ISHAM
INTERNATIONAL SOCIETY FOR
HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2016, 54, 333–352
doi: 10.1093/mmy/myv098
Advance Access Publication Date: 24 December 2015
Review Article

Review Article

Regulatory roles of phosphorylation in model and pathogenic fungi

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Received 17 June 2015; Revised 12 October 2015; Accepted 1 November 2015

Abstract

Over the past 20 years, considerable advances have been made toward our understanding of how post-translational modifications affect a wide variety of biological processes, including morphology and virulence, in medically important fungi. Phosphorylation stands out as a key molecular switch and regulatory modification that plays a critical role in controlling these processes. In this article, we first provide a comprehensive and up-to-date overview of the regulatory roles that both Ser/Thr and non-Ser/Thr kinases and phosphatases play in model and pathogenic fungi. Next, we discuss the impact of current global approaches that are being used to define the complete set of phosphorylation targets (phosphoproteome) in medically important fungi. Finally, we provide new insights and perspectives into the potential use of key regulatory kinases and phosphatases as targets for the development of novel and more effective antifungal strategies.

Key words: phosphorylation, kinases, phosphatases, human fungal pathogens, phosphoproteome.

Introduction

Protein phosphorylation is the most common type of post-translational modification in eukaryotes, including medically important fungi. Protein kinases and phosphatases mediate cellular homeostasis by the continual adjustment of complex signal transduction events in response to various internal and external environmental cues.[1–4] Protein phosphorylation is a reversible modification that is crucial for the regulation of diverse cellular processes, including metabolism, cell cycle, transcription, mating, filamentation, cell wall synthesis, maintenance of cellular integrity in stress situations (eg, in the presence of high-osmolarity and heat stresses), and virulence.[1,5–13]

The concept of protein phosphorylation was first introduced by Edmond Fischer and Edwin Krebs in the mid-1950s through studies of a special muscle system. Fischer and Krebs demonstrated a dual requirement for adenosine triphosphate (ATP) and what they described as "converting enzyme" (later called phosphorylase kinase) in the conversion of phosphorylase *b* to phosphorylase *a in vitro*.[14,15] Protein phosphatases, on the other hand, are important for the counterbalance mechanism that removes phosphate groups from various phosphorylated amino acids. Dephosphorylation mainly occurs on the hydroxyl-group-containing amino acid residues by a hydrolysis reaction. In eukaryotic cells these amino acids are typically serine, threonine and tyrosine residues.[16–18] Phosphorylation-dephosphorylation cycles serve as "on-off" switches that can trigger conformational changes of target proteins and alter their properties.[19–22]

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Protein phosphorylation has been extensively studied in the model yeast *Saccharomyces cerevisiae*. A sequence search analysis of the *S. cerevisiae* genome indicated the presence of 113 genes encoding putative protein kinases.[23] Interestingly, while a similar number of protein phosphatases was expected to counteract and maintain reversible protein phosphorylation, only 31 putative phosphatases were identified.[24] In addition, the human genome encodes about 500 protein kinases, whereas phosphatases comprise only 150 members.[25,26] Therefore, many researchers have concluded that protein phosphatases are likely to display a wider range of substrate specificity than that of protein kinases. Consistent with this notion, the structural diversity of phosphatases can be mainly attributed to an alternative regulatory subunit, which results in a diverse set of enzymes with a vast array of substrate specificities.[24,27]

In medically important fungi, the study of phosphorylation takes on an added importance since a variety of key virulence processes are controlled by this modification. These fungi include *Candida* species, which represent the 4th leading cause of hospital-acquired bloodstream infections in the United States.[28] Approximately 50% of infections can be attributed to the major human fungal pathogen *Candida albicans*, which is capable of causing a wide variety of mucosal and systemic infections in immunocompromised individuals.[29,30] Major virulence properties of *C. albicans* include phenotypic switching, biofilm formation, adhesion to host cells, secretion of degradative enzymes and the ability to undergo a reversible morphological transition from yeast to filamentous form in response to numerous host inducing signals.[31–33] *Cryptococcus neoformans* represents another major human fungal pathogen that can typically be found in a number of environmental reservoirs including the soil, compost piles and pigeon droppings.[34,35] *C. neoformans* spores are inhaled by the host, eventually leading to systemic infections that particularly target the central nervous system and can result in cryptococcal meningitis.[36,37] *C. neoformans* virulence properties include thermotolerance and melanin formation as well as formation of a protective capsule.[35,38–40] Another medically important fungus, *Histoplasma capsulatum*, is predominantly found in the soil in endemic regions such as the Ohio river valley;[41] major virulence properties include a mycelia-to-yeast transition, melanin and thermotolerance.[35,42] *Aspergillus fumigatus* also represents a major fungal pathogen that can be found in the soil as well as on decaying plant material. As a mould, *A. fumigatus* grows in the mycelial form and inhalation of conidia can lead to life-threatening pulmonary and disseminated aspergillosis.[43] *A. fumigatus* virulence properties include thermotolerance, angioinvasion, nutrient acquisition, protease secretion and gliotoxin production.[44–46] Importantly, many of the virulence properties of fungal pathogens discussed above are at least partly controlled by phosphorylation. Here, we will provide a broad and comprehensive overview of the various classes of kinases and phosphatases in pathogenic fungi and their regulatory roles, with an emphasis on enzymes that target serine (Ser)/threonine (Thr) residues (since greater than 98% of protein phosphorylation occurs on these residues).[47] Specific kinases and phosphatases of interest in model and pathogenic fungi are listed in Tables S1 and S2, respectively. We will also discuss new global approaches and efforts that have been made to define the phosphoproteome of pathogenic fungi as well as the potential that kinases and phosphatases may hold for serving as antifungal targets.

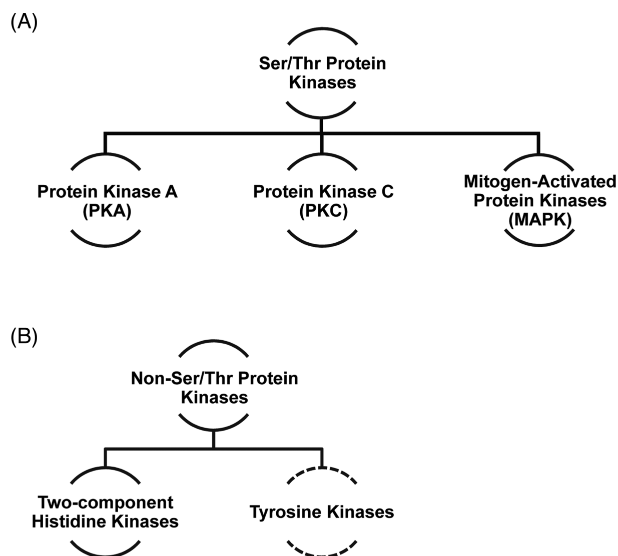

Figure 1. Major classes of kinases in model and pathogenic fungi. Major classes of Ser/Thr (A) and Non-Ser/Thr (B) protein kinases discussed in this review. Dashed line indicates that putative tyrosine kinases have been identified in several major human fungal pathogens.

