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Dissecting the Mechanism of Action of a Novel Antifungal Peptide

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Dissecting the Mechanism of Action of a Novel Antifungal Peptide

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ABSTRACT

There is an urgent need for novel treatments for *Candida* infections. The utility of antimicrobial peptides for antifungal therapy has garnered interest in recent years. One promising family of peptides is the Histatins, a family of naturally-occurring peptides secreted into the oral cavity that display antimicrobial activity. Histatin 5 is a twenty-four amino acid peptide with strong antifungal activity. Studies from our laboratory have identified a small histatin-derived peptide, KM29, that yields fungicidal activity 10-fold greater than Histatin 5 against multiple *Candida* species. Our laboratory has focused on understanding the mechanism of action of KM29 to further develop it as a therapeutic agent for oral and systemic candidiasis. To this end, a genetic screen was carried out using the available genome-wide deletion collection in *S. cerevisiae*. Our goal was to use this as a subrogates species to learn about the killing mechanism used by KM29 in *Candida* species. Analysis of the mutants revealed a significant presence of genes involved in mitochondrial function conferring increased resistance to KM29. We hypothesized that the *S. cerevisiae* mutants affected in different aspects of mitochondrial function will be more resistant to KM29 either because there is less ROS production due to their defective mitochondria, or less ATP production, which in turn may decrease peptide uptake and/or mitochondrial localization. We observed concentration dependent ROS production after exposure to KM29, however, this ROS production was loosely correlated with cell death. We also observed mitochondrial membrane potential depolarization and mitochondrial fission after exposure to KM29, indicating impairment of mitochondrial function. Additionally, we observed that the respiratory status of yeast cells inversely regulates KM29 fungicidal activity by influencing KM29 uptake. In conclusion, these studies provide valuable insights into the mechanism of action of KM29 and of cationic peptides in general.

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LITERATURE REVIEW

Candidiasis

Due to advances in healthcare causing more people to be at-risk for fungal infections, the frequency these infections has increased, making this a growing human health concern. Most fungal infections are caused by *Candida*, *Aspergillus*, or *Cryptococcus* species of fungi, with *Candida* species being the most prevalent causative agents of mycoses-related infections (1, 2). The genus *Candida* contains approximately 150 different species, with approximately 18 being medically relevant (2). As harmless commensals, *Candida* species constitute part of the normal human flora, often residing as a commensal in the gut, genitourinary tract, and skin (2). Usually, *Candida*-related opportunistic mycoses arise from conditions of reduced immune function or imbalanced bacterial microflora (2). Diseases caused by *Candida* species can range from mucosal infections to bloodstream infections, with the former occurring much more frequently (3). Although there are several clinical manifestations, mucosal candidiasis is often characterized by white plagues occurring on mucosal surfaces (4, 5). The exception to this is vulvovaginal candidiasis that has unique clinical manifestations (3). Clinical manifestations of systemic candidiasis vary greatly as *Candida* species have the potential to infect nearly every organ which results in different presentations based on what organ becomes infected (6-9). Although occurring less frequently, systemic candidiasis is perceived as the more threatening human health concern due to the associated high mortality rate. *Candida* species infections are the fourth most common cause of nosocomial bloodstream infections in the US and have attributable mortality rates estimated to be between 15% and 49% (10-12). Additionally, systemic candidiasis infections cause significant financial burdens. In 2002, it was estimated that financial costs resulting from systemic candidiasis cases amounted to $1.7 billion in the US alone (13). It is

recognized that the most common cause of all forms of candidiasis is *C. albicans*, causing 90% of vulvovaginal candidiasis cases and 50 to 60% of systemic candidiasis cases (14-16). Likewise, *Candida* species are often subdivided into two classes: *Candida albicans* and non-*albicans Candida* (NAC) species. Medically relevant NAC species include *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (2).

Candida albicans

C. albicans is a diploid, opportunistic pathogen. As stated above, *C. albicans* in the most common cause of all forms of candidiasis. Interestingly, this may be associated with the relative frequency that *C. albicans* is isolated from human subjects. In various studies, it has been shown that 30 to 60% of healthy human subjects carry *C. albicans* in their microflora (17). Various sites from which *C. albicans* has been isolated include: oral mucosa, vaginal mucosa, the gut, and the skin (2, 17, 18). Due to the diploid genome, it has been difficult to study using traditional genetic approaches, however, much has been clarified regarding the pathogenicity and virulence factors of *C. albicans*.

It comes to no surprise that *C. albicans* has a vast array of virulence factors given its ability to infect a wide range of locations and induce many different clinical manifestations. Some of its virulence factors include: dimorphism, secretion of adhesins and invasins, formation of biofilms, and secretion of hydrolytic enzymes (19).

C. albicans is a dimorphic fungus that can grow as either budding yeast or true hyphae (20). The morphogenetic switch between these two forms is regulated by several environmental cues such as pH, starvation, the presence of serum, temperature, and CO_2 (21). Although both forms of *C. albicans* have shown to be pathogenic, the transition to hyphae is believed to be crucial for pathogenicity (2). Two independent studies showed that infections caused by non-

filamentous *C. albicans* had reduced lethality (22, 23). Additionally, *C. albicans* hyphae has shown to be more invasive than the yeast form (20).

Another virulence factor of *C. albicans* is the secretion of adhesins and invasins. Adhesins are cell wall proteins that confer *C. albicans* the ability to adhere to surfaces or host cells, while invasins induce engulfment of the organism (24). Although secretion of adhesins is not a novel process with the genus *Candida*, it is important to note that *C. albicans* has been reported to have greater adherence than other *Candida* species (25, 26). Interestingly, several of the seemingly most important adhesins for *C. albicans* ability to adhere to surfaces are hyphae specific. The most notable genes implicated in adherence include those from the agglutinin-like sequence (ALS) gene family (27, 28). Eight members make up this family, with Als3 being implicated the most in adherence to host cells (28, 29). Another hyphae specific adhesin important for adherence is Hwp1 (28, 30). Apart from the hyphae specific proteins, numerous non-hyphae specific proteins have been implicated in adherence, including: Eap1, Iff4, Ecm33, Mp65, Phr1, Sap9, Sap10, and Int1 (31, 32).

Additionally, *C. albicans* secretes various invasins that can trigger both passive (endocytosis) and active (penetration) entry into host cells (31-34). Two proteins implicated in triggering passive engulfment of *C. albicans* into host cells include Als3 and Ssa1 (29, 35). Studies have shown that independent deletions of these result in reduced adherence and invasion (32, 35). In contrast, the molecular mechanisms regulating active penetration have yet to be clarified (19).

The colloquial definition of a biofilm is a “surface-associated microbial community surrounded by an extracellular matrix” (36). Biofilms can form on both abiotic (biomedical devices for example) and biotic (host cell) surfaces (19). Biofilm formation is a very relevant

topic in the fields of clinical microbiology and drug resistance. It is estimated that 80% of all microbial infections are caused by biofilms (37). Additionally, with the advent and more frequent use of biomedical devices such as pacemakers and catheters, it is particularly troubling. With regards to *C. albicans*, biofilm formation is of particular concern due to the azole antifungal resistance that is commonly associated with it (36, 38). Various explanations, such as changes in membrane composition, changes in cell wall composition, expression of multidrug resistance pumps, and presence of persister cells, have been proposed to explain the associated azole resistance with biofilm formation (36). Biofilm formation in *C. albicans* is commonly described as a progression through four distinct steps: substrate adherence, biofilm initiation, biofilm maturation, and biofilm dispersal. This progression is regulated by complex genetic pathways involving many genes encoding for various transcription factors and cell wall proteins (36).

Although there are several other virulence factors that aid in *C. albicans* pathogenicity, the last that will be discussed is the secretion of hydrolytic enzymes. Secretion of hydrolytic enzymes by *C. albicans* is thought to not only function in degrading host substrates relevant for the immune response, but also aid in acquisition of nutrients (39, 40). The secreted hydrolytic enzymes can be grouped into three classes: proteases, lipases, and phospholipases (19).

The protease class of secreted hydrolytic enzymes is largely made up of the secreted aspartic proteases (SAP) family (19). Ten members constitute this family and can further be broken down into three groups, each group associated to a particular form of candidiasis (40). Deletion of these members results in varying forms of reduced pathogenicity in animal infection models (41, 42).

C. albicans has ten lipase genes and four different classes of phospholipases (43, 44). Studies have shown that deletion of genes from both classes of enzymes results in significant reduction of pathogenicity, supporting their role as virulence factors (45, 46).

As stated before, *Candida* species infections are the fourth most common cause of nosocomial bloodstream infections in the US, with *C. albicans* being the most frequent cause (11, 47). With advances in healthcare and an increase in population of people with risk factors, the frequency of *Candida* related infections are on the rise (2). As a growing human health concern, it is imperative that more resources be funneled into studying *C. albicans* and the other medically relevant *Candida* species.

Non-*albicans Candida* species

Although *C. albicans* predominates among the species of *Candida* that can cause infection, there are other *Candida* species that impact human health. The most medically relevant non-*albicans Candida* (NAC) species include *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (2). To highlight the relevancy of these organisms, it is estimated that ~90% of all *Candida* related bloodstream infections are caused by *C. albicans* and the three NAC species mentioned (16).

Among all the *Candida* species, *C. glabrata* is the second most common species isolated in systemic candidiasis cases in the US, making this organism the most common species among all NAC species (47). *C. glabrata* is an opportunistic fungus most commonly isolated from the oral cavity (48). This organism has a haploid genome and is traditionally viewed as being more closely related to *S. cerevisiae* than other medically relevant *Candida* species (49). The haploid genome of *C. glabrata*, as well as its emergence as a significant fungal pathogen makes it an enticing organism to study.

In contrast to *C. albicans*, *C. glabrata* is not dimorphic and only grows as yeast cells (2). This is interesting due to the relative importance of the yeast-hyphae switch in *C. albicans* pathogenicity, however, this may denote the distant relation between the two. Relating to this, *C. glabrata* does not express hyphae specific adhesins but instead utilizes the *EPA* gene family that encodes for non-hyphae specific adhesins (50). Similar to *C. albicans*, *C. glabrata* is able to form biofilms which may reflect its propensity of being isolated from dentures (51). Additionally, *C. glabrata* secretes yapsin proteases, which share similarities with SAP9 and 10 proteases (52). Yapsin proteases are important for *C. glabrata* pathogenicity and survival in the host, as studies have shown deletion of genes encoding these proteases reduces survival in macrophages (53).

Of particular clinical importance is the drug resistance often associated with *C. glabrata* infections. Various clinical isolates of *C. glabrata* demonstrating resistance to azoles, caspofungin, or polyenes have been recovered (54-56). Not only does *C. glabrata* have intrinsic resistance to azoles but various mechanisms contributing to significant azole resistance have been identified (2). Although the increase in *C. glabrata* isolated from candidiasis infections is believed to be due to wide-spread use of fluconazole, there is debate whether this increase has been exacerbated by improvements in identification methods (57, 58).

The second most common NAC species isolated in systemic candidiasis cases in the US is *C. parapsilosis* (47). In different regions of the world, *C. parapsilosis* is the second most common *Candida* species isolated in *Candida* related bloodstream infections (59). Bloodstream infections caused by this organism have shown to have mortality rates ranging from 4 to 45% (60, 61). Furthermore, *C. parapsilosis* is the second most common *Candida* species isolated in *Candida* related bloodstream infections originating in NICUs (62). *C. parapsilosis* is a diploid

pathogenic fungus that can grow as yeast cells or pseudohyphae (63). *C. parapsilosis* can be a part of the normal human flora, often isolated from the skin, nails, and hands, but it can also be found in soil, natural sources of water, and domestic animals (63-65). Additionally, *C. parapsilosis* is often isolated from medical devices and thus is a common cause of nosocomial infections (65). Unlike most *Candida* infections arising from endogenous sources, infections caused *C. parapsilosis* arise from exogenous sources (66). Although the pathogenicity and virulence factors of this organism haven't been as extensively studied as they have been in *C. albicans*, it is known that biofilm formation is important for *C. parapsilosis* virulence (63). Also, various SAP gene family proteases have been identified in this organism (67). In general, antifungal resistance isn't an issue with *C. parapsilosis*, although isolated incidents of amphotericin B and azole resistance have been reported (68, 69).

The fourth most common cause of systemic candidiasis in the US is *C. tropicalis*, however, it is the second most common cause of systemic candidiasis in Latin America and the Asia-Pacific region (59). Additionally, the majority of bloodstream infections caused by this organism arise from hematologic malignancies (62). *C. tropicalis* is a diploid opportunistic pathogen that constitutes part of the normal human flora (2, 70). This organism is able to grow as yeast cells or true hyphae. The ability to grow as true hyphae is a significant virulence factor, similar to *C. albicans*, and because of this, *C. tropicalis* is believed to be one of the most virulent NAC species (70). An additional virulence factor of *C. tropicalis* includes the secretion of hyphae specific adhesins, encoded by the *ALS* and *HWF* genes (71, 72). Aiding in its virulence, *C. tropicalis* secretes SAP proteases and is able to form biofilms (70, 73). Similar to *C. parapsilosis*, incidents of azole and amphotericin B resistance have been reported in *C. tropicalis* (70, 74).

As stated above, it is estimated that ~90% of all Candida related bloodstream infections are caused by *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* (16). Additionally, even though *C. albicans* is main cause of all candida related infections, the frequency of isolation from infections of most of the NAC species has increased (16). Furthermore, *C. glabrata* and *C. parapsilosis* have been isolated from bloodstream infections occurring after specific medical procedures at a frequency that rivals, and sometimes surpasses, the frequency that *C. albicans* is isolated in those incidents (62). These data, as well as the reported incidents of antifungal resistance in the NAC species, denote the medical importance of these organisms and the need to develop new antifungals.

