Sustained cell polarity and virulence in the phytopathogenic fungus *Ustilago maydis* depends on an essential cyclin-dependent kinase from the Cdk5/Pho85 family

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Accepted 19 March 2007
Journal of Cell Science 120, 1584-1595 Published by The Company of Biologists 2007
doi:10.1242/jcs.005314

Summary

Cyclin-dependent kinases from the Cdk5/Pho85 family are thought to play important roles in morphogenesis in organisms as diverse as yeast and humans. Here we used the corn smut fungus *Ustilago maydis* to address the role of Cdk5/Pho85 kinases in the morphogenesis and virulence of dimorphic phytopathogens. We found that Cdk5 is essential for growth in *U. maydis*. A temperature-sensitive *cdk5* mutant caused cell wall and morphology defects at the restrictive temperature. Actin patches labeled with a fimbrin-GFP fusion protein were delocalized and a GFP-Myo5 fusion was directed towards the growing cell pole and rapidly dissociated from the tip. These defects were found to be due to an impairment in the maintenance of cell polarity. Our results indicated that Cdk5 is required for the activity of Rac1, probably at the level of the localization of its GEF, Cdc24. Cdk5 was required for full virulence, probably because mutant cells are unable to sustain the dramatic polar growth required for the formation of the infective structures. These results support a major role for morphogenesis in the virulence program of dimorphic fungi.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/9/1584/DC1

Key words: Cdk5, Corn smut, Cell polarity, Morphogenesis, *Ustilago maydis*

Introduction

Cell polarity is of fundamental importance for proliferation, differentiation and morphogenesis of both unicellular and multicellular organisms. The ability of cells to dynamically reorganize their cytoskeleton is crucial for rapid responses to environmental changes, as well as for the differentiation of distinct cell types during development. Therefore, cell polarity is controlled by both intra- and extracellular signals, which must be integrated to give the final shape of every particular cell. In mammals, Cdk5 represents an important example of a master element controlling different aspects of polarity, which allows efficient cytoskeleton remodeling in migrating neurons in response to external signals (Xie et al., 2006). Cdk5 belongs to a family of cyclin-dependent kinases that seem to be involved in the control of cell differentiation and morphology rather than cell division (Dhavan and Tsai, 2001). Cdk5 and its activating subunit, p35, have been implicated in neuronal differentiation (Nikolic et al., 1996; Xie et al., 2006). Loss of Cdk5 in neurons resulted in abnormal organization with no recognizable pattern of neurite arrangement (Chae et al., 1997). Among the different targets of Cdk5 there are Rho-like proteins such as Rac1 (Nikolic et al., 1998) and RhoA (Kawauchi et al., 2006), as well as cytoskeleton regulators such as Fak1 (Xie et al., 2003) and NUDEL (Niethammer et al., 2000).

Like neurons, fungal cells also provide an extraordinary example of polarized cell growth that efficiently responds to environmental cues. Interestingly, functional homologs of Cdk5 have been described in fungi (Huang et al., 1999; Nishizawa et al., 1999; Dou et al., 2003). The founding member of this family, the Pho85 cyclin-dependent kinase, was isolated in *Saccharomyces cerevisiae* because of its involvement in the regulation of phosphate-scavenging enzymes, and it has been implicated in many other cellular processes, including stress adaptation, glycogen storage, cell cycle progression and morphogenesis (for a review, see Carroll and O'Shea, 2002). The Pho85 kinase shows genetic interactions with genes encoding proteins involved in cell polarity such as Cdc42, its regulators (i.e. Bem1 and Cdc24) and downstream effectors (i.e. Cla4) (Lenburg and O'Shea, 2001; Huang et al., 2002; Moffat and Andrews, 2004). In the filamentous fungus *Aspergillus nidulans* two highly related Cdk5-like proteins, PhoA and PhoB, also play important roles integrating environmental conditions and developmental responses (Bussink and Osmani, 1998; Dou et al., 2003). Interestingly, although *S. cerevisiae* Pho85 is not essential for growth, deletion of both *A. nidulans phoA* and *phoB* caused lethality (Dou et al., 2003). The Cdk5 orthologs of *A. nidulans* seem to have roles in polarized growth. Conidia of a $phoA^{ts}/\Delta phoB$ mutant strain under restrictive conditions were unable to initiate polarized growth once germinated but instead continued isotropic growth (Dou et al., 2003).

The ability to induce strong polar growth is particularly dramatic in dimorphic fungi, which are able to produce a yeast-to-hypha transition in response to environmental conditions. Dimorphic transitions are common among pathogenic fungi and are often associated with a change in virulence (Gow et al., 2002). Therefore, proteins that control dimorphic transitions probably play major roles in microbial adaptation to new environments and in pathogenicity. Surprisingly, in spite of the apparent importance of the role of Cdk5-like kinases in morphogenesis, little is known about the role of these kinases in dimorphic fungi. To date, the presence of members of the Pho85/Cdk5 family has been described in dimorphic fungi such as *Candida albicans* (Miyakawa, 2000) and *Sporothrix schenckii* (de Jesús-Berrios and Rodríguez del Valle, 2002), but no further characterization of these regulators has been reported. In this study we took advantage of the plant pathogen *Ustilago maydis,* a known model system to study dimorphism and virulence (Bölker, 2001), to characterize the roles that Cdk5/Pho85-like kinases may have in pathogenic dimorphic fungi. During maize infection, *U. maydis* undergoes a dimorphic transition from budding, yeast-like cells to a filamentous dikaryon that proliferates in the host. This transition is regulated by mating and environmental signals. Here, we describe the identification and characterization of Cdk5, a Cdk5/Pho85 homolog in *U. maydis*. We provide evidence that this kinase is essential for growth and has a major role in the maintenance of cell polarity in this phytopathogen. Using temperature-sensitive mutants, we show that Cdk5 is required at all morphological stages of *U. maydis* – the yeast-like sporidia, the conjugation tubes and the dikaryotic hyphae – thereby affecting its ability to infect plants.

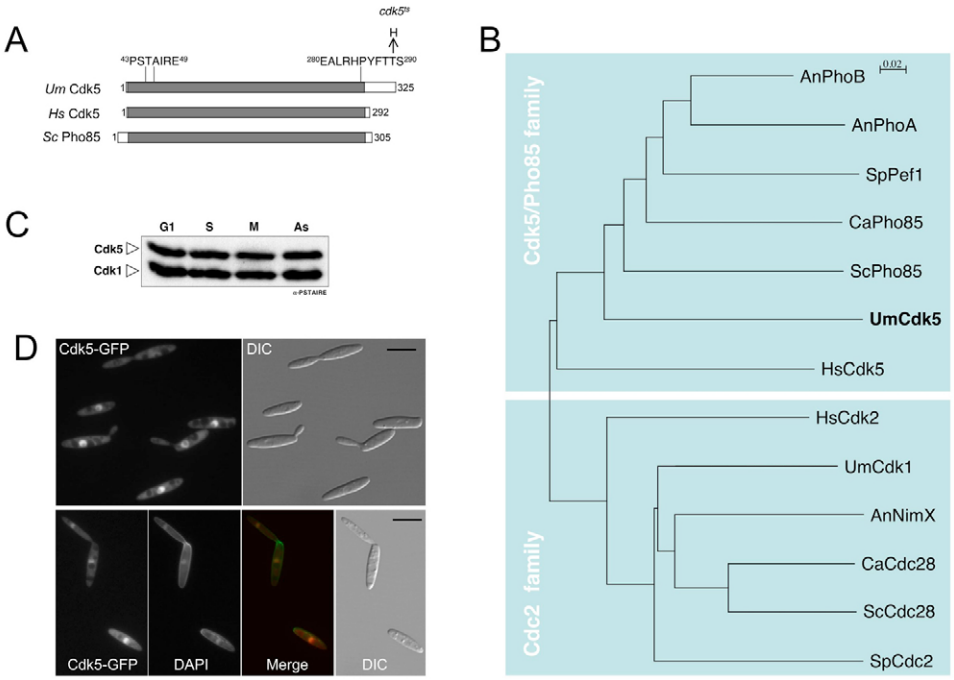

Fig. 1. Cdk5 in *U. maydis*. (A) Schematic representation of the *U. maydis* Cdk5 protein in relation to human Cdk5 (*Hs*Cdk5) and *S. cerevisiae* Pho85 (*Sc*Pho85). The catalytic kinase domains are shown as gray boxes and were identified using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de). (B) Dendrogram of CDK-like proteins. The tree was reconstructed using the ClustalW method (http://www.ebi.ac.uk/clustalw/). Bar, 0.02 substitutions per amino acid. Note that *U. maydis* Cdk5 falls into the Cdk5/Pho85 subfamily of CDKs. An, *Aspergillus nidulans*; Sp, *Schizosaccharomyces pombe*; Ca, *Candida albicans*; Sc, *Saccharomyces cerevisiae*; Um, *Ustilago maydis*; Hs, *Homo sapiens.* (C) Protein levels at different stages of the cell cycle. Extracts from FB1 arrested at S or M phase, G1 phase enriched or an asynchronous culture (As) were assayed by western blot analysis using anti-PSTAIRE antibodies. Both Cdk1 and Cdk5 kinases were detected. (D) Cdk5-GFP appears in the cytoplasm and nucleus. Cells carrying a C-terminal Cdk5-GFP fusion were grown at exponential phase in CMD medium. Subcellular distribution of Cdk5-GFP (upper panels): cells at different phases of the cell cycle showed GFP signal in the nucleus. Only cells carrying large buds (i.e. undergoing mitosis) do not show nuclear fluorescence, probably because of disassembly of the nuclear envelope. In the lower row, DAPI staining co-localizes with the GFP signal. Bar, 10 μm.

