Identification of novel molecular targets in drug resistant fungi

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Abstract

Fungal infections affect billions of people on a global scale and an increase in the more severe invasive fungal infections is observed. This increase is related to the enlarged number of hospitalized patients, including transplant patients and cancer patients. Treatment options for the fungal infections are limited to only a few drug classes, and the most commonly used drugs in the clinic includes azoles and echinocandins. However, fungal resistance towards these drugs is an increasing problem and there is an urgent need for new treatment options. This master thesis aims to address the lack of treatments of the drug resistant fungal infections. The approach used to find new treatment options is based on the identification of new molecular targets to use in the therapy of these infections. The method used to identify such targets takes advantage of the possible compensatory mechanisms activated when the fungi acquire resistance towards an antifungal agent. Targeting a possible compensatory protein could contribute to a more effective treatment of the fungal infections. Resistance towards echinocandins results from mutations in either *FKS1* or *FKS2* and identification of new molecular targets to use in treatment are based on negative genetic interactions of these genes. Interaction data was obtained from work performed by M. Costanzo et al. 2016 [1] that has been made public available. Form these data a total of 12 genes were validated in this study to have a negative genetic interaction with *FKS1* and *FKS2*. This interaction suggests that these genes could work as potential targets in the treatment of the drug resistant fungal infections and identification of drugs that inhibit the function of these genes could make the therapy more effective.

Resistance towards azoles arises due to many different mechanisms and one of them includes the overexpression of certain genes (includes *ERG11*, *UPC2* and *PDR3*). Similar to the echinocandins, a possible compensatory mechanism activated when the genes are overexpressed would be of interest to target, as this can result in a more effective treatment of the infection. Identification of targets of the azole resistant strains were in this master thesis addressed by performing an SGA screening of *S. cerevisiae* strains overexpressing *UPC2* and *PDR3*.

Acknowledgements

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Finally, I want to thank my family and boyfriend for their support and encouragement throughout my study. I couldn't have done it without them.

Linda Håkensbakken Sønsterud, December 2019

Contents

1 Introduction

1.1 Background

Globally, more than 1.5 million deaths are caused by fungal diseases and over a billion people are affected [2]. Fungal infections range from the most common and less severe ones such as mucosal (vaginal tract and oral cavity) and superficial (skin, hair, nails) fungal infections, to the more severe and life-threatening infections affecting the blood system and the lungs [2]. When a fungal infection is addressed as severe or life-threatening the term invasive fungal infection (IFI) is used. Such infections are not as frequently observed as the superficial infections, but are of a much greater concern due to the high mortality [3].The invasive fungal infections are a problem in the part of the population that already are critically ill, as these patients often have a weakened immune system as a result of various therapies. Risk factors of this are typically hematological malignancies, bone marrow transplantation, chemotherapy, HIV infection and invasive medical procedures [4, 5]. Neutropenia can contribute to a weakened immune system after chemotherapy [6]. Neutropenia refer to a lower concentration of neutrophils in the blood. The neutrophils are important because they fight off the pathogenic microbes entering the body and a lower concentration of these cells in the blood makes it possible for the microbes to cause, life-threatening, invasive fungal infections (IFI) [6, 7].

1.2 Fungal infections; A clinical point of view

Among the life-threatening fungal diseases, *Aspergillus*, *Candida*, *Cryptococcus*, *Pneumocystis* and *Histoplasma* are the main genera of species causing the infections [2, 3]. *Aspergillus* species cause infections through continuous lung exposure of the fungi [3] and invasive Aspergillosis is associated with a 50% mortality rate when diagnosed in an early stage and 100% mortality rate if the disease is not detected or detected at a late state [3]. *Cryptococcus neoformans* and *Cryptococcus gattii* are known to cause Cryptococcosis and occurs mainly after inhalation of these organisms in the lungs [3]. Pneumocystis jirovecii causes pneumonia and it is most commonly associated with immunosuppression as a result of HIV (human immunodeficiency virus) [2]. Fungal infections caused by *Candida* species go under the terminology candidiasis and are the most common species known to cause life-threatening disease in hospitalizes patients [3] . *Candida albicans* is most frequent isolated from patients with candidiasis. However, in recent years a rise in non- *Candida* albicans *Candida* (NCAC) species is observed to cause fungal disease [8]. In Europe ~15-20% of all clinical isolated *Candida* species are caused by *C. glabrata* [9]. When candidiasis causes infection in the bloodstream the term candidemia is used and such infections are associated with a mortality rate between 45-75% [10].

1.3 Candida glabrata

Candida glabrata is an opportunistic pathogen making up the normal microbiota of the oral cavity, gastrointestinal and vaginal tracts [8]. One feature that makes it possible for *C. glabrata* and other fungal species to cause invasive fungal infections is that the host is immunosuppressed [8]. This means that the immune system is not able to fight off the microbes entering the body. However, it is not only the immunosuppressed host that makes it possible for the fungi to cause an infection. Virulence factors do also contribute to the pathogenicity of the fungi [8, 11]. In *C. glabrata* virulence factors such as adhesion, biofilm formation and enzyme production take part in the pathogenicity [8]. This makes it possible for *C. glabrata* to colonize the host and resist antifungal treatment [12].

One of the features that distinguish fungal cells from the mammalian cells of the host is their cell wall. The composition of the cell wall differs between different fungal species and it is important for the characteristic of the fungi. Common for most fungi is a core region of the cell wall that is composed of branched β-(1,3) glucan, β-(1,6) glucan, and chitin [13]. The fungal cell wall of *Candida* species are rich in mannosylated proteins attached to the β-(1,6) glucan and to the β-(1,3) glucan-chitin core (Figure 1) [13].

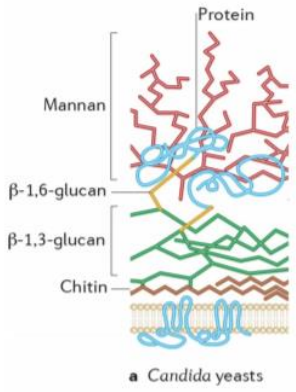


Figure 1. Composition of the cell wall of *Candida* species. The figure is obtained from Gow et al. 2017 [13].

The cell wall and the components of it represent a good molecular target due to the lack of cell wall in the mammalian cells. Treatments that take advantage of the fungal specific targets such as the cell wall will be described in more detail in later sections.

1.4 *S. cerevisiae* vs. *C. glabrata*

The budding yeast *S. cerevisiae* has been used experimentally as a model organism in the lab for decades. In the following years multiple other researchers have used the organism, that today is considered as one of the leading organisms in experimental research. The features that make *S. cerevisiae* such a good model organism includes a short generation time (divide once every 90 min), easy to grow and maintain in the laboratory, high rates of homologous recombination and it has a fully sequenced genome (by Goffeau et al. 1996) [14, 15].

S. cerevisiae is easy to work with and multiple tools are available for studying processes ranging from cell cycle analysis, meiosis and aging, to gene function and interaction [16]. The high rate of homologous recombination allows precise manipulation of the genome, making it possible tag genes and to create deletion mutants.

One other advantage of using *S. cerevisiae* in research is the two mating types (MAT) of the organism. It exists as a haploid (either MATa or MATα) or as a diploid (MATa/α). The two haploid mating types makes it possible to perform genetic analyses.

C. glabrata, on the other hand, is a challenging organism to use experimentally. One reason for this is the high degree of non-homologous end-joining (NHEJ) which repairs the double-stranded breaks in the DNA, making it hard to tag and delete genes [17, 18]. Another factor is a few available tools and the lack of a sexual life cycle, making genetic analysis like a synthetic genetic array (SGA) impossible [17]. *S. cerevisiae* is closely related to *C. glabrata* [19] and therefore makes it a good model for studying genes contributing to drug-resistance in *C. glabrata*. *C. glabrata* is more similar to *S. cerevisiae* in terms of protein conservation than other *Candida* species, e.g. *C. albicans* [17, 19] and is more closely related to *S. cerevisiae* than other *Candida* species. Because of this *S. cerevisiae* will be used as a proxy for *C. glabrata* in this study.

1.5 Treatments

Antifungal drugs are important in combating the fungal infections, as these aim to kill the fungi by targeting fungal specific features. The antifungal agents commonly target the components of the fungal cell membrane or the fungal cell wall [20].

When treating fungal infections, it is important that the drug only targets the fungi itself and not the mammalian host cells. Finding good antifungal agents is challenging because both the fungi and the host are eukaryotic organisms and will, therefore, have similar targets [21]. A drug which kills the fungi might also have toxic effects on the mammalian cells and this can have severe effects. Due to the lack of potential targets, the antifungal drugs available are therefore restricted to a few drug classes, including echinocandins, azoles, polyenes and flucytosine (5-FC) [5, 22]. These antifungal drugs classes target the cell wall (echinocandins),

the cell membrane (polyenes) or DNA/RNA synthesis (5-FC) of the fungi, and the mechanism of action of the four most common of the antifungals are shown in Figure 2. Since echinocandins and azoles are the drugs most commonly applied in the clinic today [20], these will be focused on in this study.

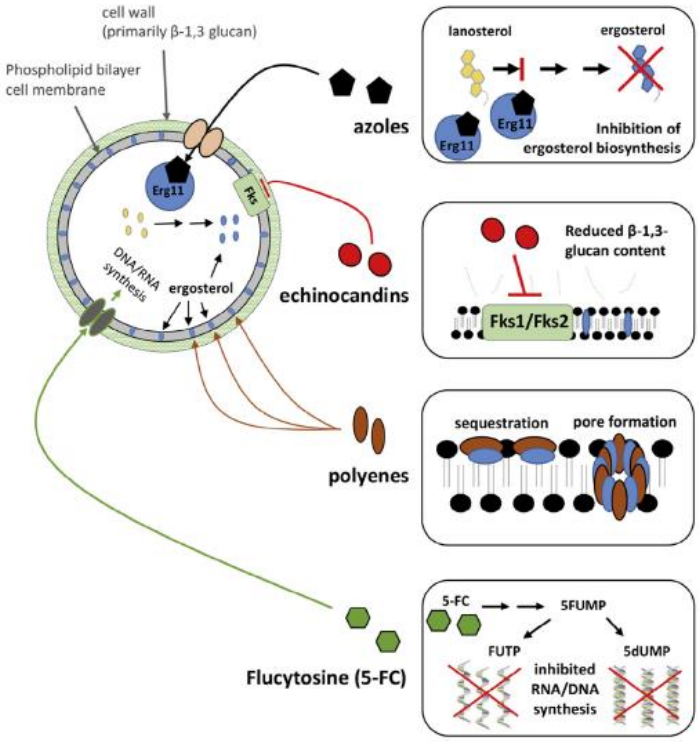


Figure 2. The four main classes of antifungal drugs and their mechanism of action. Figure from Morio et al. 2017 [22]. Copyright © 2017 Elsevier B.V. and International Society of Chemotherapy

Azoles

Azoles work as fungistatic agents, meaning that they inhibit the growth of the fungi rather than killing it, such as the fungicidal agents does. Among the azoles is fluconazole, a broad spectrum triazole that is commonly used in the clinic today as treatment of fungal infections [21]. In the clinic, fluconazole is used in the treatment of *Candida* species and *Cryptococcus neoformans* [20].

Azoles are inhibitors of the ergosterol biosynthesis. Ergosterol is a component of the fungal cell membrane and is important for membrane fluidity [5]. The enzyme converting lanosterol into ergosterol is 14α-lanosterol demethylase (*ERG11*) and it is this enzyme that is blocked by the

treatment with azoles [5, 21, 22]. This blocking leads to altered membrane stability and permeability due to the accumulation of 14α-methyl sterols [5].

Echinocandins

Echinocandins are considered as a fungicidal agent against *Candida* and included in this drug class are caspofungin, micafungin and anidulafungin [10]. The echinocandins are also considered as an efficient treatment of *Aspergillus* infections [20]. Echinocandins target the enzymes building up the fungal cell wall and more specific function by inhibiting the β-1,3-glucan synthase [10, 22], leading to alteration of the β-1,3 glucan which is a major component of the fungal cell wall [22]. The enzymes of β-1,3-glucan synthase are encoded by the *FKS* genes in *Candida,* which includes *FKS1* and *FKS2* [8, 10, 23].

1.5.1 Resistance towards the antifungal drugs

A growing concern is an evolving resistance towards antifungal treatment. Resistance, in this case, means that the drugs used in therapy no longer are effective against the infection and that the infection is able to progress despite the therapy with antifungals [24]. Over the past decade, an increase in the incidences towards the clinically used antifungals echinocandin and azoles are observed in fungal species that are causing severe invasive infections like *C. glabrata* (Figure 3). In this section, the mechanisms of the fungal resistance towards azoles and echinocandins will be addressed.

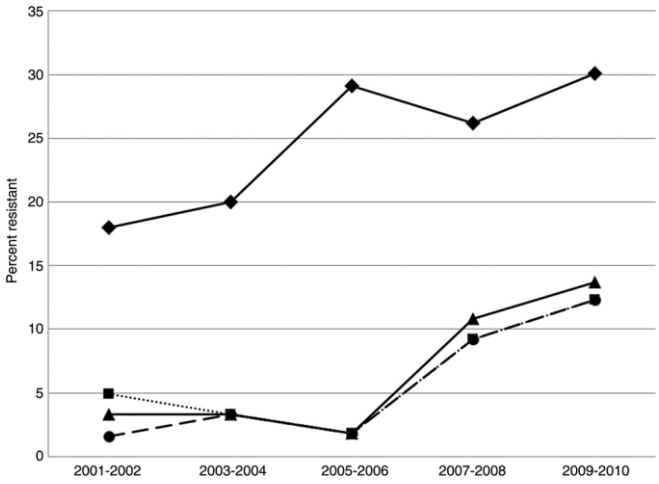


Figure 3. Antifungal drug resistance towards azoles and echinocandins in *C. glabrata*. Fluconazole (solid line with diamonds), anidulafungin (short dashed line with squares), caspofungin (solid line with triangles), and micafungin (long dashed line with circles). Barbara D. Alexander et. al 2013 [25].

Azole resistance

Resistance towards azoles is often a combination of multiple different mechanisms. Four mechanisms of action are known to give azole resistance, and these are reviewed by Morio *et al.* 2017 (Figure 4) [22]. One way in which the fungi acquire resistance is through mutations in *ERG11*, thereby inhibiting the binding of the drug because of alterations in the drug target [22]. Another mechanism is the overexpression, either directly or through its regulatory elements, of *ERG11* resulting in the need for higher dosages of the drug for inhibition, due to more 14α-lanosterol demethylase in the cell [5]. Regulatory elements that contributes to azole resistance when overexpressed include *UPC2* and *PDR3*[1] *(S. cerecisiae)* [5]. A third mechanism is the active transport of the drug out of the cell through multidrug transporters, reducing the intracellular concentration of the drug [22]. The fourth mechanism of azole resistance is metabolic bypass, meaning that the formation of sterols happens outside of the *ERG11* pathway [22].

[1] *PDR1* in *C. glabrata* (5. Cowen, L.E., et al., *Mechanisms of Antifungal Drug Resistance.* Cold Spring Harb Perspect Med, 2014. **5**(7): p. a019752.)

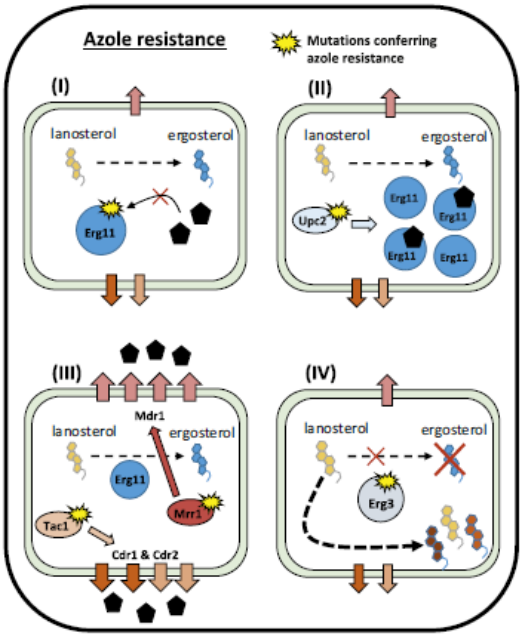


Figure 4. Mechanism in which the fungi acquires resistance towards azoles. Figure from Morio et al. 2017 [22]. Resistance towards azoles can be caused by (I) Mutation in *ERG11*, (II) Overexpression of *ERG11*, (III) Active transport or (IV) Metabolic bypass. Copyright © 2017 Elsevier B.V. and International Society of Chemotherapy

Echinocandin resistance

The resistance to echinocandins in *Candida* species is due to mutations in two conserved hot spot regions in either *FKS1* or *FKS2* [22] (Figure 5). The mutations causing resistance are random and arises during therapy as a result of repeated exposure to the drug [26].

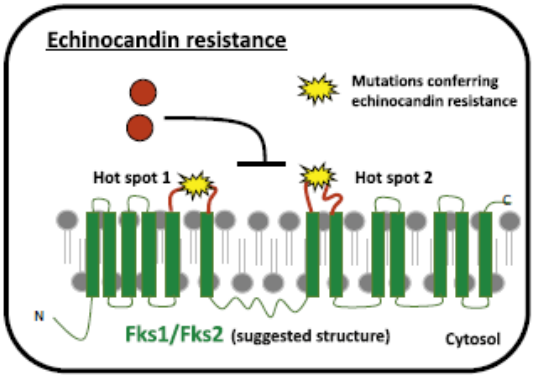


Figure 5. Mechanism in which the fungi acquires resistance towards echinocandins, showing the two hot spot regions of *FKS1* and FKS2 contributing to this resistance. Figure from Morio et al. 2017 [22]. *Copyright © 2017 Elsevier B.V. and International Society of Chemotherapy*

The mutations contributing to echinocandin resistance is observed to contribute to a fitness defect compared to yeast not acquiring the mutation [27]. A thicker cell wall is observed in the mutant strains and the composition of the cell wall is altered [27], indicating that compensatory mechanisms may be responsible for the mutated fungi being able to outcompete the WT in selective media. Targeting these possible compensatory mechanisms could provide us with more effective treatment options for drug resistant fungal infections. The approach for identification of such targets will be addressed in the following section.

1.6 Identification of new treatments

Only a limited number of drugs are available in the clinic for treatment of fungal infections. There is an urgent need for new treatment options, as more fungal infections develop resistance towards the treatment with antifungal agents.

1.6.1 Drug screening

A common way to discover new antifungal drugs is through high-throughput screenings. This is an approach where large libraries of small molecules are tested for their ability to inhibit the growth of a particular fungus [28]. The echinocandins are the newest antifungal drug class and were discovered though screening in 1970 [29]. Drug screenings are certainly an important method to discover new drugs, but it also has some disadvantages. It is not possible to predict the absolute specificity of the drug, meaning that the drug can act on other targets and thereby kill the healthy cells [30]. False positive hits are another downside, as the screening may suggest one drug that is not active against the target [30]. Other alternatives are available for the identification of new drugs and this will be described in the next section.

