Published in final edited form as:

Mol Microbiol. 2012 April ; 84(1): 130–146. doi:10.1111/j.1365-2958.2012.08016.x.

Involvement of PDK1, PKC and TOR signaling pathways in basal fluconazole tolerance in *Cryptococcus neoformans*

Hyeseung Lee¹, Ami Khanal Lamichhane¹, H. Martin Garraffo², Kyung J. Kwon-Chung¹, and Yun C. Chang^{1,*}

¹Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America

²Laboratory of Bioorganic Chemistry, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, Maryland, United States of America

Summary

This study shows the importance of PDK1, TOR and PKC signaling pathways to the basal tolerance of *Cryptococcus neoformans* toward fluconazole, the widely used drug for treatment of cryptococcosis. Mutations in genes integral to these pathway resulted in hypersensitivity to the drug. Upon fluconazole treatment, Mpk1, the downstream target of PKC was phosphorylated and its phosphorylation required Pdk1. We show genetically that the PDK1 and TOR phosphorylation sites in Ypk1 as well as the kinase activity of Ypk1 are required for the fluconazole basal tolerance. The involvement of these pathways in fluconazole basal tolerance was associated with sphingolipid homeostasis. Deletion of *PDK1*, *SIN1*, or *YPK1* but not *MPK1* affected cell viability in the presence of sphingolipid biosynthesis inhibitors. Concurrently, *pdk1Δ*, *sin1Δ*, *ypk1Δ*, and *mpk1Δ* exhibited altered sphingolipid content and elevated fluconazole accumulation compared with the wild-type. The fluconazole hypersensitivity phenotype of these mutants, therefore, appears to be the result of malfunction of the influx/efflux systems due to modifications of membrane sphingolipid content. Interestingly, the reduced virulence of these strains in mice suggests that the cryptococcal PDK1, PKC, and likely the TOR pathways play an important role in managing stress exerted either by fluconazole or by the host environment.

Keywords

PDK1; PKC; TOR; fluconazole; sphingolipid; virulence

Introduction

Cryptococcus neoformans is the most common cause of fungal meningoencephalitis. The primary predisposing factor for cryptococcosis is a compromised immune system such as the case in HIV infected patients or with other underlying conditions. Cryptococcal meningoencephalitis is fatal unless treated and its mortality rate is high even with the most advanced treatment (Kwon-Chung & Bennett, 1992, Perfect & Casadevall, 2002). Fluconazole (FLC), a triazole antifungal drug, has been the agent most widely used for prophylactic therapy as well as for the long-term management of common mycoses such as candidiasis and cryptococcosis owing to its efficacy and safety (Zonios & Bennett, 2008). Triazoles target the P450 enzyme lanosterol 14 α -demethylase, Erg11. The generally accepted mode of antifungal action of triazoles, based on the *Saccharomyces cerevisiae*

*Corresponding author Tel: 301-496-8839 Fax: 301-480-3458 ychang@niaid.nih.gov .

model, is inhibition of ergosterol biosynthesis. It is a multi-mechanistic process that is initiated by the inhibition of two cytochrome P450 enzymes involved in the catalysis of lanosterol 14 α -demethylation (Erg11) and Δ 22 desaturation (Erg3) (Kelly *et al.*, 1997). The inhibition of these enzymes results in the blockage of ergosterol biosynthesis, accumulation of toxic sterol intermediates, and the disruption of membrane integrity (Cowen & Steinbach, 2008, Anderson *et al.*, 2003, Cowen, 2008).

Long-term maintenance therapy with azoles creates a condition favorable for the emergence of resistance to the drug, and increased azole resistance *in vitro* has been shown to be predictive of treatment failures and infection relapses in *C. neoformans* (Perfect & Cox, 1999). The molecular basis of azoles resistance has been extensively characterized in *S. cerevisiae* and pathogenic *Candida* species such as *C. albicans* and *C. glabrata* (Kontoyiannis *et al.*, 1999, Lamping *et al.*, 2007, White *et al.*, 2002, Tsai *et al.*, 2006, Sanglard & Odds, 2002, Helmerhorst *et al.*, 2006, Brun *et al.*, 2004, Bennett *et al.*, 2004, Akins, 2005). Mechanisms known for the emergence of azole resistance in these fungi include: increased production of multidrug transporters (Lupetti *et al.*, 2002, Cowen & Steinbach, 2008, Cowen *et al.*, 2002), mutations in ergosterol biosynthetic pathway genes (Sanglard *et al.*, 1998, Marichal *et al.*, 1999), amplification of genomic regions that contain ergosterol biosynthetic pathway genes and transcription factors that positively regulate a subset of efflux pump genes (Selmecki *et al.*, 2006, Selmecki *et al.*, 2008), as well as activation of Hsp90 that may facilitate the cell-response to drug stress (Cowen *et al.*, 2006, Cowen & Lindquist, 2005).

C. neoformans is phylogenetically distant from these well studied fungi and the mechanism of azole resistance in this organism is poorly understood. Unlike in *Candida* species, isolation of FLC resistant mutants have rarely been reported in *C. neoformans* and the emergence of resistance has most often been documented with clinical outcomes of AIDS patients receiving azole maintenance therapy (Armengou *et al.*, 1996, Venkateswarlu *et al.*, 1997, Paugam *et al.*, 1994, Birley *et al.*, 1995, Berg *et al.*, 1998). An intriguing pattern of azole resistance called heteroresistance was reported in *C. neoformans* isolated from patients with recurrent episodes of infection (Sionov *et al.*, 2009, Mondon *et al.*, 1999). Further studies have shown that heteroresistance to azoles is intrinsic to all *C. neoformans* strains tested and heteroresistant subpopulations in each clone adapt to high concentrations of FLC by forming disomes of multiple chromosomes (Sionov *et al.*, 2010, Sionov *et al.*, 2009, Mondon *et al.*, 1999). However, the molecular mechanism(s) involved in disomy formation remains an enigma. In order to expand our knowledge on the mechanism of azole tolerance which may provide insight into our understanding of clinically observed resistance mechanisms in *C. neoformans* as well as to find ways to improve therapeutic effect of azoles for cryptococcosis, we screened a mutant library and identified strains exhibiting FLC hypersensitivity. We found homologs representing components of the signaling cascade controlled by the mammalian phosphoinositide-dependent kinase (PDK1) to be crucial for responses to FLC. PDK1 is a serine/threonine kinase that controls a complex network of signaling cascades including responses to insulin and several growth factors, glucose uptake, regulation of apoptosis, translation initiation and others (for review see (Vanhaesebroeck & Alessi, 2000, Mora *et al.*, 2004)). In addition, we found that the components of the protein kinase C (PKC) cell wall integrity signaling pathway as well as a component of the target of rapamycin (TOR) pathway were also involved in FLC response. The PKC signaling pathway is responsible for cell wall remodeling through the cell cycle and also functions in response to various stresses (Levin, 2005, Lesage & Bussey, 2006, Gerik *et al.*, 2005). TOR was first identified in *S. cerevisiae* (Heitman *et al.*, 1991) and is a highly conserved serine/threonine kinase that regulates cell growth in response to environmental changes such as nutrient availability or cellular energy status (for review see (Otsubo & Yamamoto, 2008)). In the present study, we describe a regulatory module important for basal tolerance to FLC

in *C. neoformans* that is also associated with sphingolipid homeostasis under the stress imposed by FLC.

Results

Characterization of fluconazole sensitive (FLC-s) mutants

We screened a library containing 1,201 deletion mutants of *C. neoformans* (Liu *et al.*, 2008) for strains that were sensitive to fluconazole (FLC-s) and obtained a total of 32 FLC-s mutants. We chose 9 FLC-s mutants for further studies based on their degree of drug sensitivity and the availability of annotation for each mutated gene (Fig. S1 and Table S2). Several mechanisms may contribute to alterations in FLC sensitivity as in other organisms. For instance, mutations in the FLC target, Erg11, or other enzymes involved in the ergosterol biosynthesis pathway may alter the efficacy of FLC, change the cellular sterol content, or cause accumulation of toxic sterol intermediates upon FLC treatment. Alternatively, FLC-s mutants may alter influx or efflux systems resulting in increased cellular accumulation of FLC that maximizes the impact of the drug. Therefore, we examined whether these mutants had any defect in sterol biosynthesis and/or transport of the drug. There was no noticeable reduction in ergosterol content between wild-type strain H99 and the mutants (Fig. S2). In contrast, some of the FLC-s mutants, such as *sin1*, *mpk1* and *pdk1*, accumulated greater amounts of ³H-FLC than the wild-type strain (Fig. 1A). Accumulation of FLC in the cells could have been due to a malfunction in the influx/efflux system. Since *AFR1* is the only ATP-binding cassette transporter thus far known to be involved in the efflux of FLC in *C. neoformans* and its expression reportedly increases upon FLC treatment (Sionov *et al.*, 2009, Posteraro *et al.*, 2003), we measured the expression levels of *AFR1* in these mutants. In the presence or absence of FLC, the expression levels of *AFR1* were comparable between wild-type and the FLC-s strains (Fig. S3). Although it is not known whether the protein levels or genomic location of *AFR1* has been altered in these mutants, we hypothesized that FLC influx/efflux system is likely altered in some of these FLC-s mutants by a mechanism(s) previously uncharacterized.

