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Influence of phosphatidylserine and phosphatidylethanolamine on farnesol tolerance in *Candida albicans*

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Abstract

Candida albicans is the most common human fungal pathogen. The ability to undergo the morphological transition from yeast to hyphal growth is critical for its pathogenesis. Farnesol, a precursor in the isoprenoid/sterol pathway, is a quorum-sensing molecule produced by *C. albicans* that inhibits hyphal growth in this polymorphic fungus. Interestingly, *C. albicans* can tolerate farnesol concentrations that are toxic to other fungi. We hypothesized that changes in phospholipid composition are one of the factors contributing to farnesol tolerance in *C. albicans*. In this study, we found that loss of enzymes that synthesize the phospholipids phosphatidylserine (PS) and/or phosphatidylethanolamine (PE) compromise the tolerance of *C. albicans* to farnesol. Compared to wild-type, the phospholipid mutant *cho1* / (loss of PS and decreased PE synthesis) shows greater inhibition of growth, loss of ATP production, increased consumption of oxygen, and increased formation of reactive oxygen species (ROS) in the presence of farnesol (FOH). The *cho1* / mutant also exhibits decreased sensitivity to mitochondrial ATPase inhibition, suggesting that cells lacking PS and/or downstream PE rely less on mitochondrial function for ATP synthesis. These data reveal that PS and PE play roles in farnesol tolerance and maintaining mitochondrial respiratory function.

Keywords

Candida albicans; Phospholipid; Farnesol; phosphatidylserine; phosphatidylethanolamine; phosphatidylserine synthase; phosphatidylserine decarboxylase; Reactive Oxygen Species

Introduction

Candida albicans is a polymorphic commensal fungus and one of the most important opportunistic fungal pathogens of humans (Mayer, et al., 2013). *C. albicans* normally maintains a commensal relationship with humans, but under certain conditions, especially in

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immunocompromised patients, this organism becomes pathogenic. It causes a number of health problems ranging from potentially painful oral infections to life-threatening systemic forms of disease, which display mortality rates of up to 40% (Blumberg, et al., 2001; Williams and Lewis, 2011). The frequency of candidiasis has risen over the years due to the AIDS epidemic and the use of therapeutics that lead to immunosuppression (Blumberg, et al., 2001; Giri and Kindo, 2012).

In order for *C. albicans* to switch from a harmless organism to the pathogenic form, a number of virulence factors must be expressed. For example, *C. albicans* is able to change from the yeast to the hyphal form of growth (Sudbery, 2011; Thompson, et al., 2011). This morphological flexibility has been shown to play a critical role in virulence (Thompson, et al., 2011). It has been shown that disease-associated isolates of *C. albicans* in the oral cavity, and recovered from systemic infections, switch between morphologies at a higher frequency than commensally associated isolates (Williams and Lewis, 2011). *C. albicans* utilizes several signaling pathways to regulate the switch from yeast to hyphal forms including production of quorum-sensing molecules.

C. albicans was the first eukaryotic microorganism shown to exhibit quorum-sensing through the secretion of the sesquiterpene E, E- farnesol (FOH). This molecule is generated by dephosphorylation of farnesyl pyrophosphate in the mevalonate biosynthetic pathway in mammalian and yeast cells (Hornby, et al., 2003; Nickerson, et al., 2006). Exogenous FOH inhibits yeast to hyphal formation in a concentration and time dependent manner at the earliest stage of hyphal development (Hornby, et al., 2004). FOH acts by negatively regulating hyphal specific genes (*EFG1*, *CPH1*, and *HST1*) and derepressing transcriptional repressors (*TUP1* and *NRG1*) downstream of the Ras1/cAMP/PKA and/or mitogen-activated protein (MAP) kinase signaling cascades (Kebaara, et al., 2008; Langford, et al., 2009). A good deal of research has been devoted to study the role of FOH as an inhibitor of hyphal morphogenesis, but little attention has been given to other metabolic effects of this quorum sensing molecule in *C. albicans*.

