To the Graduate Council:

I am submitting herewith a thesis written by Emily K. Bethea entitled, "The Role of the Phospholipid Regulator, *CgOPI1,* in Controlling Viability in the Pathogenic Yeast, *Candida glabrata*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in microbiology.

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The Role of the Phospholipid Regulator, *CgOPI1*, in Controlling Viability in the Pathogenic Yeast, *Candida glabrata*

A Thesis
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Abstract

The pathogenic yeast *Candida glabrata* is the second-most common cause of candidiasis in humans after *Candida albicans*. Interestingly, *C. glabrata* is phylogenetically closer to *Saccharomyces cerevisiae* than *C. albicans*. One important virulence factor in *C. glabrata* is its inherent resistance to the azole class of antifungals, necessitating the continued discovery of novel antifungal agents. Many antifungals target ergosterol or ergosterol biosynthesis. In an attempt to look for new potential drug targets in *C. glabrata*, homologues of the genes in *S. cerevisiae* that regulate the transcription of phospholipid biosythesis (the inositol regulon) were examined. The *S. cerevisiae* inositol regulon consists of a heterodimeric transcriptional activator encoded by the genes *INO2* and *INO4* and a repressor encoded by *OPI1*, none of which are essential. The most well studied target of these genes is *INO1*, whose protein product converts glucose-6-phosphate to inositol-1-phosphate for the synthesis of phosphatidylinositol in the absence of inositol. Disruption of *INO2* or *INO4* blocks transcription of several phospholipid biosynthetic genes including *INO1*, resulting in inositol auxotrophy. Disruption of *OPI1* causes overproduction of *INO1* and other genes. Surprisingly, it was found that *CgOPI1* is essential for viability in *C. glabrata*. This was found to be true for strains in both the BG2 and ATCC2001 backgrounds indicating that this is not just a strain-specific effect. This is very different from *S. cerevisiae*, where the *Scopi1*Δ mutant grows robustly. These results led to the hypothesis that the *CgOPI1* gene is necessary for viability because it causes overexpression of a target of the inositol regulon transcriptional activator CgIno2p-CgIno4p. Experimental evidence suggests that this hypothesis is true.

Disruption of *CgINO2* or *CgINO4* leads inositol auxotrophy due to the inability to transcribe regulon targets such as *CgINO1*. The *Cgopi1*Δ mutant's viability defect can be rescued by disruption of the *Cgino2*Δ gene. The *Cgopi1*Δ *Cgino2*Δ double mutant is viable in the absence of *CgOPI1* on a plasmid. These results indicate that blocking the expression of a gene that is activated by the inositol regulon can rescue the *Cgopi1*Δ mutant's viability defect. Identification of this target will help elucidate the phenotype.

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Chapter I

Background and Introduction

Background of *Candida glabrata* and *Candida* spp.

The *Candida* genus consists of a heterogeneous group of about 200 yeast species. Some *Candida* species are the causative agents of candidiasis, a disease with a wide clinical spectrum that includes superficial infections of the oral and vaginal cavities as well as systemic infections. Candidiasis in the form of oral thrush was first documented as far back as the fourth century B.C. by Hippocrates and again in 1665 by the London journalist, Samuel Pepys. The initial discovery of the thrush organism is credited to Langenbeck in 1839 who mistook it for the causative agent of typhoid fever. This organism eventually was recognized as a fungus and came to be known as *Monilia albicans* before receiving its current connotation of *Candida albicans* [1-3].

Despite the size of the *Candida* genus, relatively few of its known species are of any medical significance, and in the early twentieth century, only *Candida albicans* was thought to be of clinical importance. It was not until the latter part of the twentieth century that there was an increase in the isolation of previously nonpathogenic species from sites of infection [2]. This accompanied a general rise in nosocomial candidiasis, which was due to an increasing population of immunocompromised patients. The contributing factors to this include growing numbers of patients hospitalized for acquired immunodeficiency syndrome (AIDS) as well as medical advances such as intravenous catheters and chemotherapeutic treatments, which allow longer survival for people with otherwise fatal conditions. These patients are more susceptible to fungal infections [2, 4].

Also, the incidence of oral colonization well as the proportion of blood stream infections caused by some non-*albicans* species such as *C. glabrata* increases with age [5, 6].

Candida species have become the most important cause of opportunistic mycoses worldwide and currently rank fourth among causes of nosocomial bloodstream infections [5]. Although historically considered nonpathogenic, *Candida glabrata* is increasingly isolated in both bloodstream and mucosal infections. It is now commonly cited as second only to *C. albicans* in causing both oral and invasive candidiasis and has been associated with high mortality despite indications of low virulence [5-7]. Although *C. albicans* currently is not being challenged for its position as the primary cause of invasive candidiasis, the percentage of *C. albicans* isolates appears to be decreasing and is accompanied by a rise in non-*albicans* species [5].

Candida glabrata was originally classified in the genus *Torulopsis*. The distinguishing factor between the two genera was the ability of *Candida* to display hyphal or pseudohyphal growth and the inability of *Torulopsis* to do so. It was proposed that the two genera be merged in 1978, and this remained a controversy for several years before it was finally shown in 1997 that the basis of this separation was inadequate [8]. Although most species in the genus *Candida* do exist in both yeast and hyphal forms, *C. glabrata* has not been demonstrated to form hyphae. It was eventually shown that *C. glabrata* could undergo a morphological change by exhibiting pseudohyphal growth during nitrogen starvation [9].

The genus *Candida* belongs to the Ascomycetes class of fungi. Several species have been described to have teleomorphic forms; however, historically, species within

the genus were considered to be asexual, including the pathogenic yeast, *Candida albicans* [10]. In 1999 it was discovered that *C. albicans* contained a mating type-like (*MTL*) locus, which is similar to the *MAT* locus of *Saccharomyces cerevisiae* [11]. Further study revealed that several genes involved in mating in *S. cerevisiae* have orthologues in *C. albicans* [12]. Mating was eventually described for *C. albicans* between strains of opposite mating types that had undergone a phenotypic switch from white to opaque [13]. A sexual cycle has never been described in *C. glabrata,* but it does have conserved sexual genes, including at least 31 homologues of genes that function in meiosis in *S. cerevisiae*. Among these genes are those for α-factor as well as a putative MTL locus; however, it lacks some genes that are necessary for mating including *STE2* and *STE3,* to which α-factor and a-factor bind, and *MFA1*, which is need to make a-factor. *C. glabrata* has two silent mating loci, *MTL1* and *MTL2,* similar to *HMR* and *HML* in *S. cerevisiae,* but similar loci appear to be absent from *C. albicans* [14]. *C. glabrata* also undergoes phenotypic switching, and since this has been shown to be necessary for *C. albicans* mating, there could potentially be a cryptic sexual cycle yet to be elucidated in *C. glabrata* [13]. Interestingly, in yeasts that do have teleomorphic forms, the asexual form is the one that is isolated from sites of infection, suggesting that mating may be disadvantageous in pathogenicity [14].

Despite the clinical similarities between *C. albicans* and *C. glabrata,* the two organisms are quite different phylogenetically, with haploid *C. glabrata* actually being more closely related to *Saccharomyces cerevisiae* (Figure A.1). Orthologous proteins in *C. glabrata* and *S. cerevisiae* have an average of 65% amino acid identity, and the 38.8%

G+C content of *C. glabrata* is similar to the 38.3% of *S. cerevisiae* [15]. This same conclusion was drawn based on the homology of the 18S rRNA sequence [16]. *C. albicans* along with various *Candida* species also have a genetic code alteration in which they translate CTG codons as serine rather than leucine, but *C. glabrata* lacks this reassignment of codon usage [17]. Another difference between the two species is the inability of *C. glabrata* to assimilate any sugars other than glucose and trehalose, which accompanies its high numbers of gene loss including those genes involved in the metabolism of galactose, phosphate, nitrogen, and sulfur [10, 18].

Virulence Factors

C. albicans and *C. glabrata* differ in their known virulence factors, and *C. glabrata* lacks several of the virulence factors that had been identified in *C. albicans* such as the production of secreted proteases. Another virulence factor that *C. albicans* possesses and *C. glabrata* lacks is its ability to undergo filamentation. These morphological changes increase adherence and tissue invasion, and *C. albicans* can form both pseudohyphae and hyphae, whereas there is no clinical evidence that *C. glabrata* exhibits a change in morphology between the commensal and pathogenic forms, although it does form pseudohyphae *in vitro* [7, 15].

The virulence factors *C. glabrata* does have include cell-associated proteases and adhesins. The *C. glabrata* family of adhesions is discussed first. A mutant screen revealed that deletion of the gene *EPA1*, or epithelial adhesion 1, significantly reduced the adhesion of *C. glabrata* to human epithelial cells [19]. It has since been shown that there is actually a large family of *EPA* genes (similar to the *ALS* family in *C. albicans*),

and at least two others of these, *EPA6* and *EPA7*, are able to mediate adherence; however, they are normally kept silent via subtelomeric silencing mediated by the *RIF1* and *SIR3* genes [20]. The silencing of the *SIR* genes and subsequent induction of *EPA6* and *EPA7* occurs as a result of the limitation of nicotinic acid (NA), for which *C. glabrata* is an auxotroph. Because urine is NA-limiting, the *EPA6* and *EPA7* genes are transcribed during urinary tract infections, and their expression in this environment may also contribute to the colonization of catheters by *C. glabrata* [21].

The other potential virulence factor for *C. glabrata* is a family of GPI-linked aspartyl proteases. These genes were called *YPS* because of their homology to a group of genes by the same name in *S. cerevisiae*. In *S. cerevisiae* the *YPS* genes are involved in cell wall remodeling, and a similar function is suspected in *C. glabrata*. They are required for *in vitro* survival of *C. glabrata* during stationary phase or conditions of cell wall stress. In addition, the *C. glabrata YPS* genes were shown to be necessary for survival within macrophages, and this family of genes was identified because of its upregulation upon internalization of *C. glabrata* by macrophages [22]. *C. glabrata* proteases differ from those of other pathogenic *Candida* species in that they are not secreted extracellularly. *C. albicans* has a family of secreted aspartyl proteases (SAP) which are encoded by the genes *SAP1-10*. Several studies have correlated production of these proteases with virulence in *C. albicans*. Although different from the *YPS* proteases in *C. glabrata*, the *SAP* family does contain two genes, *SAP 9* and *SAP10* that encode sequences for GPI proteins [23].

Phospholipases could play a role in *C. glabrata* infections as well because of their potential to compromise host cell membranes. Evidence for this mechanism of action has been given in both fungal and non-fungal pathogens, including *C. albicans.* Phospholipase activity was detected in several other *Candida* species; however, there was significantly less production in non-*albicans* species compared to *C. albicans*. Nevertheless, phospholipase B was secreted by *C. glabrata* [24].

Also important in the pathogenesis of *C. glabrata* is its resistance to several antifungal drugs, particularly the azole antifungals. Azoles exist in two classes, imidazoles and triazoles, and are the most frequently used antifungal agents due to advantages such as selectivity and low toxicity. Their mode of action is to block the biosynthetic pathway of ergosterol, the principle sterol in fungal membranes. This is accomplished by inhibiting the essential enzyme product of the *ERG11* gene, lanosterol demethylase, the lack of which causes methylated sterols to replace ergosterol in the plasma membrane [25]. *C. glabrata* circumvents this by several mechanisms, but among the most important is the ability to transport drugs back out of the cell. In addition, *C. glabrata* can take up ergosterol precursors such as cholesterol from the host [10].

Drug resistance is found in several *Candida* species, but *C. glabrata* is inherently less susceptible to fluconazole than is *C. albicans*, having a minimal inhibitory concentration inhibiting 50% of the yeast population investigated (MIC_{50}) of 16µg/ml, compared to 0.25µg/ml in *C. albicans*. An increase in the frequency of *C. glabrata* isolates from patients with candidiasis has been associated with fluconazole prophylaxis. Genes encoding multidrug transporters of the ABC transporter family were identified in

C. glabrata. Transcription of the gene, *CgCDR1,* named after a similar gene in *C. albicans,* was increased in azole-resistant isolates of *C. glabrata* [26]. CgPdr1p is a transcription factor that regulates expression of the transporter genes *CgCDR1* and *CgCDR2.* A *CgPDR1* deletion will cause an increase in azole susceptibility, but gain of function mutations in this gene actually have been shown to increase azole resistance as well as the fitness of the organism, increasing its virulence [27].

Transcriptional Rewiring Among Yeasts

It is well accepted that *C. glabrata* and *S. cerevisiae* are very closely related phylogenetically, and comparative study of these two species could provide insight into why one is increasingly successful in causing infection and the other is nonpathogenic. It is widely believed that a whole genome duplication occurred in the two species' shared lineage. *C. glabrata* is thought to have lost the duplicated genes at a faster rate than *S. cerevisiae* [17, 18]. Interestingly, a number of transcription factors were retained in duplicate in *C. glabrata* while only being single-copy in *S. cerevisiae,* and this has been interpreted to suggest that *S. cerevisiae* may have a simpler transcriptional regulatory network than other yeasts [28].

Transcriptional rewiring among ascomycetous yeasts is a topic that has garnered recent attention after the publication of the genome sequences of many of these yeasts. Transcription factors can acquire and lose binding sites relatively quickly, and this flexibility could lead to the emergence of new phenotypes. One such study focused on the transcriptional regulator, Mcm1, which has a wide range of functions. While binding about 4% of the genes in *S. cerevisiae,* it has different interactions in *Kluyveromyces*

lactis (a yeast commonly used in dairy production) and *C. albicans*, where it binds about 12% of the genes in both organisms. When comparing target genes for Mcm1 homologues in all three species, only about 13%-18% of these genes are shared in common among them, so this regulator controls many different genes among the three yeasts. However, the Mcm1p cofactor interactions appear to be better conserved, and these authors suggest that while protein sequences remain fairly constant, their regulatory network has diverged significantly [29].

Rewiring of the transcriptional network has also been linked to the differing oxygen requirements between *S. cerevisiae* and *C. albicans*, with the former preferring fermentation even in the presence of oxygen, and the latter being primarily aerobic. An upstream regulatory sequence associated with genes required for rapid growth is conserved in both species but is overrepresented in *C. albicans* in the genes encoding mitochondrial ribosomal proteins. In *S. cerevisiae*, these genes are induced only during environmental stress, suggesting that a change in gene expression could cause this phenotypic difference [30].

A comparative analysis of whole-genome transcription profiles from *S. cerevisiae* and *C. albicans* yielded different amounts of conservation among groups of genes arranged into Gene Ontology (GO term) categories. Interestingly, one GO term that was not conserved was "regulation of transcription [25]." Together, these studies imply that alterations in gene transcription and in the regulators of gene transcription could drive phenotypic divergence among species. There have previously been no published data

regarding transcriptional rewiring between *S. cerevisiae* and *C. glabrata*; however, given their close relationship, such a study could prove beneficial.

Phospholipid Biosynthesis in Saccharomyces cerevisiae

Genetic Control of Phospholipid Biosynthesis

I have found that a *C. glabrata* homologue of an *S. cerevisiae* transcriptional regulator involved in this process is essential in *C. glabrata*. This led to further study of phospholipid regulation in *C. glabrata* and specifically, the *de novo* pathway of phosphatidylinositol biosynthesis. The *S. cerevisiae* phospholipid biosynthetic pathway and transcriptional regulon have served as a guide in this work, so *S. cerevisiae* phospholipid biosynthesis and regulation are described below.

Phospholipids are a major component of cellular membranes, but also play a number of other roles in yeast cells, including signal transduction and cell surface recognition. The major phospholipids found in *S. cerevisiae* are phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The pathways controlling their biosynthesis are both tightly regulated and coordinated, which is evidenced in part by all of these phospholipids being either direct or indirect products of the precursor phosphatidic acid (PA), which is metabolized to form the phospholipid precursors, CDP-diacylglycerol (CDP-DAG) or diacylglycerol (DAG) [31] (Figure A.2).

PE and PC can be synthesized from either of two pathways: the *de novo* pathway or the salvage pathway, which is also referred to as the Kennedy pathway. The first step

in the *de novo* pathway is the synthesis of PS from CDP-DAG and free serine, which is catalyzed by PS synthase, the product of the *CHO1* gene. PS is then decarboxylated to form PE by PS decarboxylase, which is encoded by the genes *PSD1* and *PSD2*. PE undergoes three methylation reactions to form phosphatidyl-monomethyl ethanolamine, phosphatidyl-dimethyl ethanolamine, and finally PC. PE methyltransferase from the *CHO2 (PEM1)* gene is involved in the first reaction, and phospholipid methyltransferase from the *OPI3 (PEM2)* gene catalyzes the final two [32].

PE and PC can also be synthesized from exogenous ethanolamine and choline via the Kennedy pathway when the enzymes from the *de novo* pathway are repressed. The enzymes ethanolamine kinase and choline kinase encoded by the *EKI1* and *CKI1* genes, respectively, are responsible for the phosphorylation of ethanolamine and choline. Phosphoethanolamine and phosphocholine are then converted to CDP-ethanolamine and CDP-choline, respectively, by cytidylyltransferases encoded by the *ECT1* and *PCT1* genes. The final step of this salvage pathway involves the formation of PE and PC through a reaction of the products from the second step with DAG [33].

PI is synthesized by the enzyme PI-synthase encoded by the *PIS1* gene, which converts CDP-DAG and inositol to PI. This is a necessary reaction, and without it, the cell cannot live, implying that PI is an essential component of the yeast cell. PI-synthase appears to have constitutive activity independent of the availability of PI precursors and this activity also does not change when cells enter stationary phase, in contrast to the activities of PS-synthase and CDP-DAG synthase [32].

Inositol for PI synthesis is derived from exogenous sources or is synthesized *de novo* from glucose-6-phosphate. The first step of the *de novo* pathway of PI biosynthesis is the conversion of glucose-6-phosphate to inositol-1-phosphate (I1P) by inositol-1-phosphate synthase. This enzyme is the product of the *INO1* gene. I1P is subsequently dephosphorylated to inositol by inositol monophosphatase, encoded by the *INM1* or *INM2* genes [34].

