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Function of Septin Proteins in *Cryptococcus neoformans* in Cell Wall and Plasma Membrane Integrity and Homeostasis

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Function of Septin Proteins in *Cryptococcus neoformans* in Cell Wall and Plasma Membrane Integrity and Homeostasis

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ABSTRACT

Cryptococcus neoformans is a pathogenic basidiomycetous yeast that causes meningitis in immunocompromised patients. This lethal fungus is sometimes referred to as the “sugar coated killer” due to its polysaccharide capsule. It is estimated that 152,000 cases of cryptococcal infection occur each year and result in 112,000 deaths. The ability of *C. neoformans* to adapt to host temperature is a main factor responsible for virulence. Septins are conserved filament-forming GTPases that are confirmed to be involved in cytokinesis and morphogenesis and have been implicated in heat stress response and virulence of *C. neoformans*. *C. neoformans* genome encodes four septins, Cdc3, Cdc10, Cdc11, and Cdc12 that form a complex at the mother-bud neck where they participate in cytokinesis through ill-defined mechanism. Our preliminary data indicate that the septin complex also forms at the plasma membrane (PM) specifically at 37℃, suggesting that septins contribute to stress response by interacting with PM and potentially supporting PM homeostasis. The *C. neoformans* septin interactome had not been previously thoroughly analyzed for potential interacting partners. In a preliminary experiment conducted by this research group, tandem-mass spectrometry was utilized to identify proteins associated with the septin complex. Based on these data, septins are hypothesized to be involved in cell wall integrity and plasma membrane homeostasis. Strains lacking septins were found hypersensitive to cell wall and PM disrupting agents, which further supports this hypothesis.

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I would like to thank Dr. Lukasz Kozubowski for his mentorship throughout the later years of my undergraduate degree. He has given me opportunities I would not have been able to have otherwise and introduced me to the research process.

I would also like to thank graduate student Stephani Martinez Barerra for her endless amount of support and guidance. Her assistance was key in completing my training and thesis project.

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I. BACKGROUND

Cryptococcus neoformans is a basidiomycetous yeast that can reproduce asexually through budding and sexually through sporulation. This fungus lives throughout the environment, but can most commonly be found in soil, decaying wood, tree hollows, and bird droppings.

The Pathogen *Cryptococcus neoformans*

C. neoformans is an opportunistic pathogen that can cause fungal meningitis in immunocompromised patients such as those with HIV. Spores of the yeast can enter a patient's lungs and spread to the central nervous system and cause meningoencephalitis (Idnurm et al, 2005). There are an estimated 152,000 cases of cryptococcosis each year resulting in 112,000 deaths. This fungus can be uniquely identified due to its ability to produce melanin and a polysaccharide capsule (Idnurm et al, 2005). Both characteristics are thought to be virulence factors.

Fungal Cell Wall and Plasma Membrane

The cell wall in fungal pathogens is necessary for cell structure and integrity and is also needed for localization of virulence factors (Baker et al, 2007). Fungal cell wall consists of chitin for wall rigidity, β-glucan to maintain shape, and mannoproteins that coat the surface and interact with the external environment (Blankenship et al, 2010). This important organelle has been proven to have a role in pathogenicity of infectious fungi. The cell wall composition is carefully controlled by signaling pathways such as the MAPK pathway in cell

wall integrity (CWI) (Blankenship et al, 2010). This pathway is connected with other signaling pathways through an intricate cross-talk to control the responses to changing external environment.

Fatty acids are commonly involved in CWI and plasma membrane function and stability. Specifically, sphingolipid intermediates have been shown to be involved in CWI pathways and lipid rafts. Sterols, specifically ergosterols, have also been implicated in these functions (Dickson, 2008). Septin proteins, previously proven to be involved in cytokinesis and morphogenesis in yeast, have been shown to interact with the plasma membrane. Septins seem to affect filament assembly and the role of phosphoinositides suggesting that they commonly associate with the plasma membrane (Bertin et al, 2010).

There are a series of compounds that can disrupt the cell wall or plasma membrane. Thanks to those activities, some of those compounds are utilized as antifungal drugs, or diagnostic tools to study the mechanisms regulating cell wall and plasma membrane homeostasis. Congo Red affects cell wall synthesis and when used creates aberrant cell wall structure (Kopecka and Gabriel, 1992). Calcofluor white inhibits growth of fungus by binding to chitin in the cell wall (Su et al, 2020). Caspofungin inhibits β-glucan synthase and is known to increase chitin production and change cell wall structure (Walker and Munro, 2020). Caffeine causes cell wall modification through excessive phosphorylation of Mpk1p (Kuranda et al, 2006). Finally, Sodium dodecyl sulfate (SDS) disrupts cell membranes while activating cell wall integrity pathways that ultimately restrict cell growth (Schroeder and Ikui, 2019). Drugs that are known to directly disrupt the plasma membrane can also be used. Myriocin inhibits serine palmitoyltransferase (SPT) which is the first enzyme involved in sphingolipid metabolism (Monasterio et al, 2022). This inhibition prevents the accumulation

of downstream intermediates such as phytosphingosine (PHS) and complex sphingolipids (Mela and Momany, 2021). PHS is a building block of complex sphingolipids that are abundant in fungi. Aureobasidin A disrupts actin assembly and affects cell membrane integrity (Endo et al, 1997). Cells treated with Myriocin or Aureobasidin A can theoretically be rescued by adding exogenous PHS. Fluconazole (FLC) can modulate membrane rigidity, heterogeneity, and water solubility (Abe et al, 2009). Cells treated with FLC can be rescued with exogenous ergosterol. Ergosterol is important for membrane integrity and has been shown to restore membrane properties in specific deletion strains and cells treated with membrane disrupting drugs (Abe and Hiraki, 2009).

