmidt 1997; Parviz and Heideman 1998), subcellular localization (Wang et al. 2004), and protein turnover (Gallego et al. 1997). Cln3 associates with Cdc28, the CDK catalytic subunit, and once the levels of Cln3/Cdc28 reach a threshold, this complex initiates the expression of a cascade of downstream cyclins that activate the next cell cycle (Cross 1995). The Cln3/Cdc28 complex inactivates Whi5 (Costanzo et al. 2004; de Bruin et al. 2004), an inhibitor of two specific transcriptional complexes, Swi4/6 Binding Factor (SBF) and Mlu-box Binding Factor (MBF). SBF, which is composed of proteins Swi4 and Swi6, binds and activates the promoters of *CLN1* and *CLN2*, encoding G1 cyclins (Andrews and Herskowitz 1989; Koch and Nasmyth 1994). MBF, which is composed of Mbp1 and Swi6, will be in charge of activation of genes encoding the S-phase cyclins Clb5 and Clb6, as well as of other proteins involved in DNA replication (Lowndes et al. 1992; Koch et al. 1993). While Cln1 and Cln2 immediately accumulate and associate with Cdc28, producing active Cln/Cdc28 complexes, Clb5 and Clb6 are submitted to two negative controls: on the one side, the accumulation of Clb5 and Clb6 is delayed by the presence of certain amounts of APC/C complex associated with the Cdh1 APC/C activator that forms one of the ubiquitin-ligase E3 complexes responsible of cyclin degradation in *S. cerevisiae* (Schwab et al. 1997; Visitin et al. 1997); and on the other, Sic1, a CKI factor specific for Clb-type cyclins, inhibits the Clb/Cdc28 complexes

that escape from Cdh1-APC/C degradation (Schwob et al. 1994). The increase in the amount of active Cln/Cdc28 complexes is responsible for the initiation of three major events: First, Cln/Cdc28 complexes promote, via activation of the Rho-like GTPase Cdc42, the polarized localization of cortical actin to the incipient bud, activating its formation (Cvrcková and Nasmyth 1993); Second, Cln/Cdc28 complexes activate the spindle-pole duplication (Benton et al. 1993); and, finally, through the inhibition and degradation of the CKI Sic1, Cln/Cdc28 liberates the Clb5,6/Cdc28 complexes, which activate DNA replication, thus starting S-phase (Amon et al. 1994; Schneider et al. 1996; Verma et al. 1997).

The burst of Clb5,6/Cdc28-associated activity not only promotes DNA replication and thereby S-phase initiation, it also elicits activation of two pairs of transcription factors -the forkhead factors Fkh1 and Fkh2- that direct the expression of the remaining B-type cyclins (Clb1–Clb4) that will be implicated in the G2/M transition (Kumar et al. 2000; Pic et al. 2000; Zhu et al. 2000). Among the roles played by these new Clb/Cdc28 complexes, the switch from polar to isotropic growth in the daughter cell will produce the round form of the bud (Tjandra et al. 1998). Also, the Clb/Cdc28 complexes will be responsible for activation of the Cdc20-APC/C complex that, via substrate degradation, will initiate mitosis (Rudner and Murray 2000). During G2, Clb/Cdc28 complexes are phosphorylated at the CDK catalytic subunit by the inhibitory tyrosine kinase Swe1, and they are dephosphorylated by the Mih1 phosphatase (Kellogg 2003). The balance of these opposed activities regulates the ability of Clb/Cdc28 complexes to reach the activity threshold required to initiate the G2/M transition. In *S. cerevisiae*, the Clb/Cdc28 tyrosine phosphorylation evolved a specialized function in execution of a morphogenesis checkpoint that is activated when cells fail to form a bud or the integrity of actin cytoskeleton is perturbed (Lew 2000). Part of this signal is mediated by the inhibition of kinase Hsl1 and its interacting partner Hsl7. Inhibition of Hsl1 and Hsl7 is thought to result in stabilization of Swe1 and in inhibition of mitosis (McMillan et al. 1999). A role for Swe1 in cell size control was suggested by the finding of a *swe1* mutant allele in a screening for *whi* phenotype (Jorgersen et al. 2002) and more recently it was shown that Swe1 delays entry into mitosis and is required for cell size control (Harvey and Kellogg 2003).

G1/S and G2/M transitions in fission yeast

The CDK complex in *S. pombe* is composed by the CDK catalytic subunit Cdc2 associated with four different cyclins: Puc1, a G1 cyclin, and three B-type cyclins, Cig1, Cig2, and Cdc13. From these, only Cdc13 is essential, while deletion of the other three have only minor effects (McNeill and Nurse 1997; Rupes 2002). The G1/S transition is mediated by the CDK catalytic subunit Cdc2 associated with the B-type cyclins (Fig. 3b). Two inhibitory activities are crucial for preventing premature initiation of S-phase: one involves the degradation of B-type cyclins via the Ste9-APC/C complex (Yamaguchi et al. 1997; Kitamura et al. 1998) in such a way that, during G1 phase, the levels of B-type cyclins (mainly Cig2) are kept low, avoiding premature entry into S-phase (Blanco et al. 2000; Yamaguchi et al. 2000). In this task, a second inhibitor, the CKI Rum1 (Moreno and Nurse 1994), keeps inactive those B-type cyclin/Cdc2 complexes that escape from degradation. The Puc1/Cdc2 complex functions upstream the B-type cyclins. At START, Puc1/Cdc2 phosphorylates and destabilizes Rum1 (Martin-Castellanos et al. 1996), allowing the activation of a small amount of B-type cyclins/Cdc2 complexes that inactivate Ste9-APC/C (Blanco et al. 2000; Yamaguchi et al. 2000). This creates a loop that contributes to the gradual accumulation of B-type cyclins. Transcriptional activation of their genes is mediated by a heteromeric complex that contains a Swi6 homolog, Cdc10, and two Swi4/Mbp1 homologs, Res1 and Res2 (Whitehall et al. 1999). Finally, the burst of B-type cyclin/Cdc2 complex activity initiates the G1/S transition.

As cells progress through S and G2, Cdc13, the main B-type cyclin slowly accumulates, but the associated Cdc13/Cdc2 activity remains inhibited by tyrosine phopshorylation mediated by two Wee1-like kinases: Mik1 (during S-phase) and Wee1 (during G2) (Lundgren et al. 1991). During G2, the activity of the Wee1 kinase is negatively controlled by the Cdr1 and Cdr2 kinases. These regulators that are homologs of *S. cerevisiae* Hsl1 are involved in the transmission of nutrient and size information during G2 phase (Wu et al. 1996; Breeding et al. 1998; Kanoh and Russell 1998). To initiate mitosis, cells must reverse Cdc2 tyrosine phosphorylation via the Cdc25 phosphatase (Millar et al. 1991). The level of Cdc25 is correlated with general synthetic activity though translational regulation (Daga and Jimenez 1999), providing a potential link between growth rate and timing of mitosis.

