Candida albicans OPI1 Regulates Filamentous Growth and Virulence in Vaginal Infections, but Not Inositol Biosynthesis

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Abstract

ScOpi1p is a well-characterized transcriptional repressor and master regulator of inositol and phospholipid biosynthetic genes in the baker’s yeast Saccharomyces cerevisiae. An ortholog has been shown to perform a similar function in the pathogenic fungus Candida glabrata, but with the distinction that CgOpi1p is essential for growth in this organism. However, in the more distantly related yeast Yarrowia lipolytica, the OPI1 homolog was not found to regulate inositol biosynthesis, but alkane oxidation. In Candida albicans, the most common cause of human candidiasis, its Opi1p homolog, CaOpi1p, has been shown to complement a S. cerevisiae opi1Δ mutant for inositol biosynthesis regulation when heterologously expressed, suggesting it might serve a similar role in this pathogen. This was tested in the pathogen directly in this report by disrupting the OPI1 homolog and examining its phenotypes. It was discovered that the OPI1 homolog does not regulate INO1 expression in C. albicans, but it does control SAP2 expression in response to bovine serum albumin containing media. Meanwhile, we found that CaOpi1 represses filamentous growth at lower temperatures (30°C) on agar, but not in liquid media. Although, the mutant does not affect virulence in a mouse model of systemic infection, it does affect virulence in a rat model of vaginitis. This may be because Opi1p regulates expression of the SAP2 protease, which is required for rat vaginal infections.

Introduction

Candida albicans is a commensal organism that lives as a benign resident of the microflora of the human oral, gastrointestinal, and vaginal tracts as well as the skin. It can shift from a commensal to a pathogenic state in response to environmental stimuli that trigger developmental programs that induce the expression of virulence factors. Virulence factors exhibited by C.
albicans include growth at 37°C, dimorphism, and production of secreted hydrolases such as proteases, lipases, and phospholipases [1, 2].

The pathways that regulate the transcription of secreted aspartyl protease (SAP) virulence factors in C. albicans are beginning to be understood, but much remains to be learned. SAPs are encoded by a family of 10 related genes (SAP1 to SAP10) [3, 4]. Unlike SAP1 to SAP8, which encode secreted proteases, SAP9 and SAP10 encode GPI-anchored proteases, located at the cell membrane or cell wall, and both are required for virulence [5]. Among this family of genes, Sap2p is the most well-studied protease since it is the major secreted protease during in vitro growth conditions. SAP2 is expressed in in vitro conditions where bovine serum albumin is the main nitrogen source [3, 4], and its regulation in these conditions has been well characterized. SAP2 is under the control of the STP1 transcription factor and STP1’s upstream GATA transcription factors GLN3 and GAT1 [6, 7].

The importance of SAP2 in pathogenesis has been discussed by several groups. For instance, De Bernardis et al. demonstrated that SAP2 is a major virulence contributor in the rat vaginitis model [8, 9]. Schaller et al. showed that SAP2 is required to cause tissue damage in an in vitro model of vaginal candidiasis [10]. In addition, Hube et al. demonstrated that SAP2 was required for virulence in a rodent model of systemic infection [11]. In contrast, Naglik et al. and Lermann and Morschhäuser found that SAP2 was not required to invade and damage oral or vaginal reconstituted human epithelium [12, 13]. Meanwhile, the effect of the aspartic protease inhibitor pepstatin A on reducing tissue damage caused by C. albicans in the reconstituted human epithelium model remains elusive. Naglik et al. showed that pepstatin A can attenuate tissue damage, while Lermann and Morschhäuser demonstrated no effect, leaving the role for the Sap family in inducing epithelial damage controversial [12, 13]. Thus, there is contradictory evidence about the role of SAP2 and other SAPs in pathogenesis.

S. cerevisiae OPI1 (ScOPI1) is a negative regulator of inositol biosynthesis, and acts to inhibit the transcription of ScINO1 along with other phospholipid biosynthetic genes in response to extracellular inositol levels [14–17]. ScINO1 encodes the inositol-3-phosphate synthase (ScIno1p) that catalyzes the conversion of glucose-6-phosphate to inositol-3-phosphate, which is then dephosphorylated by INM1 or INM2 to form inositol [17–19]. Inositol and cytidyldiphosphate-diacylglycerol (CDP-DAG) are precursors for the essential phospholipid phosphati-dylinositol (PI). ScOpi1p acts as the master regulator of ScINO1 and other target genes by inhibiting the transcriptional activators ScIno2p and ScIno4p. The mechanism by which it regulates these genes in response to extracellular inositol has been well described [15–17, 20–22].

The structural gene encoding ScINO1 is conserved between S. cerevisiae and C. albicans, and shares similar function [23]. C. albicans and S. cerevisiae INO1 homologs are similarly regulated in response to exogenously provided inositol [22]. The ScOPI1 ortholog in C. albicans (OPI1) can complement Scopi1A for INO1 regulation in S. cerevisiae [24]. We therefore hypothesized that OPI1 might function as an INO1 negative regulator in C. albicans as it does in Candida glabrata [25]. However, a report regarding the ScIno2p and ScIno4p homologs suggested that the regulation of INO1 expression in S. cerevisiae and C. albicans might not be conserved [26]. The C. albicans heterodimeric transcription factors INO2 and INO4 (related to ScINO2 and ScINO4 from S. cerevisiae) did not regulate INO1, but instead activated ribosomal protein genes such as RPL32. These results indicate that inositol regulation might be transcriptional rewired between these two related eukaryotic organisms. A previous report comparing S. cerevisiae and C. albicans Gal4p transcription factor homologs that control sugar metabolism suggests that these proteins have been rewired between these two organisms [27]. In C. albicans the Gal4p homolog activates the gluconeogenesis gene LAT1 instead of galactose metabolism genes such as GAL10, and surprisingly GAL10 was activated by another transcription factor,
Therefore, we wished to investigate if \textit{C. albicans} \textit{OPI1} has a similar role in inositol regulation to \textit{ScOPI1} and \textit{CgOPI1}, or if it has possibly been transcriptionally rewired.

