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Role of the inositol pyrophosphate multikinase Kcs1 in *Cryptococcus* inositol metabolism

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Abstract

Cryptococcus neoformans is the most common cause of deadly fungal meningitis. This fungus has a complex inositol acquisition and utilization system, and our previous studies have shown the importance of inositol utilization in cryptococcal development and virulence. However, how inositol utilization is regulated in this fungus remains unknown. In this study, we found that inositol, irrespective of the presence of glucose in the media, represses the expression of *C. neoformans* genes involved in inositol pyrophosphate biosynthesis, including the gene encoding inositol hexakisphosphate kinase Kcs1. Kcs1 was recently reported to regulate inositol metabolism in *Saccharomyces cerevisiae* and to impact virulence in *C. neoformans*. To examine the potential role of Kcs1 in inositol regulation in *C. neoformans*, we generated the *kcs1* mutant and compared its phenotype with the wild type strain. We found that Kcs1 negatively regulates inositol uptake and catabolism in *C. neoformans*, but, in contrast to Kcs1 function in *S. cerevisiae*, does not appear to regulate inositol biosynthesis. Together, these results show that Kcs1 functions to fine-tune inositol acquisition to maintain inositol homeostasis in *C. neoformans*.

Introduction

Inositol is essential for multiple cellular functions, both as a signaling molecule and as a precursor for several structural cellular components. In the human fungal pathogen *C. neoformans*, inositol plays a critical role in fungal development, mating, and virulence. We

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have previously shown that the cryptococcal inositol uptake system is required for pathogenesis, especially during infection of the central nervous system (CNS) to cause cryptococcal meningitis (Liu et al., 2013a; Liu et al., 2014; Wang et al., 2011; Xue, 2012; Xue et al., 2010; Xue et al., 2007). However, despite the increased research attention on inositol metabolism and signaling in *C. neoformans*, the molecular mechanisms of inositol regulation of sexual reproduction and fungal virulence remains incompletely understood.

The mechanisms regulating inositol homeostasis in fungi have been largely studied in the model non-pathogenic yeast *Saccharomyces cerevisiae*. In this organism, inositol metabolism is tightly regulated by transcriptional machinery that contains two helix-loop-helix (HLH) domain transcription factors Ino2 and Ino4 (Ambroziak and Henry, 1994). During high inositol abundance, transcriptional repressor Opi1 binds to the Ino2-Ino4 heterodimer to repress the expression of *INO1* and inositol transporter *ITR1*, but when inositol is insufficient, Opi1 dissociates from Ino2 and Ino4 to activate genes necessary for inositol uptake and biosynthesis (Graves and Henry, 2000; Kumme et al., 2008). However, in *C. neoformans* there are no apparent sequence homologs of Ino2, Ino4 or Opi1, and its transcriptional regulation mechanism of inositol metabolism remains to be identified.

Inositol is a precursor for several inositol polyphosphates, which are important signaling molecules that have been the focus of intense investigation both in yeast and in mammalian cells (Sziggyarto et al., 2011; Tsui and York, 2010). In *S. cerevisiae*, the inositol pyrophosphate biosynthetic pathway contains multiple inositol polyphosphate kinases, including Arg82, Ipk1, Kcs1 and Vip1 (Mulugu et al., 2007). Among them, the inositol hexakisphosphate (IP₆) kinase Kcs1 functions as a multikinase that phosphorylates inositol pentakisphosphate (IP₅) and IP₆ to produce PP-IP_{4/5}, as well as phosphorylates PP-IP₅ to produce PP₂-IP₄ (Mulugu et al., 2007; Wang et al., 2012). Kcs1 has been shown to regulate yeast autophagy, stress response, cell wall integrity and vacuolar morphogenesis (Dubois et al., 2002; Taylor et al., 2012). Kcs1 has also recently been reported to regulate inositol metabolism in *S. cerevisiae* by controlling the expression of inositol 3-phosphate synthase Ino1 (Ye et al., 2013) via direct control of *INO1* gene expression, rather than via the inositol transcriptional machinery Ino2 and Ino4 (Ye et al., 2013). *C. neoformans* genome also encodes a Kcs1 homolog, which has been shown to be important for fungal metabolic adaption and virulence (Lev et al., 2015; Li et al., 2016b). However, whether this enzyme is involved in transcriptional regulation of inositol metabolism genes in *C. neoformans*, as it is in *S. cerevisiae*, is unknown.

In this study, we performed RNA-Seq to analyze how the *C. neoformans* transcriptome is regulated by inositol and to identify genes important for inositol metabolism. Our data showed that genes involved in the inositol pyrophosphate biosynthetic pathway were down-regulated by addition of extracellular inositol. Because of the known role of Kcs1 in inositol metabolism in *S. cerevisiae* and its importance in *Cryptococcus* development and virulence, we investigated the function of Kcs1 in *C. neoformans* inositol regulation. Our data demonstrated that, in contrast to *S. cerevisiae* (Ye et al., 2013), Kcs1 in *C. neoformans* negatively regulates fungal inositol uptake and inositol catabolism, but not inositol synthesis.