What sets *Ustilago maydis* cell cycle apart from other yeast model systems?

Ustilago maydis haploid cells are cigar-shaped, around 18 μm long and 5 μm wide. Similar to *S. cerevisiae*, veg-

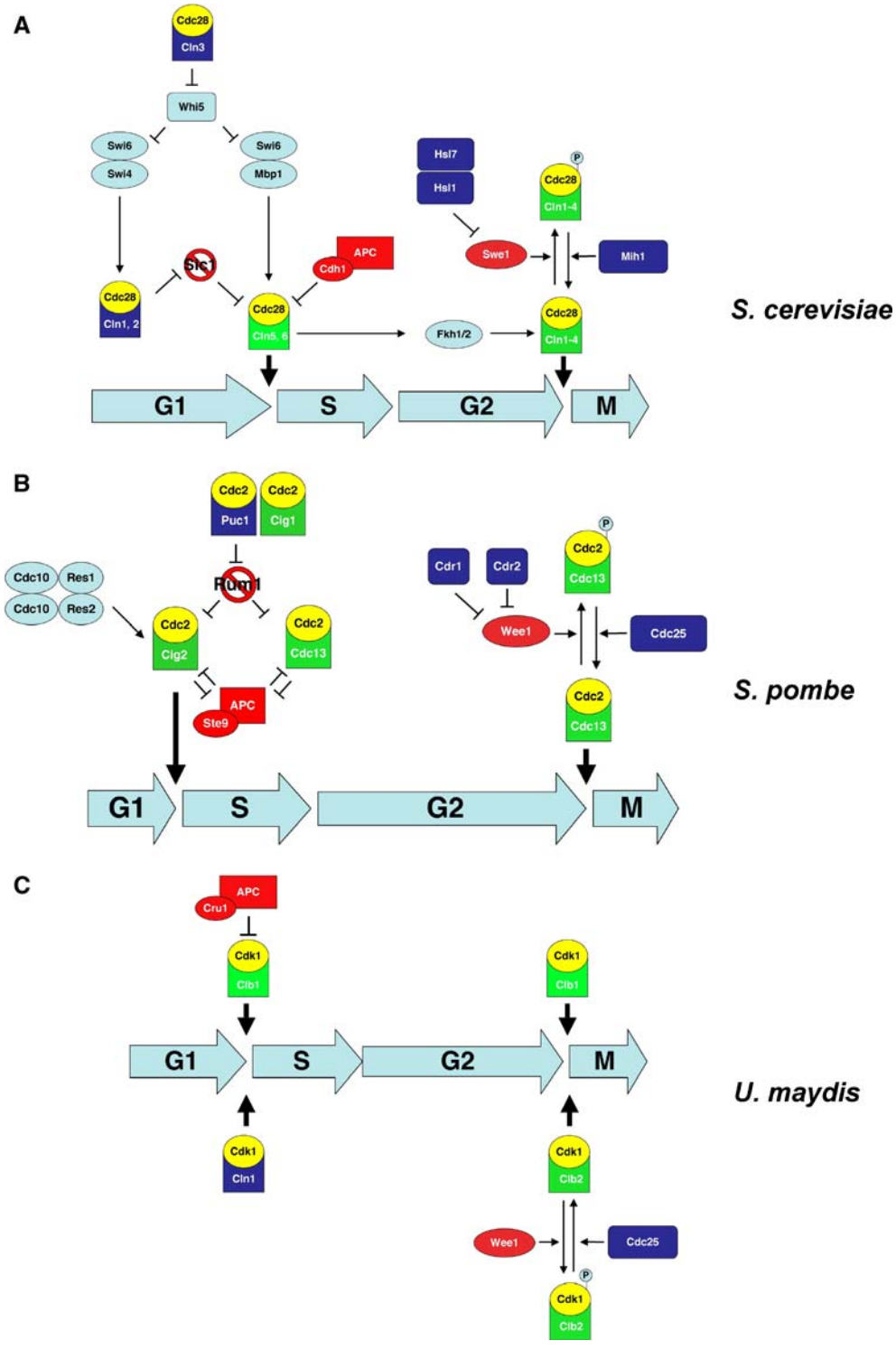

Fig. 3 Scheme of the control of cell cycle in different yeasts: **a** *Saccharomyces cerevisiae*, **b** *Schizosaccharomyces pombe*, and **c** *Ustilago maydis*. See text for a detailed description of the different elements. For simplicity, regulation of the individual components at the transcriptional, post-transcriptional, and degradation levels is not distinguished in this diagram

etative growing *U. maydis* cells normally produce one polar bud per cell cycle (Jacobs et al. 1994), which is formed at one apex, usually off center from the tip (budding angle around 35°, Straube et al. 2003). However, two main differences distinguish bud formation in both yeasts. First, studies correlating nuclear density and cell morphology showed that *U. maydis* cells complete DNA synthesis before beginning to form buds

(Snetselaar and McCann 1997). In other words, the bud formation takes place in G2 phase, in contrast to bud formation in *S. cerevisiae* that is reported to occur during S-phase (Pringle and Hartwell 1981). The second difference relates to the amount of polar growth required for bud formation. *S. cerevisiae* cells undergo a brief period of polar growth during bud emergence, but then switch to isotropic growth that occurs over the entire surface of the bud (Lew and Reed 1993). In *U. maydis*, in contrast, the growth of the bud relies almost entirely on polar growth (Steinberg et al. 2001). In fact, the growth mode of *U. maydis* is reminiscent of *S. pombe*, the fission yeast, where all growth occurs at the end of the cells (polar growth), and the activation of the growth at the new end (New End Take Off, NETO) occurs once DNA has been replicated (Mitchison and Nurse 1985). Thus, *U. maydis* appears to combine features of both yeasts.