In this communication, we report that \textit{C. albicans} \textit{OPI1} does not regulate the inositol biosynthetic gene \textit{INO1}, but affects the \textit{SAP2} expression and virulence of \textit{C. albicans} in a rat vaginitis model. In addition, \textit{OPI1} affects morphogenesis at 30°C. These results illustrate that the regulation of inositol biosynthesis in \textit{C. albicans} and \textit{S. cerevisiae} is different. From now on, in this paper, all genes from \textit{C. albicans} will be referred to by their simple names such as \textit{OPI1} or \textit{INO1}, whereas genes from other organisms such as \textit{S. cerevisiae} will be referred to as \textit{ScOPI1} or \textit{ScINO1}.

\section*{Materials and Methods}

\subsection*{Ethics Statement}

Mouse model of systemic infection studies were conducted in the animal facility at University of Tennessee (UT) in good practice as defined by the United States Animal Welfare Act and in full compliance with the guidelines of the UT Institutional Animal Care and Use Committee (IACUC). The mouse experiments were reviewed and approved by the UT IACUC under protocol number L016. Procedures involving rats and their care were conducted in conformity with national and international laws and policies. The study has been approved by the Committee on the Ethics of Animal Experiments of the Istituto Superiore di Sanita', Rome, Italy (Permit Number: DM 227/2009-B). All experimental procedures were carried out according to the ARRIVE (Animal Research: Reporting In Vivo Experiments; http://www.nc3rs.org.uk/page.asp?id=1357) and NIH (National Institutes of Health) guidelines for the ethical treatment of animals.

\subsection*{Strains and growth media}

\textit{C. albicans} strains used in this study are shown in Table 1. Media used in this study include YPD (yeast extract-peptone-dextrose: 1% yeast extract, 2% peptone, 2% glucose), defined medium 199 (M199, Invitrogen, pH 7.0 adjusted by 150mM HEPES), Spider (1% nutrient broth, 1% mannitol, 0.2% dipotassium phosphate, 1.35% agar), YPD containing 10% fetal bovine serum, YCB-BSA (1.17% yeast carbon base-Difco, 0.2% bovine serum albumin-Sigma), YCB-BSA-YE (1.17% yeast carbon base, 0.2% bovine serum albumin, 0.1% yeast extract, pH 5.0) [28, 29]. Unless otherwise stated, agar plates were solidified with 2% agar (granulated, Fisher).

\begin{table}
\centering
\caption{\textsl{Candida albicans} strains used in this study.} 
\begin{tabular}{|l|l|l|}
\hline
Strains& Genotype & Parent & Source \\
\hline
SC5314 (wild-type) & Prototrophic wild type & Clinical isolate & [47] \\
YLC58 & \textit{opi1}△::\textit{NAT1}-\textit{FLP}/\textit{OPI1} & SC5314 & This study \\
YLC85 & \textit{opi1}\textit{Δ}/\textit{OPI1} & YLC58 & This study \\
YLC86 & \textit{opi1}\textit{Δ}/\textit{opi1}△::\textit{NAT1}-\textit{FLP} & YLC85 & This study \\
YLC88 & \textit{opi1}\textit{Δ}/\textit{opi1}△ & YLC86 & This study \\
YLC117 & \textit{opi1}△/\textit{opi1}△::\textit{OPI1}-\textit{NAT1} & YLC88 & This study \\
SAP2MS4A & \textit{sap2}\textit{Δ}/\textit{sap2}\textit{Δ} & SC5314 & [37] \\
YLC223 & \textit{OPI1}/\textit{OPI1; URA3}-\textit{P}_{\text{ACT1}}\cdot\textit{SAP2} & SC5314 & This study \\
YLC226 & \textit{opi1}\textit{Δ}/\textit{opi1}△ \textit{URA3}-\textit{P}_{\text{ACT1}}\cdot\textit{SAP2} & YLC88 & This study \\
\hline
\end{tabular}
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\end{table}
Strain construction

The *C. albicans OPI1* gene (*OPI1*) was disrupted by using the *CaNAT1-FLP* cassette [30] (Table 2). For the *OPI1* disruption construct, the 379 base pair (bp) 5’ non-coding region (NCR) of *OPI1* was amplified with primers TRO522 and TRO526 (Table 3), and cloned as a Kpnl-Apal digested 228bp fragment into pJK863 5’ of the *CaNAT1-FLP* cassette (Fig. 1A). The 448 bp 3’ NCR of *OPI1* was amplified with primers TRO524 and TRO525 which introduced SacII and SacI sites, and was cloned into pJK863 3’ of the *CaNAT1-FLP* cassette (Fig. 1A). This created the *OPI1* knock out construct plasmid pYLC36 (Table 2, Fig. 1A), which was cut with Kpnl and SacI to release the disruption construct (5’ NCR of *OPI1*-CaNAT1-FLP-3’ NCR of *OPI1*) which was transformed into the wild type SC5314 strain by electroporation [31]. The disruption construct was used to sequentially disrupt both alleles of *OPI1*. The *OPI1* reconstitution construct was made by amplifying a 1.7 Kb fragment containing the *OPI1* ORF and 5’ NCR from SC5314 genomic DNA using primers (JCO12 and JCO14) that introduced Kpnl and SalI sites. This fragment was ligated into the pRS316 vector along with another 1.7 Kb fragment containing the NAT1-3’ NCR of *OPI1* amplified from plasmid pYLC36 using primers JCO50 and TRO42 which introduced SalI and SacI sites. This resulted in the *OPI1* reconstitution plasmid pYLC37 (Table 2, Fig. 1B). The 3.4 Kb Kpnl-Saci fragment from pYLC37 was transformed into the *opi1*Δ/Δ mutant (YLC88) in order to create the reconstituted *opi11*Δ/Δ::*OPI1* strain (YLC117). The SAP2 constitutive expression construct was made by cloning the dominant selectable marker NAT1 with primers JCO129 and JCO130 to NdeI-digested pAU34 [32] and resulted in pYLC219. The SAP2 ORF was then cloned to XmaI-digested pYLC219 with primers JCO131 and JCO132, and resulted in pYLC221, which can constitutively express SAP2 under the control of the *ACT1* promoter. To transform this constitutive construct into wild type and *opi11*Δ/Δ strains, a PpuMI-digested linear plasmid pYLC221 was integrated at the URA3 site of *Candida* genome, and resulted in *OPI1*/OPI1 URA3::P*ACT1*-SAP2 (YLC223) and *opi11*Δ URA3::P*ACT1*-SAP2 (YLC226).