Material and Methods

Ethics Statement

The animal studies conducted at Rutgers University were in full compliance with all of the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) and in full compliance with the United States Animal Welfare Act (Public Law 98–198). The Rutgers IACUCs approved all of the vertebrate studies. The studies were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Strains, media, and growth conditions

C. neoformans strains used in this study are listed in Table 1. Strains were grown at 30°C on yeast extract-peptone-dextrose (YPD) agar medium and synthetic (SD) medium. Modified MS medium (Murashige and Skoog medium) was used for mating and sporulation assays and prepared as previously described (Xue et al., 2007). YNB medium without inositol was purchased from MP Biomedicals. Niger-seed and L-DOPA media were used to test for melanin production. Dulbecco modified Eagles (DME) medium for assessing capsule production was prepared as previously described (Bahn et al., 2004). All other media were prepared as described previously (Alspaugh et al., 1997; Bahn et al., 2005; Granger et al., 1985; Xue et al., 2010).

RNA preparation and RNA-Seq data analysis

C. neoformans wild type strain H99 was grown at 30°C on YPD medium. The overnight culture was washed twice with 1× phosphate-buffered saline (PBS) and re-inoculated on YNB medium containing either 1% inositol (YNB + Inositol), 1% glucose (YNB + Glucose), or both (YNB + Inositol + Glucose) as carbon source, and incubated for 24 hr. Total RNAs was isolated from cultures using Trizol reagent, treated with DNase I and purified with RNA clean-up kit (Clontech, Mountain View, CA). Purified total RNAs were quantified using a Nanodrop spectrometer (Thermo Scientific, Waltham, MA) and used in the RNA-Seq and qRT-PCR experiments. The mRNA was enriched by using the oligo(dT) magnetic beads and disrupted to shorter fragments (about 200 bp). The first strand cDNA was synthesized by random hexamer-primer using the mRNA fragments as templates. The double strand cDNA was purified with QiaQuick PCR extraction kit and washed with EB buffer for end repair and single nucleotide A (adenine) addition. Finally, sequencing adaptors were ligated to the fragments. The required fragments were gel purified and enriched by PCR amplification. The library products were used for sequencing analysis via Illumina HiSeq™ 2000 at Rutgers Genomic Research Center.

The gene expression level was calculated by using RPKM method (Reads Per Kb per Million reads) as described (Mortazavi et al., 2008). The log₂-transformed transcript levels from three samples were further analyzed by Partek Genomics Suite (Partek, St. Louis, MO) for statistical analysis and to identify significantly differentially expressed genes (SDEG) between samples. The normalized RNA-Seq data from each group (n=3) were averaged and analyzed by one-way ANOVA with equal variance for significance (Subbian et al., 2013). To identify SDEG, the log₂ ratio of H99 transcript levels among different treatments was

further screened by applying an unadjusted false discovery rate (FDR) of 5% (q value 0.05). The lists of SDEG were further used to decipher the GO and network/pathway analysis.

Detection of gene expression using quantitative RT-PCR

Cultures of *C. neoformans* var. *grubii* wild type strain H99 and its mutant strains were grown on YPD for 24 hrs at 30°C or on conditions specified in the main text. Collected cells were washed with dH₂O and pellets were used for total RNA extraction. Total RNAs were extracted using Trizol reagents (Invitrogen) and purified with NucleoSpin RNA cleanup kit (Takara) following the manufacturer's instructions. First strand cDNAs were synthesized using a SmartScribe reverse transcriptase (Takara) following the instructions provided by the manufacturer. Gene expression was analyzed with the comparative C_T method using Advantage SYBR green QPCR reagents (Takara) as described previously (Xue et al., 2010).

Inositol uptake assay

The inositol uptake assay protocol was adapted in part from protocols of Jin and Seyfang (Jin and Seyfang, 2003) and Chen and Reynolds (Chen et al., 2008). In brief, *C. neoformans* wild type and its mutants were grown in YPD liquid cultures overnight at 30°C. Cells were diluted in YPD to an optical density (OD₆₀₀) of 1.0, grown at 30°C, and collected at an OD₆₀₀ of 5.0 by centrifugation at $2,600 \times g$ for 5 min. Cells were then washed twice with PBS at 4°C and resuspended in 2% glucose to a final concentration of 2×10^8 cells/ml as determined by a hemacytometer. For the uptake assay, the reaction mixture (200 µl) contained 2% glucose, 40 mM citric acid-KH₂PO₄ (pH 5.5), 0.15 µM myo-[2-³H]-inositol (1 µCi/µl; MP BioMedicals). Additional 200 µM unlabeled inositol (Sigma-Aldrich) was added to the reactions for competition assays. Equal volumes of the reaction and cell mixtures (60 µl each) were warmed to 30°C and mixed for the uptake assay, which was performed for 10 min at 30°C. Aliquots of 100 µl were removed and transferred onto pretreated Metrical filters (1.2 µm) on a vacuum manifold. The filters were washed four times each with 2 ml of ice-cold water. The washed filters were removed and added to liquid scintillation vials for measurements on a PerkinElmer TRI-CARB 2900TR scintillation counter.