Several signaling pathways are involved in FLC response in *C. neoformans*

Although none of the nine FLC-s mutants showed significant defects in the mechanisms known to be involved in FLC hypersensitivity, three of the strains harbored a mutation in either the *SIN1*, *MPK1*, or *PDK1* gene. These genes are homologs of components in the signaling cascades, TOR, MAPK, and PDK1 in mammalian and other eukaryotic systems. These genes control various cellular responses and their regulatory functions are known to be interrelated but are less known to be involved in azole susceptibility in pathogenic fungi. We, therefore, focused our attention on their role in the basal tolerance of FLC. Since *MPK1* has been extensively studied in the H99 strain (Kojima *et al.*, 2006, Kraus *et al.*, 2003), we independently deleted and complemented the *PDK1* and *SIN1* genes in H99 to obtain the strains of interest in the same genetic background. Our *sin1* and *pdk1* deletion mutants in H99 displayed a similar phenotype as the original mutants obtained from the library and complementation of their mutations restored FLC accumulation and hypersensitivity to FLC to wild-type levels (Figs. 1B and 2A and data not shown). We noted that growth of our *pdk1Δ* strain on YPD medium was considerably weaker than the original *pdk1Δ* strain (Fig. 2A vs. Fig. S1). We have isolated several independent *pdk1* deletion mutants and all of them displayed a similar phenotype (data not shown). Although poor growth of the *pdk1Δ* strain on YPD agar confounded the interpretation of FLC susceptibility test results, it was clear that the *pdk1Δ* strain was hypersensitive to FLC.

The *C. neoformans* *PDK1* gene shares similarity with the *KSG1* (kinase responsible for sporulation and growth 1) gene of *Schizosaccharomyces pombe* which is a homolog of the

mammalian *PDK1* gene (Graub *et al.*, 2003, Niederberger & Schweingruber, 1999, Inagaki *et al.*, 1999). A typical PDK1 kinase consists of a catalytic domain containing an ATP-binding site, an active site, and a substrate binding site. *C. neoformans* *PDK1* homolog encodes a putative 1,206-aa protein whose catalytic domains (281-777aa, designated by SMART program, <http://smart.embl-heidelberg.de/>) are 35% identical to the human PDK1 kinase catalytic domains. However, the *C. neoformans* Pdk1 protein has an extra 228 amino acid (347-575aa) between the ATP binding site and the active site (data not shown). *S. cerevisiae* contains two functionally redundant *PDK1* homologs, *PKH1* and *PKH2*, and these two genes share an essential role in cell growth (Inagaki *et al.*, 1999). The cryptococcal deletion mutant library contains a mutant annotated as *PKH201* which shares some similarity to *PKH2* of *S. cerevisiae*. However, Pkh201 does not contain the PDK1 catalytic domain and the *pkh201* deletion mutant was not hypersensitive to FLC (Fig. 2C).

C. neoformans *SIN1* shares 38% and 19.8% identity in their protein sequences with *S. pombe* Sin1 and *S. cerevisiae* Avo1, respectively. Sin1 of *S. pombe* was originally isolated as a Sty1 (or SAPK) interacting protein and identified as a component of the TORC2 along with Ste20 and Wat1 (Wilkinson *et al.*, 1999, Hartmuth & Petersen, 2009, Matsuo *et al.*, 2007). In *C. neoformans*, interaction of the TOR homolog with the Tor1 inhibitor rapamycin via FKBP12, a TOR binding protein, has been reported in the serotype D strain B-3501A (Cruz *et al.*, 1999). Interestingly, the *C. neoformans* *sin1Δ* strain was not only sensitive to FLC but also was hypersensitive to rapamycin (Fig. 2A and D). In *S. cerevisiae*, the rapamycin/FKBP12 complex does not bind TORC2. It is thought that components of TORC2 complex of *S. cerevisiae*, such as Avo1 (the Sin1 homolog), bound to Tor2 may hinder the binding of rapamycin/FKBP12 rendering the TORC2 complex insensitive to rapamycin (Loewith *et al.*, 2002). It is possible that *C. neoformans* Sin1 may interact with TOR complex and deletion of *SIN1* may render the strain hypersensitive to rapamycin treatment.

Mpk1 is a homolog of Slr2/MAPK kinase and its role in maintenance of the cell wall has been characterized in *C. neoformans* (Kraus *et al.*, 2003, Kojima *et al.*, 2006). Although the sensitivity to FLC of *C. neoformans* *mpk1Δ* has not been demonstrated previously, the *C. albicans* Mkc1 (a homolog of Mpk1) is phosphorylated and activated upon exposure to FLC (LaFayette *et al.*). It has been shown that protein kinase C1 (Pkc1) signaling regulates the mitogen-activated protein kinase (MAPK) cascade comprised of a linear series of protein kinases including MAPKKK (Bck1/2), MAPKK (Mkk1/2), and MAPK (Slr2) that relay signals to the terminal transcription factors in *S. cerevisiae* (Zhao *et al.*, 2007). *C. neoformans* mutants lacking components of the PKC pathway, Pkc1, Mkk2 and Bck1, also showed hypersensitivity to FLC (Fig. 2C) suggesting that PKC-MAPK cascade is required for FLC resistance.

It is noteworthy that *C. neoformans* *PKC1* contains a serine/threonine protein kinase domain with high similarity to the known PDK1 target proteins (Vanhaesebroeck & Alessi, 2000). In addition to *PKC1*, two more genes, *SCH9* and *YPK1* in the H99 genome encode proteins with high similarity to the PDK1 target proteins. These putative PDK1 targets belong to the AGC (protein kinase A/ protein kinase G/ protein kinase C) kinase family which is highly conserved and known to play myriad roles in cellular growth, proliferation, and survival in other organisms (Pearce *et al.*). The alignment of putative PDK1 target protein sequences reveals that both PDK1 and PDK2 phosphorylation sites and sequences surrounding the sites are well conserved in all three proteins (data not shown). This raised the possibility that *C. neoformans* Pdk1 may also phosphorylate and activate these putative downstream target proteins (Casamayor *et al.*, 1999). However, *sch9Δ* did not show any discernible sensitivity to FLC, suggesting that Sch9 is not a downstream target of the Pdk1 regulatory pathway associated with FLC response in *C. neoformans* (Fig. 2C). In contrast, deletion of *YPK1*

caused a substantial reduction in FLC resistance (Fig. 2C). In *S. cerevisiae*, strains lacking *YPK1* were shown to be sensitive to fluconazole (Li *et al.*, 2010). These results suggest that *C. neoformans* may respond to FLC by utilizing the PDK1 signaling pathway through the well conserved downstream targets, Pkc1 and Ypk1 (Fig. 8).

Mpk1 is phosphorylated in response to FLC treatment

It has been shown that PDK1 homologs of *S. cerevisiae*, Pkh1 and Pkh2, function upstream of Pkc1 and activate the Pkc1 kinase and the Pkc1-effector MAPK pathway in response to various stresses (Inagaki *et al.*, 1999). To determine whether *C. neoformans* responds to FLC treatment through the PDK1-PKC-MAPK pathway by phosphorylating the terminal MAPK, we monitored the status of *C. neoformans* Mpk1 phosphorylation using the commercially available MAPK-phosphorylation-specific antibody. Mpk1 phosphorylation was detected in the wild-type strain exposed to FLC (Fig. 3, lane 2). However, Mpk1 phosphorylation was also detected in the wild-type strain treated with DMSO, a commonly used solvent for FLC (Fig. 3, lane 1). When the wild-type strain was treated with FLC solubilized in water, a strong Mpk1 phosphorylation signal was detected while only a faint signal was observed in the water-treated control sample (Fig. 3 lane 3 and 4). Similarly, a faint signal was observed in the water-treated samples derived from *pdk1Δ*, *sin1Δ*, and *ypk1Δ* (data not shown). Interestingly, Mpk1 phosphorylation was diminished in *pdk1Δ* strain treated with FLC but unaffected in *sin1Δ* or *ypk1Δ* (Fig. 3 lane 5 and data not shown). These data suggest that Pdk1 is necessary for induction of Mpk1 phosphorylation and raises the possibility that Pdk1 may control the activation of PKC cell integrity pathway in *C. neoformans*. Unlike *pdk1Δ* and *sin1Δ*, however, *mpk1Δ* was not sensitive to 1M KCl and 1M NaCl (Fig. 2B), implying that Pdk1 uses downstream effectors other than the MAPK pathway in response to the stresses exerted by these compounds.

Characterization of Ypk1

S. cerevisiae Ypk1 and its homolog in *S. pombe*, Gad8, can both be phosphorylated by upstream kinases. Since *C. neoformans* Ypk1 shares 55% and 69% identity with the Ypk1 and Gad8, respectively, it is possible that *C. neoformans* Ypk1 is also phosphorylated in response to FLC treatment and relays the signal to downstream targets/effectors. To test whether *C. neoformans* Ypk1 is phosphorylated upon FLC treatment, we tagged Ypk1 with the FLAG epitope at its C-terminus. The resulting construct was transformed into a H99 *ypk1* deletion mutant to replace the deleted allele. Such transformants restored the FLC sensitivity of *ypk1Δ* to wild-type levels indicating that the Ypk1-FLAG fusion protein was functional (Fig. 4B). The expression of the Ypk1-FLAG protein was confirmed by western blot analysis with anti-FLAG antibody as the protein migrated with the expected molecular mass (64 Kda) (Fig. 4A). To test the phosphorylation status of Ypk1, the strain expressing Ypk1-FLAG was treated with 16 μg/ml FLC for various time periods. The total protein lysate was immunoprecipitated with anti-FLAG M2 magnetic beads and resolved in SDS-PAGE. In contrast to a series of slow migrating bands diagnostic of Ypk1 phosphorylation status reported in other organisms, *C. neoformans* Ypk1 migrated as a single species without any mobility shift regardless of FLC treatment (Fig. 4A). Although we used several different extraction buffers and methods, we failed to detect the slow migrating bands of Ypk1 even when the cells were treated with FLC for up to 6 hr (data not shown). In addition, since antibody specific for *C. neoformans* Ypk1 was not available, we used anti-phospho-serine and anti-phospho-threonine antibodies for immunoblotting. However, both antibodies failed to detect any phosphorylation of the Ypk1-FLAG fusion protein (data not shown).