Mitochondria are an important source of reactive oxygen species (ROS) within most eukaryotic cells. The production of ROS can cause oxidative damage to DNA, proteins, lipids and other cellular components (Machida, et al., 1998; Semighini, et al., 2006). Previous studies have shown that FOH at 50 μ M exhibits growth inhibitory effects on the yeast *Saccharomyces cerevisiae* by promoting mitochondrial ROS production (Machida, et al., 1998). FOH-mediated ROS generation occurred in a wild-type strain of *S. cerevisiae*, but not in a respiratory-deficient petite mutant (*rho*⁰), indicating a link between an active mitochondrial electron transport chain and FOH-dependent ROS production (Machida and Tanaka, 1999; Machida, et al., 1998). In contrast, *C. albicans* can tolerate and survive in high concentrations of FOH (~300 μ M) (Langford, et al., 2010). The mechanisms contributing to this protection from FOH are unknown.

Phospholipids are a major component of the membrane in eukaryotic cells, and in *C. albicans* include PS, PE, phosphatidylinositol (PI), and phosphatidylcholine (PC). We discovered that *cho1* / and *psd1* / *psd2* / (*psd1,2* /) mutants exhibit a number of pleiotropic phenotypes including mitochondrial defects (Chen et al., 2010). In this

communication we report that the *cho1* / mutant exhibits a lower tolerance to FOH. This decreased tolerance appears to be related to increased ROS production in the *cho1* / mutant, and altered electron transport chain activity, and may be related to the role for PS as a substrate to synthesize PE, which is enriched in the mitochondria.

Materials and Methods

Strains and Media

C. albicans strains used in this study include SC5314 (Gillum, et al., 1984), and mutants derived from it in (Chen, et al., 2010): *cho1* / (YLC337), *psd1* / (YLC280), *psd1,2* (YLC 375), and *cho1* / ::*CHO1* (YLC344). For all experiments, strains were grown overnight at 30 °C in rich media (YPD, 1% yeast extract, 2% peptone, 2% dextrose) under aerobic conditions and diluted into fresh media that contained dextrose or other carbon sources (see below) at OD₆₀₀ = 0.1. At least three biological replicates were carried out for all experiments.

Growth Curve and Electron Transporter Chain Inhibitors

Following overnight growth in YPD, cells from each of the strains were diluted to OD₆₀₀ = 0.1 in separate culture tubes containing 5ml YPD or YPG (1% yeast extract, 2% peptone, 2% glycerol) media with and without 100 µM FOH and/or the electron transport chain inhibitors 5µg/ml rotenone, 5µg/ml antimycin A, and 5µg/ml oligomycin. The tubes were then incubated at 30° C with shaking and the OD₆₀₀ measured every 3 hours over a 12 hour period.

Assay for ROS Detection

Intracellular reactive oxygen species (ROS) production was monitored by MitoSOX Red (Molecular Probes) that accumulates in the mitochondrial matrix, where it can be oxidized to a fluorescent product by superoxide. Cells at OD₆₀₀ = 0.1 were incubated plus/minus 100µM FOH with or without 5ug/ml oligomycin at 30 °C with shaking until they reached an OD₆₀₀ of ~0.5. MitoSOX™ Red (Invitrogen) was added to 0.4 µM for 20–30 min at 37 °C and washed 3 times with PBS. For microscopy, approximately 100 cells were counted for each experimental condition (untreated and treated). Alternatively, the resulting fluorescence was measured in phosphate buffered saline (PBS) using a BioTek plate reader set to 510_{nm} excitation and 580_{nm} emission wavelengths.

