Targets of Filastatin, A Chemical Inhibitor of Adhesion and Morphogenesis by Pathogenic Fungi

A Major Qualifying Project

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By

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Abstract:

Candida albicans is one of the most common fungal pathogens associated with opportunistic and nosocomial infections. Infection is often initiated through formation of a biofilm, which is also drug resistant. A recently discovered small molecule called filastatin shows some promise as an inhibitor of biofilm formation and adhesion to polystyrene. A high-throughput screening assay was performed using a deletion mutant library in order to determine the functional pathway of filastatin. Results indicate that our drug may play a role in disrupting the iron metabolism pathway, particularly through genes regulated through Hap43 and Rim101.

Acknowledgements

We would like to thank Professor Robert Dempski and Professor Reeta Rao for hosting this project in her laboratory at Gateway Park and for her insight into the development and planning of experiments. We would also like to thank Luca Issi, Charu Jain, and Cat Harwood for their help with experiments and informative conversations.

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Introduction:

Mycosis caused by opportunistic fungi has been observed at increasing rates in recent years especially in immuno-compromised patients. Strains of the genus *Candida* account for 8 - 10% of all nosocomial blood stream infections acquired in the hospital setting (M. A. Pfaller, 2007). The number of reported cases and deaths caused by nosocomial bloodstream infections has steadily increased by over 5,000 between 1980 and 1997 (M. A. Pfaller, 2007). In the United States, it has been observed that mycoses caused by species from the genus *Candida* are the fourth most common nosocomial blood stream infection. It has been estimated that between 7,000 and 28,000 cases of invasive candidiasis occur annually and for 40% of these patients, the infection is deadly (M. A. Pfaller, 2007). Thus, it is of great importance to the scientific and medical community to better understand the virulence strategies for *Candida*, in order to mitigate or combat nosocomial infections.

The most prevalent fungal pathogen from the *Candida* genus is *Candida albicans* (Figure 1) (U.S. Centers for Disease Control and Prevention, 2013). *C. albicans* is a polymorphic, opportunistic fungal pathogen that can cause systemic infections with high mortality rates and can also cause mucosal conditions such as thrush in immunocompromised patients and vaginitis (Mayer, Wilson, & Hube, 2013). The fungus is present as a commensal in the human epithelial lining of the gastrointestinal tract, mouth, uro-genital tract without negatively affecting the host. However, when the patient's immune system is compromised such as in neonates or infection with HIV, the fungus causes infections on the superficial region of the

body, or in rarer cases, lead to life threatening systemic infections. (Mayer, Wilson, & Hube, 2013).

Figure 1: *C. albicans*, poses a significant public health threat due to drug-resistance (U.S. Centers for Disease Control and Prevention, 2013),

The fungus is dimorphic, and can switch growth between yeast or hyphal growth (Figure 1) depending on environmental conditions. The hyphae are a straight, parallel-walled filamenting form of *C. albicans* which is considered to be more invasive and is speculated to cause tissue penetration during infection. The yeast form of *C. albicans* grows at low pH and is characterized by oval shaped cells that typically bud off at hyphal nodes. These planktonic cells are thought to be important for establishing infection at secondary locations. This morphological plasticity allows *C. albicans* to survive in a variety of environments and conditions such as adverse pH within a phagocyte or serum in the blood stream (Sudbery, Gow, & Berman, 2004). This also allows *C. albicans* to be a versatile pathogen, and is important for

virulence. Therefore, mutants that are unable to switch between morphological states are avirulent.

Another powerful virulence strategies and reason that *C. albicans* infections are so prevalent in the medical setting is the formation of biofilms (Heitman, 2012). Biofilms are the formation of yeast/hyphal cell matrix that gives the grouping a heightened form of protection from antimicrobial agents and host immune factors. To initiate the formation of a biofilm, first cells must adhere to the surface. Then proliferation of yeast cells occurs along with hyphae, which eventually escalates into an extracellular matrix (Heitman, 2012). These factors tend to form on a variety of different biotic or abiotic settings alike. In the medical setting this typically refers to objects such as catheters and other medical equipment. Since the fungus present in biofilms exhibits more resilience to antimicrobials and sterilization, the possibility of infecting patients is high in the medical setting. In addition, these biofilms exhibit resistance to antifungal drugs (Zhihong Xie, 2012), making them harder to treat than other types of fungal infections in patients. The biofilm extracellular matrix is also been linked to the resistance to neutrophils by preventing activation of reactive oxygen species (ROS) in the host (Zhihong Xie, 2012). The release of ROS is necessary for the destruction of pathogens by phagocytes and recruiting of more phagocytes to the site of infection. This study also speculates that β- glucans, an essential component of the extracellular matrix protects *C. albicans* from degradation by the immune system (Zhihong Xie, 2012). Therefore, biofilms are a strong virulence factor because they promote attachment to biotic and abiotic surfaces and render the microbial community drug resistant.

Many studies have been done to understand how to prevent and neutralize opportunistic *Candida albicans* infections. One issue that is prevalent when developing antifungal agents is that because of its eukaryotic characteristics, it is hard to find specific targets that do not exhibit a homolog with humans. The main target that most antifungal drugs utilize today targets the cell membrane (Azole class of drugs and amphotericin B) or cell wall (caspofungin) of the fungus. The two main targets are ergosterol or β-D-glucan, which are both key components for *C. albicans*. Ergosterol is targeted because it is considered a main component of fungal membranes, although its similarities to cholesterol make some antifungals toxic at high concentrations. The advantage to β-D-glucan is that it is essential to the cell wall of *C. albicans* but is not present in humans, lowering the risk of toxicity (Cowen & Steinbach, 2008). It has been the human immune system recognizes β-glucan via Dectin-1 receptors on the surface of macrophages (Brown, et al., 2002). Though β-glucan is usually expressed in high quantities in *Candida,* it is hidden by a layer mannoprotein that shields the molecule from Dectin-1 receptors. It has been shown that subinhibitory concentrations of capsofungin, a common anti-fungal drug, are capable of exposing *Candida's* β-glucan layer. This is turn is capable of producing a stronger immune response so it is possible that this method may be considered as a novel treatment for *C. albicans* infections (Wheeler & Fink, 2006).

Potential Vaccine Strategies

Researchers have also begun targeting the adhesion system of *C. albicans* in hopes of preventing many nosocomial *C. albicans* infections. The adhesion system of *C. albicans* is based on two different tactics known as induced endocytosis and active penetration. Induced endocytosis operates by the expression of proteins on the surface of the *C. albicans* that cause

the host's ligands to engulf the fungal cell, thereby bringing it into the host cell. It has been demonstrated that even dead hyphae cells are engulfed, indicating that induced endocytosis of fungus does not need viable cells, but is linked to proteins created before death (Dalle, et al., 2010). It is speculated that induced endocytosis is mediated by Als3 and Ssa1. The other form of adhesion, active penetration, is not a fully understood mechanism. However, it is postulated to operate by physical force of the hyphae in order to digest part of the hosts' cellular membrane and use the hole created as an entry way into the system (Mayer, Wilson, & Hube, 2013). It has been shown that vaccination with the recombinant N-terminus of the Als3 and related Als1 proteins protects mice against oropharyngeal, vaginal and disseminated candidiasis (Spellberg, et al., 2006). These vaccines also appear to induce both broad antibody and cell-mediated immune responses and thereby appear to provide adequate protection against *Candida* infections. Therefore, targeting adhesion proteins of *Candida* may be a viable target for vaccines and therapeutics (Spellberg, et al., 2006).