Generation of *kcs1* mutants and complemented strains

The *kcs1* mutants were generated by overlap PCR as previously described (Davidson et al., 2002). The 5' and 3' regions of each *KCS1* gene were amplified from H99 genomic DNA with primers CX120/CX121, and CX122/CX123, respectively (see Table S1 for primer sequences). The dominant selectable markers (NEO^r) were amplified with the M13 primers (M13F and M13R) from plasmid pJAF1 (Fraser et al., 2003). Each target gene replacement cassette was generated by overlap PCR with primers CX120/CX123 (Table S1). Purified overlap PCR products were precipitated onto 10 µl gold microcarrier beads (0.6 µm; Bio-Rad), and H99 was biolistically transformed as described previously (Davidson et al., 2000). Stable transformants were selected on YPD medium containing G418 (200 mg/L). To screen for mutants of *KCS1* gene, diagnostic PCR was performed by analyzing the 5' junction of the disrupted mutant alleles with primers CX126 and JH8994 (Table S1). Positive transformants identified by PCR screening were further confirmed by Southern blot analysis.

To generate complemented strains of *kcsI* mutants, a genomic DNA fragment that contains a 1.5 kb upstream promoter region, the *KCS1* ORF, and its 500 bp downstream region was amplified in a PCR using primers CX134/CX135. This PCR fragment was cloned into the plasmid pJAF13, which contains a NAT marker, by Infusion cloning (Clontech). The linearized plasmid was biolistically transformed in *kcsI* mutant strain to generate the complemented strain.

Assay for glucuronic acid production

C. neoformans overnight cultures were spun down and pellets were washed with ddH₂O twice before resuspended in 5 ml YNB+glucose or YNB+Inositol liquid medium and incubated for overnight at 30°C with shaking. Cell pellets were washed with MES buffer and resuspended in to 0.6 ml of the MES buffer. Cells were bead-beating for 5 min and then boiled for 5 min to kill all enzyme activities and denature proteins. After samples were centrifuged for 5 min at high speed, supernatants were transferred to new 2 ml tubes and kept at 4°C. The glucuronic acid concentration in each sample was measured the D-glucuronic acid assay kit (Megazyme, Ireland) following the instruction provided with the kit.

Assays for melanin production and laccase activity

Melanin production was assayed by inoculating *C. neoformans* strains into 2 ml of YPD liquid medium, incubating the culture overnight at 30°C and spotting 5 µl of each culture with ten times series dilutions on Niger-seed agar medium and L-DOPA medium. The agar plates were incubated at 30°C or 37°C for two days, and pigmentation of fungal colonies was assessed and photographed. To quantify the production of melanin by different strains, the activity of laccase enzyme was measured as previously described (Hicks et al., 2005). Single colonies of each strain were inoculated in 5 ml YPD liquid, and incubated at 30°C overnight. 10⁸ cells from each overnight culture were inoculated in to 25 ml of L-DOPA medium in 125 ml flasks, and incubated at 30°C for 16 h with 250 rpm shaking. The cultures were further incubated at 25°C for 24 h with 250 rpm shaking. One ml of each culture was centrifuged, and the supernatants were spectrophotometrically read for optical density at a wavelength of 475 nm. 1 U of laccase = optical density at 475 nm/0.001. The two-pair T-test method was used to determine the statistical significance of the difference between samples.

Results

The inositol pyrophosphate biosynthesis is down regulated by Inositol

Inositol is essential for many cellular functions in eukaryotes. Fungal cells can acquire inositol either from the environment through the inositol transporters (*ITRs*), or utilize glucose to synthesize inositol through a de novo biosynthetic pathway (Reynolds, 2009). Intracellular inositol can then be channeled into either the inositol pyrophosphate pathway or the pentose phosphate cycle (Fig. 1). To begin to understand how inositol metabolism is regulated in *Cryptococci*, we compared the transcriptional profiles of *C. neoformans* wild type strain H99 cultured in the presence of inositol, glucose, or both, as carbon sources. Total RNAs purified from these conditions were sequenced using whole transcriptome

shotgun sequencing (RNA-Seq) and transcriptional profiles of these culture conditions were compared. We found that when grown in the presence of inositol, genes involved in inositol uptake and inositol catabolism were highly up-regulated, while genes required for inositol pyrophosphate biosynthesis and inositol de novo biosynthesis were down-regulated (Fig. 2A). This gene expression pattern was observed in the absence or presence of glucose, indicating the inositol addition to the medium triggered the regulation of inositol metabolism genes irrespective of the presence of another carbon source.

