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The cAMP/protein kinase A signaling pathway in pathogenic basidiomycete fungi: Connections with iron homeostasis

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Abstract

A number of pathogenic species of basidiomycete fungi are either life-threatening pathogens of humans or major economic pests for crop production. Sensing the host is a key aspect of pathogen proliferation during disease, and signal transduction pathways are critically important for detecting environmental conditions and facilitating adaptation. This review focuses on the contributions of the cAMP/protein kinase A (PKA) signaling pathway in *Cryptococcus neoformans*, a species that causes meningitis in humans, and *Ustilago maydis*, a model phytopathogen that causes a smut disease on maize. Environmental sensing by the cAMP/PKA pathway regulates the production of key virulence traits in *C. neoformans* including the polysaccharide capsule and melanin. For *U. maydis*, the pathway controls the dimorphic transition from budding growth to the filamentous cell type required for proliferation in plant tissue. We discuss recent advances in identifying new components of the cAMP/PKA pathway in these pathogens and highlight an emerging theme that pathway signaling influences iron acquisition.

Keywords

cAMP/PKA pathway; pathogenesis; iron homeostasis; pH signaling

Introduction

As with all organisms, fungal pathogens have evolved sophisticated signaling pathways to perceive environmental conditions and respond appropriately. For pathogens, it is particularly important to mount a rapid and fitting response during the transition from the environment to host tissue. That is, pathogens must accurately perceive host conditions and adapt to differences in nutrient availability, physical conditions such as pH, oxygen, and temperature, and challenges posed by the host immune response. Perception and adaptation generally involve mitogen activated protein kinase (MAPK) pathways as well as the cyclic

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AMP (cAMP)/protein kinase A (PKA) pathway that plays a key role in adaptation to nutrient availability.

The core components of the cAMP/PKA pathway have been defined in some detail in a number of fungi with considerable information from the model yeast *Saccharomyces cerevisiae* (Thevelein and De Winde, 1999). In general, the pathway is activated in response to nutrients in the environment including glucose and amino acids that are perceived at the cell surface by G-protein coupled receptors (GPCRs). Detection of the signal results in activation of guanine nucleotide-binding proteins (G-proteins) leading to an increase in intracellular cAMP levels upon stimulation of adenylyl cyclase. The impact of elevated cAMP is to activate cAMP-dependent protein kinase A (PKA), which then phosphorylates downstream target proteins. These proteins include enzymes, structural proteins and transcription factors that carry out a myriad of responses as a result of signaling through the pathway. In fungal pathogens of animals and plants, these responses include morphological changes and the deployment of virulence factors (Mitchell and Dean, 1995; D'Souza *et al.*, 2001; Leberer *et al.*, 2001; Liebmann *et al.*, 2004).

The connections between the cAMP/PKA pathway, adaptation to the host, and regulation of virulence have been particularly well studied in two species of pathogenic fungi in the basidiomycete group, *Cryptococcus neoformans* and *Ustilago maydis*. *C. neoformans* has a worldwide distribution and is found in soil, in association with trees and in pigeon droppings (Mitchell and Perfect, 1995; Choi *et al.*, 2010). Inhalation of fungal cells from these sources leads to pulmonary infection that can spread to the central nervous system in the absence of immune containment. This fungus is an opportunistic pathogen that generally attacks people with a suppressed immune system due to HIV infection, cancer or transplantation (Park *et al.*, 2009). *C. neoformans* is able to cause disease in immunocompromised patients because: 1) it can resist the elevated body temperature of mammalian hosts; 2) it produces a polysaccharide capsule that has immunomodulatory properties and; 3) it secretes laccases to the cell wall to form protective melanin (Kronstad *et al.*, 2011b). *C. neoformans* also secretes enzymes such as urease that contribute to virulence (Kronstad *et al.*, 2011b). Interestingly, the major virulence traits of the pathogen are regulated by cAMP/PKA signaling, a process that involves activation of adenylyl cyclase via binding of the G-protein (Gpa1) leading to conversion of ATP to cAMP. The pathway responds to nutrients including glucose and amino acids (Xue *et al.*, 2006). The elevated level of cAMP promotes dissociation of the regulatory subunit (Pkr1) from the catalytic subunit (Pka1) of PKA. Defects in signaling components such as the G-protein (Gpa1), adenylyl cyclase (Cac1) or the catalytic subunit of PKA (Pka1) result in attenuated capsule elaboration, melanin formation and virulence (Fig. 1) (Alsbaugh *et al.*, 1997; D'Souza *et al.*, 2001). In contrast, deletion of the gene for the regulatory subunit of PKA (*PKRI*) results in an enlarged capsule and hypervirulence (D'Souza *et al.*, 2001). The pathway also regulates the formation of enlarged cells called titan cells and the process of mating (D'Souza *et al.*, 2001; Zaragoza *et al.*, 2010; Okagaki *et al.*, 2011). The impact of PKA on these processes was recently demonstrated with strains in which the expression of *PKA1* and *PKRI* was controlled by the glucose-repressed and galactose-activated *GAL7* promoter (Choi *et al.*, 2012). This study revealed that induction of *PKA1* expression in galactose resulted in enlarged capsule, cell

