Azole susceptibility and transcriptome profiling in the *Candida albicans* mitochondrial electron transport chain complex I mutants

by

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Running title: mitochondria, energy and azole susceptibility

Keywords: fluconazole, mitochondria, Complex I, RNA-Seq.
Abstract

Mitochondria dysfunction in pathogenic fungi or model yeast causes altered susceptibilities to antifungal drugs. Herein, we have characterized the role of mitochondria complex I (CI) of Candida albicans in antifungal susceptibility. Inhibitors of CI-CV except for CII increased the susceptibility of both patient and lab isolates, even those with a resistance phenotype. In addition, from a C. albicans library of 12 CI null mutants, 10 displayed a hypersusceptibility to fluconazole and were severely growth inhibited on glycerol implying a role for each gene in cell respiration. We chose two other hypersusceptible null mutants of C. albicans, (goa1Δ and ndh51Δ) for transcriptional profiling using RNA-seq. Goa1p is required for CI activity, while Ndh51p is a CI subunit. RNA-Seq revealed that both ndh51Δ and especially goa1Δ had significant down regulation of transporters including CDR1 and CDR2 that encode efflux proteins. In goa1Δ, down-regulation was noted of genes required for the biogenesis and replication of peroxisomes as well as metabolic pathways assigned to peroxisomes such as β-oxidation of fatty acids, the glyoxylate bypass, and acetyl CoA transferases that are known to shuttle acetyl-CoA between peroxisomes and mitochondria. The transcriptome profile of ndh51Δ did not include down regulation of peroxisome genes but had, instead, extensive down regulation of the ergosterol synthesis gene family. Our data establish that cell energy is required for azole susceptibility and that down regulation of efflux genes may be an outcome of that dysfunction. However, there are mutant-specific changes that may also increase susceptibility of both mutants to azoles in Candida species.
INTRODUCTION

Global infectious diseases caused by fungi represent a major health problem (32). New therapies, whether to potentiate immunity or kill pathogens with antifungal drugs, are slow in development. The ideal antifungal agent should have broad fungicidal activity, not select for resistant strains, and have minimal adverse effects. None of the current drugs fulfill all or even some of the criteria for an ideal agent. The azole antifungals including the newer triazoles target the ergosterol pathway by inhibiting the Erg11p 14-α-demethylase protein. Problems with their use include the selection of resistant strains due to their fungistatic property. Consequently, inherently resistant Candida spp have become more common among clinical isolates during treatment (32). Azole resistance in C. glabrata has resulted in a modification of break points for the standardized CLSI susceptibility assay to reflect its reduced susceptibility (32).

Resistance to fluconazole among Candida spp. occurs through several mechanisms or combinations of mechanisms. The Erg11p target may be overexpressed or key mutations in Erg11p may result in reduced binding of drug. The latter problem also appears to be similar to the “hot spot” mutations in the Fks1p (β-1,3-glucan synthase) observed in echinocandin-resistant strains of C. glabrata (31). Also, an inability of drugs to penetrate biofilms contributes to resistance (15). Triazole resistance also occurs as a result of an upregulation of multidrug efflux transporters of two gene families, the ABC transporters (ATP-binding cassette) and the major facilitator gene superfamily (MFS). C. albicans has two CDR genes (CDR1 and CDR2) and a single MDR1 (Candida drug
resistance/multiple drug resistance) that is not related to MFS genes of other organisms. The major facilitator efflux pump superfamily proteins transport small solutes in response to chemiosmotic ion gradients (29). Regulation of efflux protein expression can also occur by gain-of-function mutations in the transcription factors Tac1p and Mrr1p that is associated with greater Cdr1p/Cdr2p or Mdr1p expression, respectively, in resistant cells (8, 11).

An association of decreased fluconazole susceptibility with mitochondria dysfunction in C. glabrata has been reported by several laboratories (4, 5, 14, 20, 35, 44). Ferrari et al., (14) demonstrated that a mitochondrial deficiency in C. glabrata strain BPY41 caused resistance to azoles associated with an upregulation of the ABC transporter genes, CgCDR1, CgCDR2, and CgSNQ2. Strains BYP40 (azole sensitive) and BYP41 (azole resistant), both related and isolated from the same patient, were compared to determine if mitochondrial dysfunction conferred a selective advantage in virulence. While displaying a growth defect in vitro, strain BYP41 (a petite mutant) was more virulent in mice infections. The gain of fitness in strain BYP41 was an apparent result of several gene changes including increased oxidoreductive metabolism, carbohydrate metabolism, and stress responses, while genes associated with mitochondrial functions were down regulated in microarray analyses. Kaur et al., (20) screened a transposon mutant library of C. glabrata for strains altered in azole susceptibility. They identified several such mutants with altered susceptibility that were functionally associated with retrograde signaling from the mitochondria to the nucleus (CgRTG2, increased susceptibility) or mitochondrial biogenesis.
(CgSUV3, CgMRP14, and CgSHE9, decreased susceptibility). In *S. cerevisiae*, dysfunctional mitochondria activate the pleiotropic drug resistance pathway for adaptation (17).

While the relationship of mitochondrial functions toazole susceptibility has been extensively studied in *C. glabrata*, much less has been reported from *C. albicans*. Cheng *et al.*, (7) described a petite *C. albicans* (strain J5) whose decrease in susceptibility was noted to fluconazole and voriconazole but not itraconazole. The petite was originally isolated from wild type SC5314 *C. albicans* by serial passage through mice spleens and then shown to have uncoupled oxidative phosphorylation. Other *C. albicans* petite strains have been described but most are induced by harsh chemical treatments and therefore, correlations of specific mitochondria dysfunctions with azole resistance are more difficult to interpret (1,2,34). Recently, knockout strains of *C. albicans* have been constructed that lack either *GOA1* or *NDH51* (3,21,24,46). Goa1p is required for optimum electron transport chain (ETC) CI activity. Gene-deleted strains accumulate ROS, undergo apoptosis and are avirulent. *NDH51* encodes the 51-kDa subunit of the NADH dehydrogenase CI of the ETC whose deletion results in morphogenesis defects.

