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Exploring the mechanisms of action of antifungal peptides using Saccharomyces cerevisiae.

Michelle L. Mason

Exploring the mechanisms of action of antifungal peptides using *Saccharomyces cerevisiae.*

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Michelle Mason

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J. William Fulbright College of Arts and Sciences

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Abstract

Candida albicans is a normal inhabitant of the skin and mucosal membranes of humans, however, in individuals with depressed immune systems or disrupted cutaneous flora, *Candida* can overgrow and cause serious infection. *Candida* infection is the fourth leading cause of nosocomial infection in the United States. These infections are often associated with longer hospital stays and higher mortality. Current drug therapies for this infection are largely ineffective due to the increased drug resistance of *Candida* species, and for some therapeutics, high levels of drug toxicity to humans. Histatin 5 is a naturally occurring salivary peptide that has strong antifungal properties. Derivatives of this peptide, including KM12 and KM23, have been previously tested in the McNabb lab, and have shown as much as ten times more fungicidal activity than Histatin 5. Our current understanding of the mechanism by which these peptides kill has not been fully characterized. This study uses *Saccharomyces cerevisiae* as a genetically tractable model to investigate one of the proposed target mechanisms of KM23, namely respiratory metabolism. Respiratory mutants were tested to determine which gene knockouts conferred cell resistance to KM23. If resistance was present for the mutant, the corresponding gene product is part of the killing mechanism of KM23. ATP synthase mutants, *atp1Δ*, *atp2Δ*, *atp5Δ*, and *atp7Δ*, were the main focus of this study. It was found that *atp1Δ*, *atp5Δ*, and *atp7Δ* conferred resistance of the yeast cells to KM23, while *atp2Δ* did not show significant resistance. It was subsequently found that *atp2Δ* was not truly respiratory deficient, therefore, we conclude that ATP synthase function is imperative to the overall killing action of KM23, supporting prior observations that respiratory metabolism is necessary for killing by Histatin 5 and Histatin 5-related peptides.

Introduction

Candida albicans is a species of fungi that inhabits the human body on the skin and in mucosal membranes. This organism is an opportunistic pathogen, meaning that it lives on the human body harmlessly as part of the normal flora, until the host's immune response is lowered, due to other concurrent diseases or certain drug therapies. *Candida albicans* is a unicellular, diploid fungus that exists in 3 phases: budding yeast, pseudohyphae, and hyphae (Calderone & Fonzi, 2001). At room temperature and under anaerobic conditions, *Candida albicans* exists in the budding yeast form. In this state, the yeast colonies are oval-shaped, smooth, and white or cream colored (Calderone & Fonzi, 2001). However, under physiological conditions, this organism can convert to the hyphal or pseudohyphal form. In these filamentous states, the cells remain attached to each other after budding and elongate to form filaments, which are tubular, thread-like structures (Sudbery, Gow, Berman, 2004). This dimorphism is thought to aid in the virulence of *Candida albicans* (Calderone & Fonzi, 2001).

Candida infections manifest in three main types: oropharyngeal candidiasis, genital or vulvovaginal candidiasis, and systemic candidiasis. These infections occur due to an overgrowth of *Candida* species caused by a lowered host immune response or a reduction in other resident bacteria that normally compete with this organism. In intensive care units worldwide, candidemia infection has a prevalence of 6.9 per 1000 patients (Mikulska M, 2012). Individuals who are most affected by candidiasis include immunocompromised patients, such as those undergoing treatment for HIV/AIDS or cancer, low-birth-weight infants, diabetics, and individuals using corticosteroids or

broad-spectrum antibiotics (CDC 2014). Candidiasis is associated with high mortality and increased hospital stays (Paramythiotou E, 2014). It is estimated that the mortality attributable to candidiasis is 19-24% (Morgan J 2005). A yeast infection in the mouth or throat, commonly termed "thrush", if left unchecked could enter the bloodstream and spread systemically, causing further complications in a patient's condition. *Candida* infections are the fourth most common nosocomial bloodstream infection in the United States (Paramythiotou E, 2014).