Proteins Involved in Cell Wall and Plasma Membrane Organization and Homeostasis

To evaluate cell wall and plasma membrane disruption, a series of control strains were used in this study. These strains were used to demonstrate deletions in *Cryptococcus neoformans* that cause increased sensitivity to drugs affecting the cell wall or plasma membrane. Phosphoinositide-dependent kinase (Pdk1) controls a network of signaling cascades such as those involved in growth factors and regulation of apoptosis. This protein is also necessary in response and resistance to FLC. These pathways have been proven to be involved in cell wall integrity (Lee et al, 2012). Chitin synthase 3 (Chs3) integrates into the plasma membrane and causes chitin chains to be extruded across the plasma membrane and become a component of the cell wall (Banks et al, 2005). Chitin synthase regulator 2, Csr2, has been hypothesized to be a specific regulator of Chs3. Consistently, elimination of Csr2 also affects cell wall stability (Banks et al, 2005). Tricalbin proteins (Tcb) are known to regulate phosphatidylserine homeostasis in the plasma membrane (Thomas et al, 2022). This

implicated Tcb in plasma membrane integrity and repair. When studying the role of sphingolipids in the plasma membrane, the GARP complex is implicated. This complex functions as an endosome-to-Golgi retrograde vesicular transport and is needed for sphingolipid homeostasis (Frolich et al, 2015). Vps5 is a key protein in the GARP complex and when deleted causes excess sphingolipid synthesis intermediates and change in sterol distribution (Frolich et al, 2015). Finally, *ERG3* encodes sterol desaturase that ultimately creates ergosterol (Hemmi et al, 1995). Therefore, the following deletion strains will be used in this study to determine baseline susceptibility to cell wall and plasma membrane disrupters: *H99* strain (WT control), *pdk1*Δ, *chs3*Δ, *crs2*Δ, *tcb*Δ, *vps53*Δ, and *erg3*Δ.

Septin Proteins in *C. neoformans*

Septins are conserved cytoskeletal GTPases that are responsible for cell morphogenesis and cytokinesis at the mother-bud neck of yeast cells. Septins form complexes that assemble in a ring at the bud site in late G1 (Gladfelter et al, 2005). This complex prevents diffusion of membrane and cell wall-associated molecules (Gladfelter et al, 2001). Septin were first characterized in the ascomycete *Saccharomyces cerevisiae* and subsequently studied in other fungi such as *Aspergillus nidulans* and *Candida albicans*. Septins Cdc3, Cdc10, Cdc11, and Cdc12 in *S. cerevisiae* have been termed "core septins" and are also present in *Cryptococcus neoformans*. While *Saccharomyces* septins have homologs in *Cryptococcus,* their functions may not be identical between these two species. For instance, septins in *S. cerevisiae* are required for growth at 25℃ but are not essential in *C. neoformans* in non-stress conditions. However, when the temperature is raised to 37℃, elimination of Cdc3 or Cdc12 in *C. neoformans* is lethal. While the initial characterization of septins in *C. neoformans* has been

performed, their function is greatly undiscovered. It is suspected that septins share functions involving cell wall and plasma membrane stability with those in *Aspergillus nidulans*. It has been shown that septins colocalize with sterol-rich regions in the plasma membrane and recruit cell wall synthases during cell remodeling (Mela and Momany, 2021). Septins in *A. nidulans* are suspected to have roles in monitoring ergosterol and sphingolipid metabolism and coordinate cell wall integrity (Mela and Momany, 2021).

II. AIMS

Septin proteins contribute to cytokinesis and morphogenesis in *C. neoformans* through mechanisms that remain largely uncharacterized. They have been implicated in cell wall and plasma membrane stability in other fungi. We used spot growth assays to determine the role of septin proteins in cell wall and plasma membrane stability and homeostasis. In addition, we created growth curves tracking the cell density of septin deletion mutants in the presence of a plasma membrane disrupting drug. Through these experiments, novel insights into septin function in cell wall integrity and plasma membrane homeostasis were uncovered.

III. MATERIALS AND METHODS

Strains, Media, and Conditions

Strains used in this study are listed in Table 1. C. neoformans cultures were grown in yeast-peptone-dextrose (YPD) media at 25℃. Some experiments were conducted at 30℃ and 37℃, as specified in Figure legends.