1.6.2 Genetic screening

An important part of drug discovery is the identification of molecular targets to use in the treatment of the desired disease [31]. By performing genetic screenings like a synthetic genetic array (SGA) it is possible to find new targets based on interactions with other genes. A synthetic lethal interaction (negative genetic interaction) is of particular interest for this purpose. When the interaction between two genes is described as synthetic lethal it means that the combination of two mutations causes cell death, thus having a more severe fitness defect [16]. The interaction suggests that the genes function in parallel pathways and that one gene possible is compensating for the mutation of an interacting gene [32]. Identification of such negative genetic interactions could provide better treatment options, as the function of a possible compensatory protein can be inhibited.

The SGA screening offers a global analysis of synthetic genetic interactions in *S. cerevisiae* and *Schizosaccharomyces pombe* (*S. pombe*) [33]. This method works by creating deletion mutants of almost 80% of all yeast genes [34]. A double mutant is constructed from mating with one query MATα single mutant and one MATa deletion mutant, and the double mutant is then selected according to the selective markers used [34]. The growth of the constructed double mutants is scored against the respective single mutants and given a score based on whether the mutants grow less (negative genetic interaction) or more (positive genetic interaction) [33, 34]. A downside with the SGA method is that it only can be applied to *S. cerevisiae* and *S. pombe*, but since the protein sequences are well conserved between the fungi and the yeast it is reasonable to assume that the findings in yeast can be extrapolated to pathogenic fungi [19].

In this study, a strategy in which new molecular targets of echinocandin resistant fungi can be identified, take advantage of the negative genetic interactions of *FKS1* and *FKS2*. A hot-spot mutation in either *FKS1* or *FKS2* is known to contribute to the resistance towards echinocandins. However, such mutations are previously described to come with a fitness cost and affect the composition of the cell wall [27]. *C. albicans* strains with an *fks1* mutation were shown to have thicker cell walls and a higher content of chitin [27]. The alterations in the fungal cell wall caused by the mutation could indicate that other mechanisms are needed to compensate for the altered cell wall. Identification of the compensatory mechanisms is of great interest as the targeting of these could contribute to more effective treatment of the fungal infections.

1.7 Aim of study

The aim of this master thesis is to identify new molecular targets to use in the treatment of drug resistant fungal infections. The focus will be on azole and echinocandin resistant fungi and the following bullet points is addressed in this thesis:

- Identification of the negative interacting genes of *FKS1* and *FKS2* in *S. cerevisiae* that can be used as possible targets in treatment of echinocandin resistant fungal infections.
- Validation of the negative genetic interactions of *FKS1* and *FKS2* in *S. cerevisiae*
- Validation of the negative genetic interactions of *FKS1* and *FKS2* in *Candida glabrata*
- Identification of new targets to use in the treatment of azole resistant fungal infections

2 Materials and methods

2.1 Synthetic genetic array (SGA)

As we already have seen, drug resistance towards echinocandins in *C. glabrata*, arises due to mutations in *FKS1* and *FKS2*. Targeting a possible compensatory mechanism that is activated when *FKS1* or *FKS2* is mutated, could possibly reduce or inhibit the fungal growth. Discovery of potential compensatory proteins can be found based on the negative genetic interactions of *FKS1* and *FKS2*. Identification of negative genetic interactions of these genes can contribute to a more effective treatment of the fungal infections.

Negative interaction data of *FKS1* and *GSC2* (*FKS2*) used in this study were collected from the public available data[2] provided by M. Costanzo et. al. 2016 [1]. This site provides genetic interaction data in *S. cerevisiae* obtained using synthetic genetic array (SGA). The top 20 genes of both *FKS1* and *FKS2*, with a significant negative genetic interaction were chosen and these were used throughout the experiments in this study.

2.2 Strains

As mentioned in the introduction, *S. cerevisiae* works as a proxy organism for *C. glabrata* and two different yeast collections have been used in this study. The first is the DamP yeast library and the second is the yeast knock-out (YKO) collection. Wild type (WT) strains used in this study come from the Jorrit Enserink yeast (JEY) collection. Apart from the *S. cerevisiae* strains used, two *C. glabrata* strains were also used. One WT *C. glabrata* strain (JEY10028) and one *C. glabrata* strain resistant to echinocandins (JEY12725). All strains used and designed in this study are listed in Table 1, and the genotype of the different strains used are shown in table 2.

[2] www.thecellmap.org

2.2.1 DamP yeast library

The Decreased Abundance by mRNA Perturbation (DamP) yeast library from Dharmacon™ is used in the first validation experiment of the SGA data. This *S. cerevisiae* strain collection have a disruption of the 3'UTR with kanamycin resistance cassette (KanMX), resulting in a reduction of the mRNA levels due to unstable transcript. This generates hypomorphic alleles, meaning that the alleles have a reduced gene function due to the decreased mRNA levels [35, 36]. The DamP collection was used in the cell viability assay and the spot assay to check the sensitivity of the strains towards micafungin.

2.2.2 MATa knock-out library

The *S. cerevisiae* MATa library is a yest knock-out (YKO) collection were the genes are replaced with KanMX. This library was used in the construction of *S. cerevisiae* double knock-out strains.

Table 1. Overview of the strains used, their sourse and position, as well as the background of the strains made in this study. Δ indicates knock-out of a gene.

Strain number	Description	Background	Sourse/Position[3]
232	WT		JEY[4]
3023	WT		JEY
3862	WT		JEY
12507	pr_ADH_3HA::NAT-UPC2 Clone a	3862	This study
12508	pr_ADH_3HA::NAT-UPC2 Clone 1	3862	This study
12509	pr_ADH_3HA::NAT-UPC2 Clone 2	3862	This study
12510	pr_TEF1_3HA::NAT-UPC2 Clone a	3862	This study
12511	pr_TEF1_3HA::NAT-UPC2 Clone b	3862	This study
12512	pr_GAL1_3HA::NAT-UPC2 Clone 5	3862	This study
12513	pr_GAL1_3HA::NAT-UPC2 Clone 2	3862	This study
12514	pr_GAL1_3HA::NAT-ERG11 Clone 1	3862	This study
12515	pr_TEF1_3HA::NAT-ERG11 Clone 6	3862	This study
12516	pr_GAL1_3HA::NAT-PDR3 Clone 1	3862	This study
12517	pr_GAL1_3HA::NAT-PDR3 Clone 4	3862	This study
12518	pr_ADH_3HA::NAT-PDR3 Clone 1	3862	This study
1	*pkc1-damp*		DamP[5]
2	*bni4Δ*		YKO
3	*rlm1Δ*		YKO
4	*crz1Δ*		YKO
5	*cch1Δ*		YKO
6	*gsc2Δ*		YKO
7	*mid1Δ*		YKO

[3] Position – Plate Number_Row_Column
[4] JEY – *S. cerevisiae* strain in Jorrit Enserink yeast (JEY) collection
[5] https://horizondiscovery.com/products/gene-modulation/overexpression-reagents/non-mammalian/PIFs/Yeast-DAmP

8	*cnb1Δ*		YKO
9	*chs6Δ*		YKO
10	*chs7Δ*		YKO
11	*pre4-damp*		DamP
12	*pup1-damp*		DamP
13	*tif6-damp*		DamP
14	*skt5Δ*		YKO
15	*las17-damp*		DamP
16	*rpl40aΔ*		YKO
17	*msn5Δ*		YKO
18	*ybl062wΔ*		YKO
19	*yhr177wΔ*		YKO
20	*gcg1Δ*		YKO
21	*nha1Δ*		YKO
22	*fks1Δ*		YKO
23	*cdc25-damp*		DamP
24	*ydj1Δ*		YKO
25	*msc1Δ*		YKO
26	*met7Δ*		YKO
27	*smi1Δ*		YKO
28	*rpl21bΔ*		YKO
29	*ecm2Δ*		YKO
30	*ypt6Δ*		YKO
31	*atc1Δ*		YKO
32	*cox6Δ*		YKO
33	*sma2Δ*		YKO
34	*yll054cΔ*		YKO
35	*vps63Δ*		YKO
36	*aro1Δ*		YKO
37	*Arc18Δ*		YKO
38	WT		DamP
2739	*rlm1Δ*		YKO[6]/24_G_6
12701	*rlm1Δ* + Nat:GAL1p-FKS1	2739	This study
1153	*mid1Δ*		YKO/5_H_3
12702	*mid1Δ* + Nat:GAL1p-FKS1	1153	This study
2835	*chs7Δ*		YKO/15_F_11
12696	*chs7Δ* + Nat:GAL1p-FKS1 #1	2835	This study
12697	*chs7Δ* + Nat:GAL1p-FKS1 #2	2835	This study
12698	*chs7Δ* + Nat:GAL1p-FKS1 #3	2835	This study
12699	*chs7Δ* + Nat:GAL1p-FKS1 #4	2835	This study
12700	*chs7Δ* + Nat:GAL1p-FKS1 #5	2835	This study
3012	*ydj1Δ*		YKO/42_G_11
12600	*ydj1Δ + fks2Δ*	3012	This study
5882	*smi1Δ*		YKO/70_B_11
12609	*smi1Δ + fks2Δ*	5882	This study
12610	*smi1Δ + fks2Δ*	5882	This study
12611	*smi1Δ + fks2Δ*	5882	This study
5170	*vps63Δ*		YKO/22_F_11
5251	*fks1Δ*		YKO/21_H_5
6979	*fks2Δ*		YKO/44_B_3
12725	*C. glabrata* FKS1-L662W		JEY
10028	*C. glabrata* WT		JEY

[6] YKO- *S. cerevisiae* strain in MATa yeast knock-out (YKO) library

Table 2. Genotype of the strains used in this study and the background of each strain.

Strain	Background	Genotype
232	BY4741	MATa (his3Δ1, leu2Δ0, met15Δ0, ura3Δ0)
3023	Winston S288C	MATa (ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8)
3862		MATα (can1Δ::STE2pr-Sp_his5, lyp1Δ, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0)
DamP	BY4741	MATa his3Δ1/his3Δ1leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 CYH2+/cyh2)
YKO	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0

2.3 Growth media and agar plates

Different types of growth media and agar plates are needed for different purposes. The different media and plates used throughout this study are listed in Table 3, were also the composition of each are listed. Two types of media are commonly used, either rich media (YPD and YPGal) or synthetic media (CSM). These media give fast and slow growth of the yeast cells, respectively.

Each medium is prepared according to Table 3, and the volume is adjusted to 500 ml with milliQ-H_2O. The media is then sterilized by autoclaving[7]. The liquid media is cooled down at room temperature. The solid media is poured out on petri dishes and left at room temperature overnight to solidify. Nourseothricin (clonNAT) is added to cooled down[8] YPD and YPGal agar medium while stirring with a magnet to homogenize the solution, and then poured out on petri dishes.

[7] All autoclaving described in this study is performed using CertoClav Essential (cycle: 121ºC, 15min)
[8] The temperature of the bottle should be ~50ºC

Table 3. Composition of all media used in this study, their reference number (Ref.No) and the supplier of the compounds.

Growth media	Composition in a total volume of 500ml	Ref. No	Supplier
YPD medium	10g D(+)-glucose anhydrous (2%) 10g peptone (2%) 5g yeast extract micro granulated (1%)	GLU03 PEP03 YEM03	FORMEDIUM™
YPD agar plates	YPD medium added 10g Agar (2%)	AGA03	FORMEDIUM™
YPD + Nat agar plates	YPD agar medium supplemented with 500 µl clonNAT	CAS#96736-11-7	WERNER BioAgents GmbH
CSM medium	3.450g Yeast nitrogen base without amino acids 0.395g Complete supplement mixture (CSM) 10g D(+)-glucose anhydrous (2%)	CYN0405 DCS0019 GLU03	FORMEDIUM™
YPGal medium	5g yeast extract micro granulated (1%) 10g peptone (2%) 10g D(+) galactose	YEM03 PEP03 GAL03	FORMEDIUM™
YPGal + Nat medium	YPGal agar medium supplemented with 500 µl clonNAT	CAS#96736-11-7	WERNER BioAgents GmbH
YPGal agar plates	YPGal medium added 10g Agar (2%)	AGA03	FORMEDIUM™

2.4 Growth and maintenance of *S. cerevisiae* cells

All *S. cerevisiae* strains are stored in the collection at -80ºC. Growth of the yeast strains are performed by streaking out the cells from the collection with a pipette tip on to solid YPD agar plates. The agar plates are then incubated at 30ºC for 1-2 days.

The cells can be stored at 4ºC and used in the laboratory for approximately one week before they are refreshed on to a new YPD.

2.4.1 Storing new strains in collection

1. Incubate the cells overnight in YPD
2. Centrifugate cells at 3000 rpm for 3 min and remove the supernatant.
3. Prepare 20 % glycerol:

   To a 50 ml falcon tube, add 10 ml glycerol and 40 ml milliQ H_2O. Press the solution through a Whatman® filter paper, using TERUMO ® SYRINGE without needle (10 ml) to get rid of bacteria and other contaminants.
4. Add 1 ml of 20 % glycerol to the pellet and resuspend
5. Transfer solution to the CryoTube^{TM} Vials and store in collection at -80^{o}C

2.5 Cell proliferation assay; Sensitivity testing

Cell proliferation assay is a technique used in this study to measure the proliferation of the cells using optical density as a measure of cells in the culture. The number of cells is proportional to the absorbance.

Table 4. Chemicals used in the cell viability assays.

Chemical	**Catalogue number**	**Supplier**
Micafungin (16mM)	T1794	TargetMol
Ganetespib (100mM)	HY-15205	MedChemExpress
Onalespib (50mM)	S1163	Selleckchem.com

2.5.1 Overnight culture preparation and optical density measurement

S. cerevisiae cells were grown overnight in 5 ml CSM in a 50 ml falcon tube at 30°C in a Innova®44 incubator shaker.

The optical density of the cell culture (1:20 dilution) at 600nm was then measured, in duplicates (OD1 and OD2) for each sample, using the HITACHI U-1900 spectrophotometer. The average OD for each sample at 600nm was then calculated (2.1), and the final corrected OD600nm was calculated (2.2)

$$\text{OD average} = \frac{OD1 + OD2}{2} \tag{2.1}$$

$$\text{OD corrected} = \text{OD average} \times 20 \tag{2.2}$$

For the cell viability assay a final OD600nm of ~0,1 (OD final) was used. The volume of cells needed in the desired volume of CSM was then calculated according to equation (2.3).

$$\text{OD corrected} \times V1 = \text{OD final} \times V2$$

$$V1 = \frac{OD\ final \times V2}{OD\ corrected} \tag{2.3}$$

2.5.2 Micafungin gradient

1. A 100 μM batch of micafungin in DMSO was made and used in the preparation of a micafungin gradient.

2. 12 individually prepared concentrations of micafungin were then made.

    a) Each micafungin concentration was prepared in a 15ml falcon tube and the volume of the compound needed for the different concentrations were calculated according to equation (2.4).

    $$C1 \times V1 = C2 \times V2 \tag{2.4}$$

    b) The first 8 concentrations, starting at 114.8 nM, were diluted 1:1.176 in CSM. The next 3 micafungin concentrations were diluted 1:1.383, to ensure more measuring points in the IC50 area of the different DAmP strains.

    c) Final concentration was set to 1.0 nM to make sure that the more sensitive strains were detected.

3. 100 µl of each of the 12 micafungin concentrations were then transferred to a Nunc™ MicroWell™ 96-Well microplate (Thermo Fisher scientific) using a multichannel pipette.

    a) Column 1-12, row A-F, contain micafungin. Each column having its own concentration. See figure 6 for each micafungin concentration used in the different columns.

    b) Row G and H are control wells, no drug added.

4. To row A-C and D-F add 50 µl of different yeast strains with a final OD of ~0,1 Eq. (2.3). In this way technical triplicates for each strain was obtained.

5. Row G is control of strain added to row A-C, and row H is control of strain added to row D-F.

6. The OD at 600nm at timepoint 0 hours and 24 hours using the BioTek machine was then measued.

    - Prior to measure OD after 24 hours the cells were resuspended using the multichannel pipette. The cells stick to the bottom of the wells, so it's necessary to make the content of each well homogenic in order to measure the optical density.

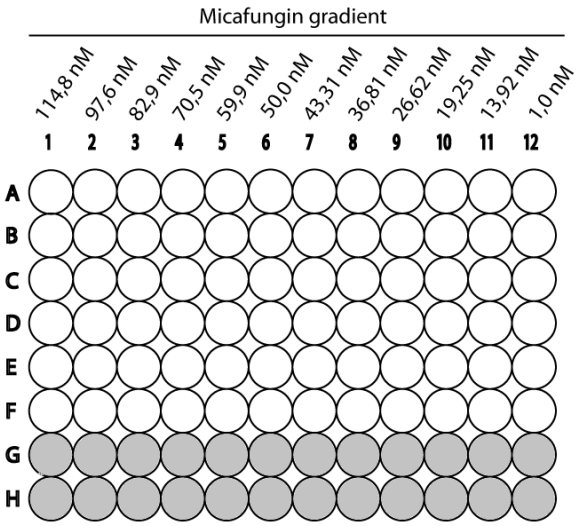


Figure 6. A 96-well plate showing the concentration of micafungin in each column. Row A-C contain technical replicates of one DAmP strain and row D-F contain technical replicates of another DAmP strain. Row G and H are controls for row A-C and D-F, respectively.

2.5.3 Ganetespib and Onalespib gradient

1. Prepare a 100µM stock of Ganestespib and Onalespib, in 1000 µL DMSO.

   The volume of Ganetespib (100mM) and Onalespib (50mM) needed to get a concentration of 100µM in 1000 µL DMSO was calculated according to the following equation:

   Ganetespib:

$$C1 \times V1 = C2 \times V2 \quad (2.5)$$
$$100\ mM \times V1 = 100\ \mu M \times 1000\ \mu L$$
$$V1 = \frac{100\mu M \times 1000\mu L}{100000\mu L} = 1\ \mu L$$

   Onalespib:

$$C1 \times V1 = C2 \times V2 \quad (2.6)$$
$$50\ mM \times V1 = 100\ \mu M \times 1000\ \mu L$$
$$V1 = \frac{100\mu M \times 1000\mu L}{50000\mu L} = 2\ \mu L$$

2. Prepare a 1:10 dilution series of Ganetespib (100µM) and Onalespib (100µM) in CSM that has a final concentration of 10µM – 5µM – 1µM – 0.5µM – 0.1µM in a 96-well with a total volume of 300µL containing 75µL Ganetespib/Onalespib, 75µL CSM and 150µL yeast cells.
3. The OD at 600nm at timepoint 0 hours and 24 hours using the BioTek machine was then measured.
   - Prior to measure OD after 24 hours the cells were resuspended using the multichannel pipette. The cells stick to the bottom of the wells, so it's necessary to make the content of each well homogenic in order to measure the optical density.