To explore the inability to detect Ypk1 phosphorylation in *C. neoformans*, we attempted to show the functional importance of the presumptive phosphorylation sites in Ypk1. *C. neoformans* Ypk1 contains putative consensus sites, T402 and S562, for Pdk1 and Pdk2

phosphorylation, respectively. We constructed Ypk1-FLAG fusion constructs containing a T402A or S562A mutation and used the constructs to replace the *ypk1Δ* allele. In transformants in which the *ypk1Δ* allele was replaced by a Ypk1(S562A)-FLAG construct, FLC resistance was recovered to levels comparable to the wild-type (Fig. 4B). This indicates that the conserved putative Pdk2 phosphorylation site, S562, is dispensable for Ypk1 function associated with FLC resistance. In contrast, transformants of Ypk1(T402A)-FLAG remained as sensitive to FLC as the *ypk1Δ* deletion mutant (Fig. 4B). The levels of Ypk1 protein, however, were lower in the T402A derivative compared to the control but were comparable to the S562A derivative (Fig. S4). These data indicate that the putative Pdk1 phosphorylation site, T402 residue, play a role in the stability and function of Ypk1.

Ypk1 kinase activity is important for FLC resistance

To test whether *C. neoformans* Ypk1 functions as a protein kinase and the ATP-binding domain is essential for its function in response to FLC, we mutated two residues of Ypk1, D386 and K274, which reside equivalently to D488 and K376 in *S. cerevisiae* Ypk1. These residues are predicted to be directly involved in anchoring of the γ -phosphate group of ATP (Knighton *et al.*, 1992). Substitution of these residues with any other amino acid abolishes the ability of Ypk1 to transfer phosphate to its target amino acids, resulting in a kinase-dead version of Ypk1 (Roelants *et al.*, 2010, Roelants *et al.*, 2002). Interestingly, both Ypk1 (D386A)-FLAG and Ypk1(K274A)-FLAG constructs failed to complement the FLC-s phenotype of the *ypk1Δ* strain (Fig. 4B), suggesting that Ypk1 kinase activity is necessary for its function in *C. neoformans*.

The importance of the TOR pathway in FLC resistance

In *S. cerevisiae*, *ypk1Δ* is hypersensitive to the TORC1 inhibitor rapamycin (Gelperin *et al.*, 2002). We found that *C. neoformans* *ypk1Δ* and *pdk1Δ* are sensitive to rapamycin (Fig 2D), which implies a regulatory link between TORC1 and Ypk1. We examined, therefore, whether the conserved TOR phosphorylation site in Ypk1 is important for response to FLC treatment in *C. neoformans*. The S543 residue of *C. neoformans* Ypk1 (corresponding to S527 of *S. pombe* Gad8) in the presumptive turn motif was substituted with alanine in the Ypk1(S543A)-FLAG construct and was expressed in the *ypk1Δ* deletion mutant. Interestingly, Ypk1(S543A)-FLAG failed to confer FLC resistance (Fig. 4B). These results imply that turn motif phosphorylation at S543 is important for Ypk1 function and support the possibility that *C. neoformans* TORC1 might be involved in Ypk1 regulation in response to FLC treatment.

To study the importance of TOR pathway in fluconazole resistance, we attempted to delete the TOR homolog in *C. neoformans*. We failed repeatedly to delete *TOR1* (CNAG_06642), suggesting deletion of *TOR1* might be lethal (data not shown). The essentiality of *TOR1* has been suggested in a serotype D strain of *C. neoformans* TOR-like (Davidson *et al.*, 2002). There is another gene, CNAG_05220 designated as *TLK1* for kinase, which shares 57% identity with Tor1 of *S. cerevisiae* but lacks the conserved FKBP-rapamycin binding domain. However, deletion of *TLK1*, did not affect the sensitivity of the strain to FLC (Fig. 2C).

In fission yeast, Tor1 forms a complex with conserved proteins, Ste20, Wat1 and Sin1. It is possible that *C. neoformans* Tor1 may form a complex with the Sin1 homolog along with other TOR complex components and relay the incoming signal to Ypk1. To examine whether Sin1 and Ypk1 function in the same pathway, we transformed the Ypk1-FLAG construct in *sin1Δ*. When the Ypk1-FLAG protein levels in the *sin1Δ* transformant (*sin1Δ* + *YPK1A*) were close to the control strain (*ypk1Δ*+*YPK1*), the *sin1Δ* transformant remained sensitive to FLC and rapamycin (Fig. 5A and B). However, the sensitivity to FLC and

rapamycin was complemented in the *sin1Δ* transformant that expressed higher amounts of Ypk1-FLAG (*sin1Δ*+*YPK1B*). This data suggests that *C. neoformans* *SIN1* is upstream of *YPK1* in FLC response. In contrast, over-expression of Ypk1 in a *pdk1Δ* deletion mutant was not sufficient to confer FLC resistance (Fig. 5), suggesting that Ypk1 is not the only downstream effector of Pdk1 or alternatively, phosphorylation at the Pdk1 site of Ypk1 by Pdk1 is absolutely essential for Ypk1 activity in the cryptococcal response to FLC.

Sphingolipid synthesis is impaired in mutants sensitive to FLC

The importance of the interaction between membrane ergosterol and sphingolipids in determining drug susceptibility has been reported in *C. albicans* (Mukhopadhyay *et al.*, 2004). Interestingly, homologs of Pdk1 and its downstream kinases are involved in sphingolipid-mediated signaling in other organisms (Sun *et al.*, 2000, Roelants *et al.*, 2010, Luo *et al.*, 2008). Furthermore, Ypk2 has been shown to regulate ceramide biosynthesis in *S. cerevisiae* (Aronova *et al.*, 2008). Since the sterol profile of the mutants did not show noticeable changes in ergosterol content, we investigated whether hypersensitivity to FLC is related to sphingolipid homeostasis and/or sphingolipid-mediated signaling. We used myriocin (MYR), aureobasidin A (ABA) and phytosphingosine (PHS), to examine the importance of the aforementioned genes in sphingolipid biosynthesis. It is known that myriocin blocks the production of endogenous phytosphingosine, aureobasidin A blocks production of the complex inositol-containing sphingolipids, and addition of exogenous phytosphingosine elevates intracellular phytosphingosine causing toxicity. Interestingly, growth of the three FLC-s mutants, *pdk1Δ*, *sin1Δ*, and *ypk1Δ*, but not *mpk1Δ*, was hampered by the inhibitors at the concentrations which do not affect growth of the wild-type strain (Fig. 6A). These observations suggest that deletion of either *PDK1*, *SIN1* or *YPK1* but not *MPK1*, is deleterious to cell viability when sphingolipid biosynthesis is compromised by these inhibitors.

To examine the possibility whether *PDK1*, *SIN1*, and *YPK1* exert FLC basal tolerance in *C. neoformans* through modulation of sphingolipid biosynthesis, we used ³H-myoinositol and thin layer chromatography to analyze the complex sphingolipids. Our experiments were carried out in the presence or absence of FLC to see the effect of each mutation as well as FLC exposure on sphingolipid biosynthesis. When the relative amounts of complex sphingolipids were compared within each strain between the FLC treated and non-treated cells, no obvious difference was observed (Fig. 6B). These data suggest that FLC treatment does not affect the accumulation of complex sphingolipids in all three deletion mutant strains as well as in the wild-type strain. However, the relative amounts of the complex sphingolipids IPC, MIPC, and MIP2C+ (a combination of M(IP)₂C and other complex sphingolipids; see materials and methods) were significantly reduced in *pdk1Δ* and *ypk1Δ* strains compared with those in the wild-type strain (Fig. 6B and C). In *sin1Δ*, MIPC was the only complex sphingolipid that showed a slight reduction compared to the wild-type strain. In *mpk1Δ*, there was an increase of IPC compared to the wild-type ($p = 0.06$ and $p = 0.05$ in the absence and presence of FLC, respectively). These results demonstrate that *PDK1*, *SIN1*, *YPK1*, and *MPK1* play a role in maintaining complex sphingolipid homeostasis.

pdk1Δ, *sin1Δ*, and *ypk1Δ* strains show reduced virulence

Since the deletion mutants showed increased sensitivity to various stresses, we investigated the impact of the strains' diminished ability to handle environmental stresses on their virulence. The growth rate at 37°C for *ypk1Δ* and *sin1Δ* was slightly slower than the wild-type (3.4 ± 0.2 h and 3.9 ± 0.3 h vs. 2.5 ± 0.3 h, respectively) and *pdk1Δ* grew significantly slower compared with the wild-type (7.8 ± 2.1 h vs. 2.5 ± 0.3 h). Expression of other major virulence factors such as capsule and melanin in the deletion mutants was comparable to wild-type (data not shown). Groups of 10 mice were challenged with different yeast strains

via tail vein injection. *pdk1Δ*, *sin1Δ*, and *ypk1Δ* strains all showed reduced virulence ($p < 0.001$ compared to wild-type H99) (Fig. 7). Furthermore, complementation of these mutations increased the virulence compared to the original deletion mutant ($p < 0.001$). It was not surprising that virulence of *pdk1Δ* is reduced since it grows much more slowly at 37°C than the wild-type strain. Significantly, *ypk1Δ* and *sin1Δ* strains showed only a slight reduction in the growth rate at 37°C and yet clearly demonstrated a reduction in virulence. Apparently, *SIN1* and *YPK1* are required by *C. neoformans* to handle the stress encountered in the host environment.