This distinct growth mode is also reflected by the different cytoskeleton organization that again points toward a combination of features between *S. cerevisiae* and *S. pombe* (Fig. 4). Interphase cells of *U. maydis* contain an extensive array of cytoplasmic microtubules (MT), which extend from one pole to the other and into the bud (Steinberg et al. 2001; Banuett and Herskowitz 2002). This organization of the MT cytoskeleton resembles that present in *S. pombe* (Hagan 1998) and differs from that in budding yeasts, which lacks a cytoplasmic network (Winsor and Schiebel 1997). Disruption of MT in *U. maydis* leads to a loss of cortical "landmarks" to tag the sites where growth must occur, resulting in lateral budding, suggesting that MTs serve similar functions in fission yeast and *U. maydis* (Steinberg et al. 2001). However, the organization of MTs by spindle pole body-independent microtubule organizing centers (MTOC) during bud growth of *U. maydis* is fundamentally different from *S. pombe*. While in fission yeasts, MTs are nucleated and stabilized by numerous MTOCs on the nuclear surface (Tran et al. 2001), in *U. maydis*, this MT nucleation occurs distantly from the nucleus at numerous nucleation sites that are clustered in the neck by the activity of dynein (Fink and Steinberg 2006). The presence of these numerous MTOCs allows MT organization independent of cell shape and might be important due to the elongated shape and the distinct budding angle, which makes it unlikely that MT reached the bud tip by random as it happens in budding yeast.

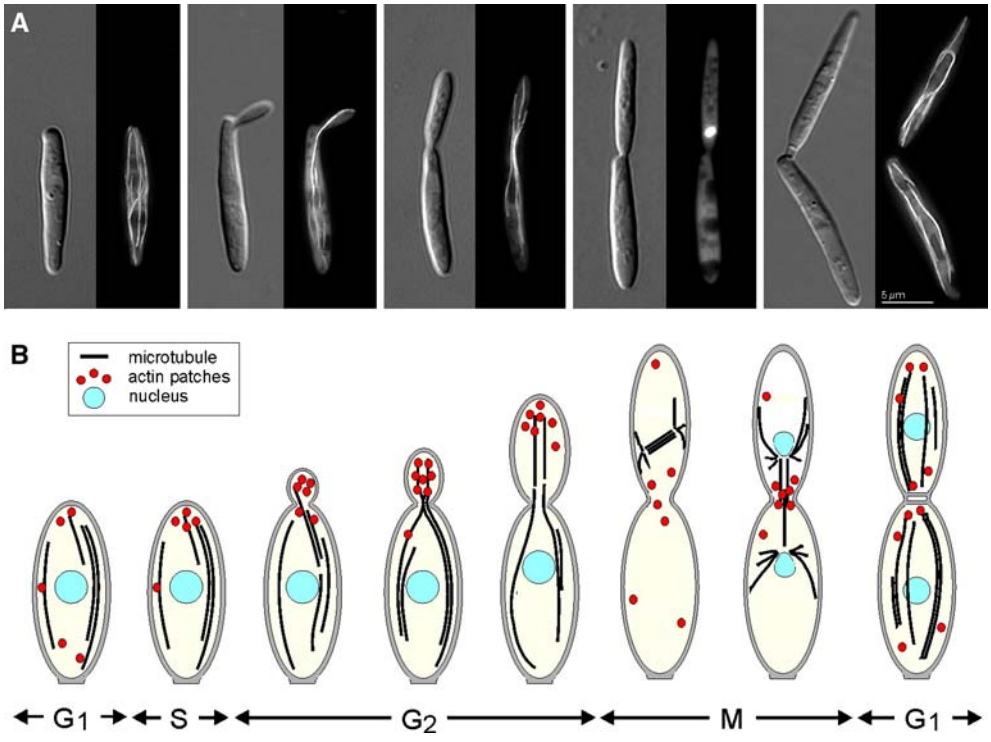

Fig. 4 Overview of the cell cycle in *Ustilago maydis*. **a** MT organization through the different stages of the cell cycle. MT arrays were visualized using a Tub1-GFP protein fusion. Observe the array of cytoplasmic MT extending from one pole to the other and into the bud during G1, S, and G2 phases, and the disassembly of the interphase network once the cell enters into mitosis. **b** Scheme of the organization of cytoskeleton and of nuclear position in *Ustilago maydis* during the cell cycle. Actin patches are shown as *red dots* and MT are *black lines*). Scheme and pictures obtained from Gero Steinberg (MPI, Marburg, Germany)

The overall organization of the actin cytoskeleton in *U. maydis*, however, resembles that of *S. cerevisiae*, where actin patches concentrate at sites of polarized growth and secretion and actin cables polarize toward these patches (Banuett and Herskowitz 2002). In addition, actin is also located as a ring in the neck region during cytokinesis. Furthermore, bud formation is strictly dependent on actin cytoskeleton as can be confirmed by the inhibition of bud growth and alteration of the shape of the cell when *U. maydis* cells are treated with lantrunculin A, which disrupts F-actin (Fuchs et al. 2005).

Control of cell size and cell cycle length also seems to combine features from budding and fission yeasts. *U. maydis* has a doubling time of ca. 120 min in complete medium. In these rapidly growing cells, G1 phase is very brief and the S-phase begins shortly after cytokinesis, because cell division produces daughter cells with a mass similar to mother cells. As other fungal cells, *U. maydis* adjusts cell cycle depending on environmental conditions. For instance, in response to poor nutritional conditions, this adjustment results in a prolongation of generation time. This cell cycle delay is caused by increasing the length of the G1 phase, until the cell reaches a minimum size to enter in a new S-phase, but the G2 phase is also lengthened, allowing the bud to reach a correct size (Snetselaar and McCann 1997; Garrido and Pérez-Martín 2003). In other words, size in *U. maydis* is monitored both during G1/S and the G2/M transitions, as it happens in higher eukaryots. In contrast, in the other yeast models, although both transitions could be used for cell size control, only one control is active while the other one is cryptic (Rupes 2002). Fission yeasts coordinate growth and division by regulating the length of time spent in G2 (Sveiczer et al. 1996), whereas budding yeast exert a size control throughout length of G1 (Jagadish and Carter 1977; Johnston et al. 1977).

The availability of the genome sequence for *U. maydis* allowed the search for the different components and regulators of cell cycle. We performed a sequence analysis of the *U. maydis* genome in search for genes encoding cell cycle regulators. Homologues of all important key factors were found (Table 1), suggesting that the basic principles of cell cycle regulation are conserved between *U. maydis* and other well-known systems.

