Defining the Role of a Putative SREBP Cleavage Activating Protein in pH Response Regulation in *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is a fungal pathogen that infects the lungs and travels to the central nervous system where it causes meningoencephalitis. Without rapid medical intervention, this is fatal for individuals with compromised immune systems. During infection, the pathogen moves from an acidic exterior environment to a slightly alkaline environment in the host. This induces alkaline-specific gene induction to maintain cellular processes for cell survival. Investigators at Duke University identified several genes that play a role in this process, including CNAG_01580, or *SCP1*, which codes for a putative SREBP cleavage activating protein. This gene was deleted in wild-type *C. neoformans* to assess alterations in virulence-related phenotypes. If this gene significantly impacts virulence, it will provide a new target for antifungal therapy.

Defining the Role of a Putative SREBP Cleavage Activating Protein in pH Response Regulation in *Cryptococcus neoformans*

Cryptococcus neoformans is an opportunistic fungal pathogen which can cause severe disease and death in immunocompromised individuals (Price et al., 2011). Through first infecting the lungs, then traveling to the central nervous system, it causes fungal meningoencephalitis (Maliehe et al., 2020). The goal of this research project was to investigate the virulence of this pathogen, through studying how *C. neoformans* adapts to pH changes that occur as it infects the slightly basic microenvironment of the human lung. Specifically, the role a putative sterol regulatory element-binding protein cleavage activating protein (SREBP-CAP), or Scp1, plays in pH adaptation pathways was studied.

Globally, approximately 223,100 cases of cryptococcal meningitis occur each year (Rajasingham et al., 2017). Despite current treatments, this disease is responsible for about fifteen percent of AIDS-related fatalities, or about 181,100 people annually (Rajasingham et al., 2017). Due to the opportunistic nature of this pathogen, this disease disproportionately affects people diagnosed with human immunodeficiency virus infection (Zono et al., 2020). Cryptococcal infections are typically observed within the HIV+ community in sub-Saharan Africa (Park et al., 2009). For example, in Togo, a 2.9% prevalence rate was observed among patients living with HIV, and in the Ivory Coast, the prevalence was 3.6% (Zono et al., 2020). It also has been determined that in Sub-Saharan Africa, the mortality rate from neuromeningeal cryptococcosis ranges from 20-50% within ten weeks of antifungal therapy (Zono et al., 2020). This fungal pathogen is deadly, and it is clear that better treatment options are needed.

Cryptococcus neoformans Mechanism of Infection

C. neoformans is typically found in the environment in soil that has been contaminated with bird droppings (Roy & Chiller, 2011). When in the form of basidiospores or desiccated yeast cells, *C. neoformans* can become airborne and be inhaled by the host organism (Coelho et al., 2014). The fungi then can become lodged in the alveoli of the lung where it will come in contact with an alveolar macrophage or dendritic cell (Coelho et al., 2014). The resulting immune response in an immunocompetent individual usually leads to the containment of the pathogen or maintenance of the pathogen in a latent state (Voelz & May, 2010). This causes no clinical manifestations. Cryptococcal infection of this kind is common. In fact, studies have shown that in urban environments, eighty percent of children were shown to have been infected by *C. neoformans*, all resulting in no apparent disease (Coelho et al., 2014).

However, for those with compromised immune systems, Cryptococcal infection has devastating effects (Maliehe et al., 2020). The infection of lung airspaces can result in pneumonia. The pathogen can then enter the bloodstream, where it has the ability to cross the blood brain barrier and impair reabsorption of cerebral spinal fluid in the brain (Maliehe et al., 2020). This results in inflammation and meningoencephalitis, which is fatal without swift medical intervention (Voelz & May, 2010).

The Rim Pathway: An Established pH Adaptation Pathway

In recent years, it has been suggested that novel treatments could be developed by targeting cryptococcal pH adaptation pathways that are activated upon infection (Brown et al., 2020). When *C. neoformans* enters the host, it encounters large changes in pH as it goes from acidic in the environment, to slightly basic in the host, back to the acidic environment of the phagolysosome. This pathogen must adapt to these pH changes in order to survive (Brown et al.,

2020). In the environment, the pathogen is typically found growing in acidic soil contaminated with uric acid due to bird droppings (Roy & Chiller, 2011; Spennemann & Watson, 2017). However, once inhaled, *C. neoformans* encounters a pH between 7.38 and 7.43 in the human lung (Effros & Chinard, 1969). When *C. neoformans* encounters this change in extracellular environment, alkaline-specific signaling pathways are induced to maintain cellular processes for survival (Brown et al., 2020). If this pH adaptation can be disrupted, the ability of *C. neoformans* to cause disease may be thwarted (Brown et al., 2020).

A well established and studied pH adaptation pathway is the Rim pathway in fungi (Ost et al., 2015). This pathway is activated by extracellular increase in pH. While the specific mechanism of this pathway is better established for ascomycete fungi, the mechanism has been found to be functionally conserved in basidiomycete species such as *Cryptococcus neoformans*. Several orthologs have been identified in the *C. neoformans* Rim pathway (Ost et al., 2015). This pathway, and also the protein kinase A pathway are responsible for activation of the *C. neoformans* Rim101 protein (Selvig & Alspaugh, 2011). The *C. neoformans* Rim101 transcription factor is activated through proteolytic processing and is localized to the nucleus. It plays a role in regulating genes involved with cell wall modification and capsule production. It also regulates genes involved in metal and ion homeostasis, capsule attachment, and formation of titan cells (Selvig & Alspaugh, 2011). Titan cells have a thickened capsule and are much larger than normal cells. This protects them from stress associated with pH change. Therefore, the Rim pathway allows *C. neoformans* to survive in the human host, despite exposure to neutral or slightly alkaline pH (Selvig & Alspaugh, 2011).

The Sterol Homeostasis Pathway: An Alternate pH Adaptation Pathway

In an attempt to identify components of the membrane sensing complex in the Rim pathway in *Cryptococcus neoformans*, a mutagenesis screen for growth inhibition at pH 8 was conducted (Ost et al., 2015). Without the Rim pathway functioning, *C. neoformans* would not be able to grow at slightly alkaline pH. Therefore, if a gene lost its functionality through an insertional mutation and it prevented the growth of *C. neoformans* at pH 8, but not at acidic pH, its gene product could play a role in the Rim pathway. Forty potential genes were identified (Ost et al., 2015).

However, through researching some of the genes identified in the screen, Brown and her colleagues at Duke University found a pH adaptation pathway completely independent of the Rim pathway. The sterol homeostasis pathway was found to be necessary for *C. neoformans* to grow in an elevated pH environment, such as the human host (Brown et al., 2020). It was found that slightly alkaline pH alone was enough to activate the sterol response element I transcription factor (Sre1) in *Cryptococcus neoformans*. This pathway leads to the production of ergosterol (Figure 1). Several details of the exact mechanism of ergosterol synthesis in *C. neoformans* remain unknown, including the sensor that activates the pathway (Brown et al., 2020). However, it is known that Sre1 is cleaved by Stp1, a basidiomycete-specific protease. This activates Sre1so it can travel to the nucleus of the cell (Figure 1). This process is also dependent on Scp1, an SREBP cleavage activating protein (Brown et al., 2020).