Spot Growth Assays for Cell Wall and Plasma Membrane Disruption

Strains *H99* (WT), *pdk1*Δ, *chs3*Δ, *csr2*Δ, *erg3*Δ, *vps53*Δ, *tcb*Δ, *cdc3*Δ, *cdc10*Δ, *cdc11*Δ, and *cdc12*Δ were grown overnight in 2 mL of YPD. These strains were refreshed in 2mL of YPD in new conical tubes. After 3 hours of growth, cells were washed with PBS and these cultures were evaluated for cell density using the hemocytometer. The cell density was calculated, and the cells were diluted with PBS to create a 10,000 cell per µL culture. A series of 10x dilutions were made resulting in cultures of 1,000, 100, and 10 cells per µL. A 2µL aliquot of every dilution in each strain was spotted on 21 types of YPD plates.

Each plate of YPD was supplemented with a different drug. Congo Red, Calcofluor white (CFW), Caffeine, SDS, Caspofungin, Myriocin, Fluconazole (FLC), and Aureobasidin A (AbA) were used to stress the cells and determine cell wall and plasma membrane disruption. The following concentrations of each drug was used: 0.5% of Congo Red, 1mg/mL of CFW, 1 mg/mL of Caffeine, 0.01% of SDS, 32µg/mL of Caspofungin, 500 ng/mL of Myriocin, 8 µg/mL and 32µg/mL of FLC, and 100 ng/mL of AbA. 15µM PHS was utilized as a rescue for Myriocin and AbA treatment while 1000nM of ergosterol was used as a rescue for FLC treatment. Plates were left to grow at 25°C (RT) for 2-5 days before images were taken.

Myriocin Growth Curve

Cells were grown overnight at 30°C in 5-ml cultures in yeast extract-peptone-dextrose (YPD) broth. Overnight cultures were refreshed by diluting 100 µl of overnight culture in 2 ml of fresh YPD Broth. The cultures were allowed to grow for 4 hours to achieve exponential phase growth. The cell cultures were then washed with water once and then OD600 was

made equal to 0.3 in YNB broth. Fifty microliters of YNB broth containing 2× the final Myriocin (Sigma) concentration was placed in each well, and then 50 µl of the OD600-adjusted cultures was placed in each well. The plate was incubated at 30°C for 72 h while shaking in a double orbital fashion, and OD600 was measured every 20 min during this 96-well plate liquid culture assay using the BioTek LogPhase600 Microbiology Reader.

IV. RESULTS

The experiments performed in this study were spot assays to determine the robustness of cell wall and plasma membrane in the absence of septin proteins. Each single deletion strain was plated onto YPD media + 20% Glucose as a control. All ten strains were then plated on YPD media supplemented with each drug or rescue of choice. The phenotype of each septin deletion mutant was analyzed in comparison to control single deletion mutants known to disrupt cell wall or plasma membrane stability. H99 (WT) was included on each plate as a control. Based on Figure 1, *pdk1*Δ*, csr2*Δ*, erg3*Δ*, cdc3*Δ, and *cdc12*Δ are sensitive to Congo Red. Similarly, *pdk1*Δ*, csr2*Δ*, erg3*Δ*, vps53*Δ*, cdc3*Δ, and *cdc12*Δ were sensitive to SDS. CFW treatment showed the same results as Congo Red with *pdk1*Δ*, csr2*Δ*, erg3*Δ*, cdc3*Δ*,* and *cdc12*Δ being affected. Finally, only *pdk1*Δ*, cdc3*Δ*,* and *cdc12*Δ were sensitive to Caspofungin.

Figure 2 includes panels with added 1 mg/mL Caffeine. Only one strong phenotype was seen in *pdk1*Δ. All other single deletion strains resembled the growth observed in *H99* (WT). When cells were exposed to 15µM exogenous PHS, strains *pdk1*Δ*, erg3*Δ*, cdc3*Δ*,* and *cdc12*Δ had limited growth (Figure 3). When 500 ng/mL Myriocin was added to the plates

containing 15 µM PHS, a severe phenotype was seen in all strains except *tcb*Δ and *cdc11*Δ (Figure 3). Also shown in Figure 3 is the growth seen in response to 100 ng/mL AbA and 500 ng/mL Myriocin alone. Both drugs resulted in a severe phenotype in every strain.

Figure 4 includes plates supplemented with 8 µg/mL FLC, 1000 nM ergosterol, and a combination of 8 µg/mL FLC and 1000 nM ergosterol. The FLC-only plates showed a strong phenotype in *pdk1*Δ*, erg3*Δ*, vps53*Δ*, cdc3*Δ, and *cdc12*Δ. Addition of 1000 nM ergosterol had its own effect on the strains: *pdk1*Δ *csr2*Δ*, cdc3*Δ*,* and *cdc12*Δ. Finally, the combination of 8 µg/mL FLC and 1000nM ergosterol resulted in a growth defect in all strains. Figure 5 shows samples supplemented with FLC at a concentration of 32 µg/mL. The 1000 nM ergosterol-only plates showed an effect in *pdk1*Δ*, cdc3*Δ*,* and *cdc12*Δ. Treating the strains with the higher concentration of FLC resulted in an overall strong phenotype. When the FLC was combined with ergosterol, a partial rescue of *H99, chs3*Δ*, tcb*Δ*, cdc10*Δ*,* and *cdc11*Δ was seen.