and vacuole size, as well as increased ploidy. Additionally, the regulated strains were used to demonstrate a positive impact for Pka1 on extracellular protease activity, a negative impact on urease activity, and a requirement for balanced PKA expression to properly regulate melanization (Choi *et al.*, 2012).

U. maydis is a pathogen of maize and teosinte, and infection of these hosts requires haploid, budding yeast cells of compatible mating types to fuse and establish dikaryotic filamentous cells that penetrate and colonize plant tissue (Banuett, 1995). Infection results in the formation of tumors at sites of infection along with stimulation of anthocyanin pigmentation. The dikaryon proliferates within tumor tissue and morphologically differentiates to form masses of abundant and highly melanized spores (teliospores). These spores disperse widely to allow colonization of new plants with subsequent germination to produce meiotic haploid progeny that can mate to reinitiate the disease cycle (Christensen, 1963; Snetselaar and Mims, 1992, 1994; Banuett and Herskowitz, 1996).

The morphological transition from budding to filamentous cells that underpins the virulence of *U. maydis* is regulated by mating and two conserved signaling pathways; a mitogen activated protein kinase (MAPK) signaling cascade and a cAMP/PKA pathway (Fig. 1) (Kronstad *et al.*, 1998). These pathways sense environmental signals such as nutrient availability, the presence of lipids, air exposure, acidic pH, and the presence of cells of opposite mating types, to regulate the dimorphic transition (Bölker *et al.*, 1995; Klose *et al.*, 2004; Martínez-Espinoza *et al.*, 2004). Mating in *U. maydis* is regulated by two unlinked mating loci, *a* and *b* (Bölker *et al.*, 1992, 1995). The *a* locus encodes a pheromone (Mfa1/2) and a pheromone receptor (Pra1/2) for cell recognition involving a MAPK pathway (Fig. 1) (Bölker *et al.*, 1992). The *b* locus encodes two homeodomain proteins (bE and bW) required for dikaryotic growth and completion of the life cycle (Kahmann *et al.*, 1995; Kronstad and Staben, 1997). The MAPK pathway leads to the activation of the pheromone response factor Prf1 (Banuett and Herskowitz, 1994; Hartmann *et al.*, 1996; Mayorga and Gold, 1999; Andrews *et al.*, 2000). Prf1 is also regulated by the cAMP/PKA pathway thus highlighting the integration of signaling pathways and mating in the regulation of filamentous growth (Gold *et al.*, 1994; Kaffarnik *et al.*, 2003; Lee *et al.*, 2003). For the cAMP/PKA pathway, the analysis of mutants with defects in signaling components indicates that high PKA activity leads to a budding phenotype, and low PKA activity results in filamentous growth.

In this review, we discuss recent findings related to the cAMP/PKA signaling pathway in *C. neoformans* and *U. maydis*, and point out similarities between the pathogens in the context of new components and shared downstream targets. In particular, there are intriguing connections between the cAMP/PKA pathway and other signaling pathways as well as shared regulatory outcomes involving iron acquisition. A number of excellent reviews are available to provide additional information on these pathogens and signaling mechanisms, as well as related topics (Kronstad *et al.*, 2011a; Vollmeister *et al.*, 2012; Coelho *et al.*, 2014).