There are multiple classes of antifungal drugs currently on the market, which attack different aspects of *Candida* species to treat infections. These drug classes include azoles, polyenes, pyrimidine analogs, and echinocandins. Azoles, such as miconazole and fluconazole, are the most commonly prescribed (Paramythiotou E, 2014). This class affects the composition of fungal plasma membranes through the inhibition of ergosterol synthesis by binding to the cytochrome P-450-dependent enzyme lanosterol demethylase in the fungal cell (Sheehan, Hitchcock, Sibley, 1999). This is an effective drug mechanism because mammalian cell membranes contain cholesterol rather than ergosterol, and because azoles have a greater affinity for the fungal enzyme associated with sterol synthesis than they do for the mammalian enzyme. However, the incidences of azole-resistant strains of *Candida* species has risen in recent years due to chronic use of fluconazole and itraconazole in prophylactic measures and to treat oropharyngeal and vaginal candidiasis (Mulu et al., 2013), making it a less effective drug to combat *Candida* infections. An alternative drug class is the polyenes, such as amphotericin B and nystatin, which bind to ergosterol within the cell membrane and create transmembrane channels,

leading to membrane instability and cell lysis (Laniado-Laborin, Cabrales-Vargas, 2009). Unfortunately, polyenes, especially amphotericin B, have a high affinity for cholesterol as well as ergosterol. For this reason, they are associated with numerous negative side effects, including renal failure after long-term use (Bondaryk, Kurzatkowski, & Staniszewska, 2013). The third class of antifungal drugs, pyrimidine analogs, like flucytosine, act like pyrimidine bases, affecting protein synthesis by incorporating into RNA and disturbing the normal coding regions (Bondaryk et al., 2013). The main enzyme that allows this drug to act, cytosine deaminase, is not present in mammalian cells, buffering them from the effects of this drug while acting on the fungal agent. However, this class of drugs is usually restricted to use in a multi-drug therapy regimen, due to its high incidence of resistance and possible negative side effects, including bone marrow suppression and liver dysfunction. The final class of major antifungal drugs is echinocandins. This class of drugs acts on the cell membrane of fungi by inhibiting the synthesis of 1,3-β-glucan synthase, eventually leading to cell wall instability and cell lysis. This drug is effective against azole-resistant *Candida* yeasts, but has many negative side effects including liver toxicity and hemolysis (Bondaryk et al., 2013). In addition, many of the drug interactions between antifungal drugs and other medications are negative or can be synergistic, leading to various complications in treatment, especially in those which candidiasis as a secondary infection.

The current methods of treatment for candidiasis are neither sufficient nor ideal due to their overall high incidence of *Candida* species resistance and adverse side effects on the patient. In addition, the increased occurrence of infection by non-*albicans Candida*

species, which can be resistant to current drug therapies, creates a need for the development and investigations of new treatment routes. Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) is a naturally occurring peptide that resides in the saliva of humans and displays strong fungicidal properties, making it a prime model for new antifungal drugs (Helmerhorst EJ, 1999). Histatin 5 contains 24 amino acid residues and is histidine-rich, making it highly cationic (Oppenheirn et al., 1988). It binds to cell wall proteins and glycans upon initial cell interactions. Histatin 5 is then taken up by yeast cells through fungal polyamine transporters in an energy dependent manner (Puri S & Edgerton M, 2014). Although histatins have been widely studied, their mechanism of killing action is not fully mapped out.

There are two current models describing the mechanism of this peptide. One proposal is that the entry of histatin 5 ultimately leads to ion imbalance in the fungal cell due to increased cell membrane permeability (Wang G, 2014). The second proposal is that the target of this peptide is mitochondrial, and that it acts to inhibit the normal respiratory pathway of the fungus, causing oxidative stress (Mochon AB and Lui H, 2008). In other studies, it has been shown that sodium azide and other respiration inhibitors protect yeast cells against the lethal activity of histatin 5 (Helmerhorst EJ, 1999). While this observation is useful, chemical manipulation of the cells could affect more than just the yeast's respiratory function. The hope is that by further elucidating the mechanism of action for histatin 5 and its derivatives, these peptides can be promoted and explored as possible novel antifungal drugs.