Our hypothesis is that mitochondria dysfunction, especially related to energy production, can potentiate azole susceptibility in *C. albicans* and other fungi. If verified, combination therapy with a fungal-specific anti-mitochondrial compound could be effective in extending the usefulness of the triazole antifungal drugs. Using specific inhibitors of the ETC complexes (CI-CV), CI mutant libraries, as
well as the NDH51 (ndh51Δ) and GOA1 (goa1Δ) knockout strains described above, we verify this hypothesis. We also look globally at mutant strains by RNA.seq analyses to define transcriptional changes that may impact susceptibility to triazoles.

MATERIALS AND METHODS

Strains, strain maintenance, and plasmids. All strains used in the present study are listed in Supplementary Table S1 and were maintained as frozen stocks in 96-well plates and propagated on yeast extract-peptone-dextrose (YPD) agar when needed (1% yeast extract, 2% peptone, 2% glucose, 2% agar). The goa1Δ and ndh51Δ nulls and their gene-reconstituted strains goa1/GOA1 and ndh51/NDH51 were grown similarly.

Antifungal Agents. Piericidin A (PdA) was purchased from Enzo life sciences. C12E8 was purchased from Affymetrix, Inc. Other ETC complex inhibitors as well as the azole antifungals were purchased from Sigma Chemical Co. These compounds were reconstituted according to the manufacturers’ directions.

Antifungal susceptibility testing. Drug susceptibility testing was carried out in flat bottom, 96-well microtiter plates (Greiner Bio One) using the broth microdilution protocol according to the Clinical and Laboratory Standards Institute M-27A methods. In addition, all strains were evaluated for growth in the presence of fluconazole and growth reported as a % of untreated cells. Overnight cultures
were prepared in YPD, washed, and \( \sim 10^3 \) cells/10 µl were inoculated into microtiter wells. CI-CV ETC inhibitors (Sigma-Aldrich) including rotenone (10µg/ml), C12E8 (4µg/ml), PdA (4µg/ml), TTFA (10µg/ml), antimycin A (10µg/ml), KCN (10µg/ml), and oligomycin (10µg/ml) were used alone or in combination with fluconazole (13,28). Growth was evaluated by measuring cell density OD<sub>595</sub> after 24h of incubation at 30°C. Experiments were repeated at least three times. Data were averaged and statistical significance among treatments determined.

MIC determinations were also done with lab stock cultures of azole susceptible and resistant strains. For these experiments, two of the most active inhibitors (C12E8 and PdA) from the previous screen were used with 0.2, 4, or 32 µg/ml concentrations of fluconazole. Strains were grown as described above. Relative growth was calculated based on OD<sub>595</sub> data and visualized using heatmap.

**Screens of C. albicans CI mutants for fluconazole susceptibility.**

Putative mitochondrial ETC complex I (CI) proteins of C.albicans were identified through the Candida Genome database using FASTA and BLASTP. All protein alignments were manually reviewed. From a morphogenesis mutant library provided by Noble and Johnson (27), 12 putative CI mutants were grown overnight in YPD broth, then plated on YPD agar medium containing fluconazole or on YPG agar plates (1% yeast extract, 2% peptone, 3% glycerol, 2% agar) to evaluate growth on a non-fermentable carbon source. Plates were incubated at 30°C for 48 h and photographed.
Drop plate assays with antifungal drugs. The susceptibility of all strains to antifungal drugs was tested by plating 5 µl of serial dilutions of 1-10 to 5x10⁴ cells onto YPD agar plates containing antifungal drugs. Yeast cells were obtained from overnight cultures grown in YPD broth at 30°C, washed with saline, and standardized by hemacytometer counts. Growth of each strain was evaluated after 48 h of incubation at 30°C.

Rapid adaptation to fluconazole, defined as growth that occurs within 48 h of treatment, was also examined. Strains of C. albicans were grown overnight at 30°C in synthetic complete (SC) medium, washed with saline, and about 10⁴ cells spread on SD agar medium with/without 128 ug/ml of fluconazole. Cultures were incubated at 30°C for 4 and 6 days and colony counts were determined. For these experiments, knock-out strains in cdr1Δ/cdr2Δ, tac1Δ, and strains that over expressed CDR1 and MDR1 were used along with goa1Δ, ndh51Δ, goa1/GOA1, and ndh51/NDH51. Goa1p is required for ETC CI activity, while Ndh51p is a subunit of the ETC CI.

RNA preparation and next-generation sequencing. Total RNA was extracted and submitted to Otogenetics Corporation (Norcross, GA) for RNA-Seq assays. In brief, the integrity and purity of total RNA were assessed using Agilent Bioanalyzer (Agilent Technologies) and OD₂₆₀/₂₈₀. One - two µg of cDNA was generated using Clontech SmartPCR cDNA kit (Clontech Laboratories Inc., Mountain View, CA, catalog# 634925) from 100 ng of total RNA. cDNA was fragmented using Covaris (Covaris, Inc., Woburn, MA., USA), profiled using Agilent Bioanalyzer, and subjected to Illumina library preparation using NEBNext
reagents (New England Biolabs, Ipswich, MA., catalog# E6040). The quality and quantity and the size distribution of the Illumina libraries were determined using an Agilent Bioanalyzer 2100. The libraries were then submitted for Illumina HiSeq2000 according to the standard operation. Paired-end 90 or 100 nucleotide (nt) reads were generated and subjected to data analysis using the platform provided by DNAnexus (DNAnexus, Inc, Mountain View, CA). Total reads of 11,279,512, 10,906,588 and 10,630,834 were obtained for wild type (SC5314), goa1Δ, and ndh51Δ, respectively.

All samples were mapped to C._albicans_SC5314_A21 with TopHat 1.3.3, and then analyzed with Cufflinks 1.2.1 for expression level of genes and isoforms. Comparison of expression levels was conducted with Cuffdiff. We compared C._albicans SC5314 with goa1Δ and ndh51Δ as well as both mutants to each other.

RNA.seq data of selected genes were validated with real time PCR.

Relative quantification of differentially expressed genes by real time PCR. Each C._albicans strain was grown overnight in 10-ml of YPD medium at 30°C. For RNA extractions, cultures were diluted to 10^8 cells per ml in 20 ml of fresh YPD medium and were grown at 30°C for 2 h and collected for subsequent RNA isolation. Total RNA was extracted using Trizol following a phosphate-buffered-saline (PBS) wash. The quality and concentration of RNAs were measured with a nano-spectrophotometer, and approximately 1 µg of the total RNA was subjected to first-strand cDNA synthesis (QuantiTect Reverse Transcription, Qiagen). Real-time PCR assays were performed with 20-µl reaction volumes that contained 1x iQ SyBR green Supermix (Bio-Rad), including
a 0.2 µM concentration of each primer (Supplemental Table S2) and 8 µl of a 1:8 dilution of each cDNA from each strain. Quantitative reverse transcription-PCR (qRT-PCR) for each experiment was performed in triplicate using Bio-Rad iQ, and the transcription level of each gene was normalized to 18S RNA levels. Data are presented as the means ± standard deviations (SD). The $2^{-\Delta\Delta CT}$ (where $C_T$ is the threshold cycle) method of analysis was used to determine the fold change in gene transcription.

