Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis* – similar inputs, different outputs

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The ability to switch between a yeast-like form and a filamentous form is an extended characteristic among several fungi. In pathogenic fungi, this capacity has been correlated with virulence because along the infection process, dimorphic transitions are often required. Two well-known organisms for which dimorphism have been studied are the pathogenic fungi *Candida albicans* and *Ustilago maydis*, which infect mammals and corn, respectively. In both cases, several signal transduction pathways have been defined. Not surprisingly, these pathways are similar to the well-known pathways involved in the pseudohyphal differentiation that some *Saccharomyces cerevisiae* diploid strains show when nutrients are starved. However, in spite of similarities at the molecular level, strikingly, fungi use similar pathways to respond to environmental inputs, but with differing outcomes.

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Current Opinion in Microbiology 2001, 4:214–221

1369-5274/01/$ – see front matter

Abbreviations

MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
PAK	p21-activated kinase
PKA	protein kinase A
ubc	*Ustilago* bypass of cyclase

Introduction

Dimorphism is a peculiar characteristic of several fungi: it is the ability to switch between a cellular yeast form and a filamentous form in response to environmental cues. Growth in the yeast form implies mitotic divisions either by budding or fission to produce two independent cells. In the filamentous form, two different modes can be seen. In one of them, the pseudohyphal mode, cells become elongated, fail to abscise following cytokinesis, and remain attached to form chains of elongated cells. In the filamentous mode, true hyphae are produced with long continuous tubes and septae separating each of the nuclei in these tubes. There is increasing evidence that pathogenic fungi utilize this characteristic to control growth between saprophytic and pathogenic forms. Furthermore, this ability to switch between different morphological states appears to be an important virulence determinant. For example, the filaments can be invasive, helping to penetrate the surface on which the cell is growing. This morphogenic switch implies true developmental programmes that are triggered by various signals *in vitro*. Many of the responses of these signals probably reflect normal interactions between the fungus and its host *in vivo*. The genes controlling the role of dimorphism in pathogenesis have been the focus of many investigations, as they have great potential as targets for novel antifungal drugs. An important aspect that people working in the field realized is that although many different signals can induce filamentous development, the strategies for connecting the external signal to the change in cell differentiation are broadly conserved among fungi. This fact helps to compare the induction of the various developmental programs in different fungi. In this review, we shall focus on the latest developments in the knowledge of these pathways in two different pathogenic fungi: *Candida albicans* and *Ustilago maydis*.