Association of RACK1-like proteins with the cAMP/PKA pathway

The Gib2 protein functions in cAMP signaling in *Cryptococcus neoformans*

Recent work revealed an interesting connection between a G β -like/RACK1 protein homolog designated Gib2 and cAMP/PKA signaling in *C. neoformans*. RACK1 (Receptor for Activated C Kinase 1) is a WD repeat protein that plays a large number of roles in eukaryotic cells including moving proteins within cells, anchoring proteins at specific locations and stabilizing protein activity (Adams *et al.*, 2011). Additionally, RACK proteins interact with a large number of proteins including ribosomal proteins, proteins in the nucleus, and cell surface receptors. As mentioned above, transmembrane GPCRs transduce signals from outside the cell by binding ligands and causing activation of a canonical G-protein complex through dissociation of the G α subunit from the G $\alpha\beta\gamma$ complex. As a RACK1-like protein, Gib2 is a non-canonical G β -like protein that shares a seven WD-40 repeat motif and that can substitute for the G β subunit in the complex (Palmer *et al.*, 2006; Adams *et al.*, 2011). Like the conventional G β sub-unit (Gpb1), Gib2 interacts with G α (Gpa1) and G γ (Gpg1 and Gpg2) to form a complex (Fig. 1) (Palmer *et al.*, 2006; Wang *et al.*, 2014; Ero *et al.*, 2015). Gpa1 is a key player in the cAMP/PKA signaling pathway which, as mentioned above, contributes to diverse phenotypes such as virulence, capsule formation, melanization, mating, titan cell formation, and secretion of specific enzymes such as protease and urease (Alspaugh *et al.*, 1997; D'Souza *et al.*, 2001; Alspaugh *et al.*, 2002; Kronstad *et al.*, 2011a). The defect in capsule formation and melanin production of the *gpa1* mutant can be restored by addition of exogenous cAMP (Alspaugh *et al.*, 1997).

The contribution of Gib2 to cAMP/PKA signaling in *C. neoformans* is complex. In addition to binding G α (Gpa1) and G γ (Gpg1 and Gpg2) proteins, Gib2 also interacts with a phosphodiesterase Pde2 and the small G-protein Ras1 (Fig. 1). Gpa1 and Ras1 both influence the activity of adenylyl cyclase (Cac1) to catalyze the synthesis of cAMP (Alspaugh *et al.*, 2002). A *cac1* mutant lacking adenylyl cyclase has the same phenotypes as a *gpa1* mutant, while a *ras1* mutant, in contrast, does not show defects in capsule formation and melanin production (Alspaugh *et al.*, 2002). Ras1 does contribute to growth at 37°C (Alspaugh *et al.*, 2000; Wang *et al.*, 2014). In this context, a *gib2* mutant also has a defect in growth at 37°C and is attenuated for virulence in mice, but the mutant is still able to produce capsule and melanin. Interestingly, Ras1 appears to negatively regulate the cAMP/PKA pathway in cells lacking Gpa1. Specifically, deletion of the *RAS1* gene in the *gpa1* mutant resulted in enhanced capsule formation. Furthermore, overexpression of *GIB2* in the *gpa1 ras1* double mutant allowed capsule formation. Adenylyl cyclase (Cac1) appears to be downstream of both Gib2 and Ras1 because disruption of *CAC1* in strains lacking Gpa1, Gpa1 and Ras1, and Gpa1 and Ras1 with overexpression of *GIB2* blocked both capsule and melanin formation. In general, the results support the novel finding that Ras1 plays a negative role in the cAMP/PKA pathway by regulating Cac1 activity (Wang *et al.*, 2014). A negative role for Ras1 in the cAMP/PKA pathway was also suggested by the comparative transcriptional analysis of the impact of mutations in *RAS1* and genes encoding components of the pathway (Maeng *et al.*, 2010). Furthermore, Gib2 physically interacts with Ras1 and elevates cAMP through inhibition of the Ras1 influence on Cac1 (Wang *et al.*, 2014). The scenario that emerges from these studies is one in which Gib2 causes

elevated cAMP by preventing the negative influence of Ras1 on Cac1 when Gpa1 is absent. More recent work determined the structure of Gib2 and further characterized its role as an adaptor or scaffold protein that interacts with a large number of proteins (i.e., 55) in *C. neoformans* (Wang *et al.*, 2014). These interactions include preferential association with ribosomal proteins and the translation machinery (Wang *et al.*, 2014).