Measurement of ATP Levels in Cultures of *C. albicans*

For intracellular ATP measurements, 100ml cultures of *C. albicans* were grown at 30°C for 2.5–3 hours in the presence of 100 µM FOH, oligomycin, a combination of each, or methanol as a vehicle control. Cells were harvested by centrifugation (7,000 × g, 5min) and washed twice with TE buffer (50 mM Tris, 2 mM EDTA, pH 7.8). Cell pellets were resuspended in 1 ml TE buffer. Ten µl of cells were diluted to 1ml with deionized water and 50ul was plated to a YPD agar plate to assess viability. The remaining cells were submerged in liquid nitrogen followed by the addition of 400 µl of boiling TE buffer. Cells were boiled for an additional 4 min, centrifuged and the supernatant collected. Cells were subjected to another freeze/boil cycle and placed on ice until assayed for ATP. For extracellular ATP

measurements, 50 μ l of the supernatant was added to 450 μ l of boiling TE buffer, boiled for 90 s, and stored on ice until assayed for ATP. Extracellular and intracellular ATP levels were measured by luminometry using an ATP Assay Kit (Sigma) according to manufacturer's instructions. Specifically, luciferin-luciferase assay mixture (100 μ l) was added to 100 μ l of cell lysates or 50 μ l of extracellular material in 96-well microtiter plates and light emission was monitored using a 1250 LKB-Wallac luminometer. Results are expressed in bioluminescence relative light units (RLU) and ATP concentrations determined from an ATP standard curve.

Seahorse Extracellular Oxygen Flux Analysis

An XF²⁴ Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) was used to measure oxygen consumption rates (OCR) in *C. albicans*. Briefly, a XF²⁴ cartridge was preincubated with 1 mL of calibration solution at 37 °C in a non-CO₂ incubator overnight, and an XF²⁴ cell culture plate was preincubated at 4 °C with 5 μ g of poly-D-lysine. Samples were prepared as follows: cells were grown overnight in 5ml YPD at 30 °C with 250 rpm shaking. The next day, cells were washed with deionized water and diluted into 10 ml fresh YPG media (1% yeast-2% peptone-2% glycerol) to achieve an OD₆₀₀ ~0.1. Cells were returned to 30 °C for 2.5–3 hours in the presence and absence of inhibitors (100 μ M FOH and +/- 5 μ g/ml oligomycin). 10 μ l of cells were diluted to 1ml with deionized water and 50 μ l was plated to a YPD agar plate to assess viability and 350 μ l of growing cells were added into 350 μ l fresh YPG in a XF²⁴ cell culture and allowed to adhere in the wells for 30 min before loading the plate into the machine for measurements. Cells were then loaded into the XF²⁴ and further equilibrated for 30 min prior to the first measurement. Data represents the average of three replicates for each strain and have been normalized by the population CFU.

Blue Native Gel Assay

Mitochondria were isolated from all strains treated with and without farnesol as described (Murakami, et al., 1988). Briefly cells were grown in YPG media until the OD reached the mid log phase. Cells were then centrifuged at 7,000 $\times$ g for 5 min, and the cell pellets were resuspended in 0.1 M Tris-SO₄, pH 9.4, containing 10 mM DTT and incubated at 30°C for 30–45 min. After centrifugation, cell pellets were resuspended in spheroplast buffer (20 mM potassium phosphate, pH 7.4, 1.2 M sorbitol; ~6 to 7 ml/g of cells), and incubated with zymolyase 100T (0.8 to 1 mg/g of cells) at 30°C until ~80–90% of cells were converted to spheroplasts. Spheroplasts were homogenized using a Dounce homogenizer with ice-cold buffer (20 mM HEPES/KOH, pH 7.5, 0.6M sorbitol, 0.1% bovine serum albumin [BSA], 1 mM EDTA, 1 mM PMSF, 50 U/ml Trasylol) containing protease inhibitors. Crude mitochondria were collected by centrifuging at 20,000 $\times$ g for 1 hr. Mitochondrial pellets were dissolved by swirling them with a small spatula in 1M aminocaproic acid and kept at -80°C. To prepare samples for Blue Native PAGE (BN-PAGE), mitochondria were defrosted in the presence of HS buffer (20 mM pH 7.4 HEPES, 0.6 M Sorbitol), and re-isolated by centrifugation at 21,000 $\times$ g for 2 min at 4°C. Supernatants were removed by aspiration and pellets were lysed in 450 μ l 1% digitonin buffer (1% digitonin, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF) prior to loading. A total of ~100 μ g of each sample was mixed with 5X BN sample buffer. BN-PAGE was carried out essentially