KM23, an inverted dimer, is the principal derivative of histatin 5 focused on in this study. In Dr. McNabb's lab, previous work has thoroughly characterized KM12 (YKRKFC-CFKRKY). This peptide is very similar to KM23 in killing activity, with slight structural differences. The LD_{50} for KM12 on *C. albicans* was determined to be 0.308±0.035 μM (Akkam Y, 2013). KM12 had similar ability to kill *S. cerevisiae* and *C. albicans*. The activity of this derivative, KM12, is ten times more active than histatin 5 alone (Akkam Y, 2013). KM23 and KM12 are inhibited in the presence of histatin 5 inhibitors, such as low temperature and sodium azide, and are therefore suggested to have a similar mode of action to histatin 5 (Akkam Y, 2013). In order to further map the mechanism of action of histatin 5 and its derivatives, the effects of KM23 on the genetically tractable model yeast, *Saccharomyces cerevisiae*, has been tested.

Saccharomyces cerevisiae serves as a good model organism for *Candida albicans* because these organisms are closely related to each other, (Li, Palecek, 2005) and they are both susceptible to the killing action of histatin 5 and our derivative peptides. *Saccharomyces cerevisiae* grows rapidly, in small, smooth, cream to tan colored colonies. This species of yeast replicates by budding like *Candida albicans*, but has a haploid instead of a diploid genome. *S. cerevisiae* serves as a prevalent model organism because it has well characterized genes and is easily manipulated due to its haploid genome. The logic of this study is to identify mutations in the electron transport chain, more specifically the ATP synthase of *S. cerevisiae*, that confer resistance to KM23. Any mutant that causes resistance to killing by KM23 is likely involved in the peptide's mechanism of killing action, which we hypothesize is localized in the mitochondria.

Unlike, chemical means of testing for killing inhibition, testing genetic knockouts focuses on a single trackable protein subunit. In other studies, it has been shown that respiratory enzyme complexes, including proteins involved in the ATP synthase gamma chain and malate dehydrogenase, were mostly down-regulated when *Candida albicans* was treated with histatin 5 (Komatsu T, 2011). *S. cerevisiae* mutants located in the ATP synthase of the electron transport chain, including *atp1*Δ, *atp2*Δ, *atp5*Δ and *atp7*Δ, were tested to examine KM23's mechanism of action.

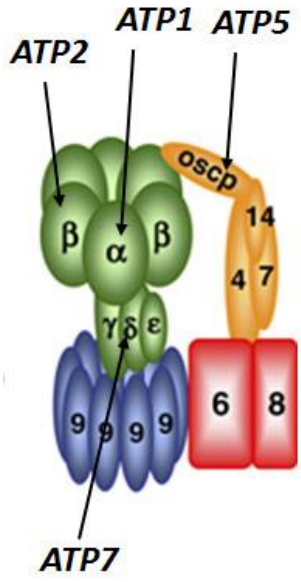

Figure 1. ATP synthase and *S. cerevisiae* respiratory mutants. Figure adapted from Rak M, 2011. Yeast mitochondrial ATP synthase protein subunits and corresponding knockout mutants.

The F_1F_0-ATP synthase of *S. cerevisiae* consists of two functional domains, the globular F_1 protein and the membrane-bound F_0 protein. These two domains couple to catalyze ATP synthesis and proton translocation across the inner mitochondrial membrane (Rak 2011). The electroosmotic potential created by the transport of protons across the

membrane is used to synthesize ATP from ADP and P_i. These two functional domains are connected by both a central and a stator stalk (Devenish 2000). This connection aids in the rotation of subunits within the enzyme that catalyzes the movement of protons across the inner membrane. The various protein subunits of the F_1F_0-ATP synthase of *S. cerevisiae* were knocked out and purchased as a library. *atp1Δ*, *atp2Δ*, *atp5Δ*, and *atp7Δ* were tested (Figure 1). While these mutants are essential to ATP synthase functioning, their deletion is not lethal to the yeast cells. These mutants are still able to undergo fermentation to produce ATP on YPD medium (Norais 1991). However, any substrate that cannot be utilized through glycolysis and alcoholic fermentation, glycerol for example, will inhibit the growth of the mutant yeast strains.

The *atp1Δ* and *atp2Δ* are subunits of the F_1 functional domain of the mitochondrial ATP synthase (Takeda 1986). They comprise an alternating hexamer of catalytic subunits that are involved in ATPase activity. *atp1Δ* is a knockout mutation of the α-subunit and *atp2Δ* is a knockout mutation of the β-subunit of the F_1 functional domain (Takeda 1986, 1985). *atp5Δ* and *atp7Δ* are mutations of subunits within the stator stalk of ATP synthase (Devenish 2000). *atp5Δ* is the OSCP or oligomycin sensitivity-conferring protein (Boyer 1997) and *atp7Δ* is a knockout mutation of the d-subunit of the stator stalk (Norais 1991). Both of these subunits are normally involved in proton translocation and rotation of the F_1 and F_0 functional domains.