Recently, Gong *et al.* (2014) reported that the Ric8 protein (resistance to inhibitors of cholinesterase 8) is also involved in the cAMP/PKA pathway and pheromone signaling during mating in *C. neoformans* (Gong *et al.*, 2014). Ric8 functions as a guanine nucleotide exchange factor (GEF) for activation of G α (Gpa1) in the cAMP/PKA pathway and deletion of *RIC8* results in capsule and melanin defects similar to those of the *gpa1* mutant. A *ric8* mutant is also attenuated for virulence in mice. Ric8 appears to function upstream of Gpa1 because expression of an activated allele of Gpa1 (GPA1^{Q284L}) suppresses the phenotypes of the *ric8* mutant (Fig. 1). Ric8 also interacts with the G α protein Gpa2 that has a role in pheromone signaling and this is consistent with the *ric8* mutant phenotype of reduced mating (Gong *et al.*, 2014). In *C. neoformans*, pheromone exchange between mating partners activates the G α protein Gpa2 by dissociating it from a G $\beta\gamma$ complex. The G β and G γ subunits (Gpb1 and Gpg2) play central roles in mating because deletion of either gene causes sterility (Hsueh *et al.*, 2007). The mating defect of the *gpb1* mutant was not restored by addition of cAMP (Wang *et al.*, 2000). In contrast, the mating defect of a *gpa1* mutant can be suppressed by exogenous cAMP (Alspaugh *et al.*, 1997; Wang *et al.*, 2000). Activated Gpa2 relays the pheromone signal via the Ste20-mediated MAPK pathway to facilitate mating (Wang *et al.*, 2000) (Fig. 1). Ric8 interacts with both Gpa1 and Gpa2 and establishes a connection between cAMP signaling via Gpa1 and pheromone signaling via Gpa1 and a MAP kinase pathway (Gong *et al.*, 2014). A connection between cAMP signaling and another MAP kinase pathway for cell wall integrity has also been established recently in *C. neoformans* (Donlin *et al.*, 2014) (Fig. 1). Overall, the recent studies with Gib2 and Ric8 add depth to our understanding of cAMP/PKA signaling at the level of the G α protein Gpa1.

The Rak1 protein functions in cAMP and MAPK signaling in *Ustilago maydis*

A RACK1-like protein encoded by the *RAK1* gene has also been characterized in *U. maydis* and found to indirectly interact with the cAMP/PKA pathway (Fig. 1) (Wang *et al.*, 2011). The *RAK1* gene was identified in a search of the *U. maydis* genome and found to complement the adhesive growth defect of an *asc1* mutant lacking a RACK1 homolog in *S. cerevisiae* (Valerius *et al.*, 2007; Coyle *et al.*, 2009; Wang *et al.*, 2011; Rachfall *et al.*, 2013). Loss of *RAK1* in *U. maydis* causes a variety of phenotypes including slow growth, sensitivity to cell wall stress, attenuated virulence and a reduction in the formation of dikaryotic hyphae upon mating. Rak1 influences the expression of genes involved in mating as revealed by a reduction in expression of the *a* and *b* mating-type genes in a *rak1* mutant. A broader study of the impact of the *RAK1* mutation on the transcriptome identified 164 up-regulated and 37 down-regulated genes relative to the wild-type strain. The down-regulated genes included components of a MAPK signaling pathway for mating including the pheromone gene *MFAL*, the pheromone receptor gene *PRA1* and the genes for two high-mobility-group-domain transcription factors Prf1 and Rop1 (regulator of Prf1) (Kaffarnik *et*

al., 2003; Brefort *et al.*, 2005). Rop1 is a direct regulator of *PRF1* expression and, interestingly, Prf1 is regulated at the post-transcriptional level by the cAMP pathway and the MAPK pathway. In particular, PKA phosphorylation pheromone-induced expression of the *a* and *b* mating-type genes (Kaffarnik *et al.*, 2003). As in *C. neoformans*, Rak1 interacts with a large number (54) of proteins including 32 ribosomal proteins (Wang *et al.*, 2011). Overall, a model emerges of Rak1 as a regulator that links the cAMP/PKA and MAPK pathways with a number of cellular processes including mating and translation in both fungi. Additional studies are needed to determine whether Rak1 participates in similar interactions with signaling pathway components as was found for Gib2 in *C. neoformans*.

Other roles for RACK1-like proteins in fungi: hints from *S. cerevisiae*

A RACK1-like protein, Asc1, was identified as a component of the 40S ribosomal subunit in *S. cerevisiae* and, as later found with Gib2 in *C. neoformans*, the protein interacts with adenylyl cyclase to reduce the production of cAMP upon glucose stimulation (Fig. 1) (Gerbasí *et al.*, 2004; Valerius *et al.*, 2007; Zeller *et al.*, 2007). In this regard, it acts oppositely to the G α protein Gpa2 in yeast that mediates the response to glucose by stimulating cAMP production (Colombo *et al.*, 1998). Recent work examined the impact of an *asc1* mutation on the proteome, transcriptome and various phenotypes (Rachfall *et al.*, 2013). The proteome analysis revealed a substantial influence on proteins for energy metabolism including glycolysis, mitochondrial functions, oxidative stress and fermentation. Proteins for cell wall biogenesis and maintenance were also down-regulated in the *asc1* mutant. The characterization of the transcriptome revealed Asc1 regulation of 80 genes that encoded functions for transposable elements and energy metabolism with the notable inclusion of proteins for glucose uptake and iron homeostasis. The latter group included functions for iron uptake such as the cell surface iron reductase Fre1 and several siderophore transporters. The phenotypes of the *asc1* mutant are consistent with the proteome and transcriptome work because the mutant is susceptible to iron limitation, nitrosative stress, and agents that challenge cell-wall integrity. Asc1 also regulates the translation of the mRNAs for several transcription factors.