Discussion

Mechanisms of azole resistance and the role of cellular signaling in fungal drug resistance have been extensively studied in *Candida* species (for review see (Cowen, 2008, Shapiro *et al.*, 2011)). However, the role of well conserved kinases in FLC basal tolerance has not been well documented. In the course of dissecting the mechanisms involved in the ability of *C. neoformans* to manage FLC stress, we found that PDK1 and PKC-MAPK signaling pathways contribute to FLC basal tolerance. Both pathways, however, showed differential involvement in response to salt and rapamycin treatment.

Azole drugs are known to block the biosynthesis of ergosterol and result in the accumulation of a toxic sterol intermediate that disrupts membrane integrity, culminating in severe membrane stress. It has also been shown that fungal azole susceptibility is related to sphingolipid metabolism. For example, deletion of genes involved in sphingolipid biosynthetic pathways such as *SLD1* encoding sphingolipid Δ^8 -desaturase, *HSX11* encoding glucosylceramide synthase, and *IPT1* encoding inositol phosphoryl transferase, results in hypersensitivity to FLC in several species (Pasrija *et al.*, 2005, Prasad *et al.*, 2005, Oura & Kajiwar, 2008, Oura & Kajiwar, 2010). In addition, an antimicrobial peptide inhibitor of the fungal plasma membrane ATPase (Pma1), which is associated with lipid rafts is known to block the azole resistance in *C. albicans* (Monk *et al.*, 2005). Moreover, acquisition of the multidrug resistance phenotype in *C. albicans* is accompanied by upregulation of genes required for normal lipid metabolism that constitute membrane rafts (Pasrija *et al.*, 2005, Mukhopadhyay *et al.*, 2004, Mukhopadhyay *et al.*, 2002). Consistent with the notion that ergosterol and sphingolipids are tightly coupled for membrane integrity and drug susceptibility, we show that FLC-s mutants, *pdk1Δ*, *sin1Δ*, and *ypk1Δ* are hypersensitive to chemicals that inhibit sphingolipid synthesis and exhibit reduced content of complex sphingolipids. We speculate that the FLC-s phenotype of our mutants can be partially attributed to impaired membrane rafts due to modified sphingolipid content.

Membrane rafts modulate signaling events via compartmentalization of signaling molecules through specific affinity between sphingolipids, sterols, and membrane proteins and/or signaling molecules (Moffett *et al.*, 2000, Wu *et al.*, 2004, Martin & Konopka, 2004). A stable interaction between ergosterol and sphingolipid is essential to maintain the stability of sphingolipid-rich microdomains (Mukhopadhyay *et al.*, 2004). Since the sphingolipid contents are altered in our mutants, integrity of the membrane rafts may have been disturbed. As a consequence, FLC causing a blockage in ergosterol biosynthesis may become more deleterious in these mutants. Little is known about how azole drugs mechanically influence membrane properties and fungal viability. Abe *et al.* showed that treatment with fluconazole decreased the rigidity of the plasma membrane in *S. cerevisiae* (Abe *et al.*, 2009). It has been postulated that lipid and sterol compositions in the plasma membrane play a role in azole accumulation (Luo *et al.*, 2008, Kohli *et al.*, 2002). Similarly, we have shown that our mutants accumulate more FLC than the wild-type strain. In light of the recent study showing that the import of FLC proceeds via facilitated diffusion (Mansfield *et al.*), it is possible that modifications in membrane composition and/or

flexibility could affect facilitated diffusion and maximize the impact of the drug simply by increased accumulation of FLC inside the cells.

The components of the PKC-MAPK signaling pathway are known to be vital for maintaining integrity of the cell, allowing full resistance to both oxidative and nitrosative stresses in *C. neoformans* (Kraus et al., 2003, Gerik et al., 2008). Recently, PKC signaling was shown to regulate FLC resistance in *C. albicans* via a circuitry comprised of the Mpk1 homolog, Mkc1 (LaFayette et al., 2010). Deletion of *SLT2/MPK1* in *S. cerevisiae* also leads to fluconazole hypersensitivity (Parsons et al., 2004). We show that deletion mutants of each component in the pathways, *PKC1*, *MKK2*, *BCK1* and *MPK1*, are hypersensitive to FLC suggesting that the PKC-MAPK cascade is involved in FLC susceptibility in *C. neoformans*. Although the sphingolipid biosynthetic pathway has been linked to Pkc1 in *C. neoformans* (for review see (Shea & Del Poeta, 2006)), it is also possible that a general cell wall deficiency rather than changes in sphingolipid composition allows the drug to enter the cell more readily and cause FLC hypersensitivity.

While Mpk1 activation is thought to be initiated by Pkc1 and carried out in a linear mode by a series of upstream kinases in the PKC pathway, the *S. cerevisiae* PDK1 homologs, Pkh1/Pkh2, function upstream of Pkc1 activating the kinase and the Pkc1 effector, the MAPK pathway (Inagaki et al., 1999). In fact, deletion of *PDK1* diminishes the Mpk1 phosphorylation suggesting the possibility that Pdk1 may function upstream of the PKC-MAPK signaling pathway in response to FLC treatment. However, we cannot rule out the possibility that Pdk1 directly phosphorylates Mpk1.

Mammalian PDK1 and its functional homologs have been shown to phosphorylate and activate a number of AGC protein kinases *in vitro*, which include p70^{S6k}, PKB, SGK (serum/glucocorticoid-inducible kinase) and PKC isoforms (Vanhaesebroeck & Alessi, 2000). All these targets contain the conserved phosphorylation sequence in their activation loop, which is termed PDK1 site (**Thr**-Phe-Cys-Gly-Thr-X-Glu-Tyr, where the bold Thr represents the phosphorylated residue and X represents any amino acid). In addition, full activation of some targets also requires the phosphorylation of a second site, PDK2 (phe-X-X-Ar-**Ser/Thr**-Ar, where the residue in bold is the phosphorylated amino acid and Ar represents an aromatic residue). Although phosphorylation of Ypk1 is yet to be confirmed biochemically in *C. neoformans*, our results are clear that mutation of the putative Pdk1 phosphorylation site in Ypk1 and kinase-dead version of Ypk1 renders the FLC-s phenotype. This strongly suggests that Ypk1 is phosphorylated by Pdk1 and Ypk1 functions as a kinase to phosphorylate the target(s) yet to be identified in *C. neoformans*.

Although we failed to delete the putative TOR homolog and directly test its functional relationship with FLC tolerance, several compelling observations led us to hypothesize that the TOR pathway might also be involved in *C. neoformans* basal tolerance toward FLC. First, deletion of the *SIN1* homolog, a known component of the TOR complex in other organisms, is hypersensitive to FLC as well as to rapamycin. Second, over-expression of Ypk1 rescues the FLC-s phenotype of *sin1Δ*. Third, the conserved TOR phosphorylation site in Ypk1 is important in *C. neoformans*' response to FLC treatment. In *S. pombe*, the Ypk1 homolog, Gad8, has been isolated as a multicopy suppressor of *tor1-g2* sterile mutant and is regulated by Tor1 as well as by Pdk1 (Matsuo et al., 2003). In addition, *S. cerevisiae* TORC2 is proposed to activate Slm1 and Slm2, phosphoinositide effectors, which then downregulate the turnover of complex sphingolipids (Tabuchi et al., 2006, Beeler et al., 1998). Given that deletion of *SIN1* or *YPK1* reduces the accumulation of complex sphingolipids, it is plausible that Tor1, along with Sin1 and Ypk1, is involved in maintaining homeostasis of complex sphingolipids in *C. neoformans*. It is known that TORC2 also regulates the Rho1/PKC cell wall integrity pathway in *S. cerevisiae* (Torres et al., 2002).

However, since the presence of rapamycin did not alter the FLC basal tolerance in the wild-type strain H99 (data not shown) and *mpk1Δ* was not hypersensitive to rapamycin (Fig. 2D), it is not clear if *C. neoformans* also channels the signal through the TORC to PKC pathway in response to FLC treatment. Figure 8 summarizes our findings on the possible involvement of PDK1, TOR and PKC-MAPK signaling pathway in *C. neoformans* FLC basal tolerance.

It is interesting to observe that deletion of *PDK1*, *SIN1*, and *YPK1* results in a significant reduction of virulence. In addition, *C. neoformans* mutants lacking Mpk1 also exhibit attenuated virulence in the mouse model of cryptococcosis (Kraus et al., 2003). Since these genes are conserved in many fungi, the importance of these genes in virulence along with FLC basal tolerance may be exploited for future drug designs to treat fungal infections. Furthermore, understanding of the FLC basal tolerance and in depth characterization of these pathways will further shed light on how *C. neoformans* handles stress. Our study provides a basis to further our understanding on clinically observed drug resistance in *C. neoformans* and may contribute towards improved designs for a new generation antifungal azoles.

Experimental procedures

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Allergy and Infectious Diseases/National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of National Institute of Allergy and Infectious Diseases (Permit Number: LCID50E).

Strains and media

C. neoformans serotype A genome sequenced strain H99 was used as the wild-type. List of strains relevant to this study is in the Table S1 and S2. Yeast extract-peptone-dextrose (YPD) and Yeast Nitrogen Base (YNB) were as described previously (Chang & Kwon-Chung, 1994). Rapamycin (Sigma R0395) is dissolved in ethanol to make 1mg/ml stock solution and added to YPD medium to 1 ng/ml. Phytosphingosine hydrochloride (PHS) (Sigma P2795) was dissolved in 95% ethanol to make 20 mM stock solution. Aureobasidin A (Clontech. Cat#630466) and Myriocin (Sigma M1177) were dissolved in methanol to make 1 mM stock solution. FLC was either dissolved in DMSO to prepare 50 mg/ml stock or in water to make 10 mg/ml stock.