Methods and Materials

***Saccharomyces cerevisiae* strains and growth media.** The wild-type *Saccharomyces* strain was BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*). In addition, four mitochondrial ATP synthase mutants, *atp1Δ*, *atp2Δ*, *atp5Δ*, and *atp7Δ* were used in this study. The mutants were isogenic derivatives of BY4741 and they were purchased from Dharmacon as part of a genome-wide S. cerevisiae knockout collection. All strains were grown in Yeast extract-peptone-dextrose (YPD) liquid culture 24 hours prior to the beginning of each assay (Guthrie and Fink, 1991). Strains were grown on YPD agar plates during each assay. Yeast extract-peptone-glycerol (YPG) was prepared as described (Guthrie and Fink, 1991).

KM23 peptide. The stock KM23 peptide was purchased from Gene Script, where it was synthesized and purified. The 50 µM stock peptide (YKRKFFKRKY) was diluted to 2 µM with 10 mM phosphate-buffered saline (PBS) for each microdilution assay.

Microdilution Assay. Prior to each independent assay, 5 mL YPD liquid medium was inoculated for each *S. cerevisiae* strain to be tested. These strains were grown at 30°C overnight. On the day of the assay, 1 mL of each strain was transferred to a 1.5 mL eppendorf tube. The tubes were centrifuged for 10 min at 14,000 rpm. The supernatant was discarded, and 1 mL of 10 mM sodium phosphate buffer (PB) was added to each tube. The pellet was resuspended, and the tubes were centrifuged for another 10 min at 14,000 rpm. The supernatant was again discarded. The pellet of each tube was resuspended a second time with 1 mL PB and vortexed. The cells were quantified on a

hemocytometer. The cell count was input into an Excel counting sheet, which computed the amount needed to have 3.6×10^6 cells in 1 mL of PB (10X stock solution of cells). This amount of cells, for each respective strain, was inoculated in 1 mL PB. Then in a second set of tubes, 900 µL of PB was inoculated with 100 µL of the prepared 10X stock of cells to bring the final concentration to 3.6×10^5 cells in 1 mL of PB. Next, 20 µL of the diluted cells were placed into two eppendorf tubes: one with 20 µL PB, the other with 20 µL 2 µM KM23. These eppendorf tubes, two for each strain, were placed in the incubator at 30°C and shaken at 320 rpm for 2 hours. After incubation, the tubes were removed, and 360 µL of yeast nitrogen base was added to each tube. The tubes were then vortexed before being plated. Two replicates of each tube were made on YPD agar plates. All of the plates were incubated at 30°C for 48 hours. After this incubation period, the colony forming units were counted on each plate. The average of the two replicates was taken and the cell viability was calculated.

Results

For evaluating the killing activity of KM23 in each of the strains, a concentration of KM23 was chosen that yielded approximately 50% cell viability when used with the wild-type strain, BY4741. Thus, 1 µM final concentration of the KM23 peptide was used in these studies. The logic for this choice was to provide for the ability to observe either increased resistance or increased sensitivity to the peptide for the various mutants evaluated.

Microdilution assays were performed for the wild-type and the *atp1*Δ, *atp2*Δ, *atp5*Δ and *atp7*Δ mutants. It should be noted that the assays were performed in duplicates for each trial and a minimum of three independent trials were performed with each mutant. For the wild-type there were thirteen independent experiments, the *atp1*Δ and *atp2*Δ mutants were tested in five independent trials, and the *atp5*Δ and *atp7*Δ mutants were evaluated in three independent trials. After conducting the microdilution assays, the data for all the mutants was compiled and statistically analyzed using an unpaired student T-test. The *atp1Δ*, *atp5Δ*, and *atp7Δ* showed significant resistance to KM23, with virtually 100% viability after exposure to 1µM KM23 as compared to the wild-type strain, which exhibited less than 50% cell viability (Figure 2). The *atp2Δ* demonstrated variable results and did not show any significant resistance to KM23 (Figure 2). This was a particularly surprising and unexpected observation. Since the *atp2Δ* mutant was one knockout strain from a large collection of over 4,900 individual *S. cerevisiae* strains, it was plausible that the knockout mutation was not correct and that the putative *atp2Δ* mutant was not as advertised by the company from where it was purchased.