Results

Cdk5, a Cdk5/Pho85-like kinase that is essential in *U. maydis*

To identify a Cdk5/Pho85 homolog in *U. maydis* we searched its genome (Kämper et al., 2006) for open reading frames showing high sequence similarity with human Cdk5 as well as *S. cerevisiae* Pho85 sequences. We found two hits with E values lower than e^{-80}. The first one corresponded to an uncharacterized protein that we named Cdk5 (GenBank accession number EF190891). The second hit corresponded to Cdk1, the mitotic cyclin-dependent kinase (CDK) from *U. maydis* (García-Muse et al., 2004). Both Cdk1 and the predicted Cdk5 protein retain the PSTAIRE domain characteristic of CDK molecules involved in cyclin binding (Fig. 1A). Western blot assays using anti-PSTAIRE antibodies, showed that Cdk5 protein levels do not change throughout the cell cycle (Fig. 1C). We believe that Cdk5 represents a true Cdk5/Pho85 homolog because a dendrogram analysis placed Cdk5 and Cdk1 in different subfamilies, with Cdk5 in the Cdk5/Pho85 subfamily of CDK proteins (Fig. 1B). To further analyze the function of Cdk5, we replaced one *cdk5* allele in the diploid FBD11 strain with a null allele carrying a nourseotricine resistance (Nat^R) marker. As a control we constructed a diploid strain carrying a Nat^R-marked *cdk5* allele producing a functional C-terminal Myc-tagged protein. After sporulation, we analyzed the meiotic progeny of these strains and we did not find any Nat-resistant cells among the haploid offspring of the diploid strain carrying the Δ*cdk5* null allele (*n*=50 spores analyzed). By contrast, roughly half of the cells in the progeny of the diploid strain carrying the Myc-tagged *cdk5* allele were Nat resistant (52.4% resistant in *n*=30 spores

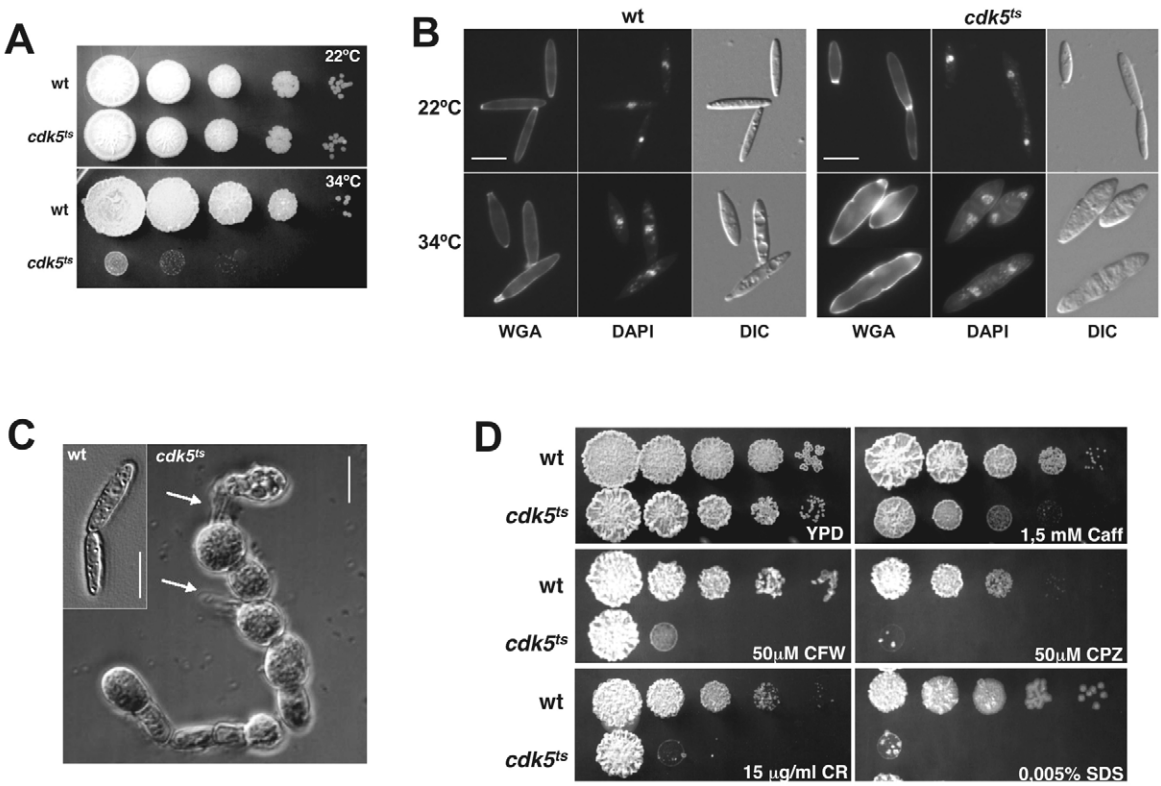

Fig. 2. Characterization of a temperature-sensitive *cdk5*^{ts} mutant. (A) Growth of FB1 (wt) and *cdk5*^{ts} strains at 22°C and 34°C in solid complete medium (CMD). At low temperature the growth of control cells and *cdk5*^{ts} is similar. However, *cdk5*^{ts} cells show severe growth defects at 34°C. (B) Morphology of *cdk5*^{ts} cells after incubation at the restrictive temperature (34°C) for 8 hours. FITC-labeled wheat germ agglutinin (WGA) signal, which marks chitin-rich areas, is restricted to the growing tips in wild-type cells at any temperature, whereas in the *cdk5*^{ts} mutant the polar staining is lost at high temperature and WGA staining is concentrated in lateral walls. Nuclei were stained with DAPI. Bars, 10 μm. (C) After 12 hours at the restrictive temperature, cells carrying the *cdk5*^{ts} allele frequently lyse as evidenced by the presence of cell 'ghosts' (arrows). By contrast, wild-type cells growing in the same conditions are cigar shaped and form polar buds (inset). Bars, 10 μm. (D) Sensitivity of *cdk5*^{ts} cells to cell wall inhibitors. Cultures of wild-type (FB1) and *cdk5*^{ts} strains were grown overnight at the permissive temperature, then diluted to an OD_{600} of 1.0. Tenfold serial dilutions were made and 2 μl of each was plated in solid YPD medium treated with the indicated compounds. The plates were grown for 4 days at 22°C.

analyzed). These results indicated that *cdk5* is essential in *U. maydis*.

To investigate the subcellular localization of the Cdk5 protein, we introduced a green fluorescent protein (GFP) fusion at the end of the chromosomal *cdk5* gene in wild-type cells. The C-terminal tagging did not interfere with the function of the Cdk5 protein and the tagged strain grew as well as wild-type cells. GFP-tagged Cdk5 was distributed throughout the cytoplasm and concentrates within the nucleus (Fig. 1D). The GFP signal seems to be associated with the nucleus through the different cell cycle phases (Fig. 1D, upper panels), although the nuclear accumulation was lost in large-budded cells (i.e. those undergoing mitosis), which could be explained by the nuclear envelope breakage that occurs during mitosis in *U. maydis* (Straube et al., 2005).

A temperature-sensitive *cdk5* mutant shows altered morphology and abnormal distribution of cell wall components

To gain further insight into the role of Cdk5 in *U. maydis* we generated a temperature-sensitive mutant allele (*cdk5*^{ts}) that contained a point mutation (Y286H) in a conserved region at the C-terminus that confers temperature sensitivity to other CDKs, such as *A. nidulans* PhoA and NimX and *S. pombe* Cdc2 (Osmani et al., 1994; Dou et al., 2003). Cells carrying the *cdk5*^{ts} allele were unable to grow in solid complete medium (CMD) at the restrictive temperature (34°C) whereas at the permissive temperature (22°C) they grew at a similar rate as the wild-type strain (Fig. 2A).

We analyzed the morphology of *cdk5*^{ts} cells in liquid complete medium (CMD). At the permissive temperature cells had a wild-type appearance, with only minor defects such as a less-elongated cell body (Fig. 2B, left panel). After they were shifted to 34°C for 8 hours, mutant cells frequently arrested their growth as ellipsoidal cell doublets (Fig. 2B, right panel), with each cell carrying a single nucleus. Fluorescence-activated cell sorting (FACS) analysis indicated a 2C DNA content in each doublet, suggesting that each nucleus was arrested at G1 phase (see supplementary material Fig. S1). In wild-type cells, FITC-labelled wheat germ agglutinin (WGA), a lectin that binds to oligomeric chitin (Nagata and Burger, 1974), accumulated at the tip of the cells as well as in newly formed septa during cell separation. By contrast, in *cdk5*^{ts} cells, WGA reacted strongly with the lateral cell walls (Fig.