The value of a prophylactic drug in preventing nosocomial infections has already been seen in earlier studies that have examined whether antifungal treatment reduces the risk of *Candida* infection in patients. In patients suffering recurrent gastrointestinal leakages, for example, only 8% of patients treated with fluconazole developed a *Candida* infection while as many as 31% of placebo-treated patients became infected. In addition, a study on patients intentionally colonized with *Candida* showed that infection only persisted in approximately 30% of fluconazole-treated patients while 70% of placebo treated patients developed infections. Lastly, it was shown that *Candida* colonization rates in liver transplant patients receiving prophylactic fluconazole decreased from 70% to 28% in comparison to those that received a

placebo. Most importantly, it was observed that mortality rate greatly decreases from 13% to 2% with prophylactic treatment (Snydman, 2003). Therefore, this study suggests that antifungal drugs have the capability to prevent *Candida* infections when administered prophylactically.

Drug Resistance

Though the use of antifungal agents as prophylactics has been shown to be effective, there is concern among some researchers that the overuse of azole drugs may lead to an increase in prevalence of other *Candida* species. For example, the use of fluconazole as a prophylactic has been correlated with a decrease in the incidence of *C. albicans* and *C. tropicalis*. However, this decrease coincided with an increase in prevalence of *C. glabrata and C. krusei,* which are more difficult to treat. Therefore, it is feared that the continued use of fluconazole as a prophylactic will lead to more-resistant and pathogenic strains becoming more prevalent. To prevent this, it has become necessary for health care workers to administer antifungals only to patients that stand to benefit the most from prophylactic treatment. Regardless, there is a growing need for the development of new antifungal agents in order to stem the proliferation of azole resistant fungi (Snydman, 2003).

Functional Drug Screen to Identify Small Molecules that Prevent Attachment to Plastics

Previous research has been done on the inhibition of *C. albicans* binding to plastics, which is a major cause of biofilm formation in health care settings. *C. albicans* has been shown to bind very strongly to polystyrene plastics (Mitchell & Nobile, 2006). Our laboratory recently reported identification of filastatin, which inhibits adhesion of *C. albicans* to polystyrene surfaces, surgical silicone mesh, and also to cultured human epithelial cells (Fazly, et al., 2013). As can be seen in Figure 2 below, *C. albicans* is capable of binding to silicone elastomers in the

presence of DMSO. However, in the presence of filastatin, *C. albicans* loses its ability to bind to the mesh. The strain *edt1* was used as a positive control in this experiment due to the fact that it lacks an adhesion protein needed for the fungi to bind to surfaces.

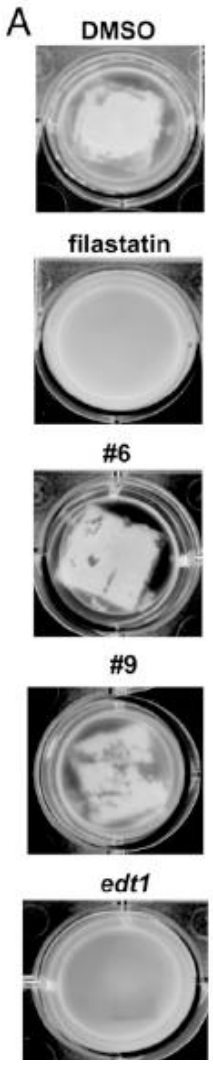

Figure 2: Adhesion of *C. albicans* to Silicone Elastomers (Fazly, et al., 2013)

Filastatin has the potential to be widely used as a prophylactic coating material for medical devices because abiotic surfaces coated with filastatin are resistant to colonization by fungal biofilm (Fazly, et al., 2013).

Filastatin was identified from a library of 30,000 small molecules for inhibition of adhesion of *C. albicans* (strain SC5314) to polystyrene. The strain *edt1*$^{-/-}$, which lacks a cell wall adhesion protein served as a positive control while SC5314 cells in the presence of DMSO alone served as a negative control. The research group then tested the ability of the filastatin to inhibit the binding of a GFP-expressing *Candida* strain to monolayers of human epithelial A549 cells. Of all of the compounds tested, it was found that filastatin was the best inhibitor of adhesion to human epithelia based on its ability to inhibit the filamentation of *Candida albicans*. Additionally, it was found that filastatin was not significantly toxic to human cells, even at concentrations as high as 250 µM. Therefore, this compound shows promise as a drug or prophylactic to fight *Candida* infections due to its ability to inhibit the fungi's adhesion to both polystyrene and human cells. The chemical structure of filastatin can be seen below in Figure 3 (Fazly, et al., 2013).

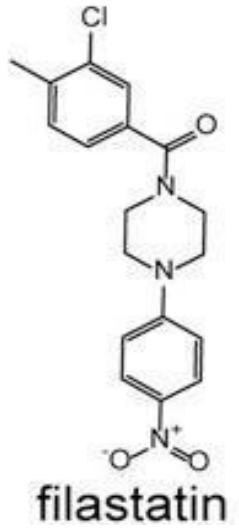

Figure 3: Chemical Structure of Filastatin (Fazly, et al., 2013).

Because of these potential clinical applications, the effect that filastatin had on *Candida* that was already bound to polystyrene was tested. It was found that filastatin did, in fact, reduce the number of *Candida* cells bound to polystyrene; however, it was more effective in this capacity when the drug and fungi were added at the same time and coincubated. Regardless of when the drug is added, these results indicated that filastatin could be effective against biofilms. Furthermore, additional experiments showed that filastatin was effective at inhibiting adhesion of three other pathogenic *Candida* strains: *C. dubliniensis C. tropicalis,* and *C. parapsilosis* (Fazly, et al., 2013).

The drug's potential use as a prophylactic against the formation of *Candida* biofilms on implanted medical devices was then tested by measuring colony formation on surgical silicone elastomers. It was observed that wild type *Candida* failed to form biofilms and instead remained dispersed in media in the presence of filastatin in both carbon-deficient Spider media and mammalian serum at low concentrations. These facts could have major clinical implications; fungal biofilms on medical devices lead to thousands of systemic and potentially life-threatening fungal infections per year. Therefore, a prophylactic drug that prevents biofilm and hyphal formation is capable of greatly reducing nosocomial *Candida* infections (Fazly, et al., 2013).

Lastly, this paper demonstrated that our drug of interest is capable of inhibiting fungal growth and infection in a variety of systems *in vivo.* When treated with filastatin, it was found that the number of *C. elegans* nematodes exhibiting the DAR phenotype caused by *C. albicans* infection decreased by approximately thirty percent. Furthermore, filastatin treatment was found to extend the life-span of *C. elegans* that had been previously infected. In addition, the

researchers found that individual yeast cells predominated in vulvovaginal candidiasis in mice that had been treated with filastatin. Therefore, this suggests that filastatin is capable of preventing hyphae and biofilm formation *ex vivo*. This finding is significant because of the number of women infected with vulvovaginal infections; 75% of women will suffer from vulvovaginal candidiasis at some point in their lifetime, with as many as 95% of these infections being caused by *C. albicans* in particular (Fazly, et al., 2013).