**Efflux of rhodamine 6G.** Approximately 1X10^6 yeast cells from each overnight culture were transferred to YPD medium and allowed to grow for 4 h. Cells were pelleted, washed twice with PBS buffer (pH 7.0, without glucose) and suspended in glucose-free PBS to 10^8 cells per ml. Cell suspensions were incubated at 30°C with shaking (200 rpm) for 120 min under glucose starvation conditions. The deenergized cells were pelleted, washed, and then suspended in glucose-free PBS to 10^8 cells per ml, to which rhodamine 6G was added at a final concentration of 10 µM. Cells were incubated for 30 min at 30°C, then washed twice, and suspended in glucose-free PBS to 10^8 cells per ml. At 10 min intervals, cells (1-ml volumes) were removed by centrifugation and absorption of triplicate 100-µl volume supernatants measured at 527 nm. Energy-dependent efflux was measured after the addition of 2% glucose (final concentration). Glucose-free controls were included in all experiments. Effluxed rhodamine 6G was calculated from a concentration curve of R6G.
RESULTS

Fluconazole susceptibility of *C. albicans* is increased by ETC Complex inhibitors. We initially compared the susceptibility of *C. albicans* SC5314 to fluconazole in the absence or presence of mitochondrial CI-V inhibitors. Included were CI inhibitors pdA (class I/A-type CI inhibitor), rotenone (class II/B-type CI inhibitor) and C12E8 (Type C CI inhibitor), TTFA (CII inhibitor), antimycin (CIII inhibitor), KCN (CIV inhibitor), and oligomycin (CV inhibitor). We observed that all inhibitors increased fluconazole susceptibility, except for TTFA (CII inhibitor) which had a minor affect (one-dilution) (*Supplemental Table S3*). The low activity of the CII inhibitor could be due to the lower amount of ATP produced via oxidoreduction of CII substrates. Of the CI inhibitors, C12E8 increased susceptibility to 0.063 μg/ml of fluconazole (MIC<sub>80</sub>), which is significantly higher than CII, CIII, CIV, and CV inhibitors. These data suggest that reduced ETC complex activity (but not CII) increases susceptibility of *C. albicans* to fluconazole.

CI inhibitors increase fluconazole hypersusceptibility of *C. albicans* lab and patient isolates. C128E or PdA CI inhibitors were assayed in combination with 0.2, 4, or 32 μg/ml of fluconazole. Controls consisted of inhibitors or fluconazole used alone (*Figure 1*). Increased susceptibility of 14 of 42 strains to fluconazole was noted when cells were incubated in the presence of PdA or C12E8 and 0.2 μg/ml of fluconazole compared to 4 strains incubated only with 0.2 μg/ml of fluconazole. The number of hypersusceptible strains increased as the concentration of fluconazole was increased to 4 and 32 μg/ml and in the
presence of either CI inhibitor. At 32 μg/ml fluconazole, a higher number of CDR over expressed isolates was hypersusceptible in the presence of PdA than C12E8. Both inhibitors were about equally effective against MDR over expressed isolates at the same concentration of fluconazole. Susceptibility was much lower in cultures treated with either inhibitor alone. These data demonstrate that susceptibility to a variety of C. albicans strains, including those that are resistant, can be increased in the presence of ETC CI inhibitors.

C. albicans mitochondrial CI null strains, fluconazole susceptibility and growth on glycerol. To further verify the enhanced fluconazole susceptibility in the presence of CI inhibitors, the susceptibility of C. albicans CI mutants to fluconazole was examined. From a library of C. albicans homozygous deletion mutants, we selected 12 lacking putative CI subunit orthologs (27). Each of the 12 mutants as well as control strains SN250 and SC5314 were grown on YPD or YPD + 16 ug/ml of fluconazole and on YP-glycerol (YPG) alone (Figure 2). Control strains SN250 and SC5314 grew on YPD alone or YPD containing 16 µg/ml of fluconazole and on YPG. Of the 12 putative CI mutants, 10 displayed hypersusceptibility to 16 µg/ml of fluconazole in YPD agar compared to control strains. Nine of the 10 CI hypersusceptible mutants did not grow on YP-glycerol (YPG) medium, demonstrating that a mitochondrial respiratory deficiency was associated with fluconazole hypersensitivity. Two CI mutants (orf19.3611 and orf19.3290) that had fluconazole susceptibilities like control strains grew on YPG agar. Based upon the growth or lack of growth on YPG, we conclude that loss of respiratory activity is associated with fluconazole hypersensitivity in most CI
mutants. The orf19.4758 mutant had increased susceptibility to fluconazole but
grew on YPG agar indicating it may not be directly involved in cell respiration
(Figure 2). Growth in the presence of glycerol may indicate that orf19.3611 and
orf19.3290 also are associated with non-respiratory mitochondrial activities.
Importantly, of these complex proteins, orf19.287 and orf19.6607 are fungal-
specific and are attenuated or avirulent (27). The others are orthologs of
mammalian mitochondrial CI proteins.

**C. albicans CI-knockout strains and azole susceptibility.** The *C. albicans*
GOA1 and NDH51 have been linked to mitochondrial functions (3,21,24,46).
Goa1p is required for optimum CI activity, while Ndh51p is a subunit of CI. The
goa1Δ has a severe deficiency in mitochondrial membrane potential and ATP
formation, overproduces reactive oxidant species (ROS), has a shorter
chronological aging pattern associated with apoptotic events, and has reduced CI
activity (3,21). Ndh51p is a subunit protein of CI and has impaired
morphogenesis (24). The association of both genes with CI functions led us to
study their antifungal susceptibility in vitro (Figure 3).