Emerging *Candida* species: *Candida auris*

Adding to the medical relevancy of *Candida* species is the recently emerging fungal pathogen, *Candida auris*. This species was first isolated in 2009 from an ear infection of a patient in Japan (75). Since then, cases involving infections caused by this species have arisen around the globe, including in locations such as Korea, Kuwait, Germany, Brazil, South Africa, and several others (76-80). Additionally, there have been several cases in the US (81). Interestingly, the seemingly explosive emergence of this species throughout many different continents is thought to be due to four different clades simultaneously emerging (82).

Various characteristics of this species have been elucidated in the recent years. *Candida auris* is positive for biofilm formation, and only some of the strains are positive for phospholipase and proteinase production (83, 84). This species is similar to *C. parapislosis* due to its ability to cause exogenous nosocomial infections (84). The most alarming characteristic however, is the drug resistance observed in *C. auris* strains. Studies have shown that *C. auris* strains have considerably high fluconazole resistance (85, 86). Although echinocandins seem to

be effective against this species, the cost and availability of these compounds may make their use limited (80).

Current antifungal therapies

The therapeutic agents used to treat superficial and systemic candidiasis are mainly limited to three classes of compounds: the polyenes, azoles, and echinocandins (87). The first major antifungal breakthrough came in 1953 with the discovery of Amphotericin B, a broad-spectrum antifungal in the polyene class of compounds (88). In 2006 two new antifungals were approved, posaconazole and anidulafungin (87, 88). Posaconazole is an azole antifungal that is active against *Zygomycetes* and anidulafungin is an echinocandin class antifungal with good activity against most *Candida* species (88).

As stated above, the first class of antifungals to be discovered were the polyenes. Amphotericin B deoxycholate is the only compound in this class that has been clinically approved to treat *Candida* infections (87). This compound has good activity against most *Candida* species, with minimal inhibiting concentration values (MIC_{90}) ranging from 0.12-4 (88, 89). The exception to this is against *C. krusei*, a NAC species, with the MIC_{90} value ranging from 0.5-8 (88, 89). Amphotericin B works by binding to ergosterol, a fungi specific cell membrane component, causing the formation of pores in the cell membrane and leading to cell death (90, 91). This compound also doesn't have any major drug-drug interactions, unlike many of the other commonly used antifungals (88). The major drawback of Amphotericin B, however, is its drug toxicity towards patients; it has been known to induce renal toxicity and damage. It is estimated that ~50% of patients who receive Amphotericin B as a therapeutic agent will experience adverse effects in the form of electrolyte abnormalities or renal damage (92). Due to the relative effectiveness of this compound against various *Candida* species, efforts have been

made to decrease the drug toxicity, most notably through the use Amphotericin B: lipid formulations. The major drawback of this, however, is cost (87, 93). Drug toxicity aside, Amphotericin B is the first choice for treatment for neonates with systemic candidiasis and for pregnant patients (88, 94).

The next class of compounds in the antifungal arsenal, and the most widely used, is the azoles (95). The first azole to be approved for treatment of fungal infections was miconazole, with many others being synthesized and approved since then, most recently posaconazole (87, 88). The four major compounds in this class include: fluconazole, itraconazole, posaconazole, and voriconazole. However, there are many other azoles available for use such miconazole, ketoconazole, and others (88).

This class is known for the presence of five-membered aromatic rings containing nitrogen atoms and the two subclasses of azoles are differentiated by the number of nitrogen atoms present (96). This structure is intimately related to the function and mechanism of action of this class of compounds as the nitrogen atoms have the propensity to bind to the heme group of cytochrome P450 14α-demethylase (87). This enzyme is encoded by *ERG11* and is vital to the production of ergosterol, thus binding inhibits ergosterol biosynthesis (98). Subsequent to inhibition of ergosterol biosynthesis, cell growth is halted (99). It is important to note the fungistatic, rather than fungicidal, nature of azoles, as this characteristic could allow for drug resistance (98).

Compounds within this class have differing spectra of activity; voriconazole and posaconazole have effective activity against all the most medically relevant *Candida* species, while fluconazole and itraconazole are effective against all except *C. glabrata* and *C. krusei* (88,

100, 101). This is demonstrated by the various MIC_{90} values for these drugs against *Candida* species.

One of the major drawbacks of this class of compounds are the various drug-drug interactions they display, making it imperative for patients to review their prescriptions before receiving antifungal therapy (88). All four major compounds within the azole class interact negatively with antiarrhythmics, antipsychotics, and migraine medications, thus combinations of these are suggested against (88). Also, three of the four major compounds interact negatively with immunosuppressants (88). Another drawback of azole therapy is the accompanied drug toxicity. Azole therapy has been shown to induce rashes, headaches, gastrointestinal complications, and hepatotoxicity (102, 103). Additionally, azole therapy has been shown to cause birth defects, hence why Amphotericin B is the preferred antifungal for pregnant women (104) .

In most instances, fluconazole is the first choice for treatment for all forms of candidiasis. This is mainly due to the other azole compounds not conferring significant advantages over fluconazole in certain manifestations of candidiasis (88). However, fluconazole can be readily replaced by the other azoles if the patient's infection is at risk of developing fluconazole resistance or being fluconazole-refractory (88, 94).

The final class of commonly used antifungal compounds are a group of semisynthetic lipopeptides called echinocandins (105). Three clinically approved compounds constitute this class: caspofungin, micafungin, and anidulafungin (106). The advent of this class is relatively recent, with the first echinocandin being discovered in the 1970s and the first echinocandin being approved for clinical use, caspofungin, in 2001 (87). Anidulafungin was the last echinocandin approved, in 2006 (Roemer, 2014). All three compounds of this class display effective activity

against all the medically relevant *Candida* species besides *C. parapsilosis* (88). These compounds also have similar activity to each other, with the MIC_{90} values ranging from .03-4 across the top four medically relevant *Candida* species for all three compounds (107).

These compounds function by inhibiting β-1,3 glucan synthase, thus inhibiting β-1,3 glucan synthesis. β-1,3 glucan is an essential cell wall component of pathogenic yeast and inhibiting the biosynthesis of this molecule results in cell death (108). These compounds have few drug-drug interactions and drug toxicity problems (88). They are believed to interact with various immunosuppressants, but the resulting effects are mild. Likewise, adverse effects due to echinocandin administration are rare and mild (88).

Various experiments have shown that echinocandin compounds are at least as effective as Amphotericin B and fluconazole (109, 110). Additionally, fewer adverse effects are associated with echinocandin administration compared to these two. Because of this, recent guidelines for treatment of systemic candidiasis are straying away from recommending fluconazole as the first choice for treatment and instead are recommending echinocandins (94).

As stated above, the antifungal arsenal is limited to three classes of compounds: the polyenes, azoles, and echinocandins (87). Each class has limitations based on drug toxicity, drug-drug interactions, cost, and effectiveness against certain *Candida* species. Considering the small number of antifungals available and the limitations of each class, it is imperative new antifungals be developed.

Drug resistance in *Candida* species

Drug resistance is a common concern in all clinical microbiology fields. For fungal infections caused by *Candida* species, it is particularly concerning due to the increase in frequency of infections caused by innately fluconazole resistant *Candida* species, *C. glabrata*

and *C. krusei*, and the increase in isolation frequency of fluconazole resistant *Candida* species (88, 107). Between 2001 and 2007, the in vitro resistance to fluconazole increased for four *Candida* species, including *C. albicans*, *C. tropicalis*, *C. guilliermondii*, and *C. lusitaniae* (107). This increase was also observed for *C. krusei* isolates between the years 1997 and 2007 (107). Although the increases in fluconazole resistant strains were minimal, the fact that there is an upward trend is concerning.

Various mechanisms of resistance for commonly used antifungals have been elucidated in the recent years. For fluconazole in particular, several molecular mechanisms of resistance spanning many *Candida* species have been clarified. Many of the mechanisms of resistance to fluconazole involve modifications of the drug target (98). As mentioned earlier, the azole class of compounds are inhibitors of the enzyme encoded by *ERG11*, 14α-demethylase (97). These alterations can take the form of upregulation of *ERG11*, point mutations of *ERG11*, and aneuploidy of chromosome 5 (98). Upregulation of *ERG11* is thought to be due to gain of function mutations in *UPC2*, an upstream regulator of sterol biosynthesis (111). The specificity for which point mutations of *ERG11* conferring increased fluconazole resistance seems to be low, with 21 different point mutations observed to confer resistance in *C. albicans* isolates (98). Regarding aneuploidy of chromosome 5, gain of this chromosome has been observed to increase fluconazole resistance (112). Each of these mechanisms of fluconazole resistance have been identified in *C. albicans* isolates, while upregulation of *ERG11* and point mutations of *ERG11* have been observed in *C. parapsilosis* and *C. tropicalis* (98). Only one of these mechanisms, point mutations of *ERG11*, have been observed in the emergent *Candida* species, *C. auris* (98).

Two *ERG11*-independent mechanisms of fluconazole resistance in *Candida* species have also been clarified. These include bypassing the *ERG11* pathway, and upregulation of drug

efflux pumps (98). Bypass of the *ERG11* pathway is usually accomplished by loss-of-function mutations in *ERG3* (113). This presumably results in a minimalization of damage caused by fluconazole (113). The mechanism of upregulating drug efflux pumps usually involves *CDR1* and *CDR2* of the ATP-binding cassette (ABC) family or *MDR1* of the major facilitator superfamily (MFS) class (98). Resistance is acquired because upregulation of these pumps results in reduced cytoplasmic levels of fluconazole (98).

Bypass of the *ERG11* pathway has been observed in *C. albicans* and *C. tropicalis* isolates (98). Upregulation of both classes of drug efflux pumps has been observed in *C. albicans, C. glabrata,* and *C. parasiplosis*. *C. krusei* isolates have been observed to have upregulation of the ABC transporters, but not the MFS transporters. In contrast, *C. tropicalis* isolates have been observed to have upregulation of the MFS transporters, but not the ABC transporters (98).

In addition to reports of increased frequency of fluconazole resistance, an increase in frequency of echinocandin resistance in *Candida* species has been reported (55, 114, 115). Although the frequencies of echinocandin resistance remain relatively low, this has the potential to be a great clinical concern if this upward trend continues (105). Adding to the concern is the fact that guidelines are now recommending echinocandins as the first choice for treatment of various forms of candidiasis due to its effectiveness, low drug toxicity, and minimal drug-drug interactions.

As stated earlier, the echinocandin class of compounds functions by inhibiting β-1,3 glucan synthase, resulting in reduced cell wall integrity (108). The major subunit of this enzyme is encoded by *FKS1* and resistance mechanisms involve point mutations of this gene (116, 117). Indeed, various point mutations of *FKS1* have been observed in various *Candida* species. The Candida species isolates that display increased echinocandin resistance due to mutations in *FKS1*

include: *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. dubliniensis* (118). Resistance has also been observed in *C. glabrata* resulting from point mutations in *FKS2*, a paralog of *FKS1* (55).

Resistance to polyenes is predicted to be rare, however, there have been reports of resistance to Amphotericin B in various *Candida* species (70, 74). It is postulated that resistance to Amphotericin B is caused by changes in cell membrane composition due to mutations of *ERG* genes (119, 120).

Although drug resistance can cause complications during antifungal therapy, the low frequency of echinocandin resistance observed should quell major concerns regarding drug resistance. It will be very important to continue conducting surveys for increased resistance, though, since the trends, as of now, point upward. Additionally, having more antifungals available will allow healthcare workers to better treat individuals affected by fungal infections, thus it is imperative to continue the development of novel antifungals.

INTRODUCTION

Fungal infections represent a growing human health concern. *Candida* species are the causative agents of the most prevalent fungal infections in the U.S., causing superficial and systemic candidiasis, the latter associated with high mortality rates (2, 121). Additionally, *Candida* species are the fourth most common cause of nosocomial bloodstream infections in the U.S (11). The therapeutic agents used to treat superficial and systemic candidiasis are mainly limited to three classes of compounds: the polyenes, azoles, and echinocandins (87). The azoles are one of the most prescribed antifungal agent even though some strains of *C. albicans* and *C. glabrata* have acquired resistance to this class of compounds while other *Candida* species, such as *C. krusei*, are innately azole-resistant at therapeutic concentrations (98, 122). In contrast, the polyenes remain highly effective, but drug toxicity has limited their usage (87, 121). Echinocandins, the most recent addition to the antifungal arsenal, have shown to be effective with minimal drug toxicity and thus a promising alternative to the former classes (87, 121). However, an increase in acquired resistance by *C. glabrata* has been reported, likely due to an increase of usage (123-125). The limitations of each class of compounds and the present and future concerns of drug resistance have brought to the forefront efforts to develop new antifungals effective against *Candida* species and other pathogenic fungi.

One area of research that has garnered interest as a source for novel antimicrobial agents is the utilization of naturally occurring antimicrobial peptides (AMPs). AMPs have been identified in a wide range of organisms, including plants, invertebrates, and vertebrates (126). These peptides often serve as first line of defense against invading pathogens and are a vital component of innate immune systems (127, 128). One characteristic that has led to the excitement surrounding these peptides is the broad range of activity they display. AMPs have

shown to have antibacterial, antifungal, antiviral, and antitumor activity (129). In addition to the broad range of activities, antimicrobial peptides display varying mechanisms of action (129). Regarding antimicrobial peptides with antifungal properties, several mechanisms of action (MOA) have been proposed, including: membrane permeabilization, mitochondrial perturbations, initiation of apoptosis, osmotic stress, and DNA damage (130-134). Due to the unique MOAs presented by antimicrobial peptides, it raises the question whether some of these could have synergistic effects with traditional antifungal agents during combination therapy and indeed, synergistic effects have been reported between AMPs and antifungal agents (135, 136).