Kcs1 regulates fungal melanin and cell integrity through activation of protein kinase C (Pkc1) in *C. neoformans*

It has been reported that in *S. cerevisiae* inositol hexakisphosphate (IP₆) kinase Kcs1, one of the enzymes in the inositol pyrophosphate biosynthetic pathway (Fig. 1), regulates inositol metabolism through its product 5PP-IP₄ in a way that is independent of the inositol transcription factors Ino2 and Ino4 (Ye et al., 2013). Given that *C. neoformans* genome does not encode Ino2 and Ino4 homologs based on sequence analysis, but does encode a Kcs1 homolog, we investigated the potential role of Kcs1 in regulation of inositol utilization in *C. neoformans* by generating the *kcs1* mutant. This mutant has been previously constructed and characterized, particularly with respect to *C. neoformans* virulence properties (Lev et al., 2015; Li et al., 2016a). Consistent with those studies, we found that Kcs1 was essential for the development of several known virulence factors (data not shown), resistance to cell wall damaging agents, and mating (Supplemental Figure S1).

One virulence factor strongly impacted by absence of Kcs1 was melanin production (Fig. 3A). To understand the mechanistic basis for this defect, we measured *LAC1* gene expression and laccase activity. We found that in the *kcs1* mutant both the expression of *LAC1* gene (Fig. 3B) and laccase activity were significantly reduced (Fig. 3C), indicating that Kcs1 controls cryptococcal melanin production by regulating the expression of *LAC1* gene. Next, we used a highly selective inositol hexakisphosphate kinase (IP6K) inhibitor, N2-(m-(trifluoromethyl)benzyl) N6-(p-nitrobenzyl) purine (TNP) (Padmanabhan et al., 2009). We found that addition of TNP clearly impaired melanin production, reducing both laccase activity and cell pigmentation (Fig. 3C and 3D), consistent with Kcs1 function as an IP6K and with TNP-mediated inhibition of Kcs1.

It has been reported that *LAC1* expression is regulated by protein kinase C (Pkc1) (Heung et al., 2005). To investigate whether Kcs1 regulates *LAC1* expression by regulating the expression of *PKC1*, we measured the expression of *PKC1* on L-DOPA medium in wild type and *kcs1* cells. Our results showed that *PKC1* gene expression was indeed reduced in the *kcs1* mutant after 4 hr incubation (Fig. 4B). Because *PKC1* expression is regulated by diacylglycerol (DAG), which can be produced by hydrolyzing PIP₂ with phospholipase C (Plc1) in other systems, including *S. cerevisiae* (Fig. 4A), we hypothesized that blocking of PP-IP₄ and/or PP-IP₅ in the *kcs1* mutant may trigger a negative feedback loop, leading to repression of *ARG1* (ortholog of *ARG82* in *S. cerevisiae*) and *PLC1* expression in the *kcs1* mutant background, which in turn reduces DAG production and *PKC1* activation. To test this hypothesis, we measured the expression of *ARG1* and *PLC1* in cells incubated on L-DOPA medium. We found no significant differences in the expression of *ARG1* and *PLC1*

between H99 and *kcs1*⁻ mutant (Fig. 4C and 4D), suggesting that the observed effect of *kcs1*⁻ on laccase and melanin production may be due to other mechanisms, such as direct cross talk between Kcs1 and Pkc1 pathways.

In addition to regulating melanin production, *C. neoformans* Pkc1 also regulates cell wall integrity through the Bck1-Mkk2-Mpk1 MAP kinase cascade (Fig. 4A) (Gerik et al., 2008; Gerik et al., 2005; Kraus et al., 2003). Thus, we examined *MPK1* expression in the *kcs1*⁻ mutant. Our data showed that *MPK1* was induced by Calcofluor white in the wild type strain but that this induction was strongly attenuated in the *kcs1*⁻ mutant (Fig. 4E). These results are consistent with the cell wall integrity defect observed in the *kcs1*⁻ mutant (Supplemental Figure S1) (Lev et al., 2015; Li et al., 2016b) and indicate that this defect is likely underpinned by the low activity of the Pkc1-Mpk1 pathway.