2.6 Spot assay

The spot assay is used in multiple of the experiments in this study to check the proliferation of cells when treated with different drugs. The spot assay was performed on both micafungin gradient plates, fluconazole plates and NAT supplemented plates.

Equipment

Equipment	Catalog number	Manufacturer
Nunc™ Omni Tray™, Single- well, w/lid	242811	Thermo Fisher scientific
96-pin multi-blot™ replicator	VP408FP6	V&P SCIENTIFIC, INC
Nunc™ MicroWell™ 96-Well microplate	167008	Thermo Fisher scientific
50 ml falcon tube	62.547.254	Sarstedt AG & Co. KG

2.6.1 Preparation of micafungin gradient plates

1. Autoclave solid YPD and let cool down while stirring with a magnet.
2. Prepare 30 ml solid YPD with a final micafungin concentration of 0.25 μg/ml and 0,4 μg/ml
3. Put the Nunc™ Omni Tray™ on the bench elevating one of the short sides of the tray. This will create a slope that makes the micafungin gradient.
4. Pour the micafungin + YPD media on to the Nunc™ Omni Tray™ and let it solidify.
5. Lay the tray flat on the bench and pour 30 ml solid YPD on top of the micafungin gradient. Let solidify.

2.6.2 Preparation of cells

1. Grow *S. cerevisiae* DAmP cells overnight in 5 ml CSM medium at 30ºC in the Innova®44 incubator shaker.
2. Measure the optical density at 600nm and the corrected OD was calculated as described in section 2.3.1. Cells with an OD final of ~1,0 were adjusted in CSM.

2.6.3 Spot assay on micafungin gradient plate

1. Transfer 200 µl of the yeast solution with OD final ~1,0 to a NuncTM MicroWell^{TM} 96-Well microplate using a multichannel pipette. Each row of the plate should then contain one yeast strain were all wells have an OD 600nm of 1,0.

2. Sterilize the 96-pin replicator by first setting it in a tray with 95 % ethanol. Then hold the replicator over a flame, and let it cool down.

3. Set the sterilized 96-pin replicator in the 96-well microplate containing the yeast cells.

4. Transfer the 96-pin replicator with the cells to the micafungin gradient plates. Be careful when setting the replicator on to the plate and when lifting it off the plate, to avoid the spots from getting spread out.

5. Leave the plates on the bench for the spots to dry before moving the plates.

6. Incubate at 30ºC for 2-3 days.

7. Image the plates using the BIO-RAD imaging machine.

2.7 Polymerase chain reaction (PCR)

Polymerase chain reaction is a method used for amplifying fragments of DNA.

The basic principles of a PCR reaction are divided into three steps. Fist the DNA is denatured at a high temperature, meaning that the two strands of the double helix are separated. Second, the temperature is lowered which allows the primers to attach to the separated strands. Third, a thermostable DNA polymerase attaches to the primers to synthetize a new DNA strand in 5’ to 3’ direction.

The templates used in the different experiments can be found in Table 5 and the reagents used for the polymerase chain reaction is listed in Table 6.

Templates

Table 5. List of the templates used in this study, showing the promoters and tags available in the plasmid (only showing the once used in this study).

Gene	Primer combination	Template	Promoter	Tag
ERG11, UPC2, PDR3	S1/S4	pYM-N8	ADH	3HA/natNT2
	S1/S4	pYM-N20	TEF1	3HA/natNT2
	S1/S4	pYM-N24	GAL1	3HA/natNT2
FKS1, FKS2	S1/S2	pFA6a-natNT2		natNT2
RLM1, MID1, CHS7, YDJ1, SMI1, VPS63	P1/P2	pFA6a-natNT2		natNT2

Reagents

Table 6. The reagents used in the polymerase chain reactions, their catalogue number and the supplier of the component.

Components	Catalogue number	Supplier
AccuPrime™ Pfx DNA polymerase	LOT 2059421	Invitrogen Thermo fisher scientific
AccuPrime™ Pfx reaction buffer	LOT 2023765	
BIOTAQ™ DNA polymerase	Cat.No. BIO-21060	Bioline
10x NH_4 reaction buffer		
50mM $MgCl_2$ solution		
DMSO[9]	LOT SZBC166AV	Sigma-Aldrich
dNTPs[10]	N/A	N/A

[9] Dimethyl sulfoxide (DMSO)
[10] Deoxyribonucleotide triphosphate (dNTP)

2.7.1 “Toolbox” Protocol

The “Toolbox” protocol provides an easy way to tag and delete genes in *S. cerevisiae*, and the system used is according to the established protocol by Janke et al. 2004 [37]. This is achieved using primers with a region aligning to both the cassette of interest and the desired gene. Because of these homologous sequences it is possible to introduce a desired sequence at any location inside, upstream or downstream of a gene due to homologous recombination [37].

In this study the “Toolbox” protocol was used to for gene deletion by exchanging a gene with the natNT2 cassette. This system was also used to amplify promoters from the cassettes that later were introduced upstream of the gene of interest.

In order to obtain a low error rate of the transcript the high- fidelity AccuPrime^{TM} Pfx DNA polymerase from Invitrogen was used. The reaction setup is shown in table 7. After mixing all reagents together the samples were run in a PCR machine from Applied Biosystems using the “Toolbox” program (se Appendix B).

*Table 7. “*Toolbox” reaction setup with AccuPrime^{TX} DNA polymerase.

	DNA templat	**milliQ H_2O**	**DMSO**	**Primer 1 (S2)**	**Primer 2 (S3)**	**AccuPrime^{TX} Pfx DNA polymerase**	**AccuBuffer with dNTPs**
Total volume 50 µl	1 µl	35 µl	5 µl	1,5 µl	1,5 µl	1 µl	5 µl

2.7.2 “Genot” protocol

“Genot” PCR, also known as genotyping, is a method performed in order to determine the insertion of a tag as well as the deletion of genes after transformation of the yeast cells. The reaction setup for this protocol is shown in Table 8 and the “Genot” PCR program used can be found in Appendix B. BIOTAQ^{TM} DNA polymerase is used in this reaction because it is considered a good choice in routine assays and gives a high yield[11].

[11] https://www.bioline.com/us/biotaq-dna-polymerase.html

Table 8. "Genot" reaction setup with BIOTAQ™ DNA polymerase

	DNA	**H_2O**	**10x NH_4 reaction buffer**	**$MgCl_2$**	**dNTPs**	**Primer 1**	**Primer 2**	**BIOTAQ™ DNA polymerase**
Total volume 25 µl	1 µl	17,15 µl	2,5 µl	2 µl	0,15 µl	1 µl	1 µl	0,2 µl

2.8 PCR analysis

In order to check the size of the amplified PCR product the QIAxcel from QIAGEN were used. This is an automated electrophoresis analysis based on separation of DNA fragments according to size through a capillary system. The fragments are further detected and can be analyzed using the QIAxcel ScreenGel software. The result can either be viewed as an electropherogram or as a gel image. The latter one will be used in this study.

2.9 Transformation of *S. cerevisiae*

Transformation was performed using the lithium acetate method. This method was first described by Ito et al. 1983, this method has been further modified several times e.g. (Gietz and Woods, 2002) to make the transformation protocol more efficient, and the transformation protocol used in this study are based on the same principles as these [38, 39].

The reagents used in the transformation of *S. cerevisiae* are shown in table 9.

2.9.1 Solutions

Table 9. Reagents used in transformation of S. cerevisiae

	Reference number	**Manufacturer**
Lithium acetate dihydrate	CAS 6108-17-4	Sigma -Aldrich
Polyethylene glycol (PEG3350)	CAS 25322-68-3	Sigma- Aldrich
DNA carrier	N/A	N/A

Preparation of solutions:

- 1M Lithium acetate was prepared by dissolving 10g of lithium acetate dihydrate in 100mL milliQ H_2O, followed by autoclaving.
- PEG3350 50% was prepared by dissolving 50g of PEG3350 in 100mL milliQ H_2O, followed by autoclaving.

2.9.2 Transformation protocol:

1. Grow cells in 5 ml YPD media o/n at 30^{o}C with shaking.
2. Refresh the o/n culture to get the cells into log phase; 1 ml of the o/n culture is used per 50 ml YPD media. Incubate at 30^{o}C with shaking for 2-3 hours.
3. Centrifuge for 2 min at 2500 rpm and remove the supernatant
4. Wash, with milliQ-H_2O
5. Add 1 ml lithium acetate (100 mM) and resuspend the pellet
6. Transfer the solution to an Eppendorf tube and spin for 2 min at 2500 rpm. Remove the supernatant.
7. Add the following to the Eppendorf tube:
    - 350 µl PEG3350 50 %
    - 40 µl lithium acetate (1M)
    - 5 µl DNA carrier
    - 15 µl PCR product
8. Resuspend the pellet by vortexing.
9. Incubate for 20 min at 30^{o}C
10. Place on water bath (42^{o}C) for 10 min
11. Transfer the cells to YPD agar plates and incubate at 30^{o}C overnight

2.9.3 Replica plating

After transformation and incubation for 24 hours the cells are able to express the gene conferring resistance.

In order to select for the cells with the inserted DNA fragment, replica plating was performed. This is a technique where cells from one agar plate are transferred to another agar plate containing a selective media to select for the positive transformants.

Place a sterile velvet or a Whatman^{TM} filter paper on the replica block and gently press the agar plate with the transformants on to the block. To the same velvet/filter paper, press a new selective agar plate. In this way the cells are replicated and makes it possible to only obtain cells with the correctly inserted PCR product.

The plates are then incubated at 30 ^{o}C for 2-5 days, depending on whether the cells are slow growing.

2.10 Microscopy

Bright field microscopy is used in this study in order to check the ratio of dead cells in different media.

ScopeA1 microscope from ZEISS with 63x/1.40 Oil lens were used and the images were obtained using Micro-Manager 1.4.22. The following settings were used: Channel; phase filter 4, exposure; 25 ms, Gain;1 and depth; 12 bits.

Images were processed using Fiji – ImageJ

2.10.1 Preparation of the cells for microscopy

1. Incubate the *S. cerevisiae* strains (*rlm1Δ, mid1Δ and chs7Δ*) + Nat:GAL1p-FKS1 in YPGal and *S. cerevisiae rlm1Δ, mid1Δ and chs7Δ* in YPD overnight at 30ºC

2. Refresh the cells by transferring 200mL of the overnight cells to 2ml YPGal or YPD, incubate at 30ºC for 2 hours

3. For the *S. cerevisiae* (*rlm1Δ, mid1Δ and chs7Δ*) + Nat:GAL1p-FKS1 strains, transfer 1mL to an Eppendorf tube.

4. Centrifugate the cells, remove the supernatant and resuspend in 1mL YPD. Repeat once more.

5. Transfer the cells to a falcon tube and incubate the cells at 30ºC for 1 hour at the time before imaging the cells.

2.11 Statistical analysis

Estimating IC50 values

The half maximal inhibitory concentration (IC50) was used in this study as a measure of how effective the treatment with micafungin was on the different *S. cerevisiae* strains tested. In order to calculate the IC50 values the values obtained from the optical density measurement was uploaded to R studio. The data from the optical density measurement was then fitted to a sigmoidal curve using the R script shown in Appendix D. The IC50 values were then estimated based on this sigmoidal curve using the same R script.

t- test

The t- test was performed using GraphPad and was used in this study to determine if there was a significant difference between the normalized IC50 of *S. cerevisiae* WT-232 and *S. cerevisiae* YKO or DAmP strains.

2.12 Normalization of the data

Normalization was performed on the cell viability data in order to compare the sensitivity of the different strains to each other.

The normalization was carried out using formula 2.7 and the IC50 of WT-DAmP for the different biological replicates was used for this purpose.

$$\text{Normalized data} = \frac{IC50\ (yeast\ strains)}{IC50\ (WT{-}DAmP)} \qquad (2.7)$$

3 Results

The identification of new targets to use in the treatment of the fungal infection could contribute to more effective therapy. In order to find new molecular targets to use in the treatment of drug resistant fungi, the synthetic genetic array was used.

The synthetic genetic array method is used for two different purposes in this study. In the first part, we want to perform SGA screening in order to find genetic interactions of azole resistant strains. As described in the literature certain genes contribute to azole resistance when they are overexpressed. These genes include *ERG11*, *UPC2* and *PDR3*. The 14α-demethylase is encoded by *ERG11* and is involved in the ergosterol synthesis, important for the stability of the cell membrane [22, 40]. *UPC2* and *PDR3* are both regulatory proteins and is involved in the regulation of ergosterol biosynthesis [22]. By performing genetic screening on yeast strains overexpressing these genes we hope to find new molecular targets that can be used in the treatment of azole resistant strains. The overexpression of *ERG11*, *UPC2* and *PDR3* are described to come with a fitness cost and to cause homeostatic alterations that could be possible to targeted [41].

In the second part, publicly available data from genetic screenings will be used to identify new targets of echinocandin resistant strains. As described in the literature, echinocandin resistance is associated with a hot-spot mutation in either *FKS1* or *FKS2*. These mutations come with a fitness cost, and the fungi is dependent on compensatory mechanisms to survive [27]. By targeting the compensatory proteins this could provide us with better treatment options. In this study, new molecular targets will be found based on the negative genetic interactions of *FKS1* and *FKS2*.

For the experiments performed in this study knock- outs of *FKS1 (fks1Δ)* and *FKS2 (fks2Δ)* are used as proxies for the mutation contributing to the drug resistance.

3.1 Identification of new targets to use in treatment of azole resistant fungi

3.1.1 Construction of azole resistant strains

In order to overexpress *ERG11*, *UPC2* and *PDR3* a strong promoter need to be inserted upstream of the gene. Three different promoters were chosen for this purpose, *pADH1*, *pTEF1* and *pGAL1*. These promoters were amplified using the "Toolbox" protocol for the polymerase chain reaction. The templates used were pYM-N8 (*pAHD*1), pYM-N20 (*pTEF1*) and pYM-N24 (*pGAL1*). Amplification of the promoter from these plasmids was carried out using the S1 and S4 primers from the "Toolbox" protocol (see Appendix A for primer sequence). The 50nt overhang of these primers aligns to a sequence upstream of the desired gene. Because of this 50nt overhang, it is possible to insert the promoter at the 5'-end of the gene by transformation through homologous recombination (Figure 7).

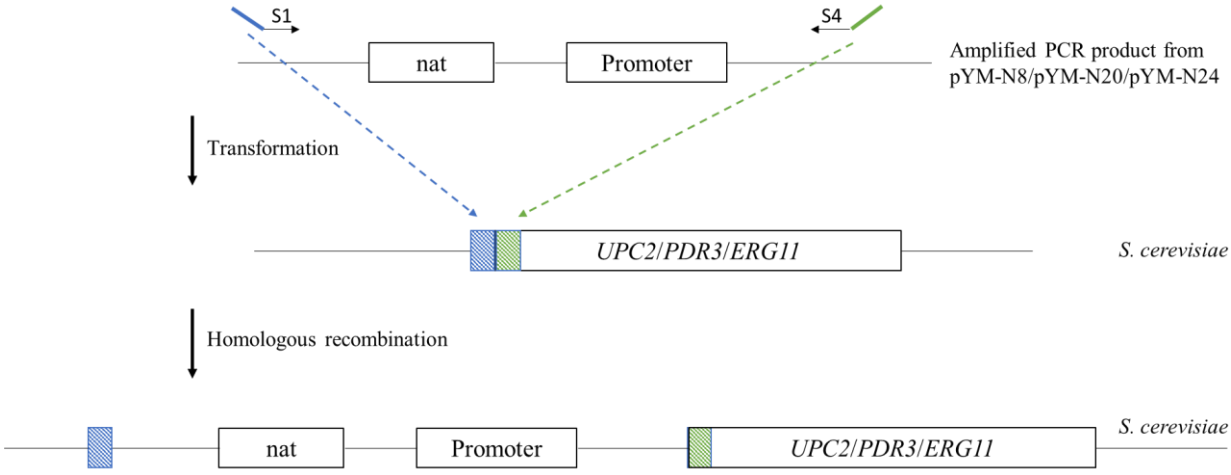


Figure 7. Insertion of promoter (p*ADH1*, *pTEF1* or *pGAL1*) upstream of the gene of interest (*UPC2*, *PDR3* or *ERG11*). First, the promoter is amplified with the S1 and S4 primer. This PCR product is then transformed into *S. cerevisiae* and incorporated into the genome of *S. cerevisiae* by homologous recombination. Blue and green color indicates the 50nt overhang aligning upstream of the gene of interest.

Amplification of promoters

The amplified PCR products from pYM-N8, pYM-N20 and pYM-N24 have the expected lengths of 2996bp, 1929bp and 1989bp, respectively. The amplification of the cassettes from the plasmids was checked by PCR analysis and the observed sizes of the PCR products were consistent with the expected sizes (Figure 8). This indicates that the amplification of the promoters has been successful.

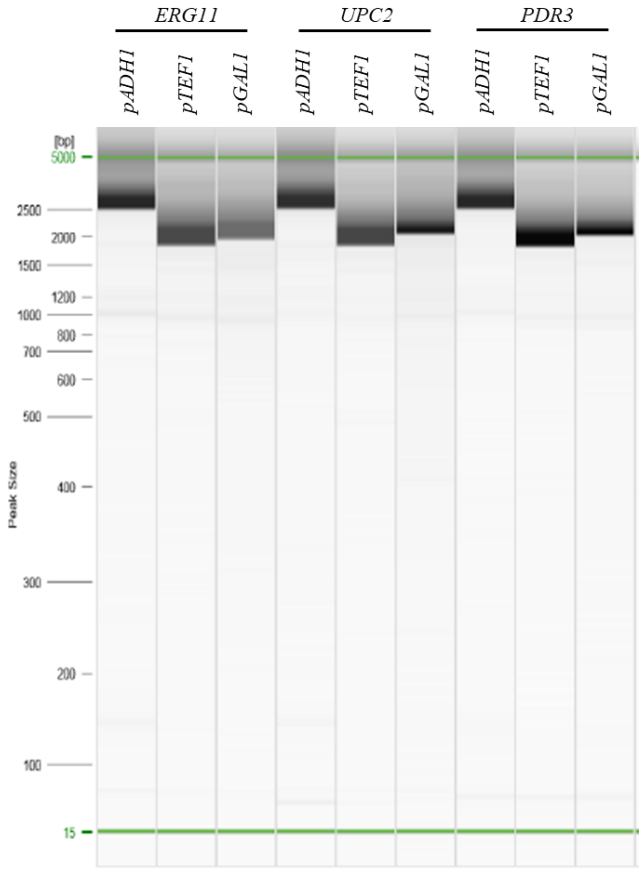


Figure 8. PCR analysis of the amplified PCR products from pYM-N8 (pAHD1), pYM-N20 (pTEF1) and pYM-N24 (pGAL1). Amplification of these cassettes were performed with S1 and S4 primers having a 50nt overhang aligning to either *ERG11*, *UPC2* or *PDR3*.

Insertion of promoter

The amplified cassettes were then transformed into *S. cerevisiae* JEY3862. This strain was used because it has the genetic markers required for the SGA screening.