AMPs share the issues many promising pharmaceuticals have, including: cytotoxicity, expensive or laborious production, low activity, and low stability (137, 138). One increasingly popular technique used to minimize or circumvent these issues is the design and synthesis of AMP analogs (137, 138). Common ways of manipulating natural AMPs include shortening of the peptide, amino acid substitution, and lipid tagging (138). Shortening of AMPs can not only reduce the cost of production but also increase activity (137, 138). A notable example of increasing activity by shortening an AMP is dhvar4, a Histatin 5 derived peptide (139). Amino acid substitution has shown to be able to improve activity and stability (140, 141). Improvement of activity is usually accomplished by substituting amino acids to increase hydrophobicity or cationicity whereas improvement of stability is accomplished by the introduction of D-amino acids or bulky side chains (138). It is thought that lipid tagging improves activity by increasing membrane association (138).

Studies from our laboratory have identified a small histatin 5-derived analog, KM29 (Y-K-R-K-F-K-R-K-Y), that yields significant fungicidal activity and is active against a variety of *Candida* species (142). Structure-function experiments were performed to identify the minimal

sequence (Y-K-R-K-F; KM Motif) needed to retain fungicidal activity (142). Moreover, a review of the literature revealed the presence of this motif in nearly every published Histatin 5-derived analog. This sequence was used to generate peptide variants with enhanced antifungal activity (142). Additionally, KM29 displayed minimal red blood cell hemolysis (142). These data denote the potential of KM29 to be utilized as a cost-effective antifungal therapeutic agent. Here we extend these studies by using *S. cerevisiae* as a genetically tractable model to elucidate the mechanism of action of KM29. We conclude by showing the conservation of MOA in the clinically relevant *Candida albicans*.

MATERIALS AND METHODS

Yeast strains

All experiments involving *S. cerevisiae* were carried with strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (143). In instances when mutant strains were used, these were obtained from the *MATa* haploid deletion library in the BY4741 background (144). Where indicated, experiments involving *C. albicans* were carried out with SC5314, LLF100 (SN152), or LLF088 (SN152). LLF100 and LLF088 originate from the SN152 strain. LLF100 is a prototrophic wild type control strain and LLF088 carries a homozygous deletion for *COQ3* (145). Unless indicated, strains were grown on standard YPD medium (1% yeast extract, 2% peptone, 2% glucose). Plates contained 2% agar (Difco).

Genome wide screen for mutants with increased resistance or increased sensitivity to KM29

Mutant strains from the *S. cerevisiae MATa* haploid deletion library were collected from frozen stocks and inoculated on OmniTray (Nunc) YPD plates for 48 hours at 30° C. Deletion strains were then transferred to 150 µl of sterile distilled water (di$H_2$0) in 96-well plates via 96-well pin replicators. The average OD_{600} of all the wells was calculated with a spectrophotometer (Eon, BioTek) and the cell count of the average OD_{600} was quantified with a hemocytometer. Cell density of individual wells was adjusted by adding more cells or adding more di$H_2$0 to achieve the average OD_{600}. Cells were diluted in di$H_2$0 and added to a 96-well plate containing 0.125X SD+Trp (synthetic dextrose + 400 µm Tryptophan) supplemented with glucose (pH 6.0), 4 µm or 2 µm KM29, and 50 µg/ml resazurin (Sigma) for a final concentration of $5.25x10^4$ cells/ml. 4 µm KM29 was used to test for increased resistance; at this concentration wild-type cells display no growth and growth of a deletion mutant would indicate increased resistance. 2 µm KM29 was

used to test for increased sensitivity; at this concentration wild-type cells display growth and lack of growth of a deletion mutant would indicate increased sensitivity. Each plate contained a well with a wild-type control (BY4741) and a negative control with no cells. Plates containing 4 µm KM29 were incubated for 43-48 hours and plates containing 2 µm were incubated for 29-32 hours at 30° C. After incubation, plates were analyzed for color change of the pH indicator, resazurin. Resazurin has a blue color at pH 6.0 and as the pH drops, due to by-products of fermentative growth in this case, transitions to orange/red. Resorufin, the acid-form of resazurin, can also be fully-reduced to form the colorless dihydroresorufin. Wells were analyzed for a transition from blue to orange/red/colorless and labeled as having growth or no growth. Each plate was repeated in duplicate for each concentration of KM29 and deletion mutants that showed growth or no growth in both trials were denoted as having increased resistance or increased sensitivity to KM29.

KM29 localization in *S. cerevisiae* and *C. albicans*

S. cerevisiae or *C. albicans* SC5314 was grown overnight in 5 ml of liquid YPD at 30° C or 37° C, respectively. The cell count of a 1:100 dilution of an overnight culture was quantified using a hemocytometer to determine the cell density. Cells from the overnight culture were pelleted and resuspended in distilled water (diH_20) to a final concentration of $1x10^8$ cells/ml. Either KM29-FITC, MitoTracker DeepRed (Molecular Probes), or both concurrently were added to cell suspensions for final concentrations of 5 uM KM29-FITC and 500 nM MitoTracker DeepRed. 3 µM KM29-FITC was used for *C. albicans* experiments to minimize oversaturation. MitoTracker DeepRed is a mitochondria specific stain that constitutively localizes to mitochondria in live cells. After incubation for 30 minutes at 30° C, cells were washed 2 times in 1X phosphate buffered saline (PBS) and visualized with a fluorescence microscope (Zeiss AxioImager M1). A

GFP filter (Zeiss set 38) was used to observed KM29-FITC localization and a Cy5 filter (Zeiss set 50) was used to observed MitoTracker DeepRed localization.

Fungicidal activity of KM29 against *S. cerevisiae* and *C. albicans*

Fungicidal activity of KM29 was probed using microdilution plate assays. *S. cerevisiae* was grown for 48 hours on YPD plates at 30° C. Single colonies of *S. cerevisiae* were resuspended in 1 ml of 5 mM sodium phosphate (NaP) buffer, pH 7.4. Cell counts were then calculated using a hemocytometer. Afterwards, cells were diluted in the same buffer to reach a cell density of $1.5x10^5$ cells/ml. Cell suspensions were made by mixing 20 µl cells and 20 µl of peptide dissolved in 5mM NaP buffer. Final peptide concentrations used were 1, 2.5, and 5 µM. These suspensions were incubated in a shaking incubator at 220 rpm for 2 hours at 30° C. Peptide reactions were stopped by adding 360 µl of yeast nitrogen base (YNB). 40 µl of cell suspension added to 100 µl of YNB was spread onto YPD plates with 6-8 glass beads and incubated for 48 hours at 30° C. Colony forming units (CFUs) were counted and percent survival was calculated as (colonies from suspension with peptide)/(colonies from suspension without peptide)x100. Each assay was repeated a minimum of three times.

This protocol was modified slightly to determine fungicidal activity of KM29 against *C. albicans* LLF100 and LLF088. Initially, *C. albicans* was grown for 24 hours on YPD at 37° C. Due to *C. albicans* increased sensitivity to KM29, 10 mM NaP buffer was used instead of 5 mM in all cases, and final peptide concentrations of 0.5, 1, and 2.5 µM were used instead of 1, 2.5, and 5 µM used for *S. cerevisiae*. These two changes allowed us to maintain a survival curve that approximately displayed 75, 50, and 25% survival, respectively. Plates were incubated for 24 hours at 37° C.

In instances that petite mutants were analyzed using microdilution assays, all incubation times for plates were extended by 24 hours to account for the slow growth of these mutants.

Measurement of ROS production

S. cerevisiae was grown overnight in 5 ml liquid YPD at 30° C. Cells from the overnight culture were resuspended in 5 mM sodium phosphate buffer (NaP) pH 7.4 to reach a cell density of ~OD_{600} 1.2 and KM29 was added to final concentrations of 56 or 139 µm. After incubation for 1 hour at 30° C, cells were washed and resuspended in 1X phosphate buffered saline (PBS). Dichlorofluorescein diacetate (DCFHDA; SIGMA) or dihydroethidium (DHE; Molecular Probes) was added to final concentrations of 10 µm or 1 µm, respectively. DCFHDA and DHE are fluorescent probes that upon oxidation by reactive oxygen species, transition to highly fluorescent molecules. For DHE related fluorescence, ethidium requires intercalating within DNA to fluoresce. These solutions were incubated for 30 minutes at 30° C in the dark in a roller drum at medium speed. Cells were then transferred to a black 96-well plate (Costar 3603) and fluorescence intensity was measured with a fluorescence spectrophotometer (Eon, BioTek). Transition from DCFHDA to DCF was measured with λ_{ex} 504 nm and λ_{em} 529 nm, and transition from DHE to ethidium was measured with λ_{ex} 518 nm and λ_{em} 606 nm. A sample of the cells was also visualized with a fluorescent microscope (Zeiss AxioImager M1). DCF fluorescence was viewed with a GFP filter (Zeiss set 38) and ethidium fluorescence was viewed with a Rho filter (Zeiss set 20).

Measurement of ROS-induced cell death

S. cerevisiae was grown overnight in 5 ml liquid YPD at 30° C. Cells from the overnight culture were resuspended in fresh YPD to reach a cell density of ~OD_{600} 1.2. N-acetyl cysteine (NAC; SIGMA) was added for a final concentration of 50 mM and incubated for 2 hours at 30° C in a

roller drum at medium speed. For cell suspensions without NAC treatment, a concomitant volume of diH_2O was added. After incubation, cells from the two experimental groups were used in microdilution killing assays as described earlier.

Assessment of mitochondrial network morphology and mitochondrial membrane potential

To assay mitochondrial network morphology and mitochondrial membrane potential (MMP), we employed the Mitoloc system (146). The MitoLoc system is a microscopy-based dual fluorescence system used to assay in vivo mitochondrial membrane potential (MMP) and mitochondrial network morphology. It consists of a plasmid constructed with GFP-tagged, preSU9 of *Neurospora crassa*, and mCherry-tagged, preCOX4 of *S. cerevisiae*; preSU9 localizes to mitochondria independent of MMP, thus robustly labeling mitochondrial structures, while preCOX4 localization to mitochondria is MMP dependent (146). Measuring colocalization between the two markers allows for single cell quantification of MMP on a pixel-by-pixel basis. *S. cerevisiae* BY4741 cells were transformed with pMitoloc using a one-step yeast transformation protocol with the addition of a 4 hour recovery in YPD and then plated on YPD containing 100 µg/ml of nourseothricin (CloNat, Werner) for plasmid selection (147). One of the resulting transformants was used to inoculate 5 ml of liquid YPD+CloNat and grown overnight. A 1:100 dilution of the overnight culture was made, and the cell count of this dilution was quantified using a hemocytometer. Cells were pelleted and resuspended in 5 mM sodium phosphate buffer (NaP) pH 7.4 to reach a cell density of $1x10^8$ cells/ml. Additionally, either carbonyl cyanide m-chlorophenyl hydrazine (CCCP; SIGMA) or KM29 was added to the cell suspensions for final concentrations of 15 µm and 166 µm, respectively. After incubation for 3 hours at 30° C, cells were washed 2 times in 1X phosphate buffered saline (PBS) and fixed with 10% formaldehyde (Mallinckrodt) for 30 minutes at room temperature. Afterwards, cells were

washed once with 1X PBS and viewed with a fluorescence microscope. The GFP filter (Zeiss set 38) was used to observe the MMP independent mitochondrial marker and the Cy5 filter (Zeiss set 50) was used to observe the MMP dependent mitochondrial marker. Image stacks using Z-spacing of 200 nm were acquired with Zeiss AxioVision image analysis software and processed by deconvolution. Image stacks were then analyzed using the ImageJ plugin yeast_correlation.

KM29 uptake in *S. cerevisiae* and *C. albicans*

S. cerevisiae or *C. albicans* (LLF100 and LLF088) was grown overnight in liquid YPD culture at 30° C or 37° C, respectively. A 1:100 dilution of the cell culture was made in either 5 mM sodium phosphate (NaP) buffer pH 7.4 for *S. cerevisiae* or 10 mM NaP buffer for *C. albicans*. Cell counts were quantified using a hemocytometer. Cells from the original culture were pelleted and resuspended in NaP buffer for a final cell concentration of $1x10^8$ cells/ml and 5 µm KM29-FITC. Cell-peptide suspensions were incubated for 30 minutes at 30° C in a roller drum at medium speed. Cells were washed twice and resuspended in 1X phosphate buffered saline (PBS). 200 µl of these cells were added to a black 96-well plate and fluorescence intensity was quantified in a fluorescence spectrophotometer at λ_{ex} 495 nm and λ_{em} 519 nm. Additionally, cells were examined with a fluorescence microscope (Zeiss AxioImager M1) with a GFP filter (Zeiss set 38).

RESULTS

Genome wide screen for mutants with increased resistance or increased sensitivity to KM29. In an effort to discern the antifungal mechanism of action of KM29 we took advantage of the *S. cerevisiae* non-essential deletion library and devised a modified MIC assay that would allow us to identify mutants that conferred either increased sensitivity of resistance to the peptide. The *MATa* haploid mutant library in the BY4741 background was screened for increased resistance and increased sensitivity to KM29 compared to the wild-type. Mutants with increased resistance were identified by the ability to grow in the presence of a KM29 concentration that completely inhibited wild-type growth, while mutants with increased sensitivity were identified by the inability to grow in the presence of a KM29 concentration that did not inhibit wild-type growth. Of the ~4900 mutants tested, 1,360 displayed increased resistance in duplicate while 508 displayed increased sensitivity in duplicate. These mutants represented ~27 and ~10% of the total number of mutants screened, respectively.