Kcs1 negatively regulates inositol uptake in *C. neoformans*

To understand whether Kcs1 regulates the expression of genes involved in inositol metabolism in *C. neoformans*, we used qRT-PCR to measure the expression of selected genes in wild type and *kcs1*⁻ mutant strains grown in the presence of either glucose or inositol. First, we measured the expression of inositol transporter (*ITR*) gene family. There are at least ten *ITR* candidates in the *C. neoformans* H99 genome. Our qRT-PCR data showed that the expression of most genes in this family were up-regulated in both wild type and the *kcs1*⁻ mutant background (Fig. 5A, 5B) in the presence of inositol. Among them, the *ITR3C* and *ITR4* expression was induced over 100-fold by inositol. This positive regulation of *ITR* expression by inositol is consistent with our RNA-Seq data (Table 1) and also our previous results based on microarray analysis (Xue et al., 2010). Interestingly, the expression of most *ITRs* was higher in the *kcs1*⁻ mutant compared to the wild type strain, and the difference in expression of *ITR2*, *ITR3* and *ITR3A* was statistically significant ($P < 0.01$), suggesting a potential negative regulation of *ITR* expression by Kcs1 in the presence of inositol. Next, we measured inositol uptake in the wild type strain and the *kcs1*⁻ mutant. Consistent with increased *ITR* expression, the *kcs1*⁻ mutant had a significantly higher inositol uptake rate than the wild type strain (Fig. 5C). These data suggest that Kcs1 negatively regulates inositol uptake, which is consistent with the RNA-seq result showing that inositol addition represses *KCS1* expression (Fig. 2).

To evaluate the specificity of Kcs1 regulation of *ITR* gene expression in response to the carbon shift from glucose to inositol, we also monitored the expression of three hexose transporters (*HXT1*, *HXT2* and *HXT3*) that were identified in our previous study (Liu et al., 2013b). Our data showed that, under the same culture conditions, there was no significant difference in the *HXT* gene expression between H99 and the *kcs1*⁻ mutant. These results demonstrate that Kcs1 specifically represses the expression of *ITR* genes, not glucose transporter genes.

Kcs1 does not regulate inositol de novo biosynthesis in *C. neoformans*

In *S. cerevisiae*, Kcs1 has been shown to positively regulate the transcription of *INO1*, which encodes inositol 3-phosphate synthase, to fine tune the regulation of inositol metabolism independent of the Ino2/Ino4 transcriptional complex (Ye et al., 2013). As a result, *S.*

cerevisiae kcs1 cells grow poorly in medium lacking inositol due to low activity of *Ino1*. We examined whether this was also the case in *C. neoformans*. First, we observed that removal of extracellular inositol from the medium failed to induce *INO1* expression in *kcs1* cells. Our qRT-PCR data also showed that the expression of *INO1* was down-regulated by inositol addition in both wild type and the *kcs1* mutant, with greater down-regulation in the mutant background (Fig. 6A). In contrast, removing inositol from the medium induced *INO1* expression in both H99 and *kcs1* at a similar level (Fig. 6B). We also compared the growth rate of H99 and the *kcs1* mutant in a growth medium with or without 75 μ M inositol. Our results showed that while the *kcs1* mutant had a general growth defect, this defect was not exacerbated when inositol was absent (Fig. 6C). As a control, we examined the growth rate of *S. cerevisiae* wild type BY4741 and its *kcs1* mutant under the same conditions. We found that the *kcs1* mutant barely grew in the absence of inositol (Fig. 6D), in consistent with a previous report (Ye et al., 2013). These data demonstrate that, in contrast to *S. cerevisiae*, in *C. neoformans* Kcs1 does not play a significant role in inositol-mediated regulation of *INO1* gene expression and is not essential for inositol synthesis.

Kcs1 negatively regulates inositol catabolism in *C. neoformans*

Finally, we measured the expression of *MIO* gens, which encode the inositol oxygenase that is the first step of inositol catabolic pathway (Fig. 1). We found that all three *MIO* genes were highly upregulated by inositol addition and, while there was no significant difference in *MIO1* expression between H99 and *kcs1* backgrounds, *MIO2* and *MIO3* induction by inositol was enhanced in the *kcs1* mutant (Fig. 7A, 7B). We then measured the production of glucuronic acid (GlcA), the product of the Mio enzyme reaction, and found that inositol significantly induced GlcA production in both wild type and the *kcs1* mutant, and the *kcs1* mutant produced slightly more GlcA when grown in inositol medium, although the difference was not statistically significant (Fig. 7C). These results demonstrated that Kcs1 suppresses *MIO2* and *MIO3* expression but does not strongly affect the overall GlcA production in *C. neoformans*, perhaps due to a dominant role of Mio1.

As a final test of a possible role for Kcs1 in inositol catabolism, we compared the growth rate of H99 and the *kcs1* mutant in growth medium containing 1% inositol or glucose as the sole carbon source. Our results showed that while H99 grew faster than the *kcs1* mutant in general, the mutant could still grow in both media. Interestingly, during first 30 hours of incubation the *kcs1* mutant grew faster in inositol medium than in the glucose medium; however, the mutant also took a longer time to reach the plateau in inositol medium than in glucose medium (Fig. 7D). Together with the enhanced induction of *MIO2* and *MIO3* gene expression in *kcs1*, this result suggests that *C. neoformans* Kcs1 also plays a role in regulation of inositol catabolism.