A growth curve of *H99*, *pdk1*Δ*, cdc3*Δ*, cdc12*Δ, and *5th septin*Δ in the presence of 5 µM myriocin was created. A control plate of cells grown without the drug was also completed. The growth of cells was analyzed based on OD600 measurements over 72 hours at 30°C. The *pdk1*Δ strain shows a lack of growth. The *cdc12*Δ and *cdc3*Δ mutants show a plateau around 24 hr with the cell density of *cdc3*Δ being slightly higher. *H99* and the mutant lacking the 5th septin show similar trends in growth over the 72 hr period (Figure 6). While *pdk1*Δ showed the lowest cell density overall, *cdc12*Δ showed the highest variability in cell density (Figure 6).

Spot assays were also completed to assess each strain's survivability at varying temperatures and high stress environments. Each strain was plated on YPD and grown at 25°C (RT) as a

control. The same strains were plated on YPD plates at 30℃ and 37℃. Figure 7 shows that while there was no extreme phenotype seen at 30℃, minimal growth of *cdc3*Δ*, cdc11*Δ*,* and *cdc12*Δ was seen at 37℃.

V. CONCLUSIONS AND DISCUSSION

The series of spot assays completed show that *cdc3*Δ and *cdc12*Δ present the strongest phenotype when treated with cell wall disrupting drugs. Congo Red, SDS, CFW, and Caspofungin present extreme phenotypes in these strains (Figure 1). This effect is similar to that in the control strain *pdk1*Δ. This deletion is known to affect cell wall integrity. The effect in growth seen in all strains on 0.01% SDS plates is assumed to be due to excessive drug concentration. When considering noncore septins, Cdc10 and Cdc11, cell wall disrupting drugs does not have much effect on growth (Figure 1). The growth of these strains is comparable to the growth of *vps53*Δ when exposed to cell wall disrupters. Caffeine, also used as a cell wall stressor, produced slightly different results. The *pdk1*Δ strain was highly affected and showed minimal growth while the core septin deletions, *cdc3*Δ and *cdc12*Δ, grew similarly to noncore septin deletions (Figure 2). Both SDS and Caffeine affect the Cell Wall Integrity (CWI) signaling pathway. While these panels showed different results, the strains treated with SDS cannot be considered in comparison to Caffeine due to the high drug concentration.

When strains are treated with known plasma membrane disrupting drugs, core septin deletion mutants do not have as strong of a phenotype as *pdk1*Δ. These septin mutants more closely resemble the control strain *erg3*Δ (Figure 3). Erg3 is responsible for ergosterol production, an

important component in the plasma membrane. Noncore septins, again, react to plasma membrane disrupters similarly to *vps53*Δ. Vps53 and Erg3 have similar functions in that they both act in pathways that produce components of the plasma membrane. However, while Erg3 plays a role in ergosterol synthesis, Vps53 is a component of sphingolipid synthesis. While results of treatments with Myriocin and AbA are interesting as they suggest plasma membrane homeostasis functions of septins, the concentration of both Myriocin and AbA are too high. Therefore, a follow-up experiment is recommended with lower concentrations of these drugs.

Strains treated with FLC were examined. When using 8µg/mL FLC, core septin deletion strains exhibited growth inhibition similar to that of *erg3*Δ and *pdk1*Δ. The similarity in response of *erg3*Δ and *pdk1*Δ and matching of core septin deletion behavior alludes to the cell wall-plasma membrane crosstalk. This similarity is also seen in plates with added exogenous ergosterol. There is a compounded sensitivity in all strains when 8 µg/mL FLC was combined with 1000nM ergosterol. When considering noncore septins, they behaved as the tcbΔ control strain (Figure 4). Tcb is known to maintain plasma membrane homeostasis and integrity. The minimal phenotype seen for the *tcb*Δ mutant suggests that while a protein is involved in plasma membrane pathways, not all membrane disrupting drugs will affect cells. Therefore, a lack of recognizable phenotype in noncore septins does not prove that they are not involved in plasma membrane homeostasis and other pathways. A panel of 32 µg/mL FLC was created. Almost no growth from any strain was seen in plates containing 32 µg/mL of FLC. However, when 1000 nM ergosterol was added to 32 µg/mL FLC, partial rescue of *H99, chs3*Δ*, tcb*Δ*, cdc10*Δ*,* and *cdc11*Δ was seen (Figure 5).

Growth curves measuring OD600 of *H99, pdk1*Δ*, cdc3*Δ*, cdc12*Δ, and *5th septin*Δ in the presence of 5 µM Myriocin were created. The *pdk1*Δ strain showed the least growth in the presence of the drug in comparison to 0 µM Myriocin. When considering septin deletion mutants, *cdc3*Δ was minimally affected by Myriocin while *cdc12*Δ shows a significant decrease in growth form the control (Figure 6). While *pdk1*Δ showed the least growth, *cdc12*Δ showed the most variability in cell growth. This shows a variable reactivity to Myriocin treatment in core septin deletion mutants while the noncore septin deletions exhibited phenotypes like *H99* (WT) (Figure 6).