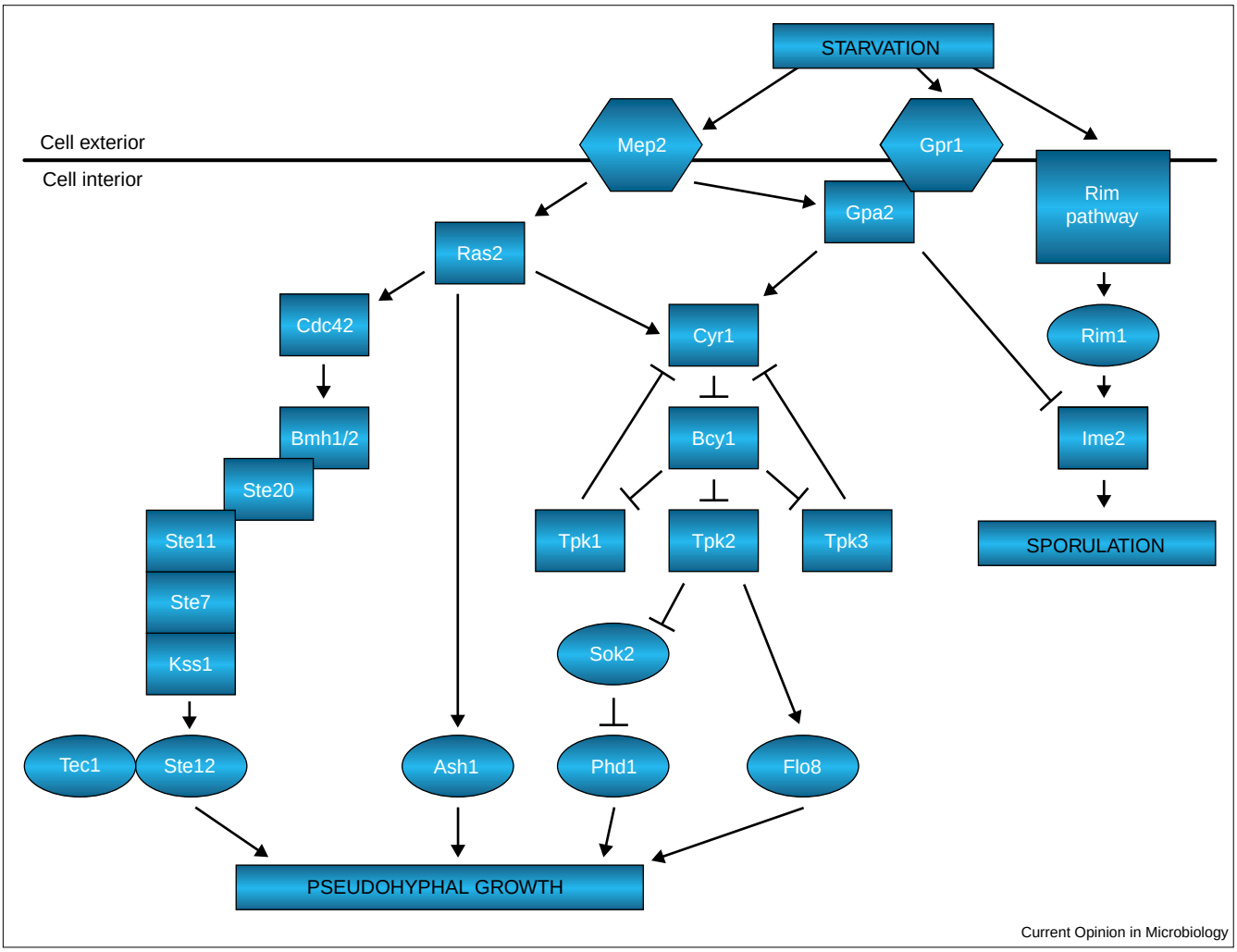
Dimorphism in *Saccharomyces cerevisiae* – the pathfinder

Certain diploid laboratory strains of *Saccharomyces cerevisiae* are able to switch from a unicellular yeast form to a pseudohyphal form on starvation of nitrogen. Although *S. cerevisiae* is a non-pathogenic organism, our ever-increasing knowledge about this organism (far more genetically tractable) has been used as a guide to explore fungal dimorphism. Studies in this organism have revealed that the signalling pathways are controlled both by cAMP and mitogen-activated protein kinase (MAPK) signal transduction pathways (Figure 1).

The MAPK pathway is composed of four central kinases, homologs of the mammalian MAPK signalling enzymes: Ste20 (a p21-activated kinase [PAK]), Ste11 (a MAPK/ERK kinase kinase [MEKK]), Ste7 (a MAPK/ERK kinase [MEK]) and Kss1 (MAPK). Quite interestingly, the three first components are shared with the mating pheromone response pathway [1]. The kinase cascade acts over the heterodimeric transcription factor composed of the Ste12 protein (which is also shared with the pheromone cascade) and the Tec1 protein. This factor binds to specific DNA sequences called filamentation/invasion response elements (FREs) [2]. It is not known how this pathway is activated, but Ras2 has some role in this step. In this model, the activation of the MAPK pathway by Ras2 occurs via the GTPase Cdc42 and a complex consisting of the 14-3-3 proteins, Bmh1 and Bmh2, and the PAK, Ste20 [3].

The cAMP pathway also controls pseudohyphal growth in *S. cerevisiae*. Two different pathways involving the small G-proteins Ras2 and Gpa2 produce an increase in cAMP level [4•] that can activate adenylate cyclase (Cyr1). cAMP binds to the regulatory subunit (Bcy1) of protein kinase A, causing its dissociation from the catalytic subunits, Tpk2, which are now able to activate downstream targets. Upstream