Discussion

In this study we showed that in *C. neoformans* extracellular inositol negatively regulates the expression of genes required for inositol pyrophosphate synthesis, including IP6 kinase Kcs1, and characterized the function of Kcs1 in regulation of inositol metabolism. We found

that, unlike in *S. cerevisiae*, cryptococcal Kcs1 negatively regulates genes involved in inositol uptake and catabolism, but not the genes involved in inositol biosynthesis. Together with the lack of the Ino2 and Ino4 homologs in this fungus, these results indicate that *S. cerevisiae* and *C. neoformans* have distinct inositol regulatory mechanisms. We also found, consistent with a previous report (Lev et al., 2015), that Kcs1 was essential for the development of *Cryptococcus* virulence factors, for sexual reproduction, and for normal cell wall integrity and stress resistance. Furthermore, our results indicate that the *kcs1* defects in melanin production and cell wall integrity are due, at least in part, to reduced *PKC1* expression and reduced activity of the Pkc1 pathway in this mutant. Together, these results show that Kcs1 functions to fine-tune inositol acquisition to maintain inositol homeostasis and inositol-dependent pathways in *C. neoformans*, adding to our understanding of the regulation of inositol metabolism and virulence factor development in this fungus.

Inositol utilization is important for *C. neoformans* development and pathogenesis, yet how inositol utilization is regulated in this fungus remains unclear. The fact that *C. neoformans* can use inositol as a sole carbon source, while *S. cerevisiae* cannot, suggests that there are significant differences in inositol utilization between the two fungi. The data presented in this manuscript elucidate several of these differences, centering in particular around the function of IP6 kinase Kcs1. In *S. cerevisiae*, besides the transcriptional regulation of inositol biosynthetic pathway and inositol uptake by the Ino2, Ino4, and Opi1 transcription factors (all of which are absent in *C. neoformans*), IP6 kinase Kcs1 has been found to regulate inositol metabolism independently of the Ino2-Ino4 complex (Ye et al., 2013). Specifically, *S. cerevisiae* Kcs1 is required for the expression of *INO1* gene, which is necessary for de novo inositol biosynthesis, and, accordingly, *kcs1* cells cannot grow on medium lacking inositol (Ye et al., 2013). In contrast, we found that although the *C. neoformans kcs1* mutant had a general slow growth phenotype, it did not exhibit inositol auxotrophy and was only modestly altered for inositol repression of *INO1* expression (Fig. 6). These experiments strongly indicate that in *C. neoformans* Kcs1 does not regulate inositol biosynthesis in *C. neoformans* and that transcriptional regulators that do regulate that process still remain to be identified.

In addition to being able to synthesize inositol de novo, fungal cells can import inositol from the environment. Interestingly, upon adding inositol to the medium, we did observe significantly increased expression of inositol transporter genes (*ITRs*) and inositol uptake activity in the *kcs1* mutant cells (Fig. 5A–C), suggesting that Kcs1 negatively regulates inositol uptake. Our previous studies showed that inositol can induce fungal mating and that mutants with reduced inositol uptake activity exhibited reduced mating hyphal production (Wang et al., 2011; Xue et al., 2010). However, instead of enhancing fungal mating, as may be expected from its enhanced inositol uptake, the *kcs1* mutant had a partial unilateral and a full bilateral mating defect (Lev et al., 2015)(Supplemental Figure S1), suggesting that Kcs1 role in regulating fungal mating is distinct from its role in inositol acquisition.

We also observed that inositol oxygenase *MIO* genes were highly upregulated by inositol and that this upregulation was affected by *kcs1*. Following inositol addition, *MIO1* expression is increased over 1000-fold compared to less than 100-fold induction of *MIO2* and *MIO3* in our qRT-PCR analysis, indicating that Mio1 likely plays a major role in

inositol oxidation. Interestingly, induction of *MIO3* and, to a lesser degree, *MIO2*, but not *MIO1*, were elevated even further in the *kcs1* strain (Fig. 7A, 7B). Despite this increase in induction of *MIO3* gene expression in the *kcs1* mutant in the presence of inositol, we observed no significant increase in the production of glucuronic acid (GlcA), the product of inositol oxidation, in this mutant (Fig. 7C). It is possible that in the context of the high abundance of Mio1 protein, the increase in *MIO3* expression in the *kcs1* mutant may not make a significant difference in the overall inositol oxygenase enzyme activity. Another possibility that remains to be tested is that GluAC production between wild type and *kcs1* may be different at other time points following inositol addition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cryptococcal inositol pyrophosphate biosynthesis is down-regulated by inositol
- IP6 kinase Kcs1 regulates melanin production via regulation of the PKC1 pathway
- Kcs1 down-regulates inositol uptake and catabolism in *C. neoformans*
- Kcs1 is dispensable for de novo inositol biosynthesis in *C. neoformans*
- Kcs1 role in inositol metabolism in *C. neoformans* is distinct from *S. cerevisiae*

Figure 1. Schematic model of the inositol acquisition, synthesis and metabolism in *C. neoformans* Myo-inositol (referred to as inositol) can be either imported from the environment *via* inositol transporters (*ITRs*) or synthesized intracellularly by converting glucose to inositol. Intracellular inositol is a precursor for producing inositol polyphosphates through PI kinase-mediated phosphorylation. Inositol in *C. neoformans* can also be converted by inositol oxygenase (*MIO*) to glucuronic acid, which can be utilized as an energy source via a catabolic pathway. Kcs1 is the inositol hexakisphosphate (IP_6) kinase, which, in *S. cerevisiae*, also activates transcription of inositol 3-phosphate synthase gene *INO1*.