Northern blot analysis

Northern blotting for SAP2 and INO1 expression was performed as described [33, 34] with the following exceptions. Strains grown in YCB-BSA or YCB-BSA-YE medium at 37°C for 12 hrs (for SAP2) and in liquid medium 199 (pH 7.0) at 37°C for 2 hrs (for INO1) were collected for total RNA extraction by the hot phenol method. The PCR product containing bps 17–571 of the SAP2 ORF (primers JCO35 and JCO36) and bps 76–581 of the INO1 ORF (primers TRO562 and TRO563) were used as probes. Expression was normalized against *C. albicans ACT1* gene expression probed on the same membrane. The *ACT1* probe was generated with the primers JCO48 and JCO49.

**Table 2. Plasmids used in this study.**

| Plasmids | Characteristics | Source |
|----------|----------------|--------|
| pJK863   | *CaNAT1-FLP* cassette carrying nourseothricin resistance gene | [30]   |
| pYLC36   | pJK863 flanked 5’ and 3’*OPI1* NCR for *OPI1* gene knock out | This study |
| pYLC37   | *OPI1* reconstitution construct | This study |
| pAU34    | Constitutively expression construct under the control of *ACT1* promoter | [32]   |
| pYLC219  | pAU34 contained NAT1 selectable marker | This study |
| pYLC221  | pYLC219 contained SAP2 ORF for constitutive expression | This study |

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RT real-time PCR

Strains were cultured overnight in YPD at 37°C, washed twice with dH2O, then diluted to 0.2 O.D600/ml and incubated in liquid YCB-BSA medium (1.17% yeast carbon base, 0.2% BSA) for 12 hrs at 37°C with shaking at 200 rpm. The 50 ml cultures were pelleted at 3000rpm at 4°C and immediately frozen with liquid nitrogen to stop cellular processes. Total RNAs were extracted with a RiboPure Yeast RNA Purification Kit (Ambion), treated with TURBO DNA-free Kit (Invitrogen), and 2 μg of DNA-free total RNAs was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR reactions of 20 μl included 6 ng cDNA (in 6 μl), 10 μl of 2x qPCR master mix (Fast SYBR Green Master Mix; Applied Biosystems), 2 μl of 2.5 μM forward primer (JC798 for SAP2), 2 μl of 2.5 μM reverse primer (JC799 for SAP2). Primer design for detecting SAPs expression was based on previous publication by Naglik et al [13]. Quantitative PCR conditions were shown below 95°C /10 min for denaturation; 95°C /3 sec, 60°C /30 sec (40 cycles); 95°C /15 sec, 60°C /60 sec, 95 °C /15 sec (melting curve). The StepOnePlus System and StepOne v2.2 (Applied Biosystems) were used to determine ΔΔCt. The bar graphs of ACT1 normalized relative quantity compared with wild-type (SC5314) were created with Prism 5.03.

Southern blot analysis

Hybridization conditions for the Southern blot analysis were similar to those for Northern blot analysis, except that the Tecne Hybrigene oven was set to 60°C for the incubation step, and 42°C and 60°C for washing steps. The cells were grown in liquid YPD at 30°C overnight. The
genomic DNA was extracted using the Winston-Hoffman method [35] and 20 μg of genomic DNA were subjected to Southern blotting. The genomic DNA of the wild type and *opi1Δ/Δ* mutants was cut by *Kpn*I and *Sph*I restriction enzymes. PCR products containing the ~500bp 3′ NCR of *OPI1* (primers TRO524 and TRO525) were used as probes for Southern blot confirmation (Fig. 1C).

**Mouse bloodstream infection studies**

Five- to six-week-old male CD1 mice (18 to 20 g) from Charles River Laboratories were used in this study. Mice were housed at five per cage. For infection, colonies from each *C. albicans* strain were inoculated into 20 ml of YPD. Cultures were grown overnight at 30°C with shaking.
in YPD, washed twice with 25 ml of sterile water, counted by hemocytometer, and resuspended at 10^7 cells per ml in sterile water. Mice were injected via the tail vein with 0.1 ml of the cell suspension (10^6 cells), and the course of infection was monitored for up to 14 days. The survival of mice was monitored twice daily, and moribund mice (body weight reduced by 30%, unable to eat/drink, or severely hunched) were euthanized with CO2. Cells were also plated on YPD to determine the viability. At least two independent infections were performed for each strain. The statistical analysis was done using Prism 5.03 software (GraphPad Software). For the mouse model of systemic infection, Kaplan-Meier survival curves were compared for significance using the Mantel-Haenszel log rank test. Statistical significance was set at \( P < 0.05 \).