as described (Schagger and von Jagow, 1991). Briefly, electrophoresis was performed with cathode buffer containing 0.02% Serva Blue G-250 anode buffer and 50 mM bis-Tris, pH 7.0 for 1 hour at 100 V at 4 °C until the protein entered the stacking gel at which time the voltage was raised to 160 V and electrophoresis continued for ~2 hours. When the Coomassie dye front was half way through the gel, the cathode buffer was then replaced with the cathode buffer without Coomassie Blue G-250, and electrophoresis continued at 160 V at 4 °C until the Coomassie Blue G had run off the gel.

Farnesol Extraction and GC –MS Measurement in *C. albicans*

To determine the extracellular level of FOH in mutants lacking phospholipids, an overnight culture of cells was inoculated into 50 ml fresh YPG media to an OD₆₀₀ of 0.1 and grown at 30 °C until they reached stationary phase. The supernatant was separated from the cells by centrifugation at 7,000 × g for 10min. The cell pellet was resuspended in 5 ml distilled water and vacuum filtered to be used for dry-weight. The cell pellets for each sample were allowed to bench dry in petri dishes and weighed regularly to calculate the dry weight. 40ml of cell-free supernatants were filtered and sterilized through a 0.2 µm filter and farnesol was extracted with the addition of 10ml volumes of ethyl acetate. The ethyl acetate extracted material was dried down under nitrogen gas and the remainder resuspended in 100 µl of ethyl acetate for derivatization. Derivatization was carried out as described by the manufacturer (BSTFA+TMCS, 99:1, Sigma (33154U)) with one ampule used for each sample. Samples were analyzed by gas chromatography/mass spectrometry (GC/MS) following one hour incubation at 55 °C in a water bath. Sample ionization was performed using electron ionization (EI). The oven temperature was initially held at 80 °C for 1 min and increased at a rate of 10 °C/minute to 250 °C where it was held for 1 minute. Nitrogen was used as the carrier gas with an inlet pressure of 39 psi.

Statistical analysis

Each experiment was performed independently and repeated at least three times. Statistical analysis was performed using GraphPad Prism 7 and the data were analyzed with Student's t test. *P* values < 0.05 were considered to be statistically significant.

Results

FOH inhibits growth of the *cho1* / and *psd1, 2* / mutants

It has been reported that the *cho1* / and *psd1, 2* / mutants have mitochondrial defects (Chen, et al., 2010; Trotter and Voelker, 1995), so we examined whether they were sensitive to farnesol (FOH) compared to wild-type. This was initially examined by comparing mutant and wild-type growth in response to FOH. As shown in Fig. 1, FOH caused ~40% inhibition of growth in wild-type and *cho1* / strains while they were growing in media containing a fermentable carbon source such as glucose (Fig. 1 A, B).

However, a difference between the strains was observed in media that contained the non-fermentable carbon source glycerol. Addition of 100 µM FOH to wild-type growing in YP glycerol (YPG) resulted in only about a 5% decrease in growth (doubling time from 2.13 hrs to 2.32 hrs). In contrast, the *cho1* / mutant suffered a 30 % decrease (doubling time from

2.48 hrs to 3.239 hrs) in growth in YPG when 100µM FOH was added (Fig. 1 C, D). The *cho1* / mutant lacks PS, but also has a decrease in PE synthesis, as PE is produced from PS by the PS decarboxylases Psd1 (mitochondria) and Psd2 (Golgi/endosome) (Gulshan, et al., 2010). Therefore, we tested the *psd1* / single mutant and *psd1,2* / double mutant for FOH sensitivity. The *psd1* / and *psd1,2* / mutants exhibited decreases in growth similar to wild-type (Fig 1).