In comparison to wild type and *goa1*/GOA1, *goa1Δ* was hypersensitive on
drop plates at 16 μg/ml of fluconazole as well as itraconazole (0.4 μg/ml),
ketoconazole (0.8μg/ml), and miconazole (1.0 μg/ml, not shown) (Figure 3A) but
equal in sensitivity to wild type cells in YPD + 5-flucytosine, amphotericin B, or
micafungin (Supplementary Figure S1). The *goa1Δ* null was also growth
inhibited in the presence of fluconazole compared to control strains (Figure 3B).
The *ndh51Δ* was also hypersensitive to fluconazole (16 μg/ml) when compared
to the gene reconstituted (*ndh51/NDH51*) and wild type strains (**Figure 3C**). Both null strains are unable to grow in YP-glycerol indicating their role in mitochondrial respiration (data not shown). Thus, hypersusceptibility to azoles is associated with deletions of ETC CI genes or inhibition of all ETC complexes except CII.

**Adaptation to fluconazole is reduced in null strains.** When large numbers of cells are plated on a high concentration of fluconazole, a rapid adaptation to decreased susceptibility has been observed in wild type cells (10). At a concentration of 128 μg/ml of fluconazole in YPD, adaptation occurred by 4-6 days in wild type (SC5314), *MDR1* and *CDR1/CDR2* over expressing strains, and the gene-reconstituted strains (*goa1/GOA1, ndh51/NDH51*) (**Figure 4**). However, *goa1Δ, ndh51Δ, tac1Δ, and cdr1/2Δ* were unable to adapt to high drug concentrations by 4 or 6 days of incubation (no increase in colony numbers) (**Figure 4**).

**RNA-Seq.** We used RNA-Seq to define transcriptional profiles of *goa1Δ* and *ndh51Δ*. For *goa1Δ*, a total of 388 genes were down regulated four-fold or higher. Gene ontology (GO) analysis of the five highest categories of down regulated genes in this mutant was associated with transmembrane transporter activity (13%), amino acid metabolism (9%), carbohydrate metabolism, (7%), mitochondria (4%), and peroxisomal functions such as β-oxidation (3%) (**Figure 5**). A significant GO term enrichment (P-value 3.93e⁻⁷) of transporter genes was calculated for those with more than a 4-fold decreased expression in *goa1Δ*. The ABC family of proteins was among the down-regulated transporter genes in
goa1Δ, suggesting that deletion of GOA1 perhaps caused a significant change in membrane fluidity or that reduced ATP levels in the null mutant may partially explain the down regulation of energy-requiring transporters (3,21).

The transcriptional profile of the ndh51Δ suggests similarities as well as differences compared to goa1Δ (Figure 6). Like goa1Δ, in ndh51Δ, a large number of transporter genes were also down regulated (Supplemental Figure S2), although the relative abundance was less than in goa1Δ (13% versus 8%). The peroxisomal gene cluster was about 4-fold less in ndh51Δ than in goa1Δ. However, the mitochondrial cluster of genes was 2-fold greater in ndh51Δ, while the sterol synthesis cluster was 4-times greater in ndh51Δ than in goa1Δ (discussed below).

**Efflux pumps and susceptibility.** The ABC drug transporters were represented among the down regulated transporters for both mutants. Their association with azole resistance is well established (19,42,43). To verify that the hypersusceptibility of goa1Δ and ndh51Δ was associated with a down regulation of CDR1 and CDR2 drug efflux transporters, we used real time PCR to measure transcription of these efflux pumps, CDR1, CDR2 and MDR1. Transcription of the ABC family members CDR1 and CDR2 but not MDR1 was significantly reduced in goa1Δ and ndh51Δ compared to wild type (SC5314) and the gene reconstituted goa1/GOA1 and ndh51/NDH51 (Figure 7).

Next, rhodamine 6G, a substrate for CDR pumps, was used to measure efflux activity in the same strains as well as an overexpressing CDR strain. Without glucose, minimal efflux of rhodamine 6G was observed in all strains. Upon the
addition of glucose to provide an energy source, an increased export of rhodamine 6G resulted over the next 60 min especially in the CDR1/2-overexpressing strain (Figure 8). In addition to the CDR1/CDR2 over expressing strain, the gene reconstituted and wild type strains also displayed high efflux activity. In comparison, both null mutants (goa1Δ and ndh51Δ) were unable to transport rhodamine 6G compared to wild type and the gene-reconstituted strains. These data point to a deficiency in rhodamine 6G transport that apparently relates in part to the lack of transcription of CDR1/2.

Other transcriptional clusters: peroxisomes and mitochondria are affected. Down regulation of a number of peroxisomal and mitochondrial genes was noted in goa1Δ (Tables 1 and 2). Peroxisomes are the cell sites of β-oxidation of fatty acids, gluconeogenesis, the glyoxylate bypass pathway, and the breakdown of peroxides (12). Many of these genes were down regulated (Table 1). Also, in goa1Δ, 15 genes, associated with either peroxisome biogenesis or replication (PEX genes), were down regulated (Table 2). Peroxisomal genes that were strongly represented among down regulated genes in goa1Δ include PEX4 (14.3-fold) and PEX13 (4.4 fold) (Table 2). However, unlike goa1Δ, neither pex4 nor pex13 null mutants were hypersensitive to fluconazole (data not shown).

Peroxisomes are functionally connected to mitochondria activities by sharing a number of common biochemical pathways (26). We next turned to exploring genes associated with these processes. Cross-talk among peroxisomes and
mitochondria requires the transport of acetyl-CoA, which is not able to cross membranes due to its amphiphilic nature and bulkiness (38, 39, 45). Acetyl-CoA is a central intermediate in carbon metabolism, which completely depends on the shuttling molecule carnitine to enter organelles in C. albicans (38, 39). In goa1Δ, genes encoding carnitine transferases (CTN1, CAT2, CTN3, CRC1) that shuttle acetyl CoA between mitochondria and peroxisomes are down regulated (Table 1). These data perhaps indicate an inability of these two organelles to coordinate acetyl-CoA transport, pending further study. The cat2Δ of C. albicans is essential for optimum β-oxidation but not required for virulence (40). In addition, the cat2Δ mutant of C. albicans was equally sensitive to fluconazole compared to wild type cells (data not shown). Although a peroxisomal carnitine transporter(s) has not been identified, the C. albicans mitochondrial carnitine carrier protein, encoded by CRC1, was found to be associated with the mitochondrial inner membrane (6). CRC1 was down regulated 4.7 fold in goa1Δ.