Figure 1



Diagrammatic representation of the different pseudohyphal pathways of *S. cerevisiae*. Two major pathways activate pseudohyphal growth. The more relevant pathway is composed of a MAPK cascade (Ste20–Ste11–Ste7–Kss1) that shares some components with the pheromone response cascade. A Ras protein that signals through the GTPase-activating protein Cdc42 and the 14-3-3 proteins Bmh1 and Bmh2 activates this cascade. The output from the MAPK cascade is received by the heterodimeric transcription factor Ste12/Tec1, which turns on promoters of genes involved in pseudohyphal growth. The second pseudohyphal pathway is composed of the adenylate cyclase protein, Cyr1, which is activated by two different G-proteins: Ras2 and Gpa2. An increase in the cAMP levels inactivates the regulatory subunit of PKA, Bcy1, releasing the three catalytic subunits (Tpk1, Tpk2 and Tpk3). Only Tpk2 is involved in the activation of pseudohyphal growth. The other two catalytic subunits are involved in adaptive mechanisms. The targets of PKA are the Flo8 and Sok2 transcriptional regulators. The first acts as a positive regulator, whereas the second acts as a negative regulator, most likely over the Phd1 transcriptional activator. Both pathways – the MAPK and the cAMP–PKA cascades – are induced by membrane receptors (Gpr1 and Mep2) that sense the nutritional status of the cell. Additional effectors are the Ash1 transcriptional regulator (which is located downstream of Ras2), and the sporulation pathway composed of the Rim pathway and the Ime2 kinase (see text for additional details). Membrane-associated receptors are represented by hexagons. Boxes represent regulatory proteins and ellipses represent transcription factors.

of Ras2 and Gpa2, there are two putative sensors of nutritional status: Gpr1 and Mep2. Gpr1 is a Gα-coupled seven-transmembrane domain receptor, which has been genetically linked to Gpa2 [5•]. Although the actual signals that are sensed by Gpr1 are currently unknown, this protein is a glucose sensor whose expression is controlled by low nitrogen concentration, suggesting a role as dual sensor of both carbon abundance and nitrogen starvation. The Mep2 protein is a high-affinity ammonium permease that has been located upstream of Ras2 and Gpa2 [6]. Downstream effectors of the cAMP pathway include several transcriptional factors. Sok2 is a Myc-like transcription factor that belongs to a large family of transcriptional factors essential for fungal differentiation [7]. Phd1, a Sok2 homolog, also affects pseudohyphal growth, although it does not appear to be a direct target of the protein kinase A (PKA)-dependent pathway [8]. It has been proposed that Sok2 is a negative regulator of Phd1, most likely via heterodimerization. A second factor to be regulated by PKA is the transcriptional regulator Flo8, which is able to activate cell surface flocculins [9].

Additional factors are known to affect pseudohyphal growth, but their relationships with the proposed signal transduction pathways are not yet clear. One of these factors is Ash1, a zinc finger transcription factor involved in daughter cell specificity in haploid cells, which has been found to affect pseudohyphal growth. Ash1 has been proposed to be downstream of Ras2, although it is not currently clear whether or not the cAMP pathway is also involved [10]. A second factor is the kinase Ime2, which is involved in sporulation and acts as a negative regulator of pseudohyphal growth. Ime2 is repressed upon interaction with Gpa2 protein. It has been proposed that in conditions of low nitrogen and high carbon content, which favor pseudohyphal growth, *S. cerevisiae* uses this pathway to shut down Ime2 activity and to avoid sporulation [11]. In addition, Ime2 helps to connect the transcriptional factor, Rim1, with pseudohyphal growth. Rim1 belongs to the PacC family of transcriptional regulators, which are involved in pH signaling in fungi [12]. Mutations in the gene that encodes Rim1 protein components of the cascade (*RIM* genes) that positively regulate this transcriptional factor do not affect dimorphism in diploid cells, but avoid invasive growth in haploid cells [13].

Dimorphism in *Candida albicans* – one step behind

C. albicans is the most common fungus identified in clinical isolates. This opportunistic pathogen causes both superficial and serious invasive infections. *C. albicans* is able to reversibly change its morphology from round budding cells to elongated hyphae or filamentous growth forms. This morphological flexibility appears to be a key contributor to virulence. *C. albicans* morphology is directly related to environmental conditions. Many conditions induce filamentous growth, though only a few have been well characterized. Among these, alterations in pH, high temperature, nutrient deprivation, and addition of serum or N-acetylglucosamine (GlcNAc) are the most commonly used conditions. Figure 2 summarizes a current interpretation of the signal transduction pathways in this organism. A MAPK pathway related to that of *S. cerevisiae* also operates in *C. albicans*. Components of this cascade include two different PAKs (Cst20 and Cla4), a MEK (Hst7) and a MAPK (Cek1). This cascade is thought to activate the Cph1 protein (the *C. albicans* Ste12 homologue) [14•]. Components still missing in this pathway (in comparison to *S. cerevisiae*) are MEKK-homologous and Tec1-homologous proteins. In both cases, the genome sequencing effort has identified homologs, but no data about function are available yet (*Candida* sequence information is available from http://alces.med.umn.edu/bin/genelist?genes). An additional component in this cascade is the protein phosphatase Cpp1, which is thought to regulate the phosphorylation status of Cek1 [15].