INO1 is a highly regulated gene that is repressed in the presence of inositol. Its transcription is activated by a heterodimeric transcriptional activator consisting of Ino2p and Ino4p [35]. In the presence of inositol, the transcriptional repressor Opi1p binds to Ino2p and prevents transcription of *INO1* [36]. *OPI1* stands for over-producer of inositol, as it was first identified among mutants that secreted inositol into the surrounding media [37]. In an *opi1Δ* mutant, transcription of *INO1* is constitutively activated, causing inositol to be produced and excreted [38]. Cells having an *ino1Δ*, *ino2Δ*, or *ino4Δ* mutation are inositol auxotrophs [39] (Figure A.3)

In the absence of inositol, Opi1p is inactivated by binding to Scs2p, an integral membrane protein, and PA, both of which are in the ER. Inositol is the rate limiting component in PI biosynthesis. The presence of inositol induces the consumption of the phospholipid precursor, PA, resulting in the release of Opi1p from the ER and its translocation to the nucleus, where it binds Ino2p [40]. Ino2p and Ino4p bind constitutively to the promoter of *INO1*, and are localized to the nucleoplasm under these repressing conditions. Dissociation of Opi1p from this complex under derepressing

conditions results in the recruitment of *INO1* to the nuclear membrane, where transcription is activated [41].

The inositol regulon controls the expression of many of the enzymes involved in phospholipid biosynthesis in response to extracellular inositol and choline levels. Genes that respond to inositol and choline contain an upstream activating sequence (UAS_{INO}) in their promoters. This sequence, CATGTGAAAT, has a basic helix loop helix (bHLH) binding site, which is essential for it to function as a UAS element and is required for the Ino2p of the Ino2p-Ino4p heterodimer to bind to *INO1*. This UAS_{INO} is also involved in the regulatory response to Opi1p. A systematic substitution of each base pair in the UAS_{INO} element revealed that this sequence is necessary for optimal function [42]. UAS_{INO} is also found in other genes that are involved in phospholipid biosynthesis and inositol-choline response including *CHO1*, *CHO2*, *CPT1*, *EPT1*, *OPI3*, *PIS1*, *PSD1*, *INO1*, as well as *INO2* and *INO4* [43].

Coordinate regulation of Phospholipid Biosynthesis

Evidence exists for the coordinate regulation of phospholipids. CDP-DAG is partitioned within the different phospholipid biosynthetic pathways depending on the available precursors. The presence of exogenous inositol shifts the utilization of CDP-DAG, causing it to be converted to PI at the expense of PS, PE, and PC via the *de novo* pathway (Figure A.2). Moreover, inositol serves as an inhibitor of PS synthase, the product of the *CHO1* gene, also driving metabolism in favor of PI [44]. Conversely, a higher concentration of PS synthase can cause CDP-DAG to be consumed, thus altering the rate of PI synthesis [32].

The *INO1* gene is the best studied and most highly expressed gene that is regulated by the interaction of these pathways. The level of *INO1* mRNA is repressed when cells are grown in inositol-containing media. *INO1* is further repressed by the addition of choline to cells already growing in inositol; however the addition of choline alone caused only a slight increase in the abundance of *INO1* RNA [38]. Repression by inositol is not limited to the products of *INO1* and *CHO1*, and the activity of CDP-DAG synthase, PS decarboxylase and the two N-methyltransferases have all been shown to be reduced in the presence of inositol [45].

An *opi1Δ* mutant is known to synthesize *INO1* constitutively and independently of inositol and choline; however, this mutant also has a high level of *CHO1*, indicating that *OPI1* also affects the *de novo* aminophospholipid pathway. The phospholipid composition of the *opi1* mutant remains stable regardless of inositol and choline levels compared to that of a wild type cell, which changes depending on the availability of inositol and choline. In addition, the activity of the methyltransferases, which are involved in the conversion of PE to PC, do not fluctuate with varying levels of inositol and choline in an *opi1* mutant, in contrast to the wild type, further indicating that *OPI1* has a pleiotropic effect on phospholipid regulation [38, 46].

INO2 and *INO4* are also considered to be pleiotropic in that the phospholipid compositions of the *ino2Δ* and *ino4Δ* mutants have a lower proportion of PC with an accumulation of the methylated intermediates involved in the synthesis of PC from PE. This suggests that Ino2p and Ino4p are required for maximum expression of the methyltransferases as they are required for derepression of Ino1p [47]. This inhibition of

the pathway that synthesizes PC in *ino2Δ* and *ino4Δ* mutants is coupled to an increase in the PI composition of the cell. Similarly, *cho1Δ* mutants that cannot synthesize PS have higher levels of PI, and *ino1Δ* mutants lacking exogenous inositol have increased PS. While PI is able to substitute for PS in *cho1Δ* mutants, the *ino1Δ* mutants under conditions of inositol starvation eventually die [32].

Phospholipid regulation has been shown to affect other cellular functions. A recent study screened mutants having the Opi- phenotype, secreting inositol into the growth medium in the absence of inositol and choline. Among the Opi- mutants were genes involved in various processes other than phospholipid biosynthesis such as protein processing and trafficking and the unfolded protein response (UPR) [48]. Inositol regulation had previously been implicated in the UPR when the transcription factor Hac1p, which promotes transcriptional activation of unfolded proteins, was demonstrated to be involved with the dissociation of Opi1p from the Ino2p-Ino4p complex [41].

Another role for the *OPI1* gene was recently determined that showed it to be involved in the activation of *FLO11*, which encodes an adhesion protein. *Opi1*- mutant *S. cerevisiae* strains were deficient for invasive growth and mat formation; however, the mechanism of this regulation remains to be elucidated [49]. The various contributions of *OPI1* to the cell suggest that it is an important regulator. The fact that *OPI1* has homologues in other species, including the close-relative pathogen, *C. glabrata*, makes it a good candidate for further study in this species, especially since *OPI1* was shown to affect an adhesin, and one of the virulence factors of pathogenic yeast species is their ability to adhere to host tissues.

On a wider scale, the *OPI1* family of genes, which has homologues across many fungal species, is gaining attention and becoming better studied in some species and is being associated with different functions. Most recently, the *OPI1* homologue in *Yarrowia lipolytica, YAS3*, was determined to play a regulatory role in the transcription of *ALK1*, a gene necessary for the oxidation of *n*-alkanes. Yas3p does not regulate the *Y. lipolytica INO1* homologue, but it does regulate *ALK1* in a manner that reflects the relationship of Opi1p, Ino2p, and Ino4p in *S. cerevisiae*. In particular, Yas3p is a repressor of *ALK1* that represses transcription by binding to the Yas2p component of the Yas1p-Yas2p heterodimeric transcriptional activator. Yas1p and Yas2p are homologs of Ino4p and Ino2p, respectively.

The sequence of Yas3p is diverged from that of Opi1p (Figure A.4A). In particular, Yas3p lacks some domains that are important for Opi1p including the polyglutamine tracts, which are often found in regulatory genes, and the two phenylalanines in an acidic tract (FFAT), which allow Scs2p to bind. Yas3p also lacks the Opi1-Sin3 interaction domain by which Opi1p contacts the pleiotropic repressor, Sin3p. [50] (Figures A.4A and A.4B). Interestingly, the Opi1p homologue in *Candida albicans* also lacks similar domains, and like Yas3p, it does not regulate *CaINO1* in *C. albicans* (Chen and Reynolds, unpublished data) (Fig A.3A).

Interestingly, the Opi1p homologue from the much more closely related *C. glabrata* contains all of the important domains that are present in *S. cerevisiae* Opi1p (Figures A.3A and A.3B). What is more, we show in this thesis that CgOpi1p also regulates *CgINO1*, *CgCHO1*, *CgCHO2*, and inositol biosynthesis in a manner very

similar to that found in *S. cerevisiae*. The surprising contrast is that despite this similarity, CgOpi1p is essential in *C. glabrata,* whereas it is not required for viability in *S. cerevisiae*. This finding is novel among the Opi1p homologuess, and CgOpi1p represents the first member of the Opi1p family to control viability. CgOpi1p's control of viability may indicate transcriptional or even metabolic rewiring in *C. glabrata* compared to *S. cerevisiae*. The work in this thesis project addresses the mechanism by which CgOpi1p controls viability in this fungus.

Chapter II

The inositol regulon controls viability in Candida glabrata

This chapter is a paper by the same name submitted for publication in the journal Molecular Microbiology in 2009 by Emily K. Bethea, Todd B. Reynolds and Billy Carver.
My contributions to this paper include (1) design of most of the yeast strains, plasmids, and primers used in the study, (2) some experimental design, (3) performance of most of the experiments, and (4) generation of most of the figures.

Abstract

Inositol is essential in eukaryotes, and must be imported or synthesized. Inositol biosynthesis in *Saccharomyces cerevisiae* is controlled by three nonessential genes that make up the inositol regulon: *ScINO2* and *ScINO4*, which together encode a heterodimeric transcriptional activator, and *ScOPI1*, which encodes a transcriptional repressor. ScOpi1p inhibits the ScIno2-ScIno4p activator in response to extracellular inositol levels. An important gene controlled by the inositol regulon is *ScINO1*, which encodes inositol-3-phosphate synthase, a key enzyme in inositol biosynthesis. In the pathogenic yeast *Candida albicans*, homologues of the *S. cerevisiae* inositol regulon genes are "transcriptionally rewired". Instead of regulating the *CaINO1* gene, *CaINO2* and *CaINO4* regulate ribosomal genes. Another *Candida* species that is a prevalent cause of infections is *Candida glabrata*; however, C. *glabrata* is phylogenetically more closely related to *S. cerevisiae* than *C. albicans*. Experiments were designed to determine if *C. glabrata* homologuess of the inositol regulon genes functioned similarly to *S. cerevisiae* or are transcriptionally rewired. *CgINO2, CgINO4,* and *CgOPI1* regulate *CgINO1* in a manner similar to that observed in *S. cerevisiae*. However, unlike in *S. cerevisiae, CgOPI1* is essential. Genetic data indicate that *CgOPI1* is a repressor that affects viability by regulating activation of a target of the inositol regulon.

Introduction

Fungi of the genus *Candida* are the most common cause of human fungal infections and can lead to both mucosal and systemic infections [51]. *Candida albicans* is the most common cause of these infections, but non-*albicans Candida* species are increasingly associated with disease [52]. One of these non-*albicans* species, *Candida glabrata* is now the second most common cause of both mucosal and systemic *Candida* infections [15].

Phylogenetically, *Candida glabrata* is more closely related to the non-pathogenic yeast *Saccharomyces cerevisiae* than most of the other common species of *Candida* associated with human disease [15]. *C. glabrata* lacks a number of the virulence factors associated with *Candida* pathogens such as secreted hydrolases and hyphal growth [15]. Despite this, *C. glabrata* is a growing challenge in clinical settings where it causes mucosal infections and is associated with approximately 15% of all *Candida*-related systemic bloodstream infections [53]. These observations are interesting in light of the fact that *C. glabrata* is more closely related to *S. cerevisiae,* but it is still a pathogen whereas *S. cerevisiae* is only very, very rarely associated with infection [54]. A clearer understanding of how *S. cerevisiae* and *C. glabrata* differ from one another may help shed light on why one is a significant human pathogen and the other is not.

A number of recent studies have shown that *Candida albicans* is transcriptionally rewired compared to *S. cerevisiae*. For example, the genes encoding enzymes of the Leloir pathway for galactose catabolism in *S. cerevisiae* are regulated by the ScGal4p transcription factor. However, in *C. albicans* these genes are regulated by the

transcription factor Cph1p, while the *C. albicans* CaGal4p homologue regulates TCA cycle genes such as *CaLAT1* [55]. Other examples of transcriptional rewiring between *C. albicans* and *S. cerevisiae* include regulatory systems controlling mating type [56], mitochondrial ribosomal genes [30], and *de novo* myo-inositol biosynthesis genes [57], (Chen and Reynolds, unpublished data). Myo-inositol will be referred to as inositol throughout the rest of this article.

Since there are several examples of transcriptional rewiring between these two more distantly related yeasts (*S. cerevisiae* and *C. albicans*), it was of interest to determine if similar rewiring is present between the more closely related yeasts *C. glabrata* and *S. cerevisiae*. The Leloir enzymes for galactose metabolism are not present in *C. glabrata*, and this yeast is unable to utilize galactose [58], so this pathway is unavailable for comparison. Mating has never been described for *C. glabrata*, thus this pathway is not useful for study either. In contrast to these pathways, the inositol regulon appears to be an excellent pathway to compare between these two yeasts. The inositol regulon is a very well studied transcriptional regulon in *S. cerevisiae* (reviewed in [43, 59]), and there are *C. glabrata* orthologs for both the transcription factors and targets of the *S. cerevisiae* inositol regulon (see results and discussion).

The inositol regulon in *S. cerevisiae* has been well-described [43, 59], and consists of three main transcription factors that regulate target gene expression in response to extracellular inositol levels (Fig 1). The roles of each of these transcription factors in controlling this regulon are described in more detail below. Transcriptional targets of the inositol regulon include a number of phospholipid biosynthetic genes, but

the most highly expressed and well-characterized of these targets is the *Saccharomyces cerevisiae INO1* (*ScINO1*) gene. *ScINO1* encodes an enzyme that occupies the rate-limiting step in *de novo* inositol biosynthesis. Inositol is essential and is required for the synthesis of phosphatidylinositol (PI) which is a precursor for several essential lipids including inositol-phosphate signaling lipids, glycosylphosphatidylinositol (GPI) anchors, and sphingolipids [60] [61].

ScIno1p is an inositol-3-phosphate synthase which converts glucose-6-phosphate to inositol-3-phosphate [62]. Inositol-3-phosphate is dephosphorylated by the inositol monophosphatases ScInm1p or ScInm2p to create inositol [34]. An *Scino1*Δ mutant cannot make inositol *de novo* and is an inositol auxotroph. In the absence of extracellular inositol (or at low concentrations like 10μM) *ScINO1* is expressed, and in the presence of higher concentrations of extracellular inositol *ScINO1* is repressed [63] (Fig 1).

The inositol regulon transcription factors ScIno2p and ScIno4p form a heterodimeric transcriptional activator that binds to the upstream activator sequence (UAS_{INO}) in the promoters of target genes such as *ScINO1* [35, 42, 64-66]. Both ScIno2p and ScIno4p are absolutely required for transcription of *ScINO1*, so *Scino2*Δ and *Scino4*Δ mutants are inositol auxotrophs.

The regulation of *ScINO1* in response to extracellular inositol is dependent on the repressor protein ScOpi1p [40, 67-70]. ScOpi1p senses the level of extracellular inositol indirectly based on the level of the PI precursor lipid phosphatidic acid (PA). Inositol is the rate-limiting metabolite in PI synthesis, and when there is abundant extracellular inositol, PI synthesis is maximal, and PA levels are lower because PA is consumed

during synthesis of PI. In these conditions ScOpi1p binds to ScIno2p and represses transcription of *ScINO1*. When extracellular inositol is not available or is greatly decreased, PI synthesis slows, and PA levels increase in the endoplasmic reticulum (ER). ScOpi1p, which binds to PA, is recruited to ER where it binds PA and the ER protein Scs2p. Thus, the ScIno2p-ScIno4p heterodimer is free to transcribe *ScINO1*. A mutation that deletes *ScOPI1* results in constitutive overexpression of *ScINO1* and of other genes carrying the UAS_{INO} sequence in their promoters.

The purpose of this study was to determine if *C. glabrata* carried an inositol regulon that is similar to that in *S. cerevisiae*, or if these yeasts are transcriptionally rewired for inositol regulation. In order to do this, *C. glabrata* homologues of the *S. cerevisiae* inositol regulon proteins were identified and disrupted. Analysis of these mutants revealed the surprising finding that *CgOPI1* is essential for growth under the experimental conditions used in this study. CgIno2p and CgIno4p (which are not essential) are activators of *CgINO1*, and CgOpi1p is a transcriptional repressor of *CgINO1*, like the situation in *S. cerevisiae*. However, unlike in *S. cerevisiae*, *CgOPI1* is required for viability. This difference from *S. cerevisiae* may indicate that the inositol regulon in *C. glabrata* has some additional or different targets than bakers' yeast, or *C. glabrata* may be metabolically rewired compared to *S. cerevisiae*.

Results

CgINO2 and CgINO4 encode transcriptional activators of the CgINO1 gene and control de novo inositol biosynthesis

A homologue of *ScINO1* was identified in *C. glabrata* by BLASTing the *ScINO1* translated protein sequence against the *Candida glabrata* genome at the Genolevures website (http://cbi.labri.fr/Genolevures/elt/CAGL). The BLAST search revealed that the gene *CAGL0I06050g* encoded a protein that was 73.9% identical to ScIno1p. We refer to *CAGL0I06050g* as *CgINO1* based on the experiments described below.

CgINO1 expression was examined in media containing or lacking inositol by Northern blotting which revealed that *CgINO1* was highly expressed in medium lacking inositol, but it was not expressed in medium containing 75μM inositol (Fig 3). This is similar to what has been observed for *ScINO1* in *S. cerevisiae* [19, 63, 71]. In order to determine if *CgINO1* was required for *de novo* inositol biosynthesis, the *CgINO1* open reading frame (ORF) was disrupted by homologous recombination using a two-step gene deletion strategy (Supplemental figure 1 and methods and materials) [19, 71]. The resulting *Cgino1*Δ mutant was unable to grow on inositol-free medium (Fig 2). However, when the *CgINO1* gene was reintegrated into the genome at the *CgINO1* locus, the reconstituted strain (*Cgino1*Δ*::CgINO1*) could grow in medium lacking inositol (Fig 2).

The *CgINO1* gene is regulated in a similar manner as the *ScINO1* gene in synthetic medium containing or lacking inositol which suggested that the inositol regulon that controls *ScINO1* in *S. cerevisiae* might be conserved in *C. glabrata*. In order to test this, homologues of the *ScINO2* and *ScINO4* transcriptional activator genes were identified by BLAST searches querying the protein sequences of ScIno2p and ScIno4p against the *C. glabrata* genome at the Genolevures website

(http://cbi.labri.fr/Genolevures/elt/CAGL). The BLAST searches revealed only one strong homologue for each protein, and these were encoded by the genes *CAGL0B01947g* for ScIno2p (35.6% identity) and *CAGL0I07359g* for ScIno4p (44.5% identity). These genes, *CAGL0B01947g* and *CAGL0I07359g*, were named *CgINO2* and *CgINO4*, respectively. *CgINO2* and *CgINO4* were disrupted using the two-step gene disruption method which completely removed the ORF of each gene. The resulting *Cgino2*Δ and *Cgino4*Δ mutants were tested to determine if they could grow in the absence of inositol in the medium. As seen for orthologous *Saccharomyces* mutants, the *Cgino2*Δ and *Cgino4*Δ mutants were unable to grow on inositol-free medium (Fig 2). These data suggested that *CgINO2* and *CgINO4* controlled the expression of the *CgINO1* gene. Northern blotting revealed that *Cgino2*Δ and *Cgino4*Δ mutants showed a complete lack of *CgINO1* expression even in inositol-free medium (Figure 3). Reconstituted *Cgino2*Δ*::CgINO2* and *Cgino4*Δ*::CgINO4* strains, conversely, grew well on medium lacking inositol (Fig 2) and regulated *CgINO1* much like the wild-type strain (Fig 3).