Ser/Thr Protein Kinases

Protein kinases in model and medically important fungi can be classified into several major groups, based on the amino acid residues that they phosphorylate, as shown in Figure 1. Ser/Thr kinases are the predominant kinase superfamily in fungi and other eukaryotes. Tyrosine kinases, by contrast, are responsible for only 0.1% of total phosphorylation events.[48] As a consequence, most research efforts have centered on describing Ser/Thr kinases in medically important fungi, particularly with respect to their roles in cell cycle, morphogenesis and pathogenicity and we will therefore focus our discussion on this family.

1. Cyclic AMP-dependent protein kinase A

Protein kinase A (PKA) is a Ser/Thr protein kinase that serves as the main intracellular target of cAMP in all

eukaryotes.[49] PKA has been well-characterized in *S. cerevisiae* as well as several pathogenic fungi and was also the first protein kinase to have its crystal structure resolved.[50] In fungal cells, cAMP levels are controlled by the interplay between a membrane-associated adenylate cyclase for synthesis, and a cAMP-specific phosphodiesterase for degradation. Activation of adenylate cyclase is usually mediated by heterotrimeric GTP-binding proteins in most fungi.[51] The regulatory subunit of PKA normally functions to inhibit the activity of the catalytic subunit. However, upon binding to cAMP, the regulatory subunit dissociates from the catalytic subunit, leading to its activation. As a consequence, specific downstream transcription factor targets of the cAMP-PKA signaling pathway are activated, leading to the induction of genes required for many aspects of fungal growth and differentiation processes.[51–53] Genes encoding the catalytic subunit of PKA have been characterized in several filamentous fungi, including *Ustilago maydis*, *Aspergillus niger*, *Blastocladiella emersonii* and *Colletotrichum trifolii*.[54–57] In *Neurospora crassa*, the regulatory subunit of PKA, encoded by the *mcb* gene, was shown to be important for polarized growth.[58] Interestingly, expression of the *C. trifolii* regulatory subunit of PKA was able to complement the *N. crassa mcb* mutant defect,[57] suggesting that the structure and function of genes encoding fungal PKAs are highly conserved.

In the major human fungal pathogen *Candida albicans*, the cAMP-PKA signaling cascade is very important for morphogenesis and many components of this pathway are required for filamentous growth under a variety of different conditions, including serum and body temperature (37°C).[59–63] The *C. albicans* PKA complex consists of two catalytic subunits, Tpk1 and Tpk2, as well as the regulatory subunit Bcy1. cAMP inhibits Bcy1, allowing Tpk1 and Tpk2 to promote filamentation by phosphorylating downstream transcription factors, which regulate the expression of filament-specific genes. Tpk1 and Tpk2 have distinct roles in promoting filamentation. Tpk1 is important for hyphal growth on solid media, whereas Tpk2 is more important for filamentation in liquid media.[64] Interestingly, while Tpk1 is not required for adherence, invasion and damage of oral epithelial cells *in vitro*, both Tpk2 and Efg1, the downstream transcription factor target of the cAMP-PKA pathway, were shown to play important roles in these processes.[65] Both *tpk2*Δ/Δ and *efg1*Δ/Δ mutants were shown to be significantly attenuated for virulence in a murine model of oropharyngeal candidiasis, although only the *efg1*Δ/Δ mutant was attenuated in a mouse systemic model. These results suggest that hyphal formation directed by cAMP-PKA-mediated signaling represents an important virulence mechanism in oropharyngeal candidiasis and that Tpk2 is more important for oral vs. systemic infections. While a role for Tpk1 in virulence remains elusive, its distinct ability to promote filamentation under solid conditions *in vitro* may suggest a more niche-specific role during infection. The highly specific roles that Tpk1 and Tpk2 play in filamentation and/or virulence also suggest that the *C. albicans* cAMP-PKA signaling pathway possesses a level of plasticity that can adapt to multiple host filament-inducing conditions.

The cAMP-PKA pathway is also important for regulating a variety of processes in another human fungal pathogen, *A. fumigatus*. The *A. fumigatus* PKA complex consists of a type II regulatory subunit and two catalytic subunits.[66,67] The catalytic subunits (PkaC1 and PkaC2) play redundant functions with respect to conidial germination and work together to control the carbon catabolic pathway. While the *pkaC1* mutant is defective for virulence in a mouse model of invasive aspergillosis, overexpression of *pkaC2* in this mutant can rescue the virulence defect. In addition, the *pkaC1* single mutant appeared to be less attenuated for virulence in this model than the *pkaC1 pkaC2* double mutant.[66] While the complementary roles of *pkaC1* and *pkaC2* in conidial germination and carbon catabolism may contribute to virulence, these results also suggest that PkaC1 and PkaC2 may have independent functions associated with pathogenesis. Deletion of the gene encoding the regulatory subunit of protein kinase A, *pkaR*, resulted in reduced *A. fumigatus* growth and germination rates, morphological abnormalities in conidiophores and reduced conidiation.[68] Consistent with findings for the *A. fumigatus* PKA catalytic subunit mutants, the *pkaR* mutant was also found to be significantly attenuated for virulence when conidia were administered intranasally in an immunosuppressed mouse model.

In the human fungal pathogen *C. neoformans*, the cAMP-PKA pathway has been found to be important for regulating many cellular processes, including capsule production, melanin formation, mating, and virulence.[69] Mutant strains lacking conserved components of the cAMP-signaling cascade such as G_α protein (Gpa1) and adenylyl cyclase (Cac1) are attenuated for virulence, most likely as a result of not showing an increase in capsule or melanin production in response to normal inducing conditions.[70–72] In addition, mutants lacking the *C. neoformans* PKA catalytic subunit, Pka1, are unable to mate, fail to produce melanin or capsule, and show reduced virulence in animal models, whereas mutants lacking the PKA regulatory subunit, Pkr1, are hyper-virulent and overproduce capsule.[73] Interestingly, hyperactivation of the PKA signaling pathway leads to enhanced virulence in *C. neoformans*[72] whereas in the plant fungal pathogen *U. maydis*, PKA hyperactivation results in defects in tumor (gall) formation and thus reduced virulence.[74,75] These studies illustrate how a conserved

phosphorylation signaling pathway has been exploited to serve related, but distinct, virulence functions in two different fungal pathogens as they evolved to adapt to different host environments.