Two clustering methods were carried out on the mutants to identify any patterns associated with increased resistance or increased sensitivity. The first clustering analysis employed was GO annotation from the SGD database (Table 1A & 2A). GO annotation grouping is based on annotations derived from published experiments in the GO database. ORF lists for mutants that displayed increased resistance or sensitivity were inputted into the Yeast GO-SlimMapper and grouped by "Process". The second clustering analysis employed was FunSpec (Table 1B & 2B). FunSpec clustering is derived from published experiments in the GO database as well as the MIPS database. ORF lists for mutants that displayed increased resistance or sensitivity were inputted into FunSpec and grouped based on various parameters such as function, process, localization, protein complexes, phenotypes, etc.

To analyze the results of the GO annotation clustering, the normalized genome frequency (cluster number/genome number x 100) was calculated for each Biological Process and ranked in descending order. As shown in Table 1A, several functional categories were enriched in the mutants that displayed increased resistance. Interestingly, a few biological processes relating to mitochondrial function were among the most enriched, including: mitochondrial translation, cellular respiration, and mitochondrion organization. These categories had normalized genome frequencies of 37, 35, and 31%, respectively. Amino acid transport and generation of precursor metabolites and energy were also heavily enriched processes. Surprisingly, sporulation had the third highest normalized genome frequency at 35%.

Further quantification of the categories enriched by the resistant mutants supported the findings by the GO annotation clustering (Table 1B). In every category, mitochondrial related annotations were the most significantly represented. Revealing is the fact that a respiratory deficiency phenotype was one of the most significantly enriched annotations. This may denote the underlying reason for why so many mitochondrial mutants were uncovered. Understanding the relationship between mitochondria and KM29s mechanism of action served as the basis for the rest of the studies presented.

Analysis of the mutants with increased sensitivity to KM29 by GO annotation clustering revealed invasive growth in response to glucose limitation, organelle inheritance, and cytoplasmic translation as the functional categories with the three highest normalized genome frequencies (Table 2A). These categories had normalized genome frequencies of 27, 23, and 22%, respectively. Interestingly, endosomal transport and vesicle organization had high normalized genome frequencies as well. Analysis of these mutants by FunSpec revealed an increased significance of annotations relating to endosomal transport and vacuolar function than

the GO annotation clustering revealed (Table 2B). In every category, an annotation relating to the endosome was significantly enriched. Additionally, translation related annotations were also present. Interestingly, the divalent cation and heavy metal sensitivity phenotype was significantly enriched by the mutants that displayed increased sensitivity to KM29.

KM29 localizes to mitochondria in *S. cerevisiae* and *C. albicans*. Among the mutants that displayed increased KM29 resistance there was a prevalence of mitochondrial annotations that prompted us to investigate the relationship between mitochondrial function and the mechanism of action of KM29. To study whether there was a direct association between KM29 and mitochondria, the internal localization of KM29 was investigated with fluorescence microscopy. *S. cerevisiae* and *C. albicans* cells were incubated with 5μM and 3μM KM29-FITC, respectively, and 500nM MitoTracker DeepRed concurrently. Preliminary work showed that labeling of KM29 with FITC did not affect the fungicidal activity of KM29, assessed by minimum inhibitory concentration assays (data not shown). KM29-FITC localized in a punctate pattern with some cells displaying increased localization near the cell periphery in *S. cerevisiae* and *C. albicans* (Fig. 1). In both organisms, partial colocalization occurred between KM29-FITC and the mitochondria-specific stain, MitoTracker DeepRed. These data indicate that KM29 is directly associated with mitochondria once internalized by *S. cerevisiae* and *C. albicans* even though some KM29 remains cytosolic, perhaps due to interaction with other cellular targets.

KM29 fungicidal activity is dependent on Electron Transport Chain function. The results from the genome wide screen and KM29 localization study demonstrated mitochondria play a role in the mechanism of action of KM29 and led us to hypothesize that the process of aerobic respiration plays an intimate role in KM29 fungicidal activity. To test this hypothesis, as well as

confirm the results of the genetic screen, we performed microdilution killing assays with *S. cerevisiae* deletion mutants encompassing the Electron Transport Chain (ETC) complexes.

Ndi1, NADH:ubiquinone oxidoreductase, is the only subunit of the yeast Complex I, responsible for transferring elections from NADH to ubiquinone in the respiratory chain (148). The *ndi1*Δ strain didn't show significantly increased resistance to KM29 in either the microdilution killing assays or the genome wide screen (Fig. 2). We next assayed Sdh2, a subunit of succinate dehydrogenase and a constituent of Complex II, which receives electrons from FADH2 (148). The *sdh2*Δ strain showed significantly increased resistance to KM29 and likewise, was uncovered to have increased resistance in the genome wide screen. It is important to note that minor amounts of respiration can still occur in single deletion mutants of each of these, and therefore, this may reflect the ability of KM29 to still have fungicidal activity against single deletion mutants of these complexes.

The next two complexes in the ETC series, Complex III (cytochrome bc1 complex) and Complex IV (cytochrome c oxidase), are essential for respiration (148). A deletion of the genes encoding essential subunits in each of these complexes (*qcr7*Δ, *cox9*Δ, and *cox12*Δ) results in significantly increased resistance to KM29, almost reaching 100% survival (Fig. 2). The three mutants also displayed high resistance in the genome wide screen. This increased resistance is presumably due to the complete lack of respiration in these mutants. *Qqcr10*Δ is a non-essential subunit in the cytochrome bc1 complex; it is the last subunit to be assembled to the complex but is not required for respiratory growth (149). According to our hypothesized relationship between aerobic respiration and KM29 mechanism of action, we would expect a *qcr10*Δ strain not to be resistant to KM29 since it is still able to respire. Indeed, *qcr10*Δ cells were sensitive to KM29, not significantly different from the wild-type strain. Interestingly, the *qcr10*Δ mutant was also

identified in the screen for increased resistance to KM29, which we interpret as a false positive, likely a result of the extreme sensitivity of the MIC assay detecting slight perturbations in growth rates. Altogether these results confirm our hypothesis that KM29 fungicidal activity is dependent on ETC function; cells defective in respiration are more resistance to KM29.

Reactive oxygen species (ROS) production in *S. cerevisiae*. The above results implicating the ETC in KM29 activity raise the possibility that perturbations of the ETC may be involved in initiating/causing cell death. One of the most common outcomes of aberrant ETC function is ROS formation. Two, common fluorescent probes suitable for measuring ROS formation include dichloroflourescein diacetate (DCF-DA) and dihydroethidium (DHE). To measure ROS formation induced by KM29, we preloaded cells with either 10μM DCF-DA or 1μM DHE and subsequently treated them with increasing concentrations of KM29. ROS formation was analyzed with fluorescent spectrophotometry and fluorescent microscopy. KM29 induced ROS formation regardless of the probe used to test it. When measured with 10μM DCF-DA ROS formation increased upon treatment with KM29 in a concentration dependent manner (Fig. 3A,B). However, when measure with 1μM DHE, the significant increase in ROS formation appears to reach a plateau with 56μm KM29 (Fig. 3C,D). This is presumably the maximum level of ROS detected due to the minimal concentration of DHE that was used to preload cells. As suggested, 1μM DHE was used to avoid artifacts caused by excessive DHE (150).

To assess whether there is a correlation between KM29-induced, ROS formation with KM29 fungicidal activity, cells were preloaded with n-acetyl cysteine (NAC), a known ROS scavenger, and subsequently used in microdilution killing assays. NAC treatment completely abolished KM29-induced, ROS formation but did not increase survival by a concomitant amount; NAC pretreatment led to ~15% increase in survival at each KM29 concentration (Fig.

3E, 3F). This is consistent with the findings from the genome wide screen as only one gene associated with the oxidative stress response was found to be more sensitive to KM29 when deleted. These results indicate that ROS formation contributes to KM29 fungicidal activity, but it is unlikely the only source of damage caused by KM29.

KM29 causes depolarization of the mitochondrial membrane potential and mitochondrial fragmentation in *S. cerevisiae*. To explore other mitochondrial perturbations possibly caused by KM29, we employed the MitoLoc system to assay in vivo mitochondrial membrane potential (MMP) and mitochondrial network morphology. A plasmid expressing GFP-tagged preSU9 and mCherry-tagged preCOX4 was introduced into wild-type and *qcr7*Δ strains. preSU9 localizes to mitochondria independent of MMP, thus robustly labeling mitochondrial structures, while preCOX4 localization to mitochondria is MMP dependent (146). Colocalization of the two markers allows for single cell quantification of MMP. Transformed wild-type cells were treated with either a non-lethal concentration of KM29 or 15μM CCCP (as a positive control for MMP disruption). The Pearson Correlation Coefficient was significantly decreased when cells were treated with either KM29 or CCCP, indicating a decrease in MMP (Fig. 4A). We included *qcr7*Δ cells as an additional control. Since these cells are respiratory-deficient we expected the Pearson Correlation Coefficient to be very low, as indeed was the case. In addition to lowering MMP, KM29-treated cells displayed clear mitochondrial fragmentation when compared with untreated cells (Fig. 4B). Also noteworthy is the fact that mitochondrial fragmentation did not occur in neither the CCCP treated cells nor the transformed *qcr7*Δ cells. These data show that the physiological status as well as the structure of mitochondria is significantly affected by KM29 treatment, consistent with KM29 localization to mitochondria and the ETC involvement in its fungicidal activity.

Respiratory status inversely regulates KM29 uptake in *S. cerevisiae*. The results thus far revealed mitochondria as one of the sites for KM29 induced perturbations, however, these findings have not clarified the mechanism by which respiratory deficiency confers cells nearly complete resistance to KM29. To investigate whether the resistance associated with respiratory deficiency is a consequence of a cytoplasmic effect or could also be affecting uptake of the peptide we measured KM29-FITC uptake in the ETC mutants presented earlier. Surprisingly, the respiratory capability of each mutant inversely regulates KM29 uptake (Fig. 5), i.e. mutants that are completely respiratory deficient have minimal KM29 uptake and mutants that have partially decreased respiratory capabilities have intermediate levels of KM29 uptake. The decrease in KM29 uptake is reflected in the killing assays presented in Fig. 2. We also looked at KM29 uptake and KM29 fungicidal activity in ATP synthase mutants. Since ATP synthase is the final complex involved in energy production from aerobic respiration, we hypothesized that these mutants would be deficient in KM29 uptake. Indeed, mutants of ATP synthase showed to have minimal KM29 uptake and ~100% survival when treated with 2.5μM KM29 (Fig. 5). Since ATP synthase isn't involved in electron shuttling nor establishment of the proton gradient, this raises the intriguing possibility that the underlying reason KM29 resistance is observed in respiratory deficient cells is due to reduced ATP production.

Respiratory status inversely regulates KM29 uptake and killing in *C. albicans*. The purpose of the genetic screen using the available genome-wide deletion collection in *S. cerevisiae* was to use this as a subrogates species to learn about the killing mechanism used by KM29 in *Candida* species. Therefore, we asked if our findings in *S. cerevisiae* would extend to the clinically relevant *Candida albicans*. To do this we analyzed KM29 uptake and killing in a *coq3*Δ mutant of *C. albicans*. *COQ3* encodes an enzyme involved in the synthesis of mitochondrial ubiquinone.

Ubiquinone shuttles electrons from Complex I & II of the ETC to Complex III (151). Absence of ubiquinone results in respiratory deficiency, thus we would expect KM29 uptake and killing to be decreased in this mutant when compared to the wild-type. The *coq3*Δ mutant had significantly decreased KM29 uptake and this is reflected in the survival when both the mutant and wild-type were treated with 1μM KM29 (Fig. 6A). In addition, the *S. cerevisiae coq3*Δ mutant shared this phenotype (Fig. 6B). Interestingly, when we compared KM29 uptake between *S. cerevisiae* and *C. albicans* wild-type cells (Fig. 6B & 6E), *C. albicans* had substantially higher KM29 uptake, which correlates with the increased sensitivity of *C. albicans* to KM29.

DISCUSSION

The goal of this study was to elucidate the fungicidal mechanism of action of the novel antifungal peptide, KM29, inspired on the naturally occurring histatins. We initially developed and performed an unbiased screen of the haploid deletion collection in *S. cerevisiae* to identify mutations that confer increased resistance or increased sensitivity to KM29 in an effort to uncover genetic pathways involved in the mechanism of action of KM29. Of the ~4,900 mutants tested, 1,360, ~27% of the total mutants displayed increased resistance while 508, ~10% of the total mutants tested displayed increased sensitivity. We hypothesized that any biological process implicated in increased resistance to KM29 may represent an intracellular target of the peptide, way of entry into the cell, or alterations in the cell wall and membranes. In contrast, we hypothesized that any biological process implicated in increased sensitivity may represent a defense system against KM29 fungicidal effects, or alterations in the cell wall and membranes. Although broad, these served as our basis for analyzing the results of the genetic screen.

Genetic pathways implicated in increased sensitivity to KM29

Regarding the mutants that conferred increased sensitivity to KM29, statistical analysis with the Yeast GO-SlimMapper revealed several biological processes. Those with the most significant representation included invasive growth in response to glucose limitation, organelle inheritance, cytoplasmic translation, endosomal transport, vesicle organization, and protein glycosylation. In addition, we performed a FunSpec analysis of these mutants which revealed endosomal and vacuolar mutants to be at the intersection of these two analyses.