CgOPI1 encodes an essential gene in Candida glabrata

In *S. cerevisiae* the *ScOPI1* gene encodes the main regulator of *de novo* inositol biosynthesis in *Saccharomyces*, and its homologue was identified in a BLAST search against the *C. glabrata* genome at Genolevures as described above. The protein encoded by *CAGL0K03267g* was found to be 52.3% identical to ScOpi1p. An attempt was made to disrupt the *CgOPI1* gene by the two-step gene disruption strategy; however, this method continuously yielded strains carrying a wild-type ORF of *CgOPI1*.

The above results suggested that *CgOPI1* might be essential. This was tested by the strategy described in figure 4. The *CgOPI1* gene along with non-coding DNA flanking both 5' and 3' of the ORF (including the transcriptional promoter and terminator, respectively) were cloned into the single-copy *CEN/ARS* vector pGRB2.1 [72], which is marked with the *S. cerevisiae URA3* gene, to create the plasmid pCgOPI1. The pCgOPI1 plasmid was transformed into the wild-type strain, and the chromosomal *CgOPI1* ORF was disrupted by homologous recombination using the construct described in figure 4 that contains the nourseothricin resistance marker *NAT1* [73]. The resulting pCgOPI1 *Cgopi1*Δ strain was then streaked onto medium containing 5-fluoroorotic acid (5-FOA). Processing of 5-FOA by the *URA3* gene product from *S. cerevisiae* or *C. glabrata* leads to production of 5-fluorodeoxyuridine which is toxic to *C.glabrata* [71, 74].

If the *Cgopi1*Δ mutation is lethal, then no *Cgopi1*Δ pCgOPI1 colonies should grow on 5-FOA medium because the 5-FOA would select against the cells carrying pCgOPI1. In figure 5 it is clear that the *Cgopi1*Δ pCgOPI1 strain cannot grow on 5-FOA. In contrast, the parental strain carrying either pCgOPI1 or the empty vector grew well on this medium.

These experiments were performed in the strain background BG2 [71], and there was concern that this phenotype was strain-specific. In order to rule this out, the above strategy was used to test the essentiality of *CgOPI1* in the strain background ATCC 2001 (a gift from Karl Kuchler). It was found that *CgOPI1* was essential in the ATCC 2001

strain as well, which suggested that the essentiality of *CgOPI1* is not just a BG2 strain-specific phenomenon (data not shown).

The regulation of viability by CgOPI1 is dependent on the CgINO2 transcription factor

Based on the model from *S. cerevisiae* (Fig 1), it was hypothesized that disruption of *CgOPI1* causes overexpression of a downstream target of the inositol regulon that then results in a loss of viability. In *S. cerevisiae*, overexpression of *ScINO1* in the *Scopi1*Δ mutant is due to unrepressed transcriptional activation by ScIno2p (Fig 1). The *Scopi1*Δ mutant's *ScINO1* overexpression phenotype can be blocked by a *Scino2*Δ mutation. The *Scopi1*Δ *Scino2*Δ double mutant acts like the *Scino2*Δ single mutant and fails to express *ScINO1* because the *Scino2*Δ mutation is epistatic to the *Scopi1*Δ mutation [63].

If the *Cgopi1*Δ mutant compromises viability due to overexpression of a downstream target of CgIno2p, then a *Cgino2*Δ mutation should restore the viability of a *Cgopi1*Δ mutant by blocking expression of this putative target. In order to test this hypothesis, the *Cgino2*Δ mutant was transformed with the pCgOPI1 plasmid, and the chromosomal ORF of *CgOPI1* was disrupted as described (Figure 4). The resulting *Cgopi1*Δ *Cgino2*Δ pCgOPI1 strain was streaked on 5-FOA medium, and it was found to grow like the wild-type strain carrying pCgOPI1 or empty vector (Fig 6). The *Cgino2*Δ strain also grew like wild-type (Fig 6).

The CgOPI1 gene product represses expression of CgINO1

The results above suggest that *CgOPI1* can act as a repressor of *CgINO2* targets such as *CgINO1*. In order to determine if *CgOPI1* could repress *CgINO1*, *CgOPI1* was place under the control of a doxycycline repressible promoter [75]. The *C. glabrata* BG14 strain (*Cgura3*Δ) was modified by disrupting both the *CgHIS3* gene (making it a histidine auxotroph) and the *CgTRP1* gene (making it a tryptophan auxotroph). Using the resulting triple auxotroph (*Cgura3*Δ *Cghis3*Δ *Cgtrp1*Δ), the promoter of the *CgOPI1* gene was replaced on the chromosome by homologous recombination with the *tetO-HOP1* promoter construct derived from plasmid p97CGH (*CgHIS3*) [75]. This strain was further modified by integration of the pINTG4 plasmid (*CgTRP1*) carrying the *tetR::GAL4AD* repressor-activator that activates the *tetO-HOP1* chimeric promoter in the absence of doxycycline, but represses it in the presence of doxycycline.

The resulting strain was then tested for growth in the presence and absence of doxycycline, and it was found that this strain showed very poor growth in 10µg/ml doxycycline, but grew quite well in the absence of doxycycline (Fig 7B). These experiments were performed on synthetic minimal medium, but similar results were seen in YPD (rich) medium (E. Bethea and T. B. Reynolds, data not shown). When *CgOPI1* levels were assessed in this strain by Northern blot, it was found that *CgOPI1* was expressed in the absence of doxycycline, but was not expressed in the presence of drug (Fig 7A).

CgOpi1p acts as a repressor of *CgINO1* expression as measured by Northern blotting. It was found that when *CgOPI1* expression is repressed by doxycycline,

CgINO1 expression increases substantially, but when *CgOPI1* is expressed in the absence of doxycycline, then *CgINO1* expression is very low or undetectable (Fig 7A). This indicates that CgOpi1p acts as a repressor of *CgINO1*. We were also able to demonstrate the regulation of *CgINO1* expression by CgOpi1p using the *C. glabrata* copper-inducible *MTII* promoter [76] as well (E. Bethea and T. B. Reynolds, data not shown).

Overexpression of CgINO1 is not responsible for the loss of viability in the Cgopi1Δ mutant

Since *CgINO1* is overexpressed in the absence of *CgOPI1*, it seemed possible that *CgINO1* overexpression is responsible for the loss of viability. This was tested by creating a *Cgopi1*Δ *Cgino1*Δ pCgOPI1 double mutant. However, when this double mutant is streaked onto 5-FOA it fails to grow (data not shown) indicating that the disruption of *CgINO1* is not sufficient to restore viability as seen with *CgINO2* (Fig 6).

CgCHO1 and CgOPI3 show altered gene expression in the CgOPI1-tetO strain

Genes that respond to inositol and choline in *S. cerevisiae* have an upstream activating sequence in their promoters called the UAS_{INO}. This sequence is found in *OPI1* as well as *INO2, INO4,* and *INO1*. It is also found in five genes in *C. glabrata*, including *CgCHO1, CgOPI3, CgCHO2, CgINO1,* and *CgITR2*. A northern blot analysis was performed using the strain containing the *CgOPI1-tetO* fusion in order to see if these two genes might be influenced by *CgOPI1*. It was found that under conditions of *CgOPI1* repression, both *CgCHO1* and *CgOPI3* showed increased expression (Fig 9).

Discussion

Transcriptional rewiring has been suggested between the inositol regulons of *S. cerevisiae* and *C. albicans*, based on the observations that *CaINO2* and *CaINO4* genes cannot regulate the *CaINO1* gene in a heterologous expression system in *S. cerevisiae* [57]. In addition, the CaIno2p and CaIno4p proteins do not appear to bind to the *CaINO1* promoter, which contrasts with ScIno2p and ScIno4p that bind the *ScINO1* promoter. Interestingly, the CaIno2p-CaIno4p complex appears to bind to a sequence in the promoters of several rRNA genes in *C. albicans.* It has even been suggested that *CaINO2* and *CaINO4* are essential in *C. albicans* based on the fact that only heterozygous mutants could be made. However, this result is not definitive as a conditional promoter was not used to confirm these assertions. Additional support for the hypothesis that the inositol regulon in *C. albicans* is rewired compared to *S. cerevisiae* is provided by the fact that the *CaOPI1* gene does not affect the expression of *CaINO1* (Chen and Reynolds, manuscript in preparation).

Candida glabrata is much more closely related to *S. cerevisiae* than *C. albicans* based on phylogenetic trees comparing 18S ribosomal sequences [15]. Our analysis suggests that the *C. glabrata* inositol regulon is not transcriptionally rewired compared to *S. cerevisiae*, at least for *CgINO1* regulation. However, a major difference between the two species is that the *OPI1* homologue in *C. glabrata* appears to be essential, whereas it is not for *S. cerevisiae.* This result suggests that there are important differences between *S. cerevisiae* either in downstream targets of the Opi1p homologues or in the lipid metabolism of these fungi.

BLAST searches against the *C. glabrata* genome revealed that it contained homologues of the *S. cerevisiae* inositol regulon components and the *ScINO1* gene. Gene disruption studies revealed that the *CgINO1* gene is required for *de novo* inositol biosynthesis indicating that it is the *C. glabrata* inositol-3-phosphate synthase gene (Fig 2). *CgINO2* and *CgINO4* encode orthologs of the *S. cerevisiae* inositol regulon components as they are required for *CgINO1* transcription and *de novo* inositol biosynthesis (Figs 2 and 3). When the expression of the *CgOPI1* gene was repressed using the doxycycline repressible promoter system, the *CgINO1* gene was overexpressed compared to the wild-type strain, indicating that CgOpi1p is a transcriptional repressor of *CgINO1* (Fig 7A).

The most interesting observation, however, was that *CgOPI1* appears to be essential for viability. Reduction of expression of the *CgOPI1* gene using the doxycycline repressible promoter prevented growth on solid agar medium (Fig 7) and decreased growth in liquid medium (data not shown).

It is not clear how CgOpi1p controls viability, but our data suggest that it causes a loss of viability by overexpressing a downstream target of CgIno2p-CgIno4p. We found that a mutation of *CgINO2* can rescue the *Cgopi1*Δ mutant's viability defect (Fig 6).

A model to explain this rescue of viability by the *Cgino2*Δ mutation is derived from the inositol regulon in *S. cerevisae*. The *ScINO2* gene has been shown to be epistatic to *ScOPI1* for the regulation of *ScINO1* and inositol biosynthesis [63]. The *Scopi1*Δ mutant overexpresses *ScINO1* and the *Scino2*Δ mutant fails to express *ScINO1*. The *Scopi1*Δ *Scino2*Δ double mutant behaves like the *Scino2*Δ single mutant and fails to

express *ScINO1*. ScOpi1p is the primary regulator of this regulon, and in a *Scopi1*Δ mutant the ScIno2p-ScIno4p complex constitutively activates target genes resulting in overexpression of *ScINO1*. ScIno2p is absolutely required for target gene expression, thus a *Scino2*Δ disruption blocks *ScINO1* expression even if *ScOPI1* is disrupted.

Using this model as a guide, it would appear that the *Cgopi1*Δ mutation causes overexpression of CgIno2p target genes and one of these targets causes a loss in viability when overexpressed. Disruption of *CgINO2* in the *Cgopi1*Δ strain rescues growth of the *Cgopi1*Δ mutant because the downstream target is no longer overexpressed.

One possible target appeared to be *CgINO1*, however disruption of *CgINO1* in the *Cgopi1*Δ strain did not rescue viability on 5-FOA plates indicating that *CgINO1* overexpression is not toxic.

There are two main models to explain the *Cgopi1*Δ mutant's loss of viability. 1) A direct downstream target gene involved in phospholipid biosynthesis is overexpressed, and *C. glabrata* is particularly sensitive to this imbalance in lipid biosynthesis and loses viability. 2) Expression of a direct target gene not involved in lipid biosynthesis is affected by *Cgopi1*Δ and results in a loss of viability.

In the first model, there are several phospholipid biosynthetic genes that may be targets of the *C. glabrata* inositol regulon based on sequence similarity to homologues in *S. cerevisiae*. Direct downstream targets of the *S. cerevisiae* inositol regulon have been identified by the presence of the UAS_{INO} consensus sequence CATGTGAAAT in their promoters and their misregulation in *Scino2*Δ, *Scino4*Δ, and *Scopi1*Δ mutants [63, 77-80]. Using these genes as a guide, there are five genes in *C. glabrata* that contain the sequence

CATGTG (the most important part of the UAS_{INO} consensus sequence [43]) in their promoters. These genes include *CgINO1, CgOPI3, CgCHO1, CgCHO2,* and *CgITR2*. In addition to these five genes, two other genes, *CgERG20* and *CgCKI1*, contain the sequence CATGTT, which differs by only one base and could possibly also respond to the inositol regulon. Preliminary data involving *CgOPI3* and *CgCHO1* (Fig 9) indicate that this model could be correct; however, further experiments including the creation of double-knockout strains of *CgOPI1* and each of these genes must be performed to provide support for this hypothesis.

In the second model, the target may be unrelated to phospholipid biosynthesis and/or may not have a homologue that is regulated by the inositol regulon in *S. cerevisiae*. Such a gene might also not be the direct cause of the loss of viability, but might itself regulate a downstream effector and cause the loss of viability. For example, if *CgOPI1* were to repress a transcriptional repressor of an essential gene then loss of *CgOPI1* could result in loss of expression of the essential gene and compromise viability.

We are currently investigating these different possibilities in order to elucidate the mechanism by which *CgOPI1* controls viability in *C. glabrata*.

Experimental Procedures

Strains and media

Strains are listed in Table 1. Integrations and manipulations were confirmed by PCR (Table 2) in most cases and by Southern blotting with the *tetO::HOP1::CgOPI1* strain. The BG14 strain, which is a uracil auxotroph (Table 1), was a gift from Brendan Cormack and was used to generate all of the strains reported in this study. Disruption of *CgINO2*, *CgINO4*, and *CgINO1* was performed in BG14 (Table 1) utilizing the two-step gene disruption strategy [19, 71, 81] with disruption constructs built in the pRS306 vector [82] (Table 3) carrying the *ScURA3* marker that can be selected for on media lacking uracil and counter-selected against using media containing 5-FOA (supplemental figure 1). Reintgrates of each of these gene disruption mutants were made by transforming the strains with *ScURA3*-marked integrating plasmids carrying the wild-type gene (Table 3).

The *Cgopi1*Δ mutant (Table 1) was generated in BG14 by transforming it with the pCgOPI1 episomal plasmid (*ScURA3* marker, Table 3), and then disrupting the *CgOPI1* gene with the *Cgopi1::NAT1* construct amplified from plasmid pCgopi1Δ (Table 3). The *Cgopi1*Δ *Cgino2*Δ double mutant was made in the same manner as the *Cgopi1*Δ mutant except it was done in the Cg5 (*Cgino2*Δ) strain.

The *tetO::HOP1::CgOPI1* strain EBCg048 (Table 1) was generated by first making BG14 a histidine and tryptophan auxtotroph (*Cghis3*Δ *Cgtrp1*Δ). This was done by disrupting *CgHIS3* in BG14 as described above for the *Cgino2*Δ gene to create the Cg33 strain. The *CgTRP1* gene was disrupted in Cg33 using a plasmid from Karl Kuchler's lab to create strain EBCg046. EBCg046 was used to create the

tetO::HOPI1::CgOPI1 strain EBCg048 by first integrating the pINTG4 plasmid carrying the *tetR::GAL4AD* repressor-activator into its genome as described [75]. Then the *tetO::HOP1::CgOPI1* construct was PCR amplified from plasmid pEB48 (Table 3) and was used to replace the *CgOPI1* locus on the chromosome.

Media used in this study included 2% agar plates or liquid medium made with YPD, YNB, or inositol free media [83] with the supplements of amino acids, nucleotides, inositol, doxycycline, 5-fluororotic acid (5-FOA), etc as described in the text. For inositol-free media Bactoagar was used because it does not contain trace amounts of inositol.

Plasmids and constructs

The gene disruption plasmids (pRS306-ino2Δ, pRS306-ino4Δ, and pRS306-ino1Δ) were made by PCR amplifying DNA corresponding to approximately 500 basepairs of noncoding DNA that flanked the 5' and 3' edges of each open reading frame (5' or 3'-NCRs), and then cloning them into pRS306 adjacent to one another to create a disrupted allele (primers and restriction sites used for cloning are listed in Table 2). The pRS306-his3Δ disruption plasmid was created in a similar manner by subcloning the *CgHIS3* 5'-NCR into the plasmid pGRB2.1 [72] upstream of the 3'NCR of *CgHIS3* contained in this plasmid. The whole 5' and 3'-NCR disruption cassette was then subcloned into the pRS306 integrating vector [82] as an *XbaI-KpnI* fragment to create pRS306-his3Δ. The disrupted alleles from all of the pRS306 disruption cassette plasmids were used to replace the respective wild-type alleles on the chromosome of

BG14 by the two-step deletion strategy [19, 71, 81]. For example, the pRS306-ino1Δ cassette was cut with *NruI* in the 5'-NCR to linearize it and target it to recombine upstream of *CgINO1* (Figure 7). PCR was used to confirm correct integration, and disruption. In a similar manner, the pRS306-ino4Δ, pRS306-ino1Δ, and pRS306-his3Δ plasmids were cut with *PmlI, SnaBI* and *BglII* respectively. Following replacement of the wild-type genes with the disrupted alleles, the mutant alleles were confirmed based on phenotype and PCR. Reintegration constructs were generated for each of the above disruptants in the vector pRS306 by PCR amplifying the corresponding ORFs plus ~500 basepair 5' and 3'-NCRs with the primers BCO3 and BCO2 for *CgINO2*, USO1 and USO4 for *CgINO4,* and MHO3 and MHO2 for *CgINO1*. Cut sites for subcloning are listed in Table 2. Plasmids were linearized with the enzymes sites mentioned above, and transformed into their respective disruptant strains. Correct integrations were confirmed by PCR with primers listed in Table 2.