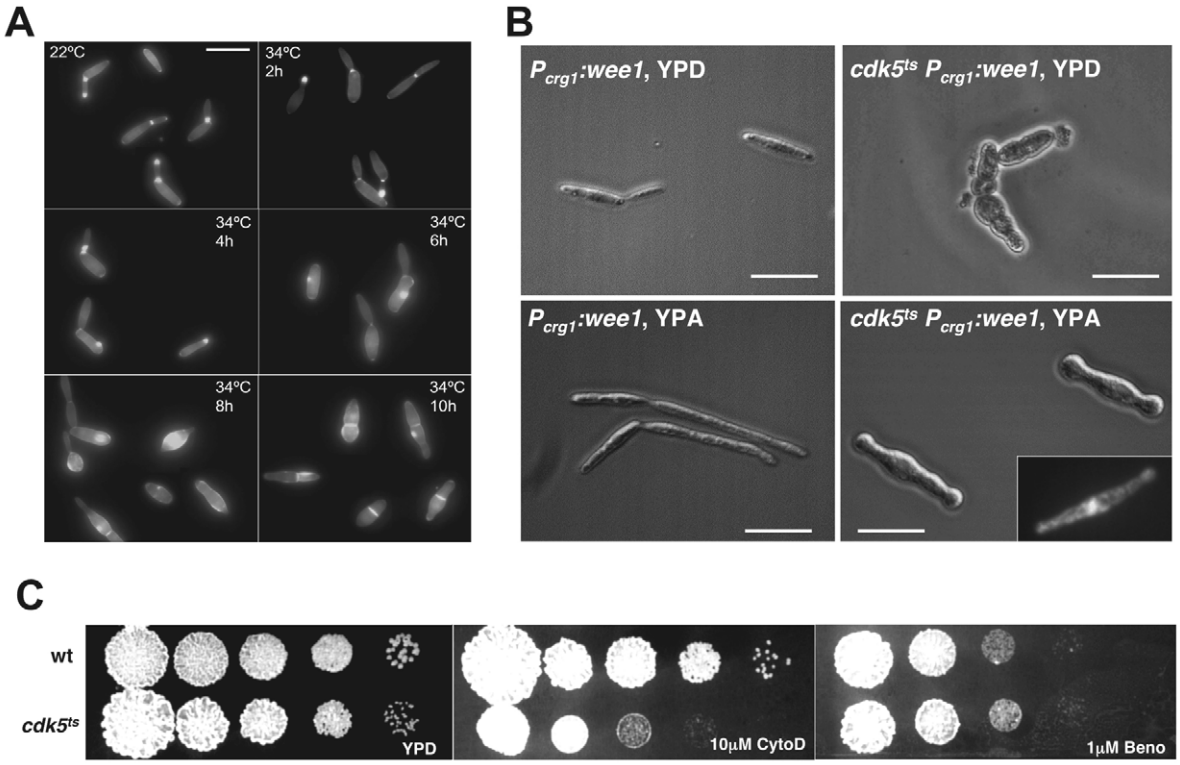

Fig. 3. *cdk5*^{ts} cells are affected in polarity. (A) Calcofluor staining of *cdk5*^{ts} cells grown in liquid CMD medium for different times at the restrictive temperature (34°C). Note that upon temperature shift, *cdk5*^{ts} cells expand their diameter and lose their bud constriction, giving cells separated by septation. Bar, 20 μm. (B) Defects in polar growth during G2 arrested *cdk5*^{ts} cells. Cells overexpressing *wee1* arrest at G2 producing elongated buds. However, in *cdk5*^{ts} cells there is no bud formation and instead two areas of isotropic growth at the poles are apparent. Inset, DAPI staining showing that in *cdk5*^{ts} cells there is only one nucleus in agreement with a G2 cell cycle arrest. Bars, 10 μm. (C) Effect of cytoskeletal inhibitors on plate growth of wild-type and *cdk5*^{ts} strains. No difference between *cdk5*^{ts} and wild-type cells was found in the presence of the MT-destabilizing drug benomyl (1 μM). By contrast, *cdk5*^{ts} cells are more sensitive to the actin inhibitor cytochalasin D (CytoD; 10 μM).

2B), suggesting an anomalous cell wall in *cdk5*^{ts} cells. To address whether the associated defects were reversed by temperature shift-down, the mutant cells grown at the permissive temperature of 22°C were shifted up to the restrictive temperature of 34°C and kept there for 6 hours, and then shifted down to 22°C and incubated for several hours. After the shift-down of the culture, the cell poles were re-established, and the morphology returned to a wild-type appearance (see supplementary material Fig. S2). However, we observed that *cdk5*^{ts} cells incubated at the restrictive temperature for long periods (more than 12 hours) had a strong lysis phenotype as evidenced by the frequent presence of cell 'ghosts' and cell debris (Fig. 2C arrows). As these results were coherent with a cell wall defect, we tested the sensitivity of *cdk5*^{ts} cells to compounds described to affect cell wall integrity (i.e. caffeine, Congo Red, Calcofluor White, chlorpromazine and sodium dodecyl sulfate) (Levin, 2005). Interestingly, at the permissive temperature, *cdk5*^{ts} cells showed a greater sensitivity than wild-type cells to all these compounds (Fig. 2D). However, we found that the temperature-sensitive growth and cell lysis defect of the mutant were not rescued by 1M sorbitol, an osmotic stabilizer known to rescue cell wall defects (see supplementary material Fig. S3B,C), suggesting that the primary defect might not be a defective cell wall structure.

cdk5^{ts} cells show defects in polar growth

To further characterize the morphogenetic defects caused by the *cdk5*^{ts} mutation, samples incubated at restrictive conditions for different times were stained with calcofluor to visualize zones of active growth (Wedlich-Söldner et al., 2000). Early in the time course (Fig. 3A), between 2 and 4 hours after the temperature shift, cells were able to bud, although the mother cell tended to swell, giving thicker cells. This morphology change was accompanied by a misplaced accumulation of calcofluor staining (around 60% of the cells showed delocalization of calcofluor deposition at 6 hours). Later in the time course, cells stopped budding, became more spherical and formed septa, as well as accumulated calcofluor in the lateral cell walls. These results suggested that the observed cell wall defects and ellipsoidal morphology could be a consequence of impaired cell polarization in the absence of Cdk5.

In *U. maydis* the formation of the bud takes place at the G2 phase, the cell cycle stage at which polar growth reaches it maximum level (Straube et al., 2003). Since we observed that *cdk5*^{ts} cells incubated at restrictive conditions arrested mostly at G1, we reasoned that the apparent defect in polarization could be a consequence of the cell cycle arrest in a cell stage not suitable for polar growth. To distinguish whether the morphogenetic defect of *cdk5*^{ts} was a direct cause of a lack of

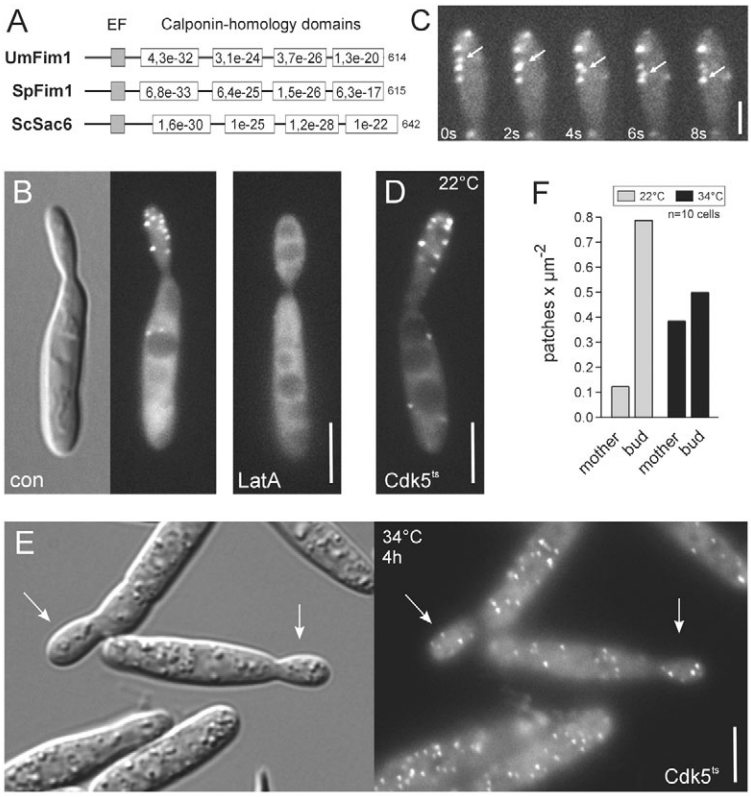

Fig. 4. Actin organization in *cdk5*^{ts} cells. (A) *U. maydis* contains a fimbrin-like gene, which is predicted to encode a protein of 615 amino acids that shares a similar domain structure with *S. pombe* Fim1 (SpFim1) and *S. cerevisiae* Sac6 (ScSac6). The *P*-values of the predicted Calponin-homology domains are given in the open boxes. (B) A Fim1-GFP fusion protein localizes to patches within the growing bud of many cells. This localization disappears after treatment with Latrunculin A, indicating that the patches are composed of F-actin. Bar, 3 μm. (C) Fim1-GFP containing actin patches are highly dynamic and move over short distances at the growth region (arrow). Elapsed time is given in seconds. Bar, 2 μm. (D) In temperature-sensitive *cdk5*^{ts} mutants at the permissive temperature (22°C) Fim1-GFP locates in the bud. Bar, 3 μm. (E) After 4-5 hours at the restrictive temperature (34°C) patches appear at the mother cell and the bud. Bar, 5 μm. (F) Statistics of E. In budding *cdk5*^{ts} mutant cells at the permissive temperature, most patches are located in the bud (22°C, gray bars), whereas are almost equally distributed between the mother cell and the bud at the restrictive temperature (34°C, black bars).

polarity or just a consequence of a G1 cell cycle arrest, we arrested cells in G2 phase by overproduction of the mitotic inhibitor Wee1 (Sgarlata and Pérez-Martín, 2005) and then shifted them to 34°C. In a strain carrying a wild-type *cdk5* allele, Wee1-induced G2 arrest resulted in a continuous polar growth in the bud apex leading to cells with elongated buds (Fig. 3B). However in the strain carrying the *cdk5*^{ts} allele, no elongated buds were observed, although a strong swelling at both cell tips was detected. These cells were G2 arrested as they showed a single nucleus (Fig. 3B, inset) with a 2C DNA content (not shown). This result indicated that depletion of Cdk5 resulted in a lack of polar growth even in the G2 phase of the cell cycle.

The polarity defective phenotypes caused by the *cdk5*^{ts} mutation could reflect an underlying defect in cytoskeletal organization or function. Therefore, we monitored the effect of cytoskeleton inhibitors on wild-type and *cdk5*^{ts} cells at the permissive temperature (Fig. 3C). In the presence of 1 μM benomyl, a microtubule destabilizer, no difference in growth on solid medium was observed between wild-type and *cdk5*^{ts} cells. By contrast, *cdk5*^{ts} cells were more sensitive than the wild type to the actin inhibitor cytochalasin D, suggesting that the actin cytoskeleton was affected.