It is interesting that these two systems are implicated in sensitivity to KM29, as they both have functions in degradation of cell membrane proteins (152). This process begins with endocytosis and formation of vesicles carrying membrane components. These vesicles coalescence to form early endosomes (152). From here, cargo in early endosomes is sorted into

those components destined for degradation in the vacuole and those destined for the golgi in order to be recycled (152). It is possible that upon contact with the cell membrane, KM29 induces damage on cell membrane components which signals these components to be endocytosed and degraded. Impairment of the endocytosis system may result in accumulation of damaged molecules on the cell membrane, thus resulting in collapse of cell membrane integrity and eventually cell death. Likewise, impairment of the system that transports these damaged molecules to the vacuole, or of the vacuole itself, may result in intracellular accumulation of damaged membrane components and cause further damage. The explanation is particularly interesting given the cationic nature of KM29 and the numerous reports of cationic molecules interacting with membranes on various cells to induce pore formation or membrane damage (153-155).

Vacuoles also play an important role in cellular homeostasis by storing various ions and molecules (156). In a scenario where KM29 induces osmotic stress, the vacuole would serve to counter this stress. Impairment of the vacuolar function, in this scenario, would lead to the cell being less able to cope with the osmotic stress caused by KM29, thus causing increased sensitivity. Confirmation of this would denote a conservation of mechanism of action between KM29 and Histatin 5, the protein from which KM29 was designed, as a study found that Histatin 5 induces osmotic stress in *C. albicans* (157). This possibility is slightly attenuated, however, by the low frequency for which genes categorized in the response to osmotic stress biological process were enriched by our genetic screen.

Supporting the implication of endosomal and vacuolar function in increased sensitivity to KM29 is the redundant categorization of endosomal-vacuolar genes into seemingly unrelated biological processes with high normalized genome frequencies in our screen. For example,

EMP70 is categorized in invasive growth in response to glucose limitation, as well as, endosomal transport. It is expected that many genes, such as this one, would have differential expression and roles in a complex process, such as invasive growth, unrelated to their basal roles within cells. Additionally, many of the genes categorized in the organelle inheritance biological process, the biological process with the second highest normalized genome frequency in our genetic screen, have functions relating to endosomes and vacuoles. *PEP12*, *VAC8*, and *VPS21* are some examples of this group. Altogether, these findings led us to hypothesize KM29 may induce cellular membrane damage and/or alterations in cellular homeostasis. Future work will be needed to confirm this.

Genetic pathways implicated in increased resistance to KM29

Regarding the mutants that conferred increased resistance to KM29, statistical analysis with the Yeast GO-SlimMapper revealed several biological processes. The most significant biological processes include mitochondrial translation, amino acid transport, cellular respiration, sporulation, mitochondrion organization, and generation of precursor metabolites and energy. In addition, we performed a FunSpec analysis of these mutants which revealed significant enrichment of mitochondrial related categories. We initially hypothesized that any biological process implicated in increased resistance to KM29 may represent a cellular target, way of entry into the cell, or alteration in the cell membrane or cell wall. We believe our results support this hypothesis by revealing amino acid transport as a possible way of entry into the cell, and mitochondria as a possible target for the peptide.

Amino acid transport had the second highest normalized genome frequency in our screen. This leads us to theorize that KM29 may hijack various amino acid permeases and transporters as a way of entry into the cell. In this scenario, it would make sense for a deletion of one of the

permeases to confer increased resistance to KM29 since there would be less cytoplasmic accumulation of KM29 and therefore, less cellular damage. To analyze whether there is specificity for the transport mechanisms KM29 can hijack, we looked closer at the genes categorized in this group. Many different amino acid transporters were present, including: a leucine permease (*BAP2*), an arginine permease (*CAN1*), a proline permease (*PUT4*), a tryptophan and tyrosine permease (*TAT2*), a cysteine transporter (*YCT1*), and a general amino acid transporter (*VBA5*). This reveals that KM29 entry into cells may be promiscuous and lacking specificity; KM29 may simply take advantage of any entry mechanism. A GABA permease (*UBA4*), was also present in this category, reflecting another transport mechanism KM29 may use for entry into cells. These data open up the intriguing possibility this nine-amino acid cationic peptide may interact with highly specific but unrelated membrane transporters in a non-specific manner to enter the cell. Alternatively, the effect is indirect; cells lacking certain transporters may undergo changes on the plasma membrane that affects permeability to KM29.

As part of our initial hypothesis, we postulated that biological processes implicated in increased resistance to KM29 would represent a cellular peptide target. Our reasoning behind this hypothesis was that if a cellular peptide target is deleted or impaired the cellular drug, then KM29 would be unable to induce damage, thereby increasing survival. Our screen identified the mitochondria as a drug target of KM29, as a myriad of genes relating to mitochondria and mitochondrial function were found to increase resistance when deleted. Four of the six biological processes with the highest normalized genome frequencies were related to mitochondrial function, including: mitochondrial translation, cellular respiration, mitochondrion organization, and generation of precursor metabolites and energy. Additionally, the FunSpec analysis of the mutants with increased resistance revealed an enrichment for mutants with a respiratory

deficiency phenotype, which may denote the process of respiration as being a specific source of KM29-induced damage. Consequences of impairment of respiration would include reactive oxygen species (ROS) production and decrease in ATP levels, both of which would have adverse effects on cells.

The possibility of mitochondria serving as the cellular target of KM29 is exciting. Several studies have highlighted the potential of developing antifungals that target this organelle and the advantages associated with it (158, 159). Among these advantages include the crippling damage mitochondrial perturbations incur on yeast cells due to the critical cell functions this organelle is involved in (158, 159). Additionally, many of the fungal mitochondrial factors that could be used as potential drug targets do not have close orthologs in humans, which would reduce drug toxicity (158). Mitochondrial dysfunction has also been implicated in hypersusceptibility to fluconazole, stressing the relevance of this organelle in the mechanism of action of antifungals (159).

The targeting of mitochondria by KM29 may also denote a conservation of mechanism of action between KM29 and Histatin 5. Studies have shown energized mitochondrion as the cellular target of Histatin 5, based on the loss of mitochondrial membrane potential and formation of ROS triggered by exposure to the peptide (160). In a broader sense, this may denote a common mechanism by which cationic peptides work. Most studies on cationic peptides focus on the membrane activity of these molecules, with little effort being put into understanding the intracellular mechanisms. Further exploration on this aspect will be of general relevance to continue developing these molecules as antifungals of therapeutic value.

Based on these data, we propose KM29 to have a multi-step mechanism of action following these sequence of events: KM29 entry into the cell via interaction with the plasma

membrane resulting in pore formation, and non-specific opportunistic use of plasma membrane transport mechanisms, cellular damage caused by KM29 interacting with the plasma membrane, followed by targeting to the mitochondria, once inside the intracellular compartment, resulting in perturbation of mitochondrial function. The possible multi-faceted mechanism by which KM29 could induce cell death makes this peptide an interesting antifungal agent as this would make it more difficult for pathogenic yeast to acquire drug resistance. Future studies will be needed, however, to clarify the exact roles of the genetic pathways implicated in increased resistance and increased sensitivity to KM29 in this peptides mechanism of action.

KM29-induced mitochondrial pertubations

To follow up on our proposed mechanism of action, we focused on the role of mitochondrial function in KM29 fungicidal activity. Our observation of KM29 localization to mitochondrion, as well as the impairment of the electron transport chain conferring increased resistance to KM29, supports our proposed mechanism. The finding that KM29 localizes to mitochondrion is interesting, as an independent study found that the cellular target of Histatin 5 to be the mitochondrion (160). Although this group proposes the localization of Histatin 5 to mitochondria to be due to structural resemblances between Histatin 5 and mitochondrial presequences, we do not believe this to be the case for KM29. An explanation for KM29's localization may be the cationic nature of this peptide. It is possible that KM29 localization to mitochondria may be electrostatic-dependent. The proton gradient formed across the inner mitochondrial membrane in actively respiring cells may serve as an attractant for KM29. This explanation is supported by other work studying the actions of cell penetrating peptides and cationic antibacterial peptides (155). This group proposed that the toxicity of these peptides was dependent on large membrane potentials. For future studies, it would be fruitful to test this

possibility by depolarizing the MMP and observing if KM29-FITC intracellular localization is affected by this. If the MMP is an attractant for KM29, we would expect prior depolarization to inhibit KM29 localization to mitochondria. Additionally, it would be interesting to examine how this might affect KM29 fungicidal activity and would provide confirmation of mitochondria serving as a site of damage.

We also observed KM29 to induce concentration-dependent ROS formation. This finding may denote conservation of mechanism between KM29 and Histatin 5, as it has been reported Histatin 5 could induce ROS formation, and this ROS formation was strongly correlated with cell death (161). However, there is controversy surrounding the role of ROS in Histatin 5 mechanism of action as a separate group did not observe ROS formation due to Histatin 5 exposure (162). In our case, KM29-induced ROS formation was not strongly linked with cell death, as pretreatment with a ROS scavenger prior to exposure to KM29 only increased survival by ~15%. This is incongruent with our finding that complete respiratory deficiency confers nearly 100% resistance to KM29, thus eliminating the possibility that ROS formation is the predominate cause of cell death. Although ROS formation did not appear to play a major role in KM29 fungicidal activity against *S. cerevisiae*, it is possible KM29-induced ROS formation may adversely affect *C. albicans* more substantially. The Crabtree effect of *S. cerevisiae*, presented by the ability to ferment in the presence of high glucose levels and repression of respiration, is quite different from the Crabtree negative *C. albicans*. Thus, optimal growth of *C. albicans* relies more heavily on cellular respiration (163, 164). The increased cellular respiration of *C. albicans* may exacerbate KM29 ability to cause ROS production. Along this line, it will be interesting in future studies to examine how KM29-induced ROS formation affects different pathogenic yeasts.

Determining the status of mitochondria requires analyzing both MMP and mitochondrial network morphology, a complex endeavor due to these two variables not being coupled to one another (146, 165, 166). We observed KM29 to induce depolarization of mitochondrial membrane potential (MMP) and mitochondrial fission. This could be a sign of mitochondrial damage, as the process of mitochondrial fission has been shown to protect healthy mitochondria, and to cause degradation of damaged mitochondria (167, 168). However, it is also possible that KM29-induced mitochondrial fission simply occurred due to loss of MMP, as rho^- have been shown to have fragmented mitochondria (146). Additionally, our observation is consistent with our finding that ROS formation did not play a major role in KM29 fungicidal activity. H_2O_2, a known inducer of oxidative stress, has been shown to induce mitochondrial fragmentation independent of MMP depolarization (146). This is in contrast with what we observed with KM29. Although these findings do not show direct damage to mitochondria caused by KM29, it is possible that depolarization of MMP caused by KM29 may indirectly affect the health of the cell by blocking the function of cellular respiration. The final step in cellular respiration is the utilization of the proton gradient formed in the previous steps by ATP synthase to produce ATP from ADP and Pi. Without a proton gradient, ATP synthase would not be able to synthesize ATP. As mentioned earlier, *S. cerevisiae* is Crabtree positive thus it is logical to assume this organism wouldn't be affected by loss of mitochondrial function under fermentative conditions, however, this doesn't wholly preclude the loss of mitochondrial ATP to adversely affect the cell.

Based on our observations, we do not believe ROS formation to be a major source of KM29-induced damage. Instead, we conclude that KM29 localizes to the mitochondria and inhibits respiration by depolarizing the MMP. In turn, this would negatively affect the energy status of the cell and possibly signal the cell to undergo cell death. It is also possible that KM29

deals direct damage to mitochondrial membranes in the process of localizing to this organelle. This explanation could account for the mitochondrial fission we observed when analyzing mitochondrial network morphology after exposure to KM29. In this case, mitochondrial fission serves to protect healthy mitochondria and the signal for degradation of damaged mitochondria. Mitochondrial fission after exposure to KM29 could indicate loss of mitochondrial integrity. This explanation would be consistent with the active functions cationic peptides have on membranes.

Respiratory deficiency and KM29 uptake

Clarification as to why respiratory deficiency confers cells nearly complete resistance to KM29 was realized when we analyzed KM29-FITC uptake in various *S. cerevisiae* electron transport chain mutants. We observed a nearly perfect inverse correlation between respiratory status and KM29-FITC uptake. This indicates the role of mitochondrial function in KM29 fungicidal activity to be two-fold: to regulate KM29 uptake and to serve as a site for KM29-induced perturbations. A possible explanation as to why mutants with varying degrees of respiratory deficiency have reduced KM29-FITC uptake could involve the energy availability in these strains. It has been reported that the plasma membrane proton ATPase is the major facilitator in establishing the plasma membrane potential of the yeast cell (169). This raises the question whether respiratory deficient mutants, because of the decrease in ATP output, have decreased proton ATPase activity and therefore, a depolarized plasma membrane potential. If true, this could explain the inability of KM29 to enter these mutants. This also aligns with our explanation of KM29 localization to mitochondria being dependent upon an established MMP. Based on this, we suggest a model of KM29 entry into yeast cells dependent upon an electrostatic attraction, and thus, an established plasma membrane potential. A similar proposal

was reported by a group studying genes important for tolerance to cationic drugs (170). This could help explain the reason why various mutants displayed increased resistance or increased sensitivity in our genetic screen. Any mutation that affects the plasma membrane potential would potentially affect the uptake of KM29. Along this line, it would be interesting to see if chemical alteration of the plasma membrane potential affects KM29 uptake. Another explanation for the reduced KM29 uptake in respiratory deficient mutants could be alterations of the plasma membrane. Mitochondria are most commonly known for "powerhouse" functions, but they are also involved in lipid synthesis (171). As a major constituent of the plasma membrane, alterations in lipid synthesis could cause alterations in the plasma membrane. This in turn could lead to alterations in KM29 entry into cells.