When a mutant strain without *SRE1* was created through gene deletion (*sre1Δ*), the strain could not grow at pH 8 (Brown et al., 2020). Rim pathway signaling remained completely intact, however. This proved the independence of the sterol homeostasis pathway from the Rim pathway. The mutant strain also proved to be avirulent in a mouse model of infection (Brown et

al., 2020). This is important to note as Sre1 in the sterol homeostasis pathway of *Cryptococcus neoformans* is necessary for the fungi to retain pathogenicity. However, it is also important to note that when the mutant strain was supplemented with ergosterol, growth was restored at pH 8 (Brown et al., 2020).

In another study, the Erg6 methyltransferase enzyme which converts zymosterol to fecosterol was studied in *Cryptococcus neoformans* (Oliveira et al., 2020). It alternatively can convert lanosterol to eburicol. This important enzyme is crucial for ergosterol synthesis in fungi. In the mutant strain *erg6Δ*, no ergosterol was detected in the cell membrane with a 0.1 μg/mg of total lipids limit of detection (Oliveira et al., 2020). This strain was also avirulent upon utilization of a wax moth model of infection. Mutant *C. neoformans* cells were not able to survive as well as the wild-type in the host and were more susceptible to stress. Ergosterol therefore must play a crucial role in *C. neoformans* pathogenicity (Oliveira et al., 2020). While this mutant strain was avirulent, it was noted that none of the classic virulence factors were affected significantly in the *erg6Δ* strain. For example, the capsule size and architecture remained very similar, and there was no alteration in melanin production when the strain was plated on L-DOPA agar (Oliveira et al., 2020).

Recently, a third study investigated sterylglucosidase 1 (Sgl1) which converts ergosterol 3β-D-glucosidase into ergosterol and glucose (Pereira et al., 2021). Deletion of this gene in *C. neoformans* results in a loss of virulence. Interestingly, it was found that this strain immunized mice against wild-type *Cryptococcus neoformans* secondary infection as well (Pereira et al., 2021). This even worked in immunodeficient mice that were $CD4^+$ T cell deficient. It is clear that targeting ergosterol synthesis in *Cryptococcus neoformans* provides an interesting target for treatment development for this devastating disease (Pereira et al., 2021).

Significance of Ergosterol to Membrane Integrity and Function

Ergosterol is a significant sterol present in the cell membrane of fungi (Oliveira et al., 2020). It serves as a rigid part of the cell membrane. The absence of ergosterol would lead to increased fluidity and permeability of the membrane. Recent studies have shown that ergosterol also has regulatory functions (Oliveira et al., 2020). The sterols can affect the organization of the cytoskeleton, signal transduction pathways, and vesicle transport (Oliveira et al., 2020). It can promote cell growth and is necessary for the maintenance of the mitochondrial DNA in *C. neoformans* (Jordá & Puig, 2020). Ergosterol can also influence virulence and innate immune system activation (Oliveira et al., 2020). Recent evidence suggests that fungal ergosterol may also induce pyroptosis of macrophages in the host (Rodrigues, 2018). Sterols in the membranes of fungi frequently form lipid rafts (Oliveira et al., 2020). These rafts have been observed on the bud tips of *Cryptococcus neoformans.* This indicates that sterols may play a role in morphology. Lipid rafts may also be able to expose membrane virulence factors (Oliveira et al., 2020).

Therefore, there are many potential reasons for why functional ergosterol synthesis pathways would be required for growth at physiological pH (Pereira et al., 2021). Increased pH would lead to alkaline membrane stress (Brown et al, 2020). As ergosterol is crucial to membrane function and integrity, it is required for membrane homeostasis under such conditions. Ergosterol synthesis pathways are required for *C. neoformans* virulence (Brown et al., 2020). Therefore, it is no surprise that pharmaceuticals have been developed to target these pathways (Pereira et al., 2021). However, there are several limitations to the current available treatments (Pereira et al., 2021).

Limitations of Current Ergosterol Targeting Antifungals

Several current antifungals work by either targeting ergosterol synthesis or by binding to sterols in the membrane (Pereira et al., 2021). The current most effective antifungal against *C. neoformans* infection is amphotericin B, a polyene drug (Kim et al., 2012). This treatment targets membrane sterols causing the creation of pores which lead to cell death through leakage of the cytoplasm. However, this antifungal is extremely damaging to the kidneys. A liposomal form of this drug was developed to help ease side effects, and this drug is also sometimes taken in combination with 5-flucytosine (Kim et al., 2012). The second most used antifungal are azole drugs such as fluconazole. These target Erg11, or lanosterol 14-demethylase. This enzyme converts lanosterol to ergosterol (Kim et al., 2012). Not only does the inhibition of this enzyme prevent the formation of ergosterol, but it also causes an unusual accumulation of 14-methylsterols. This can also lead to cytoplasmic leakage and disorganization of membrane structure. Growth is arrested in fungal cells exposed to this drug (Kim et al., 2012). While this antifungal is much safer, fungi developing resistance to it is common, and many drug interactions limit the usefulness of this treatment (Pereira et al., 2021).

A Novel Target: A Putative SREBP Cleavage Activating Protein

There are many other potential targets involved in ergosterol synthesis yet to be investigated, many of which could be viable targets for antifungal development. For example, the synthesis of ergosterol in the endoplasmic reticulum alone requires twenty-five genes (Rodrigues et al., 2018). Most genes involved in the synthesis of ergosterol are required for the survival of *Cryptococcus neoformans* (Rodrigues, 2018).

Because of the promising avenue for future antifungal therapy development, this research project explores a novel target: a putative SREBP-CAP, Scp1. This is the gene CNAG_01580 in

the *C. neoformans* genome and was identified through the mutagenesis screen done to identify pH-adaptation related mutants as described above (Ost et al., 2015). However, this gene is putatively involved in the same sterol response pathway as described by Brown and her colleagues (Brown et al., 2020). Therefore, it is proposed that this gene also affects pH adaptation independently of the Rim pathway and affects the production of ergosterol in *C. neoformans.* The purpose of this research project is to understand the role that Scp1 plays in the regulation of pH adaptation of *C. neoformans* so that more effective treatments can be designed to decrease the mortality rate associated with cryptococcal meningitis.