G1/S transition in *Ustilago maydis*

In contrast to budding and fission yeast, *U. maydis* has only three cyclins dedicated to cell cycle that are able to associate with Cdk1: Cln1, a G1 cyclin and two B-type cyclins, Clb1 and Clb2. The Clb1 cyclin associated to Cdk1 plays an essential role in the cell. It is required to perform the G1 to S as well as G2 to M transitions and, therefore, conditional *clb1* mutants arrest at restrictive conditions in G1 and G2 phases. Consistently, this protein is present from G1 to beginning of mitosis (García-Muse et al. 2004). The G1/S transition role is specific of Clb1, and no other cyclin (i.e. Cln1 or Clb2) is able to bypass the Clb1 requirement. In contrast, in *S. pombe*, where the G1/S and G2/M transitions are performed by Cig2 and Cdc13, respectively (Booher and Beach 1987; Hagan et al. 1988; Bueno and Russell 1993; Connolly and Beach 1994), Cdc13 substitutes for Cig2 to bring about the G1/S transition in cells deleted for the *cig2* gene (Fisher and Nurse 1996; Mondesert et al. 1996). How does Clb1/Cdk1 kinase first promote G1 to S transition early in the cell cycle and then prevent the reinitiation during G2? A reasonable hypothesis is that to assume that additional elements, specific for each transition, modify the scope of target molecules of this complex. Among these elements, we could hypothesize the presence of some CKI. CKIs bind to and repress the Cdk activity. In mammals, two different classes of CKIs exist: the INK4 and the Kip/Cip families, each with its own CDK binding specificity and protein structure (Sherr and Roberts 1999). No counterparts to these factors were found by sequence analysis in the *U. maydis* genome. In *S. cerevisiae*, two mitotic CKIs have been described: Sic1 and Far1, while in *S. pombe* only one CKI—Rum1—has been described (Peter and Herskowitz 1994a). In *U. maydis* by sequence analysis, no genes able to encode a protein with sequence similarity to CKI are present. It is worthy to note that there is no significant sequence similarity between the different members of the fungal CKI. Therefore, the absence of any counterpart, as defined by sequence comparison, does not necessarily mean that these proteins do not exist in *U. maydis*.

Cyclin levels are submitted to a strict control both at transcriptional as well as post-transcriptional levels. For instance, high levels of Clb1 cyclin affect viability of *U. maydis* cells, provoking defects in chromosomal segregation (García-Muse et al. 2004). Strikingly, these effects have also been reported with high levels of S-phase cyclins in other organisms. *S. cerevisiae* cells overexpressing cyclin Clb5 show an altered chromosome segregation (Sarafan-Vasseur et al. 2002), and mammalian cells overexpressing cyclin E and cyclin B1 also show defects in the segregation of the chromosomes resulting in aneuploidy (Spruck et al. 1999; Yin et al. 2001). The reasons for these defects are unknown but they may be related to unscheduled action of these

Table 1 Core cell cycle components in *Ustilago maydis* and their counterparts in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

Ustilago maydis protein	Protein type or function	*Saccharomyces cerevisiae* protein	*E*-value	*Schizosaccharomyces pombe* protein	*E*-value
Cyclin-dependent kinases					
Cdk1	Mitotic CDK	Cdc28	2×10^{-112}	Cdc2	5×10^{-110}
Cyclins					
Cln1	G1-type cyclin	Cln3	2×10^{-17}	Puc1	1×10^{-36}
Clb1	B-type cyclin	Clb2	6×10^{-71}	Cdc13	5×10^{-96}
Clb2	B-type cyclin	Clb3	2×10^{-65}	Cdc13	9×10^{-72}
CDK-activating kinases					
UM04902	Cdk7-like CDK	Kin28	4×10^{-87}	Mcs6	5×10^{-88}
UM00669	CAK subunit	Tfb3	7×10^{-35}	Pmh1	1×10^{-47}
UM05914	CAK subunit	Cak1	3×10^{-13}	Csk1	3×10^{-9}
CDK-interacting proteins					
UM03210	CDK regulator	Cks1	7×10^{-30}	Suc1	3×10^{-32}
CDK inhibitory phosphorylation					
Wee1	CDK kinase	Swe1	2×10^{-30}	Wee1	2×10^{-46}
UM03928	Wee1 kinase	Hsl1	2×10^{-83}	Cdr2	8×10^{-74}
Cdc25	CDK phosphatase	Mih1	5×10^{-20}	Cdc25	1×10^{-30}
UM03234	Polo kinase	Cdc5	3×10^{-96}	Plo1	6×10^{-93}
SCF complex					
UM01087	Cullin 1	Cdc53	6×10^{-98}	Pcu1	8×10^{-170}
UM03899	Cullin 3B	Cdc53	2×10^{-55}	Pcu3	5×10^{-138}
UM05563	Cullin 4A	Cdc53	7×10^{-24}	Pcu4	1×10^{-83}
UM05744	SCF component	Hrt1	1×10^{-35}	Rbx1	2×10^{-43}
UM04611	SCF component	Skp1	2×10^{-39}	Skp1	3×10^{-57}
UM02426	SCF component	Cdc34	3×10^{-31}	Ubc15	4×10^{-58}
UM00604	SCF component	Sgt1	1×10^{-23}	Git7	2×10^{-26}
UM05417	F-box protein	Met30	1×10^{-35}	Pof1	2×10^{-46}
UM01349	F-box protein	Grr1	2×10^{-55}	Pof1	6×10^{-84}
UM04853	F-box protein	Cdc4	4×10^{-22}	Pop1	5×10^{-28}
APC/C complex					
UM02427	APC/C subunit	Apc1	3×10^{-35}	Cut4	4×10^{-80}
UM03226	APC/C subunit	Apc2	1×10^{-8}	Apc2	9×10^{-31}
UM02089	APC/C subunit	Cdc27	7×10^{-24}	Nuc2	1×10^{-33}
UM10036	APC/C subunit	Apc5	0.03	Apc5	0.2
UM04059	APC/C subunit	Cdc16	1×10^{-87}	Cut9	4×10^{-93}
UM00651	APC/C subunit	Cdc23	5×10^{-69}	Cut23	7×10^{-81}
UM00680	APC/C subunit	Doc1	3×10^{-10}	Apc10	5×10^{-28}
UM01647	APC/C activator	Cdc20	2×10^{-66}	Slp1	5×10^{-80}
Cru1	APC/C activator	Cdh1	4×10^{-100}	Ste9	1×10^{-120}
Transcriptional regulators					
UM05338	G1 regulator	Mbp1	9×10^{-34}	Res2	8×10^{-59}
UM06196	G1 regulator	Swi4	3×10^{-32}	Res2	1×10^{-80}
Umc1	G2 regulator	Mcm1	4×10^{-21}	Map1	2×10^{-16}
UM06231	G2 regulator	Fkh1	1×10^{-8}	Fkh2	5×10^{-15}
UM01042	G2 regulator	Fkh2	1×10^{-14}	Sep1	2×10^{-22}
UM10181	M/G1 regulator	Swi5	3×10^{-17}	Prz1	3×10^{-25}
UM04774	M/G1 regulator	Ace2	1×10^{-15}	Prz1	1×10^{-25}