Transformants for each gene and promoter were obtained and PCR analysis was performed in order to check if the promoter was successfully inserted upstream of each gene (Figure 10). The primers used to check the insertion of the promoter is shown in figure 9. The primer combinations used were 1123 + X-chk1 and X-chk1 + X-chk4. (X refer to which gene the promoter is inserted upstream of, which is either *UPC2*, *PDR3* or *ERG11*). The primer combination 1123 + X-chk1 will only result in an amplified PCR product in the presence of a promoter. This is because 1123 recognizes a sequence of the NAT gene, which only is present if the promoter is inserted in the genome of *S. cerevisiae*. The primer combination X-chk1 + X-chk4 will amplify a short fragment of ~250bp (see table 10 for exact sizes) if no promoter is inserted compared to a successful insertion (Figure 9). For the results shown here (Figure 10) only the amplified products with the primer combination 1123 + X-chk1 are included.

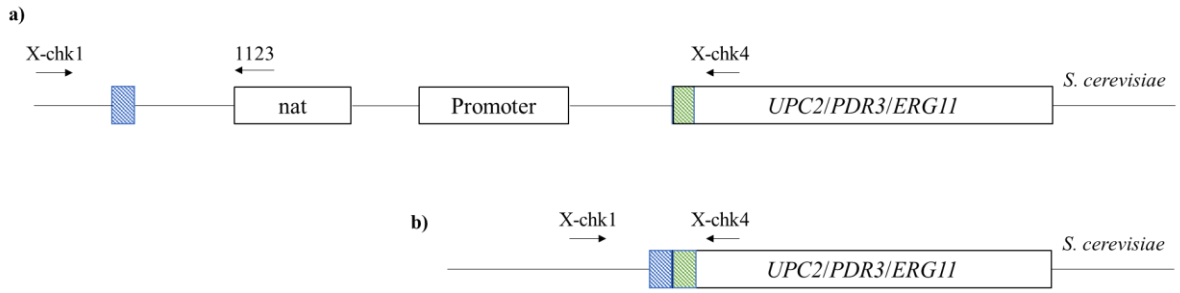


Figure 9. Alignment of primers in a) *S. cerevisiae* with a promoter upstream of the gene or b) in *S. cerevisiae* with no insertion of the promoter.

Table 10. Expected sizes of PCR products amplified with the "Genot" PCR protocol, using the listed primer combination. * corresponds to size of amplified product if promoter is not inserted. The size of the fragment if promoter is inserted is not included because this fragment is too large for the amplification by "Genot" PCR.

Primer combination	**X**	**Size**
1123 + X-chk1	*ERG11*	617bp
	PDR3	658bp
	UPC2	637bp
X-chk1 + X-chk4	*ERG11*	244bp*
	PDR3	291bp*
	UPC2	258bp*

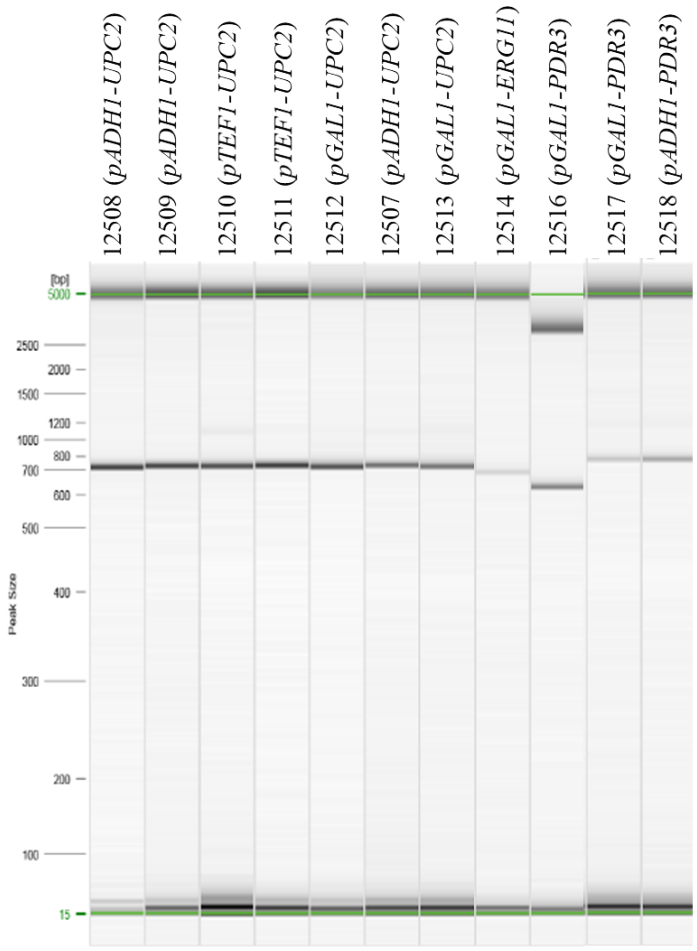


Figure 10. PCR analysis of the transformants checked by "Genot" PCR using primer combination 1123 + X-chk1. X referes to either *ERG11*, *UPC2* or *PDR3*, depending on which gene the promoter is inserted upstream of.

3.1.2 Spot assay

To test if the insertion of a promoter upstream of either *UPC2*, *PDR3* or *ERG11* leads to the overexpression of the gene, thereby contributing to azole resistance, a spot assay on fluconazole plates was performed. *ERG11* is an essential gene, while *PDR3* and *UPC2* both are non-essential genes. For the genes whose expression is controlled by the *GAL1* promoter, the spot assay was performed on YPGal plates supplemented with fluconazole, instead of YPD plates added fluconazole. This is because of the increased expression of the genes in galactose due to *pGAL1* (Figure 11).

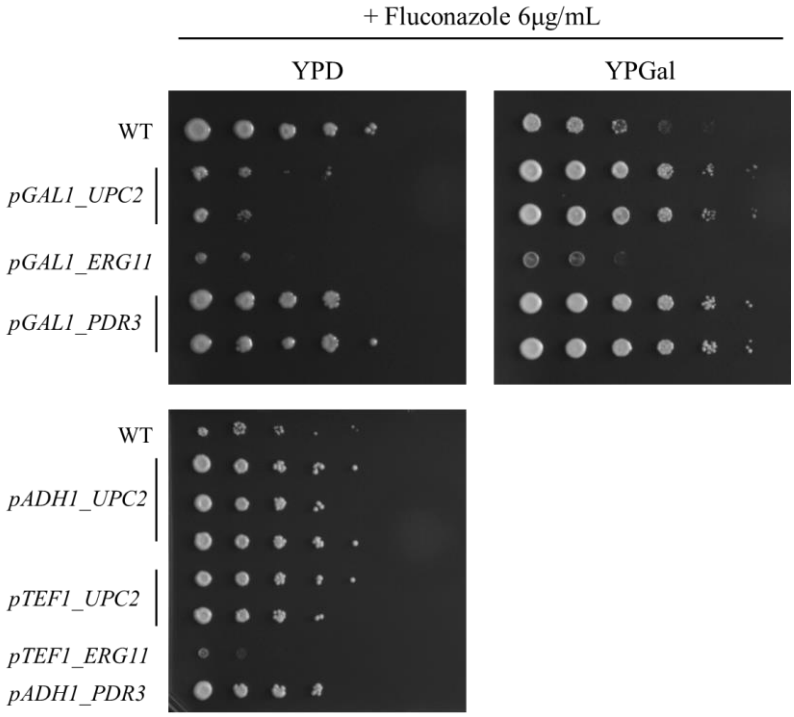


Figure 11. Spot assay of *S. cerevisiae* overexpression strains of *UPC2*, *PDR3* and *ERG11*. Multiple clones were obtained for some of the strains and this is the reason why some strains are performed in duplicates and triplicates.Spot assay of *pGAL1* overexpression strains were performed both in presence of glucose (YPD) and galactose (YPGal). Overexpression strains with *pTEF1* and *pADH1* were performed in presence of glucose.

3.1.3 SGA screening

Overexpression of *ERG11*, *UPC2* and *PDR3* contributes to azole resistance and new treatment options are needed for these azole resistant strains. By performing SGA screening of the constructed strains overexpressing *ERG11*, *UPC2* and *PDR3* new molecular targets could be found. These molecular targets may provide new treatment options for patients having fungal infections that are resistant to azoles.

From the results, the *GAL1* promoter seemed to have the strongest effect on the expression of *UPC2* and *PDR3* (Figure 9). Therefore, *pGAL1-UPC2* and *pGAL1-PDR3* overexpression strains were sent for SGA screening. The result from this screening was not ready by the time the thesis is written and is therefore not included.

3.2 Identification of new targets to use in treatment of echinocandin resistant strains

Certain hot-spot mutations in *FKS1* and *FKS2* are known to contribute to resistance towards echinocandins. New drugs against other molecular targets are needed to cope with the evolving resistance. An alternative approach to find new treatments of drug resistant fungi, were new molecular targets to use in treatment will be found based on the negative genetic interaction of *FKS1* and *FKS2*, will be described here.

3.2.1 Negative interaction genes of *FKS1* and *FKS2* in *Saccharomyces cerevisiae*

Genetic interaction data on *FKS1* and *FKS2* (*GSC2*) was obtained from M. Costanzo *et al.* 2016 [1], and the genetic interaction map of these two genes are shown in figure 12.

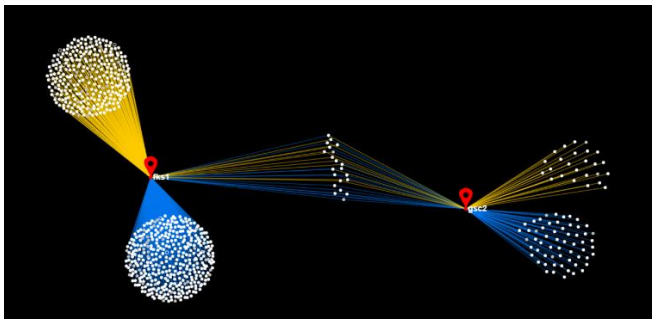


Figure 12. Genetic interaction map of FKS1 and FKS2 (GSC2). Blue color indicates a negative genetic interaction and yellow indicate a positive genetic interaction.

From the negative genetic interactions of these genes, the 20 best hits were chosen. In figure 13 the mutants for the identified genes in either the DAmP collection or in YKO are shown. Only 18 hits are available for the interaction with *FKS1* due to two different variants for *PKC1* and only one of them is included in this study, and one gene (*prp16-ts*) lacking from the collections. For the interaction with *FKS2*, only 19 hits are included because *rpb10-DAmP* was not available in the DAmP collection.

Due to the lack of growth of *yhr177wΔ (ROF1*) and *rpl21bΔ*, these genes were excluded from the experiments in this study.

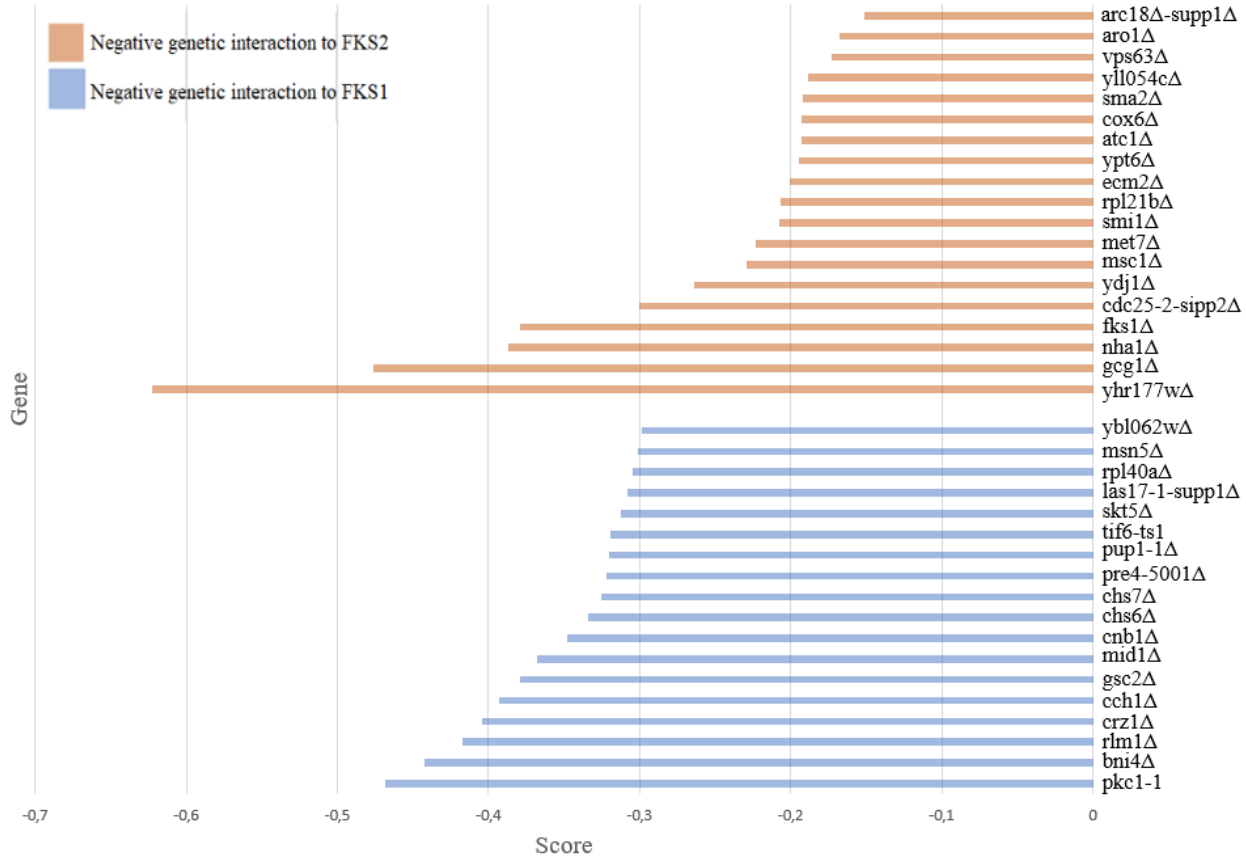


Figure 13. Negative interacting genes of *FKS1* and *FKS2* available in either the DAmP collection or in the yeast knock-out (YKO) collection.

3.2.2 Validation of the negative genetic interactions

It was necessary to validate the reported interactions of *FKS1* and *FKS2* (Figure 13). This validation was performed through cell proliferation assay and spot assay on micafungin gradient plates.

Cell proliferation

The yeast strains were treated with micafungin as a way to validate the interaction between the genes, as micafungin targets *FKS1* and *FKS2*. The hypothesis states that blocking the function of these genes, in the absence of a compensatory gene, increases sensitivity towards micafungin. An increased sensitivity could indicate a negative genetic interaction between *FKS1*, *FKS2* and their interacting genes.

Due to time limitations, only the yeast strains shown to be more sensitive to micafungin in the first biological replicates were chosen to be tested with three biological replicates.

After a 24-hour treatment of the strains with micafungin, the IC50 values were calculated. The IC50 values differed between the biological replicates. However, a bias in the data was observed and all strains in each experiment had deviating IC50 values in a similar pattern. This allowed us to normalize the data.

Because two different WTs were used it was possible to do a t-test comparing the normalized IC50 of all strains to the normalized IC50 of WT-232.

The normalized data is shown in figure 14, and we observe that multiple of the strains tested have an increased sensitivity to micafungin compared to WT.

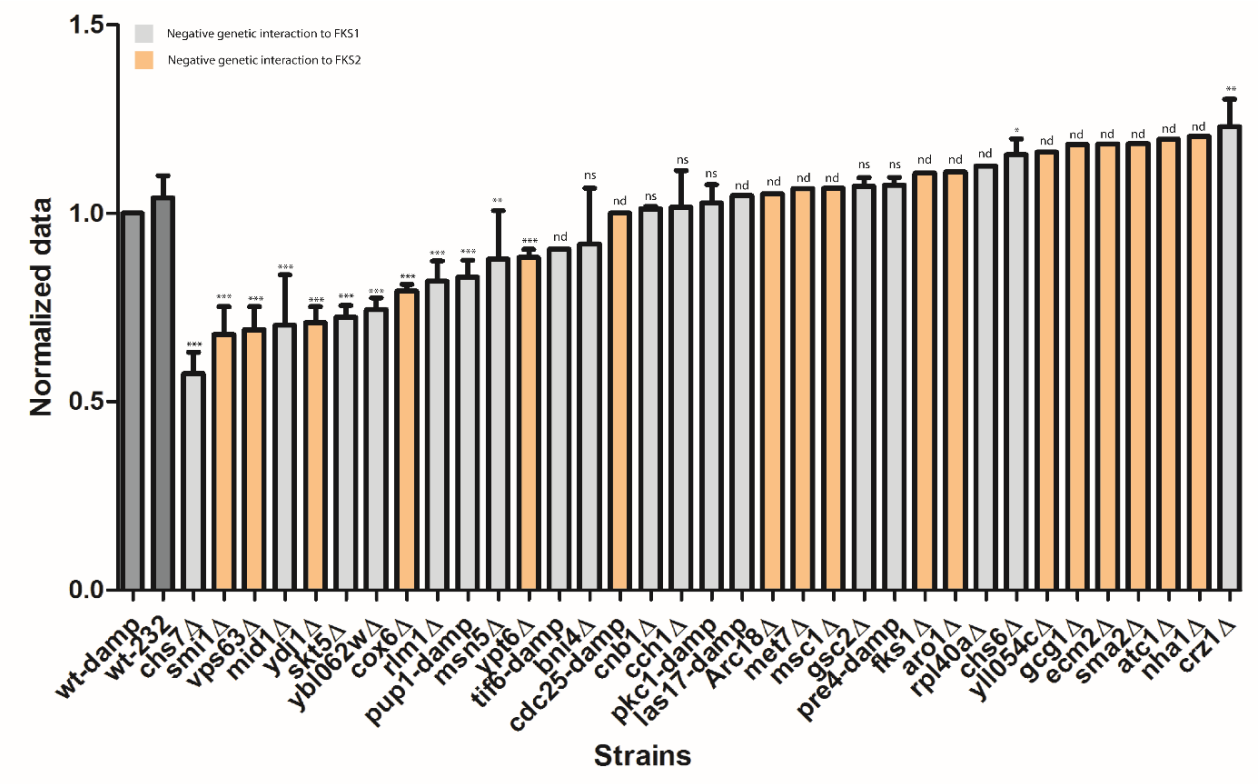
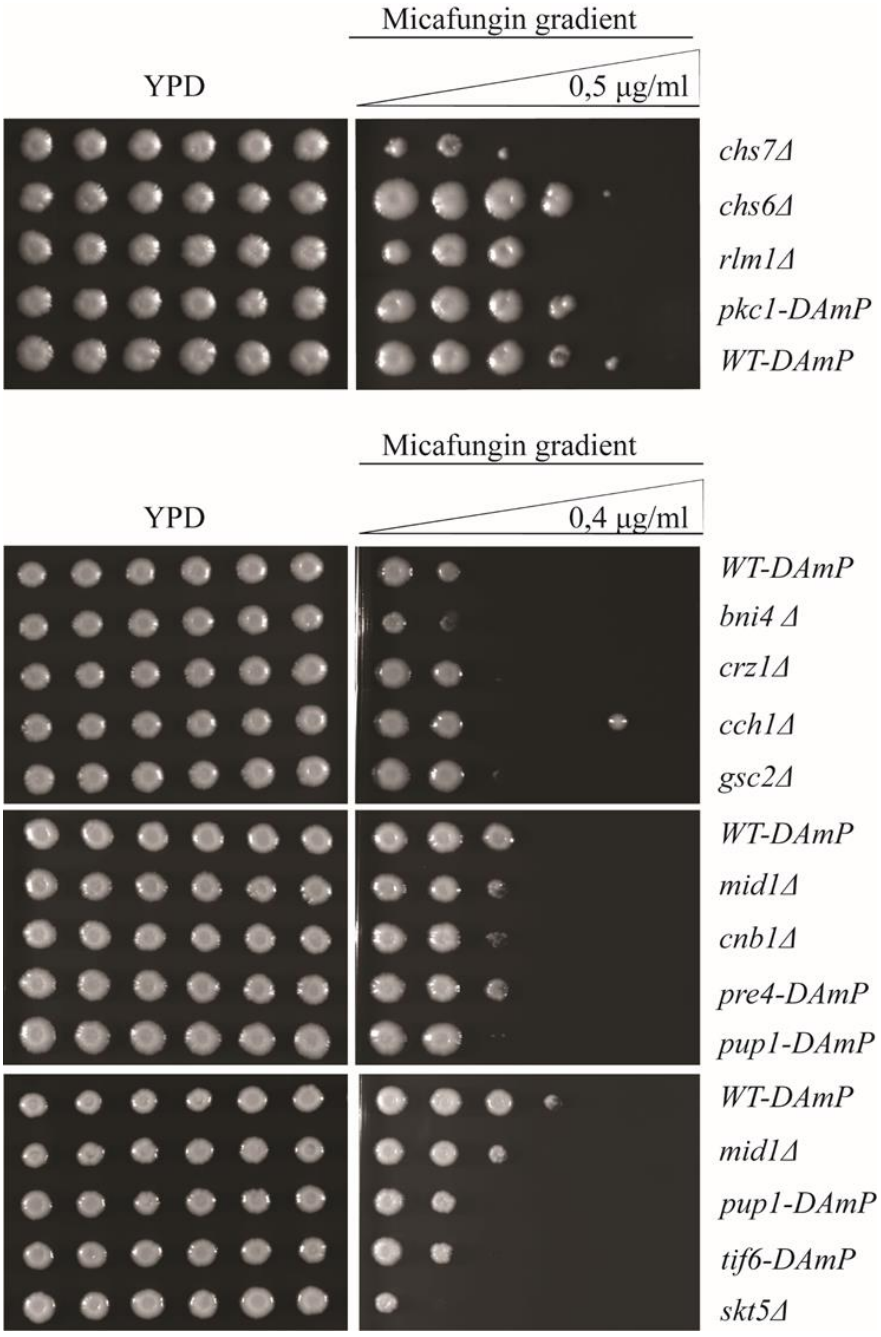