2. Protein kinase C

Protein kinase C (PKC) is a calcium/phospholipid-dependent Ser/Thr kinase, which acts as a transmitter and amplifies signal transduction pathways. PKC is a key component of the phosphoinositide cascade, which stimulates a wide variety of responses in various cell types, including cell proliferation, gene expression, membrane transport and organization of the cytoskeleton.[76,77] In fungi, genes predicted to encode PKCs have been characterized in *S. cerevisiae*,[78] *Schizosaccharomyces pombe*,[79] *C. albicans*,[80] *Trichoderma reesei*, and *A. niger*.[81] PKC orthologs are well-conserved among these fungi and appear to function as regulators of cell wall biosynthesis.[82] In *S. cerevisiae,* Pkc1 has multiple targets and is important for maintaining cell wall integrity in response to stress during growth and morphogenesis.[83–85] Interestingly, *S. cerevisiae* Pkc1 has been shown to function independently of both Ca^{2+} and phospholipids, but is regulated by autophosphorylation.[78] However, in *S. pombe* and *T. reesei* PKC activity is phospholipid-dependent, but Ca^{2+}-independent.[79,81]

In *C. neoformans*, the PKC signaling pathway is important for fluconazole tolerance as well as invasion of human brain microvascular endothelial cells.[86,87] *C. neoformans* strains deleted for *PKC1*, encoding a key component of this pathway, show altered capsule formation, reduced melanin production and are hypersensitive to oxidative and nitrosative stress, cell wall-inhibiting agents and temperature.[88] The PKC signal transduction pathway has also been shown to play a key role in controlling *C. neoformans* cell wall integrity.[89] In the filamentous fungus *A. nidulans*, the *pkcA* gene (encoding PKC) is essential and important for establishment of polarity and suppression of apoptosis under thermal stress.[90,91] In *C. albicans*, homozygous *pkc1* deletion mutants are viable and can undergo the yeast-to-hypha transition, but both yeast and hyphal cells show increased lysis defects.[92] The *C. albicans* Pkc1-activated mitogen-activated protein kinase (MAPK) cascade is conserved and has been implicated in the up-regulation of chitin synthase (*CHS*) genes in response to antifungals such as echinocandins.[92] These studies support a role for PKCs in maintaining cell wall integrity during growth and morphogenesis of both pathogenic and nonpathogenic fungi.

3. Mitogen-activated protein kinases

Mitogen-activated protein (MAP) kinase cascades are evolutionarily conserved among all eukaryotes and have been identified in a variety of organisms from fungi to humans.[93,94] MAP kinases have been shown to participate in transducing a diverse array of extracellular signals and regulating vital cellular processes such as cell differentiation, cell movement, cell division, and cell death.[95–97] MAP kinases are usually activated by dual phosphorylation of tyrosine and threonine residues by MAP kinase kinases (MAPKK), which in turn, are activated by MAP kinase kinase kinases (MAPKKK). The sequential activation of the MAPK cascade eventually results in the activation of transcription factors and the expression of specific sets of genes in response to environmental stimuli.[93]

In *S. cerevisiae*, MAP kinase signal transduction pathways have been extensively studied and shown to be involved in many cellular processes including mating, high osmolarity responses, cell wall remodeling, filamentation, and sporulation.[98,99] Adaptation to osmotic stress mainly occurs through the high osmolarity glycerol (HOG) MAP kinase pathway.[98] The *S. cerevisiae* MAP kinase pathway associated with mating is triggered by pheromones and involved in shmoo formation as well as subsequent diploid formation.[100] The *S. cerevisiae* filamentous growth MAPK pathway functions through Kss1 and is activated by the Ras2-Cdc42-Bmh1-Ste11 cascade.[101–103]

MAP kinases have also been shown to be important for virulence and/or virulence-related processes of several fungal pathogens, including *Botrytis cinerea*,[104] *Cochliobolus heterostrophus*,[105] *Fusarium oxysporum*[106] and *Ustilago maydis*.[107] In *C. albicans*, MAPK signal transduction pathways that regulate the yeast-to-hypha transition, virulence and white-opaque switching have been well-studied and characterized.[108–110] The Cek1 (homolog of *S. cerevisiae* Kss1) MAPK cascade plays an important role in the *C. albicans* yeast-hypha transition and virulence.[111] Several components of the Cek1-MAPK pathway, including *STE2*, *CST20*, *HST7*, *CEK1*, and *CPH1*, are also involved in *C. albicans* mating responses.[108,110] In addition, the *C. albicans* cell wall integrity pathway, important for virulence, is controlled by the Mkc1-MAPK pathway.[112,113]

In *C. neoformans*, the Cpk1-MAPK signaling cascade plays important roles in mating and monokaryotic fruiting, and shares many features with the well-characterized pheromone response pathway in *S. cerevisiae* described above. Both mating and monokaryotic fruiting in *C. neoformans* are mediated by Gbp1, which activates Ste20, a p21-activated protein kinase (PAK) homolog in the Cpk1-MAPK cascade.[114] Interestingly, in contrast to the case of *S. cerevisiae*, disruption of *C. neoformans STE12,* encoding a downstream pheromone response target of the Cpk1-MAPK pathway, does not abolish pheromone sensing or mating,[115,116] and additional downstream effectors for Cpk1 in this cascade have been identified.[117] A conserved Pbs2-Hog1 MAP kinase pathway has also been

shown to control morphological differentiation as well as virulence properties (eg, thermotolerance, response to oxidative stress) in the highly virulent serotype A but not a less virulent laboratory-generated serotype D, strain of *C. neoformans*.[118] Interestingly these findings suggest that fungal pathogens such as *C. neoformans* have evolved specialized MAP kinase signal transduction pathways to control virulence-related properties in more pathogenic strains. Not surprisingly, *C. neoformans* serotype A *hog1*Δ mutants were found to be attenuated for virulence in a mouse model of disseminated cryptococcosis.

In *Pneumocystis carinii*, a gene encoding a putative MAPKKKK, *PCSTE20*, has been shown to be strongly upregulated in response to binding of the pathogen to extracellular matrix proteins.[119] In *A. fumigatus* there are four known MAPKs: SakA is closely related to the HOG-MAPKs of other fungi, MpkB is similar to MAPKs involved in pheromone signaling, MpkA is similar to MAPKs involved in cell wall integrity and MpkC appears to be involved in conidial germination.[120] SakA and MpkA are both associated with *A. fumigatus* morphogenesis.[121,122] Deletion of the gene encoding the HOG-MAPK pathway component SakA results in abnormal conidial germination under different environmental conditions.[122] Overall, these studies indicate the important role that Ser/Thr signaling kinases play in morphology and mediating additional virulence-related processes in *A. fumigatus* and other pathogenic fungi.

Non-Ser/Thr protein kinases

While considerably less abundant than Ser/Thr kinases, several non-Ser/Thr protein kinases, in particular two-component histidine kinases, play important regulatory roles in human fungal pathogens. Two component histidine kinase systems are composed of a histidine kinase (HK) and a response regulator (RR) protein. In *S. cerevisiae*, histidine kinase phosphorelay systems transmit signals to activate the HOG1-MAPK pathway in response to osmotic stress.[123] In *C. albicans*, Cos1, a two-component histidine kinase, is important for hyphal development under both solid and liquid filament-inducing conditions. [124] Nik-1, a homolog of Cos1 in the filamentous fungus *N. crassa*, was also found to be important for filamentation, especially under increased osmotic pressure during growth on solid medium.[125,126] In addition, *SRR1*, a putative two-component response regulator gene in *C. albicans*, was found to be important for oxidative and osmotic stress adaptation, morphogenesis, and virulence.[127] Two-component histidine kinase systems have also been reported to be essential for stress adaptation and virulence in other pathogenic fungi, including *A. fumigatus*, *C. neoformans*, and *B. dermatitidis*.[42,128–130]

Tyrosine kinases represent a second class of non-Ser/Thr kinases. A general comparative genetic analysis of over 30 different fungal species determined that the overall representation of the tyrosine kinase group is very small.[131] While tyrosine kinases have been shown to be important for cell cycle control in *S. cerevisiae*[132] and mitotic entry/DNA damage checkpoint control in *S. pombe*,[133,134] considerably little is known about the role of these enzymes in pathogenic fungi. However, putative tyrosine kinases have been identified in several major human fungal pathogens including *C. albicans* and *C. neoformans* (http://www.candidagenome.org/, http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=Cryne_JEC21_1).