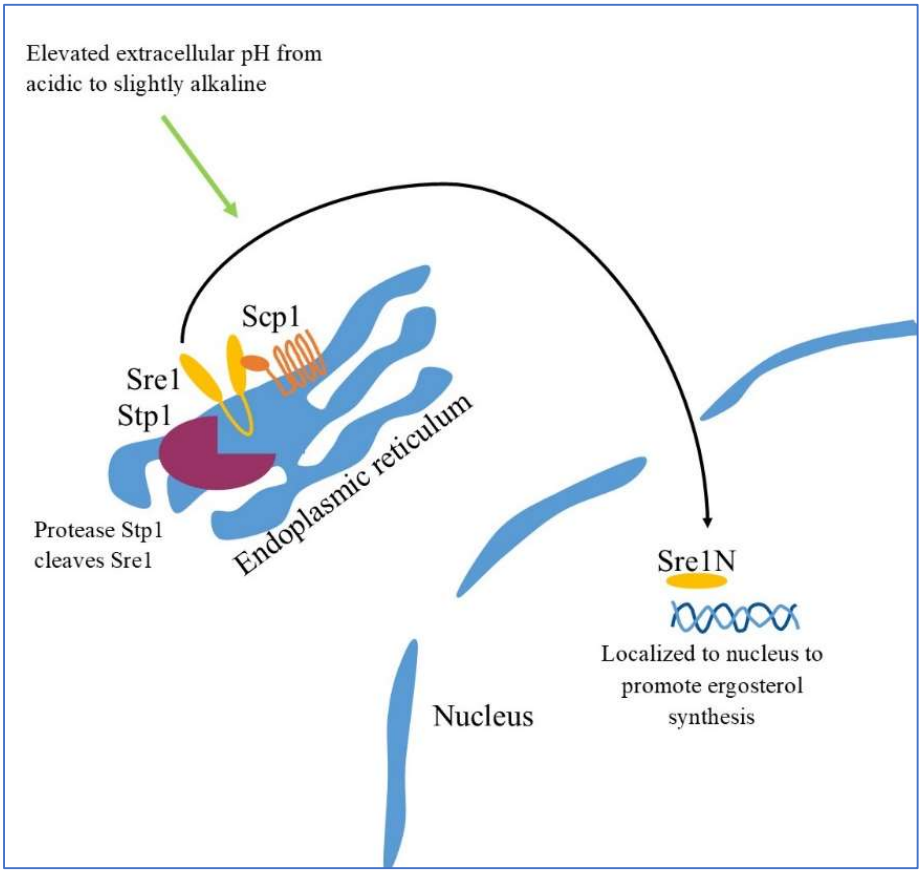
SCP1 in *C. neoformans* is homologous in sequence to the *Schizosaccharomyces pombe* gene scp1^{+}, which encodes for an SREBP-CAP (PomBase). In *S. pombe*, Scp1 and Sre1 work to sense sterol levels in the endoplasmic reticulum (ER) and coordinate the expression of genes that code for enzymes involved in the synthesis of ergosterol (Jacquier & Schneiter, 2012). These proteins are bound to the ER membrane (Porter et al., 2010). It is known that for *S. pombe*, expression of these genes increases under low oxygen or hypoxic conditions (Jacquier & Schneiter, 2012). Low oxygen has also been shown to activate sterol homeostasis pathways in *C. neoformans* as well (Brown et al., 2020).

Sre1 and Scp1 are orthologs to mammalian sterol regulators SREBP and Scap, respectively (Porter et al., 2010). In a mammalian model, cholesterol synthesis is regulated by this pathway (Figure 2). Scap detects cholesterol levels directly through actually binding to cholesterol in the ER membrane. When bound, there is a conformational change that occurs such that Scap can bind to Insig (Porter et al., 2010). This keeps the complex of the SREBP and Scap in the membrane. However, when cholesterol is not bound, and therefore present in low amounts, SREBP-Scap can dissociate from Insig (Figure 2). It travels to the Golgi, two proteases cleave an

N-terminus domain of SREBP, and this SREBP transcription factor enters the nucleus and promotes expression of cholesterol synthesis genes (Porter et al., 2010). This pathway is conserved in fission yeast like *S. pombe.* However, while functionally conserved in *C. neoformans*, the complete mechanism and how elevated pH is sufficient to activate this pathway in *C. neoformans* remains unknown (Figure 1).

Figure 1

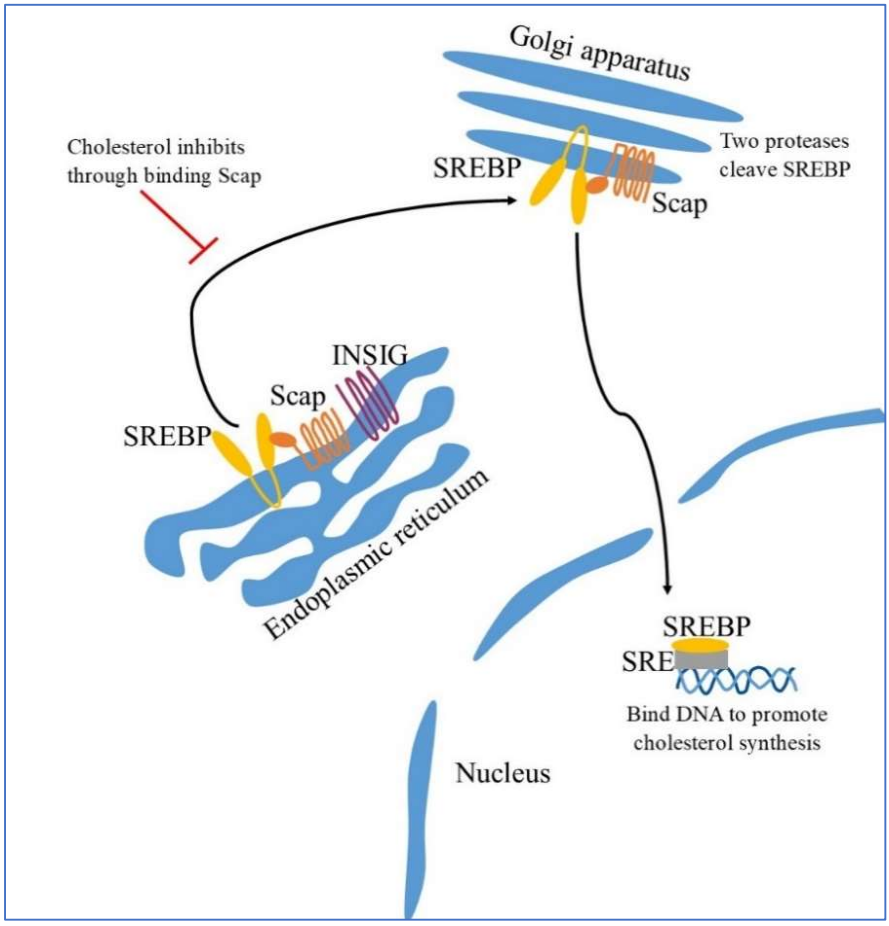
Activation of ergosterol synthesis pathways in C. neoformans



Note: The sterol homeostasis pathway in *C. neoformans* is less established than in mammalian cells. It is known that elevated pH is sufficient to activate Sre1, however, the complete mechanism remains unknown (Brown et al., 2020). Created by Hannah Finson.

Figure 2

Activation of cholesterol synthesis pathways in mammalian cells



Note: The parallel sterol homeostasis pathway in mammalian cells is well developed (Bien & Espenshade, 2010; Espenshade & Hughes, 2007). Created by Hannah Finson.