The inverse correlation between respiratory status and KM29 uptake was also observed the clinically relevant *C. albicans*. Interesting, *C. albicans* SC5314 cells had more KM29 uptake than *S. cerevisiae* wild type cells. A possible explanation for this could be differences in plasma membrane composition between these two organisms. Additionally, this could be due to differences in the Crabtree effect between the two organisms. *C. albicans* relies more heavily on cellular respiration during optimal growth therefore it is reasonable to assume it has more active mitochondria than *S. cerevisiae*. Since respiratory status has been shown to affect KM29 uptake, the more active mitochondria of *C. albicans* could result in increased KM29 uptake. This increased uptake in *C. albicans* would then be reflected in the increased sensitivity to KM29 compared to *S. cerevisiae*. Future work will revolve around understanding the increased KM29 uptake in *C. albicans* and how the metabolic differences between *C. albicans* and *S. cerevisiae* affect KM29 fungicidal activity in these two organisms.

In conclusion, we present initial steps in the elucidation of the mechanism of action of KM29 in fungal cells. By understanding the mechanism of action of KM29 in *S. cerevisiae* we are able to begin understanding how this peptide works against *C. albicans*, and provide parameters to continue developing KM29 as a potential therapeutic agent for the treatment of oral and systemic candidiasis. Additionally, we assert the necessity to consider alterations in drug uptake when studying various mechanisms of resistance in medically relevant organisms.

TABLES AND FIGURES

Table 1. **Functional pathways involved in the antifungal killing activity of KM29.** A modified MIC assay was performed on the non-essential, single deletion mutants available in the *S. cerevisiae* genome-wide deletion collection. The percentage of genes associated with a specific biological process that displayed increased resistance with respect to the frequency in the genome for specific biological processes using the Yeast GO-SlimMapper (A). A FunSpec analysis was performed to identify functional categories enriched by the mutants that displayed increased resistance (B).

A

Biological Process	Normalized Genome Frequency
Mitochondrial translation	37%
Amino acid transport	36%
Cellular respiration	35%
Sporulation	35%
Mitochondrion organization	31%
Generation of precursor metabolites and energy	30%
Biological process unknown	30%
31 other processes	20-29%
63 other processes	0-19%

B

Catergory and function	P value
Go Biological Process	
Mitochondrial translation	1.00E-14
Positive regulation of mitochondrial translation	2.67E-07
GO Cellular Component	
Mitochondrion	1.00E-14
Mitochondrial inner membrane	1.95E-08
Mitochondrial small ribosomal subunit	9.20E-08
Mitochondrial large ribosomal subunit	3.80E-07
MIPS Functional Classification	
Mitochondrion	1.76E-10
Aerobic respiration	2.14E-05
MIPS Phenotypes	
Respiratory deficiency	1.00E-14
MIPS Subcellular Localization	
Mitochondria	1.00E-14
Mitochondrial inner membrane	1.36E-06
Mitochondrial matrix	2.62E-05

Table 2. **Functional pathways involved in the antifungal killing activity of KM29.** A modified MIC assay was performed on the non-essential, single deletion mutants available in the *S. cerevisiae* genome-wide deletion collection. The percentage of genes associated with a specific biological process that displayed increased sensitivity (A) with respect to the frequency in the genome for specific biological processes using the Yeast GO-SlimMapper. (B) A FunSpec analysis was performed to identify functional categories enriched by the mutants that displayed increased sensitivity.

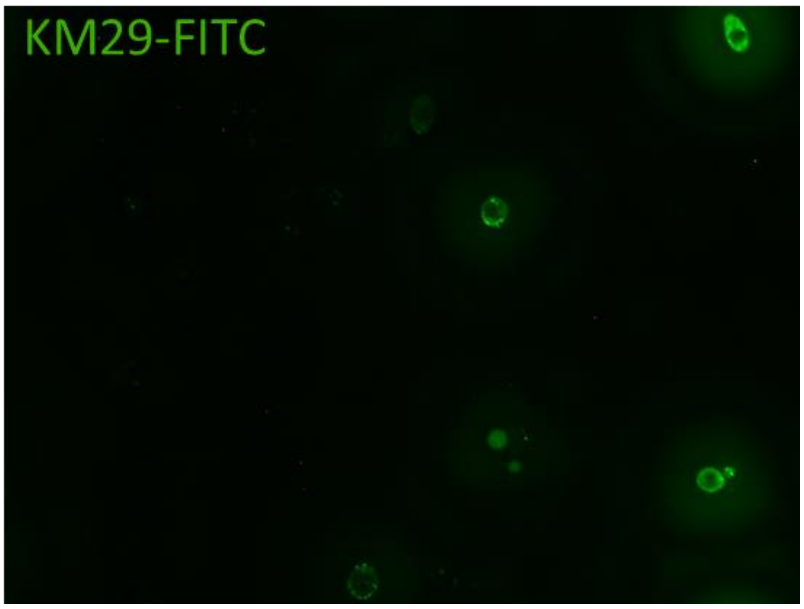
A

Biological Process	Normalized Genome Frequency
Invasive growth in response to glucose limitation	27%
Organelle inheritance	23%
Cytoplasmic translation	22%
Endosomal transport	21%
Vesicle organization	20%
Protein glycosylation	19%
46 other processes	10-19%
49 other processes	0-9%

B

Catergory and function	P value
Go Biological Process	
tRNA wobble uridine modification	4.98E-07
Protein urmylation	1.32E-06
Arginine biosynthetic process	1.50E-06
GO Cellular Component	
Endosome	2.45E-08
Cytosolic small ribosomal subunit	9.26E-08
Endosome membrane	3.56E-06
MIPS Functional Classification	
Vacuolar/lysosomal transport	2.59E-09
Ribosomal proteins	1.45E-06
Biosynthesis of arginine	1.75E-05
MIPS Phenotypes	
Other vacuolar mutants	5.70E-08
Slow-growth	5.26E-07
Divalent cations and heavy metals sensitivity	2.24E-06
MIPS Subcellular Localization	
Endosome	1.70E-05
Cytoplasm	7.14E-05
Vacuole	1.95E-04

A



B

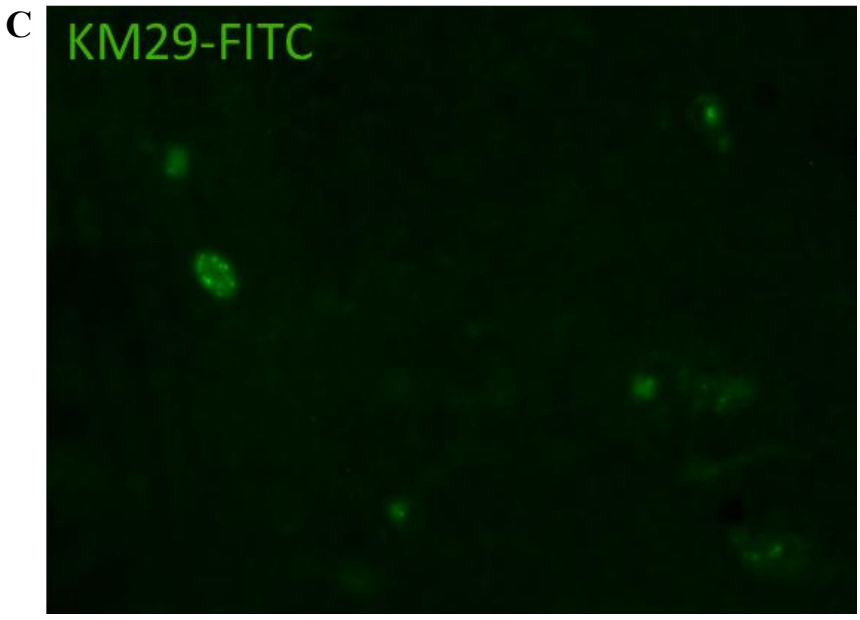


Fig. 1. **Intracellular localization of KM29-FITC in *S. cerevisiae* and *C. albicans*.** Fluorescent microscopy images of *S. cerevisiae* wild-type cells treated with KM29-FITC (A). Fluorescent microscopy images of a double labeling experiment. *S. cerevisiae* wild-typed cells were concurrently treated with 5µm KM29-FITC and 500nm MitoTracker DeepRed (MolecularProbes). Images show localization of KM29-FITC (left), localization of MitoTracker DeepRed (middle), and the double labeling (right) (B). Fluorescent microscopy images of *C. albicans* cells treated with KM29-FITC (C). Fluorescent microscopy images of a double labeling experiment. *C. albicans* wild-typed cells were concurrently treated with 3µm KM29-FITC and 500nm MitoTracker DeepRed (MolecularProbes). Images show localization of KM29-FITC (left), localization of MitoTracker DeepRed (middle), and the double labeling (right) (D).

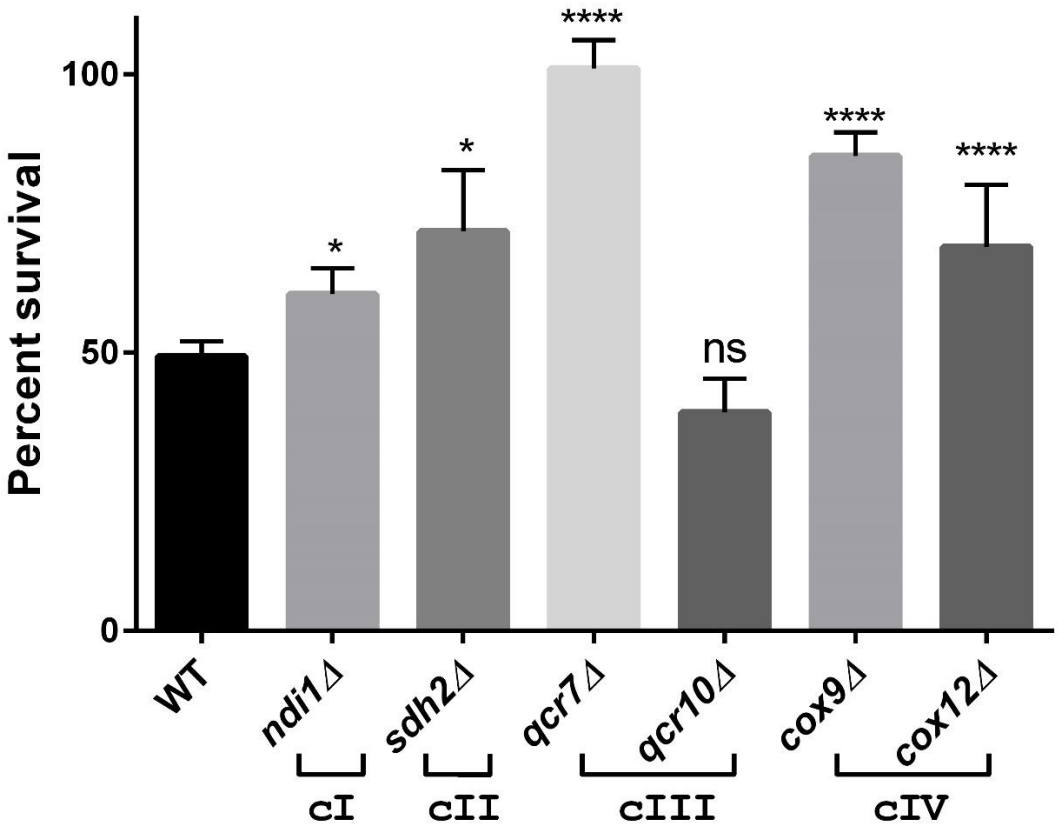
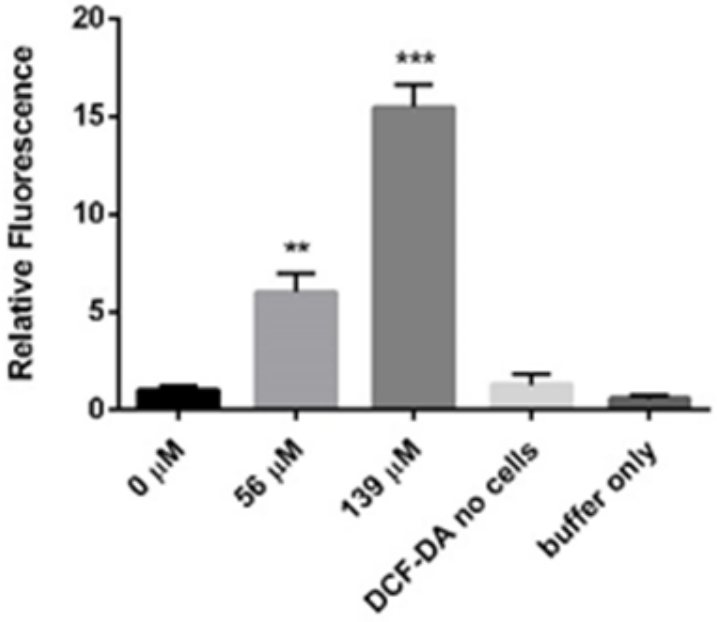
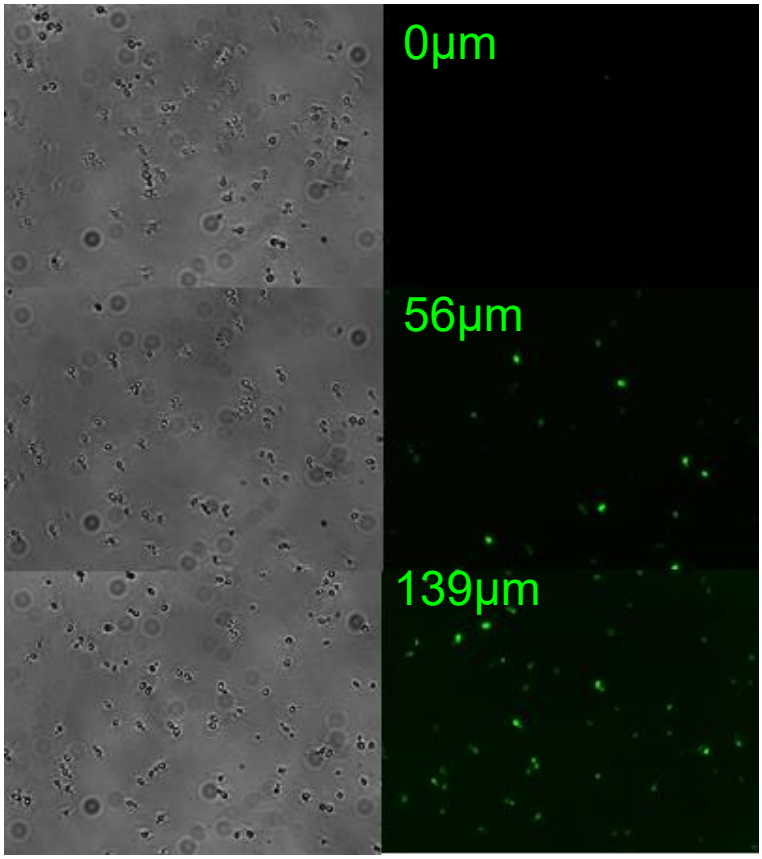


Fig. 2. **Effect of Electron Transport Chain mutations on KM29 fungicidal activity**. Microdilution assay showing cell viability of *S. cerevisiae* wild-type and mutants from each complex in the Electron Transport Chain at 2.5μM KM29.