Figure 14. Normalized data of the reported negative genetic interactions of *FKS1* and *FKS2* in *S. cerevisiae* YKO or DAmP collection. *ns (non-significant), nd (non- detectable), * (p-value ≤ 0,05), ** (p-value ≤ 0.001), *** (p-value < 0.0001)*

Spot assay

In addition to cell proliferation, a spot assay on micafungin gradient plates was also performed (Figure 15). This was to support the results obtained from the cell proliferation assay. Yeast strains that were shown to be more sensitive to micafungin in the cell viability experiment are expected to have a slower growth on micafungin gradient plates than the WT strain. The strains were grown both on YPD plates and on micafungin gradient plates. YPD plates were used to control that none of the strains tested had any fitness defect.



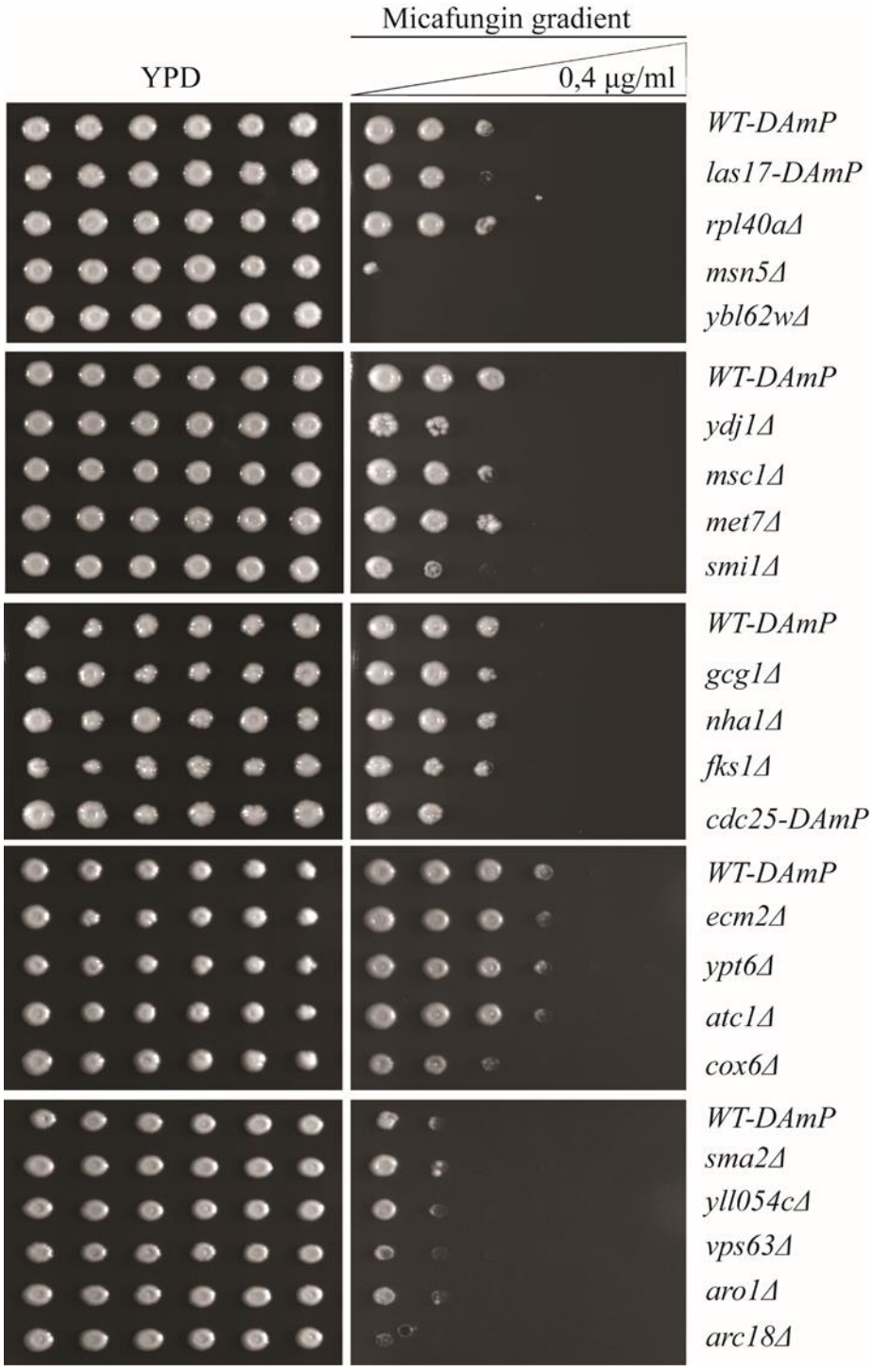


Figure 15. Spot assay on YPD plates and micafungin gradient plates (0.4µg/ml or 0.5µg/ml). The growth of all yeast strains was compared to the growth of WT-damp.

From the spot assay an increased sensitivity (Two or more spots difference from WT) is observed in *msn5Δ*, *ybl062wΔ, pup1-damp*, *tif6-damp, chs7Δ, rlm1Δ* and *skt5Δ* YKO/DAmP strains. A slightly smaller increase in the sensitivity (One spot difference from WT) is observed in *cox6Δ, vps63Δ, arc18Δ, cdc25-DAmP, smi1Δ, ydj1Δ, mid1Δ, cnb1Δ, bni4 and pkc1-damp.* All the strains shown to be more sensitive to micafungin from the normalized IC50 (Figure 14) are also shown to be more sensitive in the spot assay.

The spot assays performed are almost consistent with the cell proliferation data. Only a few more strains are shown to be more sensitive in the spot assay and these include, *pkc1-damp, tif6-damp, arc18Δ, cdc25-damp, las17-damp* and *cnb1Δ*.

3.2.3 Construction of double knock-out strains

For further validation of the genetic interactions, a set of double knock-outs was constructed. As a proxy for the mutation causing the resistance towards echinocandins, knock-outs of *FKS1* and *FKS2* were used instead. We assume that the drug resistant-associated mutation reduces the gene function and that the knock-out of the gene might have a similar effect on the cells.

Double knock out strains was constructed by replacing *FKS1* or *FKS2* with the NAT resistance cassette from pFA6a-natNT2. This cassette was amplified with the S1 and S2 primers using the "Toolbox" PCR protocol (Figure 17a). The NAT cassette was then transformed into *S. cerevisiae* YKO strain 2739 (*rlm1Δ*), 1153 (*mid1Δ*), 2835 (*chs7Δ*), 3012 (*ydj1Δ*), 5882 (*smi1Δ*) and 5170 (*vps63Δ*), replacing *FKS1*/*FKS2* by homologous recombination (Figure 16). The replacement of *FKS2* with NAT in YKO 3012 (*ydj1Δ*), 5882 (*smi1Δ*) are shown in Figure 17b and 17c.

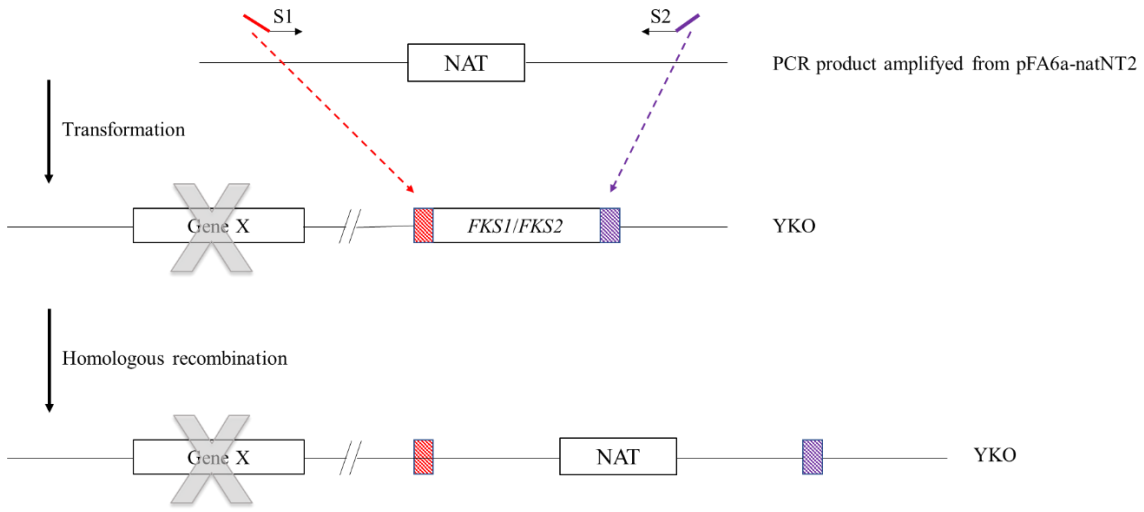


Figure 16. Replacement of *FKS1* or *FKS2* with NAT resistant cassette by homologous recombination of *S. cerevisiae* YKO strains. Following YKO strains used in construction of the double knock-outs: 2739 (rlm1Δ), 1153 (*mid1Δ*), 2835 (*chs7Δ*), 3012 (*ydj1Δ*), 5882 (*smi1Δ*) and 5170 (*vps63Δ*). Gene X refers to the gene deleted in the YKO strains. Red and purple color indicates the 50nt overhang aligning upstream and downstream, respectively, of *FKS1* and *FKS2*.

Table 11. Expected sizes of amplified PCR products

Template	Primer combination	Size
pFA6a-natNT2	FKS1-S1 + FKS1-S2	1449bp
	FKS2-S1 + FKS2-S2	
smi1Δ/fks2Δ	1124 + fks2-chk2	542bp
ydj1Δ/fks2Δ	fks2-chk3 + fks2-chk3	250bp

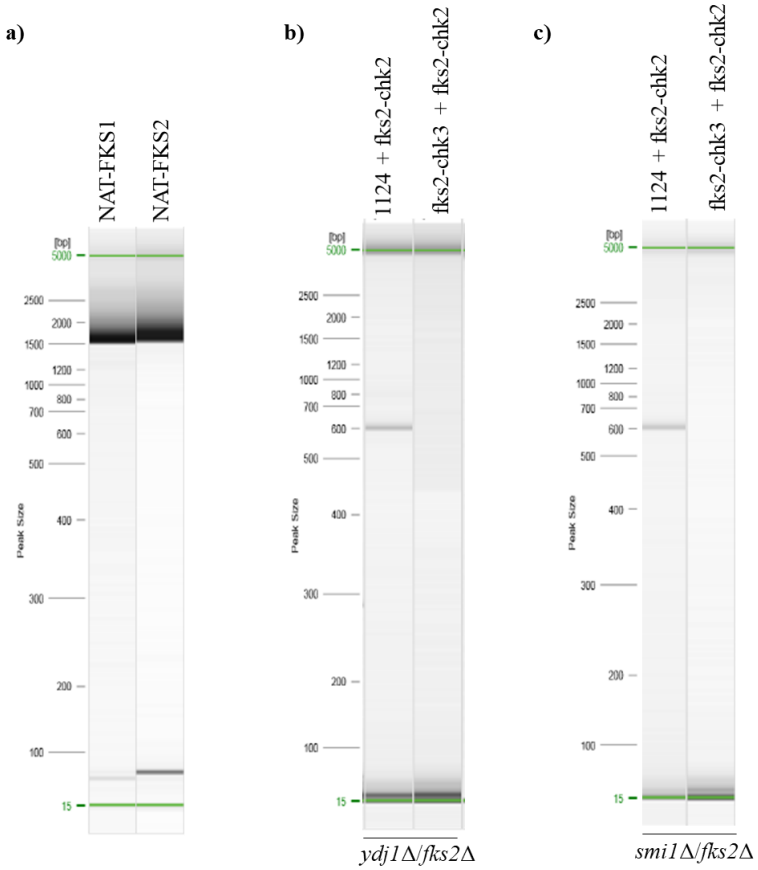


Figure 17. PCR analysis of the construction of double knock out strains in *S. cerevisiae*. a) Amplification of the NAT cassette from pFA6a-natNT2. One NAT cassette amplified with primers having a 50nt overhang aligning to *FKS1*, thereby NAT-*FKS1*. The other NAT cassette was amplified with primers having a 50nt overhang aligning to *FKS2*, thereby NAT-FKS2. b) and c) checking the deletion of *FKS2* using primer combination 1124 + fks2-chk2 and fks2-chk3 + fks2-chk3. The first primer combination confirms the deletion because the 1124 primer only aligns to the NAT gene, which replaces *FKS2*. If *FKS2* is not deleted a 250bp fragment is expected to be amplified with the fks2-chk3 + fks2-chk3 primer combination.

After obtaining the double knock-outs for *smi1Δ/fks2Δ* and *ydj1Δ/fks2Δ* the strains were grown on YPD plates (Figure 18a and 19). This was performed to check if the deletion of *FKS2* had any impact on the fitness of the yeast. No fitness defect was observed for the double knock-out strain. Because the growth of the constructed strains was not affected, the strains could be introduced to a new condition. The double knock-out strains were then grown in the presence of micafungin (Figure 19). We hypothesize that the double knock- out strains when treated with micafungin will be more sensitive than the single knock-out. This is because we inhibit a possible compensatory mechanism as well as both *FKS1* and *FKS2* (Figure 18b).

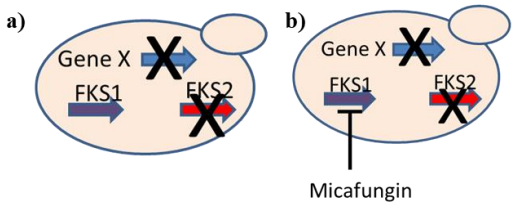


Figure 18. Visualization of the constructed double knock-out strains in *S. cerevisiae*. Gene x indicates either *ydj1Δ* or *smi1Δ*. a) Double knock-out when grown on YPD. b) Double knock-out when treated with micafungin, thereby inhibition *FKS1*.

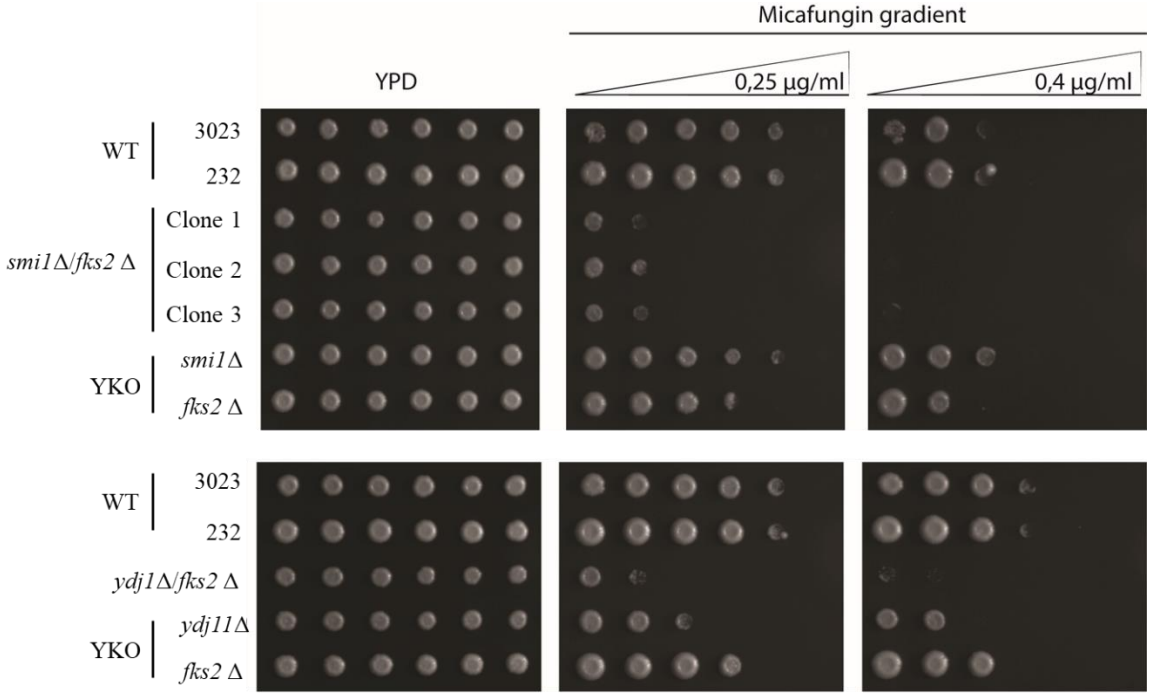


Figure 19. Spot assay of the constructed double knock-out smi1Δ/fks2Δ *and* ydj1Δ/fks2Δ, on both YPD plates and micafungin gradient plates (0.25µg/ml and 0.4µg/ml). Both *S. cerevisiae* 3023 and 232 were included as WT strains. Single knock-out strains from the YKO collection were also included in the experiment, these include 5882 (*smi1Δ*), 3012 (*ydj1Δ*) and 6979 (*fks2Δ*).