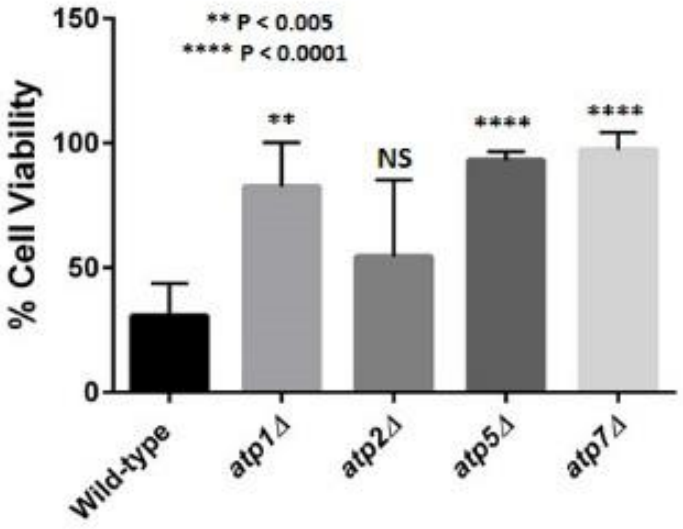

Figure 2. Mutation in the ATP Synthase confers resistance to KM23. Microdilution assays were performed as described in the Materials and Methods. Each of the strains were evaluated in duplicate with a minimum of three independent trials. The average percent cell viability is shown with the bars indicating the standard error. The data was evaluated using an unpaired student T- test and the statistical significance is indicated. NS denotes no statistical difference from the wild-type strain.

In order to address this concern, the wild-type strain and the *atp1*Δ, *atp2*Δ, *atp5*Δ and *atp7*Δ mutants were streaked on YPD medium, containing glucose as the carbon source, and YPG medium, containing glycerol as the carbon source. Strains that could grow on YPG are not respiratory deficient because they are able to generate ATP through respiration by the oxidation of glycerol, a three-carbon substrate. Strains that could only grow on YPD were able to generate ATP via fermentation of the glucose present in the YPD medium, but could not utilize non-fermentable carbon sources. It was found that the wild-type strain grew on YPG as expected. More importantly, it was observed that the *atp2Δ* mutant grew well on YPG medium confirming that it is not respiratory deficient. This data suggests that the *atp2Δ* mutant may not be truly deficient in its abilities to respire; hence, our observation that it was not resistant to the KM23 peptide. One can

also note that even on YPD, the respiration defective strains are clearly distinguishable by the difference in the growth phenotype. The respiratory mutants (*atp1Δ, atp5Δ, atp7Δ*) failed to grow as robustly as the wild-type or the *atp2Δ* mutant due to the depletion of glucose from the medium.