Rat vaginitis studies

The protocol of estrogen-dependent rat vaginal infection model adapted from De Bernardis et al. [8] was used throughout this study. Briefly, oophorectomized female Wistar rats (80–100 g; Charles River, Calco, Italy) were injected subcutaneously with 0.5 mg of estradiol benzoate (Benzatrone; Samil, Rome). Six days after the first estradiol treatment, the animals were inoculated intravaginally with 10^7 yeast cells of each C. albicans strain in 0.1 mL. The inoculum was dispensed into the vaginal cavity through a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy). The yeast cells had been previously grown in YPD broth at 28°C on a gyratory shaker (200 rpm), harvested by centrifugation (1500 g), washed, counted in a hemocytometer, and suspended to the required number in saline solution. The results of two independent experiments are each represented separately. A third experiment involving all of the strains is not shown, but gave similar trends. In each experiment, each Candida strain was inoculated into 5 rats. Kinetics of C. albicans growth in, and clearance from, the vaginal cavity was measured by colony forming unit (CFU) enumeration after culturing 100 μl of vaginal samples, taken by washing the vaginal cavity by gentle aspiration of 100 μl of sterile saline solution, repeated four times, at 1:10 serial dilutions on Sabouraud agar containing chloramphenicol (50 μg/ml). CFUs were enumerated after incubation at 28°C for 48 h.

Results

C. albicans OPI1 does not regulate INO1 expression

When heterologously expressed in an S. cerevisiae Scopi1Δ mutant, the C. albicans OPI1 gene has been demonstrated to repress expression of a reporter gene that contains the inositol/choline responsive element (ICRE) found in ScINO1 and other ScOpi1p-ScIno2p-ScIno4p target genes [24]. This data suggested that C. albicans Opi1p may regulate the cognate C. albicans INO1 gene, as its homolog does in S. cerevisiae. In order to test this both copies of the C. albicans OPI1 gene were disrupted in C. albicans using the CaNAT1-FLP cassette [30] as described in Fig. 1.

The wild type and opi1Δ/Δ strains were then compared to see if the opi1Δ/Δ mutant would fail to repress INO1, as expected, if it acts like the homologous S. cerevisiae mutant, Scopi1Δ [22]. First, the strains were grown in Medium 199, pH 7.0, which contains low levels of inositol (\(~10 \mu M\)), which should result in high expression of INO1, and it was found that both upregulated INO1 to similar levels (Fig. 2). Then, they were grown in the same medium supplemented with 75 μM inositol, which should repress INO1 expression in wild-type, but not in the opi1Δ/Δ mutant, if it cannot repress the gene. However, in both strains, INO1 was similarly repressed, suggesting that inositol biosynthesis is regulated by different transcription factors in C. albicans.
The \( \Delta \) mutant exhibits hyperfilamentous growth in filament-inducing media at 30°C

It has been shown that \( ScOPI1 \) is necessary to activate invasive growth and \( ScFLO1 \) expression in \( S. cerevisiae \) [34]. It was therefore hypothesized that \( OPI1 \) would affect filamentous growth in \( C. albicans \). Three filament-inducing media were used to test this hypothesis. In contrast to the situation with the \( Scopi1 \Delta \) mutant in \( S. cerevisiae \), it was found that the \( opi1 \Delta \Delta \) mutant exhibited hyperfilamentous growth rather than hypofilamentous growth, but only at 30°C on solid filament-inducing agar plates (Fig. 3). This effect was not observed at 37°C on similar

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**Figure 2.** \( C. albicans \) \( OPI1 \) does not regulate \( INO1 \) expression. Strains were grown for 2 hrs in Medium 199, pH 7.0 (± 75 \( \mu \)M inositol) at 37°C, collected, and subjected to Northern blotting against \( INO1 \). \( ACT1 \) was reprobed on the same membrane as a loading control.

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**Figure 3.** The \( opi1 \Delta \Delta \) mutant exhibits hyperfilamentous growth in filament-inducing conditions on agar plates. \( C. albicans \) strains from overnight cultures were diluted in sterile water and plated on the indicated medium and grown at 30°C for the indicated amount of time.

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media. These phenotypes were also not seen in liquid forms of the same filament-inducing media at either 30°C or 37°C. In order to control for a possible effect from some other unlinked mutation, a copy of the *C. albicans* *OPI1* gene was reintegrated into the *opi1Δ/Δ* mutant (Fig. 1B), and it was found that the phenotype was restored when the wild-type copy of *OPI1* was present (Fig. 3), indicating that the hyperfilamentous growth at 30°C is linked to the loss of *OPI1* gene.

**OPI1** does not affect virulence in a mouse model of systemic infection

The *opi1Δ/Δ* mutant appears to affect the ability of the fungus to repress filamentation at lower temperatures. Some hyperfilamentous mutants such as *nrg1Δ/Δ* and *tup1Δ/Δ* have been found to be attenuated in virulence in mouse models of infection [36]. Therefore, a mouse model of systemic infection was used to test the role of *OPI1* in virulence. However, the *OPI1* gene does not contribute to the virulence in this model since the *opi1Δ/Δ* mutant exhibits a similar phenotype to wild-type on the survival curves of mice (Fig. 4).

**OPI1** is involved in establishing infection in the rat vaginitis model

In addition to infections of the bloodstream, *C. albicans* can also cause infections of mucosal surfaces including the vaginal tract [8]. A rat vaginitis model was used to determine if the *opi1Δ/Δ* mutation would play a role in the establishment of infection in this host niche. It was demonstrated that *OPI1* was involved in establishing rat vaginitis. In this model *C. albicans* cells are injected into the rat vaginal tract, and then over time the level of colonization is measured based on the recovery of colony counts. It was discovered that the *opi1Δ/Δ* mutant is quickly cleared by the host compared to the wild type (Fig. 5). The *opi1Δ/Δ::OPI1* reintegrant strain and *opi1Δ/ΔOPI1* heterozygous mutant had an intermediate phenotype between the *opi1Δ/Δ* mutant and wild type (Fig. 5).

It has been shown that deletion of the *C. albicans* *SAP2* protease gene [8] causes a similar clearance to the *opi1Δ/Δ* mutant, and a *sap2Δ/Δ* mutant (*SAP2MS4A*) [37] was included in this

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**Figure 4.** *OPI1* is not required for virulence of *C. albicans* in a mouse model of systemic candidiasis.