Identification of fluconazole-sensitive mutants in the signature-tagged gene knockout mutant library

We screened the signature tag mutagenesis (STM) deletion collection of the *C. neoformans* CM018 (a serotype A strain derived from H99)(www.atcc.org) (Liu et al., 2008) to identify mutants sensitive to fluconazole. The STM strains were first replica-spotted to YPD agar media containing 16 µg/ml FLC which is the maximum concentration of FLC that allows the growth of H99 cells. Mutants that failed to grow were selected and re-tested on media containing different concentration of FLC (8, 16, and 32 µg/ml) to verify their levels of FLC sensitivity. A total of 32 FLC-sensitive (FLC-s) mutants were isolated from initial screening of 1,201 deletion mutant strains. The *afr1Δ*, *sre1Δ*, and *scp1Δ* strains were found among the 32 FLC-s mutants and previous reports showed that deletion of each of these genes results in FLC-sensitivity (Sionov et al., 2009, Chang *et al.*, 2007). Isolation of known FLC-s mutants verified this screening approach to be suitable for identification of the genes important for the azole resistance in *C. neoformans*.

Deletion of genes

Identification of *C. neoformans* homologs was performed by Blast search of the H99 genomes at Broad Institute (http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans/index.html). In order to confirm the phenotypes of the STM deletion mutants, we reconstructed the selected deletion strains in H99 and retested the phenotype. All genes were deleted by biolistic transformation with PCR fusion using a strategy similar to that described for *Clostridium difficile* (Kawayama *et al.*, 2002). Briefly, the left end and the right end of the locus were amplified with the primers listed in Table S3. The linear disruption cassette was then used to homologously integrate into the strains by biolistic transformation (Toffaletti *et al.*, 1993). Transformants were screened by colony PCR and confirmed by Southern blot hybridization. The wild-type genes *YPK1*, *SIN1*, and *PDK1* were PCR-amplified from H99 using the primers listed in Table S3. The PCR clones were sequenced, cloned into a vector containing the nourseothricin (NAT) resistance marker and transformed into deletion mutants by the biolistic method. PCR was used to identify integrative transformants containing the intact wild-type gene and Southern blot analysis was used to confirm the results.

Sterol analysis

Sterol analysis was carried out as described before (Sionov *et al.*, 2009). Briefly, the log phase YPD grown cells were harvested by centrifugation and washed once with sterile distilled water. The pellets were resuspended in 9 ml methanol; 4.5 ml 60% (wt/vol) KOH was added together with 5 µg cholesterol (used as an internal recovery standard). Cell suspensions were heated to 75°C in a water bath for 2 h to complete the saponification; the sterols were then extracted with hexane and analyzed by gas chromatography (GC) with an Agilent 6850 gas chromatograph with an HP-1 fused silica column and a flame ionization detector at a temperature programmed from 200°C (1 min) to 300°C at a rate of 10°C/min. For identification of each sterol, a GC-MS (gas chromatograph-mass spectrometer) instrument (Polaris Q, from Thermo Electron, coupled to a Focus GC) was used to obtain mass spectral data in the EI mode for each of the GC peaks. This GC-MS instrument used a Restek 5MS fused silica column (30 m length, 0.25 mm i.d., 25 µm film thickness) at a program temperature from 200°C (1 min) to 300°C at a rate of 10°C/min. Cholesterol and ergosterol were identified by comparisons with standards. The relative amount of ergosterol was determined by comparing the area under the ergosterol peak in the GC using cholesterol as internal standard.

Preparation and analysis of nucleic acid

Isolation and analysis of genomic DNA was carried out as described previously (Chang & Kwon-Chung, 1994). For gene expression analysis, overnight cultures of wild-type and each deletion mutant strain were refreshed to OD₆₀₀ = 0.2, grown in fresh YPD media for 3 hrs, treated with FLC at the final concentration of 16 µg/ml for 30 min or 2 hr. RNA was extracted from yeast cells using Trizol (Invitrogen, Carlsbad, CA), treated with RNase-free Dnase (Ambion, Austin, TX) for the removal of genomic DNA, and purified with Rneasy MinElute cleanup kit (Qiagen, Valencia, CA). cDNA was synthesized using high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) and used in real time reverse transcription PCR (RT-PCR) with *TaqMan* universal PCR master mix and the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). The primers used in RT-PCR are listed in Table S3. Data were normalized with *GPD1* level and expressed as the amount in each deletion mutant strain relative to that in H99.

Protein extraction and immunoblot analysis

Cells were grown in YPD medium under the indicated conditions. Cultures were grown overnight in YPD at 30 °C with shaking and then diluted to an optical density (OD₆₀₀) of 0.4 in 20 ml of pre-warmed, fresh YPD medium. The cells were grown with shaking for an additional 2h and then treated as indicated. The cells were harvested, resuspended in ice-cold stop buffer (0.9% NaCl, 1 mM NaN₃, 10 mM Na-EDTA, 50 mM NaF) and then washed once in ice-cold stop buffer. The cells were resuspended in 0.2 ml lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 05 mM Na-EDTA, 5 mM Na-EGTA, 0.2 mM Na₃VO₄, 50 mM KF, 30 mM Na₄O₇P₂·H₂O, 50 mM NaF, 1X protease inhibitors (Roche, no. 11836170001), and 10 µl/ml each phosphatase inhibitor cocktail 1 and cocktail 2 (Sigma, P-2850 and P-5726, respectively). Lysis was achieved with five times of 45 sec 0.5-mm zirconia/silica bead beating with intermittent cooling on ice using FastPrep-24 (Scientific Industries). Lysates were spun down at 4°C and the supernatants were collected. Protein concentrations were determined using Quick Start Bradford dye reagent (Bio-Rad, no. 500-0205). For each sample, 30 µg of total protein was loaded on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and proteins were transferred to a PVDF membrane.

For phosphorylated Mpk1 determination, a 1:1000 dilution of phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal antibody (Cell Signaling Technology, no. 9101) was used. Total Mpk1 protein was detected using a 1:2000 dilution of DYKDDDDK (FLAG) tag rabbit polyclonal antibody (Cell Signaling Technology, no. 2368). All primary antibodies were diluted in TBST (25 mM Tris-HCl (pH 8.1), 145 mM NaCl, 0.1% Tween 20) plus 5% bovine serum albumin and allowed to bind overnight at 4°C. Secondary antibodies used were goat anti-rabbit immunoglobulin G peroxidase conjugated (Sigma, A-6154) and were diluted 1:1000 in TBST. Proteins were detected using the SuperSignal West Dura kit (Thermo Scientific, no 34076).

Immunoprecipitation analysis

Protein extract was prepared and quantitated as described above. A total of 1 mg of protein extract was incubated with Anti-FLAG M2 beads (M8823, Sigma Aldrich) for 3 hr at 4 °C. The beads were collected, washed 5 times with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and eluted with 25 µl 2X SDS-PAGE sample by boiling for 3 min. Supernatant was collected for immunoblotting. Protein samples were size fractionated on Novex 4–12% Bis-TRIS gradient gels using 4-morpholinepropanesulfonic acid buffer (Invitrogen) or on regular SDS-PAGE gels with a tris-glycine buffer. Note that these different methods cause slight differences in the running behavior of proteins. Size fractionated proteins were subsequently transferred onto membranes. The membranes were incubated with specific antibodies as indicated. Bound antibodies were detected as described above.

Accumulation of [³H] fluconazole

Cultures were grown at 30°C overnight in YPD liquid media and diluted with fresh YPD broth to 10⁸/ml, as determined by optical density at 600nm. Fluconazole accumulation was measured by using the drug that had been tritiated by gas exchange to a specific activity of 629 GBq/mM (Amersham Biosciences). [³H] fluconazole was added to 6 ml cell suspension at a final concentration of 7.4 kBq/ml (0.2 µCi/ml, 3.6 ng/ml). After 60 min of incubation with rotation at 225 rpm (30°C), samples were washed three times with 6 ml PBS (pH 7.0) containing 100 µM unlabeled fluconazole and triplicates of 2 ml samples were filtered on a vacuum manifold (Millipore, Bedford, Mass.) with 24-mm-diameter GF/C glass fiber filters (Whatman, Maidstone, Kent, United Kingdom), which had been presoaked in 100 µM unlabeled fluconazole in PBS. Zero-time samples were chilled on ice for 30 min before the addition of tritiated fluconazole and kept on ice until they were washed and filtered. The filters were dried overnight at 37°C, placed in Hydrofluor scintillation fluid (National

Diagnostic, Atlanta, GA) and allowed to stand overnight, and the cells were counted with a 200CA Tricarb liquid scintillation counter (Packard, Downer's Grove, IL). Cell number was estimated based on optical density at 600 nm measured after a washing step for both 0 and 60 min samples, which allows consideration of the growth difference. Data were normalized to the level of ^3H -FLC in H99 and presented as relative % uptake.