Genes encoding the glyoxylate bypass enzymes are strongly down regulated in goa1Δ only. Those genes include isocitrate lyase, ICL1 (17.8-fold), malate synthase, MLS1 (14.6-fold), and ACO1 (2.6-fold) (Table 1). Furthermore, several mitochondrial genes functionally related to the glyoxylate cycle were also lower in goa1Δ, including citrate synthase, CIT1 (9.1-fold). Citrate is transported from mitochondria to peroxisomes and converted to isocitrate for the glyoxylate cycle. Malate dehydrogenase MDH1 (9.1-fold), an intermediate transporter SFC1 (42.5-fold), and fructose-1,6-bisphosphatase (FBP1), encoding a key enzyme of gluconeogenesis, which is one of the subsequent reactions of the glyoxylate cycle.
cycle, were down regulated by 4.3 fold in goa1Δ (Table 1). Interestingly, the majority of genes of the glyoxylate cycle and gluconeogenesis were reported to be highly induced during phagocytosis and required for adaptation during carbon starvation (22,23,33). Their down regulation in goa1Δ is consistent with our previous findings that the null was more readily killed by neutrophils and is avirulent (3). In C. albicans, beta-oxidation of fatty acids is confined to peroxisomes, with an increased number of isozymes compared with S.cerevisiae (38, 44). A majority of genes associated with fatty acid beta-oxidation are down regulated in goa1Δ (Table 1), indicating the inability to utilize fatty acids as a sole carbon source in this mutant. This hypothesis was confirmed by spot assays of goa1Δ on a variety of carbon sources including oleate and other non-fermentation carbon sources (Supplemental Figure S3).

In addition, other mitochondrial carrier protein genes were down regulated, including YMC2 (8.0-fold), TIM22 (7.0-fold), orf19.7267 (6.5-fold), orf19.3518 (3.9-fold) and orf19.4966 (3.6-fold) (data not shown). Thus, deletion of GOA1 may affect the transport between subcellular compartments of peroxisomes and mitochondria. Localization to mitochondria of nuclear encoded proteins requires functional mitochondrial membrane receptors and transporter systems of the outer and inner membranes. In this regard, the inner, mitochondrial cristae house the respiratory complexes, including CI-CV. Interestingly, orf19.6062.3, the ortholog of which has a role in the maintenance of mitochondrial cristae and inner membrane architecture (18), was down regulated more than 200-fold in
goa1Δ (data not shown). Furthermore, orf19.3089, with similar functions in the inner membrane structure, was down regulated 4.0 fold.

RNA-Seq analyses of goa1Δ are summarized in Figure 9 to indicate all possible interactions of proteins from peroxisomes and mitochondria that reflect the transcriptome of goa1Δ.

The transcription profile of ndh51Δ includes down regulation of the ergosterol pathway. Compared to goa1Δ, transcriptional changes in the peroxisomal genes were not among those significantly down regulated in ndh51Δ. However, genes of ergosterol synthesis that were down regulated in the null were much less represented in goa1Δ (Figure 10).

Discussion
Mitochondria are the major sites of ATP formation via the ETC Complexes I-V (CI-V). Indispensable for cell growth and macromolecular synthesis, they also are required for adaptation to ROS that is generated via electron flow from oxidoreduction reactions of the ETC. Antifungal drugs such as azoles also induce a stress response, and it appears that mitochondria are part of the adaptation network in S. cerevisiae and C. glabrata that leads to fluconazole resistance (17,30,42). In a C. glabrata petite mutant and gain-of-function mutations in CgPDR1, the CgPdr1 is singularly used to elevate the expression of the CgCDR1 efflux pump (ScPdr5) and resistance to fluconazole (30,42). However, a respiratory mutant of C. glabrata exhibited hypersusceptibility to several azoles (44).
In *C. albicans* differences exist compared to the fungi mentioned above. A *C. albicans* library was used in screens of *S. cerevisiae* mutants lacking either *PDR1* or *PDR3*, both regulators of multidrug transporter genes (9). The *C. albicans* CTA4, ASG1, and CTF1 each activated the transcription of the *S. cerevisiae* PDR5-lacZ reporter and conferred resistance to azoles. However, null mutants of each gene in *C. albicans* showed no changes inazole susceptibility and did not activate *MDR1*, *CDR1* or *CDR2* expression. Of the three genes, only ASG1 was required for growth on media containing acetate, ethanol or acetic acid. These data point to a rewiring of gene function in orthologs of *C. albicans*. ASG1 does not appear to be associated with a general defect in the assimilation of a carbon source, and in this regard, the asg1 null could assimilate glycerol (9). However, its association with mitochondria and non-respiratory functions has not been reported. CTA4 of *C. albicans* is most similar to the MRR1 transcription factor which regulates *MDR1*.

We have attempted to link portions of the transcriptional profiles of *goa1Δ* and *ndh51Δ* with their hypersusceptibility to fluconazole. Our conclusions are: 1) both mutants are down regulated in transporters, and of especial importance, the *CDR1/2* efflux drug pumps; 2) cell energy output is definitely reduced in *goa1Δ* (17), but less is known about this phenotype in *ndh51Δ*. Efflux pump activity requires energy; 3) in *goa1Δ*, a reduction in cell energy is associated with a CI dysfunction (17); 4) the down regulation of genes involved in gluconeogenesis, β-oxidation, acetyl CoA shuttling and cross-talk with mitochondria, and the glyoxylate cycle may also create unobtainable energy demands; and, 5) while
membrane defects have not been defined in either mutant, down regulation of the large number of transporters as well as genes of the ergosterol synthesis suggest membrane perturbation.

Are therapeutic implications relevant to this study? GOA1 is found only in the CTG clade of the Saccharomycotina, which includes most Candida species but not C. glabrata. Thus, an inhibitor of Goa1p would be narrow in spectrum but perhaps still useful therapeutically against most triazole-resistant Candida spp except C. glabrata. Ndh51p is highly conserved among fungi but also mammalian cells. Thus, target specificity is less rigorous in regard to this protein.