Given the collective information for *S. cerevisiae*, *C. neoformans* and *U. maydis*, the picture emerges of RACK1-like proteins as central regulators of signaling at the level of G proteins with an impact on signaling via the cAMP/PKA pathway and on downstream transcription factors. As discussed below, the connections with iron homeostasis revealed by the work on Asc1 may be relevant to virulence in fungal pathogens such as *C. neoformans* and *U. maydis*. In this regard, there is a need to test the role of Gib2 and Rak1 in iron homeostasis for these fungi.

Downstream targets of the cAMP/PKA pathway: Transcription factors and iron

The analysis of Asc1 in *S. cerevisiae* highlights connections between cAMP/PKA signaling and iron homeostasis that were previously identified by Robertson *et al.* (2000) in a transcriptome study of the role of each of the catalytic sub-units of PKA, Tpk1, Tpk2, and Tpk3. This analysis revealed that Tpk2 negatively regulates genes for iron uptake including

ferric reductase genes, the iron permease and ferroxidase of the high affinity uptake system, siderophore transporters and other functions. For *C. neoformans*, there is a growing body of evidence that the cAMP signaling regulates a similar set of functions and that iron regulatory proteins may also influence expression of components of the cAMP/PKA pathway. This evidence is discussed below followed by information on similar connections in *U. maydis*.

Iron uptake and cAMP/PKA signaling in *C. neoformans*

A connection between cAMP signaling and iron regulation was initially identified in *C. neoformans* during a characterization of the major iron regulator, Cir1 (Jung *et al.*, 2006). A transcriptome study that compared a *cir1* mutant with the wild-type strain indicated that Cir1 exerted a positive regulatory influence on the GPCR Gpr4 under both low iron and iron-replete conditions. A further association between the cAMP/PKA pathway and iron uptake came from characterization of the *SITI* gene encoding a siderophore transporter in *C. neoformans* (Tangen *et al.*, 2007). Deletion of the gene for the catalytic subunit of PKA resulted in elevated transcript levels for *SITI*, a pattern reminiscent of the influence of Tpk2 on the expression of siderophore transporters in *S. cerevisiae* (Robertson *et al.*, 2000; Tangen *et al.*, 2007). In a further connection, the *SITI* gene is also regulated by the transcription factor Nrg1, a candidate phosphorylation target of PKA (Cramer *et al.*, 2006). Similarly, the Alspaugh group discovered interactions between cAMP/PKA signaling, iron uptake and the response to pH as mediated by the transcription factor Rim101 (O'Meara *et al.*, 2010).

The interaction of cAMP signaling and Rim101 provides an interesting view of the complexities of the regulation and integration of signals related to cryptococcal virulence (O'Meara *et al.*, 2010). Rim101 is a Cys₂His₂ Zinc finger transcription factor that is activated by the alkaline pH-responsive Pal/Rim pathway in fungi to regulate adaptation to environmental conditions (Selvig and Alspaugh, 2011). Alkaline or neutral pH conditions result in activation of the pathway leading to proteolytic cleavage to activate Rim101. In *C. neoformans*, activation of Rim101 is required for elaboration of the polysaccharide capsule that is critical for virulence (O'Meara *et al.*, 2010). Interestingly, the Rim101 protein contains a predicted consensus sequence for PKA phosphorylation and nuclear localization is influenced by both Pka1 and Rim20, a scaffold protein required for cleavage (O'Meara *et al.*, 2010). Nuclear localization is a prerequisite for successful capsule formation. Further analysis revealed that Rim101 is specifically involved in capsule attachment to the cell wall rather than the production of capsule material (O'Meara *et al.*, 2010, 2013). Therefore, part of the influence of PKA on capsule formation could be mediated by Rim101. In addition, Rim101 contributes to the formation of titan cells, a subpopulation of enlarged cells that is observed during pulmonary infection (Okagaki *et al.*, 2011). Titan cell formation is also influenced by cAMP signaling (Choi *et al.*, 2012; Zaragoza and Nielsen, 2013). Along with a larger size, typical titan cells exhibit a thick cell wall, and increased capsule size in wild-type cells, whereas the *rim101* mutant exhibits a significant increase in cell wall thickness and altered composition without enlargement of the cells (O'Meara *et al.*, 2013). This increase might enhance survival of the *rim101* mutant within host tissue and, indeed, the

mutant was found to be somewhat more virulent in mice when compared with wild type (O'Meara *et al.*, 2010).