The episomal pCgOPI1 plasmid carrying *CgOPI1* plus 215 basepairs of upstream DNA and 439 basepairs of downstream DNA was generated by amplifying the *CgOPI1* ORF and flanking sequences from purified BG14 DNA using primers TRO605 and TRO608, and cutting the PCR product with *BamHI* and *SpeI* enzymes. The *SpeI* site was introduced by primer TRO608 and the *BamHI* site was internal to the amplified DNA fragment. This PCR product was cloned into the *ScURA3* bearing *C. glabrata CEN/ARS* plasmid pGRB2.1 [72] using *SpeI* and *BamHI*. The *Cgopi1*Δ plasmid was generated by subcloning the *NAT1* cassette from pAG25 [73] with primers TRO652 and TRO653 into an *EcoRI* site between a 469 basepair fragment containing the 5'-NCR of *CgOPI1*

(cloned in by primers TRO605 and TRO606) and a 439 basepair fragment containing the 3'-NCR of *CgOPI1* (cloned in by primers TRO607 and TRO608) both carried on the pRS306 vector. The pINT4 vector was a gift from Hironobu Nakayama [75] and was integrated into the EBCg046 genome after cutting it with *EcoRV* [75]. The pEB48 plasmid was generated by subcloning an ~500 basepair 5'-NCR of the *CgOPI1* ORF into p97CGH upstream (5') of the *HIS3 tetO-HOP1* cassette using primers EBO76 and EBO77. The *CgOPI1* ORF was then subcloned 3' to the *HIS3 tetO-HOP1* cassette using a PCR product made by primers EBO64 and EBO65. This whole segment from pEB48 containing 5'-*CgOPI1* promoter-*HIS3 tetO-HOP1-CgOPI1* ORF was then transformed into the EBCg046 strain carrying pINTG4 to replace the *CgOPI1* promoter with the *tetO-HOP1* cassette.

Northern blotting

Northern blotting was performed essentially as described [49] using probes generated from primers EBO40 and EBO41 for *CgINO1*, EBO11 and EBO12 for *CgOPI1*, TRO656 and TRO636 for *CgACT1*, EBO56 and EBO57 for *CgCHO1*, EBO54 and EBO55 for *CgOPI3*, and EBO58 and EBO59 for *CgCHO2*.

Appendices

Appendix 1: Tables

Table 1. Strains used in this study

Strains	Genotype	Reference
BG14	*Cgura3::Tn903neoR*	[71]
Cg11	*Cgura3::Tn903neoR* pCgOPI1	This study
Cg12	*Cgura3::Tn903neoR* pGRB2.1	This study
Cg14	*Cgura3::Tn903neoR Cgopi1*Δ pCgOPI1	This study
Cg5	*Cgura3::Tn903neoR Cgino2*Δ	This study
EBCg019	*Cgura3::Tn903neoR Cgino2Δ::CgINO2*	This study
Cg23	*Cgura3::Tn903neoR Cgino2*Δ pCgOPI1	This study
Cg18	*Cgura3::Tn903neoR Cgino2*Δ *Cgopi1*Δ pCgOPI1	This study
EBCg005	*Cgura3::Tn903neoR Cgino4Δ*	This study
EBCg008	*Cgura3::Tn903neoR Cgino4Δ::CgINO4*	This study
EBCg014	*Cgura3::Tn903neoR Cgino1Δ*	This study
EBCg017	*Cgura3::Tn903neoR Cgino1Δ::CgINO1*	This study
Cg33	*Cgura3::Tn903neoR Cghis3*Δ	This study
EBCg046	*Cgura3::Tn903neoR Cghis3Δ Cgtrp1*Δ	This study
EBCg048	*Cgura3::Tn903neoR Cghis3Δ Cgtrp1*Δ pINTG4 pEB48	This study

Table 2. Primers used in this study

Primer	Sequence	Restriction site	Purpose
TRO605	AAAAAAGCTTTGCCTCCTTATCGGTAACAA[a]	*HindIII*	Forward for 5'NCR[b] of *CgOPI1*l
TRO606	AAAAGAATTCTCCAGCAGCACAGTTTATTCA	*EcoRI*	Reverse for 5'NCR of *CgOPI1*l
TRO607	AAAAGAATTCCTCTTCAAATTGAAAACGTTACGAC	*EcoRI*	Forward for 3'NCR of *CgOPI1*l
TRO608	AAAAACTAGTGATTTCTGTTTGACTATTGGTTCTC	*SpeI*	Reverse for 3'NCR of *CgOPI1*l
TRO652	AAAAGAATTCCAGCTGAAGCTTCGTACGC	*EcoRI*	Forward for *NAT1* gene
TRO653	AAAAGAATTCGCATAGGCCACTAGTGGATCTG	*EcoRI*	Reverse for *NAT1* gene
VSO1	AAAACTCGAGCCTTTACACCTGTGAACTACAGTCA	*Xho1*	Forward for 5' NCR *CgINO4*
VSO2	AAAAGAATTCTCCGACTTTTGAAATGGGGT	*EcoR1*	Reverse for 5' NCR *CgINO4*
VSO3	AAAAGAATTCTGCCTGTAATTGAGAAATCCTATTG	*EcoR1*	Forward for 3' NCR *CgINO4*
VSO4	AAAAGGATCCTTGAAAAGAGAAGTTAAAACAGAGG	*BamH1*	Reverse for 3' NCR *CgINO4*
MHO3	AAAACTCGAGTGTCCCCTTTTTTTTTGCC	*Xho1*	Forward for 5' NCR *CgINO1*
MHO4	AAAACCCGGGTCGTGTGGTAAGTGTAGTTGGTCA	*Sma1*	Reverse for 5' NCR *CgINO1*
MHO1	AAAACCCGGGCAGACTTCCCAATGAGGGAAA	*Sma1*	Forward for 3' NCR *CgINO1*
MHO2	AAAACCGCGGCGTTCGTTGGCGAAACTTTT	*SacII*	Reverse for 3' NCR *CgINO1*
BCO3	AAAAAAGCTTTCGCCCGTCTGAAAAAAA	*HindIII*	Forward for 5' NCR of *CgINO2*
BCO4	AAAAGAATTCGGTTCGTGTATTAAATTAAGCACTC	*EcoR1*	Reverse for 5' NCR of *CgINO2*
BCO1	AAAAGAATTCCTACTGACTGTATGTTAGGCTGCAA	*EcoR1*	Forward for 3' NCR of *CgINO2*
BCO2	AAAAGGATCCCAAACTCTTCTTTGAATGACTTTG	*BamH1*	Reverse for 3' NCR of *CgINO2*
TRO665	AAAATCTAGAATTCCCCCATGTACCACAGTC	*XbaI*	Forward for 5' NCR of *CgHIS3*
TRO667	AAAAGGATCCTTGCTCGATGCTTCTCTTTG	*BamHI*	Reverse for 5' NCR of *CgHIS3*
EBO24	GTTGCTGTTAAGTATGTTTGA		Check insertion of pEB13 (ino4Δ)
EBO27	TGGCAACTAGAATTTTTCACATGC		Check insertion of pEB19 (ino1Δ)
TRO668	TACGTTGTTACCCACACGATT		Check insertion of pRS306-his3Δ
TRO623	ACAGTCATCCAAAGGTGACTCTCAT		Check insertion of pRS306-ino2Δ
TRO536	CCAGGGTTTTCCCAGTCA		Reverse for primers EBO24, EBO27, and TRO623
TRO614	GAAGGAATCTCAAAAGTGCGGA		Check insertion of pCgopi1Δ
TRO461	GTGCGCAGAAAGTAATATC		Reverse primer for TRO614
EBO64	AAAAGAATTCACCATGGACACAAGGCGTG	*EcoR1*	Forward for *CgOPI1* tetO promoter
EBO 65	AAAAGGGCCCGTAGATGTAGGTTCTCCTTTTCATTA	*Apa1*	Reverse for *CgOPI1* tetO promoter
EBO40	ATGACTGTGAATAAAGGTATTAGCATTC		Forward for *CgINO1* probe
EBO41	CTATTTCAATCTTTCTTCGAATCTCAG		Reverse for *CgINO1* probe
TRO656	GCCGGTTTCGCCGGTGACG		Forward for *CgACT1* probe
TRO636	CCAAAGCGACGTAACATAGCTTT		Reverse for *CgACT1* probe
EBO12	GACACAAGGCGTGGGTG		Forward for *CgOPI1* probe
EBO11	TCATACCTTTTGTAAATGCATA		Reverse for *CgOPI1* probe

a Underline shows the restriction site

b NCR-non-coding region that is either 5' or 3' to the designated ORF

Table 3. Plasmids used in this study.

Plasmid	description	type	restriction site	Reference
pRS306-ino2Δ	*CgINO2* knockout	integrating	*SnaBI*	This study
pRS306-CgINO2	*CgINO2* reintegration	integrating	*SnaBI*	This study
pEB19	*CgINO1* knockout	integrating	*Nru1*	This study
pEB21	*CgINO1* reintegration	integrating	*Nru1*	This study
pEB13	*CgINO4* knockout	integrating	*Pml1*	This study
pEB17	*CgINO4* reintegration	integrating	*Pml1*	This study
p*CgOPI1*	contains *CgOPI1*	episomal		This study
pCgopi1Δ	*Cgopi1*Δ	integrating		This study
pRS306-his3Δ	*CgHIS3* knockout	integrating	*BglII*	This study
pGRB2.1	*ScURA3* vector	episomal		[72]
pAG25	*NAT1* cassette	PCR template		[73]
pEB48	*tetO::HOP1::CgOPI1*	Integrating		This study
pINTG4	*tetR::GAL4AD*	Integrating		[75]
pRS306	Shuttle vector	Integrating		[82]

Appendix 2: Figures

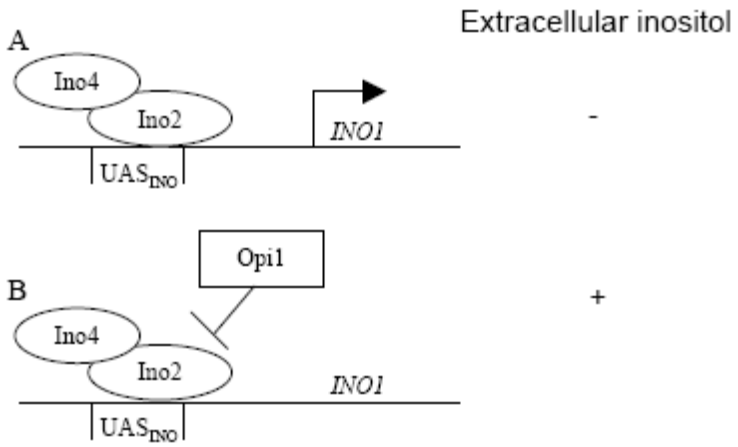


Figure 1. The Inositol Regulon in *S*. cerevisiae.

The inositol regulon in *S. cerevisiae* controls transcription of phospholipid biosynthetic genes like *ScINO1* in response to the level of extracellular inositol. (A) The ScIno2p-ScIno4p heterodimer activates transcription of *ScINO1* and other target genes in the absence of extracellular inositol. ScIno2p-ScIno4p binds the upstream activating sequence for inositol regulation (UAS_{INO}) present in the promoters of genes like *ScINO1*. (B) In the presence of extracellular inositol ScOpi1p binds to ScIno2p and prevents it from activating transcription of *ScINO1* and other targets.

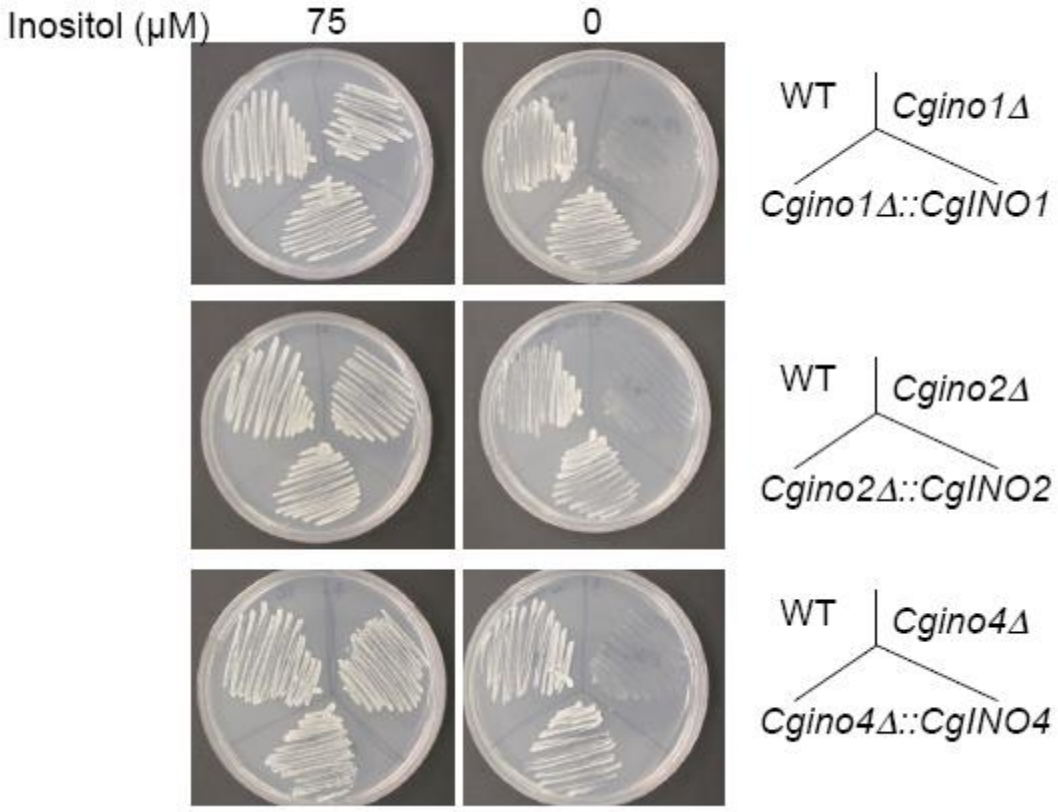


Figure 2. Inositol Auxotrophy of *Cgino1Δ*, *Cgino2Δ*, and *Cgino4Δ*.

The *CgINO1*, *CgINO2*, and *CgINO4* genes are all involved in de novo inositol biosynthesis, like their *S. cerevisiae* homologuess. The *Cgino1*Δ, *Cgino2*Δ, and *Cgino4*Δ mutants and their respective reconstituted strains (along with the wild-type control) were streaked onto inositol free media supplemented with 0 or 75μM inositol, and grown at 30˚C for 3 days.

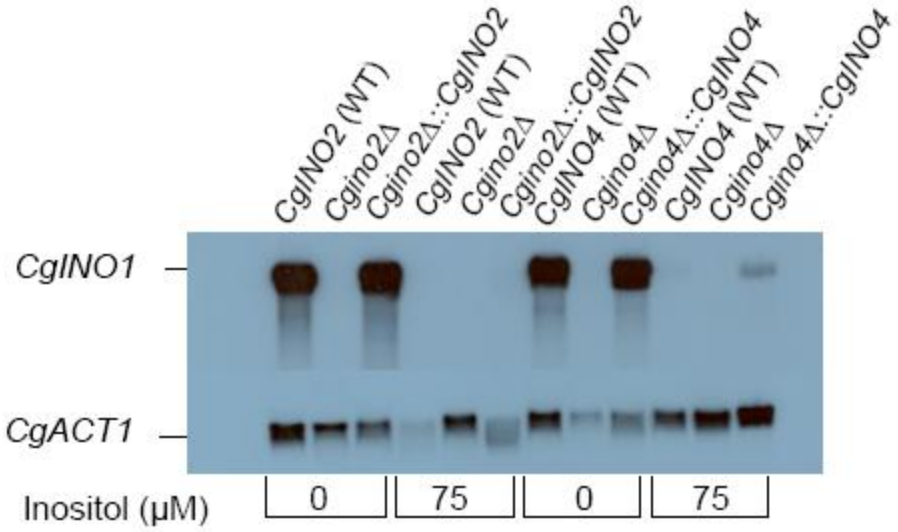


Figure 3. *CgINO1* Expression in Mutants.

CgINO2 and *CgINO4* are required to express *CgINO1* in the absence of exogenous inositol. Northern blotting was used to assess the expression of *CgINO1* in wild-type (WT), mutant, and reconstituted strains in the presence or absence of exogenous inositol. *CgACT1* was used as a loading control. Strains were grown overnight at 30˚C in inositol-free medium supplemented with 75μM inositol, washed with water, and resuspended in inositol-free media containing either 0 or 75μM inositol, after which the cultures were incubated with shaking for 6 hours before collecting total RNA for Northern blotting.

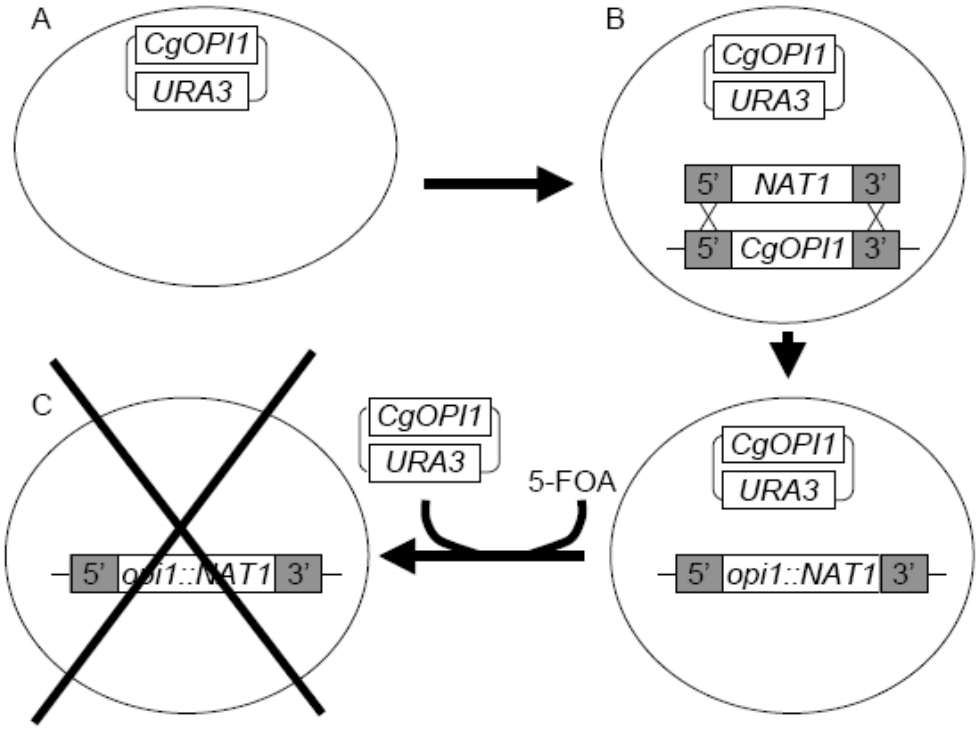


Figure 4. Diagram of molecular technique used to show that *CgOPI1* is essential.