We observe from the spot assay performed that the deletion of both *FKS2* and its interacting gene (*smi1* of *ydj1*) is a lethal combination when the cells are treated with micafungin. Both single knock-outs of *smi1, ydj1* and *fks2* are capable of growing in the presence of micafungin, suggesting that *smi1* and *ydj1* could be possible molecular targets of drug resistant fungi.

3.2.4 Conditional knock-down of *FKS1*

In the previous section double knock-out strains were constructed, only two out of six double knock-outs were obtained. A possible reason for this is that the double mutant might have a non-viable phenotype. To test this hypothesis, the conditional knock-down of *FKS1* was performed. *FKS2* is not included due to time limitations.

For the conditional knock-down the expression of *FKS1* was controlled by the *GAL1* promoter. This means that *FKS1* is expressed in the presence of galactose and shut down in the presence of glucose. Conditional knock-down of *FKS1* in YKO *chs7Δ, rlm1Δ* and *mid1Δ* was constructed.

The "Toolbox" PCR protocol was used to amplify the *GAL1* promoter from pYM-N24. The primer combination FKS1-S1 + FKS1-S4 was used for this purpose and result in a fragment of 1989bp (Figure 21a).

The amplified cassette containing both *GAL1* promoter and the NAT marker was then transformed into *S. cerevisiae* the YKO strains 2835 (*chs7Δ*), 2739 (*rlm1Δ*) and 1153 (*mid1Δ*). Due to the 50nt overhang of the primers used in the amplification of the cassette the promoter could be incorporated into the genome of *S. cerevisiae* by homologous recombination (Figure 20).

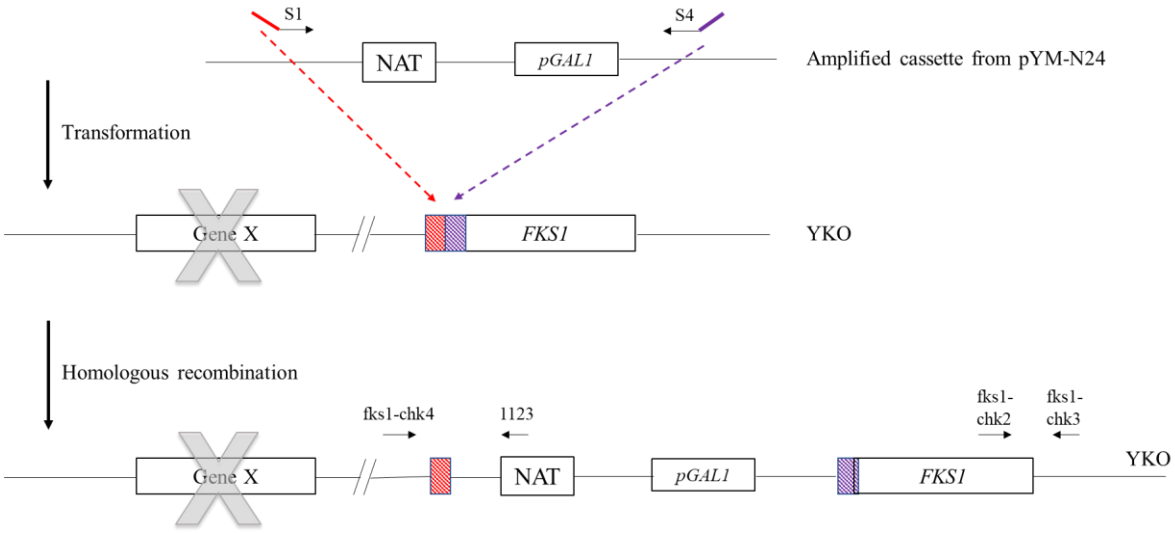


Figure 20. Insertion of GAL1 promoter in YKO strain 2835 (*chs7Δ*), 2739 (*rlm1Δ*) and 1153 (*mid1Δ*). GAL1 promoter is first amplified with the "Toolbox" protocol using the S1 and S4 primer for *FKS1*. The amplified cassette is then transformed into the YKO strains and inserted into the genome by homologous recombination. This process is possible due to the 50nt overhang of the primers that align upstream of *FKS1*, indicated by red and purple color in the figure. Gene X refers to the gene deleted in the YKO strains. The primers used to check the insertion of the promoter and where they attach are indicated in the figure.

The insertion of the *GAL1* promoter was followed by "Genot" PCR and PCR analysis of the obtained transformants (Figure 21b). This was performed to check that the amplified cassette had been successfully inserted into the genome of *S. cerevisiae*. The following primer combinations were used: A: fks1-chk4 + 1123 and B: fks1-chk2 + fks1-chk3. Combination A is expected to produce a 591bp fragment and will only be amplified if the *GAL1* promoter is inserted. Combination B confirms that *FKS1* is present and is expected to amplify a 264bp fragment.

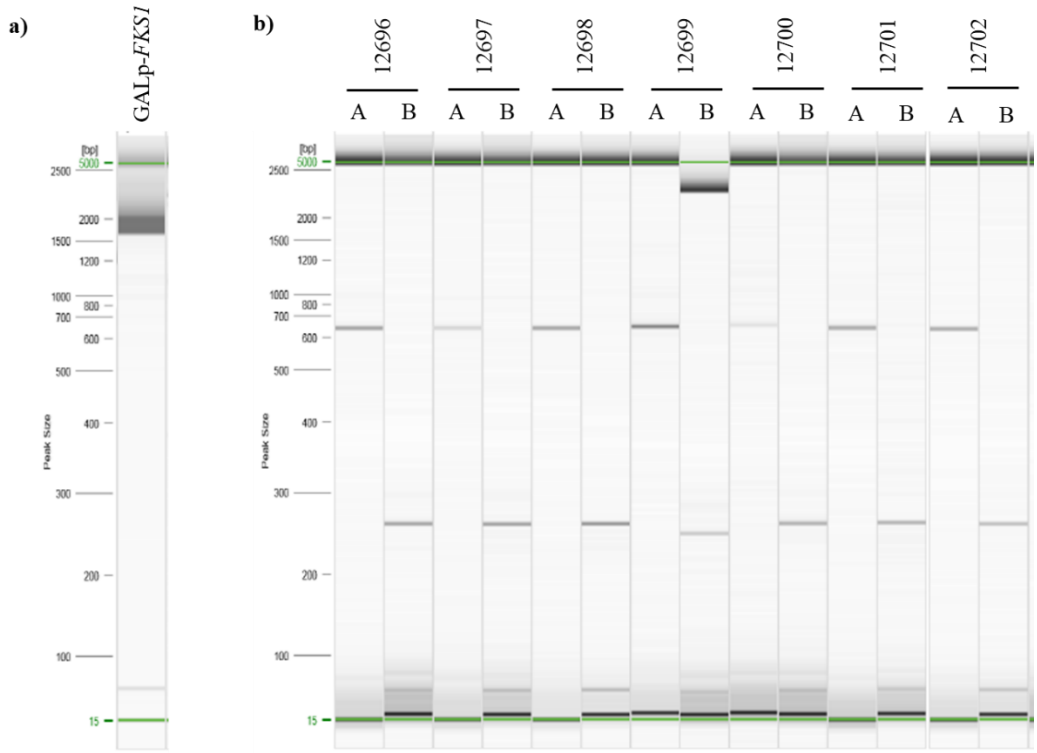


Figure 21. PCR analysis a) of GAL1 promoter (1989bp) amplified using "Toolbox" PCR protocol and b) checking the insertion of GAL1 promoter in S. cerevisiae by "Genot" PCR protocol. Primer combination A: fks1-chk4 + 1123 (591bp), B: fks1-chk2 + fks1-chk3 (264bp). S. cerevisiae strain 12696- 12700: chs7Δ/GAL1p-FKS1, 12701: rlm1Δ/GALp-FKS1, 12702: mid1/GAL1p-FKS1.

To check that the insertion of the *GAL1* promoter does not affect the fitness of the cells, a spot assay on plates with galactose (YPGal plates) and glucose (YPD) was performed. No fitness defect was observed in the conditional knock-down strains compared to the single knock-out strains (Figure 22). The following spot assay was performed in the presence of glucose (YPD plates) instead of galactose. This leads to the downregulation of *FKS1* and the effect of this is shown in figure 20.

From the result, we observe that knock-down of *FKS1* results in a reduction in the growth (*rlm1Δ/GAL1p-FKS1 and mid1Δ/GAL1p-FKS1)* or full inhibition of growth (*chs7Δ/GAL1p-FKS1*) compared to their respective single knock-out strains. This reduction in growth could be the reason why it is not possible to obtain double knock-out of more than *smi1Δ*/*fks2Δ* and *ydj1Δ/fks2Δ*.

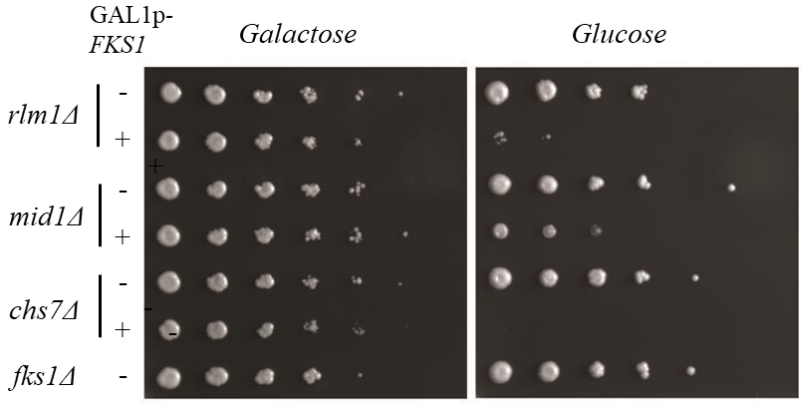


Figure 22. Spot assay of conditional knock-down strains of FKS1 (GAL1p-FKS1 +) constructed in YKO strain 2835 (*chs7Δ*), 2739 (*rlm1Δ*) and 1153 (*mid1Δ*). The spot assay was performed on YPGal (Galactose) and YPD (Glucose) plates.

3.2.5 Microscopy analysis

Microscopy of the yeast with downregulation of *FKS1* was then performed to get an indication of whether the cells stop proliferating or if they die.

The conditional knock-down strains were first grown in the presence of galactose (YPGal) for the yeast cells to be able to express *FKS1*. Each strain was then split into two different batches, one grown in galactose and one grown in glucose (YPD). The yeast cells were then imaged after 1 hour and after 2 hours in both media (Figure 23). As a control, the single knock-out strains 2835 (*chs7Δ*), 2739 (*rlm1Δ*) and 1153 (*mid1Δ*) in the YKO collection was grown in YPD and imaged after 1 hour (Figure 24). The percentage of dead cells observed from the microscopy were then quantified (Figure 25).

From the results (Figure 23-25) an increase in the ratio of dead cells is observed with increasing exposure in glucose. This is true for the strains were *FKS1* is under the control of the *GAL1* promoter. In the strains were *FKS1* is not downregulated, no growth defect is observed and the percentage of dead cells are minimal.

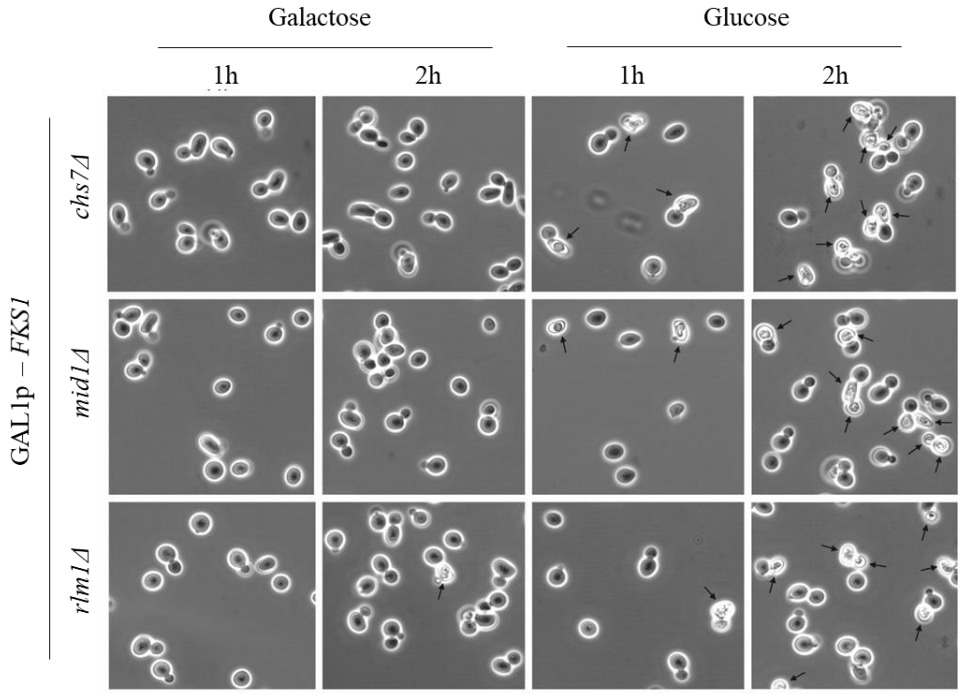


Figure 23. Microscopy of the conditional knock-down strains of *FKS1* constructed in this study. Images of the yeast cells were obtained after 1 hour and 2 hours incubation in medium containing either galactose (YPGal) or glucose (YPD). Arrows indicates dead cells.

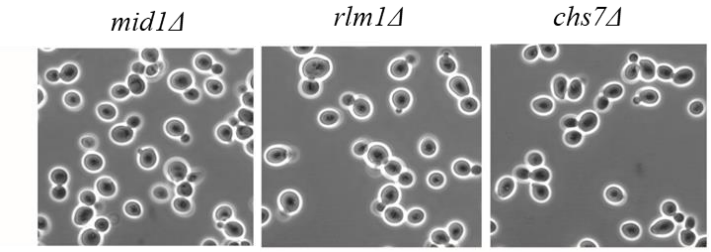


Figure 24. Microscopy of the YKO strain 2835 (*chs7Δ*), 2739 (*rlm1Δ*) and 1153 (*mid1Δ*) after 1 hour incubation in YPD.

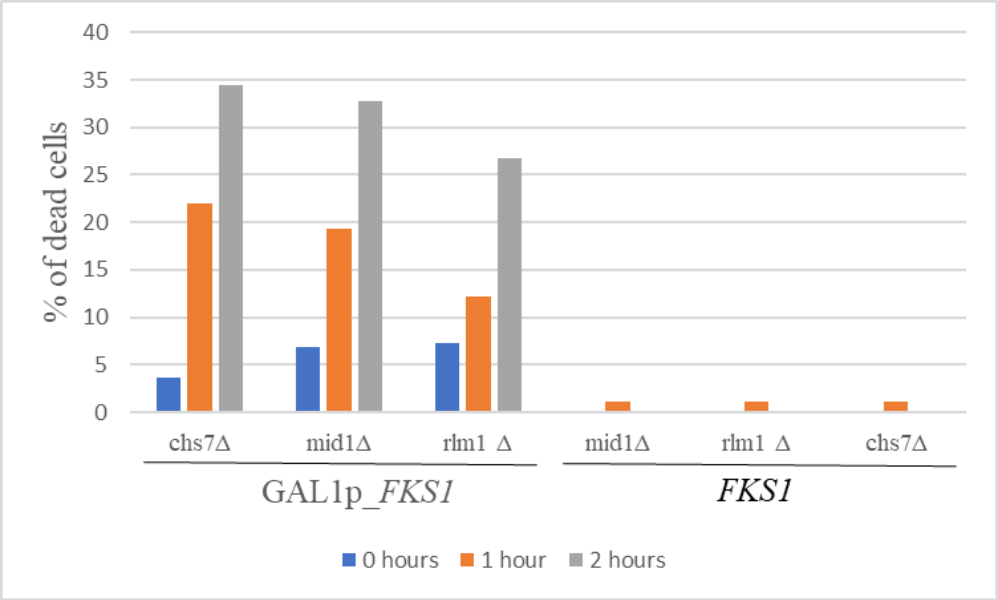


Figure 25. Quantification of the percentage of dead cells when FKS1 expression is downregulated. Hours indicates the time in glucose, meaning that 0 hours is the percentage of dead cells in presence of galactose.

3.2.6 HSP90 inhibitors in treatment of echinocandin resistant fungi

One of the genes shown to interact with *FKS2*, and affect the sensitivity to treatment with echinocandin, was the *YDJ1* gene. This gene is a type 1 HSP40 co-chaperone and is known to be involved in the regulation of HSP90. We hypothesized that echinocandin resistant strains are more sensitive to HSP90 inhibitors and that *YDJ1* and HSP90 could be possible targets for the treatment of drug resistant fungi.

Therefore, we tested two HSP90 inhibitors on the *fks1Δ* YKO strain and WT *S. cerevisiae* cells (Figure 26). The HSP90 inhibitors used were Onalespib and Ganetespib. Testing of the *fks2Δ* YKO strain was not included due to time limitations.

An increase in the sensitivity towards treatment with HSP90 inhibitors in *S. cerevisiae fks1Δ* compared to WT was observed. Onalesbib and Ganetespib showed the same sensitivity towards the two strains tested and is the reason why there is only one IC50 curve for the strains tested.

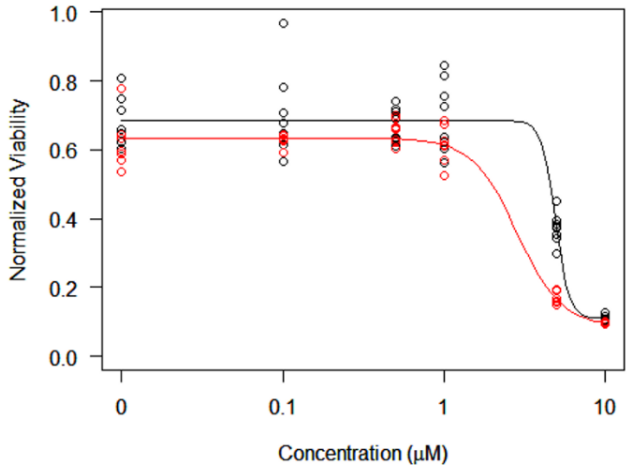


Figure 26. IC50 curves of *S. cerevisiae* WT (black line) and *S. cerevisiae fks1Δ* (red line) treated with HSP90 inhibitor (Onalespib and Ganetespib) for 24 hours.