Results

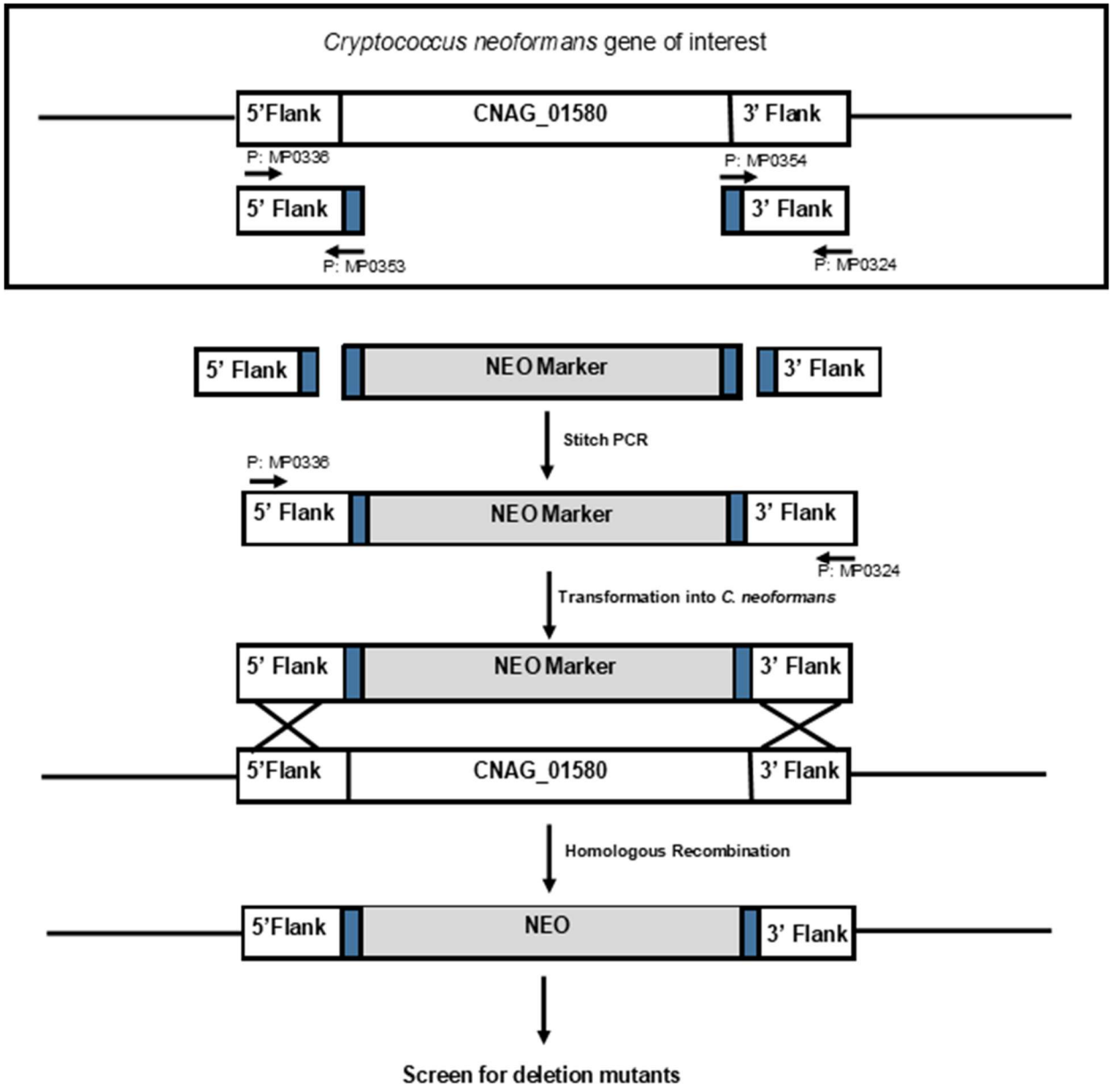
In order to determine the function *of SCP1* in *Cryptococcus neoformans*, gene deletion was used to create a mutant strain, *scp1Δ*. First, a gene deletion construct for the putative *C. neoformans SCP1* gene was made. A region of DNA upstream of the target gene was amplified in order to create a 5' homology region. The same was done downstream of the gene to create a 3' homology region. A gene coding for neomycin and antifungal G418 resistance (NEO) was

also amplified. Then, using Polymerase Chain Reaction (PCR), the gene deletion construct was ligated together (Figure 3). The construct was confirmed through gel electrophoresis (Figure 4). The construct was approximately 4 kilobases in length, which was consistent with the combined length of the three ligated pieces.

Following purification of the PCR product from the gel, the deletion construct was then introduced into wild-type *C. neoformans* strain KN99α via biolistic transformation. Because of the homology regions on either side of the construct, homologous recombination could occur to replace *SCP1* with NEO (Figure 4). The NEO resistance gene acted as a dominant selectable marker as it permitted growth on the antifungal G418. Therefore, *C. neoformans* transformants were plated on G418 containing plates. Colonies expressing the NEO resistance gene grew on the plates while wild-type *C. neoformans* could not. The observance of colonies suggested that the transformation was successful (Figure 4).

Figure 3

Model of Homologous Recombination to Create Mutant Strain scp1Δ

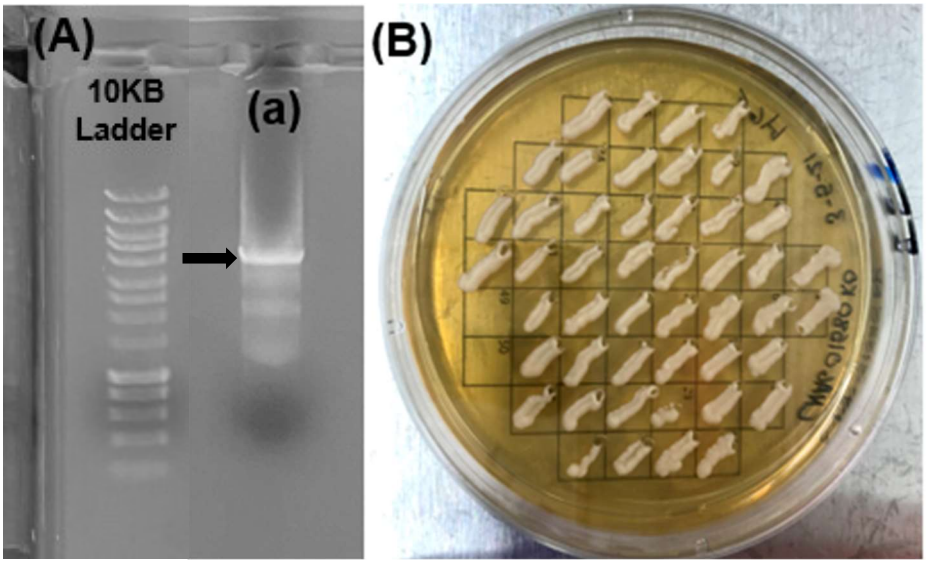


Note. This model demonstrates the homologous recombination that occurred upon biolistic transformation of the NEO gene knock-out construct into wild-type *Cryptococcus neoformans* strain KN99α. The 5' flank upstream of *SCP1* was amplified using primers MP0336 and

MP0353 (Table 1). The 3' downstream flank was amplified with primers MP0354 and MP0324. These were ligated to either side of the NEO gene using polymerase chain reaction. This construct replaced *SCP1* in the *C. neoformans* genome via homologous recombination. Created by Hannah Finson.