Ser/Thr protein phosphatases

Ser/Thr protein phosphatases represent more than 90% of all phosphatases and play essential regulatory roles in all eukaryotes.[135] An increasing number of Ser/Thr protein phosphatases have been discovered and characterized in fungi, several of which play important cellular functions including cell cycle regulation, growth, protein synthesis, filamentation and maintenance of cellular integrity.[19,135,136] Interestingly, biochemical analyses of Ser/Thr protein phosphatases in certain filamentous fungi have provided evidence for functional similarities with those studied in higher eukaryotes.[137–139] For example, in the presence of calmodulin, a highly conserved catalytic subunit of a *N. crassa* calmodulin-dependent protein phosphatase showed equivalent phosphatase activity to that of bovine brain calcineurin.[138] In addition, a protein phosphatase-1 (PP1) inhibitor has also been shown to effectively inhibit both mammalian and *N. crassa* PP1.[137] While Ser/Thr protein phosphatase catalytic domains are remarkably similar, enzyme structural diversity within subfamilies is mainly attributed to regulatory subunit specificities.[23,25] Ser/Thr protein phosphatase complexes consist of multiple combinations of the conserved catalytic subunit and numerous regulatory subunits that control a broad spectrum of signaling pathways.[18,22,139–143] Due to the 'eccentric' functionality of these enzymes, relatively few Ser/Thr phosphatases control the specific dephosphorylation of thousands of phosphoprotein substrates.[144] Ser/Thr protein phosphatases are classified biochemically based on substrate specificity and sensitivity to endogenous inhibitors[141] and are divided into two broad groups, type-l and type-2. The type-2 enzymes are further separated into three subgroups, 2A, 2B, and 2C, based on their structure and regulation[19,23] as illustrated in Figure 2. Next, we will discuss the role of specific Ser/Thr phosphatase subfamilies in controlling a variety of biological processes in model and medically important fungi.

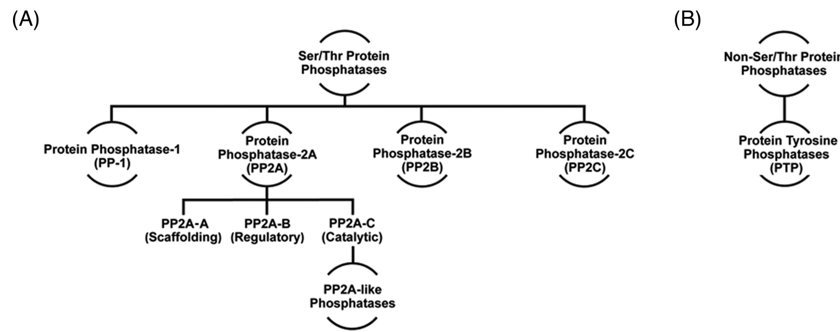

Figure 2. Major classes of phosphatases in model and pathogenic fungi. Major classes of Ser/Thr (A) and Non-Ser/Thr (B) protein phosphatases discussed in this review.

1. Protein phosphatase 1

Protein phosphatase 1 is one of the major eukaryotic Ser/Thr protein phosphatase classes that regulates an enormous variety of cellular functions. This is believed to occur by interaction of the catalytic subunit of this enzyme with multiple regulatory subunits.[145–147] In contrast to the protein Ser/Thr kinases,[148] PP-1 does not display obvious consensus sequence selectivity, dephosphorylating multiple substrates both *in vivo* and *in vitro*.[149]

From fungi to mammals, PP-1 has been shown to play an evolutionarily conserved role in controlling cell cycle progression.[150–153] *S. pombe DIS2*, which encodes PP-1, is required for chromosome disjoining during mitosis.[154] In *S. cerevisiae*, multiple studies have also suggested that PP-1 is important for reversing phosphorylation of aurora kinases, a family of mitotic Ser/Thr kinases, during mitosis and meiosis.[155–159] In *A. nidulans*, *BIMG11*, which encodes PP-1, is required for completion of anaphase in the cell cycle.[160] More recent work in *S. cerevisiae* has suggested that PP-1 is also important for regulating the spindle checkpoint during chromosome segregation in the cell cycle.[153,161]

In addition, PP-1 is known to control protein synthesis in a wide range of eukaryotes.[162,163] Phosphorylation of eIF2α is the principal mechanism yeast cells use to inhibit protein synthesis under a variety of stress conditions including amino acid starvation. PP-1, however, restores protein synthesis by dephosphorylating eIF2α.[164] PP-1 has also been shown to be important for controlling glycogen accumulation in yeast as well as metabolism and glucose regulation in mammals.[165–167]

In *C. albicans*, few PP-1 enzymes have been identified and characterized. A study has determined that the PP-1/Glc7 regulator, Shp1, plays important roles in *C. albicans* morphogenesis, cell cycle progression and DNA damage response.[168] In *S. cerevisiae* Bni4 represents the PP-1/Glc7 phosphatase targeting subunit and is involved in bud-neck localization of chitin synthase.[169,170] A *C. albicans* strain deleted for the *BNI4* homolog formed lemon-shaped yeast cells, had a 30 % reduction in cell-wall chitin, and showed reduced hyphal formation under filament-inducing conditions.[171] These results suggest an important role for PP-1 in *C. albicans* cell wall maintenance and filamentation. A *C. albicans* mutant for Sal6, a PP-1-related phosphatase, has been reported to have a slight to moderate virulence defect in a silkworm infection model.[172] Overall, PP-1 plays critical roles in dephosphorylating substrates to control a variety of cellular processes in fungi, including mitosis, meiosis, cell division, filamentous growth, protein synthesis and glycogen metabolism.[24]

2. Protein phosphatase-2B

Protein phosphatase 2B (PP2B), also known as calcineurin (CaN), is a highly conserved Ca^{2+}/calmodulin-regulated Ser/Thr protein phosphatase present in many organisms from yeast to humans.[173] Calcineurin is typically composed of a catalytic calmodulin-binding A subunit and a regulatory Ca^{2+}-binding B subunit.[174] The regulatory B subunit functions to promote the activity of the catalytic A subunit. Like other Ser/Thr protein phosphatases, calcineurin also has broad substrate specificity. Calcineurin functions in many pathogenic fungi to control a broad spectrum of cellular processes, including cation homeostasis, morphogenesis, and virulence[175,176] and is considered to be a key regulator of cellular stress responses in eukaryotes.[176]

In *S. cerevisiae*, both genes encoding calcineurin catalytic subunits (*CNA1* and *CNA2*) are not essential for viability.[177] However, calcineurin is required for cellular adaptation under a variety of environmental stresses. Once activated, calcineurin dephosphorylates the transcription factor Crz1, which, in turn, activates genes involved in a wide variety of processes, including signal transduction and cell wall integrity.[178] In *C. albicans*, calcineurin is not essential. However, this phosphatase is critical for mediating cell survival during membrane stress.[179] Calcineurin can be pharmacologically inhibited in *C. albicans* by the combination of either cyclosporine A or tacrolimus (FK506) with fluconazole.[179] Homozygous deletion of *C. albicans CMP1*,

which encodes the calcineurin A (CNA) subunit, resulted in hypersensitivity to serum and antifungal agents that target ergosterol biosynthesis *in vitro*, as well as attenuated virulence in a mouse model of systemic candidiasis.[180,181] These findings suggest that calcineurin plays a key role in the ability of *C. albicans* to adapt to serum and stress conditions in the host environment and the observed virulence defect may be attributed to a reduced ability to respond to environmental stresses during infection. Deletion of the calcineurin target, *CRZ1,* in *C. albicans* results in hypersensitivity to membrane stress conditions. Interestingly, *crz1* homozygous deletion mutants are not defective for virulence in a mouse model of systemic candidiasis.[182,183] Deletion of *CRZ1* only partially reduces azole resistance in *S. cerevisiae*, whereas deletion of *CNB1*, a regulatory subunit of calcineurin, completely blocks resistance.[182,184] These results suggest that additional downstream effector(s) of the calcineurin-signaling cascade, besides Crz1, regulate azole resistance.