Polar distribution of the actin patches is affected in *cdk5*^{ts} mutant cells

It was previously demonstrated that polar growth of *U. maydis* requires an intact actin cytoskeleton (Fuchs et al., 2005). Therefore, we labeled F-actin by tagging the endogenous copy of the fimbrin-like gene *fim1* (accession number XM755822.1). *U. maydis* Fim1 is 61% identical to *Schizosaccharomyces pombe* Fim1 and 62% identical to *S. cerevisiae* Sac6, and shares a similar domain structure (Fig. 4A; *P*-values for the predicted calponin-homology domains are indicated). Both ScSac6p and SpFim1 are components of actin patches (Adams et al., 1989; Huckaba et al., 2004; Pelham and Chang, 2001) in which they are thought to organize F-actin (Adams et al., 1991). Consistent with its assumed role in actin patches, a fusion protein of UmFim1 and GFP localized to patches in the growing bud in wild-type background cells (Fig. 4B, 'con') and only a minor fraction was found in the mother cell (not shown). After treating the cells with 50 μM Latrunculin A for 30 minutes, this polar localization of Fim1-GFP was lost (Fig. 4B, LatA), suggesting that Fim1-GFP concentrated in actin patches. Consistent with this conclusion, Fim1-GFP patches showed a high turn-over (not shown) and occasionally moved within the cell (Fig. 4C, arrow), a behavior that is typical for actin patches in *S. cerevisiae* (Huckaba et al., 2004). In *cdk5*^{ts} mutant cells at 22°C, actin patches containing Fim1-GFP also concentrated in the bud (Fig. 4D), but this distribution was lost after 4-5 hours at the restrictive temperature and patches were randomly distributed within the mother cell and the bud (Fig. 4E,F; bud indicated by arrow).

Cdk5 is important for the stabilization of polarity

The loss of polarization of actin patches observed in *cdk5*^{ts} cells could be explained either by a defect in the initiation of polar growth (i.e. establishment of polarity) or by a defect in the maintenance of the polar growth. To distinguish between these possibilities, we introduced a fusion protein of GFP and Myo5, a class-V myosin from *U. maydis* (Weber et al., 2003), into the *cdk5*^{ts} mutant background. In wild-type cells (Fig. 5A), the GFP-Myo5 protein concentrated at the bud tip and transiently appeared at the distal cell pole, where it is thought

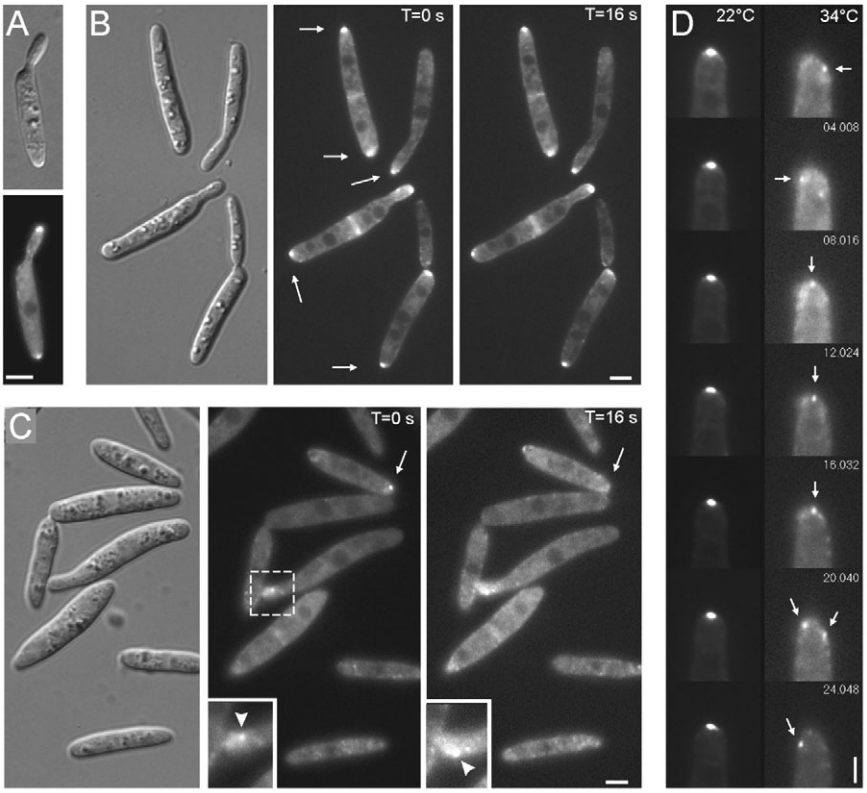

Fig. 5. Distribution of GFP-tagged myosin 5 in *cdk5*^{ts} mutants. (A) In the control strain FB1, a biologically active GFPMyo5 fusion protein localizes to the growing tip of the bud and the distal cell pole, in which it is thought to prepare the next bud site. Bar, 3 μm. (B) In budding *cdk5*^{ts} mutant cells at the permissive temperature, GFP-Myo5 is also concentrated at the growing cell poles (arrows). In addition, GFP-Myo5 is found at septa that are, in contrast to control cells, often found in the middle of the mother cell. The polar localization of GFP-Myo5 remains constant over time (compare T=0s and T=16s). Bar, 3 μm. (C) At restrictive temperature (34°C, 4-5 hours), some *cdk5*^{ts} cells still contain a polar cluster of GFP-Myo5 (arrow at T=0s and box). However, cells only transiently accumulate GFP-Myo5 at the cell pole (arrow in T=16s). In cases where the GFP-Myo5 spot remains at one cell pole, it rapidly rearranges at the putative growth region (arrowheads in detail). Bar, 3 μm. (D) Time series of cell poles of *cdk5*^{ts} cells show that the GFP-Myo5 accumulation at the growth region remains stationary at the permissive temperature (left series, 22°C), but undergoes rapid rearrangement at restrictive temperature (arrow in right series, 34°C). Elapsed time is given in seconds:milliseconds. Bar, 2 μm.

to mediate actin-dependent secretion (Weber et al., 2003). A similar distribution was found in *cdk5*^{ts} cells at 22°C (Fig. 5B, arrows) in which the polar GFP-Myo5 cluster remained stationary over time (Fig. 5B, compare T=0s and T=16s). By contrast, at the restrictive temperature (34°C, 4-5 hours) GFP-Myo5 polar accumulation was absent in many cells (Fig. 5C). Occasionally, GFP-Myo5 concentrated at cell poles but this localization was only transient (arrow in Fig. 5C, compare T=0s and T=16s). A more detailed analysis revealed that at the restrictive temperature, GFP-Myo5 signal appeared at the cell poles, but disappeared rapidly (Fig. 5C, inset; Fig. 5D, 34°C), a behavior that was never found in control cells (not shown) or *cdk5*^{ts} mutants at the permissive temperature (Fig. 5D, 22°C; see also supplementary material Movie 1). These results indicate that Cdk5 does not seem to be involved in the initial recruitment of Myo5 to polarization axes, but it is required for its subsequent retention. In other words, these data strongly suggest that Cdk5 is required to stabilize the polarization of the cell.

Cdc24 overexpression bypassed the requirement of Cdk5 for polar growth induction but did not suppress *cdk5*^{ts} allele lethality

The amplification of polarization signals and subsequent stabilization of polarity axes have been recently shown in *S. cerevisiae* to be dependent on the Rho-like protein Cdc42 (Nern and Arkowitz, 2000; Irazoqui et al., 2003; Wedlich-Söldner et al., 2003). In dimorphic fungi, the Rho-like proteins Rac1 and Cdc42 play essential roles in cytoskeleton organization and polarization (Hurtado et al., 2000; Ushinsky et al., 2002; Boyce et al., 2003; Vallim et al., 2005; Bassilana and Arkowitz, 2006; Mahlert et al., 2006). In *U. maydis*, Rac1 is required to sustain polar growth, whereas Cdc42 has a role in cell separation (Mahlert et al., 2006). Since Cdk5 seems to be required for the stabilization of polarity axes in *U. maydis*, we wondered whether it acts in the same genetic cascade as Rac1. *rac1* overexpression is sufficient to induce filament formation in *U. maydis* (Mahlert et al., 2006) (Fig. 6A). However, the overexpression of *rac1* in a strain carrying the *cdk5*^{ts} allele at the restrictive temperature did not result in filament formation (Fig. 6A). Therefore, Cdk5 was required for Rac1-induced polar growth. To address whether Cdk5 is a downstream effector or an upstream activator of Rac1 we took advantage of the constitutively active *rac1*^{Q61L} allele, whose expression in wild-type cells produces a delocalized isotropic cell wall extension (Mahlert et al., 2006) (Fig. 6A). We overexpressed the *rac1*^{Q61L} allele in *cdk5*^{ts} cells and we found that it produced the same effect in *cdk5*^{ts} mutant cells as in wild-type cells (Fig. 6A). As *rac1*^{Q61L}-mediated activity is independent of upstream activation, this result suggests that Cdk5 might be acting as an upstream activator of Rac1. Rho-like proteins cycle between an inactive GDP-bound and an active GTP-bound state. GDP/GTP cycling is regulated positively by a guanine-nucleotide exchange factor (GEF). The *U. maydis* Cdc24 homolog is highly likely to be a Rac1 GEF: it localizes at the bud tip in growing cells; physically interacts with Rac1; and induces filamentation upon overexpression in a *rac1*-dependent manner (I.A.-T. and J.P.-M., unpublished results) (see also Mahlert et al., 2005). We wondered whether Cdk5 was acting at the level of Cdc24, and therefore we overexpressed *cdc24* in cells carrying the *cdk5*^{ts} allele. Strikingly, we found that *cdc24* overexpression was able to induce polar growth in *cdk5*^{ts} cells (Fig. 6A), supporting the notion that Cdk5 promotes Rac1 activation via Cdc24. To