To determine if the growth defect correlates with a loss of viability, we analyzed the viability of *cho1* / , *psd1,2* / , and wild-type strains based on their ability to exclude methylene blue (Fig 2). At 50 µM FOH, ~33% of the *cho1* / mutant cells stained positive for methylene blue, and this increased to 39% and 65% in 100µM and 200µM FOH, respectively, while only 10% to 20% of the wild type cells were methylene blue positive for these concentrations. The *psd1,2* / mutant behaved much like the *cho1* / mutant, but was not as hypersensitive. The double mutant was only significantly different from FOH-treated wild-type at 100µM and 200µM FOH, whereas *cho1* / showed a significant, three-fold increase over wild-type, even at 50µM FOH. The *psd1* / single mutant was similar to wild-type. The FOH effect on viability as judged by methylene blue staining for the *psd1* / mutant and wild type showed a partially dose-dependent relationship (Fig 2).

Farnesol Induces ROS in *cho1* / and *psd1,2* / Strains

As FOH treatment induces oxidative stress in *S. cerevisiae*, we tested to see if it does so in the *cho1* / and *psd1,2* / strains compared to wild-type. We monitored intracellular reactive oxygen species (ROS) levels after treatment with FOH using MitoSOX™ Red, a dye used to measure superoxide production in the mitochondrial matrix. The percentages of cells exhibiting ROS in the FOH treated *cho1* / and *psd1,2* / mutants were 55% and 33%, respectively, whereas wild-type was only 1% (Fig 3E). This suggests that the lack of PS and/or decreased levels of PE synthesis result in an altered mitochondrial response to FOH that culminates in excess ROS production. The reintroduction of *CHO1* into the *cho1* / strain returned the strain to near wild-type level of ROS production following exposure (Fig 3D).

Farnesol and Electron Transporter Chain Inhibitors

FOH causes ROS production in our mutant strains, and may do so via interference with the mitochondrial electron transport chain. Therefore, we next sought to determine whether other compounds that disrupt the mitochondrial electron transport chain would inhibit growth in the *cho1* / , *psd1* / , or *psd1,2* / mutants similarly to what was observed with FOH. Therefore, we treated all the strains with oligomycin, antimycin A and rotenone. Treatment of *cho1* / and *psd1,2* / strains with the mitochondrial electron transport chain inhibitors did not decrease the growth significantly compared to wild-type (Fig 4A–E). However, oligomycin inhibited wild-type more than it did *cho1* / . The *psd1,2* / mutant showed a modest similarity to *cho1* / in response to oligomycin. With regards to ROS production in *cho1* / , none of these other inhibitors caused as great of an increase in ROS as FOH (Fig 4F).

Since oligomycin did not strongly inhibit *cho1* / , but did inhibit wild-type, we tested to see if it would rescue the growth defect in the *cho1* / mutant caused by FOH. We also tested this because oligomycin could rescue some FOH-induced defects in *S. cerevisiae* (Machida and Tanaka, 1999). We found that incubation of FOH-treated *cho1* / cells with oligomycin almost completely reversed the growth defect (Fig 5 A, B). The *psd1,2* / mutant co-incubated with FOH and oligomycin was also partially rescued for its growth defect. There is also a slight rescue in the wild-type and *psd1* / mutant as well (Fig 5 A, D), although they do not show strong growth defects with FOH. We hypothesized that this rescue in *cho1* / might correlate with a loss of FOH-induced ROS production, so we stained *cho1* / cells treated with FOH +/- oligomycin with MitoSOX™ Red to measure any protection from ROS production. The result for staining indicates that there is not a significant decrease in FOH-induced ROS production in *cho1* / or *psd1,2* / in the presence of oligomycin (Fig 4F).

Given that phenotypes seen in the *cho1* / mutant are often also seen in the *psd1,2* / double mutant, but less pronounced, we focused our attention on the *cho1* / mutant for the rest of these studies.