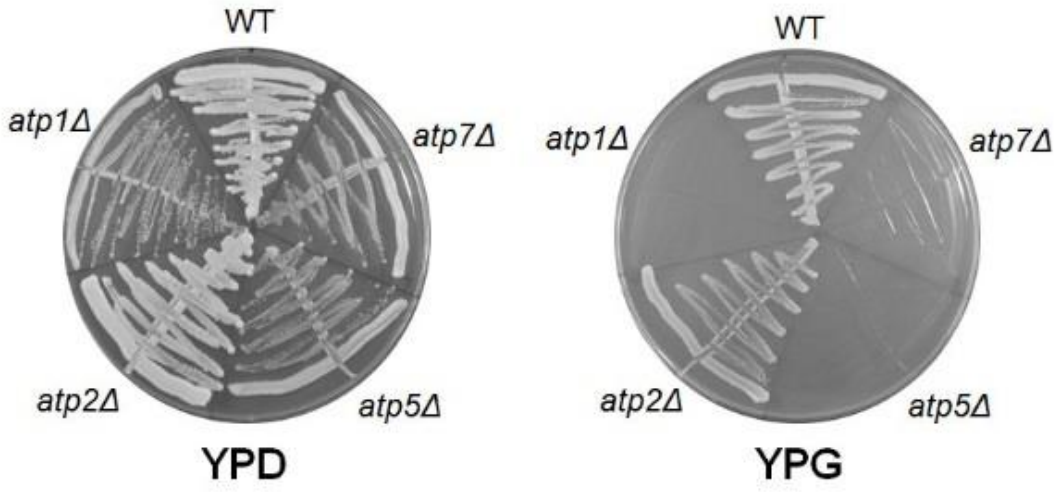

Figure 3. The respiratory phenotype of the putative ATP Synthase mutants. The wild type, *atp1*Δ, *atp2*Δ, *atp5*Δ and *atp7*Δ mutants as indicated were streaked on either YPD medium or YPG medium and grown at 30ºC for 3 days.

Discussion

Multiple other studies have referenced the possible importance of the mitochondrion in the killing mechanism of histatin 5 and its derivatives (Mochon AB and Lui H, 2008) (Helmerhorst EJ, 1999). Previous studies used chemical methods, which have led to uncertain conclusions and the need for a more targeted approach. Through testing respiratory mutants of several subunits within the ATP synthase in *S. cerevisiae,* we were able to specifically target the electron transport chain within the mitochondria. The mutants we used were gene knockouts for each subunit within the F_1F_0-ATP synthase of *S. cerevisiae*. KM23 cannot act without this molecular target within the yeast cell. Mutants that conferred resistance to KM23, the histatin 5 derivative peptide, are involved in the killing mechanism of the peptide. *atp1Δ*, *atp5Δ*, and *atp7Δ* show this trend, having significant resistance to KM23 in comparison to the cell viability of the mutants in the presence of the buffer. *atp2Δ* was not significantly resistant to the lethal effects of KM23; however, it was also shown to not be respiratory deficient. Interestingly though, this mutant still provided an unknown internal control for our studies and further emphasized the importance of respiration in the killing action of our peptide.

The inability of the *atp2Δ* mutant to protect itself against KM23 acts as unintentional support for our hypothesis. After testing the respiratory abilities of *atp2Δ* by growing the mutants on fermentable and non-fermentable carbon sources, we found that the *atp2Δ* strain was not in fact a full gene knockout. It was able to grow on YPG, forcing the cells to respire using glycerol as the primary carbon source and thereby, the electron transport chain to produce energy (Figure 3). Therefore, the *atp2Δ* strain that we assumed did not

have a functional ATP synthase, did in fact have the enzyme. KM23 was able to bind to and act on this strain, just as it could in the wild-type yeast, because the intracellular target was present. This study shows that ATP synthase activity in *S. cerevisiae* is crucial to the killing activity of KM23.

Further support for the role of the mitochondria in the mechanism of KM23 activity comes from the recent Honors thesis of Gabriela Morris, another student in the McNabb laboratory. In those studies, the KM23 peptide was fluorescently labeled and fluorescent microscopy was used to determine the intracellular localization of the peptide. It was found that KM23 localizes exclusively to the mitochondria, lending further support to the model that respiratory metabolism is important for the killing mechanism of KM23.

The question arises as to whether other mutations in the electron transport chain would yield similar KM23-resistant phenotypes or whether this is exclusively associated with the ATP synthase. Future studies should be directed toward examining mutants in subunits of Complex I, Complex II, Complex III and Complex IV of the electron transport chain to determine whether those mutations confer resistance to KM23. Nevertheless, identifying the target pathways involved in peptide resistance does not provide details on the mechanism by which the KM23 peptide is fungicidal. Since *S. cerevisiae* does not require functional respiration for survival, it poses the question as to why mutations that abolish respiration result in resistance to the peptide. Is abundant ATP production essential? Does reduced ATP synthesis influence other pathways, such as energy-dependent transporters, that may be the actual target of KM23 killing?

In order to further study the mechanism of KM23 it must be determined whether respiration in general is required for the lethal activity of KM23, or whether the intracellular target is more specific. In the McNabb lab, students are currently screening a library of approximately 5000 *S. cerevisiae* mutants to determine all possible genetic pathways involved in the fungicidal activity of KM23. This includes mutations within the other complexes of the electron transport chain. In addition, it will be important in future studies to test respiratory mutants in *C. albicans* and other *Candida* species to compare the pathways affected by KM23.

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