Each strain was used to infect mice by injecting 10⁶ *C. albicans* yeast-form cells into the tail-vein of each mouse. The mice were then assessed over the course of 14 days. The number of mice used for a specific strain is indicated in parentheses. The data obtained here are from a single experiment.

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experiment as a control. Our results confirmed that an independently constructed sap2Δ/Δ mutant (gift from Joachim Morschhäuser), behaved like a previously constructed sap2Δ/Δ mutant, and exhibits reduced colonization in the rat vaginal tract (Fig. 5), suggesting the importance of SAP2 in the rat vaginitis model. Our results also indicate that OPI1 plays a critical role in establishing infection in the rat vaginal tract (Fig. 5).

**OPI1 affects rat vaginal establishment through regulating SAP2**

The similarity of the phenotypes of the opi1Δ/Δ mutant with the sap2Δ/Δ mutant suggested that OPI1 might act through SAP2. In wild-type cells, SAP2 is upregulated in bovine serum albumin (BSA) media. We performed reverse transcriptase (RT) real-time PCR to detect if OPI1 controls SAP2 expression in YCB-BSA medium. The opi1Δ/Δ mutant showed 5.5 fold reduced SAP2 expression compared to wild type (Fig. 6), indicating that OPI1 controls SAP2 expression. The opi1Δ/Δ::OPI1 reintegrant strain can restore the SAP2 expression and actually shows ~3 fold higher expression of SAP2 than the wild type.

In order to test if OPI1 affects colonization of the rat vaginal tract through SAP2, an epistasis experiment was performed in which the SAP2 gene was overexpressed in the opi1Δ/Δ mutant via the ACT1 promoter (PACT1::SAP2). This overexpression was confirmed by Northern blotting (S1 Fig.). If opi1Δ/Δ blocked rat colonization by compromising SAP2 expression, then overexpression of SAP2 from an independent promoter should suppress the phenotype. In contrast to the opi1Δ/Δ mutant, the opi1Δ/Δ::URA3::PACT1::SAP2 mutant was suppressed for its defect in rat vaginal infection, and behaved similarly to the wild type (Fig. 7). This implicates the OPI1 gene as a regulator of SAP2 in the vaginal tract of the rat.

**Discussion**

Our results show that the OPI1 gene of *C. albicans*, unlike its homologs in *S. cerevisiae* and *C. glabrata* [22, 25], does not affect INO1 expression, but does repress filamentous growth at
low temperature (Fig. 3) and regulates virulence in the rat vaginitis model (Figs. 5 and 7). The latter phenotype appears to be mediated by changes in \( \text{SAP2} \) expression. The \( \text{opi1}^{\Delta/\Delta} \) mutant exhibits reduced \( \text{SAP2} \) expression compared with the wild type in liquid YCB-BSA medium (Fig. 6), and \textit{in vivo} SAP2 overexpression can restore the \( \text{opi1}^{\Delta/\Delta} \) mutant’s vaginal colonization defect, when under the control of the constitutive \( \text{ACT1} \) promoter. Epistasis experiments are inherently challenging to interpret, as overexpression of a target gene such as \( \text{SAP2} \) could lead to enhanced colonization by a mechanism that bypasses the actual defect caused by the \( \text{opi1}^{\Delta/\Delta} \) mutation. Based on our data, this possibility cannot be completely ruled out. It is also possible that \( \text{OPI1} \) controls colonization in the rat vaginal tract by regulating one of the other SAPs (e.g. \( \text{SAP1, SAP3--10} \)). However, as the differential expression of nine other SAPs in the \( \text{opi1}^{\Delta/\Delta} \) mutant compared to the wild type using the same condition (i.e. YCB-BSA liquid medium and \textit{RT} real time PCR) was not detected, we do not know if these others are affected \textit{in vivo}, and this remains to be examined (unpublished data). Meanwhile, further studies will be needed to test if \( \text{SAP4, SAP5 and SAP6} \) genes are regulated by \( \text{OPI1} \) under hypha-inducing conditions since \( \text{SAP4--6} \) are hypha-specific genes [38, 39].

In \textit{S. cerevisiae}, \textit{ScOpi1p} is the master regulator of \textit{ScINO1} and other phospholipid genes [15–17, 20]. \textit{ScOpi1p} controls expression in response to cellular inositol levels by binding to

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**Figure 6. SAP2 expression is regulated by OPI1 in \textit{C. albicans}.** RT real-time PCR was used to assess the SAP2 expression levels in the wild-type (SC5314), \( \text{opi1}^{\Delta/\Delta} \), \( \text{opi1}^{\Delta/\Delta}::\text{OPI1} \) and \( \text{sap2}^{\Delta/\Delta} \) mutants. Strains were cultured overnight in YPD at 37°C, washed twice with dH2O. Then strains were diluted to 0.2 O.D_{600}/ml and incubated in liquid YCB-BSA medium (1.17% yeast carbon base, 0.2% BSA) for 12 hrs at 37°C. The error bars represented the standard errors of the mean. The data obtained here are from a representative single experiment with technical triplicates. \( P \) value was determined by t tests and \(< 0.05\) was considered statistically significant.

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Ino2p in the Ino2p-Ino4p heterodimer and repressing its activation of ScINO1, among other targets. When inositol is plentiful, PI is efficiently synthesized from CDP-DAG and inositol by the ScPis1p enzyme [40, 41]. In this circumstance, the endoplasmic reticulum (ER) localized pool of phosphatidic acid (PA), which is the precursor for CDP-DAG, is consumed, and ScOpi1p is translocated to the nucleus. There, it binds ScIono2p and represses ScINO1 with help from the global repressor Sin3p via a direct interaction involving the N-terminal Sin3p binding domain of ScOpi1p [16, 24]. When inositol is not plentiful in the environment, cellular stores drop and PI synthesis slows causing a build-up of precursors including PA. ScOpi1p binds to PA in the ER via its basic domain, and the ER membrane protein Scs2p via its FFAT domain [20, 42]. This sequesters ScOpi1p to the ER, and then Ino2p-Ino4p activate transcription of ScINO1 so inositol can be synthesized for PI production.