Still, there does not seem to be precedent to pursue mitochondrial proteins as anti-fungal drug targets. At least five reasons compel such a study, including: 1) at least two and probably more CI fungal specific proteins exist, and other fungal-specific targets are very likely part of the pathogen genome. 2) mitochondria are energy conduits that are needed for many cell processes; 3) conceivably, specific antifungal drugs that target mitochondria could act in synergy with triazoles; and, 4) there are new initiatives to identify compounds that attenuate or boost mitochondrial functions in cancer, neurodegenerative diseases, and type II diabetes (16,25,37,41). Assays for identifying these compounds are relatively high throughput using a two-tier system of growth in the presence of glucose and galactose, the latter to identify mitochondrial respiratory inhibitors in mammalian cells (16). In regard to fungi, readers are directed to a review on mitochondria as drug targets (36).
ACKNOWLEDGEMENTS

The authors wish to thank the Fungal Genetics Stock Center and Susan Noble for providing the C. albicans mutant library. Thanks are also given to Theodore White, Joachim Morschauser, Dominique Sanglard, Patrice LePape, and David Perlin for providing azole-resistant mutants. We wish to also thank Dr. Margaret Hostetter for providing the isogenic set of parental, gene-reconstituted and the NDH51 null. LZ is an awardee of the National Distinguished Young Scholar Program in China. Nuo Sun received a Georgetown University Graduate student grant to support this research. The authors also wish to thank the Biomedical Graduate Research Organization of the GUMC for research funds.
Table 1. RNA Seq data of genes down regulated in *goa1Δ*.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene ID(s)</th>
<th>Description</th>
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<tr>
<td><strong>β-oxidation</strong></td>
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<tr>
<td>Acyl CoA synthetase</td>
<td>orf19.7156</td>
<td>FAA2-3 2.8 Predicted acyl CoA synthetase</td>
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<tr>
<td></td>
<td>orf19.272</td>
<td>FAA21 3.9 Predicted acyl CoA synthetase</td>
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<tr>
<td></td>
<td>orf19.7379</td>
<td>FAA2 13.0 Putative acyl CoA synthetase</td>
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<tr>
<td></td>
<td>orf19.4114</td>
<td>FAA2-1 2.3 Predicted long chain fatty acid CoA ligase</td>
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<td>Acyl-CoA oxidase</td>
<td>orf19.5723</td>
<td>POX1 2.6 Predicted acyl-CoA oxidase</td>
</tr>
<tr>
<td></td>
<td>orf19.1655</td>
<td>PXN2 10.5 Putative acyl-CoA oxidase</td>
</tr>
<tr>
<td></td>
<td>orf19.1652</td>
<td>POX1-3 3.5 Predicted acyl-CoA oxidase</td>
</tr>
<tr>
<td>3-hydroxyacyl-CoA epimerase</td>
<td>orf19.1288</td>
<td>FOX2 7.4 3-hydroxyacyl-CoA epimerase, required for fatty acid beta-oxidation</td>
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<tr>
<td>Acetyl-CoA C-acyltransferase</td>
<td>orf19.7520</td>
<td>POT1 4.8 Putative peroxisomal 3-oxoacyl CoA thiolase</td>
</tr>
<tr>
<td></td>
<td>orf19.1704</td>
<td>FOX3 3.3  Putative peroxisomal 3-oxoacyl CoA thiolase</td>
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<td></td>
<td>orf19.2046</td>
<td>POT1-2 6.0  Putative peroxisomal 3-ketoacyl CoA thiolase</td>
</tr>
<tr>
<td>Dodecenoyl-CoA delta-isomerase</td>
<td>orf19.6443</td>
<td>orf19.6443 15.0 Has domain(s) with predicted catalytic activity and role in metabolic process</td>
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<td></td>
<td>orf19.6445</td>
<td>ECI1 2.8 Protein similar to S. cerevisiae Eci1p, which is involved in fatty acid oxidation</td>
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<tr>
<td>Glyoxylate cycle</td>
<td>orf19.6844</td>
<td>ICL1 17.8  Isocitrate lyase; enzyme of glyoxylate cycle; required for virulence in murine infection</td>
</tr>
<tr>
<td></td>
<td>orf19.4833</td>
<td>MLS1 14.6  Malate synthase; glyoxylate cycle enzyme; no mammalian homolog</td>
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<tr>
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<td>orf19.5323</td>
<td>MDH1-3 2.0  Predicted malate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>orf19.4393</td>
<td>CIT1 9.1  Citrate synthase</td>
</tr>
<tr>
<td></td>
<td>orf19.6385</td>
<td>ACO1 2.6  Aconitase</td>
</tr>
<tr>
<td>Carnitine acetyl transfer</td>
<td>orf19.4551</td>
<td>CTN1 27.1  Predicted carnitine acetyl transfer; required for growth on nonfermentable carbon sources</td>
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<td></td>
<td>orf19.4591</td>
<td>CAT2 3.1  Major carnitine acetyl transfer localized in peroxisomes and mitochondria; involved in intracellular acetyl-CoA transport</td>
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<td></td>
<td>orf19.2809</td>
<td>CTN3 4.8  Predicted peroxisomal carnitine acetyl transfer</td>
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<tr>
<td></td>
<td>orf19.2599</td>
<td>CRC1 4.7  Mitochondrial carnitine carrier protein</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td>orf19.7514</td>
<td>PCK1 4.9  Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>Gene</td>
<td>Fold</td>
<td>Description</td>
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<tr>
<td>-----------</td>
<td>------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
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<tr>
<td>orf19.6178</td>
<td>4.3</td>
<td>Fructose-1,6-bisphosphatase, key gluconeogenesis enzyme involved in carbohydrate metabolism</td>
</tr>
<tr>
<td>FBP1</td>
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**Table 2. Down regulated genes (PEX) of peroxisomes in goa1Δ.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEX4</td>
<td>14.3</td>
<td>Putative peroxisomal ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>CAT1</td>
<td>12.2</td>
<td>Catalase; hydrogen peroxide detoxification in the peroxisomal and mitochondrial matrix</td>
</tr>
<tr>
<td>PXA2</td>
<td>6.0</td>
<td>Putative peroxisomal, half-size adrenoleukodystrophy protein subfamily ABC transporter</td>
</tr>
<tr>
<td>PXA1</td>
<td>4.8</td>
<td>Putative peroxisomal, half-size adrenoleukodystrophy protein subfamily ABC transporter</td>
</tr>
<tr>
<td>PEX6</td>
<td>4.7</td>
<td>Ortholog(s) have protein heterodimerization activity, ATPase activity, protein import into peroxisome matrix</td>
</tr>
<tr>
<td>orf19.5575</td>
<td>4.5</td>
<td>Protein required for peroxisomal protein import mediated by PTS1 and PTS2 targeting sequences</td>
</tr>
<tr>
<td>PEX13</td>
<td>4.4</td>
<td>Ortholog(s) have 2,4-dienoyl-CoA reductase (NADPH) activity, role in ascospore formation, fatty acid catabolic process and peroxisomal matrix localization</td>
</tr>
<tr>
<td>orf19.3684</td>
<td>4.0</td>
<td>Ortholog(s) have ubiquitin-protein ligase activity, role in protein import into peroxisome matrix and peroxisomal membrane localization</td>
</tr>
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<td>PEX5</td>
<td>3.8</td>
<td>Required for PTS1-mediated peroxisomal protein import, fatty acid beta-oxidation</td>
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<tr>
<td>orf19.5660</td>
<td>3.4</td>
<td>Ortholog(s) have ubiquitin-protein ligase activity, role in protein import into peroxisome matrix and peroxisomal membrane localization</td>
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<td>PEX12</td>
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<td>Ortholog(s) have ubiquitin-protein ligase activity, role in protein import into peroxisome matrix and integral to peroxisomal membrane localization</td>
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<td>orf19.1933</td>
<td>3.2</td>
<td>Ortholog(s) have role in peroxisome organization and peroxisomal membrane localization</td>
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<tr>
<td>PEX7</td>
<td>2.9</td>
<td>Ortholog(s) have peroxisome matrix targeting signal-2 binding activity, role in protein import into peroxisome matrix, docking and cytosol, peroxisome localization</td>
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<td>PEX1</td>
<td>2.8</td>
<td>Ortholog(s) have protein heterodimerization activity, ATPase activity, role in protein import into peroxisome matrix</td>
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<tr>
<td>PEX19</td>
<td>2.6</td>
<td>Role in ER-dependent peroxisome organization, protein exit from endoplasmic reticulum, protein import into peroxisome membrane, protein stabilization</td>
</tr>
<tr>
<td>PEX11</td>
<td>2.5</td>
<td>Putative protein involved in fatty acid oxidation</td>
</tr>
<tr>
<td>PEX2</td>
<td>2.4</td>
<td>Ortholog(s) have ubiquitin-protein ligase activity, role in protein import into peroxisome matrix and peroxisomal membrane localization</td>
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<tr>
<td>orf19.2092</td>
<td>2.3</td>
<td>Putative peroxisomal cystathionine beta-lyase</td>
</tr>
<tr>
<td>PEX3</td>
<td>2.2</td>
<td>Putative peroxisomal protein involved in targeting proteins into peroxisomes</td>
</tr>
<tr>
<td>PEX22</td>
<td>2.2</td>
<td>Putative peroxin</td>
</tr>
<tr>
<td>PEX8</td>
<td>2.0</td>
<td>Putative peroxisomal biogenesis factor</td>
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Figure legends