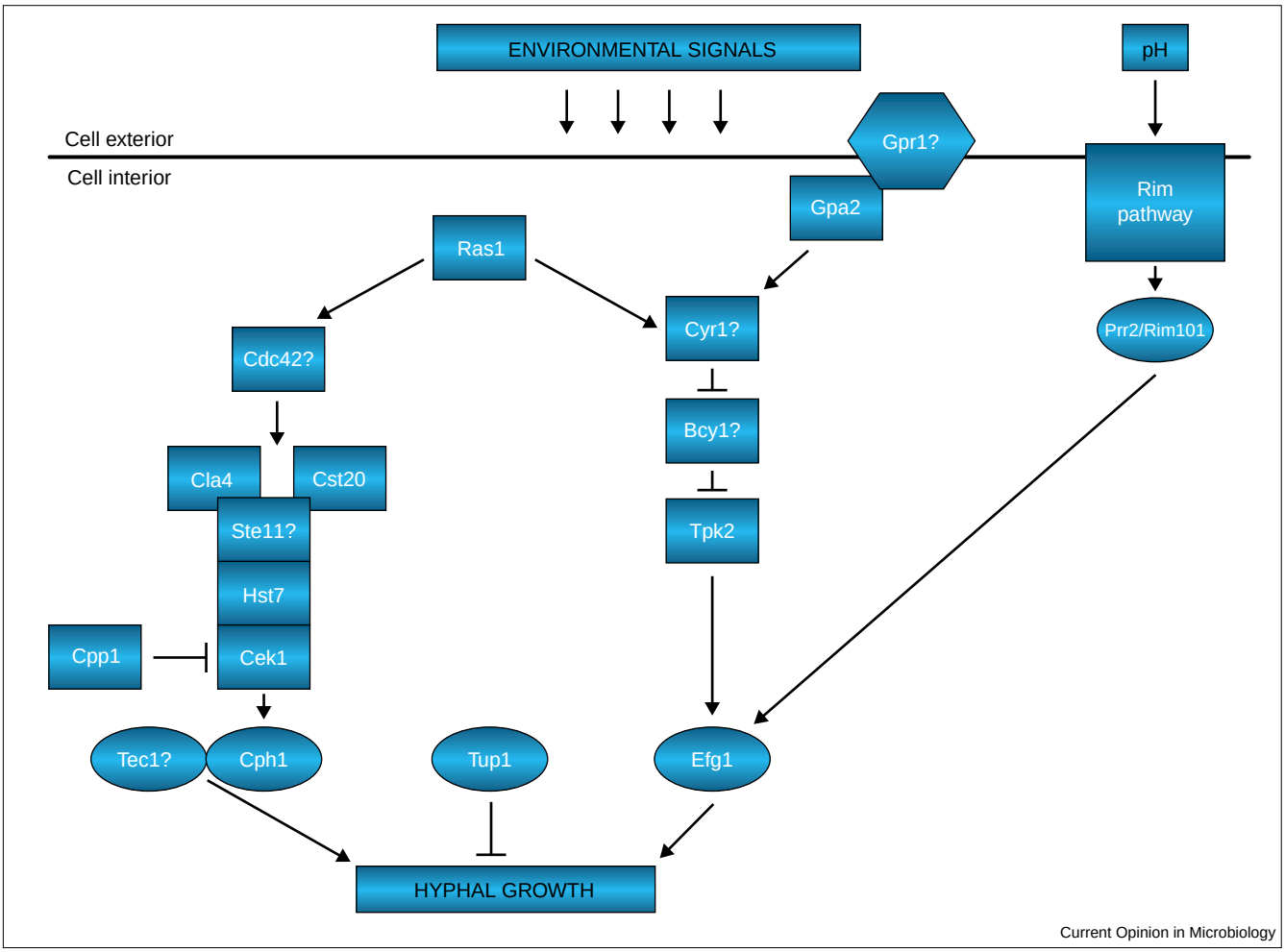
In addition to MAPK kinases, cAMP influences hyphal development. Added cAMP has been reported to induce hyphal growth [16]. The recent cloning of one of the catalytic subunits of PKA (Tpk2) clearly demonstrated that there is a cAMP pathway operating in *C. albicans* dimorphism [17••]. A defect in the Tpk2 isoform partially impairs hyphal growth, suggesting the presence of additional PKA isoforms (actually, there is at least a second catalytic subunit, Tpk1, as indicated by the genome sequencing project). The Efg1 protein (highly homologous to Sok2 and Phd1) appears to be downstream of PKA. Efg1 is a major player in dimorphism [18,19]. Analysis of gain and loss of function mutants of Efg1 suggests that Efg1 is a direct target of PKA activity, in a similar way to Sok2, although the proposed role of Efg1 is a positive one with respect to hyphal growth (JE Ernst, personal communication). Upstream of both the MAPK cascade and the cAMP pathway is the Ras1 protein (similar to *S. cerevisiae* Ras2) [20•]. We have recently cloned a Gα-protein with sequence similarities to Gpa2, and preliminary data suggest that it will be involved in dimorphic transitions (C Sánchez-Martínez, J Pérez-Martín, unpublished data).