A role for Rim101 in the regulation of iron uptake and homeostasis was also revealed by the comparison of transcriptome profiles between a *rim101* mutant and the wild-type strain (O'Meara *et al.*, 2010). This experiment identified a subset of genes involved in iron uptake and homeostasis that showed down-regulated transcript levels in the *rim101* mutant. The regulated genes encoded functions such as the cell wall mannoprotein Cig1, the siderophore transporter Sit1, the ferroxidase Cfo1, and the iron permease Cft1, which are responsible for heme uptake, siderophore uptake, and high-affinity iron uptake in *C. neoformans*, respectively. Reduced transcript levels of these genes were well correlated with the growth deficiency of the *rim101* mutant in low-iron media (O'Meara *et al.*, 2010). Furthermore, a series of recent studies showed that both the *cig1* mutant and the *rim101* mutant were defective in heme uptake and that Rim101 plays a role in heme utilization via the ESCRT machinery, although evidence for a Rim101-independent heme uptake pathway was also obtained (Cadieux *et al.*, 2013; Hu *et al.*, 2015).

A recent comparative transcriptome analysis with *pka1* and *rim101* mutants also revealed that 1,077 genes are commonly regulated by Pka1 and Rim101, thus strengthening the position of Rim101 as a key downstream target of cAMP signaling (O'Meara *et al.*, 2014). Transcripts for genes encoding cell wall biosynthesis and remodeling functions were prominent among the set regulated by both PKA and Rim101. In contrast, the transcript levels for some genes were distinctly regulated by only one factor. For example, the *ENAI* gene encoding a sodium transporter was regulated specifically by Rim101 and this finding was consistent with the shared growth defects of *enal* and *rim101* mutants on media with high salt or high pH (O'Meara *et al.*, 2014). In this context, it has previously been shown that the expression of *ENAI* is induced in response to salt and osmotic stress, and that this response is dependent on the Hog1 MAPK pathway in *C. neoformans* (Jung *et al.*, 2012). The transcriptome analysis of O'Meara *et al.* (2014) was also coupled with an examination of the binding of Rim101 to the promoters of regulated genes. These included genes for cell wall biosynthesis, the *ENAI* gene, the *HAPX* gene (encoding a regulator of iron homeostasis), the *CFT1* gene for the high-affinity iron permease, and the *CTR4* gene encoding a copper transporter. Chromatin immunoprecipitation with a Gfp-Rim101 protein detected binding at the promoters of all of these genes.

Iron acquisition functions such as the high-affinity iron uptake system encoded by *CFT1* and *CFO1*, and the heme uptake function of Cig1 are important for the virulence of *C. neoformans* in a mouse model of cryptococcosis (Jung and Kronstad, 2008; Jung *et al.*, 2009; Han *et al.*, 2012; Cadieux *et al.*, 2013). A screen for functions needed for growth on heme revealed that proteins of the endosomal sorting complex required for transport (ESCRT) participate in iron acquisition, as well as in capsule formation and virulence (Hu *et al.*, 2013, 2015). Interestingly, ESCRT functions are also required in fungi for the activation of Rim101 (Selvig and Alspaugh, 2011; Ost *et al.*, 2015). In this context, mutants defective in ESCRT functions share a number of phenotypes with a *rim101* mutant including impaired growth on heme as the sole iron source and a reduced capsule size (Hu *et al.*, 2013, 2015). Most of the impact of the ESCRT machinery on capsule occurs through participation

with PKA in the activation of Rim101. However, PKA appears to make a contribution to capsule elaboration in addition to its influence on Rim101 because expression of the N-terminal (activated) portion of the transcription factor only partially restores capsule in a mutant lacking both ESCRT function and the regulatory subunit of PKA. Also, the cAMP/PKA pathway regulates the expression of genes for capsule production (Pukkila-Worley *et al.*, 2005). For iron acquisition, the ESCRT machinery make a contribution both through an influence of Rim101 activation and, independently, perhaps through endocytosis of heme (Hu *et al.*, 2015).