Figure 1. Susceptibility profiles of *C. albicans* strains in the presence of fluconazole or the CI inhibitors C12E8 or PdA are displayed alone or in combination with fluconazole (0, 2, 4, and 32 µg/ml). Relative growth is indicated as in Figure 1 (at the right). Susceptibility was strain dependent, but the combination of 4 µg/ml of fluconazole and C12E8 or PdA increased the susceptibility of the largest number of strains, including those that were resistant to fluconazole. *Left.* Strain numbers. *Right.* Strains are clustered according to their susceptibility, source, and/or resistance mechanisms. The combination of fluconazole with either C12E8 or PdA increased fluconazole susceptibility compared to inhibitor or fluconazole alone.

Figure 2. The growth of wild type (SC5314 and SN250) and CI mutants of *C. albicans* are shown with their susceptibilities to fluconazole. (*left panel*): YPD lacking fluconazole (*middle panel*), and on YP-glycerol (*right panel*). Of the 12 CI mutants, 10 have increased susceptibility to fluconazole and 9 are also growth-defective on YP-glycerol. Two strains (orf19.3611 and orf19.3290) have wild type susceptibilities to fluconazole and are able to grow on YP-glycerol. Growth of the two resistant mutants on YP-glycerol indicates that their corresponding genes are probably not part of the respiratory pathway of mitochondria.

Figure 3. *A*) Antifungal susceptibility profiles of *C. albicans* wild type, *goa1Δ*, and *goa1/GOA1* are shown in drop plate assays compared to YPD alone. Concentrations of each drug are indicated per panel. Susceptibility profiles were also determined for 5-flucytosine, micafungin, and amphotericin B (Supplemental Figure S1). *B*) Susceptibility of wild type strain, *goa1Δ*, and *goa1/GOA1* to
fluconazole (μg/ml). Data are presented as the percentage of growth compared with untreated cells (mean ± s.d. of three independent experiments).

C). Susceptibility profiles of wild type, $ndh51\Delta$, and $ndh51/NDN51$ to fluconazole are shown. The $ndh51\Delta$ is hypersensitive to fluconazole compared to control strains.

Figure 4. Rapid acquisition of adaptation to fluconazole in strains of $C. albicans$. All strains were grown overnight in SD medium, standardized to cell number and plated on SD medium containing 128 μg/ml of fluconazole. Cells from treated or untreated cultures were grown for 4 or 6 days on YPD agar and the colony numbers of each strain determined. The $goa1\Delta$ and $ndh51\Delta$, $\Delta cdr1/2$, and $\Delta tac1$ were unable to grow in the presence of fluconazole, while wild type (SC5314) and $CDR1/2$ and $MDR1$ over expressing strains adapted to fluconazole 4 and 6 days following incubation with the drug.

Figure 5. RNA-Seq. transcription profile of $goa1\Delta$. Functional categories are indicated as a % of the total down regulated genes.

Figure 6. RNA-Seq. transcription profile of $ndh51\Delta$. Functional categories are indicated as a % of the total down regulated genes.

Figure 7. Real time PCR of $CDR1$, $CDR2$, and $MDR1$ is shown for wild type, $goa1\Delta$, $ndh51\Delta$, and $goa1/GOA1$, and $ndh51/NDH51$. Expression of both $CDR1$ and $CDR2$ is significantly reduced in both mutant strains but not in the wild type or gene-reconstituted strains.