The focus of our project is to identify the biological target of filastatin in *C. albicans*, which would allow for a better understanding of its mechanism. We will use loss-of-function reverse genetics approaches to identify potential targets of filastatin by screening a *C. albicans* homozygous deletion library using the adhesion assay described previously (Noble, et al., 2010). In short, loss of target gene function is expected to mimic filastatin treatment and prevent attachment of *C. albicans* to plastic surfaces as shown in **Error! Reference source not found.** below. Therefore, mutants that lack the drug target will not show an altered phenotype in when treated with filastatin (**Error! Reference source not found.** green). Additionally, it was hypothesized that filastatin might have multiple targets (Fazly, et al., 2013). Such genes are also likely to be identified in this screen as mutants that show a synergistic effect upon treatment with filastatin. The identity of the target gene will be confirmed by complementing the deletion mutant by replacing a single copy of the gene at a chromosomal location. The complemented heterozygous strain is predicted to restore the ability of the mutant to adhere to plastic.

The library will be tested on polystyrene plates through a crystal violet assay, to determine susceptibility to filastatin and how adhesion is affected by different deletion

mutations. As shown in **Error! Reference source not found.**, two different types of phenotype s would be the most ideal to discover.

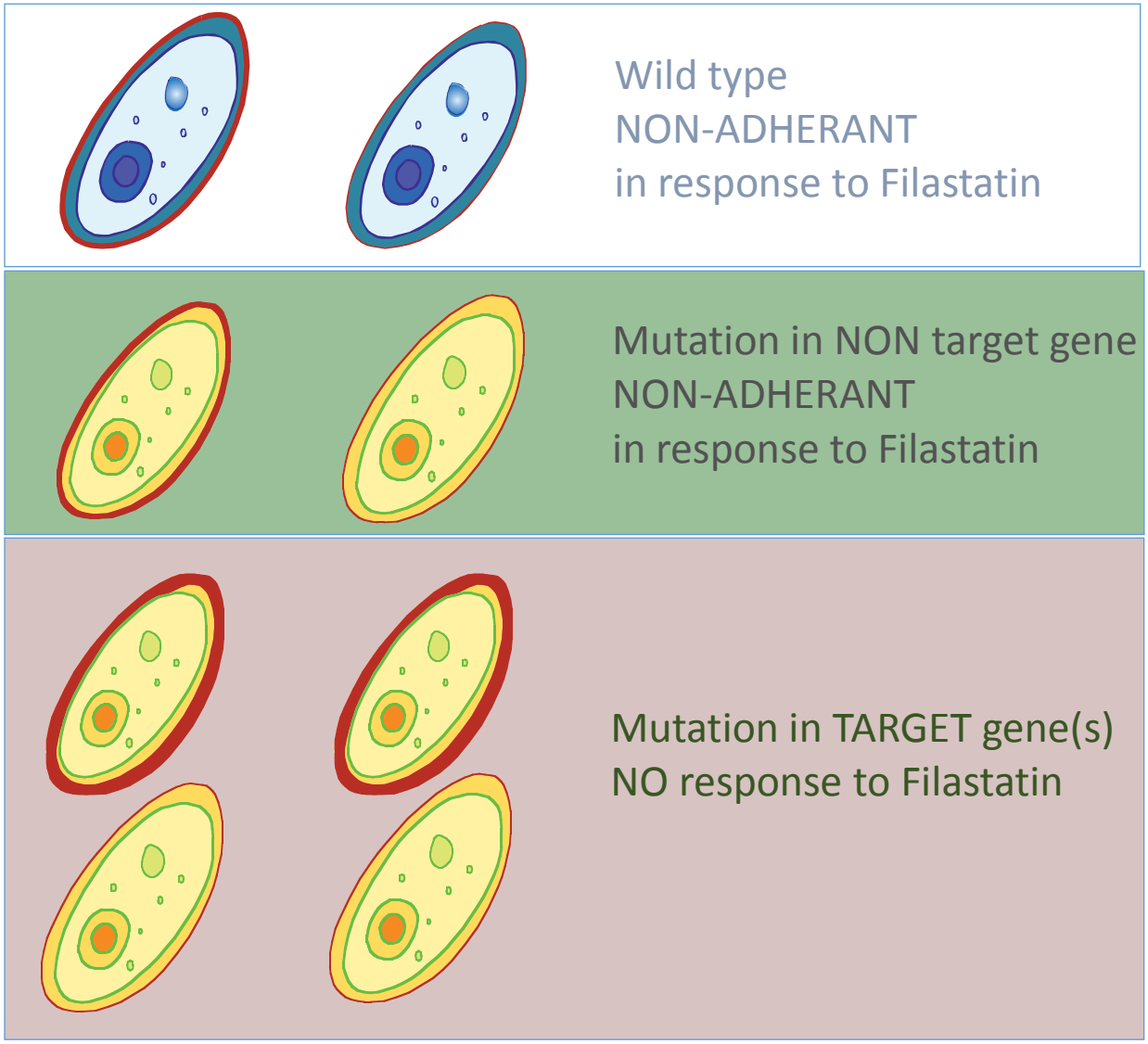

Figure 4: Expected Adhesion Patterns of Mutants to Filastatin

Before mutants showing significance are further studied, the phenotype being observed will be shown to be a result of the intended deletion. To do this, reverse complementation will take place by replacing a single copy of the deletion gene back into the chromosomal location. The

complemented strain should rescue the mutant phenotype and phenocopy the wild type by restoring adhesion to plastic surfaces. This will allow for the mutant to be used for testing with model *in vitro/ in vivo* assays such as *C. elegans* or mitochondria. The end conclusion will help give a greater understanding of how filastatin affects *C. albicans* and give a greater understanding of the underlying mechanisms that help prevent adhesion.

Materials and Methods

Primary Screening

For the primary screen, single colonies were picked and inoculated into 5 mL cultures of SC+ 0.15% glucose media and grown overnight in a rotating wheel. The OD of the cultures was then measured at OD600nm. Cultures were spun down and resuspended in fresh SC +0.15% glucose to a final concentration of 0.5 OD/mL. 200 uL of 0.5 OD/mL *C. albicans* strains were pipetted onto 96 well flat-bottom polystyrene plates. Filastatin or DMSO was added to cultures at appropriate concentrations, never exceeding 1% DMSO. Plates were covered with aluminum foil and incubated at 37 degrees for 4 hours. After 4 hours of incubation, plates were decanted and 40 uL of 0.5 % Crystal Violet Dye was added to each well. The plates were covered and left at room temperature to incubate for 45 minutes. Plates were then decanted and washed by submersion 10 times in an ice bucket filled with dH20, changing the water every 5 washes. Plates were tapped onto a paper towel to remove residual dH20 and 200 uL of 75% MeOH was added to each plate and incubated at room temperature for 30 minutes. The plates were then read at 590 nM to detect relative crystal violet fluorescence.

Data analysis

The primary screening was analyzed using Microsoft Excel. All data was normalized to the wild type (SN250). If a mutant exploited RFU that was two times the standard deviation either higher or lower than that of the wild type and then did not show a change in adhesion with the addition of filastatin, it was considered a hit.