Figure 2. Expression profile of genes involved in inositol metabolism
A) Intensity plot of genes involved in inositol synthesis, catabolism and metabolism. Gene expression of wild type H99 was compared under conditions of inositol (Ino), glucose (Glu), or both (Ino+Glu), as carbon sources. The fold changes of inositol related genes between inositol-containing medium versus glucose-containing medium (Ino vs Glu) and between both glucose- and inositol-containing medium versus glucose-containing (Ino+Glu vs Glu) are shown. The scale bar ranges from +3 (red) to -3 (blue) Log2s. B) Inositol phosphate metabolism pathway as defined in the KEGG pathway database. Genes encoding enzymes highlighted in blue were down-regulated by inositol, those highlighted in yellow were not significantly altered, and those highlighted in red were strongly (2-fold or higher) induced by inositol.

Figure 3. Melanin production is dependent on *KCS1* and can be blocked by adding an IP6K inhibitor

A) Color development by H99, *kcs1* Δ and its complementary strain (*kcs1* + *KCS1*) was evaluated after cultures were grown on L-DOPA medium for 48 hr at 37°C. B) The expression of the *LAC1* gene at 1, 4 and 24 hours after incubation was determined by qRT-PCR and its relative expression changes to time 0 were calculated using Δ CT method. * denotes $P < 0.01$ in a Student's t-test. C) Laccase activity for each strain was quantified after 48 hr incubation in L-DOPA liquid medium at 30°C, with or without the IP6K inhibitor TNP. D) Melanin production in L-DOPA medium after 24 hr incubation in the presence of different concentrations of TNP. The pigment level of each culture was quantified by measuring the OD₄₇₅.

Figure 4. Kcs1 regulates *PKC1* and *MPK1* expression

A) Schematic of the signaling pathways downstream of PIP_2 involving Kcs1, Pkc1, melanin production, and cell integrity. B–D) Cultures of H99 and *kcs1Δ* were incubated in L-DOPA medium for 1, 4, and 24 hrs. Expression of *PKC1* (B), *PLC1* (C), *ARG1* (D) and *MPK1* (E) was determined with qRT-PCR and the relative expression changes to time 0 were quantified by CT method. * denotes $P < 0.01$ in a t-test.

Figure 5. Kcs1 negatively regulates the expression of *ITR* expression and inositol uptake
 A) and B) The fold changes of expression of the *ITR* gene family members were compared in H99 and *kcs1* following the change of carbon sources. Cultures grown on YNB containing 1% glucose (Glu) were switched to YNB containing 1% inositol (Ino) for 4 hrs (A) or 24 hrs (B). The *GAPDH* gene was used as a reference. C) Inositol uptake assay for H99, *kcs1* and its complemented strain was performed using ³H-labelled inositol. Cultures were incubated for 10 mins before being collected and measured in a scintillation counter for radioactive signal. D) The fold changes of the expression of three hexose transporters (HXT) were compared in H99 and *kcs1* following the change of carbon sources (from glucose to inositol). Error bars indicate standard deviation for three biological replicates. * denotes P<0.01 in a t-test.

Figure 6. Kcs1 does not play an important role in regulating *INO1* expression in *C. neoformans*
 A) Cultures of wild type (H99) and isogenic *kcs1* strains incubated in YNB with 1% glucose were switched to YNB with 1% inositol and incubated for 4 hrs and 24 hrs. Fold changes of *INO1* gene expression induced by inositol were compared between H99 and the *kcs1* mutant. B) Cultures of H99 and *kcs1* incubated on YNB with 1% glucose and 1% inositol were switched to YNB with 1% glucose and incubated for 4 hrs. Fold changes of *INO1* gene expression induced by inositol removal were compared between H99 and the *kcs1* mutant. C) Growth curves for H99 and *kcs1* cells grown in YNB with or without 75 μm inositol (Ino+ and Ino- respectively). D) Growth curves for *S. cerevisiae* BY4741 and *kcs1* cells grown in YNB with or without 75 μm inositol (Ino+ and Ino- respectively). The error bars indicate the standard deviation of at least three biological replicates.