Spot assays on only YPD plates were completed and grown at three different temperatures, 25, 30, and 37℃. All septin deletions except *cdc10*Δ were inhibited for growth at 37℃. Specifically, *cdc3*Δ and *cdc12*Δ strains showed extreme sensitivity to 37℃ (Figure 7). This shows that septin proteins, especially core septin proteins, are essential for growth at host temperature. This contrasts with septins in *S. cerevisiae* where septins are necessary for viability at all temperatures. Thus, *C. neoformans*' septin dependent pathways while not necessary at 25℃ (RT), become essential at 37℃. This implicates septins in unique stress-related functions that could also be conserved in other eukaryotes.

VI. TABLES

Strain	Genotype	Sources
H99	(α) WT	(Perfect, et al. 1993)
pdk1Δ	(α) CNAG_02915::NAT	Madhani Collection (Chun & Madhani, 2010)
chs3Δ	(α) CNAG_05581::NAT	Madhani Collection (Chun & Madhani, 2010)
csr2Δ	(α) CNAG_07636::NAT	Madhani Collection (Chun & Madhani, 2010)
erg3Δ	(α) CNAG_00519::NAT	Madhani Collection (Chun & Madhani, 2010)
vps53Δ	(α) CNAG_00368::NAT	Madhani Collection (Chun & Madhani, 2010)
tcbΔ	(α) CNAG_01824::NAT	Madhani Collection (Chun & Madhani, 2010)
*cdc3*Δ	(α) CNAG_05925::NAT	(Kozubowski & Heitman, 2010)
*cdc10*Δ	(α) CNAG_01373::NAT	This study
*cdc11*Δ	(α) CNAG_02196::NAT	Madhani Collection (Chun & Madhani, 2010)
*cdc12*Δ	(α) CNAG_01740::NEO	(Kozubowski & Heitman, 2010)

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Kozubowski L, Heitman J. Septins enforce morphogenetic events during sexual reproduction and contribute to virulence of *Cryptococcus neoformans*. Mol Microbiol. 2010;75(3):658–75. pmid:19943902

Chun and Madhani. 2010. Methods in Enzymology 470: 797 (PMID 20946836)

Table 1: Strains Used

VII. FIGURES

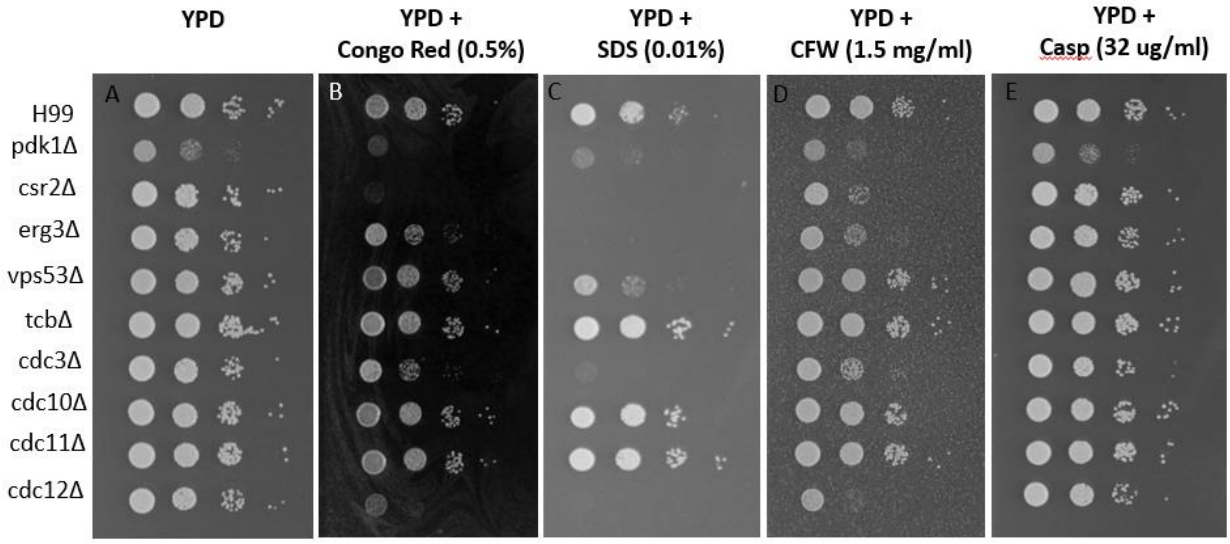

Fig. 1: **Cell Wall Disrupting Drugs**

A) All strains were plated on YPD media with no drug supplement. All strains show normal growth and morphology. **B)** Growth is inhibited in some strains when YPD is supplemented with 0.5% Congo Red. These strains include *pdk1*Δ, *csr2*Δ, *cdc3*Δ, and *cdc12*Δ. The most severe phenotype was seen in *cdc12*Δ. **C)** Strains were treated with 0.01% SDS. Several strains were affected including *pdk1*Δ, *csr2*Δ, *erg3*Δ, *cdc3*Δ, and *cdc12*Δ. **D)** Cells were treated with 1.5 mg/mL of CFW. Severe phenotypes were seen in *pdk1*Δ, *csr2*Δ, *erg3*Δ, *cdc3*Δ, *cdc12*Δ. **E)** Cells were treated with 32 µg/mL of Caspofungin (Casp). A severe phenotype was only seen in *pdk1*Δ.