In *C. neoformans*, calcineurin plays a central role in regulating virulence and morphogenesis.[185,186] Pharmacological inhibition of calcineurin by FK506 renders cells unable to mate. Calcineurin is also required for *C. neoformans* hyphal elongation in diploid strains and asexual monokaryotic fruiting of *MATα* cells in response to nitrogen limitation.[187] Indeed, calcineurin is required for virulence in both a rabbit model of cryptococcal meningitis as well as a murine systemic model.[185,188] These virulence defects can most likely be attributed to the inability of *C. neoformans* calcineurin mutant strains to survive under *in vitro* conditions similar to those of the host environment (alkaline pH, high temperature, 5% CO_2). Cbp1, a calcineurin-binding protein in *C. neoformans*, functions as a targeting subunit to regulate mating-dependent filamentation.[186,189] However, *cbp1* mutants show no defects during haploid fruiting and only a modest virulence defect in mice, suggesting that additional targeting proteins(s) interact with calcineurin to regulate these processes.

The calcineurin pathway is also important for morphology in *A. fumigatus*. Both pharmacological and genetic inhibition of *A. fumigatus* calcineurin impairs filamentation, resulting in delayed hypha production.[190] Strains bearing mutations in *cnaA*, which encodes the *A. fumigatus* calcineurin catalytic subunit, display improper polarized growth, reduced filamentation, and decreased virulence in a mouse model of invasive aspergillosis;[191,192] virulence defects are most likely at least partly attributed to reduced filamentation. Similar defects are observed upon mutation of *A. fumigatus crzA* (the *CRZ1* homolog).[193,194]

Interestingly, recent studies have demonstrated that calcineurin plays a key role in the dimorphic transition and virulence of *Mucor circinelloides*.[195,196] *M. circinelloides* is a causative agent of mucormycosis, a frequently lethal, but uncommon human fungal infection.[197] Deletion of the gene encoding the calcineurin regulatory B subunit of *M. circinelloides* resulted in a mutant locked in yeast phase growth.[195] Similar results were also observed when *M. circinelloides* was grown in the presence of the calcineurin inhibitor FK506. The calcineurin regulatory B subunit gene deletion mutant was also attenuated for virulence in a wax moth larvae model, suggesting that the *M. circinelloides* yeast-hyphal dimorphic transition is important for this process. More recent work with this yeast-locked mutant has showed that phagosome maturation occurs in the presence of yeast but not spores.[196] Surprisingly, *M. circinelloides* mutants for *cnaA*, encoding the calcineurin A catalytic subunit A, showed larger size spores and increased virulence in the wax moth larvae model.[195] One possible explanation for this unexpected finding is that calcineurin phosphatase negatively regulates other kinases in the cell that are important for virulence. Consistent with this notion, in *U. maydis* and *S. cerevisiae* there is an established antagonistic relationship between calcineurin and PKA.[198,199] Interestingly, mutants in the *M. circinelloides* calcineurin A catalytic subunit B showed several functional differences when compared to *cnaA* mutants (eg, greater sensitivity to cyclosporine A and inability to produce hyphae in the presence of this compound) and were not attenuated for virulence in the wax moth larvae model.[196] As in the case of PKA, these findings suggest that *M. circinelloides* calcineurin catalytic subunits play related, but distinct, roles with respect to morphology, virulence and/or response to the host environment.

3. Protein phosphatase-2C

Protein phosphatase-2C (PP2C) is a class of Mg^{2+}-dependent Ser/Thr phosphatases that are highly conserved, present in both prokaryotes and eukaryotes and involved in a wide variety of key cellular processes, including proliferation, metabolism, and cell death.[200–202] In contrast to other Ser/Thr phosphatases, PP2C phosphatases are monomeric enzymes and share no structural homology with PP-1, PP2A, or PP2B.[202] PP2C phosphatases are not associated with multiple regulatory subunits and their function is usually achieved by multiple catalytic isoforms. For example, more than 14 genes encoding PPC phosphatases were identified in humans, and up to 80 PP2C proteins have been predicted in *Arabidopsis thaliana*.[203,204] These multiple catalytic isoforms are likely to provide the structural basis for functional specificity. In *S. cerevisiae* there are seven identified PP2C-encoding genes (*PTC1-7*),[205,206] which are involved in diverse cellular functions. Homologs of several of these genes play important roles in medically important fungi.

Ptc1 phosphatase is the best-characterized of the PP2C isoforms in yeast. Several genetic studies have demonstrated that Ptc1 is a negative regulator of the HOG pathway and associates with components of this pathway in *S. cerevisiae*.[207–209] Ptc1 phosphatase has also been linked to the yeast MAPK cell wall integrity (CWI) pathway (Slt2/Mpk1) via interaction with Pck1 kinase.[210] Ptc1 plays an important role in the regulation of mating in *S. cerevisiae*[211] and is likely to be involved in controlling numerous additional processes in yeast since *ptc1* mutant strains are hypersensitive to heavy metals, alkaline pH, calcium ions, and exhibit fragmented vacuoles, a random budding pattern, as well as defects in both vacuolar and cortical ER inheritance.[210,212–214] Ptc2 and Ptc3 have been implicated in regulating progression through the yeast cell cycle.[205,215] *S. cerevisiae* Ptc6 has been shown to be necessary for survival of stationary phase cells and is also involved in the mitochondrial degradation process known as mitophagy.[216,217]

The *C. albicans* homozygous null *ptc1* mutant is more resistant than a wild-type strain to the cell wall stressor Congo red and the antifungal terbinafine.[172] However, this mutant also shows hypersensitivity to the echinocandin-derived antifungal micafungin, reduced hyphal growth both *in vitro* and *in vivo* and a significant attenuation in virulence in both silkworm and mouse models of disseminated candidiasis.[172] *C. albicans* cells deleted for *PTC2* are sensitive to azole antifungals and SDS, as well as the DNA synthesis inhibitor hydroxyurea and the DNA methylation agent methylmethane sulphonate (MMS).[218] Ptc2 is also associated with mitochondria and these findings suggest that this phosphatase has multiple functions in *C. albicans*, including checkpoint recovery from DNA damage and the control of mitochondrial physiology. Disruption of other Ptc isoforms, such as *PTC7*, does not affect growth or filament development in *C. albicans*.[219] A new member of the PP2C family, Ptc8, has also been characterized in *C. albicans*.[220] *PTC8* is induced in response to growth in the presence of high osmolarity as well as serum at 37°C. The *ptc8*Δ/Δ mutant is defective for hyphal formation[220] but has not been linked to any known filamentous growth signaling pathways.