No receptors of environmental signals have been specifically linked to any of these pathways. Moreover, there is no a clear correlation between environmental conditions and specific pathways, with the exception of ambient pH. *C. albicans* develops hyphal growth in neutral-basic pH. The control of pH-dependent gene expression has been most extensively studied with *Aspergillus nidulans* (see [12] and references therein). Seven genes critical for this regulation have been identified. Six of them, called *pal* genes, are involved in signal transduction, whereas *pacC* encodes a transcriptional factor containing a zinc finger domain. The data obtained so far suggests a clear conservation of pH-responsive pathway in different fungi. This fact has prompted the search for homologs in *Candida albicans*. These homologs have been found both for *pal* genes [21,22•,23•] and for the *pacC* gene [21,23•,24•]. Furthermore, deletion analysis has shown that these genes are involved in pH signaling [22•,23•]. As in *A. nidulans* PacC protein, the activation of the *C. albicans* homolog, called Prr2/Rim101 protein, appears to be due to proteolytic processes, as it has been suggested by dominant active alleles in the transcriptional factor [25••]. Quite interestingly, it has been shown that pH-regulated dimorphism but not pH-dependent expression requires the transcription factor Efg1 [25••].

Dimorphism in *Ustilago maydis* – do the same, but the contrary

U. maydis, the causative agent of corn smut disease, exhibits a dimorphic switch from budding to filamentous growth in reponse to mating interactions and environmental conditions. Haploid cells can form filaments in response to nutrient starvation or acidic pH. Two different pathways appear to control the dimorphic switch (Figure 3). The first one implicates cAMP and, in contrast to that in *S. cerevisiae* and *C. albicans* cells, low cAMP levels stimulate the transition from yeast-like cells to the filamentous form [26]. The most upstream effector known