A previous report demonstrated that OPI1 from C. albicans could complement an Scopi1Δ mutant in S. cerevisiae, and it could repress the ICRE promoter element found on ScINO1 and other phospholipid biosynthetic genes when expressed heterologously in S. cerevisiae [24]. However, we found that in C. albicans, OPI1 does not regulate INO1 expression. This overlap in function of CaOPI1 when expressed heterologously in S. cerevisiae, but not endogenously in C. albicans, may be due to the conservation of some key domains required for ScOpi1p function, but not the conservation of other domains (S2 Fig.). In particular, the C. albicans Opi1p has very little conservation with ScOpi1p in the large N-terminal ScSin3p binding domain [16]. However, CaOpi1p does have some conserved sequences with the C-terminal ScIono2p interaction domain of ScOpi1p, including two out of three residues (ScOpi1p aas 358–360) that were shown to be crucial for ScIono2p-ScOpi1p interactions in S. cerevisiae [16]. In contrast, CaOpi1p shares very few residues in common with ScOpi1p in the PA-binding basic domain, and no residues of the FFAT domain that binds to Scs2p [20, 42]. It does, however, carry a leucine zipper motif with some isoleucine substitutions that has been shown to be crucial for ScIono2p-ScOpi1p interactions [15]. Thus, this conservation of some domains, but not others may help explain why CaOpi1p can complement a Scopi1Δ mutant for ScINO1 repression [24].

Figure 7. OPI1 affects rat vaginal establishment through regulating SAP2. For each strain 5 rats were inoculated on day 0 with 10^7 blastospores, and vaginal CFUs were counted by plating at the indicated time points. The error bars represented the standard errors of the mean in each group. The number of rats used for a specific strain is indicated in parentheses and the data obtained here are from a single experiment.

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but not act the same within *C. albicans* itself. Further support for our findings comes from the observation that *CaINO2* and *CaINO4* do not appear to regulate *CaINO1* either, but may actually regulate ribosomal genes [26]. This is in marked contrast to *CgOPI1* from *C. glabrata*, which does regulate *CgINO1* with help from *CgINO2* and *CgINO4* [25]. Consistently, *CgOpi1p* has close conservation of all of the important regulatory domains of *ScOpi1p* (S2 Fig.). Interestingly, one other *ScOpi1p* homolog has been characterized, and this is *Yas3p* from *Yarrowia lipolytica*. *Yas3p* also does not have a number of domains conserved with *ScOpi1p*, and like *CaOpi1p* does not regulate *YlINO1*, but does, along with *Ino2p* and *Ino4p* homologs *Yas1p* and *Yas2p*, respectively, regulate hexane metabolism genes [43]. The *C. albicans* regulators of *CaINO1* are currently unknown, and this will be interesting to elucidate, as expression of *CaINO1* is regulated by extracellular inositol levels, but not by *CaOpi1p* and apparently not by *Calno2p* or *Calno4p* either.

Finally, the role *Opi1p* in repressing filamentation at 30°C on solid media remains elusive (Fig. 3). The *opi1Δ/Δ* mutant exhibits hyperfilamentous growth in filament-inducing agar plates including medium 199, spider, and 10% serum at 30°C, but not 37°C. These results indicate that *OPI1* might be a low temperature repressor of filamentous growth. It has been demonstrated that *C. albicans* *CPP1*, a tyrosine phosphatase, is required for repression of the hyphal transition at 23°C in contact with solid surfaces [44, 45]. The *cpp1Δ/Δ* mutant exhibited hyperfilamentous growth on spider and a wide variety of rich and defined solid media including Lee’s medium, YPD, YPM, and 10% serum at 23°C, but not at 37°C. The germ tube formation defect of the *cpp1Δ/Δ* mutant was not observed at liquid culture at 37°C, an effect similar to *opi1Δ/Δ* mutant. In contrast to *opi1Δ/Δ*, the *cpp1Δ/Δ* mutant exhibited reduced virulence in mouse systemic infection and mouse mastitis models [44–46]. The relationship between *Opi1p* and *Cpp1p* is unknown and needs further studies in *C. albicans*. Taken together, our data suggest that, when compared to its homolog in *S. cerevisiae*, *C. albicans* has a transcriptionally re-wired regulator, *OPI1*, which does not regulate *INO1* expression but affects morphogenesis, *SAP2* expression and virulence in a rat vaginitis model. It also makes it clear that identification of *ScOpi1p* homologs in other fungi does not clearly implicate them for roles in regulating inositol biosynthesis in these microbes. Rather, the *Opi1p* family members, which are conserved in a wide variety of fungi appear to have a diversity of functions.

**Supporting Information**

**S1 Fig.** *SAP2* is overexpressed in the *PACT1-SAP2* construct. Expression was tested by Northern blotting in YPD media, and it was confirmed that the *PACT1-SAP2* construct overexpressed *SAP2*.

(TIF)

**S2 Fig.** Multiple sequence alignment comparing *Opi1p* homologues of *S. cerevisiae* (S.C.), *C. glabrata* (C.G.), *Y. lipolytica* (Y.L.), and *C. albicans* (C.A.). This alignment was performed using Clustal W. 2.0.1.0. Asterisk represents conservation among all four species. Various domains represented by different colors, and boxes highlight particularly conserved regions between species. Blue: *Opi1-Sin3* interaction domain. Gold: phosphatidic acid (PA)-binding domain. Red: Leucine zipper. Green: FFAT (2 phenylalanines and an acid tract). Purple: Polyglutamine tract. Orange with black boxes: *Ino2p* activator interaction domain.

(TIF)
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Author Contributions

Conceived and designed the experiments: YLC TBR. Performed the experiments: YLC FB SJY SS SK RT EB. Analyzed the data: YLC FB SJY. Wrote the paper: YLC FB SJY TBR.