In *Fusarium graminearum*, the major causal agent of *Fusarium* head blast disease on barley and wheat, *PTC1* was found to play an important role in the ability of mycelial growth to resist lithium toxicity.[221] Deletion of *PTC1* attenuates *F. graminearum* virulence on wheat coleoptiles but not on wheat heads.[221,222] While these results may suggest that mycelial growth mediated by Ptc1 plays a specialized role in directing virulence against specific niches on wheat, another possible explanation is that independently constructed versions of the *ptc1* deletion strain were used to test for virulence in the wheat coleoptile vs. head models. We conclude that PP2C phosphatases play an integral role in an array of key cellular processes in pathogenic and nonpathogenic fungi, including cell wall integrity, filamentous growth, and virulence.

4. Protein phosphatase-2A

Type 2A protein phosphatases (PP2A) constitute a diverse family of Ser/Thr phosphatases that are ubiquitously expressed in eukaryotic cells and perform multiple functions in cellular signaling.[141] PP2A is a multiprotein complex composed of three distinct subunits. The A subunit (PP2A-A) is the structural subunit that serves as a scaffold to accommodate the other two subunits. The C subunit (PP2A-C) is the catalytic subunit and the B subunit (PP2A-B) is the regulatory subunit which dictates substrate specificity and intracellular localization of the enzyme.[141] It is considered the most structurally diverse subunit. To date, four unrelated protein families of PP2A regulatory subunits have been identified: B, B′, B″, and B‴.[223–225] In higher eukaryotes (eg, mammals) each family is encoded by multiple genes and some transcripts of these genes undergo alternative splicing to generate an even greater number of isoforms.[226,227] The functional involvement of PP2A in so many diverse biological processes can be largely attributed to the B subunit. PP2A exists in two different forms: dimeric form ($PP2A_D$) and trimeric form ($PP2A_T$). The dimeric form is known as the core enzyme and is composed of the catalytic and scaffold subunits, while the trimeric form is an active heterotrimeric holoenzyme complex which consists of all three subunits.[139,141,228]

Regulation by PP2A and PP2A catalytic, scaffold and regulatory subunits

PP2A phosphatases are highly conserved from fungi to humans and involved in a variety of functions in multiple species, including cell differentiation, cell cycle, oncogenic transformation, signal transduction, and filamentous growth.[139,229] Not surprisingly, PP2A phosphatases are also tightly regulated by post-translational modifications. These modifications mainly involve methylation at the carboxyl terminus of the catalytic subunit[230,231] and phosphorylation.[232]

In *S. cerevisiae*, loss of both PP2A catalytic subunits (*PPH21* and *PPH22*) impairs growth, but is not lethal.[233,234] In comparison, *PPA1* and *PPA2*, which encode PP2A catalytic subunits, are essential for growth in *S. pombe*.[235] In *A. nidulans,* deletion of *PPHA*, a PP2A homolog, leads to slow growth, delayed germ tube emergence and mitotic defects at low temperature.[236] The *S. cerevisiae* PP2A scaffolding subunit is encoded by the gene *TPD3*. Deletion of *TPD3* is not lethal but renders yeast cells cold-sensitive. Following a shift to 13°C, *tpd3* mutant cells

become multibudded and multinucleate, suggesting a defect in cytokinesis.[237] The *tpd3* deletion mutants are also sensitive to high temperature (eg, 37°C), and this temperature sensitivity phenotype is most likely attributed to a defect in RNA polymerase III transcription.[237] A recent BLAST search has indicated the presence of orthologs for Tpd3 in major human fungal pathogens, including *C. albicans*, *A. fumigatus*, *H. capsulatum,* and *C. neoformans* (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The PP2A regulatory subunit (PP2A-B) is encoded by one gene in *S. cerevisiae*, *CDC55*. *S. cerevisiae* strains deleted for *CDC55* showed multi-budded and multi-nucleated yeast cells (similar to the *tpd3* phenotype), suggesting a role for PP2A in cell cytokinesis.[238] Genetic analysis indicates that Cdc55 is involved in at least two steps during the cell cycle: the metaphase-anaphase transition and mitotic exit.[239–241] Orthologs for Cdc55 are present in multiple human fungal pathogens such as *H. capsulatum, A. fumigatus*, *C. neoformans* and *C. albicans* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In *N. crassa*, PP2A-B is required for completion of macroconidiation. A study has found that *RGB-1,* encoding a putative PP2A-B regulatory subunit, is a regulator of budding during the macroconidiation process; *rgb-1* mutants, which are defective for macroconidiation budding, instead undergo arthroconidiation.[242] In *A. nidulans*, two PP2A-B regulatory subunit homologs, *parA* and *pabA*, have been recently identified and characterized.[243] Deletion of *parA* causes hyper-septation, while overexpression of *parA* abolishes septum formation. Interestingly, this study also showed that *parA* deletion is capable of suppressing septation defects in *pabA* mutants,[243] suggesting that ParA counteracts PabA during the septation process. However, both PP2A-B regulatory subunits act synergistically during hyphal growth, since a double mutation of *parA* and *pabA* led to synthetic defects in colony growth at 42°C.[243]

PP2A-like phosphatases

PP2A-like phosphatases show a degree of sequence identity to PP2A enzymes but are not sufficiently identical to be classified as homologs.[233,234,244,245] In addition, in yeast, a PP2A-like protein has been shown to only partially complement defects of strains deleted for *PP2A*.[233] PP2A-like phosphatases can form complexes with regulatory subunits and are highly conserved from yeast to humans.[246–249] Three different PP2A-like phosphatases have been identified in fungi: Sit4, Pph3 and Ppg1.[245]

Sit4 plays a key role in cell growth and proliferation in *S. cerevisiae*.[233,245,250] Deletion of *SIT4* also causes cell cycle arrest at late G_1, suggesting that the Sit4 phosphatase is required for the G_1/S transition.[244] Another study has suggested that Sit4 is required for expression of the G_1 cyclins, *CLN1* and *CLN2*.[251] *S. cerevisiae SIT4* has also been shown to be involved in the Pkc1-MAPK signaling pathway, which is important for the transcriptional response to stresses that alter cell wall integrity.[84,252] In *C. albicans*, disruption of *SIT4* causes a significant reduction in growth rate, hyphal formation and virulence in a mouse model of systemic candidiasis.[253] Consistent with these findings, a more recent study has indicated that the *C. albicans sit4* null mutant is defective for morphogenesis on solid Spider medium.[254] *C. albicans sit4* cells also displayed reduced transcript levels for genes encoding HOG1-MAPK pathway components in a DNA microarray experiment.[253]

A second PP2A-like phosphatase is Pph3. In *C. albicans*, Pph3 and its regulatory subunit, Psy2, control dephosphorylation of Rad53, a putative component of the cell cycle checkpoint, and cell morphogenesis during recovery from DNA damage.[246,255] Deletion of *PPH3* or *PSY2* results in hypersensitivity to DNA-damaging agents, such as cisplatin and MMS.[246] In addition, *pph3*Δ/Δ and *psy2*Δ/Δ mutant cells show robust filamentation under genotoxic stress.[246] Interestingly, more recent studies in *S. cerevisiae* have linked the activity of Pph3 to both the nonhomologous end-joining (NHEJ) pathway as well as cell cycle progression.[256] Consistent with the later finding, a recent study has determined that the *C. albicans* Pph3–Psy2 phosphatase complex is important for Rfa2 dephosphorylation during G_1-phase and under DNA replication stress.[257] Rfa2 is a key subunit of the replication protein A (RPA) heterotrimeric complex, which functions in DNA replication, repair and recombination pathways in eukaryotes.[258,259] Altogether, these studies suggest that the Pph3/Psy2 complex plays key roles in cell morphogenesis, cell cycle progression and/or recovery from DNA damage in *S. cerevisiae* and *C. albicans*. However, little is known about the function of Pph3/Psy2 complexes in other fungal systems.