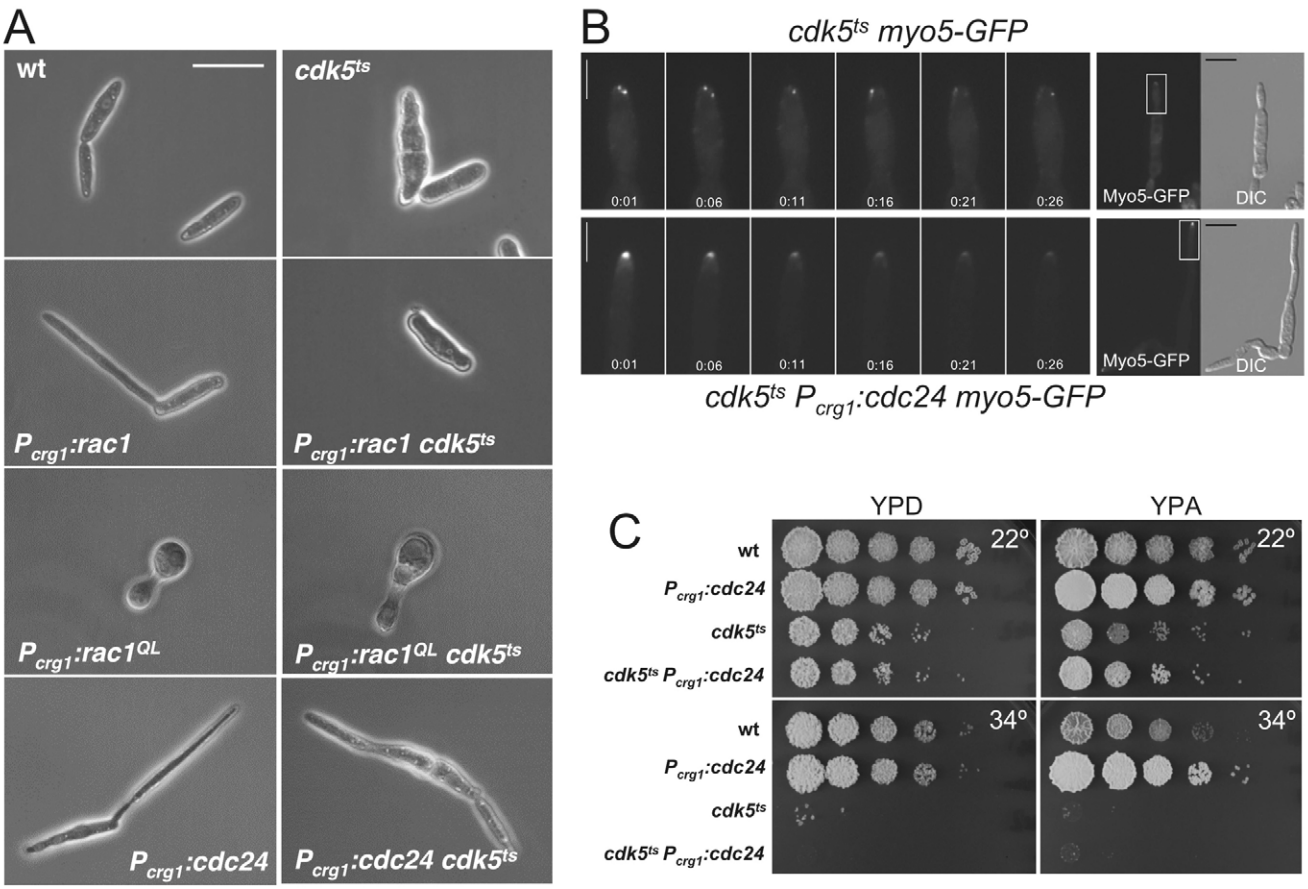

Fig. 6. Cdc24 overexpression bypassed the requirement of Cdk5 for polar growth. (A) Overexpression of *cdc24* induces polar growth in *cdk5*^{ts} cells. Wild-type and *cdk5*^{ts} cells, as well as derivates carrying constructions for overexpression under the *crg1* promoter (repressed in glucose, induced in arabinose) of *rac1*, *rac1*^{Q61L} and *cdc24* were grown at the permissive temperature until OD_{600}=0.2 and then shifted to the restrictive temperature (34°C) in arabinose-containing complete medium for 5-6 hours. (B) Time series of cell poles of a *cdk5*^{ts} *myo5-GFP* cell as well as a *cdk5*^{ts} *myo5-GFP* cell overexpressing *cdc24* at the restrictive temperature. Note that in the *cdk5*^{ts} cell the GFP-Myo5 accumulation at the growth region undergoes rapid rearrangement at the restrictive temperature, but when overexpressing *cdc24*, the GFP-Myo5 signal remains stationary. Elapsed time is given in minutes:seconds. Bars, 3 μm. (C) Lack of suppression of temperature-sensitive growth of *cdk5*^{ts} cells after *cdc24* overexpression. Wild-type and *cdk5*^{ts} cells carrying a construction overexpressing *cdc24* under the control of the *crg1* promoter were spotted in inducing (YPA) and non-inducing (YPD) conditions and incubated at permissive (22°C) and restrictive (34°C) conditions.

address whether *cdc24* overexpression might counterbalance the failure of *cdk5*^{ts} cells to maintain the polarity, we analyzed the localization of Myo5-GFP in the *cdc24*-overexpressing cells. We found that high levels of Cdc24 suppressed the inability of the *cdk5*^{ts} cells to retain the Myo5-GFP signal at the growing tip (Fig. 6B and supplementary material Movies 2 and 3).

Since *cdc24* overexpression was able to bypass the requirement of Cdk5 for the maintenance of the polar growth in *U. maydis* cells, we analyzed whether the cell growth defect of *cdk5*^{ts} was rescued by high levels of Cdc24. However, *cdc24* overexpression did not suppress the growth defect of *cdk5*^{ts} cells at restrictive temperature (Fig. 6C). It is noteworthy that although *cdk5*^{ts} cells growing at 34°C and expressing high levels of *cdc24* were able to polarize, they failed to form long filaments. Instead they lysed shortly after polarization has occurred (data not shown), suggesting that Cdk5 has additional roles, most likely related with the ability to stabilize the cell wall.

Localization of Cdc24 at the growing tip is dependent on Cdk5

We analyzed the localization of Cdc24 to the incipient bud in *cdk5*^{ts} cells, as it could be a key determinant in the establishment and maintenance of an axis of polarity through activation of Rac1. Strikingly, in *cdk5*^{ts} cells incubated under permissive conditions it was possible to find Cdc24 at the growing pole of the cell, as in wild-type cells, whereas under restrictive conditions fluorescence was dispersed throughout the cell with no discrete accumulation at the cell tip (Fig. 7A,B). The levels of Cdc24-GFP fusion protein were not affected by the temperature shift, as assessed by western blot (not shown). These results supported the notion that Cdk5 could be required to stabilize Cdc24 at the cell tip. The ability to keep Cdc24 at the tip could be involved in the induction of a positive feedback loop that stabilizes polarity axes in this fungus, as it has been proposed for Cdc24 in budding yeast (Butty et al., 2002).

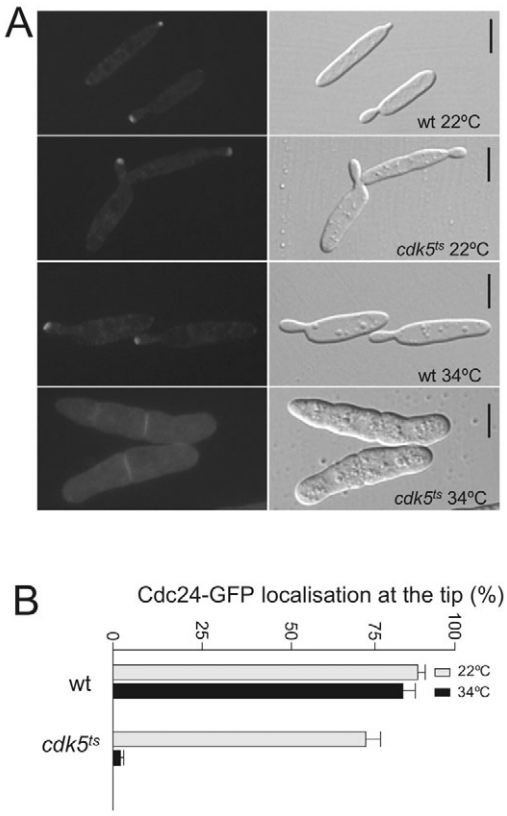

Fig. 7. Cdk5 is required for polar localization of Cdc24. (A) Localization of Cdc24-GFP was analyzed in wild-type and *cdk5*^{ts} cells grown in CMD at the permissive and restrictive temperatures. In wild-type and *cdk5*^{ts} cells at the permissive temperature, Cdc24-GFP accumulates at the cell pole. By contrast, in *cdk5*^{ts} cells at the restrictive temperature, Cdc24-GFP appears to be absent from the bud tip, although a diffuse fluorescence signal was present in the cytoplasm. (B) Quantification of Cdc24-GFP localization at the tip (mean ± s.e.m.; *n*=80 cells).

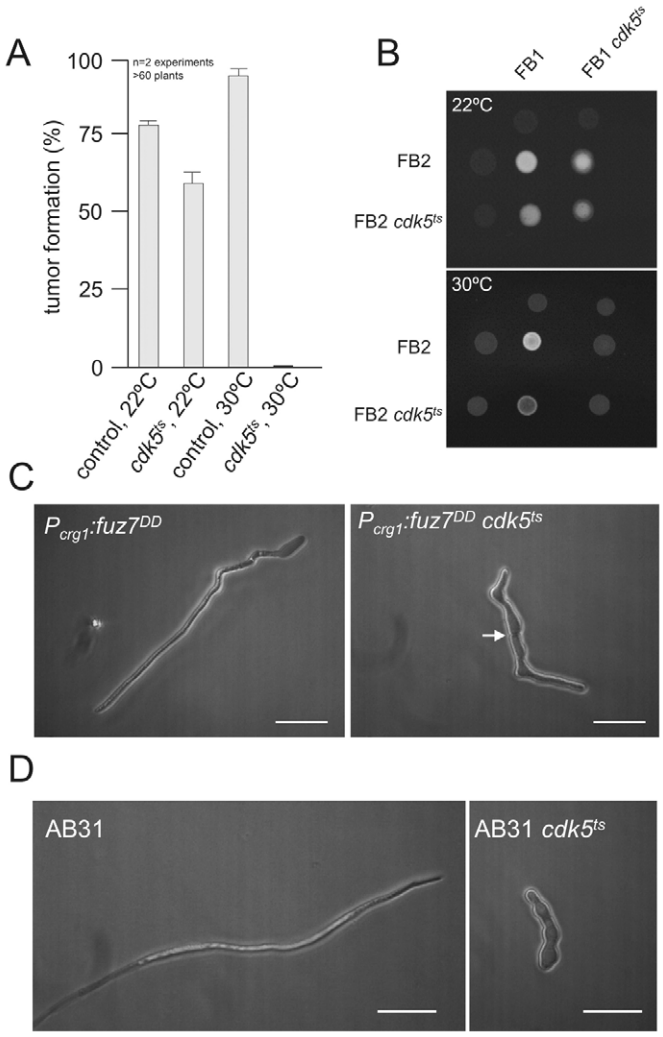

Fig. 8. Cdk5 is required for virulence. (A) Maize plants were infected with wild-type control strains and *cdk5*^{ts} mutants and incubated at 22°C and 30°C. The graph shows the quantification (mean ± s.e.m.) of tumor formation on infected maize plants at 22°C and 30°C after 14 days. (B) Mating assays of control strains FB1 × FB2, and *cdk5*^{ts} mutant strains FB1 *cdk5*^{ts} × FB2 *cdk5*^{ts} on charcoal-containing agar plates at 22°C and 30°C. (C) Formation of conjugation hyphae after Fuz7DD overexpression in cells carrying a wild-type *cdk5* allele (*Pcrg:fuz7DD*) and a *cdk5*^{ts} allele at 30°C. The image on the right shows a *cdk5*^{ts} cell doublet separated by a septum (arrow). Bar, 20 μm. (D) Formation of infective hyphae after bE/bW overexpression in cells carrying a wild-type *cdk5* allele (AB31) and *cdk5*^{ts} allele at 30°C. Bars, 20 μm.