Figure 4

Confirmation of Gene Deletion Construct and Transformation Results



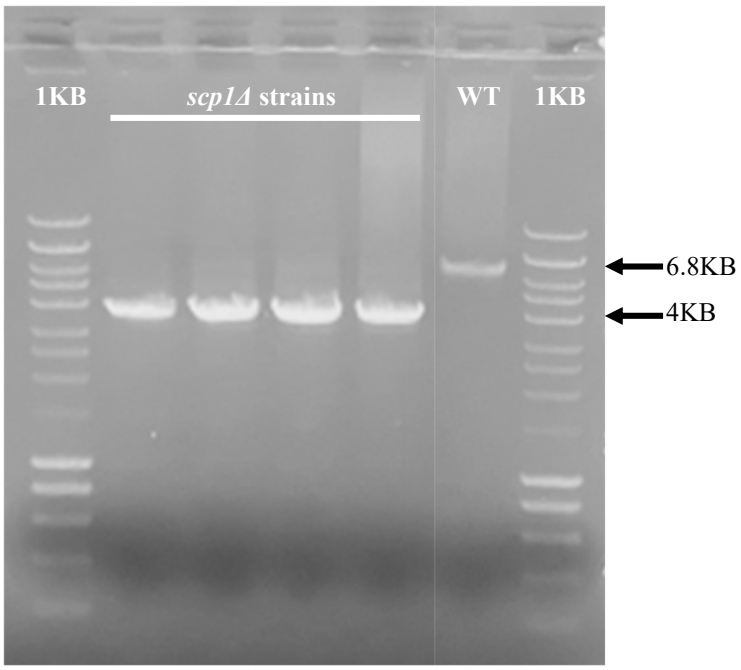
Note. (A) The knock-out construct created for deletion of *SCP1* in *Cryptococcus neoformans* was confirmed by running the amplified construct on a 0.7% agarose gel. The 5' homology region, 3' homology region and the neomycin resistance gene (NEO) ligated together is approximately four kilobases in length. A band at 4 KB in lane (a) indicated by an arrow therefore demonstrated that the gene deletion construct was successfully created. (B) Transformation was done using Biolistic transformation to replace CNAG_01580 with the knock-out construct containing NEO. Transformants were plated on selective media containing antifungal G418. Since colonies were observed, it can be concluded that the NEO resistance gene marker successfully integrated into the genome. Created by Hannah Finson.

Following this, the transformants were plated on pH 8 plates, and many transformants were found to not grow. These colonies were selected to undergo further testing as they were predicted to be successful transformants. Growth inhibition at pH 8 upon loss of function of *SCP1* was already established in the mutagenesis screen (Ost et al., 2015). Since ergosterol plays a large structural and regulatory role in the membrane, disruption of ergosterol synthesis should put stress on the membrane (Oliveira et al., 2020). Testing for susceptibility to osmotic stress through plating the mutant on high salt plates is one way to measure stress to the membrane. Growth inhibition was observed on plates containing 1.5 M NaCl. Some increased inhibition was observed for the transformants as compared to wild-type. The *sre1Δ* mutant was plated as a positive control, and a very similar pattern of growth inhibition to the transformants was observed, as expected. This suggests that the mutant strains are more susceptible to osmotic stress.

While the screen indicated that transformation was successful, integration of the gene deletion construct at the correct locus of the genome was confirmed using PCR (Figure 5). Primers on either side of the wild-type location of *SCP1* were used to amplify the locus of interest. This revealed a 4 KB band, the length of the gene deletion construct. If *SCP1* was still present in the genome, the band would have been 6.8 KB. Therefore, the mutant *C. neoformans* strain *scp1Δ* was successfully created (Figure 5). The wild-type locus was amplified as a control, and the expected 6.8 KB band was observed.

Figure 5

Confirmation of Successful Transformation



Note. Integration of the NEO gene knock-out construct into the correct genomic locus was confirmed via polymerase chain reaction and subsequent gel electrophoresis on a 0.7% agarose gel. Primers on either side of the locus of interest were chosen to amplify where *SCP1* would be located in wild-type *C. neoformans* and where the construct was presumed to enter the genome. A 4 KB band, as labeled by arrow above, indicated that the gene deletion construct replaced *SCP1* successfully. If wild-type *SCP1* was still present in the genome, the band would have appeared at 6.8 KB in length. A 6.8 KB band would have meant the NEO gene inserted into the genome ectopically. The wild-type *C. neoformans* locus of interest was run as a control, as represented by WT. A band at approximately 6.8 KB was observed, as expected. This is labeled above by an arrow. Created by Hannah Finson.

In order to determine the function of *SCP1* in pH adaptation and virulence, phenotypic tests were conducted on the mutant strain. Alterations in virulence-related traits were first

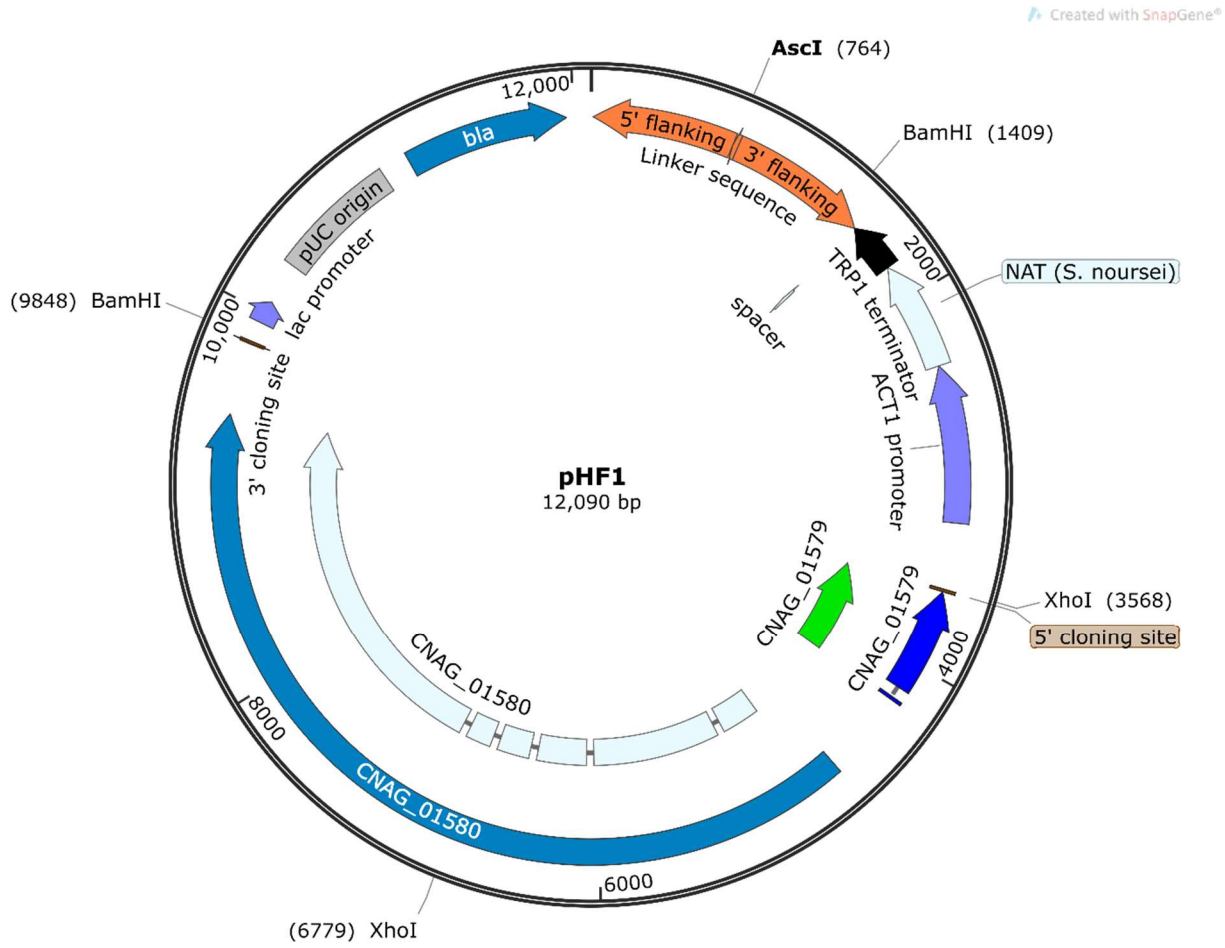
assessed. One main virulence factor as described above is the production of melanin. Many avirulent strains of *C. neoformans* can no longer produce melanin in the presence of dopamine (Casadevall et al., 2000). However, *scp1Δ* was still able to produce melanin on L-DOPA plates. This test independently cannot determine whether or not the mutant strain is virulent. Further testing of virulence-related factors is required.