The final PP2A-like phosphatase that we will discuss is Ppg1. *PPG1* was first cloned and identified in *S. cerevisiae* based on sequence similarity to other Ser/Thr phosphatases.[245] *S. cerevisiae ppg1* deletion mutants are viable, but show a decrease in glycogen accumulation.[245] Recently, a transposon mutagenesis screen has suggested a role for Ppg1 in ethanol and heat tolerance in *S. cerevisiae*.[260] In *S. pombe* Ppa3, a Ppg1 ortholog, was found to be involved in regulating two of the SIN (septation initiation network) pathway kinases, Cdc7 and Sid1, important for actomyosin ring maturation and stability.[261] A more recent study, however, demonstrated that the *S. pombe ppg1*Δ strain shows normal cell morphology.[262] *C. albicans* Ppg1 was first identified in a screen of orthologs of previously annotated *S. cerevisiae* protein phosphatases.[172] A systematic screen of a *C. albicans* homozygous deletion library

has demonstrated that the *ppg1*Δ/Δ mutant strain is defective for morphogenesis and shows reduced kidney fungal burden in a mouse model of systemic candidiasis.[254] A recent study has also demonstrated that both a *ppg1*Δ/Δ mutant as well as a mutant specifically defective for Ppg1 phosphatase activity show reduced filament extension and invasion as well as highly attenuated virulence in the mouse systemic model.[136] In addition, *C. albicans* Ppg1 appears to function via the cAMP/PKA filamentous growth pathway. While the *ppg1*Δ/Δ virulence defect is most likely attributed to defects in filamentation and invasion, Ppg1 may control other virulence-related processes which have yet to be elucidated. Within *C. albicans*, the Ppg1 catalytic subunit is highly conserved among other PP2A and PP2A-like phosphatases.

Non-Ser/Thr protein phosphatases

Three protein tyrosine phosphatases (PTPs) have been identified in *S. cerevisiae*: Ptp1, Ptp2, and Ptp3.[263,264] Ptp2 and Ptp3, but not Ptp1,[265] are involved in regulation of various MAPK cascades. Ptp2 and Ptp3, however, differ in their ability to dephosphorylate yeast MAP kinases. Ptp2 preferentially dephosphorylates Hog1 and Mpk1 (involved in the cell wall integrity pathway), whereas Ptp3 preferentially dephosphorylates Fus3 (involved in the pheromone response pathway).[264,266,267] A *ptp2*Δ/Δ *ptp3*Δ/Δ double mutant shows significantly decreased sporulation efficiency in *S. cerevisiae*.[21] In human fungal pathogens, the role of PTPs in controlling virulence and virulence-related properties is poorly understood. A recent study, however, has found that *PTP1* and *PTP2* are important for both *C. neoformans* differentiation and pathogenicity.[268] Consistent with results in *S. cerevisiae*, *C. neoformans* Ptp2 suppressed the hyperphosphorylation of Hog1. *C. neoformans* Ptp2 was also found to be involved in mediating vegetative growth, sexual differentiation, stress responses, and antifungal drug resistance. In contrast, *C. neoformans* Ptp1 was not essential for Hog1 regulation. However, *PTP1* overexpression could rescue or partially rescue *ptp2* mutant defects in thermotolerance, as well as resistance to H_2O_2, flucytosine and $CdSO_4$. Importantly, this study also determined that Ptp2 is important for virulence in a murine model of systemic cryptococcosis. The observed virulence defect can most likely at least partly be attributed to one or more of the *in vitro ptp2* mutant defects listed above. It is hoped that future studies will identify and characterize PTPs in other human fungal pathogens. While protein histidine phosphatases (PHPs) represent an important class of non-Ser/Thr phosphatases, their potential role in medically important fungi remains elusive.

Global analyses of fungal pathogen phosphoproteomes

Recent advances in proteomics have made it possible to define the complete set of proteins in human fungal pathogens which are phosphorylated (phosphoproteome). Typically, proteins isolated from cultures grown *in vitro* are digested with trypsin, subjected to titanium dioxide-based enrichment and analyzed by mass spectrometry. A recent phosphoproteomic study in the model filamentous fungus *A. nidulans*[269] identified 1801 phosphosites corresponding to 1637 unique phosphorylated peptides. Further analysis indicated an enrichment among the phosphoproteins for gene ontology (GO) terms related to fungal morphogenesis, including "site of polarized growth," "vesicle-mediated transport," and "cytoskeleton organization." The majority of phosphoproteins were targets of the CDK and CK2 kinase families. A significant number of substrates for kinases that control hydrolytic enzyme secretion were also identified by this analysis.[269]

A recent phosphoproteomic analysis of *C. neoformans*[270] has identified 1089 phosphopeptides from 648 protins, including 45 kinases. Similar to the case of *A. nidulans*, most CDK substrates were phosphorylated, as indicated by a motif enrichment analysis. Among the phosphoproteins, enriched GO terms included "metabolism," "transport," "signal transduction," "transcription," "cell cycle progression," and "stress response." Phosphorylated kinases identified by this study were known to control the cell cycle, metabolic processes and virulence. Kinases included components of the cAMP/PKA and MAPK pathways. Phosphorylation of cAMP/PKA components is known to be important for controlling *C. neoformans* capsule size and melanin biosynthesis.[271] Additional phosphoproteins included components of the PKC MAPK signaling pathway, important for cell wall integrity and thermotolerance.[88,89,270] Four phosphopeptides corresponding to Sp1, a transcription factor important for resistance to nitrosative stress, maintenance of cell wall integrity and virulence,[272] were also identified in this study.[270] In addition, two members of the p21-activated protein kinase (PAK) family, important for mating, cytokinesis and virulence in serotypes A and D,[273] were shown to be phosphorylated. Finally, phosphopeptides corresponding to Ypk1, important for the ability of *C. neoformans* to tolerate fluconazole treatment were also identified.[270] Altogether, results from this study strongly suggest that a wide variety of processes important for *C. neoformans* virulence appear to be controlled by phosphorylation.