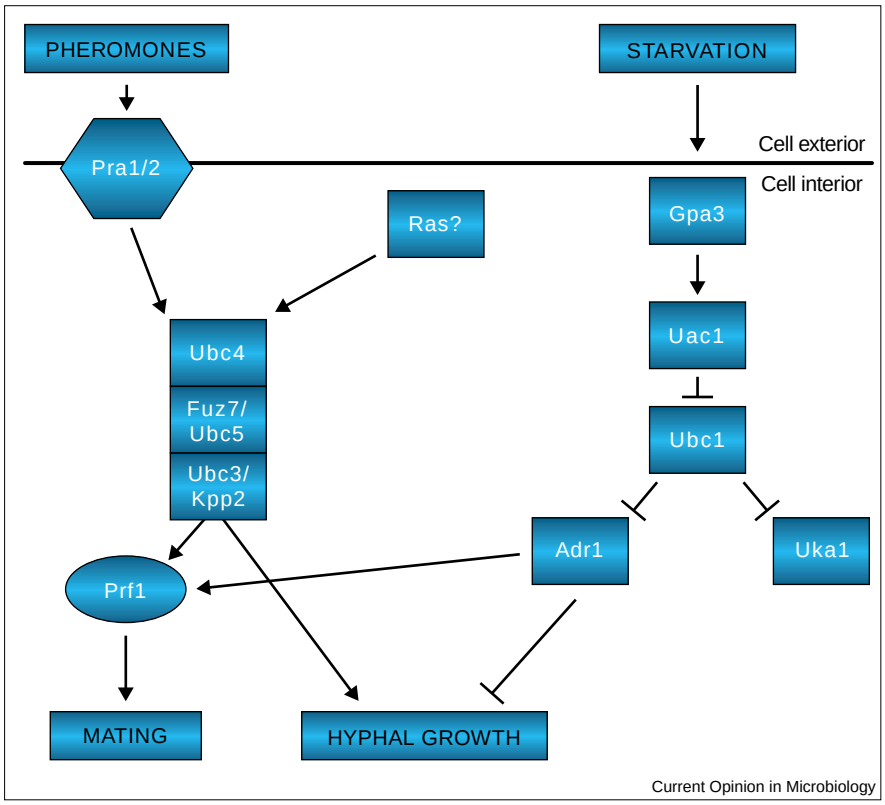
Figure 2



Schematic model of signalling pathways to hyphal growth in *C. albicans*. At least three pathways trigger hyphal growth. In contrast to *S. cerevisiae*, the more important pathway in *C. albicans* is the cAMP–PKA route. In this pathway, only the PKA catalytic subunit Tpk2 has been characterized. The adenylate cyclase (Cyr1) and the regulatory subunit (Bcy1) have been located in the sequencing project. Tpk2 positively regulates the transcription factor Efg1, which is a major player in hyphal growth. The components of the MAPK pathway are better characterized, although some components are not well known yet. Finally, the pH response is dictated by the Rim pathway, which feeds into the transcriptional regulator Prr2/Rim101. Hyphal growth in response to pH requires both Prr2/Rim101 and Efg1 (see text for details).

so far is the Gα-protein, Gpa3. Although originally described as a component of the pheromone-response pathway [27], recent experiments have concluded that Gpa3 transduces a signal that activates the cAMP pathway [28], probably acting as a positive regulator of Uac1, the *Ustilago* adenylate cyclase. Mutants in the *uac1* gene as well as in the gene *adr1* are filamentous [26]. The gene *adr1* encodes one of the three catalytic subunits of PKA in *U. maydis* [29]. The isolation of second-site suppressor mutants of the constitutive filamentous growth of Uac1-defective mutants enabled the isolation of the *Ustilago* bypass of cyclase (*ubc*) genes [30]. From this screening, mutations in one regulatory subunit of the PKA enzyme, called ubc1, were obtained stressing the role of cAMP in the dimorphic switch [26]. More interestingly, other genes obtained from this screening were the genes *ubc3* (also known as *kpp2*) [31••,32••], *ubc4* and *ubc5* (previously cloned as *fuz7*) [33,34•], which encode members of a MAPK cascade. Interestingly, the components of this MAPK cascade are also involved in the pheromone signal transduction pathway required for mating [35•]. The interpretation of the genetic data suggests that, in *U. maydis*, the cAMP pathway and the MAPK pathway work in an opposing manner. The cAMP–PKA pathway represses hyphal growth, whereas the MAPK cascade acts as a positive effector of hyphal growth. The interplay of the cAMP and MAPK signal transduction pathways is reinforced by the fact that Prf1, an HMG-like factor involved in the activation of genes upon response to pheromone [36], is regulated post-transcriptionally by the pheromone and cAMP signals [37••] (HMG-like factors are a family of proteins that share a protein domain responsible for DNA binding). However it is not known whether or not Prf1 is required for filamentous growth in haploid cells.

Figure 3



Model of dimorphic development in *Ustilago maydis*. Two opposite pathways dictate the hyphal growth in the corn smut. The cAMP–PKA pathway is well known and is composed of a Gα–protein, which activates the adenylate cyclase (Uac1). cAMP inactivates the PKA regulatory subunit (Ubc1), releasing the catalytic subunits (Adr1 and Uka1). High levels of Adr1-dependent PKA activity repress hyphal growth. Prf1 has been proposed to be downstream of PKA activity. The MAPK cascade is shared with the pheromone response cascade. It is not known whether the pheromone-responsive transcription regulator, Prf1, has some role in hyphal growth. A Ras protein has been hypothesized, but there is no available proof for its existence (see text for details).