PCR Design

PCR primers were designed for Hap43 and Rim101, using the IDT primer design application. In order to confirm full integration of gene, amplicons were designed to have 100-200 bp overhangs on both sides of the gene. The amplicon was also designed to complement 20 bp of PS105 plasmid (Hap43: F= 5'-GTAAATTACAATTGGTATTTTCAACCAATATT ACCACACCAACTAGCCACGTCGTTCC-3' R= 5'-CTATCTCTCTTTTTTTTGCCCATTTGAGTTAG TGCATGACGATGCAGATGACGCGGATTC-3', Rim101: F= 5' - GTAAATTACAATTGGTAT TTTCAACCAATATTACCACACTGGCAATCAATTCCCAGACA03; R=5' ATCTCTCTTTTTTTTGCCCATTTGA GTTAGTGCATGACAGAGACCGGTAACACTACTTTC-3'). Long amp polymerase was used for all PCR reactions. For confirmation of insertion back into *C. albicans*, primers were designed to check for the insertion into the genome. Primers were designed to aplify a small region of the 5' region of the mutant of interest and also amplify the arginene resistant gene (Hap43: F= 5' - CTACACCAAAGTGATCCCACA-3' R= 5' - CATCACTTGACGTGGCATTATC-3', Rim101: F= 5' -ATTACC ACACTGGCAATCAATTC-3', R= 5' CTCTCACTTGCACCGGTATT-3').

Complementation analysis

One colony of *S. cerevisiae* was picked and inoculated into 3 mL of YPD media and incubated at 30 C overnight in a tube roller. The next morning, 1.5 mL of cultures were used to inoculate 50 mL of fresh YPD media and grown in a shaker at 30 C until OD600 1-1.5 was met. The culture was then centrifuged at 3000 rpm for 5 minutes at room temperature and washed with 5 mL LiOAC mix (1x TE ph 8, 100 mM lithium acetate). This mixture was then centrifuged at 3000 rpm for 5 minutes and the pellet was resuspended in 1 mL LiOAC. 100 uL of washed cells was then mixed with 10 uL single strand DNA carrier and 5 uL of the PCR product being used for

gap repair. Then 500 uL of the PEG Mix (1x TE ph 8, 100 mM lithium acetate, 40 % PEG 3350) was added into the solution. The mixture was mixed well by pipetting and incubated for 30 minutes at room temperature. The mixture is then heat shocked at 42C for 5 minutes and centrifuged at approximately 3600 rpm for 3 minutes. The PEG mixture was decanted and the cells were washed with 0.5 mL YPD. The pellet was then resuspended in 100 uL of ddH20 and plated on selective media. The mixture was grown for 48 hours at 30 C. To confirm integration of gene of interest into plasmid, conformational PCR was run after plasmid purification.

High Efficiency Electrotransformation of E. coli

The competent *E. coli* cells were thawed on ice and then 40 uL of the cell suspension and 5 uL of purified plasmid from previously described gap repair transformation were placed into a pre-chilled 1.5 mL microfuge tube, gently mixing with pipette tip (do not mix by pipetting up and down). The mixture was allowed to incubate on ice for approximately one minute. The cells were transferred to a cold electroportion cuvette and the MicroPulser was set to "Ec2". The cuvette was then placed into the chamber slide, were it is seated between the contacts in the base of the chamber. One pulse was administered and then 1 mL of SOC medium was immediately added to the cuvette and gently resuspended with a Pasteur pipette. The cells were then transferred to a microfuge tube and incubated at 37 C for 1.5 hours shaking at 225 rpm. Then 100 uL of cell suspension was plated onto LB+AMP plates and incubated overnight at 37C. The plasmid was purified using the qiagen mini-prep plasmid purification strategy and stored for later use at -20C.

Transformation of C. albicans

One colony of the knockout *C. albicans* strain of interest was grown in 5 mL of YPD overnight at 30 C in a rotating wheel. The following morning 1.5 mL of the overnight culture was inoculated into 50 mL of fresh YPD media and incubated in a 30 C shaker for 5-6 hours until the OD_{600} reaches between 0.5 -1. The culture was then centrifuged for 2 min at 1000g and the supernatant was discarded. The cells were then resuspended at 900 uL LiOAC/TE and transferred to a microcentrifuge tube. The mixture was then pelleted for 1 minute at 1000g, followed by two more washes with 900 uL LiOAC/TE, to be finally resuspended in 400 uL LiOAC/TE. In a separate microfuge tube, the following reagents were mixed. First 10 uL of 10mg/mL Herring sperm (or Salmon sperm) DNA was administered after being denatured by boiling for 2 minutes and then snap cooled on ice. Then 1 ug (20-50 uL of digested plasmid) to be transformed into *C. albicans* genome was added into the solution. Followed by 200 uL washed cells in LiOAC/TE and 1 mL PEG mix. This mixture was incubated overnight at room temperature while inverting.. The mixture was then heat shocked at 42C for 1 hr the following morning and pelleted for 1 minute at 1000g. The supernatant was discarded and the pellet was washed with 1 mL sterile water. The pellet was resuspended in 150 uL ddH20 and plated on YNB-ARG media. The plates were allowed to incubate at 30 C for 2-3 days to allow for the growth of colonies. Genomic integration was confirmed by running conformational PCR analysis and confirmation of prospective phenotype.

Colony morphology Assay

Mutants of interest and wild type (*C. albicans* SN250) were grown overnight in SC+ AA+ 0.15% glucose. Culture concentrations were measured at OD600 nm. The cultures were then spun down at 3000 rpm and resuspended in ddH20 to give a final concentration of 1 OD. A 96

well plate was used to make a series of 10-fold serial dilutions using ddH20. Then 5 uL of the dilutions were incubated at 37 C on solid Spider media for six days.

Sensitivity Assay

Mutants of interest and wild type (*C. albicans* SN250) were grown overnight at 30 C in SC+AA+ 0.15% glucose. Cultures were spun down at 3000 rpm and resupsended in ddH20 to give a final concetration of 1 OD. A 96 well plate was then used to make a series of 10-fold serial dilutions using ddH20. Then 5 uL of the dilutions were incubated at 30 C on SC+ 1 mM CuSO4 + AA+ glucose agar plates and incubated overnight at 30 C.

Results:

Primary Screening

In this MQP, a reverse genetic screen was performed using a knockout mutant library (Noble, et al., 2010). To gain a better understanding of how the anti-adhesion compound filastatin decreases the ability of the pathogen *Candida albicans* to adhere to plastic surfaces, mutants were allowed to form biofilms on a polystyrene surface of a 96 well plate. Biofilms were treated with filastatin or the carrier DMSO and stained with crystal violet to visualize adhesion.

Data Analysis

The mutants were analyzed with the use of Microsoft excel and normalized to the wild type, which was present on every plate. After normalization took place, the mutant was compared to the wild type and if it was greater or less than 2 times the standard deviation, it went onto the next round of analysis. If the change in adhesion was less than 10%, the mutant was added as a hit. An example of a non hit v. both versions of a hit (more adhesive and less adhesive) can be seen below in figure 5.