A comprehensive analysis of the *C. albicans* phosphoproteome in hyphal form cells has also recently been carried out.[274] In sum, 15,906 unique phosphosites were identified

on a total of 2,896 proteins. Serine and threonine phosphosites were highly represented (80.01% and 18.11%, respectively) and, as expected, tyrosine phosphosites were a small minority (1.81%) of the total. Interestingly, several differences were noted in GO enrichment for Tyr vs. Ser/Thr phosphorylated proteins. For example, a greater fraction of Tyr-phosphorylated proteins were enriched for "kinase," "DNA-binding," and "signal transducer" Molecular Function categories. Proteins important for maintaining and establishing cytoskeletal polarity, as well proteins associated with hyphal growth, were among the most highly phosphorylated. These proteins included Gin4, a Ser/Thr protein kinase involved in septum formation, and the related kinase Hsl1. Bud neck and septin ring formation proteins, including Spa2, Bni3, and Bud4 were also highly phosphorylated. As expected, numerous components and targets of the *C. albicans* Ras cAMP/PKA filamentous growth pathway were also found to be phosphorylated. In addition, the Mediator complex, important for RNA polymerase II transcription, was highly phosphorylated. Several of these phosphorylation events were found to be mediated by Cbk8, a kinase component of Mediator important for stress resistance, metabolism and hyphal growth.[274]

Although relatively few phosphoproteomic analyses have been performed to date in pathogenic fungi, these studies have highlighted the importance of phosphorylation for controlling multiple virulence-related processes and are beginning to provide new insights into the global impact of phosphorylation on fungal pathogenesis. The recent emergence of new and more quantitative proteomic techniques should facilitate this process. Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) involves metabolic incorporation of stable isotope-labeled amino acids, such as ^{15}N-arginine, into the proteome of cells grown in culture.[275–277] Equal quantities of protein extracts from cells grown in both "light" medium, containing natural isotope amino acids, and "heavy" medium, containing labeled isotope amino acids, are mixed, digested into peptides and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The advantage of this technique is that the ratio of signal intensities from "light" and "heavy" samples provides a highly accurate quantitation of relative protein abundance. In addition, unlike previous techniques which involve examining ^{32}P-labeled bands on 1D or 2D gels or Westerns with phosphosite-specific antibodies, SILAC can be used to very accurately and quantitatively detect novel phosphosites and dynamic changes in phosphorylation events that occur on a global scale across the whole proteome.[16,277,278] In addition to SILAC, high-accuracy MS technology, phosphopeptide enrichment techniques based on affinity chromatography and recently developed bioinformatics tools[277,279] are likely to greatly facilitate the identification and analysis of fungal pathogen phosphoproteomes. While experiments utilizing many of these techniques should provide a wealth of new information about global phosphorylation events associated with fungal pathogen virulence properties that can be assessed *in vitro* (eg, filamentation and biofilm formation), greater challenges are likely to be encountered in the assessment of phosphorylation patterns during infection *in vivo*. For example, it may be difficult to obtain sufficient quantities of fungal pathogen proteins for phosphoproteomic analysis from infected tissues. In addition, in the case of SILAC it may be difficult to obtain a sufficiently high level of stable isotope labeling for fungal pathogen proteins during an infection. Overall, however, future phosphoproteomic studies in medically important fungi are likely to provide valuable information and could lead to the identification of important kinase/phosphatase substrates, interacting partners and potential targets for the development of new and more effective antifungal strategies.

Perspectives and future directions: targeting kinases and phosphatases for the development of new antifungal strategies

Given the variety of key functions that kinases and phosphatases play in controlling morphology, virulence and a variety of virulence-related processes in pathogenic fungi, could these enzymes serve as effective antifungal drug targets? Probably the best example of such a potential drug target is the calcium/calmodulin-dependent protein phosphatase calcineurin. As discussed previously, calcineurin plays an important role in filamentation, virulence, stress response, antifungal drug tolerance and/or mating of a variety of medically important fungi, such as *C. neoformans*, *A. fumigatus,* and *M. circinelloides* and multiple *Candida* species (including *C. albicans*).[175,176,179–181,185–187,190–192,195] Two pharmacological inhibitors of calcineurin, cyclosporin A and FK506, are effective against a variety of fungal pathogens, especially when combined with azole or echinocandin treatments.[179,186,280–283] However, these inhibitors also have immunosuppressive effects and are unlikely to serve as viable antifungals in the clinic. Future studies to identify cyclosporin A or FK506 analogs, or other inhibitors of the calcium-calcineurin signaling pathway, with fewer side effects may hold promise.[282,283] As previously discussed, the calcineurin target, Crz1, controls virulence-related processes in multiple fungal pathogens[182,183,193,194] and has also been regarded as a promising antifungal target. However, Crz1 does not appear to be universally required for virulence in human fungal pathogens and is thus less likely to serve as a more broad-spectrum antifungal target.[282] It

is hoped that future phosphoproteomic studies, mentioned above, will elucidate more promising calcineurin targets for antifungal development.

A recent BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search has also indicated the presence of homologs for several important kinases (PKC, Cek1-MAPK, PKA) and phosphatases (calcineurin, Ptc1, and Ppg1) in additional major human fungal pathogens including *Coccidiodes immitis*, *H. capsulatum*, and *Blastomyces dermatitidis* (homologs for the kinases listed above were also identified in the more distantly related fungal pathogen *P. carinii*). Several of these enzymes are likely to play roles in pathogenicity.

In particular, *C. albicans* Ppg1 has recently been shown to play a critical role in filamentation and virulence[136] and could serve as a promising drug target. Importantly, the catalytic activity of Ppg1 has specifically been shown to be required for pathogenesis and future studies to screen small molecule libraries for inhibitors of Ppg1 phosphatase activity may hold promise. One concern, however, is that the Ppg1 catalytically active binuclear center is highly conserved in mammalian PP2A phosphatases, raising the possibility that such inhibitors may have detrimental side effects if used as therapeutics. Ultimately, future studies to solve the crystal structures of Ppg1, and other critical phosphatases/kinases in medically important fungi may allow for the identification of fungal-specific active site subregions that could be targeted by small molecule inhibitors. However, an easier approach may be to target kinases and phosphatases which are entirely fungal-specific and not conserved in mammalian hosts. A recent large-scale comparative genomics study has identified 222 *C. albicans* proteins with catalytic activity which are unique when compared to the human proteome.[284] Crk1, a Cdc2-related protein kinase important for *C. albicans* hyphal development and virulence,[285] was among these proteins and could eventually serve as a novel antifungal target. Additional fungal-specific kinases and phosphatases involved in metabolic processes which are essential for viability are also likely to represent promising targets. Future studies, which focus on the identification and characterization of fungal-specific kinases and phosphatases (or kinase/phosphatase substrates) that are critical for viability and/or virulence in human fungal pathogens are therefore likely to hold significant therapeutic potential.

Acknowledgements

We thank Brian Wickes for useful comments on the manuscript. M.T.A. was supported by a National Institute of Dental and Craniofacial Research Ruth L. Kirschstein National Research Service Award for Individual Postdoctoral Fellows (F32DE023471). D.K. was supported by a Voelcker Young Investigator Award from the Max and Minnie Tomerlin Voelcker Fund as well as a grants 5R01AI083344 and 1R21AI117299 from the National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, National Institute of Allergy and Infectious Diseases or National Institute of Dental and Craniofacial Research.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary Material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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