The Oxygen Consumption Rate (OCR) of the *cho1* / mutant is greater than in wild-type in the presence of farnesol

Since mitochondrial electron transport chain activity is associated with ROS production and ROS differs between wild-type and mutant strains in the presence of FOH, we wanted to determine if mitochondrial electron transport chain activity differed between the strains. Furthermore, we wanted to determine if oligomycin impacted mitochondrial electron transport chain activity with respect to FOH treatments. Mitochondrial electron transport chain activity can be determined by measuring the mitochondrial oxygen consumption rate (OCR), so we measured OCR in all strains in YPD, and YPG, before and after treatment with FOH, oligomycin, or a combination of FOH and oligomycin. No significant differences were observed in OCR between wild type and mutant cells grown in YPD medium (data not shown). However, in YPG, the level of OCR was significantly higher in the *cho1* / mutant when it was treated with FOH. This was not the case in wild-type (Fig 6). Treatment with oligomycin did cause a decrease in OCR in the presence of FOH, which was expected, as treatment of oligomycin should decrease mitochondrial electron transport chain activity (Li, et al., 2011; Ogasawara, et al., 2006)

Since mitochondrial electron transport chain activity is higher in the *cho1* / mutant in the presence of FOH, we wanted to determine if the accumulation of cytosolic ATP levels in wild type and *cho1* / mutants follows the observed differences in OCR. Thus, ATP levels were measured. As shown in figure 7A, ATP levels were similar between the strains grown in YPG media, however when challenged with 50 or 100 μM FOH, the intracellular ATP level of *cho1* / decreased 60% after 3 hours incubation with FOH, whereas wild-type decreased by only 25%. The wild-type had a greater loss of ATP with oligomycin treatment (~50% inhibition) than FOH treatment (no significant decrease), whereas the *cho1* / and *psd1,2* / mutants showed no loss of ATP with oligomycin alone (Fig 7B).

In contrast to the effect of oligomycin on *cho1* / treated with FOH regarding growth or OCR, oligomycin did not rescue the FOH-induced loss of ATP (Fig 7B). As oligomycin is an ATP synthase inhibitor, these data might suggest that the *cho1* / mutant is less efficient at ATP synthesis through respiration.

Mitochondria ETC Complexes are not Grossly Affected in the *cho1* / Mutant

The above data suggested that perhaps the ATP synthase complex function is disrupted in the *cho1* / mutant. In order to determine if the ATP synthase or any other complex is disrupted, we used Blue Native PAGE to separate the mitochondrial membrane complexes. This technique separates complexes without dissociating them into their constituent polypeptides (Schagger, 2002). Mitochondrial electron transport chain complexes were evaluated in mitochondria isolated from wild-type and mutant strains +/- FOH treatment. No significant differences were detected between wild type and mutants strains at this level (Fig. 8).

GC/MS Analysis of Cell-free *C. albicans* Supernatants

To address the possibility that our mutants secrete FOH at different levels, wild-type and mutant strains were grown in liquid media containing glucose or glycerol. Cultures were pelleted and the cell free supernatant analyzed for FOH by gas chromatography/mass spectroscopy (GC/MS as described in Hornby *et al* (Hornby, et al., 2003)). Commercial FOH was used as a control. No significant differences were observed in FOH level in wild type and mutant (Fig. 9) indicating that the sensitivity of the *cho1* / mutant to FOH is not related to the level of FOH produced by this strain.

Discussion

In this communication, we describe that mutants lacking PS synthase and/or having PE synthesis defects are hypersensitive to FOH (Figs 1&2). The *cho1* / and *psd1,2* / mutants exhibit these phenotypes in medium where they are forced to use a non-fermentable carbon source (*ie.* YPG). In this medium, strains are forced to use the mitochondrial electron transport chain for ATP production more than in medium containing a fermentable carbon source. We found that the *cho1* / mutant's growth defects in the presence of FOH correlate best with its consumption of oxygen (OCR) as demonstrated in Fig 6, suggesting an interaction between PS and FOH for controlling the mitochondrial electron transport chain. The *cho1* / mutant, but not wild-type, exhibits an increase in OCR upon FOH treatment, but this is reversed by treatment with ATP synthase poison oligomycin (Fig 6). This correlates with its growth defect, where the *cho1* / mutant exhibits an ~8 fold decrease in growth when treated with FOH alone, but undergoes an ~4 fold improvement in growth when co-treated with oligomycin and FOH (Fig 5). Wild-type treated with FOH exhibits little growth inhibition, and when wild-type is treated with oligomycin and FOH it exhibits only a modest 1.2 fold increase in growth over treatment with FOH alone.