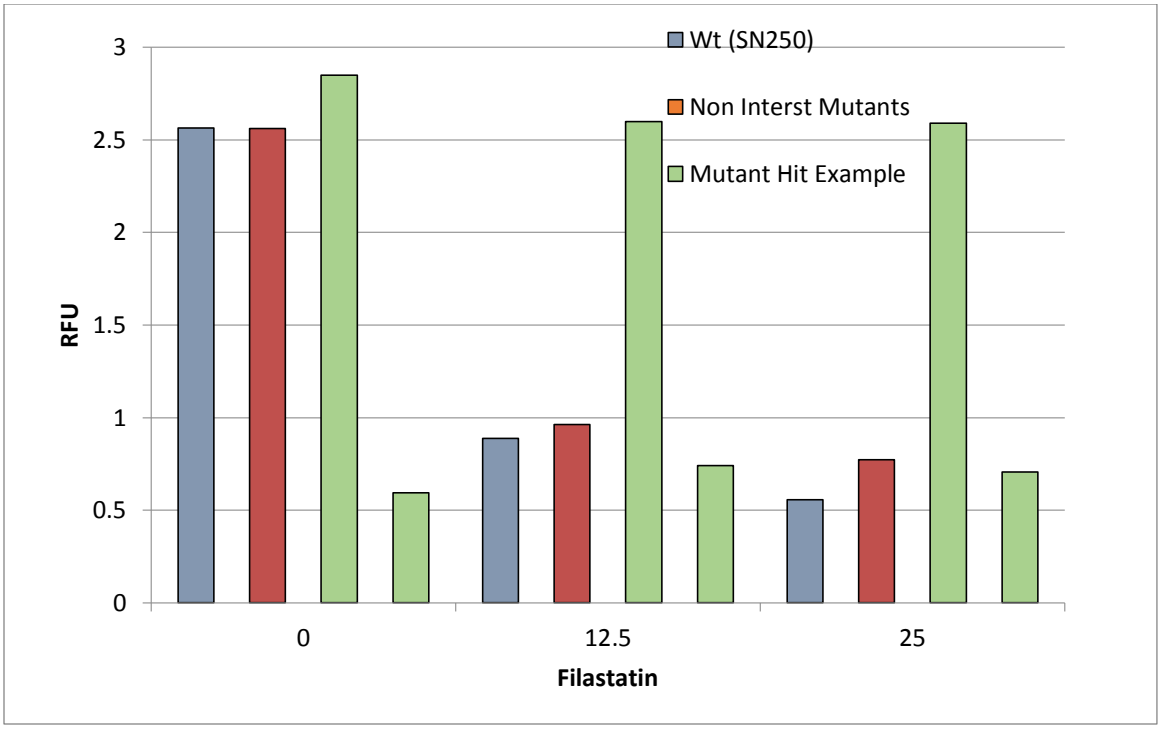

Figure 5: Example of hit for lower and higher adhesion in Crystal Violet assay

Out of the ~650 mutants surveyed, 42 of the mutants were identified as potential targets, or pathways that regulate targets of filastatin. This list of 42 hits constituted to be approximately 6% of all total mutants screened. Out of the forty two putative mutants, fifteen resulted from mutants that showed higher adherence to the polystyrene plates than the wild type and twenty seven of the hits resulted in mutants that showed a lower adherence than that of the wild type. These hits were organized according to their adherence patterns in **Error! Reference source not found.** below.

Table 1: List of Hits from Primary Screen with Descriptions, Organized According to Higher or Lower Adherence

Adheres More Than WT	
PRN4	protein with similarity to pirins, flow model biofilm repressed
CSA2	Extracellular-associated protein; repressed by Rim101 at pH 8; regulated by Tsa1, Tsa1B in minimal media at 37 deg; induced by ketoconazole, nitric oxide, Hap43; required for normal RPMI biofilm formation; Bcr1 induced in RPMI
ORF19.3919	RNI-like superfamily domain-containing protein; early-stage flow model biofilm induced; Spider biofilm induced
LIP2	Secreted lipase; member of a differentially expressed lipase gene family; expressed in alimentary tract, but not oral tissue, during mouse oral infection; may have a role in nutrition and/or in creating an acidic microenvironment
RBT5	GPI-linked cell wall protein; hemoglobin utilization; Rfg1, Rim101, Tbf1, Fe regulated; Sfu1, Hog1, Tup1, serum, alkaline pH, antifungal drugs, geldamycin repressed; Hap43 induced; required for RPMI biofilms; Spider biofilm induced
HAK1	Putative potassium transporter; similar to Schwanniomyces occidentalis Hak1p; amphotericin B induced; induced upon phagocytosis by macrophage; Hap43-repressed; rat catheter biofilm repressed
HET1	Putative sphingolipid transfer protein; involved in localization of glucosylceramide which is important for virulence; Spider biofilm repressed
ORF19.6637	Predicted glycosyl hydrolase; hypoxia induced; flow model biofilm induced
HEX1	Beta-N-acetylhexosaminidase/chitobiase, highly glycosylated enzyme that is secreted to the periplasm and culture medium; required for full virulence; may have role in carbon or nitrogen scavenging; possibly an essential gene (UAU1 method)
NPR2	Putative urea transporter; induced during infection of murine kidney, compared to growth in vitro; has murine homolog
ORF19.587	Ortholog(s) have transcription export complex localization
ORF19.649	
CFL11	Protein similar to ferric reduc (Inglis DO, 2012)tase Fre10p; flucytosine repressed; possibly adherence-induced; possibly an essential gene, disruptants not obtained by UAU1 method; rat catheter biofilm repressed
ORF19.2726	Putative plasma membrane protein; Plc1-regulated; Spider biofilm induced
HYR1	GPI-anchored hyphal cell wall protein; macrophage-induced; repressed by neutrophils; resistance to killing by neutrophils, azoles; regulated by Rfg1, Efg1, Nrg1, Tup1, Cyr1, Bcr1, Hap43; Spider and flow model biofilm induced
Adheres Less than WT	
ORF19.380	Protein of unknown function; induced by alpha pheromone in SpiderM medium
BTA1	Betaine lipid synthase
ORF19.1365	Putative monooxygenase; mutation confers hypersensitivity to toxic ergosterol analog; constitutive expression independent of MTL or white-opaque status
HWP2	GPI-anchored, glycosylated cell wall protein; required for biofilm formation, adhesion, filamentous growth on some media; expressed in hyphae; mutant delayed in virulence; regulated by Efg1, Tup1; similar to Hwp1 and Rbt1 domains
MTS1	Sphingolipid C9-methyltransferase; catalyzes methylation of the 9th carbon in the