Metabolic Radio-labeling and analysis of complex sphingolipids

Overnight cultures were transferred to YNB without inositol for 2 hrs at 30°C. ^3H -Myo-inositol (NET1177005 at 1 mCi/ml) was added at a concentration of 5 $\mu\text{Ci}/2 \times 10^7$ cells/ml for 1 hr before adding FLC (16 $\mu\text{g}/\text{ml}$). Cultures with or without FLC treatment were harvested 2hrs later. Cells suspensions at same OD_{600} were stopped with 0.1 mg/ml BSA and 5% TCA and put on ice for 10min. Cells were centrifuged and resuspended in 1 ml of ethanol/ H_2O /diethylether/pyridine (30:30:10:2). Samples were incubated at 60°C in a water-bath for 1 hr and sonicated two times during the incubation. The extracted lipids were dried and dissolved in chloroform/methanol (5:4) and 100 μl of the extract was spotted on silica TLC plate (EMD Chemical Inc. 60W HX733723) using a spotter. The lipids were resolved in chloroform/methanol/4.5N ammonium hydroxide (90:45:10) for 75 min. Radioactive bands were visualized by X-ray film after spraying with En^3Hance (NEN Life Science Products 6NE970C). Sphingolipids and phosphatidylinositols (PI) were distinguished by treating the extracted lipids with monomethylamine/methanol/ H_2O /butanol (5:4:3:1) at 53°C for 45 min. The same volume of water and butanol/petroleum ether (b.p. 40-60°C)/ethyl formate (20:4:1) were added to the sample after cooling. The sample was mixed vigorously and spun. The lipid layer was dried and analyzed on TLC alongside samples without deacylation. Inositol phosphorylceramide (IPC), mannosyl-inositol phosphorylceramide (MIPC), mannosyl-di(inositol phosphoryl) ceramide ($\text{M}(\text{IP})_2\text{C}$) were identified using a strain of *S. cerevisiae* as a reference based on previous publication since complex sphingolipids of *C. neoformans* have not been analyzed under the same experimental conditions. We noted that at least two hitherto unidentified complex sphingolipids were detected on TLC in *C. neoformans* but not in *S. cerevisiae* (Fig. 6B). For quantitative analysis of sphingolipids, X-ray films obtained from various exposure times of TLC plates were scanned with GS-800 Imaging Densitometer using Quantity One 1-D analysis software (BioRad). The relative amounts of lipid in each strain are expressed as a ratio between the signal density of each lipid and the corresponding lipid signal in the FLC untreated H99 sample. Because of the poor resolution of the signals for $\text{M}(\text{IP})_2\text{C}$ and the slower migrating bands of unknown complex sphingolipids, the signals of $\text{M}(\text{IP})_2\text{C}$ and all the slower migrating bands were combined and designated as MIP2C^+ in our comparison.

Virulence study

Female BALB/c mice (6-8 weeks old) were injected via the lateral tail vein with 0.2 ml of a suspension of each yeast strain ($2.5 \times 10^4/\text{ml}$) as described previously (Chang & Kwon-Chung, 1994) and the mortality was monitored. Kaplan-Meier analysis of survival was performed with JMP software for Macintosh (SAS Institute, Cary, NC).

Spot Assay

Exponentially growing cultures ($\text{OD}_{600} = 0.5-1.0$) were washed, resuspended in 0.9% NaCl and adjusted to $\text{OD}_{600} = 0.1$. The cell suspensions were serially diluted, spotted onto the indicated media, and incubated for 3-4 days at 30°C

Plasmids

The FLAG epitope was inserted in frame at the carboxy terminus of *YPK1* by PCR amplification and the nourseothricin (NAT) resistance marker from *pAI3* was cloned into the

3' flanking region of *YPK1*. The resulting plasmid (pHL164) was sequenced to confirm that no errors had been introduced during amplification and used to transform *pdk1Δ*, *sin1Δ* as well as *ypk1Δ*. Transformants were first screened for loss of G418 resistance and for gain of nourseothricin resistance to select integrative transformants containing the fusion construct at the *YPK1* locus. PCR and Southern blot analysis were employed to confirm the integration event.

To generate a catalytically inactive (kinase-dead) version of Ypk1(K376A) and Ypk1(D386A), Lys376 (AAA) was replaced with Ala (GCT) and Asp386 (GAT) was replaced with Ala (GCT) respectively by PCR. These positions correspond to conserved residues critical for recognition of Mg^{2+} -ATP substrate in all protein kinases (Hanks & Hunter, 1995). Likewise, to attempt to generate phosphorylation defective Ypk1 derivatives – T402A, S562A, and S543A, three presumptive phosphorylation sites, Thr402 (ACC), Ser562 (TCC), and Ser543 (TCG) that match the consensus sequence for phosphorylation by PDK1, PDK2 and TOR1, respectively, were replaced with alanine (GCT). Primers were designed to incorporate each mutation/substitution as well as restriction enzymes to swap the resulting fragment into pHL164. Each fragment containing the corresponding mutation was amplified from pHL164 by fusion PCR, cloned into pCR2.1, and sequenced to verify the sequences. The fragment of each construct was cloned into pHL164 to replace the wild-type corresponding fragments using internal restriction enzyme sites. 1.3kb *NdeI-SalI* fragment of Ypk1 from K274A mutant, 1.8kb *NdeI-XhoI* fragments of D386A and T402A, 1kb *SalI-SnaBI* fragments of S543A and S562A were used to replace corresponding segments in pHL164 to create the final constructs, pHL177-181, respectively. Each construct was transformed into the *ypk1Δ* deletion mutant and the transformants were first screened for the loss of G418 resistance and gain of nourseothricin resistance to select integrative transformants containing the fusion construct at the *YPK1* locus. PCR and Southern blot analysis were used to confirm the integration event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Y. J. Kim, T. Balla, M. A. Singh, and M. Del Poeta for suggestion and technical help in analysis of complex sphingolipids, Andy Han for technical help in initial screening of the mutants and A. Varma for critical discussions and reading of the manuscript. This study was supported by funds from the intramural program of the National Institute of Allergy and Infectious Diseases, NIH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- Abe F, Usui K, Hiraki T. Fluconazole modulates membrane rigidity, heterogeneity, and water penetration into the plasma membrane in *Saccharomyces cerevisiae*. *Biochemistry*. 2009; 48:8494–8504. [PubMed: 19670905]
- Akins RA. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Med Mycol*. 2005; 43:285–318. [PubMed: 16110776]
- Anderson JB, Sirjusingh C, Parsons AB, Boone C, Wickens C, Cowen LE, Kohn LM. Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. *Genetics*. 2003; 163:1287–1298. [PubMed: 12702675]
- Armengou A, Porcar C, Mascaro J, Garcia-Bragado F. Possible development of resistance to fluconazole during suppressive therapy for AIDS-associated cryptococcal meningitis. *Clin Infect Dis*. 1996; 23:1337–1338. [PubMed: 8953097]

- Aronova S, Wedaman K, Aronov PA, Fontes K, Ramos K, Hammock BD, Powers T. Regulation of ceramide biosynthesis by TOR complex 2. *Cell metabolism*. 2008; 7:148–158. [PubMed: 18249174]
- Beeler T, Bacikova D, Gable K, Hopkins L, Johnson C, Slife H, Dunn T. The *Saccharomyces cerevisiae* TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca²⁺-sensitive csg2Delta mutant. *J Biol Chem*. 1998; 273:30688–30694. [PubMed: 9804843]
- Bennett JE, Izumikawa K, Marr KA. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrob Agents Chemother*. 2004; 48:1773–1777. [PubMed: 15105134]
- Berg J, Clancy CJ, Nguyen MH. The hidden danger of primary fluconazole prophylaxis for patients with AIDS. *Clin Infect Dis*. 1998; 26:186–187. [PubMed: 9455533]
- Birley HD, Johnson EM, McDonald P, Parry C, Carey PB, Warnock DW. Azole drug resistance as a cause of clinical relapse in AIDS patients with cryptococcal meningitis. *Int J STD AIDS*. 1995; 6:353–355. [PubMed: 8547418]
- Brun S, Berges T, Poupard P, Vauzelle-Moreau C, Renier G, Chabasse D, Bouchara JP. Mechanisms of azole resistance in petite mutants of *Candida glabrata*. *Antimicrob Agents Chemother*. 2004; 48:1788–1796. [PubMed: 15105136]
- Casamayor A, Torrance PD, Kobayashi T, Thorner J, Alessi DR. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr Biol*. 1999; 9:186–197. [PubMed: 10074427]
- Chang YC, Bien CM, Lee H, Espenshade PJ, Kwon-Chung KJ. *Cryptococcus neoformans* sterol regulatory element binding protein (Sre1p), a virulence factor that functions in oxygen sensing and sterol homeostasis. *Mol Microbiol*. 2007; 64:614–629. [PubMed: 17462012]
- Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol*. 1994; 14:4912–4919. [PubMed: 8007987]
- Cowen LE. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. *Nat Rev Microbiol*. 2008; 6:187–198. [PubMed: 18246082]
- Cowen LE, Anderson JB, Kohn LM. Evolution of drug resistance in *Candida albicans*. *Annu Rev Microbiol*. 2002; 56:139–165. [PubMed: 12142485]
- Cowen LE, Carpenter AE, Matangkasombut O, Fink GR, Lindquist S. Genetic architecture of Hsp90-dependent drug resistance. *Eukaryot Cell*. 2006; 5:2184–2188. [PubMed: 17056742]
- Cowen LE, Lindquist S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science*. 2005; 309:2185–2189. [PubMed: 16195452]
- Cowen LE, Steinbach WJ. Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. *Eukaryot Cell*. 2008; 7:747–764. [PubMed: 18375617]
- Cruz MC, Cavallo LM, Gorlach JM, Cox G, Perfect JR, Cardenas ME, Heitman J. Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in *Cryptococcus neoformans*. *Mol Cell Biol*. 1999; 19:4101–4112. [PubMed: 10330150]
- Davidson RC, Blankenship JR, Kraus PR, de Jesus Berrios M, Hull CM, D'Souza C, Wang P, Heitman J. A PCR-based strategy to generate integrative targeting alleles with large regions of homology. *Microbiology*. 2002; 148:2607–2615. [PubMed: 12177355]
- Gelperin D, Horton L, DeChant A, Hensold J, Lemmon SK. Loss of ypk1 function causes rapamycin sensitivity, inhibition of translation initiation and synthetic lethality in 14-3-3-efficient yeast. *Genetics*. 2002; 161:1453–1464. [PubMed: 12196392]
- Gerik KJ, Bhimireddy SR, Ryerse JS, Specht CA, Lodge JK. *PKC1* is essential for protection against both oxidative and nitrosative stresses, cell integrity, and normal manifestation of virulence factors in the pathogenic fungus *Cryptococcus neoformans*. *Eukaryot Cell*. 2008; 7:1685–1698. [PubMed: 18689526]
- Gerik KJ, Donlin MJ, Soto CE, Banks AM, Banks IR, Maligie MA, Selitrennikoff CP, Lodge JK. Cell wall integrity is dependent on the PKC1 signal transduction pathway in *Cryptococcus neoformans*. *Molecular Microbiology*. 2005; 58:393–408. [PubMed: 16194228]