The *cho1* / mutant also exhibits other phenotypes when treated with FOH that reflect defects in mitochondrial function, but they are not reversed by oligomycin co-treatment and therefore may not fully explain the growth defects, although they may contribute to them.

For instance, the *cho1* / mutant treated with FOH exhibits increased reactive oxygen species (ROS) formation (Fig 3) and decreased ATP levels (Fig 7). However, these phenotypes are not reversed by oligomycin co-treatment (Figs 4 & 7). Thus, the effects on OCR may be the most important contributors to growth defects.

It is possible that *cho1* / either has defects in electron transport chain efficiency or it is partially decoupled for oxidative phosphorylation, which in either case would make it more difficult to derive ATP from respiration. Neither defect could be extremely severe, as OCR of *cho1* / in the absence of FOH treatment seems to be unaffected (Fig 6). A strong decoupling effect should increase OCR and a strong decrease in electron transport chain function should decrease OCR (Cheng, et al., 2007). However, this mild defect may be exacerbated when cells are grown in a non-fermentable carbon source and treated with FOH, and this may help explain why *cho1* / is not adversely affected by oligomycin, as it is not as reliant on the electron transport chain for ATP synthesis. A less efficient electron transport chain would explain the increased production of ROS and decreased production of ATP in the presence of FOH.

We favor the hypothesis that oxidative phosphorylation is decoupled, as this may better explain all of our data. It has been suggested in *S. cerevisiae* that FOH generates ROS by interfering with the electron transport chain at complex III (Machida and Tanaka, 1999; Machida, et al., 1998). It is possible that FOH in *C. albicans cho1* / causes electrons to be shuttled into the alternative oxidase pathway, which could increase oxygen consumption at the cost of ATP synthesis (Alonso-Monge, et al., 2009). This would help explain the dramatic loss of ATP synthesis upon FOH treatment. The wild-type may be resistant to this because it does not have altered electron flow through the electron transport chain to begin with. Alternatively, this may indicate that in wild-type *C. albicans* PS and/or PE are protective against FOH interfering with electron transport chain function or complex III in particular.

If our hypothesis above holds true, then the gradient across the inner membrane of the mitochondria established by pumping protons is diminished in *cho1* / due to partial decoupling, and the addition of FOH may cause a decreased gradient due to shuttling electrons away from complexes III and IV. This may compromise the ψ which will affect viability. However, addition of oligomycin, which decreases proton dissipation by blocking passage of protons through the ATPase may help to re-establish the gradient, thus rescuing the cell's growth, despite not rescuing ROS production or ATP synthesis. In this model, *cho1* / is more resistant to the effects of oligomycin compared to wild-type because it does not depend so much on ATP synthesis through the ATPase to begin with. The oligomycin rescue of OCR from FOH treatment (Fig 6) may simply be because the decreased ATPase activity causes a more general feedback inhibition of the electron transport chain (Ocampo, et al., 2012).

Based on this model, we suggest that *cho1* / and *psd1,2* / cells have increased permeability to protons across the mitochondrial membrane. The *cho1* / mutant may have greater phenotypes than *psd1,2* / because both PS and PE play a role in this. The roles of PE and/or PS in the membrane as proton barriers is unclear. More work will need to be done

to fully resolve this model, but our work here sets the stage for better understanding how these phospholipids impact the mitochondrial ETC and membrane structure.

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Figure 1.

Effect of farnesol on *C. albicans* cell growth in different media. Cultures were grown aerobically in media containing glucose (YPD, panels A, B) or glycerol (YPG, panels C, D) at 30°C in the absence (panels A, C) and presence (panels B, D) of 100μM FOH. The measured optical density at 600nm is plotted for *C. albicans* SC5314 (WT); *cho1*Δ/Δ; *psd1*Δ/Δ; *psd1,2*Δ/Δ homozygous deletion mutant, and *cho1*Δ/Δ/*CHO1* reconstructed strain. Values shown are the average of triplicate experiments ± SD.