	long chain base component of glucosylceramides; glucosylceramide biosynthesis is important for virulence; Spider biofilm repressed
FLO9	Putative adhesin-like cell wall mannoprotein; repressed during the mating process; mutation confers hypersensitivity to toxic ergosterol analog; decreased transcription is observed upon fluphenazine treatment
ORF19.5449	Predicted integral membrane protein; Spider biofilm induced
PGA10	GPI anchored membrane protein; utilization of hemin and hemoglobin for Fe in host; Rim101 at ph8/hypoxia/ketoconazole/ciclopirox/hypha-induced; required for RPMI biofilm formation, Bcr1-induced in a/a biofilm; rat catheter biofilm repressed
KRE5	UDP-glucose:glycoprotein glucosyltransferase; 1,6-beta-D-glucan biosynthesis, hyphal growth, virulence in mouse IV model; partially complements S. cerevisiae kre5 mutant defects; flow biofilm repressed, Bcr1-repressed in RPMI a/a biofilms
ORF19.2484	Has domain(s) with predicted peptidase activity and role in proteolysis
GZF3	GATA-type transcription factor; oxidative stress-induced via Cap1; mutant has abnormal colony morphology and altered sensitivity to fluconazole, LiCl, and copper; Spider biofilm induced
ORF19.3108	Putative DNA repair methyltransferase; induced by nitric oxide independent of Yhb1; Spider biofilm induced
ORF19.3226	Ortholog(s) have role in intracellular sterol transport and extracellular region, fungal-type vacuole lumen localization
HYR4	Putative GPI-anchored adhesin-like protein; Rim101-repressed; constitutive expression independent of MTL or white-opaque status
ORF19.3404	Protein of unknown function; transcription repressed by fluphenazine treatment
ORF19.3763	Has domain(s) with predicted serine-type endopeptidase activity and integral to membrane localization
GYP1	Putative Cis-golgi GTPase-activating protein; transcript regulated by Nrg1, Mig1, and Tup1
KIS1	Snf1p complex scaffold protein; similar to S. cerevisiae Gal83p and Sip2p with regions of similarity to Sip1p (ASC and KIS domain); interacts with Snf4p; mutants are hypersensitive to caspofungin and hydrogen peroxide; Hap43p-repressed gene
ORF19.4195	Ortholog of *C. dubliniensis CD36* : Cd36_60540, *C. parapsilosis CDC317* : CPAR2_602830, *Candida tenuis NRRL Y-1498* : CANTEDRAFT_108530 and *Debaryomyces hansenii CBS767* : DEHA2F15532g
CAS5	Transcription factor; cell wall damage response; required for adherence, response/resistance to caspofungin; repressed in core stress response; mutants have reduced CFU in mice, hyphal defect in C. elegans infection; Spider biofilm induced
ORF19.4805	Putative membrane protein; induced by alpha pheromone in SpiderM medium; Hap43-induced gene; Spider biofilm induced
IFF11	
ORF19.5406	
PTC7	Protein phosphatase, type 2C; has S/T phosphatase activity, Mn2+/Mg2+ dependent; predicted membrane-spanning segment and mitochondrion-targeting signal
MBP1	Putative component of the MBF transcription complex involved in G1/S cell-cycle progression; non-periodic mRNA expression; predicted, conserved MBF binding sites upstream of G1/S-regulated genes

ORF19.6318	Ortholog(s) have endoplasmic reticulum, mitochondrion localization
ORF19.6449	
ORF19.6654	Predicted membrane transporter, member of the L-amino acid transporter-3 (LAT3) family, major facilitator superfamily (MFS)

Analysis of Pathways that Regulate Filastatin Action

To identify genes and cellular components that regulate biological function of filastatin Gene Ontology (GO) analysis was performed (Inglis et al, 2012).This analysis showed that several genes were related in regards to the biological functions (such as metabolism, biofilm formation, etc) that they regulate. 7 of the 42 mutant genes identified were defined by the category "*single species biofilm formation",* giving significance between 1E-4 and 0.5. 2 out of the 42 mutants fall under the category hemoglobin import, also showing the significance of 1.0E-4 to 0.5. The results of this analysis can be viewed in Table 2 below.

Table 2: CGD GO analysis results for Process Ontology (Inglis et al, 2012)

Terms from the Process Ontology					
Gene Ontology term	**Cluster frequency**	**Background frequency**	**Corrected P-value**	**False discovery rate**	**Genes annotated to the term**
Hemoglobin import	2 out of 42 genes, 4.7%	2 out of 6517 background genes, 0.0%	0.00769	4.00%	PGA10, RBT5
Single-species biofilm formation	6 out of 42 genes, 14.0%	132 out of 6517 background genes, 2.0%	0.03674	6.00%	CAS5, CSA2, HWP2, HYR1, PGA10, RBT5
Biofilm formation	6 out of 42 genes, 14.0%	141 out of 6517 background genes, 2.2%	0.05262	5.33%	CAS5, CSA2, HWP2, HYR1, PGA10, RBT5

GO analysis was also used to compare the cellular components of the genes of interest. Out of the 42 genes that were run through the Go analysis, six of the genes fell under external encapsulating structures with a significance of 1.0E-4 to 0.5. Another cellular component that showed a similar level of significance were genes that fell into the extracellular region, which encompassed seven of the forty two genes. The results of this analysis are shown in Table 3 below.

Table 3: CGD GO analysis results for Component Ontology (Inglis et al, 2012)

Terms from the Component Ontology					
Gene Ontology term	**Cluster frequency**	**Background frequency**	**Corrected P-value**	**False discovery rate**	**Genes annotated to the term**
Extracellular region	7 out of 42 genes, 16.3%	133 out of 6517 background genes, 2.0%	0.00105	2.00%	CSA2, HEX1, HYR1, IFF11, LIP2, RBT5, orf19.3226
Cell wall	6 out of 42 genes, 14.0%	142 out of 6517 background genes, 2.2%	0.01450	14.00%	HWP2, HYR1, HYR4, IFF11, PGA10, RBT5
Fungal-type cell wall	6 out of 42 genes, 14.0%	142 out of 6517 background genes, 2.2%	0.01450	9.33%	HWP2, HYR1, HYR4, IFF11, PGA10, RBT5
External encapsulating structure	6 out of 42 genes, 14.0%	144 out of 6517 background genes, 2.2%	0.01563	7.00%	HWP2, HYR1, HYR4, IFF11, PGA10, RBT5

Of the forty two hits that were found, 24% or 10 mutants were regulated by the transcription factor Hap43 and 12% were regulated by Rim101. There is an overlap of 7% between these genes and which are thought to be regulated by both Hap43 and Rim101. These genes are organized graphically in the Venn diagram in Figure 6 below.