The ambient pH is able to affect dimorphic transitions, although *U. maydis* cells respond differently to the way in which *C. albicans* cells do. Acid pH induces hyphal growth, whereas basic pH represses it [38]. No gene function has been associated so far with this response, although the isolation of mutants whose ability to produce hyphal growth is affected at acid pH has been reported[39].

Cross talk between pathways – introducing order in here

In addition to a molecular characterization of the signal transduction pathways, several important issues need to be addressed in dimorphic transitions. One of them is the relative importance of each pathway with respect to the others. This is particularly clear in organisms such as *S. cerevisiae* or *C. albicans*, in which two different pathways positively regulate the dimorphic transition. In *S. cerevisiae*, the two morphogenetic pathways, cAMP and MAPK, do not seem equally important in directing pseudohyphal growth. In this organism, defects in the MAPK pathway and particularly in the downstream effectors Ste12 and Tec1 have a major effect on dimorphism, whereas the cAMP pathway seems to be less important. In contrast, in *C. albicans*, the major player in dimorphic transitions appears to be the Efg1 factor, whereas Cph1 has only a minor role specific to some stimuli, suggesting that the MAPK pathway has a secondary role. An interesting aspect emanating from this issue in *C. albicans* is the way in which all the signals are integrated to produce a developmental outcome. Two different models of the regulatory circuit can be used to explain this aspect [40••]. In the first model, a central control model, a master regulator that integrates signals from upstream pathways provides a single output that controls filamentous growth. The second model, a network model, proposes a network of connections between regulatory pathways and downstream genes. Studies of epistasis analysis with different regulatory mutants [40••] suggest a network model operating in *C. albicans* cells, with the existence of several distinct types of filamentous forms, each dependent on a particular set of environmental conditions and each expressing a unique set of outcome proteins. This conclusion is supported by the study of particular hyphal-induced genes like *ALS8*, in which the promoter includes regulatory regions to respond to different hyphal-inducing stimuli (AJP Brown, personal communication). Studies in the *FLO11* gene from *S. cerevisiae* indicate a similar conclusion [41••]. In this case, the regulatory region is unusually large and evidence indicates that distinct transcription factors and promoter elements receive the MAPK and cAMP signals.

An additional issue is the way in which these different pathways regulate each other. From *S. cerevisiae*, it is clear that a single effector, Ras2 feeds into both the MAPK and the cAMP pathway. However, downstream, it is not clear whether or not the preferential role of one pathway over the

other is because of cross-downregulation, or whether or not there is a cross-upregulation regardless of which pathway has been activated. In agreement with this idea, in this organism, there are three PKA catalytic subunits that are specialized with respect to pseudohyphal differentiation. While Tpk2 is a positive effector of filamentous growth, the other catalytic subunits, Tpk1 and Tpk3, have an inhibitory effect, regulating the PKA pathway via a negative feedback loop that inhibits cAMP production [4•]. This negative function may serve to avoid filamentous growth in rich media, or to return filamentous cells to normal budding cells.

In *C. albicans* cells, future studies should address whether or not there is crossregulation among pathways. Some data suggest that this could be the case. One of the first transcriptional regulators cloned in *C. albicans* was the *TUP1* gene [42], which encodes a transcriptional repressor. This regulatory protein controls filamentation as well as other functions unrelated to dimorphism [43]. The morphogenetic factor, Efg1, has additional roles in microaerophilic conditions: it is required for chlamydospore formation, but represses hyphal growth under these conditions, probably acting over the regulator Czf1 [44,45]. Additional support of some crosstalk between pathways is found in the requirement of Efg1 factor to pH-regulated dimorphism [25••], although whether or not the Rim pathway directly affects Efg1 or feeds into the cAMP pathway must be addressed.