(A) *CgOPI1* was expressed episomally from a single-copy (*CEN/ARS*) vector carrying a *URA3* marker (pCgOPI1). (B) The chromosomal copy was then disrupted with a *Cgopi1*Δ disruption cassette by homologous recombination. (C) The resulting *Cgopi1*Δ*::NAT1* pCgOPI1 strain was then streaked on 5-FOA medium to select for cells that lost the pCgOPI1 plasmid. Those which did loose the plasmid could not grow because the chromosomal copy was missing as well and *CgOPI1* is essential.

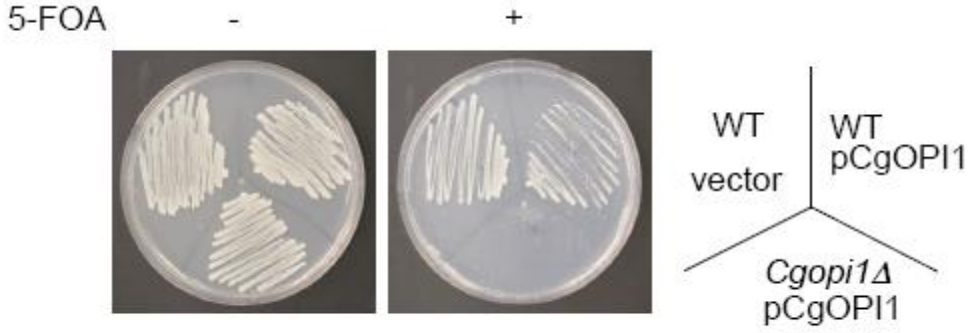


Figure 5. The *CgOPI1* gene is essential.

Wild-type (WT) and *Cgopi1*Δ strains carrying *CgOPI1* on a *URA3* plasmid (pCgOPI1) were grown for 3 days on media +/- 5-FOA at 30˚C.

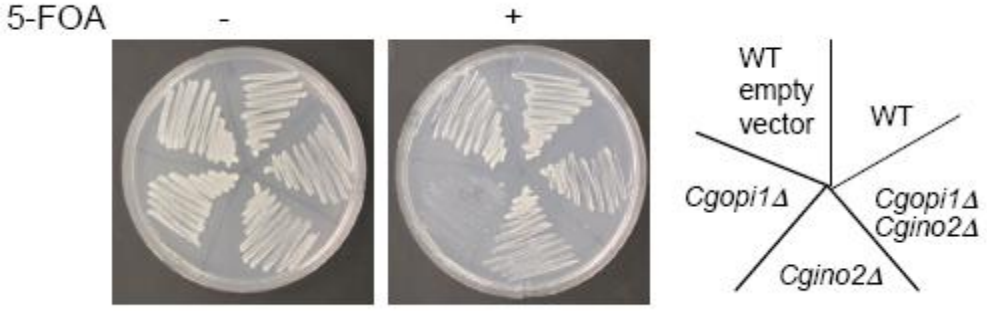


Figure 6. A *CgOPI1-CgINO2* double knockout rescues growth.

The *Cgopi1*Δ mutant's viability defect is dependent on the *CgINO2* transcriptional activator. The wild-type (WT), *Cgopi1*Δ, *Cgino2*Δ, and *Cgopi1*Δ *Cgino2*Δ strains containing the pCgOPI1 plasmid (*URA3*-based) were streaked

onto synthetic media plates ± 5-FOA. The WT strain containing the empty vector was included as a control.

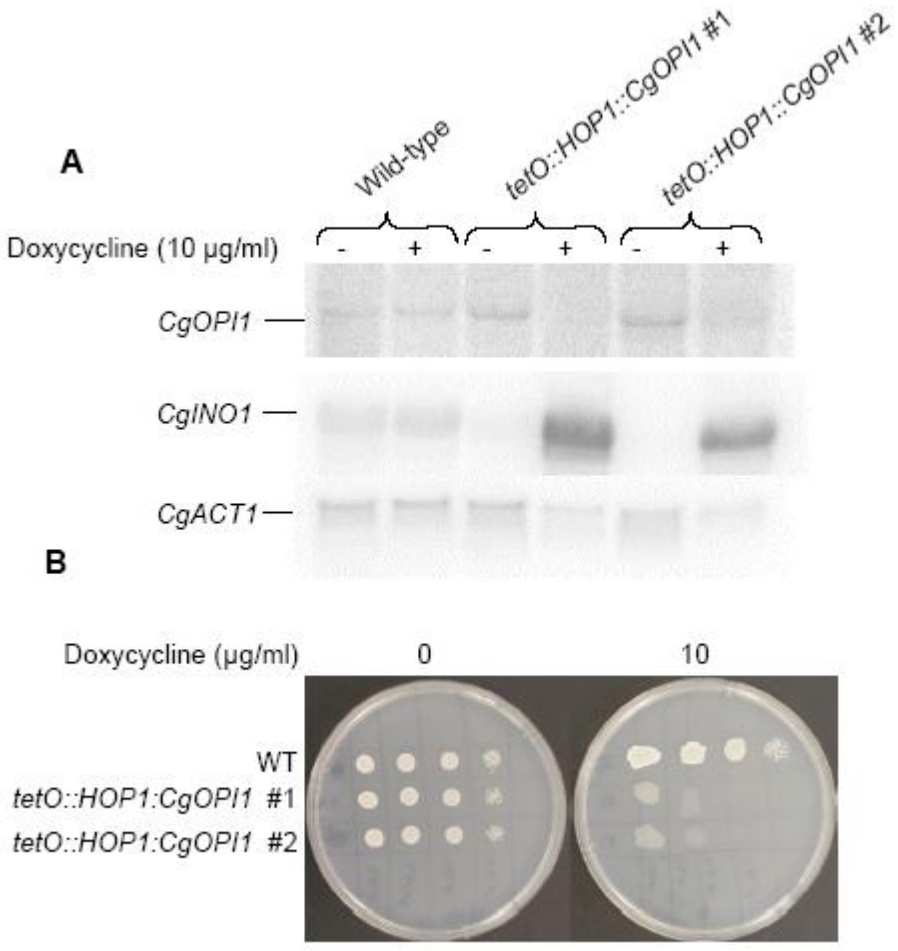


Figure 7. CgOpi1p is a transcriptional repressor of *CgINO1*.

The promoter of the chromosomal copy of *CgOPI1* was replaced with the doxycycline-repressible *tetO::HOP1* promoter. (A) Expression of *CgOPI1*, *CgINO1*, and *CgACT1* (loading control) were tested by Northern blotting in the presence and absence of doxycycline. Cells were grown to saturation overnight in

synthetic medium (contains ~11 µM inositol) lacking doxycycline, washed with water, and then resuspended in fresh synthetic media containing 0 or 10 µg/ml doxycycline and grown for ~6 hours at 30˚C with shaking at which time samples were taken for Northern blotting. (B) Cells were grown on plates containing 0 or 10µg/ml doxycycline to confirm that loss of *CgOPI1* expression decreased viability. Two different transformants were tested for doxycycline repressible promoter.

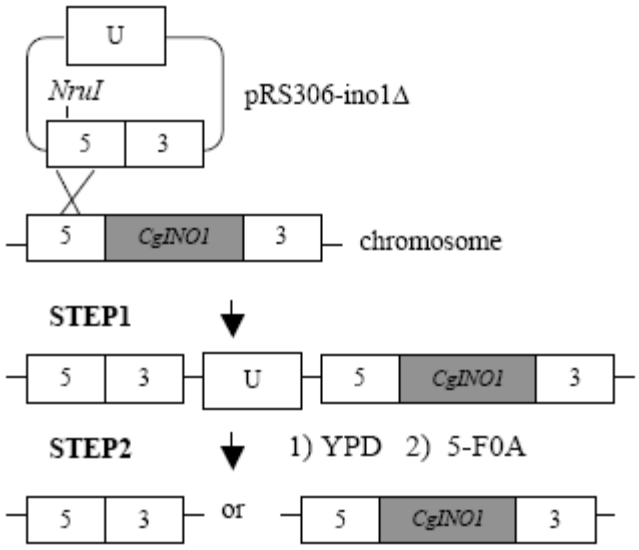
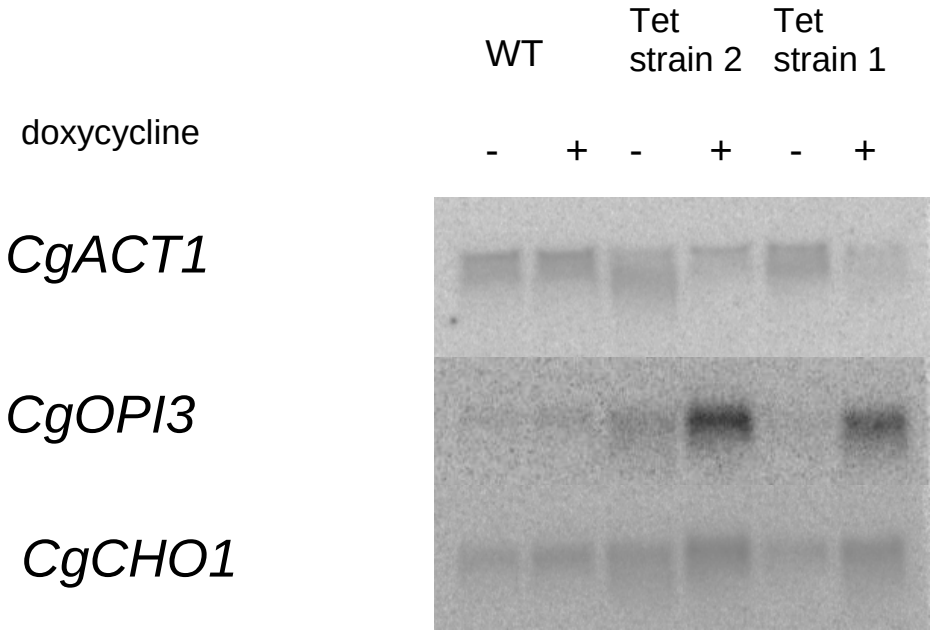


Figure 8. Two-step gene deletion method for disrupting *CgINO1* in *C. glabrata*.

The pRS306-*ino1*Δ plasmid was cut with *NruI* and transformed into the BG2 strain (*ura3*Δ) and selected on SC-ura medium. The resulting transformant was then grown in YPD and plated on 5-FOA medium to select for strains where the integrated plasmid had recombined out of the chromosome and was lost. PCR was used to determine which allele (wild-type or disruptant) was left behind. The *CgINO1* ORF is shown in gray. The 500 base-pair 5' and 3'-non-coding regions (NCRs) flanking the ORF are labeled 5 and 3, respectively. The *URA3* gene is labeled U.

A


WT
Tet strain 2
Tet strain 1
doxycycline
- + - + - +
CgACT1
CgOPI3
CgCHO1

B

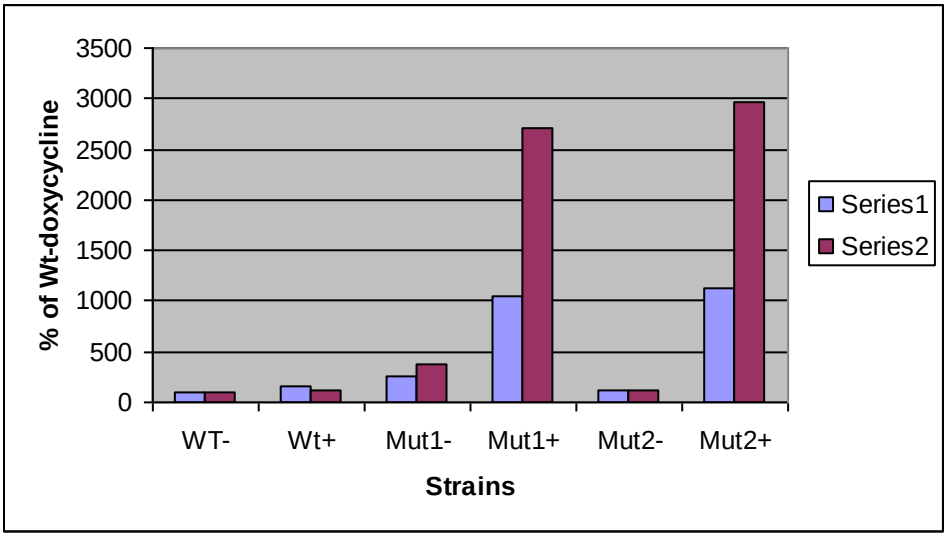

3500
3000
2500
2000
1500
1000
500
0
% of Wt-doxycycline
WT-
Wt+
Mut1-
Mut1+
Mut2-
Mut2+
Strains
Series1
Series2

Figure 9. Expression of *CgOPI3* and *CgCHO1*.

A. Northern blot measuring gene expression of *CgOPI3* and *CgCHO1* by wild type and two strains having the *CgOPI1* gene fused to the tet system. This was measured in the presence and absence of 10µg/ml doxycycline. B. Quantification of northern blot analysis normalized against *CgACT1*. Series 1 corresponds to *CgCHO1* and Series 2 corresponds to *CgOPI3.*

Acknowledgements

We gratefully acknowledge Brendan Cormack for supplying us with strains, plasmids, and a great deal of advice without which this work would not have been possible. We also are grateful to Karl Kuchler for sending us strains and plasmids. We are grateful to Hironobu Nakayama for sending us the doxycycline repressible plasmid system and Fritz Muhlschlegel for the *MTII* promoter plasmids. This work was supported by grants AHA 0765366B and NIH-1R03AI071863-01A1.

Chapter III

Additional Work in *Candida glabrata*

Designing Conditional Mutants in *Candida glabrata*

There are several vectors that are utilized in yeast to conditionally control gene expression. One such vector contains the promoter of the *MtII* gene from *C. glabrata*, which is induced in the presence of copper. When the *MtII* promoter was fused to the *lacZ* gene of *Escherichia coli* and transformed into *C. glabrata*, β-galactosidase activity was detected with increasing concentrations of copper ($CuSO_{4)}$ [76].

A vector carrying this inducible-conditional promoter (Figure A.5) was used to control expression of the *CgOPI1* gene, which is essential for the viability of the organism. Although a lack of CuSO4 in the medium led to a lack of detectable expression of *CgOPI1* from the *MtII* promoter as measured by Northern blotting (Figure A.6A), the conditional mutant remained viable (Figures A.6B and A.6C). This promoter did serve to show that *CgOPI1* controls *CgINO1* expression, since in the absence of detectable *CgOPI1* (0 μM $CuSO_4$), the *CgINO1* gene was overexpressed (Figure A.6A). In as little as 20μM $CuSO_4$, no expression of *CgINO1* was detected, while *CgOPI1* was strongly expressed (Figure A.6A).

The above analysis was performed using the *MTII* promoter on an episomal plasmid, pEB9 (Figure A.5). It was hypothesized that the episomal plasmid, although centromeric, may be present in multiple copies per cell and may cause enough expression of *CgOPI1* to explain the strain's viability even in the absence of extra $CuSO_4$. *CgOPI1* expression level was not detectable by Northern blotting, but since the cells were viable,

CgOPI1 was believed to be present based on the fact that a complete *CgOPI1* deletion mutant is inviable (see chapter 2). Therefore, the *MTII* promoter-*CgOPI1* construct was subcloned into an integrating vector and integrated into the strain at the *MTII* locus. This resulted in several transformants, some of which failed to grow on plates in the absence of $CuSO_4$, and others did grow in the absence of $CuSO_4$ (data not shown). It was hypothesized that the strains that did grow had multiple copies of the plasmid integrated and the strains that did not grow had only one copy. There are three virtually identical *MTII* genes so the multiple integrations seemed possible. However, Southern blot analysis was inclusive, thus we do not know why some strains grew and others did not.

In order to confirm the essential nature of the gene as well as to obtain a useable conditional mutant of *CgOPI1*, we needed to utilize a vector that would provide a tight enough shutoff of the gene to eliminate its viability. In *Candida albicans,* the *MET3* promoter can be used to repress gene expression in the presence of cysteine and/or methionine [84]. Since *C. glabrata* has a homologue of this gene, it was hypothesized that this could serve as a conditional promoter in this organism. Again, the viability of a strain of *C. glabrata* containing this vector was not diminished. When a northern blot experiment was performed, the results were ambiguous, leading to the conclusion that the *C. glabrata* homologue of *MET3* does not make an efficient conditional promoter (all data not shown).

Sufficient control of *CgOPI1* expression was eventually obtained using the doxycycline-repressible system, indicating that it is probably the most effective conditional promoter currently available for *C. glabrata* (See chapter 2 for details).

CgCHO1

Genes involved in phospholipid biosynthesis are beginning to be studied in pathogens in the context of virulence. In *Mycobacterium tuberculosis*, a mutant of the *ino1* gene was attenuated for virulence. It was quickly cleared from murine macrophages in one experiment and unable to cause mortality in mice in another [85]. In *Candida albicans*, the PS synthase-encoding gene, *CHO1*, has been implicated in virulence, as *cho1Δ/cho1Δ* mutants are avirulent in mice compared to the wild type. A knockout of both genes responsible for PS decarboxylase, *psd1Δ/ psd1Δ* and *psd2Δ/psd2Δ* also resulted in reduced virulence (Reynolds and Chen, unpublished data).

In order to see the phenotype of the *Cgcho1Δ* mutant, this gene was disrupted in *Candida glabrata*. The gene was initially thought to be essential because it was not possible to obtain mutants using the two-step gene deletion approach. Therefore, the *Cgcho1Δ* mutant was constructed by the same technique used for the *Cgopi1Δ* mutant, which is depicted in figure 4. This *Cgcho1Δ* mutant carrying the pEB41 plasmid could not grow on medium containing 5-FOA unless the media was supplemented with 5mM concentrations of ethanolamine or choline. However, even in 5mM ethanolamine and choline, cells grew poorly and unexpectedly could not be subcultured to a new plate containing ethanolamine or choline, suggesting that in some way the *Cgcho1*Δ mutant was very sick (data not shown). In addition, these strains cannot be recovered from the -80˚C freezer. Only a strain carrying *CgCHO1* on the p41 plasmid could be recovered from the -80˚C freezer. In order to clear this up *CgCHO1* was expressed from the *MTII* promoter which was integrated into the genome at the *CgCHO1* locus using plasmid

pEB51. This was done by transforming the strain carrying pEB41 and *Cgopi1::NAT1* with pEB51, and then selecting on 5-FOA. This strain could not grow in the absence of $CuSO_4$ without exogenous ethanolamine and/or choline (Figure A.7). However, it grew very poorly in the presence of 5mM ethanolamine and choline in the absence of $CuSO_4$, and it was very poorly subcultured to a new plate containing 5 mM ethanolamine and choline, but not CuSO4. This strain grew much better when plated on media containing $CuSO_4$. (Figure A.8).