cyclins in further cell cycle stages. The levels of Clb1 are kept under control during G1 phase by the action of the Cru1-associated APC/C. This feature is conserved in both budding and fission yeast. In *S. pombe*, Ste9 is required for degradation of mitotic cyclins during G1 phase (Blanco et al. 2000) and cells of *S. cerevisiae* defective in the APC/C activator Cdh1 have substantial amounts of mitotic cyclins during G1 phase (Schwab et al. 1997). In *U. maydis*, the use of cell cycle arrest/release experiments has shown that, in *cru1*Δ cells, the Clb1 cyclin accumulates faster than in wild-type cells, and that this accumulation correlates with a premature entry into S-phase (Castillo-Lluva et al. 2004). The ability to delay G1/S transition could be important for small cells that have to lengthen the G1 phase until they reach the minimum cell size required

to initiate DNA replication or to prevent entry into mitosis from G1. Such a role explains the reported smaller size of *cru1*Δ cells, as well as the absence of adjustment of the G1 phase length in nutrient-poor medium (Castillo-Lluva et al. 2004). A simple way to signal nutrient availability to cell cycle is controlling the levels of Cru1, since *cru1* mRNA levels depend on nutritional conditions: the poorer the nutrients, the higher the Cru1 levels and, subsequently, the longer the G1 phase (Castillo-Lluva et al. 2004).

Among the positive elements controlling G1 phase, the *U. maydis cln1* gene encodes a protein with high sequence similarities to G1 cyclins from other fungi (Castillo-Lluva and Pérez-Martín 2005). 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* (Castillo-Lluva and Pérez-Martín 2005). Furthermore, the *cln1* gene is preferentially expressed in G1 phase. In agreement with this view, cell cycle arrest/release experiments indicate that cells lacking Cln1 have a delayed G1 phase with respect to wild-type cells (Castillo-Lluva and Pérez-Martín 2005).

The current model on how a G1/S transition proceeds in *U. maydis* involves the pivotal role of Clb1/Cdk1 complex (Fig. 3c). The accumulation of this complex depends on Cru1-APC/C levels, which respond directly to nutritional condition. We also propose the presence of a putative CKI that is predicted to negatively regulate the Clb1/Cdk1 complex, and by analogy, to what happens in budding and fission yeasts, Cln1/Cdk1 might be involved in the removal of this putative CKI and in the activation of S-phase. This role has been conserved for G1 cyclins in fungi as well as D-type cyclins in mammalian, and it seems to be pivotal in regulating G1/S transition. For instance, in *S. cerevisiae*, the deletion of all three G1 cyclin genes is lethal, but can be achieved if the gene encoding the CKI Sic1 is also removed (Richardson et al. 1989).

The apparent minor role of Cln1 triggering the G1/S transition in *U. maydis* contrasts with a major role at morphogenetic level. The absence of Cln1 produces cell aggregates that are composed of cells that lose their ability to divide by budding, remain attached after cytokinesis, and often lose their polarity. In opposition, high levels of Cln1 provoke a strong polar growth that results in filaments composed of cells separated by septa (Castillo-Lluva and Pérez-Martín 2005). 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 occasionally cause significant bud elongation (Lew and Reed 1993; Loeb et al. 1999a). In *C. albicans*, G1 cyclins 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). By contrast, deletion of *CLN3* produces hyphal growth in the absence of environmental inducing conditions (Bachewich and Whiteway 2005; Chapa y Lazo et al. 2005).

G2/M transition in *Ustilago maydis*

Once DNA replication ends, the formation of the bud marks the beginning of G2. Once the proper bud size is reached, mitosis is induced. The onset of mitosis requires both Clb1/Cdk1 and Clb2/Cdk1. The Clb2 cyclin appears to be specific for G2/M transition, and it seems to be a rate-limiting regulator for entering into mitosis. Cells carrying a conditional *clb2* allele growing in restrictive conditions are arrested in G2 phase with an elongated bud displaying active polarized growth. On the contrary, high levels of Clb2 expression result in short cells that divide by septation, resulting in hyphal-like growth of the cells (García-Muse et al. 2004). In *U. maydis*, the G2 phase is characterized by polar growth that results in the elongation of the bud. It could well be that the levels of Clb2 mark the length of G2 and thus the size of the bud. This could be related to a G2/M size control, operating through the levels of Clb2/Cdk1. The postulated role of Clb2 is reminiscent of the roles proposed for cyclin A in humans (Furuno et al. 1999) and for cyclin B2 in plants (Weingartner et al. 2003) as promoters of mitosis. In human cells, it has been demonstrated that cyclin A/CDK2 activity is a rate-limiting component for entering into mitosis because exogenous active cyclin A/CDK2 drives cells prematurely into mitosis (Furuno et al. 1999). In plants, induction of ectopic cyclin B2 expression drives cells to enter mitosis earlier, causing developmental abnormalities in transgenic plants (Weingartner et al. 2003).

The B-cyclin/Cdk1 complex activity seems to be regulated via inhibitory phosphorylation of Cdk1 during the length of G2 phase. In *U. maydis*, this tyrosine phosphorylation depends on the Wee1 kinase. As expected, the *U. maydis wee1* gene functions as a dose-dependent inhibitor of mitosis (Sgarlata and Pérez-Martín 2005a). When *wee1* is overexpressed in *U. maydis*, uninuclear cells with 2C DNA content undergo arrest

during G2 phase. In agreement with a specific role controlling G2/M transition, phosphorylated Cdk1 is associated to B-type cyclins, but it is not found in complexes with Cln1, indicating that, as in other organisms, different cyclins target CDKs for distinct negative regulatory controls in *U. maydis* (Devault et al. 1992; Booher et al. 1993; Watanabe et al. 1995). The primary target for Wee1 is the Clb2/Cdk1 complex. Thus, *clb2* overexpression produces a phenotype that resembles those produced by down-regulation of *wee1*. In contrast, overexpression of *clb1* produces lethal chromosome missegregation (García-Muse et al. 2004). Furthermore, high levels of Wee1 overcome the effects on cellular morphology imposed by high levels of Clb2, while the toxic effect of high levels of Clb1 (García-Muse et al. 2004) cannot be suppressed by an abundance of Wee1 (Sgarlata and Pérez-Martín 2005a). Inhibitory phosphorylation seems to be essential for cell cycle regulation in *U. maydis*. This is similar to *S. pombe*, in which two related kinases (Wee1 and Mik1) phosphorylate Cdc2, the deletion of either gene being lethal (Lundgren et al. 1991). On the other side, the gene encoding the Wee1-like kinase in *S. cerevisiae* (*SWE1*) is dispensable for growth (Booher et al. 1993). The requirement in *U. maydis* for *wee1* reflects the importance of controlling G2 phase length in this organism. Once DNA replication has occurred, *U. maydis* cells must decide whether to bud or to enter in a mating program, a decision that is taken in response to external stimuli (García-Muse et al. 2003). Controlling the length of G2 seems to be primordial for *U. maydis* for making the correct decision. In addition, these evidences are in accordance with an interesting model proposed by Kellogg (2003), which suggests that Wee1-related kinases monitor the total amount of polar growth that occurs. Fission yeast and *U. maydis* are rod-shaped cells and all growth occurs at the ends of the cell (polar growth). In contrast, *S. cerevisiae* cells undergo a brief period of polar growth during bud emergence, but then growth occurs over the entire surface of the bud (isotropic growth). In agreement with Kellogg (2003), the loss of *wee1* function causes a more severe phenotype in *U. maydis* than in *S. cerevisiae*, despite the fact that both organisms divide by budding. However, *U. maydis* relies almost entirely on polar growth that occurs during G2, whereas budding yeast only undergo a brief period of polar growth and then switch to isotropic growth.