- Graub R, Hilti N, Niederberger C, Schweingruber ME. *Ksg1*, a homologue of the phosphoinositide-dependent protein kinase 1, controls cell wall integrity in *Schizosaccharomyces pombe*. *J Basic Microbiol.* 2003; 43:473–482. [PubMed: 14625898]
- Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *Faseb J.* 1995; 9:576–596. [PubMed: 7768349]
- Hartmuth S, Petersen J. Fission yeast Tor1 functions as part of TORC1 to control mitotic entry through the stress MAPK pathway following nutrient stress. *J Cell Sci.* 2009; 122:1737–1746. [PubMed: 19417002]
- Heitman J, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science.* 1991; 253:905–909. [PubMed: 1715094]
- Helmerhorst EJ, Venuleo C, Sanglard D, Oppenheim FG. Roles of cellular respiration, CgCDR1, and CgCDR2 in *Candida glabrata* resistance to histatin 5. *Antimicrob Agents Chemother.* 2006; 50:1100–1103. [PubMed: 16495279]
- Inagaki M, Schmelzle T, Yamaguchi K, Irie K, Hall MN, Matsumoto K. PDK1 homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast. *Mol Cell Biol.* 1999; 19:8344–8352. [PubMed: 10567559]
- Kelly SL, Lamb DC, Baldwin BC, Corran AJ, Kelly DE. Characterization of *Saccharomyces cerevisiae* *CYP61*, sterol delta22-desaturase, and inhibition by azole antifungal agents. *J Biol Chem.* 1997; 272:9986–9988. [PubMed: 9092539]
- Knighton DR, Pearson RB, Sowadski JM, Means AR, Ten Eyck LF, Taylor SS, Kemp BE. Structural basis of the intrasteric regulation of myosin light chain kinases. *Science.* 1992; 258:130–135. [PubMed: 1439761]
- Kohli A, Smriti, Mukhopadhyay K, Rattan A, Prasad R. In vitro low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. *Antimicrob Agents Chemother.* 2002; 46:1046–1052. [PubMed: 11897588]
- Kojima K, Bahn YS, Heitman J. Calcineurin, Mpk1 and Hog1 MAPK pathways independently control fludioxonil antifungal sensitivity in *Cryptococcus neoformans*. *Microbiology.* 2006; 152:591–604. [PubMed: 16514140]
- Kontoyiannis DP, Sagar N, Hirschi KD. Overexpression of Erg11p by the regulatable GAL1 promoter confers fluconazole resistance in *Saccharomyces cerevisiae*. *Antimicrob Agents Chemother.* 1999; 43:2798–2800. [PubMed: 10543768]
- Kraus PR, Fox DS, Cox GM, Heitman J. The *Cryptococcus neoformans* MAP kinase Mpk1 regulates cell integrity in response to antifungal drugs and loss of calcineurin function. *Mol Microbiol.* 2003; 48:1377–1387. [PubMed: 12787363]
- Kuwayama H, Obara S, Morio T, Katoh M, Urushihara H, Tanaka Y. PCR mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res.* 2002; 30:E2. [PubMed: 11788728]
- Kwon-Chung, KJ.; Bennett, JE. *Medical Mycology*. Lea & Febiger; Philadelphia: 1992.
- LaFayette SL, Collins C, Zaas AK, Schell WA, Betancourt-Quiroz M, Gunatilaka AA, Perfect JR, Cowen LE. PKC signaling regulates drug resistance of the fungal pathogen *Candida albicans* via circuitry comprised of Mkc1, calcineurin, and Hsp90. *LID - e1001069 [pii]. PLoS Pathog.* 2010; 6
- Lamping E, Monk BC, Niimi K, Holmes AR, Tsao S, Tanabe K, Niimi M, Uehara Y, Cannon RD. Characterization of three classes of membrane proteins involved in fungal azole resistance by functional hyperexpression in *Saccharomyces cerevisiae*. *Eukaryot Cell.* 2007; 6:1150–1165. [PubMed: 17513564]
- Lesage G, Bussey H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev.* 2006; 70:317–343. [PubMed: 16760306]
- Levin DE. Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev.* 2005; 69:262–291. [PubMed: 15944456]
- Li X, Gianoulis TA, Yip KY, Gerstein M, Snyder M. Extensive in vivo metabolite-protein interactions revealed by large-scale systematic analyses. *Cell.* 2010; 143:639–650. [PubMed: 21035178]
- Liu OW, Chun CD, Chow ED, Chen C, Madhani HD, Noble SM. Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. *Cell.* 2008; 135:174–188. [PubMed: 18854164]

- Loewith R, Jacinto E, Wullschlegel S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*. 2002; 10:457–468. [PubMed: 12408816]
- Luo G, Gruhler A, Liu Y, Jensen ON, Dickson RC. The sphingolipid long chain base-Pkh1/2-Ypk1/2 signaling pathway regulates eisosome assembly and turnover. *J Biol Chem*. 2008; 283:10433–10444. [PubMed: 18296441]
- Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S. Molecular basis of resistance to azole antifungals. *Trends Mol Med*. 2002; 8:76–81. [PubMed: 11815273]
- Mansfield BE, Oltean HN, Oliver BG, Hoot SJ, Leyde SE, Hedstrom L, White TC. Azole drugs are imported by facilitated diffusion in *Candida albicans* and other pathogenic fungi. LID - e1001126 [pii]. *PLoS Pathog*. 6
- Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC, Bossche HV. Contribution of mutations in the cytochrome P450 14 α -demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology*. 1999; 145(Pt 10):2701–2713. [PubMed: 10537192]
- Martin SW, Konopka JB. Lipid raft polarization contributes to hyphal growth in *Candida albicans*. *Eukaryot Cell*. 2004; 3:675–684. [PubMed: 15189988]
- Matsuo T, Kubo Y, Watanabe Y, Yamamoto M. *Schizosaccharomyces pombe* AGC family kinase Gad8p forms a conserved signaling module with TOR and PDK1-like kinases. *Embo J*. 2003; 22:3073–3083. [PubMed: 12805221]
- Matsuo T, Otsubo Y, Urano J, Tamanoi F, Yamamoto M. Loss of the TOR kinase Tor2 mimics nitrogen starvation and activates the sexual development pathway in fission yeast. *Mol Cell Biol*. 2007; 27:3154–3164. [PubMed: 17261596]
- Moffett S, Brown DA, Linder ME. Lipid-dependent targeting of G proteins into rafts. *J Biol Chem*. 2000; 275:2191–2198. [PubMed: 10636925]
- Mondon P, Petter R, Amalfitano G, Luzzati R, Concia E, Polacheck I, Kwon-Chung KJ. Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans*. *Antimicrob Agents Chemother*. 1999; 43:1856–1861. [PubMed: 10428902]
- Monk BC, Niimi K, Lin S, Knight A, Kardos TB, Cannon RD, Parshot R, King A, Lun D, Harding DR. Surface-active fungicidal D-peptide inhibitors of the plasma membrane proton pump that block azole resistance. *Antimicrob Agents Chemother*. 2005; 49:57–70. [PubMed: 15616276]
- Mora A, Komander D, van Aalten DM, Alessi DR. PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol*. 2004; 15:161–170. [PubMed: 15209375]
- Mukhopadhyay K, Kohli A, Prasad R. Drug susceptibilities of yeast cells are affected by membrane lipid composition. *Antimicrob Agents Chemother*. 2002; 46:3695–3705. [PubMed: 12435664]
- Mukhopadhyay K, Prasad T, Saini P, Pucadyil TJ, Chattopadhyay A, Prasad R. Membrane sphingolipid-ergosterol interactions are important determinants of multidrug resistance in *Candida albicans*. *Antimicrob Agents Chemother*. 2004; 48:1778–1787. [PubMed: 15105135]
- Niederberger C, Schweingruber ME. A *Schizosaccharomyces pombe* gene, *ksg1*, that shows structural homology to the human phosphoinositide dependent protein kinase PDK1, is essential for growth, mating and sporulation. *Mol Gen Genet*. 1999; 261:177–183. [PubMed: 10071224]
- Otsubo Y, Yamamoto M. TOR signaling in fission yeast. *Crit Rev Biochem Mol Biol*. 2008; 43:277–283. [PubMed: 18756382]
- Oura T, Kajiwaru S. Disruption of the sphingolipid Delta8-desaturase gene causes a delay in morphological changes in *Candida albicans*. *Microbiology*. 2008; 154:3795–3803. [PubMed: 19047747]
- Oura T, Kajiwaru S. *Candida albicans* sphingolipid C9-methyltransferase is involved in hyphal elongation. *Microbiology*. 2010; 156:1234–1243. [PubMed: 20019081]
- Parsons AB, Brost RL, Ding H, Li Z, Zhang C, Sheikh B, Brown GW, Kane PM, Hughes TR, Boone C. Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nature biotechnology*. 2004; 22:62–69.
- Pasirja R, Prasad T, Prasad R. Membrane raft lipid constituents affect drug susceptibilities of *Candida albicans*. *Biochem Soc Trans*. 2005; 33:1219–1223. [PubMed: 16246085]