In a gene deletion study, it is imperative to reintroduce the gene of study back into the genome of the mutant strain in order to observe reemergence of wild-type phenotypes. First, *SCP1* was amplified from the wild-type KN99α *Cryptococcus neoformans* genome using PCR. This was subcloned into the plasmid pSDMA25 containing a nourseothricin resistance gene (NAT). This created the plasmid pHF1 (Figure 6). Correct cloning was confirmed through linearizing the plasmid with two restriction enzymes separately: BamHI and XhoI. pHF1 has two cut sites for BamHI and two cut sites for Xho1 (Figure 6). When cut with BamHI, the expected band sizes were 3651 base pairs and 8439 base pairs. When cut with Xho1, expected band sizes were 3211 and 8879 base pairs. Gel electrophoresis of the cut plasmid revealed expected fragment lengths (Figure 7).

pHF1 was then linearized with Asc1, such that it could be introduced into the safe haven I region of the *C. neoformans* genome. This is a region of DNA in which it has been shown to be safe to insert genes there without altering the functionality of other genes (Arras et al., 2015). sgRNA was also amplified from pBHM2329 using two sets of primers with tags that overlap in the middle of the two amplified areas, such that stitch PCR can be used to create the twenty-base-pair guide for CRISPR-Cas9 (Huang et al., 2021). Cas9 was also amplified from the plasmid pBHM2403. This was done such that Transient CRISPR-Cas9 coupled with Electroporation could be completed in the future (Lin et al., 2020).

Figure 6

Creation of pHF1 plasmid

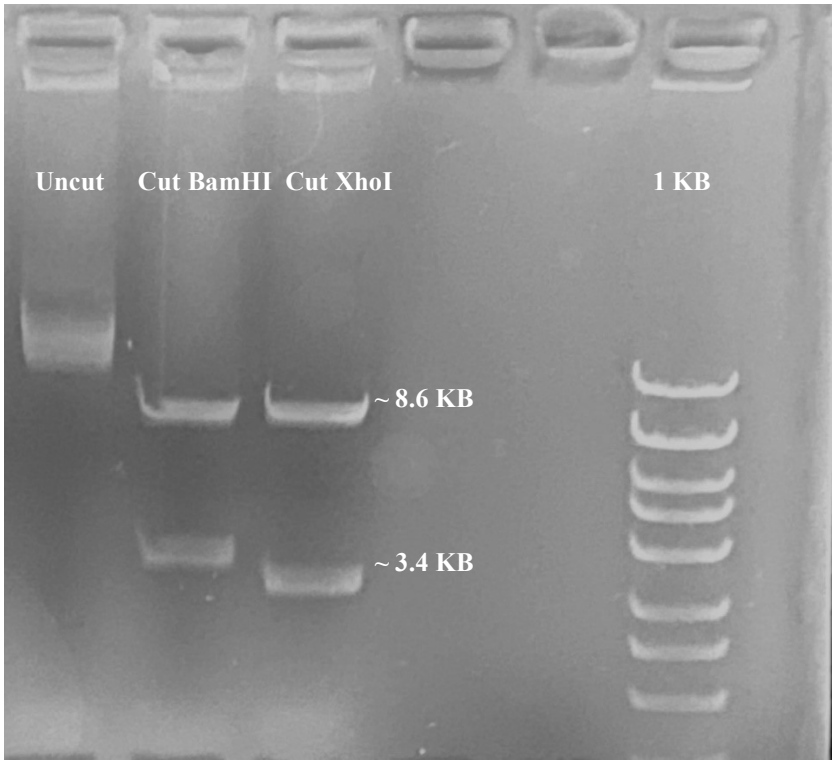


Note. This is a schematic of the pHF1 plasmid which was created to reintroduce *SCP1* into *scp1Δ*. *SCP1* was amplified from wild-type *Cryptococcus neoformans* and introduced into pSDMA25. There is also a nourseothricin resistance gene (NAT) labeled above. There are several restriction digest cut sites, including BamHI and XhoI which were used to confirm correct cloning of the plasmid. There is also an AscI cut site labeled above. This was used to linearize the plasmid for reintroduction into *scp1Δ*. The gene labeled "bla" above is an ampicillin

resistance gene, which allowed for growth on LB + Ampicillin plates. Created by Hannah Finson.

Figure 7

Confirmation of Successful Creation of pHF1



Note. Restriction digest of the construct for reintroduction of *SCP1* confirmed success in creating the plasmid. Enzymes BamHI and Xho1 were used. Upon digestion for one hour, bands of expected length were produced with run on a 0.7% agarose gel. Created by Hannah Finson.

Discussion and Future Work

The construct containing *SCP1*, pHF1, will be introduced into *scp1Δ*. pHF1, Cas9, and a guide RNA will be introduced into *scp1Δ* by electroporation. Integration of pHF1 into safe haven I will occur by CRISPR gene editing. Recently, this method has been developed for use in targeted mutagenesis in *Cryptococcus neoformans* (Lin et al., 2020). This method combines

electroporation, which has very high efficiency, and CRISPR-Cas9 technology which has high efficiency for DNA integration. This system is called TRACE (Transient CRISPR-Cas9 coupled with Electroporation) (Lin et al., 2020).

Further phenotypic testing of *scp1Δ* is required to further elucidate the function of *SCP1*. More virulence-related factors will be screened for such as production of capsule and survival in macrophages. While these virulence factors are important to test for before using animal models of infection, it is important to note that in study of *erg6Δ*, none of the main *C. neoformans* virulence factors were affected, yet the strain was still found to be avirulent in a wax moth model of infection (Oliveira et al., 2020). It will also be tested whether supplementing exogenous ergosterol rescues growth of *scp1Δ* at physiological pH. It would be expected that growth would be rescued as that was found to be true for *sre1Δ* (Brown et al., 2020).