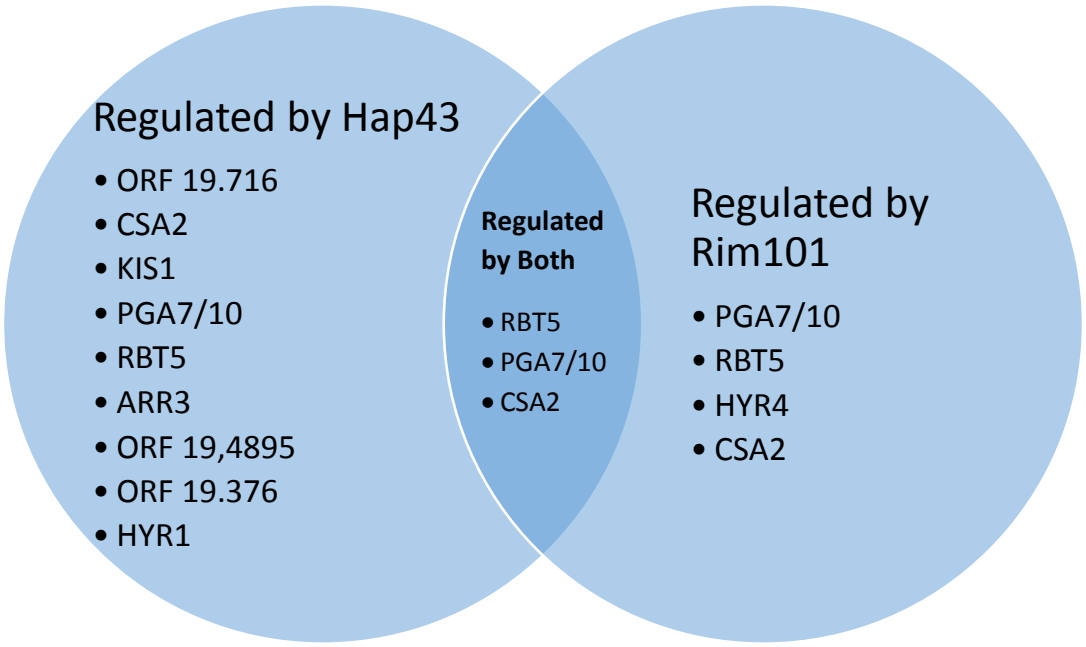

Figure 6: Genes Regulated by Hap43 and/or Rim101

Validation of mutants identified

Since a high number of hits were associated with both Hap43, a CCAAT-binding factor-dependent transcription factor that is required for survival for low iron response, and Rim101, a transcription factor needed for alkaline-induced hyphal growth (Inglis et al, 2012), these two transcription factors were chosen for further analysis. The knock out mutants of Hap43 and Rim 101 were selected for complementation to confirm that they play a role in the biological function of filastatin. The Rim101 knock out mutant (taken from the Noble Library) does not show the presence of filamentation when put through the Hyphal growth profiling experiments with the use of spider inducing media, when compared to the wild type SN250.

The transcription factor knockout Hap43 has previously shown to have higher copper metal resistance than that of the wild type (Homann et al, 2009) and was chosen to test if the

complementation was successful. The Rim101 and Hap43 genes were then complemented back into their respective knockout mutants using the previously described method. Rim101 complementation was checked using PCR and phenotypic analysis.

Discussion:

Our results have identified two pathways that may regulate filastatin activity. In our assay, we considered mutants that showed little to no decrease in adhesion in the presence of filastatin relative to the negative control to be 'hits' as described in **Error! Reference source not found.** above. The rationale was that if the mutant lacks a target of filastatin, then adding filastatin will have no effect on how well the fungi can adhere to the plastic. Therefore, the differences in absorbance between wells that have filastatin and those that do not will be statistically insignificant. If these values were within 10% of each other during the primary screen, we selected that strain for retesting in our secondary screen. Conversely, 'non-hits' were strains which showed significantly lower binding in the presence of filastatin in comparison to the negative control. In theory, these strains have deletions in genes that are unrelated to the targets of filastatin; as a result, filastatin is still capable of being effective and reducing adhesion and biofilm formation. Our screening produced 42 potential genes of interest, of which we focus on three: Rim101, Hap43 and PGA10.

One gene that we believe may be a target of filastatin is Rim101 or one of the several pathways which it regulates. Rim101 has been previously shown to regulate gene expression responses to extracellular changes in pH. Specifically, Rim101 has been shown to induce hyphal formation in response to neutral or alkaline pH conditions. In addition, this gene is known to be a member of the Rim101/PacC family of C2H2 zinc finger transcription factors, which play a number of different roles in regulating the virulence of different fungal pathogens (Penalva and Arst, 2004). A number of studies have been performed showing the role of Rim101 in hyphal formation and the fungal virulence. One study found that Rim101 mutants formed shorter and

fewer true hyphae but more pseudohyphae in comparison to the wild type. The same study also looked at the ability of a Rim101 mutant to invade oropharyngeal epithelial cells. It has been previously known that *Candida albicans* invades this particular type of epithelial cell by damaging them and inducing their own endocytosis. However, mutants lacking Rim101 have a significantly decreased capability of being endocytosed by epithelial cells, which suggests that Rim101 plays an important role in initiating infections of the epithelia. Lastly, mice infected with Rim101 mutants had significantly lower fungal burden levels than mice infected with the wild type or with strain in which the Rim101 gene was complemented. Therefore, these studies show that this gene plays an important role in the initiation of infection by *C. albicans* in epithelial cells. One current hypothesis regarding the exact role of Rim101 in virulence is that it is responsible for activating and regulating hyphae-specific genes. In fact, it has been found that many of the most Rim101-dependent genes, such as SOD5, CSA2, PGA7/RBT6, ECE1, CSA1, SAP5, HYR1 and RBT5 are specific to hyphae formation. Our drug filastatin has been previously shown to inhibit hyphal formation in *C. albicans* in both biotic and abiotic environments. Therefore, one hypothesis explaining the effects of filastatin is that it could interact with Rim101 or one of the hyphal-specific genes that it regulates, thereby inhibiting the formation of hyphae. Furthermore, Rim101 is known to also regulate genes that play a role in biofilm adherence, such as Pga10 and Csa1. Therefore, it is also possible that filastatin may inhibit the formation of biofilms by disrupting one of these pathways as well. Currently, more tests are needed to determine whether or not filastatin has an effect on Rim101 or any of its pathways (Nobile, et al., 2008).

Of all of the genes of interest identified in the screen, a significant portion of them were found to be regulated by a transcription factor known as Hap43. Hap43 has previously been indicated in iron-metabolism due to the fact that mutants lacking Hap43 were unable to survive on iron-deficient media. However, Hap43 mutants displayed a normal ability to uptake iron in various assays, suggesting that Hap43 does not play a major role in iron acquisition but rather iron utilization. Quantitative gene analysis revealed that Hap43 is actually responsible for repressing genes that utilize iron under iron deprivation and upregulating iron acquisition genes. Most significantly, however, Hap43 mutants were shown to be considerably less virulent in comparison to the wild-type or heterozygotes in a mouse model of disseminated candidiasis. It has been long known that the human body (and mice) has extremely low concentrations of free-ionic iron in order to promote proper functioning of the immune system and to inhibit the growth of pathogens. Therefore, this suggests that the iron metabolism pathways regulated by Hap43 also play a critical role in fungal virulence. Because Hap43 has been shown to regulate many of our genes of interest, this seems to suggest that filastatin may play a role in disrupting the fungi's ability to survive and switch to its virulent form in low iron conditions (Hsu, et al., 2011).