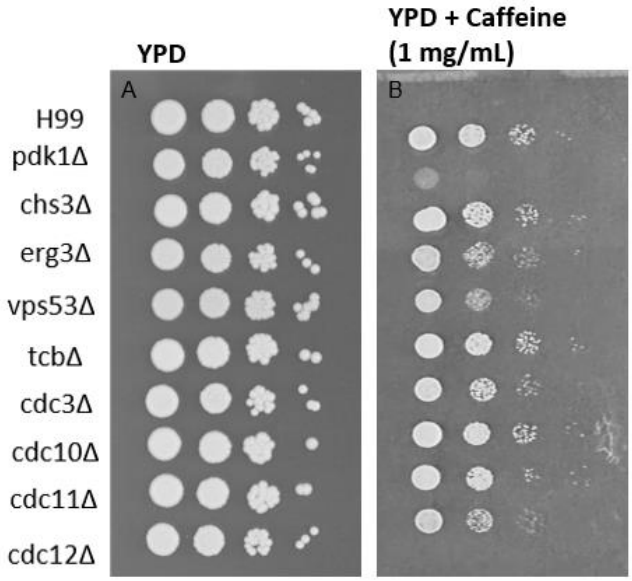

Figure 2: Effect of Caffeine

A) All strains were grown on YPD as a control. **B)** Strains were grown on YPD supplemented with 1 mg/mL Caffeine. The most severe phenotype was seen in *pdk1*Δ.

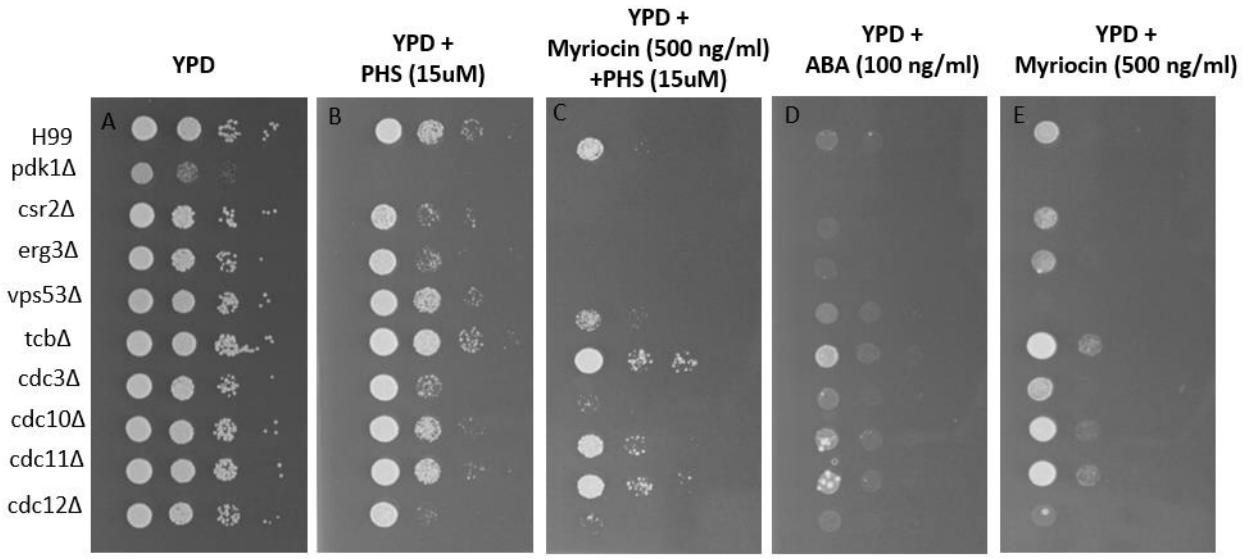

Figure 3: Cell Membrane Disrupting Drugs

A) Strains grown on YPD media show normal growth. **B)** Media with added exogenous PHS was used to grow each strain. Severe phenotypes were seen in *pdk1*Δ*, erg3*Δ*, cdc3*Δ, and *cdc12*Δ. **C)** Cells were treated with 500 ng/mL of Myriocin and 15 µM of PHS. A severe phenotype can be seen in all strains except *tcb*Δ and *cdc11*Δ. **D)** YPD supplemented with AbA showed a severe phenotype in every strain. **E)** YPD supplemented with Myriocin showed a severe phenotype in every strain.