3.3 Validation of negative genetic interactions in *Candida glabrata*

Next, the negative interacting genes of *FKS1* and *FKS2* found to have an impact on the sensitivity to echinocandin treatment in yeast needed to be validated in *C. glabrata*. Even though knock out of *FKS1* and *FKS2* in *S. cerevisiae* might work as a good proxy for the mutations contributing to resistance in *C. glabrata*, a validation in *fks1* mutant in *C. glabrata* would be of great interest.

The approach used for this validation was executed by replacing the gene of interest with the NAT gene instead of the NAT cassette. Because NAT was amplified without its promoter, NAT will only be transcribed if inserted correctly at the 3'- end of the promoter of the deleted gene (Figure 27).

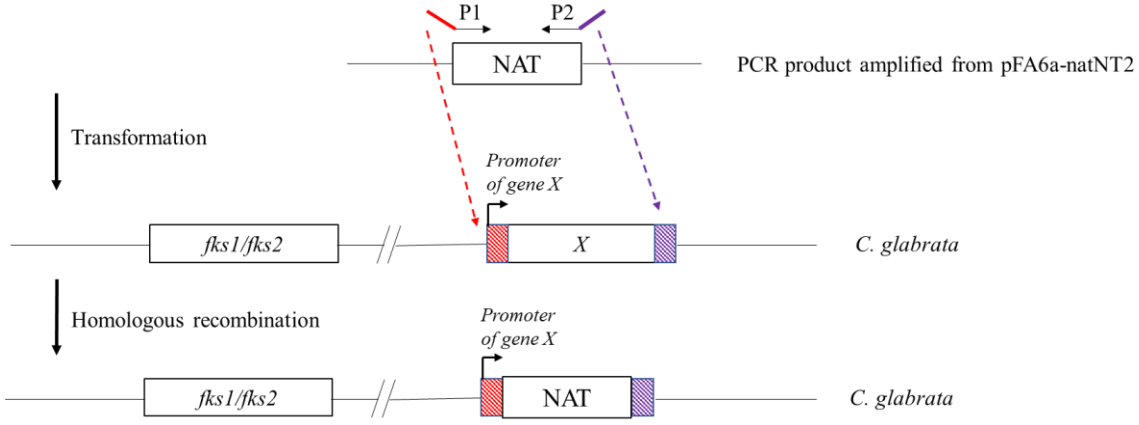


Figure 27. Deletion of gene in *C. glabrata* strain resistant to echinocandin, by replacing it with NAT through homologous recombination. This is possible due to the 50nt overhang of the P1 and P2 primers used to amplify NAT, shown as the red and purple color. The 50nt overhang align to sequences upstream and downstream of gene X.
X refer to the gene (*RLM1*, *CHS7*, *MID1*, *YDJ1*, *SMI1* or *VPS63*) having a negative genetic interaction to either *FKS1* or *FKS2*.

3.3.1 Sensitivity of *C. glabrata* to NAT

Before deleting the gene of interest, the tolerance of which *C. glabrata* can grow in the presence of NAT was necessary to be established. This is because transformants with the gene deleted (replaced by the NAT gene) is selected based on the growth in NAT. The minimal concentration of NAT required for inhibition of growth was therefore exanimated. A spot assay of *C. glabrata* WT and FKS1-L662W on YPD plates supplemented with NAT was therefore performed (Figure 28).

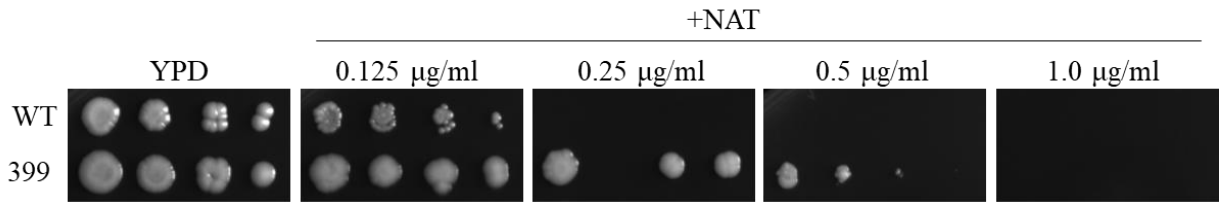


Figure 28. Spot assay of *C. glabrata* WT and 399 (*C. glabrata* FKS1-L662W) on YPD plates supplemented with NAT (0.125 µg/ml, 0.25 µg/ml, 0.5 µg/ml and 1.0 µg/ml). Note that one spot is missing for C. glabrata 399 from YPD plate supplemented with 0.25 µg/ml NAT, due to an uneven replicator used in the experiment.

A concentration of 1.0 µg/ml NAT was required for inhibiting the growth of both *C. glabrata* WT and *C. glabrata FKS1-L662W*.

3.3.2 Construction of *C. glabrata* deletion strains

To delete the genes (*RLM1, CHS7, MID1, YDJ1, SMI1* or *VPS63*) from *C. glabrata* the NAT gene from pFA6a-natNT2 was amplified using the "Toolbox" protocol (Figure 29). The amplification of the NAT gene was performed using the P1 and P2 primers. The amplified PCR product of NAT is expected to be a fragment of 676 bp.

Amplification of the NAT gene (Figure 29) was successful for NAT amplified with P1 and P1 primer aligning to *MID1*, *SMI1* and *VPS63*.

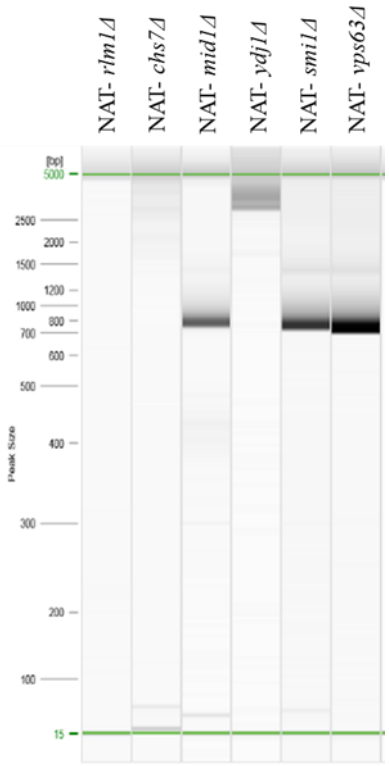


Figure 29. PCR analysis of the NAT gene amplified with the P1 and P2 primers having a 50nt overhang aligning to either *RLM1, CHS7, MID1, YDJ1, SMI1* or *VPS63*. Successful amplification of the NAT gene is expected to give a 676 bp fragment.

The successfully amplified NAT gene aligning to either *MID1*, *SMI1* or *VPS63* was then transformed into both *C. glabrata* WT strain and the echinocandin resistant *C. glabrata* strain *fks1-L662W*. Transformants were obtained for all combinations; *mid1Δ*, *smi1Δ* and *vps63Δ* in both *C. glabrata* WT and *C. glabrata fks1-L662W*. However, due to the time limitations of this master thesis, validation of these *C. glabrata* deletion strains remains to be performed.

4 Discussion

A rise in the resistance towards drugs used in the clinic for treating fungal infections is increasing and it is critical to develop new treatment options. Different strategies can be used in the development of new antifungal agents and screening of small synthetic molecules is one of the most common ways [28]. To screen and develop new drugs, the identification of new molecular targets are essential [31]. The goal of this study was to identify new molecular targets of drug resistant fungi. With a focus on azole and echinocandin resistant fungi, as these are the drugs that are most commonly used in the clinic. The approach used in this master thesis to identify new targets was based on genetic screenings and the findings will be addressed in the following segments.

4.1 Identification of new targets to use in treatment of azole resistant fungi

Resistance towards azoles can be caused by several different mechanisms. One of the mechanisms known to contribute to azole resistance is the upregulation of certain genes. Among the genes contributing to azole resistance when overexpressed are for instance *ERG11*, *UPC2* and *PDR3 (PDR1 in C. glabrata)* [5, 42]. However, it seems like the overexpression comes with a fitness cost [41]. This implies that the fungi depend on compensatory mechanisms to overcome the fitness defect. This allows us to identify new targets based on the compensatory mechanisms that are activated when the fungi have developed resistance to azoles.

The results confirm that overexpression of *UPC2* and *PDR3* contribute to azole resistance as these strains are more resistant to fluconazole. Contrary to the expectation that overexpression of *ERG11* would be more resistant to fluconazole, we do not observe any increase in the resistance. The fact that *ERG11* is an essential gene under normal growth conditions could be one reason for this. Another possible reason is the *GAL1* promoter is weaker than the promoter of *ERG11* itself, thereby contributing to reduced gene expression *of ERG11*.

The results from the SGA screening of *S. cerevisiae* strains overexpressing *UPC2* and *PDR3* remains to be analyzed. The hypothesis is that the genetic interaction data from this screening can reveal some potential targets that can be used in the treatment of azole resistant fungi.

4.2 Identification of new targets to use in treatment of echinocandin resistant strains

Echinocandin resistance is associated with hot-spot mutations in either *FKS1* or *FKS2*, and such mutations are known to have an effect on the fitness of the fungi [27]. This means that the fungi depend on compensatory mechanisms in order to cope with the reduced fitness. This can be used as an advantage to find new targets that can be used in the treatment of the echinocandin resistant fungi. The approach used in this study to identify new molecular targets were based on genetic interaction data performed by M. Costanzo *et al.* 2016 [1]. These data provide us with information about negative genetic interactions of *FKS1* and *FKS2* that could be possible targets to use in the therapy of drug resistant fungal infections. Here it is important to note that these data are based on knock-outs of the genes rather than the mutated *fks1* and *fks2*. For this study, we therefore assumed that the mutation contributing to echinocandin resistance to some extent would suppress the gene function and that the knock-out of the genes would have a similar effect.

A total of 12 genes with a negative genetic interaction to *FKS1* and *FKS2* from the genetic interaction data were shown to have an impact on the sensitivity towards echinocandin. Among the genes validated to interact with *FKS1* and *FKS2* we find *CHS7, SMI1, VPS63, MID1, YDJ1, SKT5, YBL062W, COX6, RLM1, PUP1, MSN5* and *YPT6* (See Table 12 for molecular function). According to the genetic interaction data, all the genes tested should have been more sensitive to echinocandin treatment. However, this differs from the observations, as only a limited amount of the genes were shown to interact with *FKS1* and *FKS2*.

Even though the negative interactions only are validated in *S. cerevisiae* it is reasonable to believe that the same genes will be of importance in *C. glabrata.* The work performed by Caplan et al. 2018 [43] identified genes involved in cellular responses to echinocandin treatment in *C. albicans*. Among the identified genes, HSP90 and *PKC1* were found to have an effect on the sensitivity towards echinocandin treatment in *C. albicans*. *PKC1* is one of the genes shown in the Costanzo [1] data to interact with *FKS1* and thereby indicates that this could be a possible target of echinocandin resistant fungi. The Caplan data [43] showing that *PKC1* plays an important role in *C. albicans* treated with echinocandin suggests that our findings are relevant for *C. glabrata.* HSP90 was also observed in this study to be of importance in the sensitivity

towards echinocandins as *YDJ1*, a regulator of HSP90 [44], was shown to have a negative genetic interaction to *FKS1 and FKS2*.

Some of the genes validated to have a negative genetic interaction to either *FKS1* or *FKS2* were further validated by checking the sensitivity of double knock-out strains with echinocandin treatment. The double knock-out strains of *smi1Δ/fks2Δ* and *ydj1Δ/fks2Δ* were not capable of growing in the presence of 0,4 μg/ml micafungin, indicating that *SMI1* and *YDJ1* are important for maintaining fungal growth. These results suggest that *SMI1* and *YDJ1* could work as good targets in the treatment of echinocandin resistant fungi.

In addition to the constructed double knock-out strains *smi1Δ/fks2Δ* and *ydj1Δ/fks2Δ,* double knock-outs of *rlm1Δ, mid1Δ, chs7Δ* and *vps63Δ* were also tried, without any success. A possible reason for this is that the genes play an important part in maintaining growth when *FKS1* or *FKS2* are deleted. In order to test this hypothesis, the conditional knock-down of *FKS1* was performed in knock-out strains of *RLM1*, *MID1* and *CHS7*. From the result we observed that knock- down of *FKS1* led to a reduction in the growth or no growth of the cells at all, thereby explaining the reason for the missing double knock-outs of these genes. An increased number of dead cells were observed when *FKS1* was downregulated compared to the functional *FKS1*.

Table 12. Molecular function of the negative interacting genes of *FKS1* and *FKS2 shown to affect the sensitivity to echinocandin treatment*. * The open reading frame (ORF) of YBL062W overlaps with *SKT5*.

Gene	**Molecular function**	**Reference**
CHS7	Chitin synthesis	[45, 46]
SMI1	Cell wall integrity	
VPS63	Vacuolar protein sorting	[47]
MID1	Ca^{2+} influx and Sensor for mechanical stress	[48, 49]
YDJ1	HSP70 and HSP90 regulator	[44, 50]
MSN5	Nuclear import and export of proteins	[51]
YBL062W/ SKT5*	Chitin synthesis	[52]
COX6	Oxygen regulation	[53]
RLM1	Cell wall integrity	[54]
PUP1	Protein degradation	[55]
YPT6	Endosome to Golgi transport	[56]

In addition to identify new molecular targets, it is also important to find drugs that can inhibit the function of these targets. One of the genes found in this study to have an impact on the sensitivity towards echinocandins was the *YDJ1* gene. *YDJ1* is a type 1 HSP40 co-chaperone a known regulator of HSP90 [44, 50]. HSP90 inhibitors are already established as a treatment in cancer patients and are available for clinical use [57, 58]. The two HSP90 inhibitors, Onalespib and Ganetespib, were tested in this study and *S. cerevisiae fks1Δ* strain was shown to be more sensitive to the treatment. This suggests that HSP90 inhibitors could be used as a possible treatment option for echinocandin resistant fungi.

The validated interactions could be possible targets to use in the treatment of echinocandin resistant fungi. Many of these possible targets have the advantage that they are fungal specific, meaning that the same targets do not exist in the mammalian cells. A drug targeting a protein that the gene encodes will therefore not be expected to have toxic effects on the patient. The molecular function of the genes that have to do with for example cell wall integrity and chitin synthesis, such as *CHS7* and *SMI1*, are among the targets of interest as these are not found in mammalian cells.

5 Conclusions

The limited number of antifungal drugs to use in the treatment of fungal infections is a problem and so is the increasing resistance towards the clinically available antifungal drugs. Finding new treatment options is therefore essential in order to cope with the drug resistant fungi. In this master thesis, we addressed this problem by the means of identify new molecular targets.

Upregulation of certain genes in *Candida* species is known to contribute to azole resistance and they include *ERG11*, *UPC2* and *PDR3*. *S. cerevisiae* overexpressing *UPC2* and *PDR3* was shown in this study to be more resistant to fluconazole. Identification of new targets to use in the treatment of azole resistant strains were in this study carried out using SGA screening and the results remain to be analyzed. We hypothesize that negative genetic interaction of *UPC2* and *PDR3* overexpression strains could be good targets to use in the treatment.

A total of 12 genes were validated to have an impact on the sensitivity towards echinocandin, suggesting that these genes could be used as possible targets in treatment of the echinocandin resistant fungal infections. These genes affect cellular functions like chitin synthesis, cell wall integrity and mechanical stress, that are important for the fungi to be able to maintain cell growth. One gene of particular interest is *YDJ1* as this gene takes part in the regulation of HSP90. Inhibition of HSP90 could possibly block the function of *YDJ1* and in this way inhibit fungal growth. Two HSP90 inhibitors (Ganetespib and Onalespib) was in this study observed to increase the sensitivity towards *S. cerevisiae fks1Δ* compared to WT, thereby suggesting HSP90 inhibitors as a treatment option for echinocandin resistant fungi.

5.1 Future perspectives

5.1.1 New targets of azole resistant fungi

The data from the SGA screening of the *UPC2* and *PDR3* overexpression strains needs to be analyzed. Hypothesize that these genetic interaction data can provide us with new targets to use in the treatment of azole resistant fungal infections.

5.1.2 Hot- spot mutation

Knock-out strains seem to work as good proxies for the hot-spot mutation in *FKS1* and *FKS2* that contribute to echinocandin resistance. However, it would be of great interest to validate the genetic interaction data in strains having the hot-spot mutation that is causing the echinocandin resistance.

5.1.3 Candida

The findings of the negative genetic interactions in *S. cerevisiae* needs to be validated in *Candida glabrata* and other *Candida* species.

5.1.4 Drug interaction testing

The use of two drugs at the same time could provide us with a more effective treatment option of the drug resistant fungal infections. Testing the combination of micafungin and the HSP90 inhibitors would therefore be of interest.

5.1.5 Drug discovery through drug protein interaction

Some of the genes shown to interact with *FKS1* and *FKS2* that contribute to increased sensitivity towards echinocandin could be possible targets to use in the treatment of the fungal infections. Drugs that bind to these targets are of great interest to identify. Identification of novel drugs without screening thousands of molecules can be obtained using machine learning [59].