Cdk5 is required for virulence in *U. maydis*

To determine the importance of Cdk5 in virulence, we infected maize plants with a mixture of compatible control strains FB1 and FB2 as well as the temperature-sensitive mutant strains FB1*cdk5*^{ts} and FB2*cdk5*^{ts}. To circumvent the associated growth defects of *cdk5*^{ts} cells at the restrictive temperature (34°C), infected plants were incubated at both permissive (22°C) and semipermissive (30°C) temperatures. We found that at 30°C, *cdk5*^{ts} cells were able to grow, the cell lysis defect was avoided but they still displayed clear morphological defects (see Fig. S3A in supplementary material). At 22°C, infection symptoms (measured as a percentage of tumor formation after 14 days, Fig. 8A) were found at roughly similar rates in plants infected with both wild-type and mutant cells. However, at the semipermissive temperature, *cdk5*^{ts} mutant strains failed to induce symptoms, whereas normal infection was observed in plants infected with control strains (Fig. 8A).

Plant infection by *U. maydis* requires the formation of conjugative hyphae, which, after cell fusion, establish an infective filament that invades the epidermis and continues the pathogenic program inside the plant. The formation of dikaryotic filaments can be monitored on charcoal-containing agar plates (Holliday, 1974). To further analyze the impaired virulence in *cdk5*^{ts} strains, we performed mating assays using compatible mutant strains as well as compatible control strains. At the permissive temperature, both compatible control and *cdk5*^{ts} strains fused to form a fuzzy white colony consisting of dikaryotic filaments. By contrast, at 30°C only the control cells formed fuzzy colonies, whereas *cdk5*^{ts} mutants were unable to form dikaryotic hyphae (Fig. 8B).

As polarized growth of conjugative and infecting hyphae is

proposed to play a key role during infection, we sought to analyze the role of Cdk5 in these processes. For this, we introduced the *cdk5*^{ts} allele into FB1Fuz7DD and AB31 haploid strains. In FB1Fuz7DD cells, the overexpression of an activated form of the MAPK kinase Fuz7 induces the formation of conjugative hyphae mimicking an active pheromone signaling (Fig. 8C, left panel) (Müller et al., 2003). In AB31 cells, overexpression of a compatible bW/bE heterodimer induces the formation of infective hyphae (Fig. 8D, left panel) (Brachmann et al., 2001). Strikingly, upon Fuz7DD or bW2/bE1 induction, a clear defective polar growth was observed in cells carrying the *cdk5*^{ts} allele growing under semipermissive conditions (30°C, Fig. 8C,D, right panels). These results support the notion that Cdk5 is required for sustained polar growth in *U. maydis* during pathogenic conditions, and they help to explain the absence of virulence of *cdk5*^{ts} strains.

Discussion

The functional characterization of the Cdk5/Pho85 homolog in *U. maydis* reported in this work indicated that it has an essential role most likely related to the cellular morphogenesis. Previous work has identified in *U. maydis* another essential cyclin-dependent kinase, Cdk1, a functional homolog of the cell-cycle-specific Cdc2/Cdc28 kinase (García-Muse et al., 2004). Although Cdk1 has a clear role in cell cycle progression in *U. maydis*, we believe that Cdk5 has a major role in the regulation of polarity in this fungus. In budding yeast, Pho85 appears to be required for cell cycle progression, collaborating with G1 cyclin-associated Cdc28 kinase in the degradation of the CDK inhibitor Sic1 (Nishizawa et al., 1998). We observed that *U. maydis cdk5*^{ts} cells incubated at the restrictive temperature arrested mostly at G1, suggesting a cell cycle defect. However, the accumulation of arrested cells occurs after several hours upon temperature shift, and we believe that the observed defects in cell integrity in this mutant could be responsible for the apparent G1 cell cycle arrest. Therefore, although we cannot discount a role of Cdk5 in cell cycle progression in *U. maydis*, we favor the hypothesis that defects in cell integrity could activate a sort of checkpoint mechanism arresting the cell cycle at G1 in *cdk5*^{ts} cells incubated at the restrictive temperature.

The reason for the essentiality of Cdk5-like kinases in *A. nidulans* and *U. maydis* versus the non-essential role of Pho85 in *S. cerevisiae* is unclear. An interesting possibility to explain this difference is the different amount of polar growth that occurs in these organisms. *A. nidulans* grows in a polar manner over its entire cell cycle. By contrast, budding yeast undergoes a brief period of polar growth during bud emergence, but then switches to isotropic growth, over the entire surface of the bud. In *U. maydis*, during the yeast-like phase, the bud expansion relies almost entirely on sustained polar growth (Steinberg et al., 2001). Assuming a major role of fungal Cdk5-like kinases in sustained polar growth, it is plausible that the absence of Cdk5-like activity will be more deleterious in *A. nidulans* and *U. maydis*, which depend on continued polar growth, than in budding yeast, which have only a brief period of polar growth. An alternative possibility may rely on functional redundancy with other cellular components. In *S. cerevisiae* it is well established that Pho85 becomes essential in the absence of the G1 Cdc28-associated cyclins Cln1 and Cln2 (Measday et al., 1994). In *U. maydis* there is a single G1 cyclin, which interacts with Cdk1 and appears to have cell cycle and morphogenetic roles (Castillo-Lluva and Pérez-Martín, 2005). However, we found that overexpression of this cyclin does not suppress the growth defects of *cdk5*^{ts} cells (J.P.-M., unpublished observations), suggesting that no such redundant function is present in *U. maydis*.

Our results indicated that Cdk5 is required for the accumulation of Cdc24 at the cell pole. We also observed that high levels of Cdc24 bypassed the requirement of Cdk5 to induce polarization. We believe that some of the polarization defects associated with *cdk5*^{ts} mutants at the restrictive temperature can be explained by defects in the localization of Cdc24. Cdc24 is essential for growth in *U. maydis* and cells carrying a conditional *cdc24* allele, and growing at restrictive conditions recapitulates some of the phenotypes of *cdk5*^{ts} cells grown at 34°C, including swollen cells, often in form of doublets, with a highly delocalized deposition of chitinous material in the cell wall (see supplementary material Fig. S4). In *S. cerevisiae*, polarity mutants defective in *cdc24* function exhibit similar chitin deposition as a consequence of a defect in the targeted localization of vesicles containing the enzymatic apparatus and precursors for chitin biosynthesis (Adams et al., 1990). The mechanism behind the Cdk5-dependent stabilization of Cdc24 at the cell pole is not understood. One possibility is that Cdc24 is a target of Cdk5, as it has been proposed for *S. cerevisiae* Pho85 (Moffat and Andrews, 2004), and its phosphorylation allows or enhances its interaction with some anchor structure at the membrane. Alternatively, Cdk5 may activate an anchor protein that mediates Cdc24 recruitment at the cell pole. We tried to demonstrate physical interaction between Cdc24 and Cdk5 with no reproducible results, suggesting some indirect interaction (J.P.-M., unpublished observations). Furthermore, we have no evidence of phosphorylation of Cdc24 by Cdk5 (J.P.-M., unpublished observations). In *S. cerevisiae*, the scaffolding protein Bem1 maintains Cdc24 at sites of polarized growth (Butty et al., 2002), and it is subjected to phosphorylation by different kinases (Loog and Morgan, 2005; Han et al., 2005). There is a putative *U. maydis* Bem1 homolog (accession number EAK85966), and future effort will be directed to address whether it plays a role in Cdc24 localization and whether it could be a target of Cdk5.

Strikingly, in spite of the inability to localize Cdc24 to the cell pole, in *cdk5*^{ts} cells growing at the restrictive temperature, Myo5 could be found at the cell poles, although this localization was only transient and usually disappeared rapidly. This result indicates that actin-mediated transport is still present in *cdk5*^{ts} cells at the restrictive temperature, most likely because other polarity determinants – for instance formins – can assemble actin cables. However, it seems that the elements transported to the cell end cannot be stabilized at the pole and they therefore diffuse freely. This could explain why actin patches concentrate at the tip of the bud in wild-type cells whereas in *cdk5*^{ts} mutant cells at the restrictive temperature they are distributed throughout the entire cell. It also may explain the progressive delocalization of the calcofluor staining in *cdk5*^{ts} cells incubated at restrictive conditions. In fungi, septins are diffusion barriers that specify the boundaries of the apical growth zone (Barral et al., 2000). The *U. maydis* genome contains four septin genes, from which only one (*sep3*) has

been characterized (Boyce et al., 2005). Interestingly, deletion of *sep3* results in cells with aberrant morphology which resembles that observed in *cdk5*^{ts} mutants (Boyce et al., 2005), and therefore it is tempting to propose that septins in *U. maydis* could be regulated, albeit indirectly, by Cdk5. Proper stabilization of septin assembly in *S. cerevisiae* requires the activity of the Cla4 kinase (Dobbelaere et al., 2003; Schmidt et al., 2003; Versele and Thorner, 2004), which is activated through binding to Cdc42-GTP. In *U. maydis* it is thought that Cla4 – which is required for sustained polarity and is activated by Rac1 (Leveleki et al., 2004) – could be involved in septin apparatus stabilization (Leveleki et al., 2004). Therefore, we believe that the inability to properly activate Rac1 because of the delocalization of Cdc24 in *cdk5*^{ts} cells, could result in the loss of diffusion barriers. We found that *cdk5*^{ts} shows a synthetic growth defect at the permissive temperature in cells carrying deletion of *rac1* or *cla4* (J.P.-M., unpublished observations).