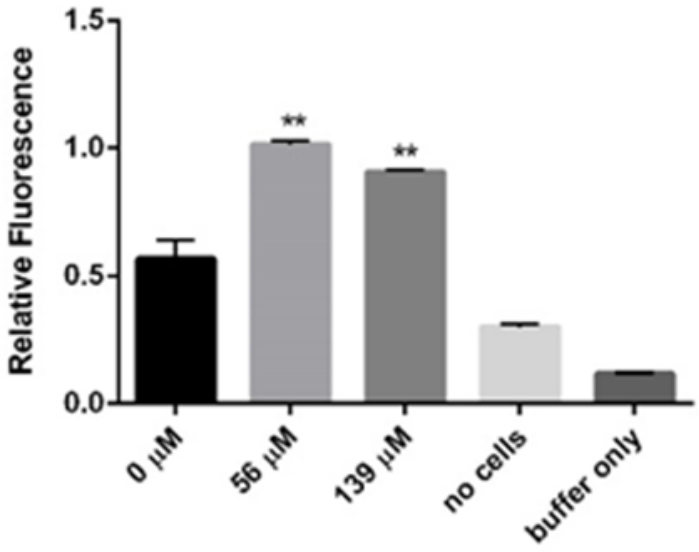
A


Relative Fluorescence
20
15
10
5
0
**
***
0 µM
56 µM
139 µM
DCF-DA no cells
buffer only

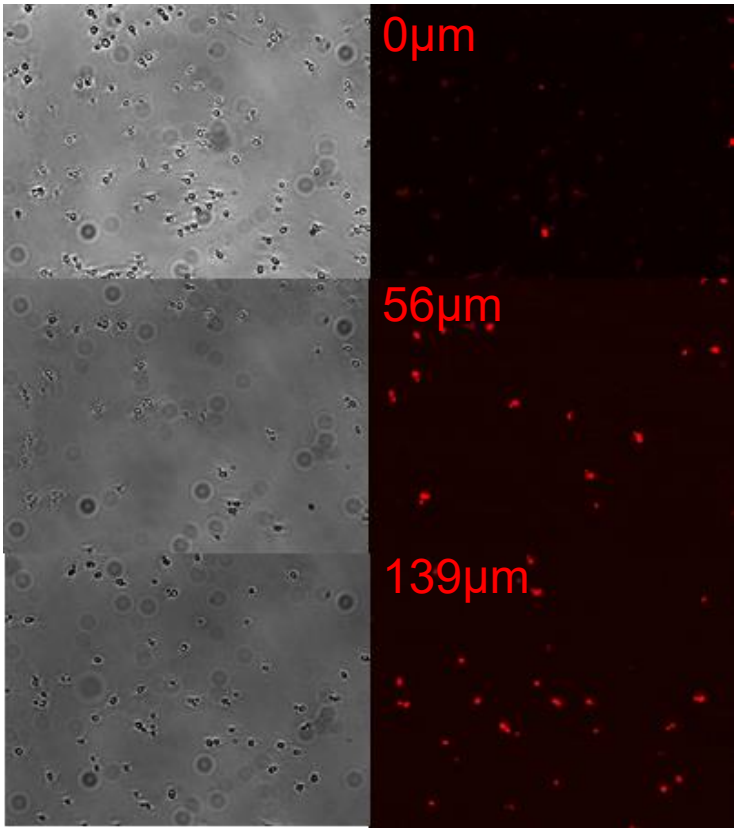
B


0µm
56µm
139µm

C


Relative Fluorescence
1.5
1.0
0.5
0.0
**
**
0 µM
56 µM
139 µM
no cells
buffer only

D


0µm
56µm
139µm

E

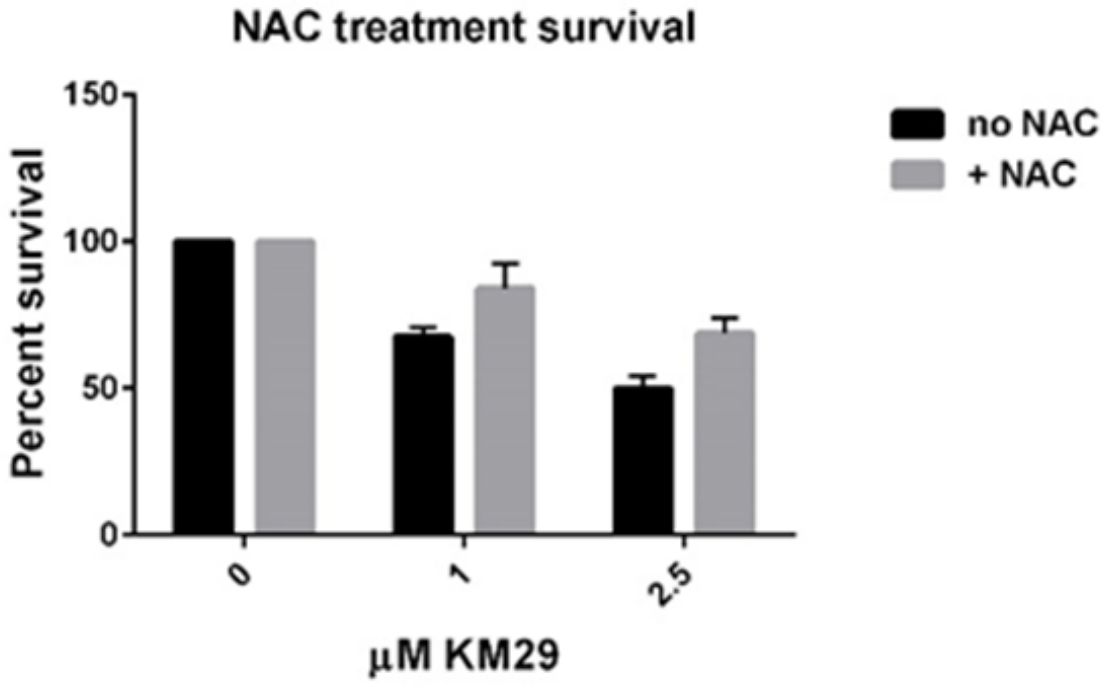


Fig. 3. **KM29 concentration-dependent ROS formation.** Fluorescent spectrophotometry and corresponding fluorescent microscopy images of S. cerevisiae wild-type cells preloaded with 10µm DCF-DA (A, B) or 1µm DHE (C, D) and treated with increasing concentrations of KM29 for 30min. Microdilution assay comparing cell viability of *S. cerevisiae* wild-type cells pretreated with ROS scavenger, n-acetylcysteine, and non-pretreated cells after exposure to increasing concentrations of KM29 (E).

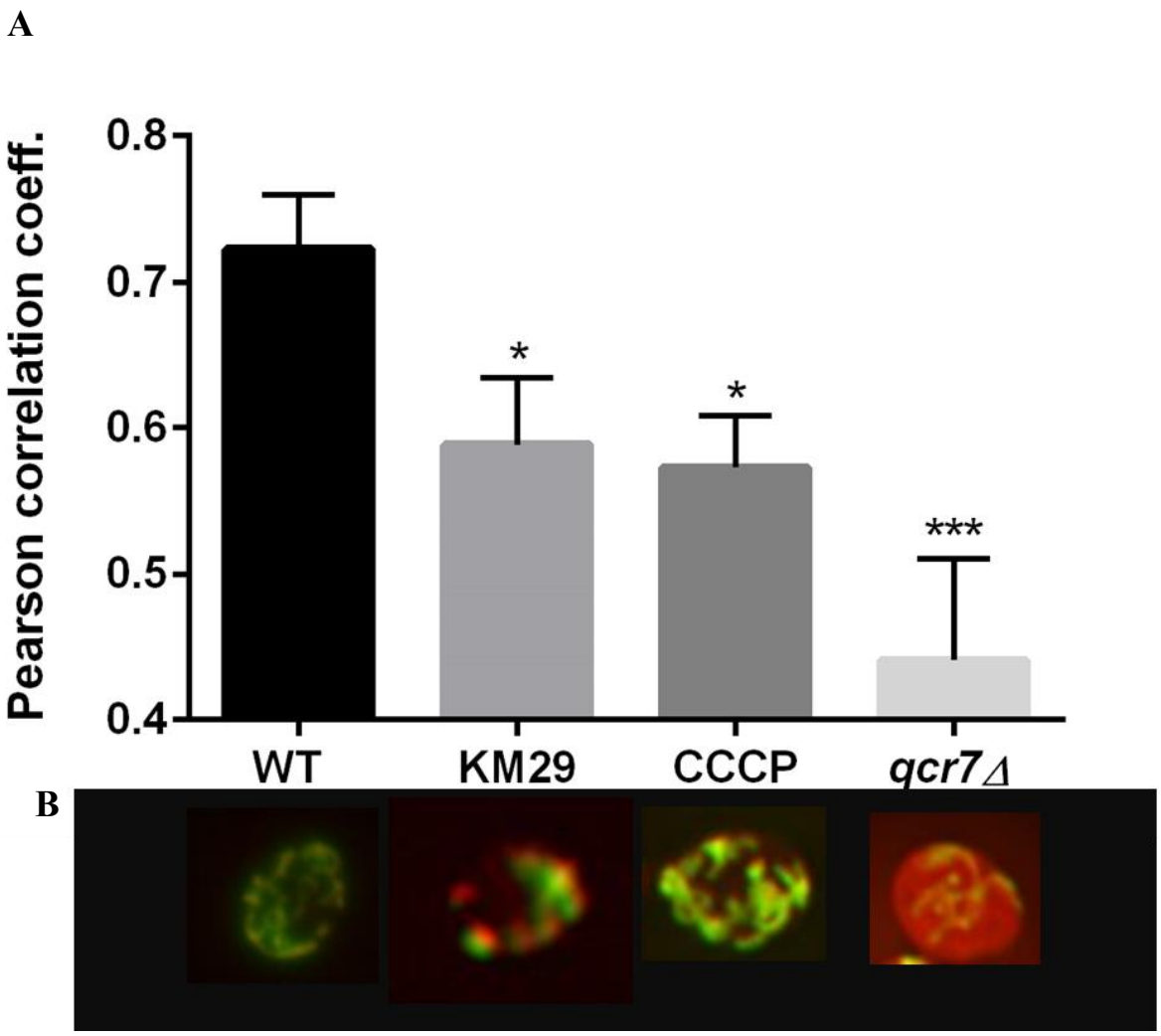
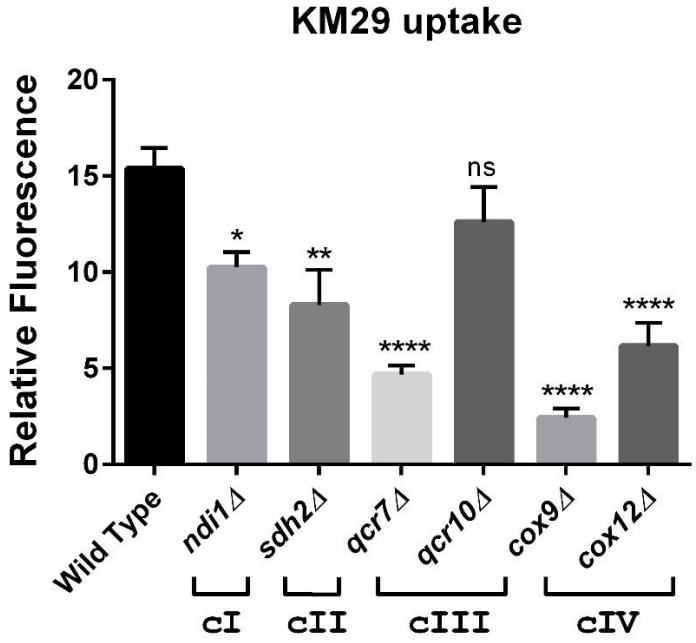
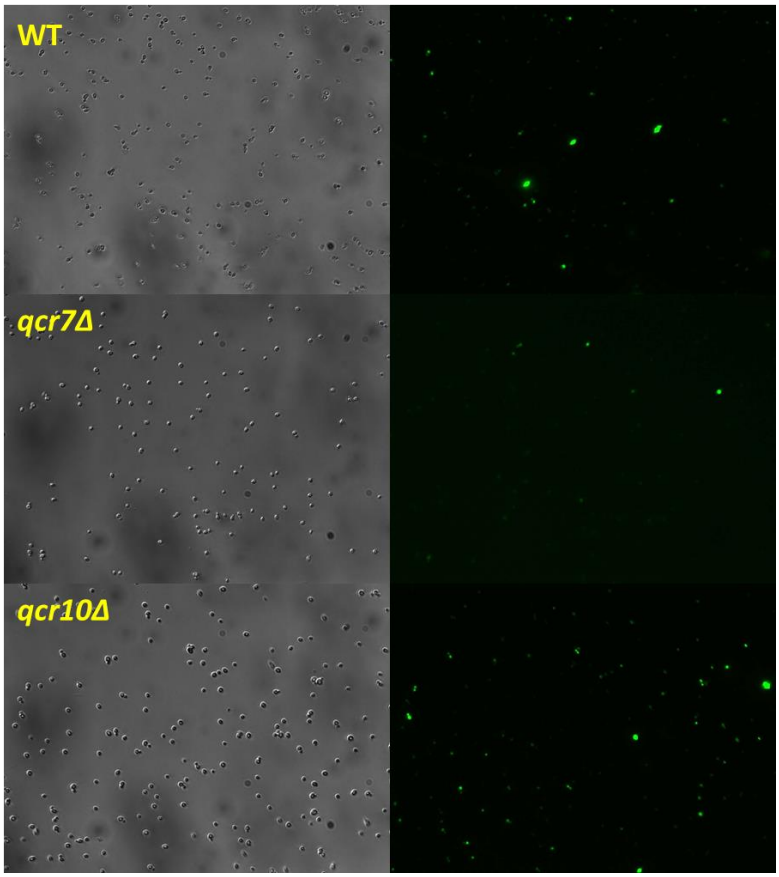