By focusing on our hypothesis that filastatin affects adhesion and virulence via disruption of *C. albicans'* iron metabolism pathways, we identified PGA10 (which is also known as RBT51) as a particular gene of interest. During our screening process, we found that PGA10 was one of three genes that were regulated by Hap43 and Rim101 that was known to play a role in iron-acquisition and metabolism. In particular, PGA10 has been found to be especially important in hemin and hemoglobin-iron acquisition. *C. albicans* mutants containing a deletion

of PGA10 showed no growth on media in which hemoglobin was the only available iron source; however, when the PGA10 gene was complemented back into the mutant, the wild type phenotype was fully restored. In addition, heterozygotes for this gene demonstrated a reduced growth pattern on this type of media, suggesting a haploinsufficiency of strains possessing a single copy of the gene. PGA10 has also been shown to be induced in conditions of iron-starvation; considering that several of the genes we found in our screening played a role in iron acquisition, this finding further strengthens our hypothesis that filastatin disrupts one or more iron metabolism pathways in the cell. Lastly, PGA10 has been shown to be a glycoprotein that localizes at the cellular envelope due to the presence of a GPI consensus sequence at its C-termini and a predicted signal sequence at its N-termini (Weissman and Kornitzer, 2004). By utilizing this knowledge, we were able to suggest a potential hypothesis as to how filastatin works, which is summarized below in Figure 99. We suggest that filastatin and hemoglobin compete for binding sites on PGA10 at the surface of cells. In low-iron conditions, PGA10 is upregulated and expressed via Rim101 and Hap43 in order to acquire hemoglobin from its surroundings. Binding triggers the endocytosis of hemoglobin and PGA10 into the cell along with initiating signal transduction pathways (which are currently unknown to us) that induce hyphal growth and other virulence factors. In addition, several PGA10 receptors from different *C.albicans* cells can bind to a single molecule of hemoglobin. Therefore, a relatively large number of yeast cells are capable of binding to a small number of hemoglobin complexes. As a result, the cells begin to aggregate and form sheets as they begin to create an extracellular matrix. We speculate that filastatin disrupts the binding of PGA10 to hemoglobin. If this were the case, the presence of filastatin would prevent *C. albicans* from agglomerating into biofilms.

We hypothesize that filastatin disrupts the binding of hemoglobin to PGA10, thereby inhibiting iron-acquisition, biofilm formation and other virulence factors. However, additional evidence is required before this hypothesis can be supported. A number of different assays that can be performed in the future in order to better understand the mechanism of filastatin will be discussed in detail later in this report.

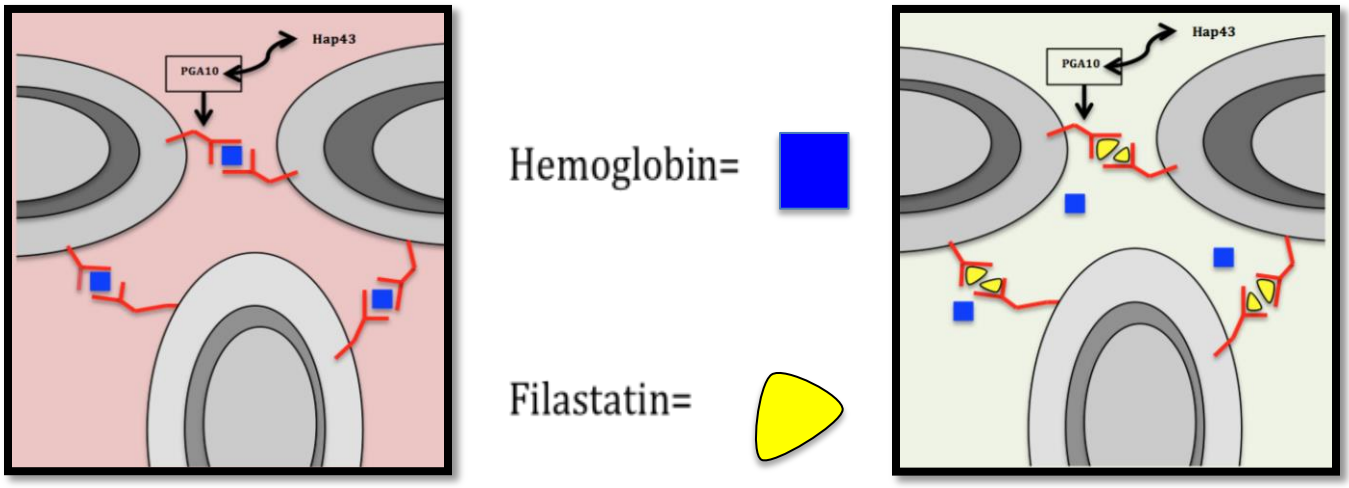

Figure 7: Example of a possible single target interaction with PGA10, which is regulated by Hap43

Though this proposed mechanism would explain how filastatin disrupts the formation of biofilms *in vivo*, it does not explain how filastatin can prevent and reverse biofilm on abiotic surfaces such as polystyrene. It is possible that this may not be the exact mechanism that filastatin utilizes or that filastatin may perform its function through a number of different pathways. Therefore, a number of different experiments should be performed to thoroughly elucidate the mechanism of action of filastatin in both biotic and abiotic environments. In addition, it may be necessary to perform a screen using another deletion library to study the effect of filastatin on non-transcription factor mutants.

There a number of experiments that should be performed in the future to better understand the mechanism of filastatin. To test our PGA10 hypothesis mentioned earlier, it may be beneficial to observe whether or not adding varying concentrations of hemoglobin in the presence of filastatin would be able to restore adhesion and biofilm formation capability to the wild type, SN250. Our hypothesis states that filastatin disrupts binding of PGA10 to hemoglobin, which in turn prevents cells from adhering to each other and forming biofilms. If this were true, adding hemoglobin should, in theory, lower the probability of filastatin binding to PGA10. As a result, this would restore the ability of *Candida* to agglomerate and form biofilms. One potential experimental model would be to use a double gradient; for example the concentration of filastatin could be varied down a single row on a 96 well plate while hemoglobin concentration could be varied down a single column. This set up would make it possible to study the effect of both hemoglobin and filastatin at a number of different concentrations. If our hypothesis is true, we would predict that as the concentration of hemoglobin is increased at a particular concentration of filastatin, binding of *Candida* to polystyrene should increase. Conversely, at low levels of hemoglobin and high levels of filastatin we would expect adhesion levels to be very low. This assay alone would not be able to strongly support our hypothesis; a number of additional experiments would also be needed improve our understanding of the relationship between PGA10, filastatin and hemoglobin. For example, a similar assay could be performed using iron (II) or iron (III) to determine if filastatin disrupts the interaction between PGA10 and the entire hemoglobin complex or only the central iron atom.

In order to better understand and further validate the hits of interest that were found through our primary screening, it will be important to compliment and rescue the phenotypes that have been seen through the screening. This will involve taking some of the more promising candidates and using the previously described complementation protocol to place the gene of interest back into the genome at a highly expressed region. Then, the adhesion assay would need to be revisited, to show that to a degree of upmost certainty, the mutants that we are interested in are causing the phenotype exhibited.

After the knocked out gene has been replaced in the mutant's genome, further testing can be done to help further validate the authenticity of our results. There are a variety of tests and possibilities for future testing, ranging from *in vivo* and *in vitro.* One possible test at the *in vivo* level could be the use of a *C. elegans* survival assay with the use of our complimented strains and knockouts. This type of testing would be helpful in gaining a better understanding of how our knockouts and complemented strains affect a multi-cellular organism. Another possible experiment set to explore would be the use of an *in vitro* biofilm growth assay to gain a better understanding of how our knockouts adhere to silicone, which is similar to the material of implanted medical devices (Fazly, et al., 2013). This MQP has shown much promise into gaining a further understanding of how filastatin functions; with further testing these promising leads can be further validated.

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