Iron uptake and cAMP/PKA signaling in *U. maydis*

The analysis of the impact of cAMP/PKA signaling on iron uptake has not been studied to the same extent in *U. maydis* compared with *C. neoformans*. For example, Rim101 has been characterized in *U. maydis* but a functional analysis of the influence of the factor on iron uptake has not been performed (Arechiga-Carvajal and Ruiz-Herrera, 2005; Antonio *et al.*, 2010; Franco-Frias *et al.*, 2014). However, a transcriptome analysis of a *rim101* mutant versus wild type does implicate Rim101 in the regulation of iron uptake functions (Franco-Frias *et al.*, 2014). Specifically, the transcript level for the *FER3* gene encoding a siderophore peptide synthetase was positively regulated by Rim101 while the transcripts for a siderophore transporter (*FER7*) and a ferric reductase (*FRE4*) were negatively regulated. In addition, the transcript for the GATA transcription factor *Urbs1* that regulates siderophore biosynthesis was also positively regulated by Rim101 (2.5 fold). *Urbs1* shows sequence similarity to the iron regulator *Cir1* in *C. neoformans* (Jung *et al.*, 2006). Thus, the connection between Rim101 and iron regulation that is observed in *C. neoformans* may also be present in *U. maydis*, although functional studies are needed to assess the ability of a *rim101* mutant to grown under iron limited conditions.

Interestingly, the *FER3* and *FER7* genes that are regulated by Rim101 are found in one of three gene clusters that were previously shown to be regulated upon induction of the *ADRI* gene encoding the catalytic subunit of protein kinase A (Eichhorn *et al.*, 2006). The transcripts for the genes in the clusters were demonstrated to be down-regulated upon growth in iron-replete conditions, and a transcriptome analysis with an *urbs1* mutant revealed an impact of the transcription factor on the transcript levels of the genes. Eichhorn *et al.* (2006) went on to assess the impact of a defect in the cAMP pathway (by deletion of the *UAC1* gene encoding adenylyl cyclase) and exogenous cAMP on the expression of some of the genes in the clusters. Importantly, the expression analysis identified the *FER2* gene, encoding a high-affinity iron uptake permease, as being completely dependent on an intact cAMP signaling pathway; deletion of this gene attenuated virulence in maize plants. Taken together, these studies link Rim101 to the regulation of iron uptake in *U. maydis* and reveal a definitive connection between cAMP signaling and iron in this pathogen. The results also suggest that the iron regulator *Urbs1* may be a target of PKA phosphorylation (Eichhorn *et al.*, 2006).

Conclusions

Recent studies on G β -like/RACK1 protein homologs in *C. neoformans* and *U. maydis* add depth to our understanding of the cAMP/PKA signaling pathway and its interconnections with MAPK signaling pathways. Importantly, detailed studies of the G β -like/RACK1 protein homolog Asc1 in *S. cerevisiae* support the linkage of the cAMP/PKA pathway with the control of iron uptake functions in the basidiomycete pathogens. This linkage is strengthened by emerging information on the pH-responsive transcription factor Rim101 and the iron regulators Cir1 and Urbs1 that may be targets of the cAMP/PKA pathway. These transcription factors integrate cAMP/PKA signaling with other functions including the pH response pathway and the MAPK pathways for pheromone response and cell wall integrity. There is a wealth of emerging systems biology information on the functions of transcription factors in *C. neoformans* and this work will likely support the identification of additional targets of cAMP/PKA signaling (Jung *et al.*, 2015; Maier *et al.*, 2015). Overall, these findings set the stage for future work to identify new signaling components including transcription factor targets as well the molecular mechanisms of regulation and cross talk.