In *S. cerevisiae*, respiratory deficiency is correlated with increased levels of *CHO1* transcript. PE is a major component of mitochondrial membranes, and it is necessary that cells synthesize it in order to grow on nonfermentable carbon sources. The *cho1Δ* mutant as well as *psd1Δ* and *psd1Δpsd2Δ* mutants in *S. cerevisiae* are more likely to form petites, or respiratory deficient cells [86]. Respiratory function was also affected in *cho1Δ, psd1Δ,* and *psd1Δpsd2Δ* mutants of the non-petite-forming yeast, *C. albicans* (Reynolds and Chen, unpublished data). Since *C. glabrata* and *S. cerevisiae* can both form petites, the mitochondrial activity of *Cgcho1Δ* mutants was observed using fluorescence microscopy with the mitochondria-staining fluorophore, Mito-tracker. This also yielded equivocal results; there was clearly a difference between the wild type and the *CgCHO1* mutant, but the mutant fluoresced more than expected (Figure A.9). In an attempt to understand these observations, petite mutants of *C. glabrata* were created, and these failed to fluoresce when stained with Mito-tracker (data not shown). The *Cgcho1*Δ mutant clearly has some respiratory activity, but it is not clear in what way it is defective.

Appendix

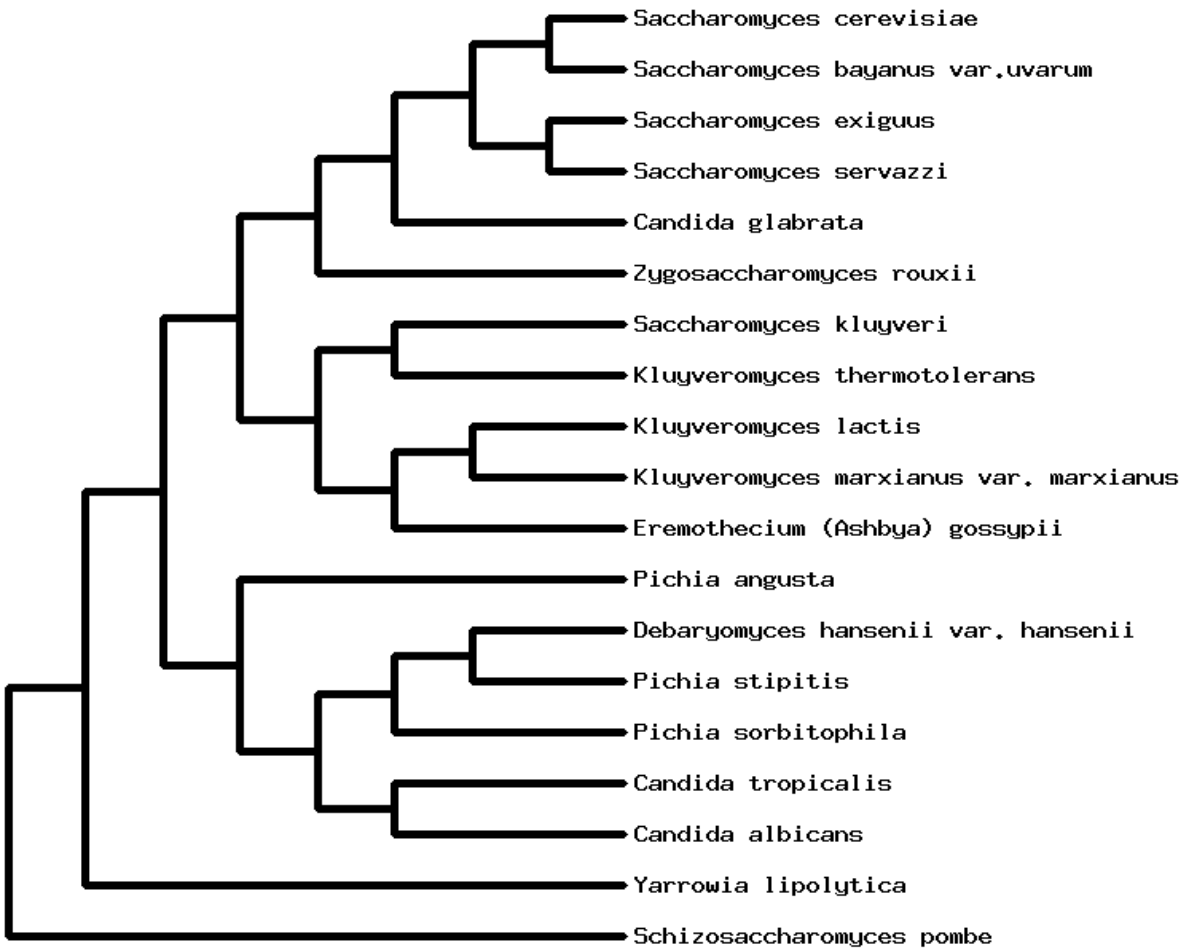


Figure A. 1. Relationships among various yeast species.

This figure is from the website http://cbi.labri.fr/Genolevures/. It is based on the combined results from multigene concatenation and supertree analyses performed by Fitzpatrick, *et al*. 2006 [87].

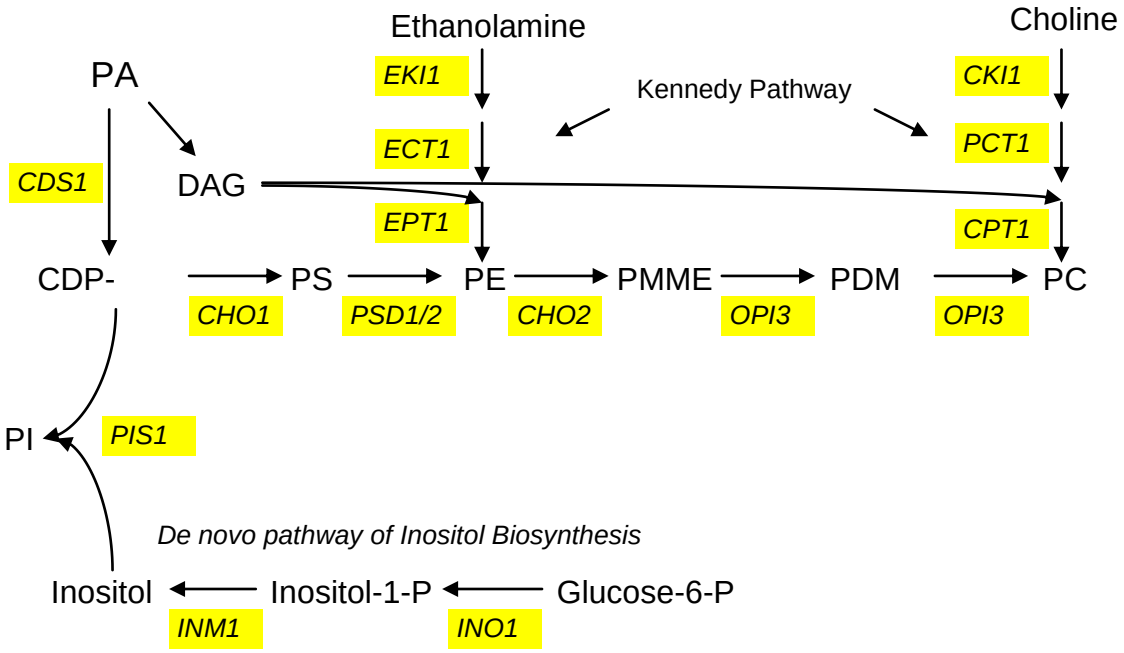


Figure A. 2. Phospholipid biosynthetic pathways of *Saccharomyces cerevisiae.*

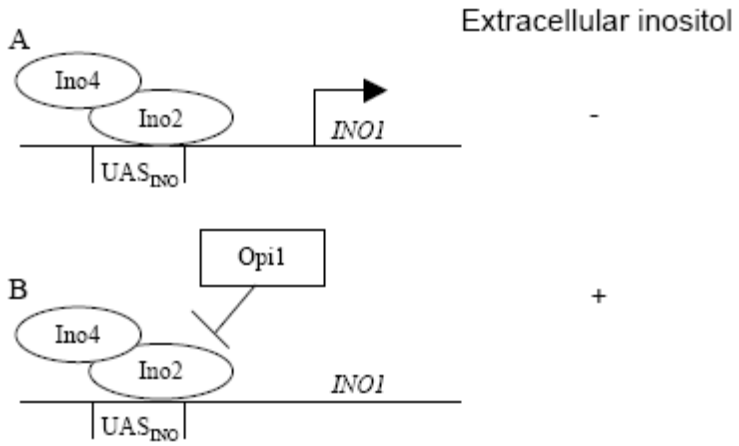


Figure A. 3. The inositol regulon in *S. cerevisiae.*

The inositol regulon in *S.* cerevisiae controls transcription of phospholipid biosynthetic genes like *ScINO1* in response to the level of extracellular inositol. (A) The ScIno2p-ScIno4p heterodimer activates transcription of *ScINO1* and other target genes in the absence of extracellular inositol. ScIno2p-ScIno4p binds the upstream activating sequence for inositol regulation (UAS_{INO}) present in the promoters of genes like *ScINO1*. (B) In the presence of extracellular inositol ScOpi1p binds to ScIno2p and prevents it from activating transcription of *ScINO1* and other targets.

A.

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CLUSTAL 2.0.10 multiple sequence alignment


S.C.Opi1p       ------------------------------------------------------------
C.G.Opi1p       ------------------------------------------------------------
Y.L.Yas3p       MRKTDPAIEGEEIRGAEVSSSAVIAFCVVITTSHALCPRTCLWCNCTFPPNAPNPNLNPK 60
C.A.Opi1p       ------------------------------------------------------------


S.C.Opi1p       ------------------------------------------------MSENQ----- 5
C.G.Opi1p       ----------------------------------------------MDTRRGWIHN 10
Y.L.Yas3p       LHKTHERDTANFPSDKGAVSISSQHNHISNTNTTFATSGAQSQYHRLSNNTKTFTRDSPY 120
C.A.Opi1p       ----------------------------------------------MTAPSISNQQ 10


S.C.Opi1p       ---RLGLSEEEVEAAEVLGVLKQS------------CRQKSQPSEDVS-------QADKM 43
C.G.Opi1p       YISFSGLSEEDVEAAEALDVLRNSHVDADVEVEEDRSKKRVRDGEDEDGGHVRRRKASVR 70
Y.L.Yas3p       HSRTPSVDDDGIRQASFLNRVYQSSKSYSPRFRYGAEIVERWASSENTNAATPSASTTNS 180
C.A.Opi1p       LPPPPYSKTDNLPTSNYQSSLNGSVQGQDQ--QVQAQVQGQAPELVSAAETLTSLTRNGT 68
                       . : :  :.  . :  *

S.C.Opi1p       PASESSTTPLN--------------------------------ILDRVS---NKIISNVVT 69
C.G.Opi1p       DRSDSRMSQLSNVSNASTEERVVRVNSHEDSQSKRQEE--ESLFDKVCRNSNEILTNMGS 128
Y.L.Yas3p       NNSNHSNTPVSTTPSSTTMPKALSSKSLDAASIHMASNGAPPLIQKSSLHDFERLKSGID 240
C.A.Opi1p       PPSDADTVAMDDAQSPVILP-----------PIHPQHQRHPIVSTVSMVARHPIVMNAVK 117
                  *:     :.                              :           : .

S.C.Opi1p       FYDEINTN-----------------------------KRPLKSIGRLLDDDDE----HD 96
C.G.Opi1p       FFEEMNSNVFMEGGSD---------VPEEHPNGYVRPRRDSRSISTTGADADTESQGTQG 179
Y.L.Yas3p       DIQNSDTNGTQYSVDVGSQGLRMRIQTQGYAPSGNSNRGSPVPPSPALSTSTSSGSTSAA 300
C.A.Opi1p       YYETSKRNYPSFNYAAG---------------IVESAAIPVVNNIEAKLNTRHQTRQASA 162
                  :  . *                                          .

S.C.Opi1p       DYD----------YNDDEFFTNKRQKLSRAIAKGKDNLKEYKLNMSIESKKRLVTCLHLL 146
C.G.Opi1p       SYNGKWDTWSSRSYNDRQYYS-KRKALSEALAKGRYNLREYKLTMSIESKKRLITCLHLL 238
Y.L.Yas3p       ASRSTSSPVPQQPQTAATAAGGPRPGQPRSAWQEVLISATSLASLSQDSRKRLRYCLHLL 360
C.A.Opi1p       VSSTDITPTNSNFGDYKIQHDGKNSLQKKRRFSASSQTTNISSYSSIDTKKRLQFCINIL 222
                                  .    .   .            * :::***  *:::*

S.C.Opi1p       KLANKQLSDKISCLQDLVEKEQVHPLHKQDGNARTTTGAGEDETSSDEDDDDEEFFDASE 206
C.G.Opi1p       KLANKQLSDKVAYLQDAVEKEQELANGEE---VKKETRALNGEHSGPQNDDDLEFYDASE 295
Y.L.Yas3p       KLANAHLASTVTKLQGAIAEETAYSLAQS------------IAANHVPHNERQAYLHQPP 408
C.A.Opi1p       KLANTNISSKVEFLQEKIDETEIAVKEER------------------EKLIAQKSHDSNS 264
                **** :::..:  **  : :       :                    .       .

S.C.Opi1p       QVNASEQSIVVKMEVVGTVKKVYSLISKFTANSLPEPARSQVRESLLNLPTNWFDSVHST 266
C.G.Opi1p       SVDQN--SGDLGLEIVGTVKKVYSLISKYTGSSLPEPARSQVRESLLNLPSNWNTSVHNG 353
Y.L.Yas3p       SAEPALSITALKADVVSTIRKIIKVVSQYAGNSLPEPARSHIRTYILGLPSRWASTTAST 468
C.A.Opi1p       TNTTEQATQKTKTEIVGTVKKIIHLISNFRPSSLGDTSVTNGLSPVSSNGSQSNQDFELK 324
                          ::*.*::*:  ::*::  .** :.: ::    : .  :.

S.C.Opi1p       SLPHHASFHYANCEEQKVEQQQQQQQQQQQQQLLQQQLLQQQQQKRNKDGDDSASPSSSV 326
C.G.Opi1p       FKNNGTGIMTASS-------------------------------STDSLSSYSSILPV 380
Y.L.Yas3p       NITPTRSPAGSQSPVGSPKECVTPDHQGPPLPTN----TVSSPVPEGPSDEQLSRHRIEV 524
C.A.Opi1p       NAIRDIIMSLPQS-------------------------------LQQQQQSQQSGPPSN 352
                         ...                                      .  .

S.C.Opi1p       TANGKVLILAKESLEMVRNVMGVVDSTLGKAEEWVKQKQEVKEMIRERFLQQQQQYRQQ- 385
C.G.Opi1p       SSNGKYLILAKESLNMVQSVIDVVDSTLGRAEEWVKQKQELKEMIKKKFLEQQEHQKMLG 440
Y.L.Yas3p       EAGGKVLILANEALDMLGNIISIVDGTLERAEGWCEGINRVKQRVGLGETAPGAAGEPGA 584
C.A.Opi1p       SQDDVIFKFAKESLVMISKLTQIFTEKLDQVEHWVNGDEEQEQKLQSQSQSPEGLEKQQE 412
                  ..  : :*:*:* *: .:  :.  .* :.* * :  :. :: :           .

S.C.Opi1p       -----QQKDGNYVKPSQ-----------------DNVDSKD------ 404
C.G.Opi1p       IGTESLDQNESSVKKEQPTTHISISSITNSTNTLDTVKEEDMHLQKV 487
Y.L.Yas3p       STEASAEASAEASASAAAAYS-----------AVDTSTDQDVEMGDA 620
C.A.Opi1p       DEREEENLAAETKRMKLDGSV-------------------------- 433
                             :    .
```

B.

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CLUSTAL 2.0.10 multiple sequence alignment


S.C.Opi1p       MSENQ--------RLGLSEEEVEAAEVLGVLKQS------------CRQKSQPSEDVS-- 38
C.G.Opi1p       MDTRRGWIHNYISFSGLSEEDVEAAEALDVLRNSHVDADVEVEEDRSKKRVRDGEDEDGG 60
                *. .:          *****:*****.*.**::*            .::: : .** .

S.C.Opi1p       -----QADKMPASESSTTPLN----------------------------ILDRVS---N 61
C.G.Opi1p       HVRRRKASVRDRSDSRMSQLSNVSNASTEERVVRVNSHEDSQSKRQEEESLFDKVCRNSN 120
                     :*.    *:*  : *.                              ::*:*.   *

S.C.Opi1p       KIISNVVTFYDEINTN-------------------KRPLKSIGRLLDDDDDE----HDD 97
C.G.Opi1p       EILTNMGSFFEEMNSNVFMEGGSDVPEEHPNGYVRPRRDSRSISTTGADADTESQGTQGS 180
                :*::*: :*::*:*:*                    :*  :**.    * * *    :..

S.C.Opi1p       YD----------YNDDEFFTNKRQKLSRAIAKGKDNLKEYKLNMSIESKKRLVTCLHLLK 147
C.G.Opi1p       YNGKWDTWSSRSYNDRQYYS-KRKALSEALAKGRYNLREYKLTMSIESKKRLITCLHLLK 239
                *:          *** :::: **: **.*:***: **:****.*********:*******

S.C.Opi1p       LANKQLSDKISCLQDLVEKEQVHPLHKQDGNARTTTGAGEDETSSDEDDDDEEFFDASEQ 207
C.G.Opi1p       LANKQLSDKVAYLQDAVEKEQELANGEE---VKKETRALNGEHSGPQNDDDLEFYDASES 296
                *********:: *** *****  .  ::   .:. * * :.* *. ::*** **:****.

S.C.Opi1p       VNASEQSIVVKMEVVGTVKKVYSLISKFTANSLPEPARSQVRESLLNLPTNWFDSVHSTS 267
C.G.Opi1p       VDQN--SGDLGLEIVGTVKKVYSLISKYTGSSLPEPARSQVRESLLNLPSNWNTSVHNGF 354
                *: .  *  : :*:**************:*..******************:**  ***.

S.C.Opi1p       LPHHASFHYANCEEQKVEQQQQQQQQQQQQQLLQQQLLQQQQQKRNKDGDDSASPSSSVT 327
C.G.Opi1p       KNNGTGIMTASS-----------------------------------STDSLSSYSSILPVS 381
                  : :.:  *..                                   ..*. .* *.  .*:

S.C.Opi1p       ANGKVLILAKESLEMVRNVMGVVDSTLGKAEEWVKQKQEVKEMIRERFLQQQQQYRQQ-- 385
C.G.Opi1p       SNGKYLILAKESLNMVQSVIDVVDSTLGRAEEWVKQKQELKEMIKKKFLEQQEHQKMLGI 441
                :*** ********:**:.*:.*******:**********:****:::**:**:: :

S.C.Opi1p       ----QQKDGNYVKPSQ-----------------DNVDSKD------ 404
C.G.Opi1p       GTESLDQNESSVKKEQPTTHISISSITNSTNTLDTVKEEDMHLQKV 487
                     ::: . ** .*                *.*..:*
```

Figure A. 4. ClustalW analysis.