Inhibitory phosphorylation is removed at the onset of mitosis by the activity of the Cdc25 phosphatase. The *U. maydis* Cdc25-like phosphatase has a C-terminal domain that contains all the conserved motives unique to Cdc25 phosphatases (Eckstein et al. 1996). Cdc25 is essential for growth in *U. maydis*, and cells lacking this regulator arrest their cell cycle at G2 phase, in accordance with the role of these phosphatases as mitotic inducers in other systems. The phenotype found in conditional *U. maydis* Cdc25-ablated cells is a phenocopy of that found when *U. maydis wee1* is overexpressed, consisting of single, uninuclear cells with a bud-like protrusion that continue elongating over time and are arrested in G2 phase (Sgarlata and Pérez-Martín 2005b). Furthermore, overexpression of *cdc25* is, at morphological level, indistinguishable from that observed in conditional *wee1* cells in restrictive conditions or from the overexpression of the inhibitory resistant $cdk1^{AF}$allele (Sgarlata and Pérez-Martín 2005b). In summary, these observations support a model in which G2/M transition in *U. maydis* could be controlled by the balance between Wee1 and Cdc25 activities, thereby controlling the Tyr-15 phosphorylation levels of Cdk1 associated to Clb2 (Fig. 3c).

Fission yeast, where Cdc25 is also essential, can survive the absence of Cdc25 if the mitotic inhibitor *wee1* is also deleted. However, in *U. maydis*, the double mutant *cdc25*Δ and *wee1*Δ is lethal. In the case of *S. pombe*, the ability to survive a double *cdc25*, *wee1* deletion depends on the presence of the back-up phosphatase Pyp3 (Millar et al. 1992) and the Wee1-like kinase Mik1 (Lundgren et al. 1991). In the genome search performed in this work, we were unable to find a clear homolog of Pyp3 and Mik1 in *U. maydis*, suggesting the absence of such a back-up system in this fungus.

While in metazoan cells Cdc25 activity seems to require stimulation by Polo kinase in allowing entry into mitosis, it does not seem the case for budding and fission yeasts (Lee et al. 2005). In fission yeast, Polo kinase appears to be downstream of mitotic CDK activity, and there is no evidence that budding yeast Polo kinase regulates entry into mitosis. However, in *U. maydis*, inactivation of the Polo kinase-encoding gene arrests cells in G2, being unable to enter into mitosis, suggesting that Polo kinase is part of a conserved mechanism that regulates entry into mitosis (N. Mielnichuk and J. Pérez-Martín, in preparation). Whether this mechanism operates through Cdc25 activation or inhibition of Wee1 is currently unknown.

An accurate control of G2/M transition seems to be important in regulating cell length in *U. maydis*. In order to maintain a constant size during cellular proliferation, cell growth must be coordinated with the rate of cell division. Progression through the cell cycle is regulated principally before the onset of S-phase and before the onset of mitosis. In both cases, a critical cell mass must be attained before progression occurs, and it is thought that cells use cell-size checkpoints in coordinating growth and division (Rupes 2002). In higher

eukaryotes, size is monitored both during G1/S and G2/M transitions. Fission yeasts coordinate growth and division by regulating the length of time spent in G2 (Sveiczer et al. 1996), whereas budding yeasts exert a size control throughout the length of G1 (Jagadish and Carter 1977; Johnston et al. 1977). In *U. maydis*, both G1/S and G2/M control points are actively used. Alterations in either G1 or G2 duration affect cell length. Cells lacking the gene encoding the APC/C activator Cru1 are shorter because they are unable to prolong the G1 phase (Castillo-Lluva et al. 2004). In a similar way, low levels of Wee1 or overexpression of *cdc25* cause a brief G2 phase, resulting in shorter cells (Sgarlata and Pérez-Martín 2005a, b). Furthermore, it seems that *U. maydis* cells compensate for induced alterations in the length of one cell cycle phase by altering the length of the other phase, and that simultaneous impairment of both G1/S and G2/M controls are deleterious to cells (Sgarlata and Pérez-Martín 2005b). Such a compensatory mechanism has been recently described at molecular level in *Drosophila melanogaster* wing disc cells (Reis and Edgar 2004), implying the cell cycle-specific transcriptional regulator dE2F1 and *string*/*cdc25*.

Induction of the pathogenicity program requires a cell cycle arrest

One of the prominent features of *U. maydis* is that virulence and sexual cycle are intimately intertwined. The pathogenic form, the filamentous dikaryon, is established after mating of two haploid cells. As it happens in other fungal systems, pheromone recognition blocks cell cycle progression in *U. maydis* cells in order to prepare mating partners for conjugation. This may ensure that both cells are at the correct stage of the cycle at the time of conjugation. However, in contrast with the previously characterized pheromone-induced G1 cell cycle arrest in *S. cerevisiae* and *S. pombe* (Sprague and Thorner 1992; Davey 1998), *U. maydis* cells undergo arrest in G2 phase after pheromone recognition (García-Muse et al. 2003).

In the budding yeasts, a Mitogen Activated Protein Kinase (MAPK) pathway is involved in the recognition of mating pheromones. The MAP kinase Fus3 phosphorylates and activates the CKI Far1 (Chang and Herskowitz 1990; Peter et al. 1993; Peter and Herskowitz 1994b). This phosphorylation facilitates binding of Far1 to Cdc28 associated with the G1-specific Cln cyclins, blocking cell cycle progression at G1. A pheromone-dependent reduction in expression of cyclin genes also contributes to cell cycle arrest (Valdivieso et al. 1993).