References

1. Heitman J, Filler SG, Edwards JE, Mitchell AP (2006) Molecular principles of fungal pathogenesis. Washington DC: ASM Press.
2. Calderone R, Clancy C (2012) Candida and Candidiasis. Washington DC: ASM Press.
3. Naglik JR, Challacombe SJ, Hube B (2003) Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67: 400–428, table of contents. doi: 10.1128/MMBR.67.3.400-428.2003 PMID: 12966142
4. De Bernardis F, Sullivan PA, Cassone A (2001) Aspartyl proteinases of Candida albicans and their role in pathogenicity. Med Mycol 39: 303–313. doi: 10.1080/mmy.39.4.303.313 PMID: 11556759
5. Albrecht A, Felk A, Pichova I, Naglik JR, Schaller M, et al. (2006) Glycosylphosphatidylinositol-anchored proteases of Candida albicans target proteins necessary for both cellular processes and host-pathogen interactions. J Biol Chem 281: 688–694. doi: 10.1074/jbc.M509297200 PMID: 16269404
6. Dabas N, Morschhauser J (2008) A transcription factor regulatory cascade controls secreted aspartic protease expression in Candida albicans. Mol Microbiol 69: 586–602. doi: 10.1111/j.1365-2958.2008.06297.x PMID: 18547391
7. Martinez P, Ljungdahl PO (2005) Divergence of Stp1 and Stp2 transcription factors in Candida albicans places virulence factors required for proper nutrient acquisition under amino acid control. Mol Cell Biol 25: 9435–9446. doi: 10.1128/MCB.25.21.9435-9446.2005 PMID: 16227594
8. De Bernardis F, Arancia S, Morelli L, Hube B, Sanglard D, et al. (1999) Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for Candida vaginitis. J Infect Dis 179: 201–208. doi: 10.1086/314546 PMID: 9841840
9. De Bernardis F, Cassone A, Sturtevant J, Calderone R (1995) Expression of Candida albicans SAP1 and SAP2 in experimental vaginitis. Infect Immun 63: 1887–1892. PMID: 7729898
10. Schaller M, Bein M, Korting HC, Baur S, Hamm G, et al. (2003) The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium. Infect Immun 71: 3227–3234. doi: 10.1128/IAI.71.6.3227-3234.2003 PMID: 12761103
11. Hube B, Sanglard D, Odds FC, Hess D, Monod M, et al. (1997) Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of Candida albicans attenuates virulence. Infect Immun 65: 3529–3538. PMID: 9284116
12. Lermann U, Morschhauser J (2008) Secreted aspartic proteases are not required for invasion of recombinant human epithelia by Candida albicans. Microbiology 154: 3281–3295. doi: 10.1099/mic.0.2008/022525-0 PMID: 18957582
13. Naglik JR, Moyes D, Makwana J, Kanzaria P, Tsichlaki E, et al. (2008) Quantitative expression of the Candida albicans secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. Microbiology 154: 3266–3280. doi: 10.1099/mic.0.2008/022293-0 PMID: 18957581
14. White MJ, Hirsch JP, Henry SA (1991) The OPI1 gene of Saccharomyces cerevisiae, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper. J Biol Chem 266: 863–872. PMID: 1985968
15. Wagner C, Blank M, Strohmann B, Schuller HJ (1999) Overproduction of the Opi1 repressor inhibits transcriptional activation of structural genes required for phospholipid biosynthesis in the yeast Saccharomyces cerevisiae. Yeast 15: 843–854. doi: 10.1002/(SICI)1097-0061(199907)15:10<843::AID-YEAC24%3E3.0.CO;2-M PMID: 10407264
16. Wagner C, Dietz M, Wittmann J, Albrecht A, Schuller HJ (2001) The negative regulator Opi1 of phospholipid biosynthesis in yeast contacts the pleiotropic repressor Sin3 and the transcriptional activator Ino2. Mol Microbiol 41: 155–166. doi: 10.1046/j.1365-2958.2001.02495.x PMID: 11454208
17. Henry SA, Kohlwein SD, Carman GM (2012) Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190: 317–349. doi: 10.1534/genetics.111.130286 PMID: 22345606

18. Donahue TF, Henry SA (1981) myo-Inositol-1-phosphate synthase. Characteristics of the enzyme and identification of its structural gene in yeast. J Biol Chem 256: 7077–7085. PMID: 7016881

19. Murray M, Greenberg ML (2000) Expression of yeast *INM1* encoding inositol monophosphatase is regulated by inositol, carbon source and growth stage and is decreased by lithium and valproate. Mol Microbiol 36: 651–661. doi: 10.1046/j.1365-2958.2000.01886.x PMID: 10844654

20. Loewen CJ, Gaspar ML, Jesch SA, Delon C, Klistakis NT, et al. (2004) Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. Science 304: 1644–1647. doi: 10.1126/science.1096083 PMID: 15192221

21. Klig LS, Hoshizaki DK, Henry SA (1988) Isolation of the yeast *INO1* gene, a positive regulator of phospholipid biosynthesis. Curr Genet 13: 7–14. doi: 10.1007/BF00365749 PMID: 2834106

22. Klig LS, Homann MJ, Carman GM, Henry SA (1985) Coordinate regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*: pleiotropically constitutive *opi1* mutant. J Bacteriol 162: 1135–1141. PMID: 3888957

23. Chen YL, Kauffman S, Reynolds TB (2008) *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. Infect Immun 76: 2793–2801. doi: 10.1128/IAI.01514-07 PMID: 18268031

24. Heyken WT, Wagner C, Wittmann J, Albrecht A, Schuller HJ (2003) Negative regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae* by a *Candida albicans* orthologue of *OPI1*. Yeast 20: 1177–1188. doi: 10.1002/yea.1031 PMID: 14587102