In *U. maydis*, the opposite roles of the cAMP and MAPK pathways suggest some kind of negative crosstalk, although there is no data about the level on which it may happen. It is tempting to speculate about the existence of a filamentous pathway downstream of cAMP and MAPK pathways, which receives negative and positive signals in a continuous way, integrating the metabolic status of the cell. Interestingly, the MEKK Ubc4 has in its amino-terminal section a region with homology to the Ras interaction region of the *Saccharomyces pombe* MEKK Byr2 [46]. Although no Ras protein has been reported so far in *Ustilago*, an appealing possibility is that a Ras effector feeds into the two pathways, retaining the equilibrium that can be displaced to either side by the contribution of additional effectors/signals like Gpa3. Interestingly, *adr1* and *uka1* [29], two genes encoding PKA catalytic subunits, have been identified, but only one of them has positively affected filamentation, reminiscent of the *S. cerevisiae* case (see above).

Conclusions: PKA, the pathmaster

C. albicans, *S. cerevisiae* and *U. maydis* are organisms that exploit the same signal transduction pathway — the cAMP–PKA pathway — to respond to similar environmental conditions (nutrient limitation). However, they produce different responses — filamentous growth in *C. albicans* and *S. cerevisiae*, and budding growth in *U. maydis*. In addition, the relationships between the different transduction pathways are different: in *C. albicans* and *S. cerevisiae,* the cAMP–PKA pathway collaborates with the MAPK pathway, whereas in *U. maydis*, it acts in the opposite way. We think that the explanation for these divergences in otherwise conserved pathways has to do with the different possible choices the organisms have to cope with during nutrient starvation.

C. albicans is a diploid organism with no known sexual cycle in nature. The dimorphic transition can be interpreted as being the way in which cells from this organism evade starvation conditions (hyphal growth enables otherwise sessile cells to forage for nutrients at a distance from their point of colonization). It makes sense, then, that every transduction pathway produces the same output: to forage for food using any kind of hyphal or pseudohyphal growth.

S. cerevisiae exists in the environment primarily as diploid. In *S. cerevisiae*, mating is inhibited in diploid cells and dimorphic transitions do not take place in haploid cells. In contrast to *C. albicans* cells, diploid *S. cerevisiae* cells have two mutually exclusive choices upon nutrient starvation: pseudohyphal growth or sporulation. Nitrogen starvation induces pseudohyphal growth, but both carbon and nitrogen starvation trigger sporulation. In this decision, the cAMP–PKA pathway plays a central role, because conditions that activate this pathway repress the sporulation fate, inducing the pseudohyphal one. Then, in budding yeast, there is a hierarchy of responses that depends on the cAMP–PKA pathway.

U. maydis cells are haploid in the environment. The mating in *U. maydis* cells is induced in response to nutrient deprivation. In fact, one of the roles of the cAMP–PKA cascade in this organism is to switch on all the mating apparatus (receptors and pheromones) [35•]. The presence of compatible partners in the vicinity enables the *U. maydis* cells to enter a mating program. Only when no compatible partner is found does the cell enter a filamentation program. Because the two different programs involve morphological changes that are quite different, they are mutually exclusive. Again, there is a hierarchy of decisions and it is the cAMP–PKA pathway that decides which one should be taken.

Comparisons of the three different organisms give us some conclusions. In all cases, the MAPK pathway acts positively over hyphal growth in response to nutrient starvation. The cAMP–PKA pathway, however, dictates the final fate of the cells. In *C. albicans*, there is only one choice — hyphal growth — and then both pathways collaborate. In *S. cerevisiae*, there are two choices — pseudohyphal growth and sporulation — that depend on the nitrogen–carbon balance, and the cAMP–PKA pathway integrates this balance. In *U. maydis*, the default is mating, and then the cAMP–PKA pathway represses hyphal growth, inducing the mating apparatus. In the previous cases, the status of the cAMP–PKA pathway is utilized to decide which fate the organism will take. We can propose, then, that the role of cAMP–PKA pathway in all these organisms is actually the same: to integrate and to dictate the preferred pathway, whether it is filamentation, sporulation or mating. This role is maintained even when the outcomes look so different.

Acknowledgements

We would like to thank Joachim Ernst and Allistair JP Brown for sharing with us their unpublished work. We apologize for not including other interesting data because of space constraints. Work in the authors' laboratory is supported by grants from Comisión Interministerial de Ciencia y Tecnología (BIO99-0906) and Comunidad Autónoma de Madrid (07B/0030/1998 and 08.2/0023/1998).

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