The mechanism of G1 arrest in fission yeasts is far less understood. The mating pheromone is also transmitted by a MAPK cascade (Davey 1998), and causes a cell cycle arrest by inducing the degradation of both Cig2 and Cdc13, the cyclins required for G1/S transition. The initial target is the complex Cig2/Cdc2. Precise details on how the mating pheromone induces the degradation of Cig2 are yet to be defined but it is largely independent of the CKI Rum1, and can be saturated by over-expressing Cig2 (Stern and Nurse 1997). The loss of Cig2/Cdc2 complex causes a transient G1 arrest that is soon released, unless Cdc13/Cdc2 is also inhibited. This is achieved by Rum1-dependent targeting of Cdc13 to the proteasome. Although barely detectable during exponential growth, Rum1 quickly accumulates during the transient G1 arrest caused by loss of Cig2/Cdc2. It then binds to Cdc13 and targets it to the APC/C complex for degradation. As predicted from this mechanism, a non-degradable form of Cdc13 prevents pheromone-induced G1 arrest (Stern and Nurse 1998).

The mechanism of G2 arrest in *U.maydis* is yet to be characterized. As in budding and fission yeasts, a common conserved MAP kinase pathway transduces in *U. maydis* the pheromone signal from membrane-located pheromone receptors (Müller et al. 2003), and the integrity of this pathway is required for the induction of the cell cycle arrest (García-Muse et al. 2004). However, no downstream effector of this pathway being specifically required for cell cycle arrest has been characterized so far. It appears that the target of cell cycle arrest is the Clb1/Cdk1 complex. Activation of the pheromone MAPK cascade in *U. maydis* causes a down-regulation of the *clb1* expression, and mutations in the C-terminal end of Clb1 avoids the pheromone-induced cell cycle arrest (I. Flor-Parra and J. Pérez-Martín, unpublished observations).

Regardless of the mechanisms underlying the pheromone-induced cell cycle arrest in *U. maydis*, the different cell cycle stage affected set apart *U. maydis* from the other yeasts. Although the reasons for such a different behavior are not completely understood, we propose the following explanations. A plausible one is related to the specific characteristics of the sexual reproduction in *U. maydis*, not shared with these other well-known yeast systems. Sexual recognition in cells of either budding or fission yeasts causes them to block in G1 phase, after which two haploid mating partners undergo conjugation to form a diploid zygote. In this process, cell fusion is closely followed by nuclear fusion. In contrast, in *U. maydis* cells, nuclei of opposite mating type do not fuse after cell fusion (Bölker et al. 1992; Müller et al. 1999). The resulting dikaryotic

hypha is stable and karyogamy is delayed until the dikaryotic mycelium proliferates inside the infected plant and develops diploid teliospores (Kahmann and Kämper 2004). We speculate that the absence of karyogamy immediately after cell fusion is responsible for the observed differences between ascomycete yeasts and *U. maydis*. A G2 state could have several advantages. For instance, the first cell division after cell fusion would be faster, as the cell does not have to pass through S-phase. This could be advantageous because the first division of the infective dikaryon takes place inside the plant, once the infection begins. Alternatively, this specific cell cycle arrest in *U. maydis* may have a mechanistic reason. In *U. maydis*, the G2 phase is characterized by polar formation of a bud (Snetselaar and McCann 1997), which requires the rearrangement of the cytoskeleton and involves specialized set of motors that support the polar extension of the cell, such as kinesins (Schuchart et al. 2005; Lenz et al. 2006) and most likely dynein (Straube et al. 2001; Wedlich-Söldner et al. 2002). In other words, in the G2 phase, the cytoskeletal growth machinery is set up to support polar growth. Assuming that the formation of a conjugation tube is based on similar mechanisms as polar bud growth, a prolonged G2 phase is best suited in supporting tip growth during tube formation. We could then postulate that, in *U. maydis*, a point of decision exists once DNA has been replicated in such a way that, in response to external stimuli, the cell decides either to bud or to enter in a mating program. A decision-taking point, in which the cell must choose between alternative developmental fates, is consistent with the previous observation that cells responding to pheromone do not form buds (Spellig et al. 1994).

Mitosis or mating: two options, one choice

While the response to pheromone and the activation of the mating program induces a cell cycle arrest in *U. maydis*, there are several reports suggesting that entering into the sexual/virulence program seems to be incompatible with an active mitotic cell cycle (Castillo-Lluva et al. 2004; Castillo-Lluva and Pérez-Martín 2005). The induction of the sexual development in *U. maydis* initiates with the expression of the *a* mating-type locus, which includes the genes encoding the pheromone precursor and the receptor. Such expression depends on the activity of the transcriptional regulator Prf1 (Hartmann et al. 1996) and is regulated by various signals, including nutritional conditions (Hartmann et al. 1999). Different results suggest that cyclin/Cdk1 complexes may repress for the induction of sexual determinants. For instance, high levels of *cln1* expression impair the expression of *mfa1*, which encodes the precursor of sexual pheromone a1, while down-regulation of *cln1* enables the expression of *mfa1*, even in nutritional conditions that repress the expression of the *a* mating-type genes (Castillo-Lluva and Pérez-Martín 2005). Similarly, the APC/C activator Cru1 is required for *a* locus expression, and this requirement 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 on the same target. The deletion of *cln1* fails to rescue the *mfa1* expression in *cru1*Δ cells, suggesting that either the inhibitory function of Cln1 is upstream of Cru1 activating function or that Cru1 functions "in parallel" to the Cln1 cyclin in regulating pheromone expression, but that Cru1 requirement has a stronger effect on the process (Castillo-Lluva and Pérez-Martín 2005). The inability to express *mfa1* seems to be related with the absence of *prf1* expression when Cru1 is absent or *cln1* is overexpressed. Moreover, both the effect of the absence of *cru1* and the overexpression of *cln1* on *mfa1* expression are suppressed by heterologous expression of *prf1* (Castillo-Lluva and Pérez-Martín 2005). 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 Upstream Activating Sequences (UAS, Hartmann et al. 1999; Garrido et al. 2004), and Rop1, which binds the Rop1 Recognition Sites (RRS; Brefort et al. 2005). Whether the cyclin/Cdk1 complexes act by inhibiting any of these regulatory inputs or via new regulatory players is currently unknown.

Why are mating and mitosis incompatible options in *U. maydis*? In Nature, *U. maydis* is a saprophytic organism that enters the sexual cycle only in particular conditions (e.g. the surface of its host plant), most likely when nutrients are limited. Under favorable nutritional conditions, *U. maydis* cells prefer to reproduce asexually by means of the mitotic cell cycle. An active mitotic cycle correlates with high cyclin/Cdk1 activity. In contrast, when they experience starvation, for instance on the plant surface, the cell cycle is turned down and therefore an opportunity for mating process is opened by the induction of sexual development. Consequently, 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/Cdk1, Clb1/Cdk1) and negative (Cru1-APC/C) 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).