Figure 2.

Effect of farnesol on *C. albicans* cell death. After 6 hrs of FOH treatment, cell viability was assessed microscopically using 0.01% methylene blue in YPG media. Percentage of methylene blue positive cells (proxy for dead cells) in the treated cultures increased with FOH concentration.

* $P < 0.002$, compared to wild-type at the same concentration of FOH, ** $P < 0.05$ compared to wild-type with no FOH treatment.

Figure 3.

Mitochondrial superoxide production in *C. albicans* treated with FOH for 6 hr. Fluorescent microscopic images of cells stained with MitoSOXTM Red indicator with (+) and without (–) 100 μ M FOH. (A) Wild type (B) *cho1* / (C) *psd1, 2* / (D) *cho1* / ::*CHO1*. (E) Percentage of reactive oxygen species (ROS) production in different strains. Approximately 100 cells were counted for each strain per experiment by microscopy and the percentages of cells that stained with MitoSOX Red were determined. Experiments were repeated three times. Although the *psd1,2* / cells stained more brightly than the *cho1* / cells, this was not found to be statistically significant when analyzed by Image J. *p<0.05, compared to wildtype.

Figure 4. Effects of mitochondrial electron transport chain inhibitors on growth and superoxide generation. Growth curves were measured for (A) wild-type (B) *cho1* / (C) *cho1* / *CHO1* (D) *psd1* / (E) *psd1,2* / strains treated with oligomycin, antimycin and rotenone. (F) Mitochondria superoxide was stained with MitoSOX™ Red after 6 hrs of growth. Relative fluorescence units were detected by microplate reader. *p<0.05, compared to untreated cells. # p<0.05 compared to oligomycin treated cells.

Figure 5.

Protective effect of oligomycin on FOH-induced growth inhibition. Growth of (A) Wild-type (B) *cho1* Δ (C) *cho1* Δ *CHO1* (D) *psd1* Δ (E) *psd1,2* Δ was measured in presence of FOH (black bar) and FOH + oligomycin (gray bar) after 4 hr exposure. Partial rescue of the *cho1* Δ growth defect is detected after 4 hr co-incubation with both oligomycin and FOH. * $p < 0.05$, compared to FOH alone.

Figure 6.

Evaluation of FOH effects on oxygen consumption rate (OCR) in wild-type (SC5314) and *cho1* Δ cells. OCR was monitored through Seahorse XF-24 Extracellular Flux Analyzer with and without 100 μ M FOH and +/- 5 μ g/ml oligomycin at the starting inoculation after baseline rate measurement in YPG media. Values shown are the average of triplicate experiments $\pm$ SD. * $P < 0.05$ compared to untreated cells.

Figure 7.

ATP levels are decreased in *cho1* Δ/Δ cells treated with FOH. Intracellular ATP levels were measured after a 4 hr incubation of each strain +/- oligomycin and FOH at 30°C. A)

Comparing the level of ATP production in wild type, *cho1* Δ/Δ , *psd1* Δ/Δ and *psd1,2* Δ/Δ in presence of 50 or 100 μ M FOH. B) Measurement of ATP levels in wild type, *cho1* Δ/Δ , *psd1* Δ/Δ , *psd1,2* Δ/Δ in the presence of 100 μ M FOH and oligomycin. Values shown are the average of triplicate samples per experiment $\pm$ SD, *p<0.05, compared to untreated strain.

Figure 8. Effect of FOH on respiratory super-complexes. Migration by blue native electrophoresis of electron transport chain complexes of mitochondria isolated from wild type and *cho1* / strains with and without 100μM FOH exposure. 100 μg of protein loaded per well.

Figure 9.
GC-MS analysis of ethyl acetate extracts from cell- free supernatants of different strains.
Cells were grown overnight at 30°C with starting OD=0.1 prior to GC-MS in different media
(noted in figure). Concentration was measured as nanomole per gram dry weight of cells.