Figure 8. Efflux of fluorescent rhodamine 6G, a substrate of Cdr1p and Cdr2p pumps. All strains were grown overnight in YPD, starved for 2 h in PBS buffer, incubated with rhodamine 6G, and then transferred to buffer. At 30 min glucose was added to cultures and efflux of fluorescent rhodamine was measured subsequently for a total of 90 minutes. The CDR-over expressing strain exhibited
the greatest amount of rhodamine efflux, followed by the gene-reconstituted strains goa1/GOA1 and ndh51/NDH51 (intermediate efflux). The goa1Δ and ndh51Δ did not cause efflux of rhodamine.

Figure 9. Down regulation of genes (brown) in goa1Δ of peroxisomes (left) or mitochondria (right) are shown. See the text for a description of genes indicated. The relative level of down regulation is shown as circles that vary in diameter.

Figure 10. The ergosterol synthesis pathway is shown (left) along with transcription levels that correspond to each pathway gene. Down regulated genes (green) or minimal change (black) are shown. All genes are aligned on the right for both strains. Down regulation of ERG genes is more common in ndh51Δ than goa1Δ.
REFERENCES


Figure 1
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Figure 2

<table>
<thead>
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<th>FLC 16 µg/ml</th>
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<th>YPG</th>
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<tr>
<td>SC5314</td>
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<td>orf19.6007Δ</td>
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<td>SN250</td>
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Figure 2. The growth of wild type (SC5314 and SN250) and CI mutants of *C. albicans* are shown with their susceptibilities to fluconazole. (left panel): YPD lacking fluconazole (middle panel), and on YP-glycerol (right panel). Of the 12 CI mutants, 10 have increased susceptibility to fluconazole and 9 are also growth-defective on YP-glycerol. Two strains (orf19.3611 and orf19.3290) have wild type susceptibilities to fluconazole and are able to grow on YP-glycerol. Growth of the two resistant mutants on YP-glycerol indicates that their corresponding genes are probably not part of the respiratory pathway of mitochondria.
Figure 3A

Wild type, goa1A, and goa1/GOA1 strains were tested for their growth in YEPD and FLC (16 μg/ml) media. The images show the colony formation at different dilutions: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}.

- **YEPD**
  - **Wild type**
  - **goa1A**
  - **goa1/GOA1**

- **FLC (16 μg/ml)**
  - **Wild type**
  - **goa1A**
  - **goa1/GOA1**

- **Itraconazole (0.4μg/ml)**
  - **Wild type**
  - **goa1A**
  - **goa1/GOA1**

- **Ketoconazole (0.8μg/ml)**
  - **Wild type**
  - **goa1A**
  - **goa1/GOA1**
Figure 3. A) Antifungal susceptibility profiles of *C. albicans* wild type, goa1Δ, and goa1/GOA1 are shown in drop plate assays compared to YPD alone. Concentrations of each drug are indicated per panel. Susceptibility profiles were also determined for 5-flucytosine, micafungin, and amphotericin B (Supplementary Figure S1).
Figure 3B

Fluconazole

- wild type
- \( \text{goa1} \Delta \)
- \( \text{goa1}/GOA1 \)

Relative growth (% of Control)

0 100
0.01 0.1 1 10 100 (\( \mu \)g/ml)
B). Susceptibility of wild type strain, goa1Δ, and goa1/GOA1 to fluconazole (μg/ml). Data are presented as the percentage of growth compared with untreated cells (mean ± s.d. of three independent experiments).
C). Susceptibility profiles of wild type, ndh51Δ, and ndh51/NDN51 to fluconazole are shown. The ndh51Δ is hypersensitive to fluconazole compared to control strains.
Day 4
Day 6

Figure 4

Colony numbers (>1 mm)

Wild type

goa1Δ

= 0

goa1/GOA1

= 0

ndh51Δ

ndh51/NDH51

tac1Δ

cdr1 Δ/cdr2 Δ

= 0

CDR1/2

MDR1

OE

OE

Day 4

Day 6
Figure 4. Rapid acquisition of adaptation to fluconazole in strains of C. albicans. All strains were grown overnight in SD medium, standardized to cell number and plated on SD medium containing 128 μg/ml of fluconazole. Cells from treated or untreated cultures were grown for 4 or 6 days on YPD agar and the colony numbers of each strain determined. The goa1Δ and ndh51Δ, Δcdr1/2, and Δtac1 were unable to grow in the presence of fluconazole, while wild type (SC5314) and CDR1/2 and MDR1 over expressing strains adapted to fluconazole 4 and 6 days following incubation with the drug.
Figure 5. RNA-Seq. transcription profile of $goa1\Delta$. Functional categories are indicated as a % of the total down regulated genes.
Figure 6

- Stress response: 5%
- Oxidoreductase: 2%
- Ferric reductase: 1%
- DNA metabolism: 3%
- Sterol metabolism: 4%
- RNA metabolism: 3%
- Carbohydrate metabolism: 5%
- Transcription factors: 1%
- Amino acid metabolism: 7%
- Mitochondrial metabolism: 8%
- Glyoxylate cycle: 1%
- Cell wall organization: 6%
- Others: 46%
Figure 6. RNA-Seq. transcription profile of ndh51Δ. Functional categories are indicated as a % of the total down regulated genes.
Figure 7. Real time PCR of CDR1, CDR2, and MDR1 is shown for wild type, goa1Δ, ndh51Δ, and goa1/GOA1, and ndh51/NDH51. Expression of both CDR1 and CDR2 is significantly reduced in both mutant strains but not in the wild type or gene-reconstituted strains.
Figure 8

![Graph showing the effects of glucose on Rhodamine 6G production for different genetic backgrounds.](image)

- **Wild type**
- **goa1Δ**
- **goa1/GOA1**
- **ndh51Δ**
- **ndh51/NDH51**
- **CDR1/2 OE**

Glucose is added at time 0 minutes.
Figure 8. Efflux of fluorescent rhodamine 6G, a substrate of Cdr1p and Cdr2p pumps. All strains were grown overnight in YPD, starved for 2 h in PBS buffer, incubated with rhodamine 6G, and then transferred to buffer. At 30 min glucose was added to cultures and efflux of fluorescent rhodamine was measured subsequently for a total of 90 minutes. The CDR-over expressing strain exhibited the greatest amount of rhodamine efflux, followed by the gene-reconstituted strains goa1/GOA1 and ndh51/NDH51 (intermediate efflux). The goa1Δ and ndh51Δ did not cause efflux of rhodamine.
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