- Paugam A, Dupouy-Camet J, Blanche P, Gangneux JP, Tourte-Schaefer C, Sicard D. Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin Infect Dis*. 1994; 19:975–976. [PubMed: 7893896]
- Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol*. 11:9–22. [PubMed: 20027184]
- Perfect JR, Casadevall A. Cryptococcosis. *Infect Dis Clin North Am*. 2002; 16:837–874. v–vi. [PubMed: 12512184]
- Perfect JR, Cox GM. Drug resistance in *Cryptococcus neoformans*. *Drug Resist Updat*. 1999; 2:259–269. [PubMed: 11504497]
- Posteraro B, Sanguinetti M, Sanglard D, La Sorda M, Boccia S, Romano L, Morace G, Fadda G. Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole. *Mol Microbiol*. 2003; 47:357–371. [PubMed: 12519188]
- Prasad T, Saini P, Gaur NA, Vishwakarma RA, Khan LA, Haq QM, Prasad R. Functional analysis of CaIPT1, a sphingolipid biosynthetic gene involved in multidrug resistance and morphogenesis of *Candida albicans*. *Antimicrob Agents Chemother*. 2005; 49:3442–3452. [PubMed: 16048959]
- Roelants FM, Baltz AG, Trott AE, Fereres S, Thorner J. A protein kinase network regulates the function of aminophospholipid flippases. *Proc Natl Acad Sci U S A*. 2010; 107:34–39. [PubMed: 19966303]
- Roelants FM, Torrance PD, Bezman N, Thorner J. Pkh1 and pkh2 differentially phosphorylate and activate ypk1 and ykr2 and define protein kinase modules required for maintenance of cell wall integrity. *Mol Biol Cell*. 2002; 13:3005–3028. [PubMed: 12221112]
- Sanglard D, Ischer F, Calabrese D, Micheli M, Bille J. Multiple resistance mechanisms to azole antifungals in yeast clinical isolates. *Drug Resist Updat*. 1998; 1:255–265. [PubMed: 16904408]
- Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis*. 2002; 2:73–85. [PubMed: 11901654]
- Selmecki A, Forche A, Berman J. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science*. 2006; 313:367–370. [PubMed: 16857942]
- Selmecki A, Gerami-Nejad M, Paulson C, Forche A, Berman J. An isochromosome confers drug resistance in vivo by amplification of two genes, *ERG11* and *TAC1*. *Mol Microbiol*. 2008; 68:624–641. [PubMed: 18363649]
- Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol Mol Biol Rev*. 2011; 75:213–267. [PubMed: 21646428]
- Shea JM, Del Poeta M. Lipid signaling in pathogenic fungi. *Current Opinion in Microbiology*. 2006; 9:352–358. [PubMed: 16798065]
- Sionov E, Chang YC, Garraffo HM, Kwon-Chung KJ. Heteroresistance to fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence. *Antimicrob Agents Chemother*. 2009; 53:2804–2815. [PubMed: 19414582]
- Sionov E, Lee H, Chang YC, Kwon-Chung KJ. *Cryptococcus neoformans* overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes. *PLoS Pathog*. 2010; 6:e1000848. [PubMed: 20368972]
- Sun Y, Taniguchi R, Tanoue D, Yamaji T, Takematsu H, Mori K, Fujita T, Kawasaki T, Kozutsumi Y. Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. *Mol Cell Biol*. 2000; 20:4411–4419. [PubMed: 10825204]
- Tabuchi M, Audhya A, Parsons AB, Boone C, Emr SD. The phosphatidylinositol 4,5-bisphosphate and TORC2 binding proteins Slm1 and Slm2 function in sphingolipid regulation. *Mol Cell Biol*. 2006; 26:5861–5875. [PubMed: 16847337]
- Toffaletti DL, Rude TH, Johnston SA, Durack DT, Perfect JR. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. *J Bacteriol*. 1993; 175:1405–1411. [PubMed: 8444802]
- Torres J, Di Como CJ, Herrero E, De La Torre-Ruiz MA. Regulation of the cell integrity pathway by rapamycin-sensitive TOR function in budding yeast. *J Biol Chem*. 2002; 277:43495–43504. [PubMed: 12171921]

- Tsai HF, Krol AA, Sarti KE, Bennett JE. *Candida glabrata* PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. *Antimicrob Agents Chemother*. 2006; 50:1384–1392. [PubMed: 16569856]
- Vanhaesebroeck B, Alessi DR. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*. 2000; 346(Pt 3):561–576. [PubMed: 10698680]
- Venkateswarlu K, Taylor M, Manning NJ, Rinaldi MG, Kelly SL. Fluconazole tolerance in clinical isolates of *Cryptococcus neoformans*. *Antimicrob Agents Chemother*. 1997; 41:748–751. [PubMed: 9087482]
- White TC, Holleman S, Dy F, Mirels LF, Stevens DA. Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrob Agents Chemother*. 2002; 46:1704–1713. [PubMed: 12019079]
- Wilkinson MG, Pino TS, Tournier S, Buck V, Martin H, Christiansen J, Wilkinson DG, Millar JB. Sin1: an evolutionarily conserved component of the eukaryotic SAPK pathway. *Embo J*. 1999; 18:4210–4221. [PubMed: 10428959]
- Wu M, Holowka D, Craighead HG, Baird B. Visualization of plasma membrane compartmentalization with patterned lipid bilayers. *Proc Natl Acad Sci U S A*. 2004; 101:13798–13803. [PubMed: 15356342]
- Zhao X, Mehrabi R, Xu JR. Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryot Cell*. 2007; 6:1701–1714. [PubMed: 17715363]
- Zonios DI, Bennett JE. Update on azole antifungals. *Semin Respir Crit Care Med*. 2008; 29:198–210. [PubMed: 18366001]

Fig. 1. Characterization of FLC-s mutants

A. [^3H] Fluconazole accumulation of initially identified FLC-s mutants from the library screen. [^3H] fluconazole was added to the overnight culture for 60 min and the amount of [^3H] in each strain was measured. **B.** [^3H] Fluconazole accumulation of indicated deletion mutants generated in our laboratory and the corresponding complemented strains. Data were normalized to the level of ^3H -FLC in H99 at 0 min and 60 min, respectively, and presented as % of relative ^3H FLC levels. Bars indicate standard deviation.

Fig. 2. Spot assays for the effect of various agents

Cells of exponentially growing cultures from each strain were serially diluted and spotted onto media containing indicated reagents and incubated for 3-4 days at 30 °C (**A-D**). FLC and rapamycin concentrations are 8 μ g/ml and 1 ng/ml, respectively. The *pkc1* Δ was grown on media containing 1 M sorbitol since *pkc1* Δ grew poorly on YPD medium.

Fig. 3. Mpk1 phosphorylation

Total proteins were isolated from indicated strains that were treated with either DMSO (lane 1; D), 16 μ g/ml of FLC dissolved in DMSO (lane 2; D+F), water (lane 3; W), or 16 μ g/ml of FLC dissolved in water (lane 4-6). Mpk1 phosphorylation status was detected using the MAPK-phosphorylation-specific antibody.

Fig. 4. Characterization of Ypk1

A. Western blot of Ypk1-FLAG. Ypk1 was tagged with FLAG and protein extracts before and after IP were analyzed by western blot using anti-FLAG antibody. Lanes 1-4 correspond to samples treated with 16 μ g/ml FLC for 0, 30, 60 and 90 min, respectively. **B.** Spot assay of *ypk1* mutants. Cells of each indicated strain were spotted on YPD or YPD plus 8 μ g/ml FLC and incubated at 30°C for 3 days.

Fig. 5. *SIN1* is epistatic to *YPK1*

A. Spot assay. Cells of exponentially growing culture of each indicated strain were spotted on YPD or YPD plus 8 μ g/ml FLC or 1 ng/ml of rapamycin incubated at 30°C for 3 days. **B.** Western blot of Ypk1-FLAG. The Ypk1 expression levels were analyzed from the indicated strains expressing Ypk1-FLAG using anti-FLAG antibody.

Fig. 6. Homeostasis of complex sphingolipids synthesis

A. Spot assays. Cells of exponentially growing culture of indicated strains were spotted on YPD, 64 μ M aureobasidin A (ABA), 100 μ M myriocin (MYR) or 10 μ M phytosphingosine (PHS). **B.** TLC analysis of complex sphingolipids. Lipids were extracted from indicated strains labeled with 3 H-myoinositol and analyzed by TLC. “–” and “+” denote that cells were grown in the absence or presence of FLC, respectively. “S” denotes the sample from *S. cerevisiae*. **C.** Quantitative analysis of TLC results. The amounts of each species of lipids separated on TLC were measured and expressed as amounts relative to that of the FLC untreated wild-type samples. Data represent an average of three independent experiments. Bars indicate standard errors. * denotes $p < 0.05$ compared to H99 (student t-test). MIP2C+ represents the combined signals derived from M(IP) $_2$ C and all the slower migrating lipids.

Fig. 7. Virulence studies

Mice were injected with the indicated strain via the lateral tail vein with a suspension of each yeast strain (2.5×10^4 /ml) and the mortality was monitored (A-C).

Fig. 8. Schematic drawing of three signaling pathways, PKD1, PKC and TOR that are involved in regulating FLC basal tolerance in *C. neoformans* based on the present study.