A.) ClustalW 2.0.10 multiple sequence alignment comparing Opi1p homologues of *S. cerevisiae* (S.C.), *C. glabrata* (C.G.), *Y. lipolytica* (Y.L.), and *C. albicans* (C.A.). Asterisk represents conservation among all four species. Various domains represented by different colors. Blue: Opi1-Sin3 interaction domain. Gold: PA-binding domain. Red: Leucine zipper. Green: FFAT. Purple: Polyglutamine Tract. Orange: Activator Interaction Domain.

B.) Same as 3A, but comparing only *S. cerevisiae* and *C. glabrata.*

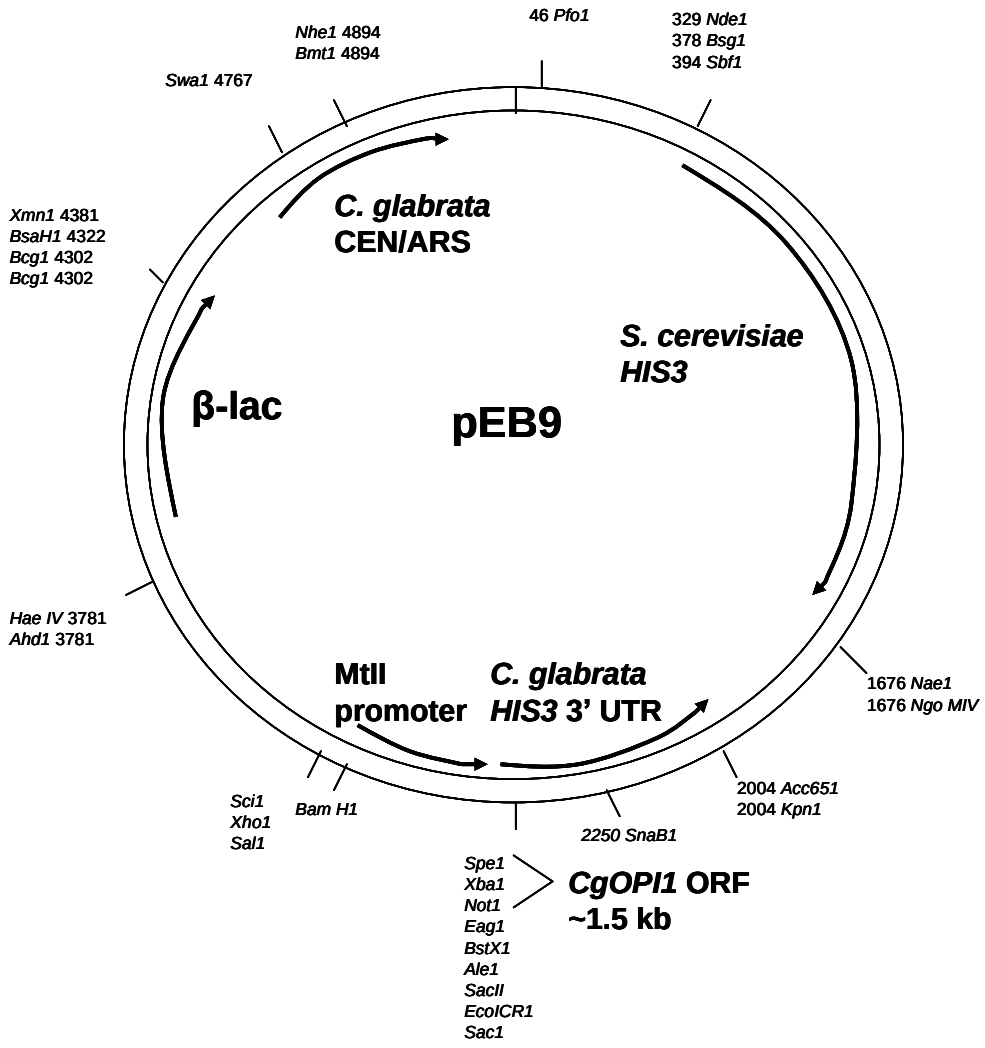
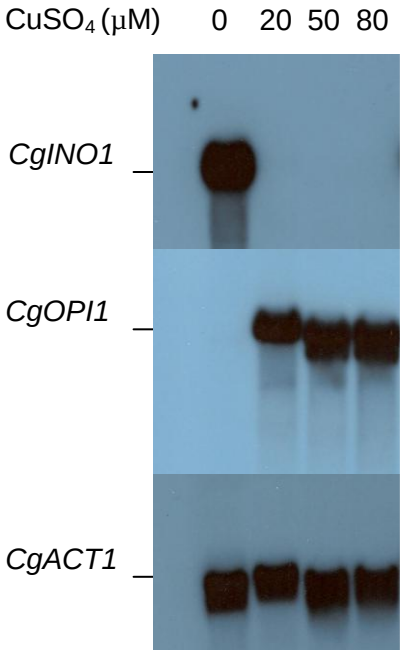


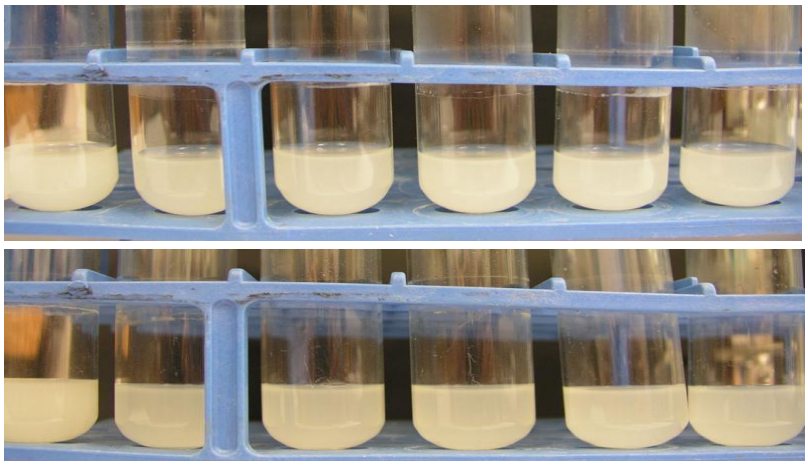
Figure A. 5. Plasmid initially used to create MtII-*CgOPI1* fusion.

pEB9 is an episomal plasmid that was transformed into a strain containing the *CgOPI1* gene on another plasmid with a *URA3* marker. The chromosomal *CgOPI1* was replaced with a nourseothricin-resistance cassette. The plasmids used for integrating MtII-*CgOPI1* and MtII-*CgCHO1* fusions (pEB37 and pEB51, respectively) are similar to this one but lack the *C. glabrata* CEN/ARS region.

A



B



C

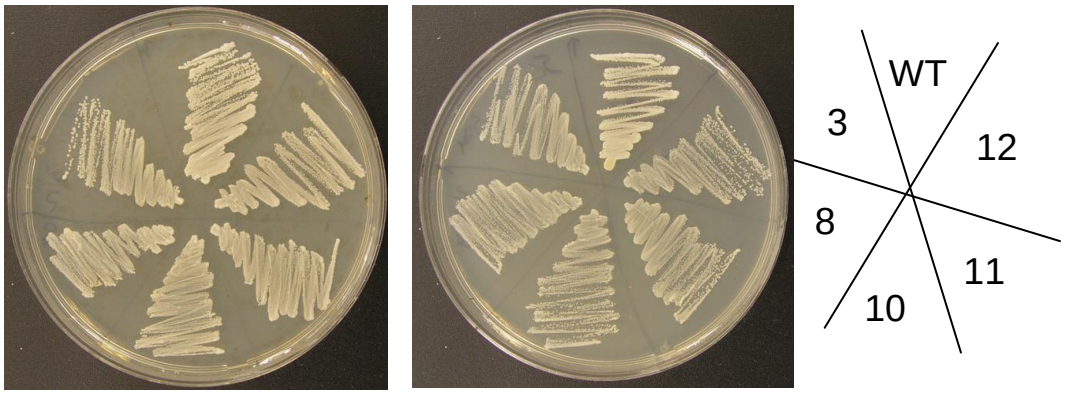


Figure A. 6. *CgOPI1* with MtII promoter integrated into the chromosome.

A.) Northern Blot analysis of the expression of *CgOPI1* and *CgINO1* in the presence of varying concentrations of $CuSO_4$. B.) Strains with *CgOPI1*-MtII construct grown in SC liquid media in the absence (top) and presence (bottom) of 50 mM $CuSO_4$. Strains are in order from left to right Cg1 (WT), EBCg049-3, EBCg049-8, EBCg049-10, EBCg049-11, EBCg049-12. C.) *CgOPI1*-MtII constructs grown on SC solid media in the absence (left) and presence (right) of 50 mM $CuSO_4$.

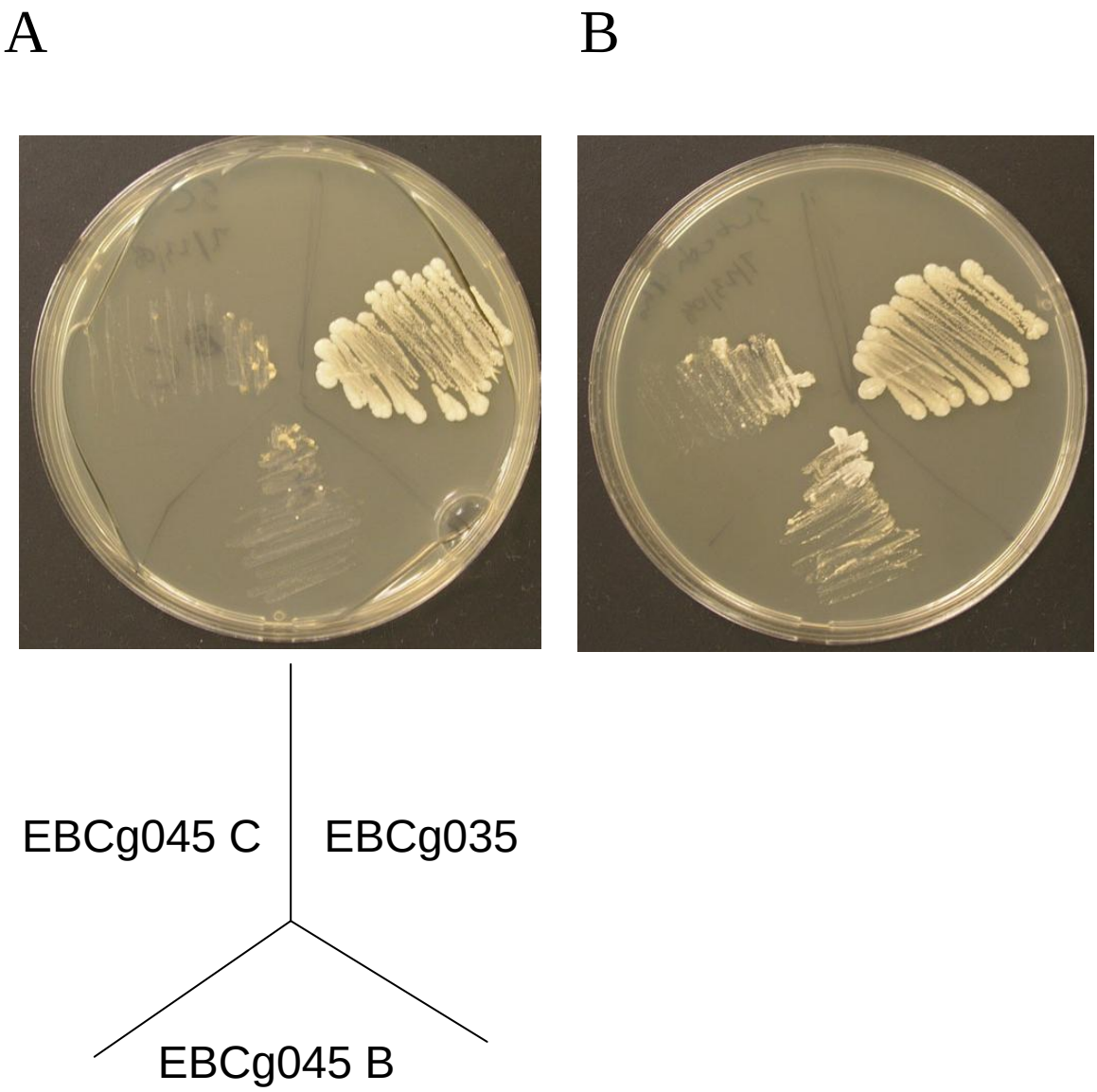


Figure A. 7. *CgCHO1Δ* mutant is an ethanolamine and choline auxotroph.

Plated on SC media without $CuSO_4$. EBCg045 B and C are two isolates that carry integrating plasmid, pEB51, which has *CgCHO1* fused to the MtII promoter. A.) 0 µm ethanolamine and 0µm choline. B.) 5µm ethanolamine and 5µm choline.

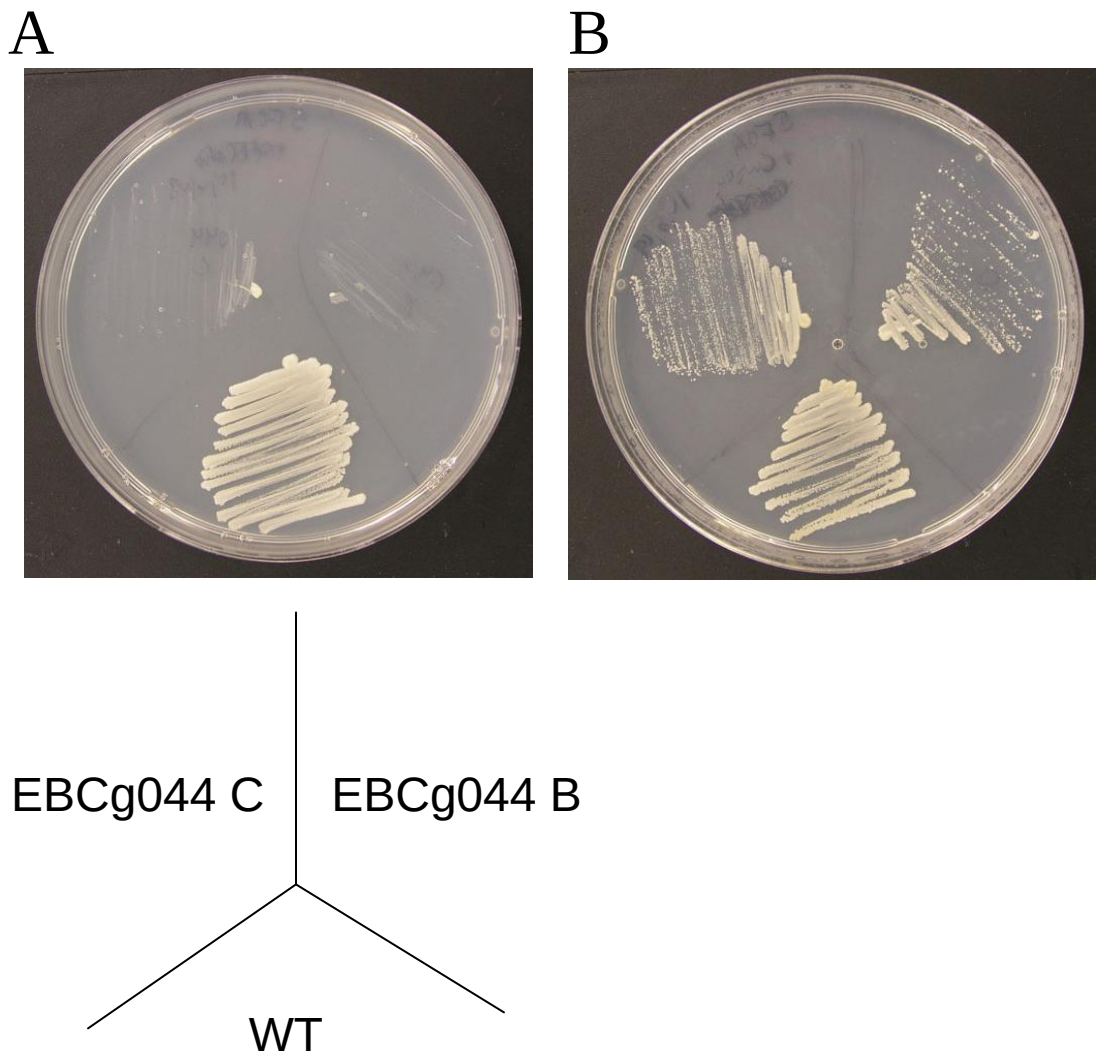


Figure A. 8. MtII promoter controls expression of *CgCHO1*

.

C. glabrata strain containing integrating plasmid pEB51 carrying the MtII promoter fused to *CgCHO1* along with *CgCHO1* on an episomal plasmid, pEB41, containing the *URA3* gene. Plated on 5-FOA. A.) 0µm $CuSO_4$. B.) 50µm $CuSO_4$.