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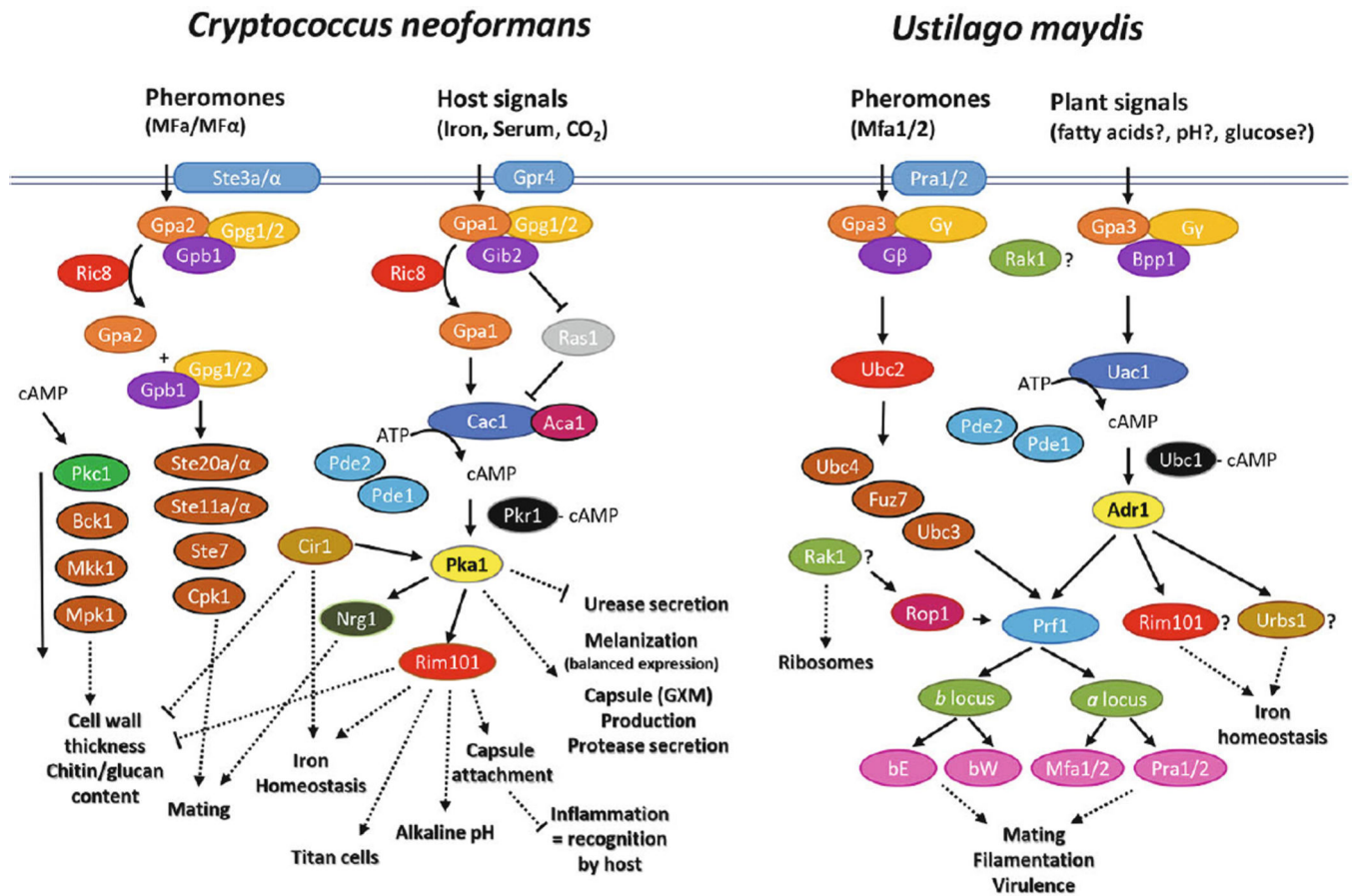


Fig. 1. The pheromone response MAPK and cAMP signaling pathways in *C. neoformans* and *U. maydis*

The known input signals are indicated at the top of each pathway (at the level of the plasma membrane) and the key identified components of the pathways are shown below as ovals. The position of Rak1 in *U. maydis* is shown as influencing ribosomes and the transcription factor Rop1 (Wang *et al.*, 2011). A speculative position for Rak1 at the level of G protein signaling is also shown for *U. maydis* and indicated with a question mark based on the findings in *C. neoformans* (Wang *et al.*, 2014). In addition, Rim101 and Urbs1 are positioned as potential downstream targets of the protein kinase A catalytic subunit ADR1. Solid arrows indicate the flow of signals through the pathways with dotted arrows representing the influence on downstream target phenotypes. The representations of the signaling pathways are based on previous reviews and the recent findings (Regenfelder *et al.*, 1997; Muller *et al.*, 2004; Arechiga-Carvajal and Ruiz-Herrera, 2005; Klosterman *et al.*, 2007; Kozubowski *et al.*, 2009; Wang *et al.*, 2011, 2014; Kronstad *et al.*, 2011a; Kozubowski and Heitman, 2012; Donlin *et al.*, 2014; Roach *et al.*, 2014). Details on additional signaling components can be found in these reviews.