Figure 7. Regulation of inositol catabolism by Kcs1

A) and B) Cultures of H99 and *kcs1* strains incubated in YNB with 1% glucose were switched to YNB with 1% inositol and incubated for 4 hrs (A) and 24 hrs (B). Fold changes of *MIO1*, *MIO2*, and *MIO3* gene expression relative to time 0 were compared between H99 and the *kcs1* mutant. * denotes $P < 0.01$ in a t-test. C) The production of glucuronic acid (GluAC) was measured to determine inositol oxygenase enzyme activity. GluAC production was compared between H99 and the *kcs1* mutant under two different culture conditions, YNB with 1% inositol and YNB with 1% glucose. D) Growth rates of H99 and *kcs1* were measured in YNB with 1% glucose or YNB with 1% every 30 mins at 30°C. Error bars indicate inositol. The cell density was determined by OD₆₀₀ standard deviations for three replicates.

Table 1

Expression profile of genes involved in inositol metabolism

	Locus	Gene	Ino/Glu	Ino+Glu/Glu
Inositol uptake	CNAG_00097	<i>ITR1</i>	2.28302	1.71354
	CNAG_04552	<i>ITR1A</i>	1.98406	2.19533
	CNAG_00864	<i>ITR2</i>	1.05826	2.22661
	CNAG_05377	<i>ITR3</i>	-1.55892	1.44987
	CNAG_00867	<i>ITR3A</i>	-3.68902	1.60298
	CNAG_05667	<i>ITR3B</i>	-3.80998	-1.60194
	CNAG_05381	<i>ITR3C</i>	30.5358	25.8554
	CNAG_05662	<i>ITR4</i>	12.4597	13.8718
	CNAG_04024	<i>ITR5</i>	1.07981	1.62615
	CNAG_03910	<i>ITR6</i>	1.07981	1.62615
Inositol biosynthesis	CNAG_01539	<i>INO1</i>	-4.21058	-5.1748
	CNAG_01823	<i>INM1</i>	-1.12981	-1.21511
	CNAG_03262	<i>INM2</i>	-1.44061	-1.21405
Inositol metabolism	CNAG_05384	<i>PIS1</i>	-1.24315	-1.13229
	CNAG_03821	<i>PI3K1</i>	-6.22329	-1.12548
	CNAG_04335	<i>PI3K2</i>	-3.13056	1.00329
	CNAG_05771	<i>PI3K3</i>	-4.38881	-1.1348
	CNAG_07744	<i>PI4K1</i>	-2.83262	-1.44291
	CNAG_06161	<i>PI4K2</i>	-2.40952	1.0078
	CNAG_01209	<i>PI5K1</i>	-3.58724	-1.00483
	CNAG_00388	<i>PI5K2</i>	-3.85494	-1.28289
	CNAG_02867	<i>PLC1</i>	-2.37392	-1.05082
	CNAG_02802	<i>ARG1</i>	-4.25842	-1.15421
	CNAG_02897	<i>KCS1</i>	-1.81765	-1.18569
	CNAG_01294	<i>IPPK</i>	-4.8533	-1.49437
	CNAG_00390	<i>CNM1</i>	-1.58482	1.14841
	CNAG_07354	<i>INPP1</i>	-1.52501	-1.49287
	CNAG_02109	<i>INPP2</i>	-3.45535	-1.11818
	CNAG_01532	<i>INPP3</i>	-1.62614	-1.00029
	CNAG_03657	<i>INPP4</i>	-2.48048	1.42827
	CNAG_07317	<i>INPP5</i>	1.00826	-1.00511
	CNAG_02001	<i>INPP6</i>	-1.07712	1.44921
	CNAG_06967	<i>MINPP1</i>	-2.20861	-1.172
	CNAG_01495	<i>IDH1</i>	23.4837	37.1694
	CNAG_05321	<i>IDH2</i>	-3.37034	1.07878
	CNAG_05329	<i>IDH3</i>	-2.1077	-1.00248
	CNAG_07626	<i>IDH4</i>	2.03336	1.63199
	CNAG_05320	<i>IDH5</i>	-1.62082	-1.45
	CNAG_06530	<i>IDH6</i>	23.7249	11.1206

	Locus	Gene	Ino/Glu	Ino+Glu/Glu
Inositol catabolism	CNAG_06836	<i>IDH7</i>	−2.60601	1.90868
	CNAG_01878	<i>IDH8</i>	−2.64142	1.02043
	CNAG_06623	<i>MIO1</i>	155.001	127.324
	CNAG_05316	<i>MIO2</i>	1.14051	1.50548
	CNAG_03277	<i>MIO3</i>	143.403	1.14841
	CNAG_06051	<i>GUK1</i>	3.90581	2.85293
	CNAG_06001	<i>GUK2</i>	1.66901	−1.0589
	CNAG_03946	<i>GUK3</i>	1.32337	1.2702
	CNAG_02748	<i>USP1</i>	−1.79376	−1.49111
	CNAG_05828	<i>USP2</i>	−3.18518	−1.38563

Note: Ino, Inositol; Glu, Glucose