Lastly, a murine infection model will be used to analyze the effect this gene has on virulence. If knocking out this particular gene inhibits the ability of *C. neoformans* to cause disease in the host, this may present interesting new insights into the virulence of this fungal pathogen. It is expected that *scp1Δ* infection will not lead to increased mortality rates when compared to the control. This is because *SCP1* putatively plays a role in ergosterol synthesis in *C. neoformans.*

Cryptococcus neoformans is a fungal pathogen that has devastating effects for people with immunodeficiency disorders such as HIV (Rajasingham et al., 2017). Despite current antifungal treatment, the mortality rate from cryptococcal meningitis remains high (Park et al., 2009). This study investigated how *C. neoformans* adapts to the pH change from the acidic environment to the slightly alkaline pH of the human lung. Specifically, the link between sterol response pathways in *C. neoformans* and pH adaptation as the pathogen infects the host was

studied. Ergosterol is a crucial membrane component in *C. neoformans* as it affects membrane fluidity and permeability. It also has been recently shown to have important regulatory functions. Ergosterol can promote cell growth and help induce the pyroptosis of macrophages in the host. Therefore, targeting of ergosterol synthesis pathways may affect virulence of this pathogen.

In this study, a putative SREBP cleavage activating protein was investigated as a potential new target for future development of novel treatments. Functional analysis of the gene was carried out via deletion of *SCP1* in wild-type *C. neoformans.* Analysis of virulence-related traits was done. The gene will also be reintroduced into the mutant strain to observe reemergence of wild-type phenotypes. If future work proves that *scp1Δ* is avirulent in a mouse model of infection, this will provide fascinating insights into *C. neoformans* infection. Eventually, these insights could lead to effective treatments for cryptococcal meningitis.

Methods

For this study, wild-type strain KN99α *Cryptococcus neoformans*, a gracious gift from the lab of Dr. Andrew Alspaugh, was used unless noted otherwise. Also, YPD media (1.0% yeast extract, 2.0% peptone, 2.0% agar, 2.0% glucose) was used for plating unless otherwise specified. DNA was prepared using QIA Spin Miniprep Kit through Qiagen (cat nos. 27104 and 27106) and given Quick-start protocol unless otherwise specified.

Creation of a Gene Knock-out Construct

Amplification of a 5' flanking region of *SCP1* from wild-type *C. neoformans* was done utilizing primers MP0353 and MP0336 (Table 1). The Polymerase Chain Reaction protocol was as follows: 96℃ for four minutes, then repeated 35X: 96℃ for fifteen seconds, 51℃ for fifteen seconds, and 72℃ for two minutes, followed by 72℃ for five minutes, then hold at 12℃. The product was run on a 0.4% agarose gel at 100V for approximately forty-five minutes. Then, the

band was excised and purified using the Zymoclean Gel DNA Recovery Kit through Zymoresearch Genesee Scientific (Cat no: #11-301) and its provided quick protocol.

A 3' flanking region of *SCP1* was also amplified from wild-type *C. neoformans.* The primers used for this PCR were MP0354 and MP0324 (Table 1). The Polymerase Chain Reaction protocol was as follows: 95℃ for two minutes, then repeated 35X: 95℃ for fifteen seconds, 53℃ for fifteen seconds, and 72℃ for one minute, followed by 72℃ for five minutes, then hold at 10℃. The product was run on a 0.7% agarose gel at 100V for thirty-five minutes. This band was also excised from the gel and purified using the same DNA Recovery Kit as aforementioned.

The third piece of the gene knock out construct was the dominant selectable marker, a neomycin resistance gene, NEO. This was amplified out of the pJAF1 plasmid, a gracious gift from the lab of Dr. Andrew Alspaugh. Primers MP0351 and MP0352 were used (Table 1). The PCR protocol was as follows: 95℃ for two minutes, then repeated 35X: 95℃ for fifteen seconds, 49.5℃ for fifteen seconds, and 72℃ for two minutes, followed by 72℃ for five minutes, then hold at 12℃. Gel electrophoresis was done on the product using a 0.7% agarose gel run at 100V for forty-five minutes. This band was excised from the gel and purified as before.

In order to ligate the three amplified pieces, polymerase chain reaction, or "Stitch PCR" was used. Equimolar amounts of the three pieces were used for the deletion construct as described by Davidson and colleagues (Davidson et al., 2002). Primers MP0351 and MP0352 (Table 1) were used as well as the following PCR protocol: 96℃ for four minutes, then repeated 35X: 96℃ for fifteen seconds, 51.5℃ for fifteen seconds, and 72℃ for four minutes, followed by 72℃ for five minutes, then hold at 12℃. The polymerase chain reaction product, the gene

knock out construct, was run on a 0.7% agarose gel at 100V for ninety minutes. The band was excised and purified using the Zymoclean Gel DNA Recovery Kit. All amplified DNA in each step were quantified on a Nanodrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Table 1

List of primers utilized in this study

Primer name	Sequence	Description
MP0324	ACGACAAACAAAATTTCCCC	CNAG_01580-6; Right Primer for Downstream Flank
MP0336	GGTTTATTTGACGGATTTCA	CNAG_01580-1a; Left Primer for Upstream Flank
MP0351	GGA TAT CTG CAG AAT TCG	NEO Infusion Forward Linker
MP0352	CTC GGA TCC ACT AGT AAC	NEO Infusion Reverse Linker
MP0353	GTT ACT AGT GGA TCC GAG CAA TAC ACC ATC ACA TCC AA	CNAG_01580 5' Flank Reverse Primer
MP0354	CGA ATT CTG CAG ATA TCC CCT CAC ATA GCT ATT CAC CC	CNAG_01580 3' Flank Forward Primer
MP0395	TCG ACG GTA TCG ATA GGT TTA TTT GAC GGA TTT CA	CNAG_01580 Forward Primer for Infusion Cloning into PSDMA25 at HINDIII
MP0396	AGG AAT TCG ATA TCA CAC CTT ATA TGC AGA ATC GA	CNAG_01580 Reverse Primer for Infusion Cloning into PSDMA25 at HINDIII
MP0379	TTTGCATTAGAACTAAAAAC AAAGCA	sgRNA Forward Primer; U6P-F
MP0380	TAAAACAAAAAAGCACCGA CTCGGTGCC	sgRNA Reverse Primer; sgRNA-R
MP0383	ATCTCTTTCACCACAGCACT, AACAGTATACCCTGCCGGTG	SH1 sgRNA: U6 promoter R
MP0384	AGTGCTGTGGTGAAAGAGAT, GTTTTAGAGCTAGAAATAGC AAGTT	SH1 sgRNA: sgRNA scaffold F
M13F	GTAAAACGACGGCCAG	Forward Primer for Cas9
M13R	CAGGAAACAGCTATGAC	Reverse Primer for Cas9

Note: The NEO gene was amplified from the plasmid pJAF1. sgRNA was amplified from the plasmid pBHM2329. Remaining polymerase chain reactions amplified from *Cryptococcus neoformans* wild-type strain KN99α. Created by Hannah Finson.