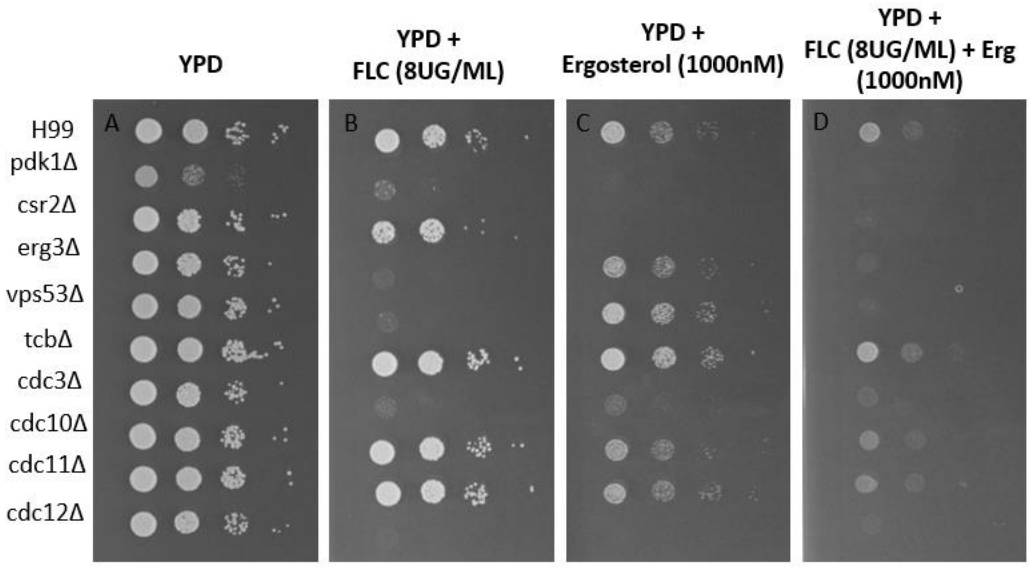

Figure 4: Low Concentration FLC and Ergosterol

A) Strains grown on control YPD plates **B)** Strains grown on YPD media with 8 µg/mL FLC. A severe phenotype was seen in *pdk1*Δ*, erg3*Δ*, vps53*Δ*, cdc3*Δ, and *cdc12*Δ. **C)** 1000 nM ergosterol was added to YPD media. An extreme phenotype was seen in *pdk1*Δ *csr2*Δ*, cdc3*Δ, and *cdc12*Δ **D)** 8 µg/mL of Fluconazole and 1000nM of ergosterol were added to YPD media. A severe phenotype was seen in all strains.

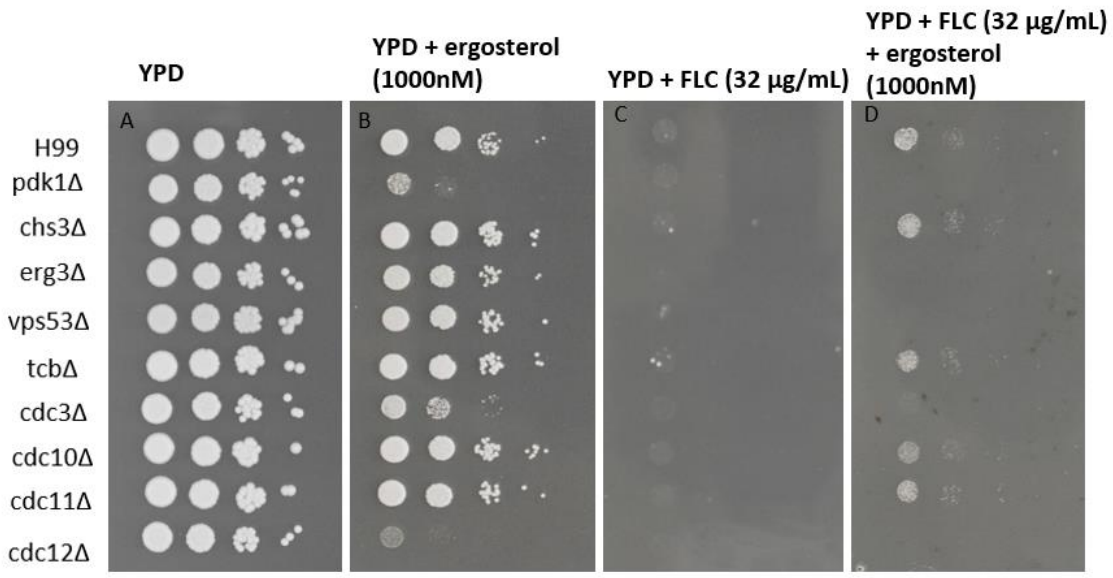

Figure 5: High Concentration FLC and Ergosterol

A) All strains were grown on YPD media as a control. **B)** 1000 nM of exogenous ergosterol was added. An effect was seen in *pdk1*Δ*, cdc3*Δ, and *cdc12*Δ. **C)** Strains grown in 32 µg/mL FLC all had a severe response to the drug. **D)** Plates with 32 µg/mL FLC and 1000nM of ergosterol show partial rescue in some strains. *H99, chs3*Δ*, tcb*Δ*, cdc10*Δ, and *cdc11*Δ show this partial rescue.