Fig. 4. **Mitoloc employed to assess KM29 effect on mitochondrial membrane potential and mitochondrial morphology.** *S. cerevisiae* wild-type cells transformed with pMitoloc were treated with 166μm KM29 or 15μm CCCP for 3 hrs. and single cells were analyzed using ImageJ plugin yeast_correlation. Transformed *qcr7*Δ (respiratory deficient) cells served as a positive control. De co-localization of the two markers is reflected by a decrease in correlation coefficient, PCC (A). Fluorescent microscopy images of a representative cell from each experimental condition (B).

A


KM29 uptake
Relative Fluorescence
20
15
10
5
0
*
**
****
ns
****
****
Wild Type
ndi1Δ
sdh2Δ
qcr7Δ
qcr10Δ
cox9Δ
cox12Δ
cI
cII
cIII
cIV

B


WT
qcr7Δ
qcr10Δ

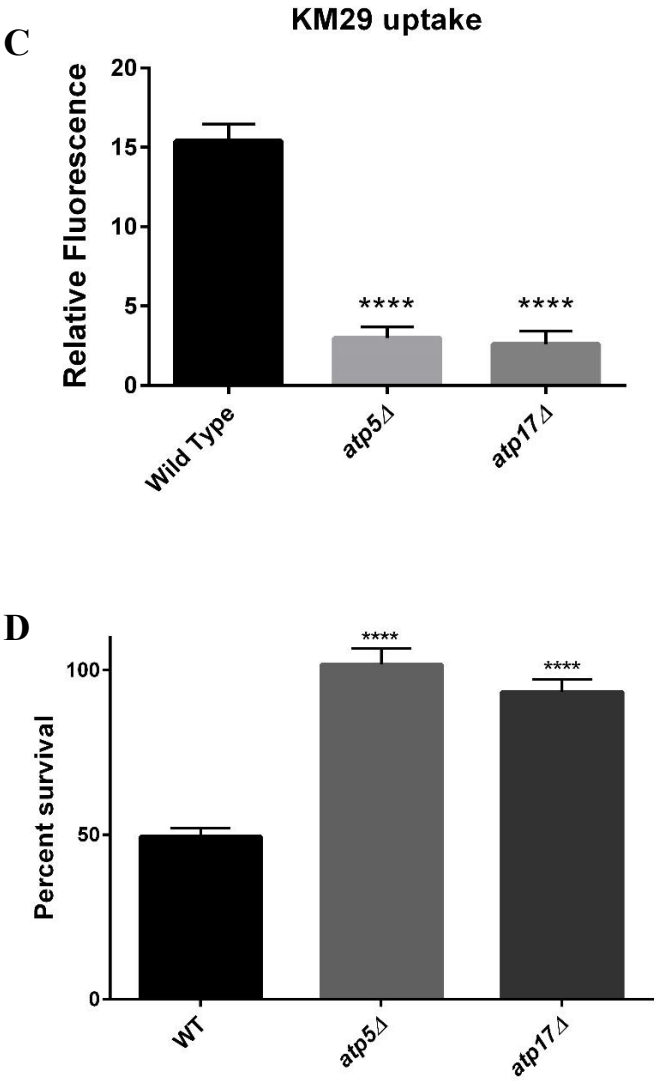
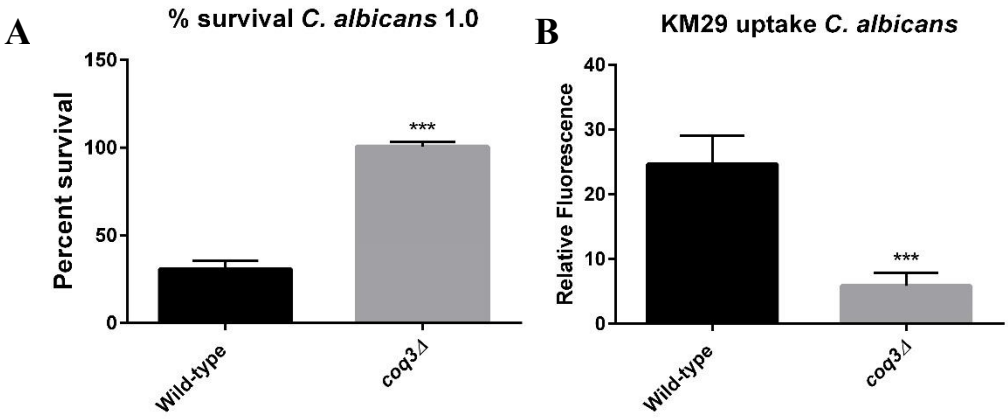
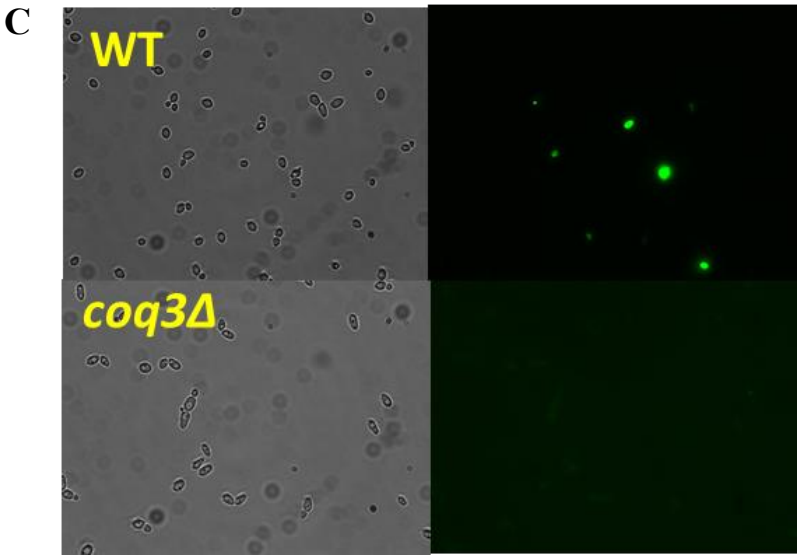


Fig. 5. **Comparison of KM29 uptake and killing in single deletion mutants of the yeast ETC.** Fluorescent spectrophotometry showing uptake of 5µm KM29-FITC in *S. cerevisiae* wild-type and mutants (A). Fluorescent microscopy images of KM29-FITC uptake in *S. cerevisiae* wild-type (respiratory capable; top), *qcr7*Δ (respiratory deficient; middle), and *qcr10*Δ (respiratory capable; bottom) cells (B). Fluorescent sprectrophotometry showing uptake of 5µm KM29-FITC in S. cerevisiae wild-type, *atp5*Δ, and *atp17*Δ (C). Microdilution assay showing cell viability of *S. cerevisiae* wild-type, *atp5*Δ, and *atp17*Δ at 2.5µM KM29 (D).


A
% survival C. albicans 1.0
Percent survival
150
100
50
0
***
Wild-type
coq3Δ
B
KM29 uptake C. albicans
Relative Fluorescence
40
30
20
10
0
***
Wild-type
coq3Δ


C
WT
coq3Δ

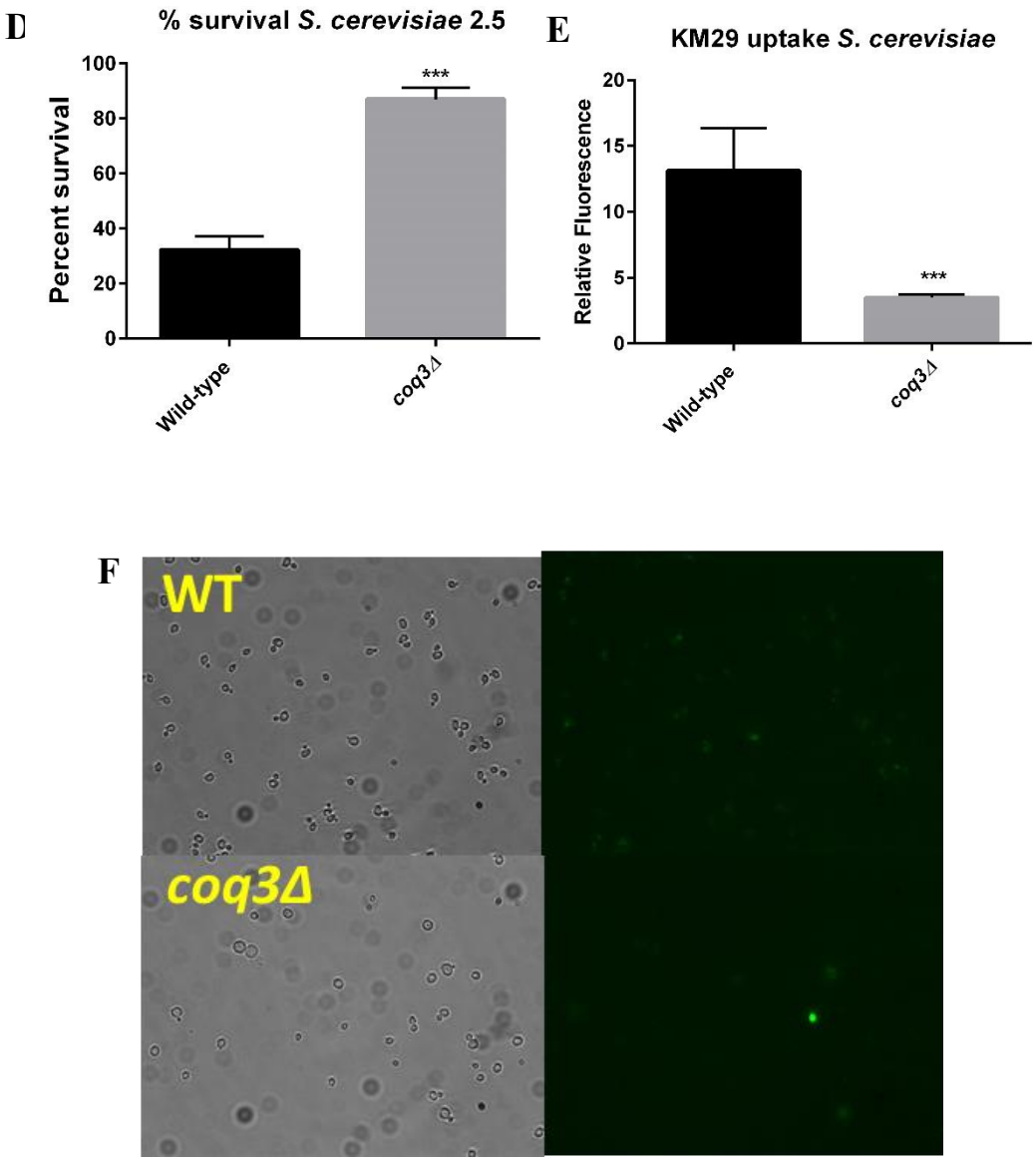


Fig. 6. **Effect of respiratory deficiency on KM29 uptake and killing in *S. cerevisiae* and *C. albicans*.** Microdilution assay showing cell viability of *C. albicans* LLF100 (prototrophic wild-type) cells and *C. albicans* LLF088 (*coq3*Δ; respiratory deficient) cells when treated with 1.0µM KM29 (A). Fluorescent sprectrophotometry showing uptake of 5µm KM29-FITC in *C. albicans* LLF100 (prototrophic wild-type) cells and *C. albicans* LLF088 (*coq3*Δ; respiratory deficient) cells (B). Fluorescent microscopy images of KM29-FITC uptake in *C. albicans* LLF100 (prototrophic wild-type) cells and *C. albicans* LLF088 (*coq3*Δ; respiratory deficient) cells (C). Microdilution assay showing cell viability of *S. cerevisiae* wild-type cells and *coq3*Δ when treated with 2.5µM KM29 (D). Fluorescent sprectrophotometry showing uptake of 5µm KM29-FITC in *S. cerevisiae* wild-type and *coq3*Δ (E). Fluorescent microscopy images of KM29-FITC uptake in *S. cerevisiae* wild-type and *coq3*Δ (F).

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