Transformation of the Gene Deletion Construct into *Cryptococcus neoformans*

The gene deletion construct was introduced into wild-type *Cryptococcus neoformans* via Biolistic Transformation as described previously (Toffaletti et al., 1993, Davidson et al., 2002). Transformants were kept on YPD + 1 M sorbitol plates for four hours before transferring to selection plates following transformation. The selection plates were YPD + 200 μg/mL G418. The individual colonies were plated and grew up on patch plates, then were passaged into YPD broth for stabilization. A second serial passage was done in YPD broth using 96-pin replication and incubated for a couple days.

Once stabilized, the transformants were plated on YPD plates and YPD pH 8 (YPD plus 75 μM HEPES pH 8 buffer, then brought to pH 8 with NaOH) plates. These plates were incubated for two days at 30℃. Meanwhile, the 96-well-plates were supplemented with 100 μL of 30% glycerol and were frozen at -80℃. The number of colonies unable to grow at pH 8 were recorded. Colonies that failed to grow at pH 8 were suspected transformants and underwent further testing to confirm successful transformation. Four of these mutants, along with wild-type and *sre1Δ* as controls, were then plated in five serial dilutions on YPD + 1.5 M NaCl plates and YPD plates and incubated for two days at 30℃.

Verification of the Mutant Strain, *scp1Δ*

Polymerase chain reaction was used to confirm *scp1Δ* mutants. An overnight culture was grown up, the cells were pelleted, resuspended in sterile water, and transferred to a microcentrifuge tube. Cells were pelleted, and resuspended in 0.5 mL phenol chloroform isoamyl alcohol, 0.5 mL TENTS buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8, 100 mM NaCl, 2% Triton X-100, 1% SDS), and 0.2 mL 0.45 mm glass beads. This was vortexed for ten minutes, then the aqueous phase was extracted and 50 μL of 3 M sodium acetate and 1 mL of

100% ethanol were added. This solution was centrifuged for at least twenty minutes at 4 ℃. The pellet was washed with ethanol and dried, then dissolved in 100 µL TE + 10 µg/mL RNase A and incubated for thirty minutes at room temperature.

Polymerase chain reaction was done using primers MP0336 and MP0324 (Table 1) with 5% of the reaction mix being DNA. Four mutants along with a wild-type control were used. The PCR protocol was as follows: 96℃ for four minutes, then repeated 35X: 96℃ for fifteen seconds, 51℃ for fifteen seconds, and 72℃ for seven minutes, followed by 72℃ for five minutes, then hold at 12℃. Gel electrophoresis with a 0.7% agarose gel was done on the product.

Testing of Virulence-Related Phenotypes

Melanin production was tested for using L-DOPA agar (2.0% bacto-agar, 0.1% L-asparagine, 0.1% glucose, 0.3% KH2PO4, 0.025% MgSO4-7H2O, 0.01% L-DOPA, pH 5.6, 0.0001% thiamine-HCl, $5x10^{-9}$% biotin). Four strains of *scp1Δ* were plated onto L-DOPA agar alongside wild-type *Cryptococcus neoformans* strain KN99α as a control. This was incubated at 30℃ overnight.

Cloning of *SCP1* into pSDMA25 for Future Reintroduction into the Genome

First, *SCP1* was amplified from wild-type *C. neoformans.* This was done using primers MP0395 and MP0396 (Table 1). The PCR protocol was 96℃ for four minutes, then repeated 35X: 96℃ for thirty seconds, 51℃ for thirty seconds, and 72℃ for seven minutes, followed by 72℃ for ten minutes, then hold at 12℃. The product was run on a 0.7% agarose gel for forty-five minutes at 100V and the band was excised, purified, and quantified.

The goal was to insert amplified *SCP1* into the plasmid pSDMA25, a gracious gift from the lab of James Fraser. DNA assembly was done using the Hifi DNA assembly protocol through

NEB. This was done alongside a control containing no amplified *SCP1*. The reactions were incubated for fifteen minutes at 50℃ before being placed in the -20℃ freezer for storage. Then the High Efficiency Transformation protocol was done (New England Biolabs protocol, C2987H/C2987I). The product was plated on LB/Carb (made from powder mix, + 75µg/mL carbenicillin) plates and incubated at 37℃ overnight. The given plasmid contains an ampicillin resistance gene which allowed for the use of selective media. pSDMA25 also contained a nourseothricin resistance gene (NAT) that will be used as a dominant selectable marker upon introduction into *scp1Δ.* The new plasmid with *SCP1* was named pHF1.

Verification of Successful Cloning

Colonies were grown up, put in an overnight culture, miniprepped, and quantified. In order to confirm cloning, restriction digest was performed using BamHI and XhoI separately. The protocol used was from New England BioLabs. The reactions were incubated along with uncut controls for one hour at 37℃. The products were run on a 0.7% gel for forty-five minutes at 100V.

Preparation for CRISPR-Cas9 Transformation and Reintroduction of *SCP1*

The pHF1 plasmid was linearized using the enzyme Asc1. The protocol for linearization was from New England BioLabs. The reaction was incubated at 37℃ for two hours. A control without restriction enzyme was incubated alongside the reaction as well. Gel electrophoresis was conducted on the product to confirm linearization using a 0.7% agarose gel, run at 100V for forty-five minutes. This band was excised and purified using the Zymoclean Gel DNA Recovery Kit through Zymoresearch Genesee Scientific (Cat no: #11-301) and its provided quick protocol.

To prep for CRISPR-Cas9, sgRNA was amplified from the plasmid pBHM2329, a gracious gift from Dr. Hiten Madhani. Two adjacent pieces of DNA were amplified using

primers MP0379 and MP0383, as well as MP0380 and MP0384 (Table 1). The Polymerase Chain Reaction protocol for both the U6 sgRNA and the Scaffold sgRNA was as follows: 96℃ for four minutes, then repeated 35X: 96℃ for fifteen seconds, 55℃ for fifteen seconds, and 72℃ for thirty seconds, followed by 72℃ for one minute, then hold at 12℃. The products were run on a 2.0% agarose gel at 100V for forty-five minutes. The bands for U6 sgRNA and Scaffold sgRNA were excised and purified using the same DNA Recovery Kit as aforementioned. Then polymerase chain reaction was used to ligate U6 sgRNA and Scaffold sgRNA. The molar ratio for each segment was 1:1, and the primers MP0379 and MP0380 were used (Table 1). The same PCR protocol as was used to amplify each piece was used for this PCR. The product was run on a 2.0% agarose gel at 100V for one hour, then purified and quantified.

Cas9 was amplified from the plasmid pBHM2403, a gracious gift from the lab of Dr. Hiten Madhani. Primers M13F and M13R were used (Table 1). The Polymerase Chain Reaction protocol was as follows: 98℃ for thirty seconds, then repeated 20X: 98℃ for ten seconds, 57℃ for fifteen seconds, and 72℃ for four minutes. Then, repeated twelve times: 98℃ for ten seconds, 49℃ for fifteen seconds, then 72℃ for four minutes. After this was 72℃ for seven minutes then a hold at 12℃. The product was run on a 0.7% agarose gel at 100V for one hour. The band was purified and quantified.

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