25. Bethea EK, Carver BJ, Montedonico AE, Reynolds TB (2010) The inositol regulon controls viability in *Candida glabrata*. Microbiology 156: 452–462. doi: 10.1099/mic.0.030072-0 PMID: 19875437

26. Hoppen J, Dietz M, Warsow G, Rohde R, Schuller HJ (2007) Ribosomal protein genes in the yeast *Candida albicans* may be activated by a heterodimeric transcription factor related to Ino2 and Ino4 from *S. cerevisiae*. Mol Genet Genomics 278: 317–330. doi: 10.1007/s00438-007-0253-x PMID: 17588177

27. Martchenko M, Levitin A, Whiteway M (2007) Transcriptional activation domains of the *Candida albicans* *Gcn4p* and *Gal4p* homologs. Eukaryot Cell 6: 291–301. doi: 10.1128/EC.00183-06 PMID: 17158732

28. Chen YL, Montedonico AE, Kauffman S, Dunlap JR, Menn FM, et al. (2010) Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. Mol Microbiol 75: 1112–1132. doi: 10.1111/j.1365-2958.2010.07018.x PMID: 20132453

29. Styles C (2002) How to set up a yeast laboratory. Methods Enzymol 350: 42–71. doi: 10.1016/S0076-6879(02)50955-1 PMID: 12073328

30. Shen J, Guo W, Kohler JR (2005) CaNAT1, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida* species. Infect Immun 73: 1239–1242. doi: 10.1128/IAI.73.2.1239-1242.2005 PMID: 15664973

31. De Backer MD, Maes D, Vandoninck S, Loghme M, Contreras R, et al. (1999) Transformation of *Candida albicans* by electroporation. Yeast 15: 267–272. doi: 10.1002/(SICI)1097-0061(199911)15:15%3C267::AID-YEA485%3E3.0.CO;2-P PMID: 10572258

32. Uhlen MA, Johnson AD (2001) Development of *Streptococcus thermophilus* lacZ as a reporter gene for *Candida albicans*. Microbiology 147: 1189–1195. PMID: 11320212

33. Kohler K, Domdey H (1991) Preparation of high molecular weight RNA. Methods Enzymol 194: 398–405. doi: 10.1016/0076-6879(91)90420-0 PMID: 2171180

34. Reynolds TB (2006) The *Opi1p* transcription factor affects expression of *FLO11*, mat formation, and invasive growth in *Saccharomyces cerevisiae*. Eukaryot Cell 5: 1266–1275. doi: 10.1128/EC.00022-06 PMID: 16896211

35. Hoffman CS, Winston F (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57: 267–272. doi: 10.1016/0378-1119(87)90131-4 PMID: 3319781

36. Murad AM, Leng P, Straffon M, Wishart J, Macaskill S, et al. (2001) *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. EMBO J 20: 4742–4752. doi: 10.1093/emboj/20.17.4742 PMID: 11532938

37. Staub P, Lemmann U, Blass-Warmuth J, Degel B, Wurzner R, et al. (2008) Tetracycline-inducible expression of individual secreted aspartic proteases in *Candida albicans* allows isoenzyme-specific inhibitor screening. Antimicrob Agents Chemother 52: 146–156. doi: 10.1128/AAC.01072-07 PMID: 17954688
38. Chen YC, Wu CC, Chung WL, Lee FJ (2002) Differential secretion of Sap4–6 proteins in Candida albicans during hyphae formation. Microbiology 148: 3743–3754. PMID: 12427964

39. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, et al. (2002) Candida albicans hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. Infect Immun 70: 3689–3700. doi:10.1128/IAI.70.7.3689-3700.2002 PMID: 12065511

40. Chen M, Hancock LC, Lopes JM (2007) Transcriptional regulation of yeast phospholipid biosynthetic genes. Biochim Biophys Acta 1771: 310–321. doi: 10.1016/j.bbalip.2006.05.017 PMID: 16854618

41. Fischl AS, Carman GM (1983) Phosphatidylinositol biosynthesis in Saccharomyces cerevisiae: purification and properties of microsome-associated phosphatidylinositol synthase. J Bacteriol 154: 304–311. PMID: 6300035

42. Loewen CJ, Roy A, Levine TP (2003) A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. EMBO J 22: 2025–2035. doi:10.1093/emboj/cdg201 PMID: 12727870

43. Hirakawa K, Kobayashi S, Inoue T, Endoh-Yamagami S, Fukuda R, et al. (2009) Yas3p, an Opi1 family transcription factor, regulates cytochrome P450 expression in response to n-alkanes in Yarrowia lipolytica. J Biol Chem 284: 7126–7137. doi: 10.1074/jbc.M806864200 PMID: 19131334

44. Schroppel K, Sproesser K, Whiteway M, Thomas DY, Rollinghoff M, et al. (2000) Repression of hyphal proteinase expression by the mitogen-activated protein (MAP) kinase phosphatase Cpp1p of Candida albicans is independent of the MAP kinase Cek1p. Infect Immun 68: 7159–7161. doi:10.1128/IAI.68.12.7159-7161.2000 PMID: 11083847

45. Csank C, Makris C, Meloche S, Schroppel K, Rollinghoff M, et al. (1997) Derepressed hyphal growth and reduced virulence in a VH1 family-related protein phosphatase mutant of the human pathogen Candida albicans. Mol Biol Cell 8: 2539–2551. doi: 10.1091/mbc.8.12.2539 PMID: 9398674

46. Guhad FA, Csank C, Jensen HE, Thomas DY, Whiteway M, et al. (1998) Reduced pathogenicity of a Candida albicans MAP kinase phosphatase (CPP1) mutant in the murine mastitis model. APMIS 106: 1049–1055. doi: 10.1111/j.1699-0463.1998.tb00257.x PMID: 9890266

47. Shepherd MG (1985) Pathogenicity of morphological and auxotrophic mutants of Candida albicans in experimental infections. Infect Immun 50: 541–544. PMID: 3902649