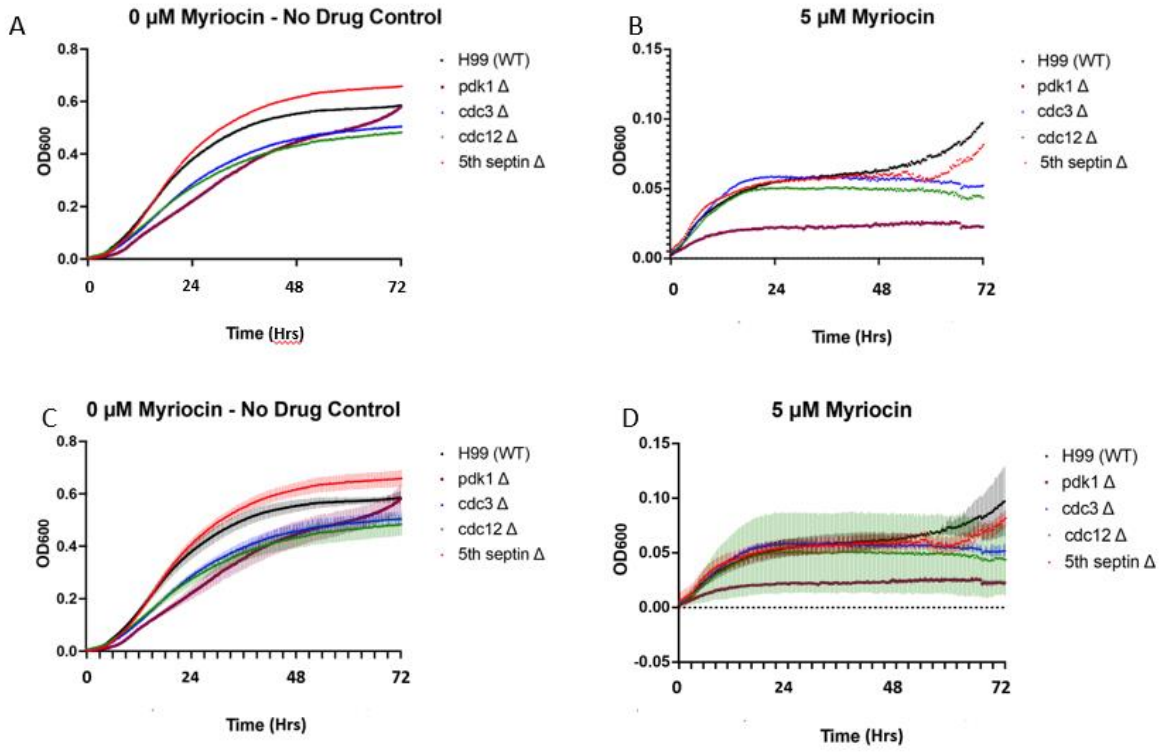

Figure 6: Myriocin Growth Curves

Growth curves were created for five strains with and without myriocin. **A)** The normal growth of cells without myriocin treatment. **B)** The growth of strains was tracked over time when treated with 5 µM Myriocin. An extreme phenotype was seen in the *pdk1*Δ strain. **C)** The control experiment with error bars is shown with no myriocin treatment. **D)** The error margin as shown for strains treated with 5 µM myriocin. There was wide variability in the growth of *cdc12*Δ strains.

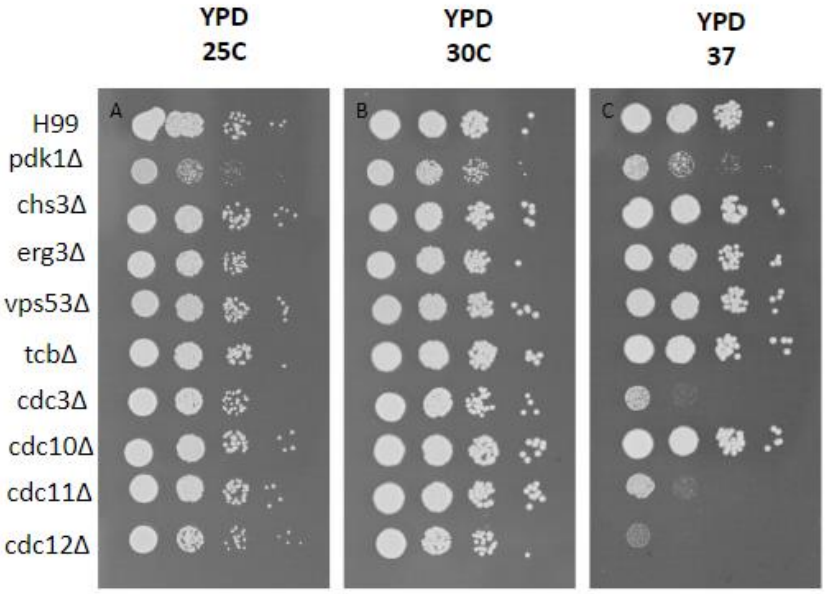

Figure 7: Heat Stress Spot Assays

A) Strains grown on YPD media at 25℃ show normal growth. **B)** The growth temperature was raised to 30℃ and no severe phenotype was shown. **C)** Strains were grown at 37℃ and growth phenotypes were seen in *pdk1*Δ, *cdc3*Δ, *cdc11*Δ, and *cdc12*Δ.

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