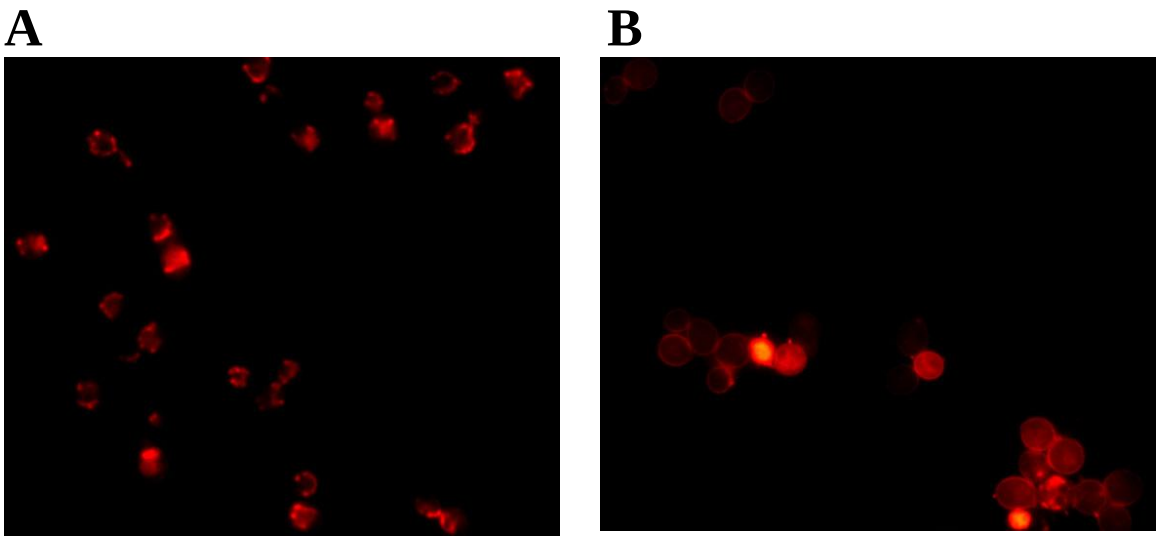


Figure A. 9. Fluorescence microscopy of cells stained with Mito-tracker.

A.) Cg1 wild-type cells. B.) *Cgcho1Δ* mutant.

Table 4. Plasmids used for data in Chapter III.

Plasmids	Type	Description
pEB9	episomal	MtII-*CgOPI1*, *HIS3*, CgCEN/ARS, AmpR
pEB37	integrating—Sma1	MtII-*CgOPI1*, *HIS3*, AmpR
pEB41	Episomal	CgCEN/ARS, AmpR, URA3, CgCHO1 ORF + 5',3' NCRs
pEB51	integrating	MtII*CgCHO1*, *HIS3*, AmpR

Table 5. Strains used for data in Chapter III.

Strain	Genotype
EBCg044	ura3Δ::Tn903 NeoR, his3Δ, pEB41, pEB51, CgCHO1::NatR
EBCg045	Ura3Δ::Tn903 NeoR, his3Δ, CgCHO1::NatR, pEB51
EBCG035	Ura3Δ::Tn903 NeoR, his3Δ, pEB41, CgCHO1::NatR
EBCg049	Ura3Δ::Tn903 NeoR, his3Δ, pEB37, CgOPI1::NatR, pCgOPI1—URA3
EBCg039-42	Petite mutants

References

1. Kwon-Chung, K.J. and J.E. Bennett, *Medical Mycology*. 1992, Philadelphia: Lea & Febiger.
2. Calderone, R.A., ed. *Candida and Candidiasis*. 2002, ASM Press: Washington D.C.
3. Barnett, J.A., *A history of research on yeasts 12: medical yeasts part 1, Candida albicans.* Yeast, 2008. **25**(6): p. 385-417.
4. Fisher-Hoch, S.P. and L. Hutwagner, *Opportunistic candidiasis: an epidemic of the 1980s.* Clin Infect Dis, 1995. **21**(4): p. 897-904.
5. Pfaller, M.A. and D.J. Diekema, *Epidemiology of invasive candidiasis: a persistent public health problem.* Clin Microbiol Rev, 2007. **20**(1): p. 133-63.
6. Li, L., S. Redding, and A. Dongari-Bagtzoglou, *Candida glabrata: an emerging oral opportunistic pathogen.* J Dent Res, 2007. **86**(3): p. 204-15.
7. Fidel, P.L., Jr., J.A. Vazquez, and J.D. Sobel, *Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans.* Clin Microbiol Rev, 1999. **12**(1): p. 80-96.
8. Odds, F.C., et al., *Candida and Torulopsis: a blinded evaluation of use of pseudohypha formation as basis for identification of medically important yeasts.* J Clin Microbiol, 1997. **35**(1): p. 313-6.
9. Csank, C. and K. Haynes, *Candida glabrata displays pseudohyphal growth.* FEMS Microbiol Lett, 2000. **189**(1): p. 115-20.
10. Bialkova, A. and J. Subik, *Biology of the pathogenic yeast Candida glabrata.* Folia Microbiol (Praha), 2006. **51**(1): p. 3-20.
11. Hull, C.M. and A.D. Johnson, *Identification of a mating type-like locus in the asexual pathogenic yeast Candida albicans.* Science, 1999. **285**(5431): p. 1271-5.
12. Tzung, K.W., et al., *Genomic evidence for a complete sexual cycle in Candida albicans.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3249-53.
13. Miller, M.G. and A.D. Johnson, *White-opaque switching in Candida albicans is controlled by mating-type locus homeodomain proteins and allows efficient mating.* Cell, 2002. **110**(3): p. 293-302.
14. Wong, S., et al., *Evidence from comparative genomics for a complete sexual cycle in the 'asexual' pathogenic yeast Candida glabrata.* Genome Biol, 2003. **4**(2): p. R10.
15. Kaur, R., et al., *A yeast by any other name: Candida glabrata and its interaction with the host.* Curr Opin Microbiol, 2005. **8**(4): p. 378-84.
16. Barns, S.M., et al., *Evolutionary relationships among pathogenic Candida species and relatives.* J Bacteriol, 1991. **173**(7): p. 2250-5.
17. Scannell, D.R., G. Butler, and K.H. Wolfe, *Yeast genome evolution--the origin of the species.* Yeast, 2007. **24**(11): p. 929-42.
18. Dujon, B., et al., *Genome evolution in yeasts.* Nature, 2004. **430**(6995): p. 35-44.
19. Cormack, B.P., N. Ghori, and S. Falkow, *An adhesin of the yeast pathogen Candida glabrata mediating adherence to human epithelial cells.* Science, 1999. **285**(5427): p. 578-82.

20. Castano, I., et al., *Telomere length control and transcriptional regulation of subtelomeric adhesins in Candida glabrata.* Mol Microbiol, 2005. **55**(4): p. 1246-58.
21. Domergue, R., et al., *Nicotinic acid limitation regulates silencing of Candida adhesins during UTI.* Science, 2005. **308**(5723): p. 866-70.
22. Kaur, R., B. Ma, and B.P. Cormack, *A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of Candida glabrata.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7628-33.
23. Naglik, J.R., S.J. Challacombe, and B. Hube, *Candida albicans secreted aspartyl proteinases in virulence and pathogenesis.* Microbiol Mol Biol Rev, 2003. **67**(3): p. 400-28, table of contents.
24. Ghannoum, M.A., *Potential role of phospholipases in virulence and fungal pathogenesis.* Clin Microbiol Rev, 2000. **13**(1): p. 122-43, table of contents.
25. d'Enfert, C. and B. Hube, eds. *Candida: Comparative and Functional Genomics*. 1 ed. Vol. 1. 2007, Caister Academic Press: Norfolk. 428.
26. Sanglard, D., et al., *The ATP binding cassette transporter gene CgCDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents.* Antimicrob Agents Chemother, 1999. **43**(11): p. 2753-65.
27. Ferrari, S., et al., *Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence.* PLoS Pathog, 2009. **5**(1): p. e1000268.
28. Scannell, D.R., et al., *Multiple rounds of speciation associated with reciprocal gene loss in polyploid yeasts.* Nature, 2006. **440**(7082): p. 341-5.
29. Tuch, B.B., et al., *The evolution of combinatorial gene regulation in fungi.* PLoS Biol, 2008. **6**(2): p. e38.
30. Ihmels, J., et al., *Rewiring of the yeast transcriptional network through the evolution of motif usage.* Science, 2005. **309**(5736): p. 938-40.
31. Steiner, M.R. and R.L. Lester, *In vitro studies of phospholipid biosynthesis in Saccharomyces cerevisiae.* Biochim Biophys Acta, 1972. **260**(2): p. 222-43.
32. Paltauf, F., S.D. Kohlwein, and S.A. Henry, eds. *Regulation and Compartmentalization of Lipid Synthesis in Yeast*. The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression, ed. E.W. Jones, J.R. Pringle, and J.R. Broach. Vol. 2. 1992, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.
33. Daum, G., et al., *Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae.* Yeast, 1998. **14**(16): p. 1471-510.
34. Lopez, F., et al., *The yeast inositol monophosphatase is a lithium- and sodium-sensitive enzyme encoded by a non-essential gene pair.* Mol Microbiol, 1999. **31**(4): p. 1255-64.
35. Ambroziak, J. and S.A. Henry, *INO2 and INO4 gene products, positive regulators of phospholipid biosynthesis in Saccharomyces cerevisiae, form a complex that binds to the INO1 promoter.* J Biol Chem, 1994. **269**(21): p. 15344-9.

36. Wagner, C., et al., *The negative regulator Opi1 of phospholipid biosynthesis in yeast contacts the pleiotropic repressor Sin3 and the transcriptional activator Ino2.* Mol Microbiol, 2001. **41**(1): p. 155-66.
37. Greenberg, M.L., B. Reiner, and S.A. Henry, *Regulatory mutations of inositol biosynthesis in yeast: isolation of inositol-excreting mutants.* Genetics, 1982. **100**(1): p. 19-33.
38. Hirsch, J.P. and S.A. Henry, *Expression of the Saccharomyces cerevisiae inositol-1-phosphate synthase (INO1) gene is regulated by factors that affect phospholipid synthesis.* Mol Cell Biol, 1986. **6**(10): p. 3320-8.
39. Donahue, T.F. and S.A. Henry, *Inositol Mutants of SACCHAROMYCES CEREVISIAE: Mapping the ino1 Locus and Characterizing Alleles of the ino1, ino2 and ino4 Loci.* Genetics, 1981. **98**(3): p. 491-503.
40. Loewen, C.J., et al., *Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid.* Science, 2004. **304**(5677): p. 1644-7.
41. Brickner, J.H. and P. Walter, *Gene recruitment of the activated INO1 locus to the nuclear membrane.* PLoS Biol, 2004. **2**(11): p. e342.
42. Bachhawat, N., Q. Ouyang, and S.A. Henry, *Functional characterization of an inositol-sensitive upstream activation sequence in yeast. A cis-regulatory element responsible for inositol-choline mediated regulation of phospholipid biosynthesis.* J Biol Chem, 1995. **270**(42): p. 25087-95.
43. Greenberg, M.L. and J.M. Lopes, *Genetic regulation of phospholipid biosynthesis in Saccharomyces cerevisiae.* Microbiol Rev, 1996. **60**(1): p. 1-20.
44. Kelley, M.J., et al., *Regulation of phospholipid biosynthesis in Saccharomyces cerevisiae by inositol. Inositol is an inhibitor of phosphatidylserine synthase activity.* J Biol Chem, 1988. **263**(34): p. 18078-85.
45. Carman, G.M. and G.M. Zeimetz, *Regulation of phospholipid biosynthesis in the yeast Saccharomyces cerevisiae.* J Biol Chem, 1996. **271**(23): p. 13293-6.
46. Klig, L.S., et al., *Coordinate regulation of phospholipid biosynthesis in Saccharomyces cerevisiae: pleiotropically constitutive opi1 mutant.* J Bacteriol, 1985. **162**(3): p. 1135-41.
47. Loewy, B.S. and S.A. Henry, *The INO2 and INO4 loci of Saccharomyces cerevisiae are pleiotropic regulatory genes.* Mol Cell Biol, 1984. **4**(11): p. 2479-85.
48. Hancock, L.C., R.P. Behta, and J.M. Lopes, *Genomic analysis of the Opi- phenotype.* Genetics, 2006. **173**(2): p. 621-34.
49. Reynolds, T.B., *The Opi1p Transcription Factor Affects Expression of FLO11, Mat Formation, and Invasive Growth in Saccharomyces cerevisiae.* Eukaryot Cell, 2006. **5**(8): p. 1266-75.
50. Hirakawa, K., et al., *Yas3p, an Opi1-family transcription factor regulates cytochrome P450 expression in response to n-alkanes in Yarrowia lipolytica.* J Biol Chem, 2009.
51. Calderone, R.A., *Candida and Candidiasis*. 2002, Washington, DC: ASM Pewaa. 451.

52. Coleman, D.C., et al., *Importance of Candida species other than Candida albicans as opportunistic pathogens.* Med Mycol, 1998. **36 Suppl 1**: p. 156-65.
53. Pfaller, M.A. and D.J. Diekema, *Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of Candida.* Clin Microbiol Infect, 2004. **10 Suppl 1**: p. 11-23.
54. Piarroux, R., et al., *Are live saccharomyces yeasts harmful to patients?* Lancet, 1999. **353**(9167): p. 1851-2.
55. Martchenko, M., et al., *Transcriptional rewiring of fungal galactose-metabolism circuitry.* Curr Biol, 2007. **17**(12): p. 1007-13.
56. Tsong, A.E., et al., *Evolution of a combinatorial transcriptional circuit: a case study in yeasts.* Cell, 2003. **115**(4): p. 389-99.
57. Hoppen, J., et al., *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, 2007. **278**(3): p. 317-30.
58. Kreger-vav Rij, N.J.W., *The yeast-a taxonomic study*. 3rd ed. 1984, Amsterdam: Elsevier.
59. Chen, M., L.C. Hancock, and J.M. Lopes, *Transcriptional regulation of yeast phospholipid biosynthetic genes.* Biochim Biophys Acta, 2007. **1771**(3): p. 310-21.
60. Strahl, T. and J. Thorner, *Synthesis and function of membrane phosphoinositides in budding yeast, Saccharomyces cerevisiae.* Biochim Biophys Acta, 2007. **1771**(3): p. 353-404.
61. Dickson, R.C. and R.L. Lester, *Yeast sphingolipids.* Biochim Biophys Acta, 1999. **1426**(2): p. 347-57.
62. Majumder, A.L., M.D. Johnson, and S.A. Henry, *1L-myo-inositol-1-phosphate synthase.* Biochim Biophys Acta, 1997. **1348**(1-2): p. 245-56.
63. Graves, J.A. and S.A. Henry, *Regulation of the yeast INO1 gene. The products of the INO2, INO4 and OPI1 regulatory genes are not required for repression in response to inositol.* Genetics, 2000. **154**(4): p. 1485-95.
64. Lopes, J.M. and S.A. Henry, *Interaction of trans and cis regulatory elements in the INO1 promoter of Saccharomyces cerevisiae.* Nucleic Acids Res, 1991. **19**(14): p. 3987-94.
65. Nikoloff, D.M. and S.A. Henry, *Functional characterization of the INO2 gene of Saccharomyces cerevisiae. A positive regulator of phospholipid biosynthesis.* J Biol Chem, 1994. **269**(10): p. 7402-11.
66. Schwank, S., et al., *Yeast transcriptional activator INO2 interacts as an Ino2p/Ino4p basic helix-loop-helix heteromeric complex with the inositol/choline-responsive element necessary for expression of phospholipid biosynthetic genes in Saccharomyces cerevisiae.* Nucleic Acids Res, 1995. **23**(2): p. 230-7.
67. Heyken, W.T., et al., *Constitutive expression of yeast phospholipid biosynthetic genes by variants of Ino2 activator defective for interaction with Opi1 repressor.* Mol Microbiol, 2005. **56**(3): p. 696-707.

68. Jiranek, V., J.A. Graves, and S.A. Henry, *Pleiotropic effects of the opi1 regulatory mutation of yeast: its effects on growth and on phospholipid and inositol metabolism.* Microbiology, 1998. **144 (Pt 10)**: p. 2739-48.
69. Loewen, C.J., A. Roy, and T.P. Levine, *A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP.* Embo J, 2003. **22**(9): p. 2025-35.
70. Loewen, C.J. and T.P. Levine, *A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins.* J Biol Chem, 2005. **280**(14): p. 14097-104.
71. Cormack, B.P. and S. Falkow, *Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen Candida glabrata.* Genetics, 1999. **151**(3): p. 979-87.
72. Frieman, M.B., J.M. McCaffery, and B.P. Cormack, *Modular domain structure in the Candida glabrata adhesin Epa1p, a beta1,6 glucan-cross-linked cell wall protein.* Mol Microbiol, 2002. **46**(2): p. 479-92.
73. Goldstein, A.L. and J.H. McCusker, *Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae.* Yeast, 1999. **15**(14): p. 1541-53.
74. Boeke, J.D., et al., *5-Fluoroorotic acid as a selective agent in yeast molecular genetics.* Methods Enzymol, 1987. **154**: p. 164-75.
75. Nakayama, H., et al., *A controllable gene-expression system for the pathogenic fungus Candida glabrata.* Microbiology, 1998. **144 (Pt 9)**: p. 2407-15.
76. El Barkani, A., et al., *Candida glabrata shuttle vectors suitable for translational fusions to lacZ and use of beta-galactosidase as a reporter of gene expression.* Gene, 2000. **246**(1-2): p. 151-5.
77. Bailis, A.M., et al., *Cis and trans regulatory elements required for regulation of the CHO1 gene of Saccharomyces cerevisiae.* Nucleic Acids Res, 1992. **20**(6): p. 1411-8.
78. Jackson, J.C. and J.M. Lopes, *The yeast UME6 gene is required for both negative and positive transcriptional regulation of phospholipid biosynthetic gene expression.* Nucleic Acids Res, 1996. **24**(7): p. 1322-9.
79. Lai, K. and P. McGraw, *Dual control of inositol transport in Saccharomyces cerevisiae by irreversible inactivation of permease and regulation of permease synthesis by INO2, INO4, and OPI1.* J Biol Chem, 1994. **269**(3): p. 2245-51.
80. Li, Z. and M. Brendel, *Co-regulation with genes of phospholipid biosynthesis of the CTR/HNM1-encoded choline/nitrogen mustard permease in Saccharomyces cerevisiae.* Mol Gen Genet, 1993. **241**(5-6): p. 680-4.
81. Rothstein, R., *Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast.* Methods Enzymol, 1991. **194**: p. 281-301.
82. Sikorski, R.S. and P. Hieter, *A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae.* Genetics, 1989. **122**(1): p. 19-27.
83. Styles, C., *How to set up a yeast laboratory.* Methods Enzymol, 2002. **350**: p. 42-71.

84. Care, R.S., et al., *The MET3 promoter: a new tool for Candida albicans molecular genetics.* Mol Microbiol, 1999. **34**(4): p. 792-8.
85. Movahedzadeh, F., et al., *The Mycobacterium tuberculosis ino1 gene is essential for growth and virulence.* Mol Microbiol, 2004. **51**(4): p. 1003-14.
86. Birner, R., et al., *Roles of phosphatidylethanolamine and of its several biosynthetic pathways in Saccharomyces cerevisiae.* Mol Biol Cell, 2001. **12**(4): p. 997-1007.
87. Fitzpatrick, D.A., et al., *A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis.* BMC Evol Biol, 2006. **6**: p. 99.

Vita

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