It is noteworthy that although *cdk5*^{ts} cells growing at 34°C and expressing high levels of *cdc24* were able to polarize, the *cdc24* overexpression did not suppress the growth defect of *cdk5*^{ts} cells at the restrictive temperature. This result indicates that the Cdk5 kinase is important for some step additional to polarization, such as cargo delivery or fusion to the growth site, or local function of the cell wall remodeling machinery. During periods of polarized growth, cell wall remodeling must be carried out in a highly regulated manner: the growth site is loosened enough to allow expansion but not so much as to risk rupture. Additional roles of Cdk5 related with cell wall integrity are consistent with the finding that the *cdk5*^{ts} strain was more sensitive to cell wall stressors than the wild-type strain, even at the permissive temperature. We also found a synthetic lethality at the permissive temperature in strains carrying the *cdk5*^{ts} allele and a deletion of *mpk1*, encoding a MAPK kinase involved in the cell wall integrity cascade in *U. maydis* (N. Carbó, S.C.-L. and J.P.-M., unpublished results). This kind of interaction has been previously reported between *PHO85* and genes encoding components of the cell wall integrity cascade in *S. cerevisiae* (Lenburg and O'Shea, 2001; Huang et al., 2002).

A speculative model is that Cdk5 stabilizes polar growth in *U. maydis* acting at least at two levels. On one hand, regulating the polar localization of Cdc24 it could allow the activation of Rac1 that, via Cla4, could help septin assembly. Septins may control the establishment of a specialized cortical domain facilitating the generation of positional information. However, our results also suggest that Cdk5 may be involved in the correct synthesis of the cell wall, although its role has not yet been established.

From our analysis, we found that *cdk5*^{ts} mutants showed drastically reduced virulence, probably because of its involvement in the induction of the dramatic polar growth required for the infection process. Pathogenic development of many fungi requires a yeast-hypha transition, which enables the pathogen to be passively spread or to actively invade substrates by polar hyphal growth (Gow et al., 2002). These morphological changes link polarized growth to pathogenesis, emphasizing the importance of growth dynamics regulation in dimorphic fungi. Based on this report we propose that Cdk5-like kinases may play a primordial role during the infective process in pathogenic fungi, and therefore may represent an 'Achilles' heel' that can be exploited to limit fungal growth, as it has been proposed with other polarity regulators (Harris, 2006).

Materials and Methods

Strains, growth conditions and plant infections

All strains had the genetic background FB1 (*a1b1*) or FB2 (*a2b2*) (Banuett and Herskowitz, 1989) and are summarized in supplementary material Table S1. *U. maydis* cells were grown in YEP-based or complete medium (CM) (Holliday, 1974). The expression of genes under the control of the *crg1* and *nar1* promoters, FACS analysis, western blot and cell cycle arrest were all performed as described previously (Brachmann et al., 2001; García-Muse et al., 2003; García-Muse et al., 2004; Garrido et al., 2004).

Strain and plasmid constructions

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.

Deletion of *cdk5* in the diploid strain FBD11 was performed by the construction of a deletion cassette consisting of a pair of DNA fragments obtained by PCR flanking the *cdk5* open reading frame (ORF) and ligated to a nourseothricin (Nat) resistance cassette. The 5′ fragment spans nucleotide –218 to nucleotide +1 (considering the adenine in the ATG as nucleotide +1) and the 3′ fragment spans nucleotide +632 to nucleotide +1000. This cassette was introduced into the native *cdk5* locus by homologous recombination.

To construct the *cdk5-myc* allele the *cdk5* ORF without the stop codon was ligated to three copies of the *myc* epitope, marked with a Nat-resistance cassette, flanked at the 3′ end by 1 kb of the 3′ region downstream of the *cdk5* open reading frame and introduced by homologous recombination into the *cdk5* locus. The *cdk5-GFP* allele was constructed in a similar way, but instead of three copies of the *myc* epitope, the sequence encoding GFP was cloned at the 3′ end of the Cdk5 ORF.

The temperature-sensitive allele *cdk5*^{ts} was generated by PCR using a two-step mutagenesis protocol. The TAC codon (Tyr) at nucleotide position 856-858 was replaced by CAC (His). In addition, a silent point mutation (G to C) was introduced at position 870 to create a diagnostic *Sac*II site. The mutated gene was marked with a Nat resistance at the 3′ end and flanked at the 3′ end of the Nat-resistance cassette by 1 kb of the 3′ region downstream of the *cdk5* ORF and introduced by homologous recombination into the *cdk5* locus of corresponding strains.

To overexpress *rac1* under the control of *crg1* promoter, the *rac1* ORF plus 100 bp of the region downstream, were amplified by PCR from genomic DNA. The ATG codon start was exchanged with a *Bam*HI site inserted in the same translational phase as a T7 epitope cloned under the control of *crg1* in the pRU11T7 plasmid (S.C.-L., unpublished results). The T7-Rac1 protein was as functional as a wild-type untagged Rac1 protein. To construct the *rac1*^{QL} version we followed the two-step mutagenesis protocol as described (Mahlert et al., 2006) using T7-*rac1* as a template.

To overexpress *cdc24*, a fragment carrying the *cdc24* ORF as well as 160 bp of the 3′ downstream region was amplified by PCR, and cloned into the pRU11 plasmid (Brachmann et al., 2001) under the control of *crg1* promoter. These plasmids were inserted by homologous recombination into the *cbx* locus as described in Brachmann et al. (Brachmann et al., 2001).

A bipartite cassette composed of a *gfp* ORF and an antibiotic resistance gene was used to construct the *fim1-GFP* and *cdc24-GFP* alleles. To this end, around 1000 bp fragment of the 3′ end of the corresponding ORFs without the stop codon were amplified by PCR and inserted in the same translational phase as *gfp* in the bipartite cassette. A second flanking region, corresponding to the 3′ region downstream of *fim1* and *cdc24* open reading frames was cloned in the 3′ end of the cassette and introduced by homologous recombination into the native loci of corresponding strains.

Microscopy and staining procedures

For microscopic analysis, cells were grown overnight at 22°C, 200 r.p.m., shifted to 34°C for 4-5 hours, and mounted on a 2% agar cushion for observation in a Zeiss Axioplan II imaging microscope (Oberkochen, Germany). Standard FITC fluorescent filters and a 100× Apochromat objective prewarmed to 34°C were used. For the analysis of GFP-Myo5 dynamics images series of 60 frames at 1-second intervals were taken. A CoolSNAP-HQ CCD camera (Photometrics, Tucson, AZ) controlled by the imaging software Metamorph (Universal Imaging, Downingtown, PA) was used.

Plant infections

To test for mating, compatible strains were co-spotted on charcoal-containing PD plates that were sealed with Parafilm and incubated at 22°C or 30°C for 48 hours (Holliday, 1974). Plant infections were performed with the maize cultivar Early Golden Bantam as described previously (Old Seeds, Madison, WI) (Gillissen et al., 1992). For the isolation of progeny and meiotic studies, spores from tumors were

collected 10-14 days after inoculation and plated on plates containing 2% water agar. After 2 days, microcolonies (with about 200 cells) were removed and resuspended in YPD and dilutions plated onto YPD plates with or without nourseothricine as a selective agent.

The authors thank P. Sudbery for critical reading of the manuscript. The helpful suggestions of two anonymous reviewers are also thanked. This work was supported by Grants to J.P.M. from the Spanish government (BIO2005-02998) and EU (MRTN-CT-2005-019277). The authors declare no competing financial interests.

References

Adams, A. E., Botstein, D. and Drubin, D. G. (1989). A yeast actin-binding protein is encoded by *SAC9*, a gene found by suppression of an actin mutation. *Science* **243**, 231-233.

Adams, A. E., Johnson, D. I., Longnecker, R. M., Sloat, B. F. and Pringle, J. R. (1990). CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **111**, 131-142.

Adams, A. E., Botstein, D. and Drubin, D. G. (1991). Requirement of yeast fimbrin for actin organization and morphogenesis in vivo. *Nature* **354**, 404-408.

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.

Barral, Y., Mermall, V., Mooseker, M. S. and Snyder, M. (2000). Compartmentalization of the cell cortex by septins is required for maintenance of cell polarity in yeast. *Mol. Cell* **5**, 841-851.

Bassilana, M. and Arkowitz, R. A. (2006). Rac1 and Cdc42 have different roles in *Candida albicans* development. *Eukaryotic Cell* **5**, 321-329.

Bölker, M. (2001). *Ustilago maydis* – a valuable model system for the study of fungal dimorphism and virulence. *Microbiology* **147**, 1395-1401.

Boyce, K. J., Hynes, M. J. and Andrianopoulos, A. (2003). Control of morphogenesis and actin localization by the *Penicillium marneffei RAC* homolog. *J. Cell Sci.* **116**, 1249-1260.

Boyce, K. J., Chang, H., D'Souza, C. A. and Kronstad, J. W. (2005). An *Ustilago maydis* septin is required for filamentous growth in culture and for full symptom development on maize. *Eukaryotic Cell* **4**, 2044-2056.

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.

Bussink, H. J. and Osmani, S. A. (1998). A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. *EMBO J.* **17**, 3990-4003.

Butty, A. C., Perrinjaquet, N., Petit, A., Jaquenoud, M., Segall, J. E., Hofman, K., Zwahlen, C. and Peter, M. (2002). A positive feedback loop stabilizes the guanine-nucleotide exchange factor Cdc24 at sites of polarization. *EMBO J.* **21**, 1565-1576.