Cell cycle regulation inside the plant

Hyphal growth within the plant is a dynamic process that involves several stages of differentiation and the infection process surely requires an accurate control of the cell cycle. On the plant surface, the initial mating reaction results in the formation of a dikaryotic hypha. This filament differentiates and forms appressoria, a specific structure that appears as a swelling of the hyphal tip, which redirects the fungal growth in a 90° angle to penetrate the plant. Formation of appressoria appears to be connected with cell cycle. *U. maydis* cells defective in *biz1*, a gene that encodes a transcriptional regulator that represses the transcription of *clb1* under pathogenic conditions, are severely impaired in appressoria formation (Flor-Parra et al. 2006). Once the fungus penetrates the plant cuticule, the dikaryotic hypha continues rapid, unbranched growth without mitotic divisions, leaving behind old compartments, which become devoid of cytoplasm, sealed off, and collapse. Around 2 days later, fungal cells activate the mitotic program and hyphae start branching and are filled with cytoplasm. The trigger of this switch is presently unknown but supposedly it may be a plant signal. In subsequent stages, branching increases profusely. Around 9 days post-infection, the fungal hyphae fragmentate into segments of one to several elongated cells. Karyogamy probably occurs at this stage, and is followed by maturation of individual rounded cells to yield the diploid teliospores.

It seems obvious that, in each of these different developmental steps, fungal cells have to take distinct decisions in response to the surrounding conditions. Studies from both yeast and mammalian systems indicate that G1 is the phase of the cell cycle in which cells respond to extracellular signals to commit to another round of cell division, to withdraw temporarily from cell cycle and become quiescent, or to terminally differentiate (Pardee 1989). Hence, it is likely that the control of the length of G1 plays an important role in pattern formation and differentiation. Supporting this idea, it has been reported that cells lacking the *cru1* gene are impaired at several steps during infection (Castillo-Lluva et al. 2004). The role of Cru1 in the cell seems to be to lengthen G1 (Castillo-Lluva et al. 2004). A short G1 phase could mean a short window to load a new developmental program and therefore little time to adapt to changing environmental conditions. In fact, it has been proposed that the role of Cru1 in *U. maydis* during pathogenic development is to keep an accurate G1 length to elaborate the appropriate response (Castillo-Lluva et al. 2004). In other words, cells need time to "think" about the next step and this time could be provided in G1 phase by the activity of Cru1-APC/C. In the absence of Cru1, due to a narrow G1 phase, only a limited number of cells would be able to respond correctly; so, the further they progress in the infection, the lower the probability to take the right decision, resulting in a dramatic drop in the infectivity of the mutant population. In this sense, Cru1 might act as a car gear, keeping the G1 phase running while environmental signals are received and the responses are elaborated.

Control of G2 also seems to be quite important. For instance, fungal cells with high levels of Clb2 do not progress in the infection to symptoms beyond chlorosis, while infection of the plant with fungal cells carrying half a dose of Clb2 induces the production of anthocyanin by the plant, although tumor production does not occur (García-Muse et al. 2004). As the cyclin Clb2 levels correlate with the length of G2 phase -the higher the levels of Clb2, the shorter the length of G2 phase- these observations indicate that an appropriated G2 length is crucial for fungal progression inside the plant. In accordance with the importance of the control of the G2 phase during the pathogenic development, Tyr-15 phosphorylation of Cdk1 is also required for the infection to progress inside the plant (Sgarlata and Pérez-Martín 2005a). Taken together, these data indicate that the accurate control of G2/M transition seems to be important for successful infection by *U. maydis*. It is worth bearing in mind that *U. maydis* cells grow in yeast-like unicellular form in saprophytic conditions. The induction of the pathogenic phase requires two compatible haploid cells to fuse and the generation of an infective dikaryotic filament that invades and proliferates inside the plant (Kahmann et al. 2000).

Studies of pseudohyphal development in *S. cerevisiae* have revealed the importance of controlling the G2/M transition for the production of the filamentous growth (Rua et al. 2001).

Pathocycles: perspectives

In *U. maydis*, an accurate control of the cell cycle must be required not only in cells growing in vegetative conditions, but also during the infection process. This is not an isolated case, and in general, all pathogenic fungi have in common the requirement of accurate developmental decisions for the induction of the virulence program. Developmental decisions often involve differentiation processes that need the reset of the cell cycle and the induction of a new morphogenetic program. Recent progress in the molecular analysis of signal transduction pathways during virulent development in pathogenic fungi has not been equaled by a corresponding increase in knowledge of the effectors that mediate such a response. Clearly, the cell cycle machinery is an attractive target by which signaling may coordinately regulate fungal morphogenesis and cell-cell interactions, and thereby virulence. Therefore, the fungal capability to modify its cell cycle must be an important determinant in carrying out a successful infection. The challenge of this new view to define virulence in fungi is to put together the expertise in well-known fungal model systems and pathogenic fungi to coin a new research topic: regulation of the fungal pathogenic cell cycle (Pathocycle). For this goal to be achieved, it is necessary to understand which events regulate progression through the pathogenic cell cycle, what makes non-pathogenic cell types to exit the cell cycle to differentiate into the pathogenic cell types, and how gene networks contribute to the formation of infection structures. From this field, an important increase in our knowledge of the pathogenic process and specially the definition of new targets to design antifungal therapies is expected. To understand how cell cycle is modified during pathogenic development will contribute significantly in settling the basis for a better understanding of the fungal pathogenic process and how to cope with it.

Acknowledgments This review summarizes the work performed in the laboratory of JPM since 2000. Along these years, the continuous and generous support of Regine Kahmann by providing technical help, material, and discussion has been crucial and determinant for the development of the topic. To enter into *Ustilago maydis* field represented for JPM an acceptance with open arms and friendship by the entire *Ustilago* community. JPM want to thank members of Max-Planck-Institut für Terrestrische Mikrobiologie at Marburg for hospitality and the availability to discuss any topic related to *Ustilago*. JPM really appreciate the friendship of Gero Steinberg, who helped me enter into the cell biology area; Michel Feldbrügge, who provided me with vectors and lots of ideas; Jöerg Kämper, for a lot of productive discussions and Andreas Brachmann, for smashing my head with questions. JPM also acknowledges the gift of Fig. 5 and critical reading of the manuscript from Gero Steinberg and the help with the English language from my wife, Loreto Carmona. The careful editing labor of one of the anonymous reviewers is also appreciated. The work of JPM is supported by the MCyT (BIO2005-02998).

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