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Appendices

Appendix A
Primer sequences

Appendix B
“Toolbox” and “Genot” PCR programs

Appendix C
Homolog proteins of the negative interacting genes of FKS1 and FKS2 in C. glabrata and H. sapiens

Appendix D
R scripts

Appendix A

Table 13 – Primer sequences

Experiment	PCR	Primer name	Direction	Sequence (5'→3')	Supplier
Construction of double knock-outs in *S. cerevisiae* - Deletion of *FKS1* and *FKS2*	" TOOLBOX"	Fks1-S2	Reverse	GGATAGAATATCAGTAAAATCAAGCGTTCAAGCAAGTATTGATTGTATTAATCGATGAATTCGAGCTCG	Eurofins
		Fks2-S2	Reverse	GCGTGATAAACTTGCTTAGAAACAAAAATAGATTGTAAACTAAAAAAATCAATCGATGAATTCGAGCTCG	Eurofins
		Fks1-S1	Forward	AAATAAGCAAGTAGCTGAAATCAAGTCTTTCATACAACGGTCAGACCATGCGTACGCTGCAGGTCGAC	Sigma Aldrich
		Fks2-S1	Forward	AAAAAAATAAAAAGTGGACAATAAATAATTATTAAACTGTCATAGTTATGCGTACGCTGCAGGTCGAC	Sigma Aldrich
	" GENOT"	Fks1-chk2	Forward	GTCCTGCTGTAGCCTCTGC	Eurofins
		Fks1-chk3	Reverse	GCAACATCTTGAGAGTTTCTGGTC	Eurofins
		Fks2-chk2	Reverse	CACATCCAAATATGTTGCAGATCC	Eurofins
		Fks2-chk3	Forward	GATACAGGGTCCCAGATGTC	Eurofins
Validation of the negative interacting genes in *C. glabrata*	" TOOLBOX"	P1-rlm1	Forward	ATTCAAACTTTATTTAAAATCTAACACATTTCATTTAAGAAGATAAGATGGGTACCACTCTTGACGAC	Eurofins
		P2-rlm1	Reverse	GAGATTCTTTGAGATTTCAACAGCACCCCTGGTATTTATAGATGGTATTAGGGGCAGGGCATGC	Eurofins
		P1-mid1	Forward	CACTGACATCTCCCTAATTGGCATTCAGATACATTATCAGGGATAAGATGGGTACCACTCTTGACGAC	Eurofins
		P2-mid2	Reverse	TTTAAATTATATAAGAAAAGCATACTCATGGCCTGCATAAGAGAAAATTAGGGGCAGGGCATGC	Eurofins

		P1-chs7	Forward	CCTATATCAATATACTAGAATAAGTCAAGTCACAAAGAGTATACCAGATGGGTACCACTCTTGACGAC	Eurofins
		P2-chs7	Reverse	TTTAATATTCTGTTGAGTTGTCCTCTTTTTTTTCTATCCCTATATGGTTAGGGGCAGGGCATGC	Eurofins
		P1-ydj1	Forward	GTGGGCCGAACGATAAATTCAAGGTGGAGTCTAACAAGCGAGCTAAGATGGGTACCACTCTTGACGAC	Eurofins
		P2-ydj1	Reverse	TTACTCCCATTAAGCCTCAAATAATTTTCAGAAGAATTCTAAGAAAATCATTAGGGGCAGGGCATGC	Eurofins
		P1-smi1	Forward	GGACAAGAATAAAAGAAAAATAACGCGTGTCTCGCACAAAGGAAAGAATGGGTACCACTCTTGACGAC	Eurofins
		P2-smi1	Reverse	AATTATGGGATAAATAAATGTGGAATAAAAGGGCGATGCAATGCTTATTATTAGGGGCAGGGCATGC	Eurofins
		P1-vps63	Forward	CTTGGGTTATCTCAGACAAGCAGAAGAAACAGAAAACTGAGGAAATAATGGGTACCACTCTTGACGAC	Eurofins
		P2-vps63	Reverse	GTATCTTTATATCTTATGCATTAATGCTATTGTCGGATATCCGGTACTTATTAGGGGCAGGGCATGC	Eurofins
Validation of the negative interacting genes in *C. glabrata*	" GENOT"	rlm1-chk1	Forward	CCTCAGTTTCTTCGTCAGTCCTTCTGAG	Eurofins
		rlm1-chk2	Reverse	CCAACTATAGTATCTGAAATATCATAAGTGTATTCGATTCTTTCC	Eurofins
		mid1-chk1	Forward	CCCAGTCCAGTGGCTTGTCTGTC	Eurofins
		mid1-chk2	Reverse	GAAGAATTGAAAAACGCTGAATCACTTTATCAGC	Eurofins
		chs7-chk1	Forward	CGGCGGACTGAAAATAATAAAAATCTTAGAAACTAAAATATAGTAACTC	Eurofins
		chs7-chk2	Reverse	CATCTAGTAAAATGCCCCTGATTTCATAGGGG	Eurofins
		ydj1-chk1	Forward	GAGCAGCACACATTGGAAGAGACAC	Eurofins

		ydj1-chk2	Reverse	GGACATAAAAAATGATAAGAATAATTCTATATAATATTCCTTTAATGCTCCATAACTAC	Eurofins
		smi1-chk1	Forward	CGAAAAAAAAATTTGAATCGCAAATTGGGAAGC	Eurofins
		smi1-chk2	Reverse	GCAACGTATAATATGGTAGTTATTTGTTTTATAGAAATACGGC	Eurofins
		vps63-chk1	Forward	GAATACGAATTATCACACGATCACTCGTACAAG	Eurofins
		vps63-chk2	Reverse	GCTTGCGCAGTTAACAGGCAATGTC	Eurofins
Overexpression of ERG11, UPC2 and PDR3 in *S. cerevisiae*	“ TOOLBOX”	S1-ERG11	Forward	AATTGCAGCAGGCTTGAATAGAAACAGAACAAACGAGTAATACAAGGATGCGTACGCTGCAGGTCGAC	Eurofins
		S4-ERG11	Reverse	CCAATGTTTACGTATTCCAATGCCTCTCCAACGATTGACTTGGTAGCAGACGATGAATTCTCTGTCG	Eurofins
		S1-UPC2	Forward	ATAGTGAATCAAAAAAAGTTAAGTACAAATATTTACAGTTCAGCAGTATGCGTACGCTGCAGGTCGAC	Eurofins
		S4-UPC2	Reverse	CTTCTGGGTTTTGTCACCGCTTTCTTGTGATTCTGTATACCGACTTCGCTCGATGAATTCTCTGTCG	Eurofins
		S1-PDR3	Forward	CAACTGCATCAGCAGTTTTATTAATTTTTTCTTATTGCGTGACCGCAATGCGTACGCTGCAGGTCGAC	Eurofins
		S4-PDR3	Reverse	CAATTGACACATGCTGTCGAAACTTTTGATCTAGTTGATTTCTTCACTTTCGATGAATTCTCTGTCG	Eurofins
	GENOT	ERG11-chk1	Forward	GATTGATAAGCAGTATCGTTCAGCGTGTG	Eurofins
		ERG11-chk4	Reverse	GAGATTCTTTGGGCCAATGGTAAAGCC	Eurofins
		UPC2-chk1	Forward	GGGTGCGATAGTGCTGACTGTTC	Eurofins
		UPC2-chk4	Reverse	CCGTCCACTTCAATTAGCTCGATGAC	Eurofins

		PDR3-chk1	Forward	CCGCGGAATAATAAATGAACTATCACAGTGAG	Eurofins
		PDR3-chk4	Reverse	CAGTTGGTACATGGATATTTACCTGTGCATTTG	Eurofins
Align within the NAT marker	GENOT	1123	Reverse	GGTAAGCCGTGTCGTCAAGAGTG	N/A
		1124	Forward	GCAGGCGCTCTACATGAGCATG	N/A
Introducing GAL1 promoter upstream of *FKS1* and *FKS2*	TOOLBOX	FKS1-S4	Reverse	GGTCCCTGGGTATAGTCCGTTTGGCCCTGATAAGGTTGTTGATCAGTGTTCGATGAATTCTCTGTCG	Eurofins
		FKS2-S4	Reverse	CCATCACCGTTACTGTAATACTGTCCATTCAAGTTTGGATCGTTGTAGGACGATGAATTCTCTGTCG	Eurofins
	GENOT	FKS1-chk4	Forward	GGAGAAAATACTGTCATTGGACTGATAG	Eurofins
		FKS2-chk4	Forward	CGCCGCATATATTTTCTGCAG	Eurofins

Appendix B

TOOLBOX PCR SETUP

Table 14. PCR program for the "Toolbox" protocol

		Temperature	Time (hh:mm:ss)	Cycles
Stage 1	Initial denaturation	95,0ºC	00:01:00	1x
Stage 2	Denaturation	95,0 ºC	00:00:30	40x
	Annealing	56,0 ºC	00:00:30	
	Elongation	68,0 ºC	00:02:30	
Stage 3	Final elongation	68,0 ºC	00:07:00	1x
	Hold	4,0 ºC		

GENOT PCR SETUP

Table 15. PCR program for the "Genot" protocol

		Temperature	Time (hh:mm:ss)	Cycles
Stage 1	Initial denaturation	95	00:01:00	1x
Stage 2	Denaturation	95	00:00:30	30x
	Annealing	56	00:00:30	
	Elongation	72	00:01:30	
Stage 3	Final elongation	72	00:07:00	1x
	Hold	4,0 ºC		

Appendix C

Homologs of negative genetic interactions of *FKS1* and *FKS2* in *Candida glabrata* and *Homo sapiens*

The protein sequence for the negative interacting genes of *FKS1* and *FKS2* was obtained from www.yeasstgenome.org. The protein sequence of all the genes was then used to perform a BLAST P search. Organisms chosen for the search was Candida glabrata and Homo sapiens. The default parameters for the algorithm was used, having the BLOSUM62 matrix and gap costs 11 (existence 11, extension 1).

Table 16. Homologs of negative interacting genes of FKS1 in C. glabrata and H. sapiens

Allele	ORF	Molecular function	C. glabrata homolog		H. sapiens homolog	
			Name	ID	Name	ID
pkc1-damp	YBL105C	Protein kinase C activity	Protein kinase C-like 1	KTA98553.1	protein kinase C epsilon type isoform X9	XP_011531285.1
bni4Δ	YNL233W	Protein binding	Protein BNI4	KTB11319.1	No significant similarity found	
rlm1 Δ	YPL089C	DNA binding	CAGL0H05621g	XP_447040.1	myocyte-specific enhancer factor 2B isoform b	NP_005910.1
crz1 Δ	YNL027W	DNA binding	Transcriptional regulator CRZ1	KTB04268.1	myoneurin isoform X4	XP_005247681.1
cch1 Δ	YGR217W	Calcium channel activity	Calcium-channel protein CCH1	KTB16352.1	unnamed protein product	BAG54350.1
gsc2 Δ	YGR032W	1,3-beta-D-glucan synthase activity	CAGL0G01034g	XP_446406.1	CHD9 protein, partial	AAH33770.2
mid1 Δ	YNL291C	Calcium channel activity	related to Stretch-activated cation channel MID1	SLM10770.1	No significant similarity found	
cnb1 Δ	YKL190W	Calcium dependent protein serine/ threonine phosphatase activity	CAGL0L00605g	XP_448800.1	calcineurin subunit B type 1	NP_000936.1

chs6 Δ	YJL099W	Unfolded protein binding	CAGL0L03608g	XP_448930.1	TTC7B protein, partial	AAH35865.1
chs7 Δ	YHR142W	Unfolded protein binding	Chitin synthase export chaperone	KTB23343.1	No significant similarity found	
pre4-damp	YFR050C	Molecular functions-elemental activities	CAGL0A04719g	XP_444973.1	prosome beta-subunit	AAB31085.1
pup1-damp	YOR157C	Endopeptidase activity	CAGL0L04312g	XP_448962.1	Proteasome subunit beta type 7	AAH00509.1
tif6-damp	YPR016C	Ribosomal large subunit binding	CAGL0K02497g	XP_448334.1	eukaryotic translation initiation factor 6 isoform a	NP_002203.1
skt5 Δ	YBL061C	Enzyme activator activity	B1J91_A04411g	OXB45455.1	protein sel-1 homolog 2 isoform X10	XP_006723715.1
las17-damp	YOR181W	Actin binding	Proline-rich protein LAS17	KTB23444.1	unnamed	BAF82484.1
rpl40a Δ	YIL148W	Protein tag/Structural constituent of ribosome	ubiquitin-60S ribosomal protein L40 fusion protein	XP_446470.2	ubiquitin-60S ribosomal protein L40 isoform 1 precursor	NP_003324.1
msn5 Δ	YDR335W	Nuclear export signal reporter / protein binding	CAGL0M01144g	XP_449394.1	exportin 5, isoform CRA_b	EAX04207.1
ybl062w Δ	YBL062W	Molecular function	No significant similarity found		No significant similarity found	

Table 17. Homologs of negative interacting genes of FKS2 in C. glabrata and H. sapiens

Allele	ORF	Molecular function	C. glabrata homolog		H. sapiens homolog	
			Name	ID	Name	ID
gcg1 Δ	YER163C	Gamma-glutamylcyclotransferase activity	Glutathione-specific gamma-glutamylcyclotransferase	KTA97936.1	glutathione-specific gamma-glutamylcyclotransferase 2 isoform 1	NP_001008708.1
nha1 Δ	YLR138W	Antiporter activity	Na(+)/H(+) antiporter	KTB12859.1	No significant similarity found	
fks1 Δ	YLR342W	1,3-beta-D-glucan synthase activity	CAGL0G01034g	XP_446406.1	EWSR1/ATF1 fusion protein type 2	ADX41458.1
cdc25-damp	YLR310C	Ras guanyl-nucleotide exchange factor activity	Cell division control protein 25	KTA95061.1	Chain A Rem-Cdc25	2II0_A
ydj1 Δ	YNL064C	ATPase activator activity	CAGL0J09966g	XP_448143.1	dnaJ homolog subfamily A member 1 isoform 1	NP_001530.1
msc1 Δ	YML128C	Molecular function-elemental activities (catalysis or binding)	Meiotic sister chromatid recombination protein 1	KTB03535.1	E3 ubiquitin-protein ligase RNF103 isoform 1	NP_005658.1
met7 Δ	YOR241W	Tetrahydrofolylpolyglutamate synthase activity	CAGL0J03762g	XP_447868.1	folylpolyglutamate synthetase	AAA35852.1
smi1 Δ	YGR229C	Molecular function-elemental activities (catalysis or binding)	CAGL0L06534g	XP_449059.1	No significant similarity found	
ecm2 Δ	YBR065C	Molecular function-elemental activities (catalysis or binding)	CAGL0L07458g	XP_449102.1	pre-mRNA-splicing factor RBM22	NP_060517.1

ypt6 Δ	YLR262C	GTPase activity	GTP-binding protein YPT6	KTA95680.1	ras-related protein Rab-6A isoform b	NP_942599.1
atc1 Δ	YDR184C	Molecular function-elemental activities (catalysis or binding)	Protein ATC1/LIC4	SLM15913.1	No significant similarity found	
cox6 Δ	YHR051W	Mitochondrial electron transport	CAGL0J00429g	XP_447730.1	cytochrome c oxidase subunit 5A	NP_004246.2
sma2 Δ	YML066C	Molecular function-elemental activities (catalysis or binding)	CAGL0L11286g	XP_449259.1	No significant similarity found	
yll054c Δ	YLL054C	DNA-binding transcription factor activity, RNA polymerase II-specific	CAGL0D02486g	XP_445523.1	No significant similarity found	
vps63 Δ	YLR261C	Cellular Component	No significant similarity found		No significant similarity found	
aro1 Δ	YDR127W	3-dehydroquinate dehydratase activity	Pentafunctional AROM polypeptide	KTB23807.1	No significant similarity found	
Arc18 Δ	YLR370C	Molecular function	CAGL0G04895g	XP_446573.1	actin-related protein 2/3 complex subunit 3 isoform 2	NP_001274151.1

Appendix D – R scripts

IC50 values for sensitivity of yeast strains towards micafungin

```
#Libraries load

library(ggplot2)
library(GRmetrics)
library(plyr)
library(drc)
library(PharmacoGx)

df <- read.table("~/UiO- master molekylær biovitenskap/Master prosjekt/S. cerevisiae (C. glabrata)/OD measues/03.07.2019/Plate 1, sample 14, 24hrs.csv", header=TRUE, sep=";")
df2 <- read.table("~/UiO- master molekylær biovitenskap/Master prosjekt/S. cerevisiae (C. glabrata)/OD measues/03.07.2019/Plate 2, sample 17 + 18, 24hrs.csv", header=TRUE, sep=";")
df3 <- read.table("~/UiO- master molekylær biovitenskap/Master prosjekt/S. cerevisiae (C. glabrata)/OD measues/03.07.2019/Plate 3, sample 30 + 32, 24hrs.csv", header=TRUE, sep=";")

colnames(df2)
df <- rbind(df, df2, df3)
rm(df2,df3)
df <- na.omit(df)
df <- subset(df, df$OD.time.0 != 0)

df$Time <- as.numeric(df$Time)
df$Replicate <- as.character(df$Replicate)
df$Concentration <- as.numeric(df$Concentration)
df$OD.final<-as.numeric(df$OD.final)
df$OD.final<- df$OD.final-df$OD.time.0
df$OD.ctrl <- df$OD.ctrl - df$OD.time.0
df$OD.normal <- df$OD.final/df$OD.ctrl
df$OD.normal[which(df$OD.normal < 0)] <- 0

par(mfrow=c(3,4))
strain <- as.character(unique(df$Strain))[1]
demo <- drm(data = subset(df, Time=="24" & Strain==strain),
OD.normal~Concentration,fct=LL.4(),na.action = na.omit, lowerl = c(-Inf,0,0,-Inf))

df.IC50 <- data.frame(t(summary(demo)$coefficients[4,1:4]))
df.IC50$Strain <- strain
plot(demo, type = "all", ylab="Normalized Viability", xlab =expression(paste ("Concentration (", n, "M")),ylim = c(0,max(df$OD.normal)))
title(main=strain)
```

```
for(i in 2:length(unique(df$Strain))){

        strain <- as.character(unique(df$Strain))[i]
        demo <- drm(data = subset(df, Time=="24" & Strain==strain),
        OD.normal~Concentration,fct=LL.4(),na.action = na.omit)#, lowerl = c(-Inf,0,0,-Inf))
        a <- data.frame(t(summary(demo)$coefficients[4,1:4]))
        a$Strain <- strain
        df.IC50 <- rbind(df.IC50, a)
        plot(demo, type = "all", ylab="Normalized Viability", xlab =expression(paste ("Concentration
        (", n, "M")),ylim = c(0,max(df$OD.normal)))
        title(main = strain)
        rm(a, demo)
        }
```

IC50 curve for HSP90 inhibitor testing

```
#Libraries load

library(ggplot2)
library(GRmetrics)
library(plyr)
library(drc)
library(PharmacoGx)


df<- read.csv("D:/Nacho-Private/Dropbox/Labo Oslo/Linda Data/drugresponsehsp90.csv", sep = ";")

df$rep <- as.character(df$rep)
df$conc <- as.numeric(df$conc)
df$value<-as.numeric(df$value)


df$OD.normal[which(df$OD.normal < 0)] <- 0

strain <- as.character(unique(df$strain))

demo <- drm(data = subset(df,  strain=="wt"), value~conc,fct=LL.4(),na.action = na.omit, lowerl = c(-
Inf,0,0,-Inf))
demo2 <- drm(data = subset(df,  strain=="fks1"), value~conc,fct=LL.4(),na.action = na.omit, lowerl =
c(-Inf,0,0,-Inf))

#,names = c("Hill","Limit","Baseline", "EC50")
#lowerl = c(-Inf,0,0,-Inf)
#IC50 <- summary(demo)$coefficients[4,1]
df.IC50 <- data.frame(t(summary(demo)$coefficients[4,1:4]))
df.IC50$Strain <- strain[1]

plot(demo, type = "all", ylab="Normalized Viability", xlab =expression(paste ("Concentration (", mu,
"M")),ylim = c(0,max(df$value)))
par(new=TRUE)
plot(demo2, type = "all", ylab="Normalized Viability", xlab =expression(paste ("Concentration (", mu,
"M")),ylim = c(0,max(df$value)), col="red")
```