Carroll, A. S. and O'Shea, E. K. (2002). Pho85 and signaling environmental conditions. *Trends Biochem. Sci.* **27**, 87-93.

Castillo-Lluva, S. and Pérez-Martín, J. (2005). The induction of the mating program in the phytopathogen *Ustilago maydis* is controlled by a G1 cyclin. *Plant Cell* **17**, 3544-3560.

Chae, T., Kwon, Y. T., Bronson, R., Dikkes, P., Li, E. and Tsai, L. H. (1997). Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* **18**, 29-42.

de Jesús-Berríos, M. and Rodríguez-del Valle, N. (2002). Expression of a Pho85 cyclin-dependent kinase is repressed during the dimorphic transition in *Sporothrix schenckii*. *Fungal Genet. Biol.* **37**, 39-48.

Dhavan, R. and Tsai, L. H. (2001). A decade of Cdk5. *Nat. Rev. Mol. Cell Biol.* **2**, 749-759.

Dobbelaere, J., Gentry, M. S., Hallberg, R. L. and Barral, Y. (2003). Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Dev. Cell* **4**, 345-357.

Dou, X., Wu, D., An, W., Davies, J., Hashmi, S. B., Ukil, L. and Osmani, S. A. (2003). The PHOA and PHOB cyclin-dependent kinases perform an essential function in *Aspergillus nidulans*. *Genetics* **165**, 1105-1115.

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.

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

Gow, N. A., Brown, A. J. and Odds, F. C. (2002). Fungal morphogenesis and host invasion. *Curr. Opin. Microbiol.* **5**, 366-371.

Han, B. K., Bogomolnaya, L. M., Totten, J. M., Blank, H. M., Dangott, L. J. and Polymenis, M. (2005). Bem1p, a scaffold signaling protein, mediates cyclin-dependent control of vacuolar homeostasis in *Saccharomyces cerevisiae*. *Genes Dev.* **19**, 2606-2618.

Harris, S. D. (2006). Cell polarity in filamentous fungi: shaping the mold. *Int. Rev. Cytol.* **251**, 41-77.

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

Huang, D., Patrick, G., Moffat, J., Tsai, L. H. and Andrews, B. (1999). Mammalian Cdk5 is a functional homologue of the budding yeast Pho85 cyclin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **96**, 14445-14450.

Huang, D., Moffat, J. and Andrews, B. (2002). Dissection of a complex phenotype by functional genomics reveals roles for the yeast cyclin-dependent protein kinase Pho85 in stress adaptation and cell integrity. *Mol. Cell. Biol.* **22**, 5076-5088.

Huckaba, T. M., Gay, A. C., Pantalena, L. F., Yang, H. C. and Pon, L. A. (2004). Live cell imaging of the assembly, disassembly, and actin cable-dependent movement of endosomes and actin patches in the budding yeast, *Saccharomyces cerevisiae*. *J. Cell Biol.* **167**, 519-530.

Hurtado, C. A., Beckerich, J. M., Gaillardin, C. and Rachubinski, R. A. (2000). A Rac homolog is required for induction of hyphal growth in the dimorphic yeast *yarrowia lipolytica*. *J. Bacteriol.* **182**, 2376-2386.

Irazoqui, J. E., Gladfelter, A. S. and Lew, D. J. (2003). Scaffold-mediated symmetry breaking by Cdc42p. *Nat. Cell Biol.* **5**, 1062-1070.

Kämper, J., Kahmann, R., Bölker, M., Ma, L.-J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Müller, O. et al. (2006). Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **444**, 97-101.

Kawauchi, T., Chihama, K., Nabeshima, Y. and Hoshino, M. (2006). Cdk5 phosphorylates and stabilizes $p27^{kip1}$ contributing to actin organization and cortical neuronal migration. *Nat. Cell Biol.* **8**, 17-26.

Lenburg, M. E. and O'Shea, E. K. (2001). Genetic evidence for a morphogenetic function of the *Saccharomyces cerevisiae* Pho85 cyclin-dependent kinase. *Genetics* **157**, 39-51.

Leveleki, L., Mahlert, M., Sandrock, B. and Bolker, M. (2004). The PAK family kinase Cla4 is required for budding and morphogenesis in *Ustilago maydis*. *Mol. Microbiol.* **54**, 396-406.

Levin, D. E. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **69**, 262-291.

Loog, M. and Morgan, D. O. (2005). Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature* **434**, 104-108.

Mahlert, M., Leveleki, L., Hlubek, A., Sandrock, B. and Bölker, M. (2006). Rac1 and Cdc42 regulate hyphal growth and cytokinesis in the dimorphic fungus *Ustilago maydis*. *Mol. Microbiol.* **59**, 567-578.

Measday, V., Moore, L., Ogas, J., Tyers, M. and Andrews, B. (1994). The PCL2 (ORFD)-PHO85 cyclin-dependent kinase complex: a cell cycle regulator I yeast. *Science* **266**, 1391-1395.

Miyakawa, Y. (2000). Identification of a *Candida albicans* homologue of the *PHO85* gene, a negative regulator of the PHO system in *Saccharomyces cerevisiae*. *Yeast* **16**, 1045-1051.

Moffat, J. and Andrews, B. (2004). Late-G1 cyclin-CDK activity is essential for control of cell morphogenesis in budding yeast. *Nat. Cell Biol.* **6**, 59-66.

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

Nagata, Y. and Burger, M. M. (1974). Wheat germ agglutinin. Molecular characteristics and specificity for sugar binding. *J. Biol. Chem.* **249**, 3116-3122.

Nern, A. and Arkowitz, R. A. (2000). G proteins mediate changes in cell shape by stabilizing the axis of polarity. *Mol. Cell* **5**, 853-864.

Niethammer, M., Smith, D. S., Ayala, R., Peng, J., Ko, J., Lee, M. S., Morabito, M. and Tsai, L. H. (2000). NUDEL is a novel substrate that associates with LIS1 and cytoplasmic dynein. *Neuron* **28**, 697-711.

Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F. and Tsai, L. H. (1996). The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev.* **10**, 510-522.

Nikolic, M., Chou, M. M., Lu, W., Mayer, B. J. and Tsai, L. H. (1998). The p35/cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* **395**, 194-198.

Nishizawa, N., Kawasumi, M., Fujino, M. and Toh-e, A. (1998). Phosphorylation of Sic1, a cyclin-dependent kinase (CDK) inhibitor, by CDK including Pho85 kinase is required for its prompt degradation. *Mol. Biol. Cell* **9**, 2393-2405.

Nishizawa, N., Kanaya, Y. and Toh-e, A. (1999). Mouse cyclin-dependent kinase Cdk5 is a functional homologue of a yeast Cdk, Pho85 kinase. *J. Biol. Chem.* **274**, 33859-33862.

Osmani, A. H., Van Peij, N., Mischke, M., O'Connell, M. J. and Osmani, S. A. (1994). A single $p34^{cdc2}$ protein kinase ($nimX^{cdc2}$) is required at G1 and G2 in *Aspergillus nidulans*. *J. Cell Sci.* **107**, 1519-1528.

Pelham, R. J. and Chang, F. (2001). Role of actin polymerization and actin cables in actin-patch movement in *Scizosaccharomyces pombe*. *Nat. Cell Biol.* **3**, 235-244.

Schmidt, M., Varma, A., Drgon, T., Bowers, B. and Cabib, E. (2003). Septins, under Cla4 regulation, and the chitin ring are required for neck integrity in budding yeast. *Mol. Biol. Cell* **14**, 2128-2141.

Sgarlata, C. and Pérez-Martín, J. (2005). Inhibitory phosphorylation of a mitotic cyclin-dependent kinase regulates the morphogenesis, cell size and virulence of the smut fungus *Ustilago maydis*. *J. Cell. Sci.* **15**, 3607-3622.

Steinberg, G., Wedlich-Soldner, R., Brill, M. and Schulz, I. (2001). Microtubules in the fungal pathogen *Ustilago maydis* are highly dynamic and determine cell polarity. *J. Cell Sci.* **114**, 609-622.

Straube, A., Brill, M., Oakley, B. R., Horio, T. and Steinberg, G. (2003). Microtubule

organization requires cell cycle-dependent nucleation at dispersed cytoplasmic sites: polar and perinuclear microtubule organizing centers in the plant pathogen *Ustilago maydis*. *Mol. Biol. Cell* **14**, 642-657.

Straube, A., Weber, I. and Steinberg, G. (2005). A novel mechanism of nuclear envelope break-down in a fungus: nuclear migration strips off the envelope. *EMBO J.* **24**, 1674-1685.

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.

Ushinsky, S. C., Harcus, D., Ash, J., Dignard, D., Marcil, A., Morchhauser, J., Thomas, D. Y., Whiteway, M. and Leberer, E. (2002). CDC42 is required for polarized growth in human pathogen *Candida albicans*. *Eukaryotic Cell* **1**, 95-104.

Vallim, M. A., Nichols, C. B., Fernandes, L., Cramer, K. L. and Alspaugh, J. A. (2005). A Rac homolog functions downstream of Ras1 to control hyphal differentiation and high-temperature growth in the pathogenic fungus *Cryptococcus neoformans*. *Eukaryotic Cell* **4**, 1066-1078.

Versele, M. and Thorner, J. (2004). Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK Cla4. *J. Cell Biol.* **164**, 701-715.

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.

Wedlich-Söldner, R., Bolker, M., Kahman, R. and Steinberg, G. (2000). A putative endosomal t-SNARE links exo- and endocytosis in the phytopathogenic fungus *Ustilago maydis*. *EMBO J.* **19**, 1974-1986.

Wedlich-Söldner, R., Altschuler, S., Wu, L. and Li, R. (2003). Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. *Science* **299**, 1231-1235.

Xie, Z., Sanada, K., Samuels, B. A., Shih, H. and Tsai, L. H. (2003). Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell* **114**, 469-482.

Xie, Z., Samuels, B. A. and Tsai, L. H. (2006). Cyclin-dependent kinase 5 permits efficient cytoskeletal remodeling – a hypothesis on neuronal migration. *Cereb. Cortex* **16**, i64-i68.