

Leveraging an *In Vitro* Assay to Perform Chemical, Genomic, and Genetic Analyses of the Molecular Mechanisms Underpinning the Yeast to Titan Transition in *Cryptococcus neoformans*

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A thesis submitted to the University of Birmingham for the degree of
DOCTOR OF PHILOSOPHY

School of Biosciences
College of Life and Environmental Sciences
University of Birmingham
January 2022

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Abstract

Recognition of the threat that fungal pathogens impose on human health is necessary, especially when there is a lack of funding and knowledge behind the species and the diseases they cause. *Cryptococcus neoformans* infection has increased in occurrence over the past 40 years particularly among the immunocompromised, and yet our understanding of *C. neoformans* pathogenesis and drug resistance remains limited. Moreover, long-term use of azoles has led to the development of drug resistance via poorly understood mechanisms. During infection, *C. neoformans* undergoes an unusual morphological transition: formation of titan cells during which yeast grow isotropically to over 10-100 µm, forming highly polyploid Titan cells. Previously it was difficult to study the molecular mechanism behind titanisation and its induction due to titan cells only being seen *in vivo*, making the knowledge behind this quite limited. Now thanks to the development of our *in vitro* model I can successfully study this phenomenon without the use of mice, and allow for a better understanding of the mechanisms behind this transition. The role of titanisation in drug resistance has not yet been explored.

In this thesis, it was shown through the use of paired naïve and heteroresistant clinical isolates that exposure to fluconazole can impart or remove the capacity to form titan cells. While Titan cell growth itself does not necessarily lead to drug resistance, fluconazole does impact genome plasticity and polyploidy, which in turn contribute to increased resistance in *C. neoformans*. Alongside this, our study showed how there are many pathways, such as cAMP pathway and production of mitochondrial superoxide, that may be facilitating the way fluconazole affects *C. neoformans* genome plasticity in turn facilitating this resistance. Reducing titanisation using certain FDA approved drugs appeared to increase fluconazole resistance.

In addition, *in silico* analyses of the sequences of clinical isolates and mutant libraries were used to explore the mechanisms of titanisation. The results identified an additional pathway, the trehalose biosynthesis pathway, which negatively regulates titanisation through Tps1. To date this pathway has not been known to play a role in this morphological transition.

This work has helped expand our understanding of titan cell formation and its molecular mechanisms whilst also calling for further investigation into the new pathways and the causes of drug resistance.

Acknowledgements

Whilst a PhD is thought to be a degree where one is independent, this is not possible without the support and encouragement of those around them. I learnt this throughout my 3 years in the lab, particularly during Covid-19.

I want to express my sincere thanks to the NC3Rs for funding my PhD research, but also for the support throughout including the summer school. Alongside this, thank you to the university for providing the environment for me to grow as a researcher, 3 years here have been an incredible experience for me.

I count myself so incredibly lucky to have the most supportive supervisors. I could not thank Dr Elizabeth Ballou enough for being the most supportive, trusting and encouraging supervisor. Thank you, Liz, for showing me that women are incredible leaders, this is something I will keep for life. For always keeping your door open to us when we want to share our troubles or laughs. I also want to thank Professor Robin May for also being a very supportive supervisor and for the guidance given. Your down to earth persona with your passion for research has always been very inspiring to me during my time here.

A fear of mine was whether I would feel lonely in the lab, but I was very fortunate to work in a lab that truly was a team/family. I never once felt that I could not approach someone for help, and I am very grateful for this. The HAPI lab truly reflects its name. I also want to say a big thank you to Diana Tamayo and Phoebe Xin Zhou. Thank you for celebrating my wins and helping me in my troubles without a second

thought. You both played such a big part of this PhD. I will truly miss working in this lab, and in this team.

From the bottom of my heart I would like to say a special thank you to my family. To my wonderful mama and papa who never once stopped me from reaching my goals. I never could have gotten to this stage without your constant love, support and duas. To Waleed and Amna for being my cheerleaders and helping me realise that I was capable of this when I felt like I wasn't.

Also, a special thanks to my friends who are practically my sisters. Thank you to Farhana Chowdhury who would pick up the phone no matter what the time was to help me in any situation, big or small, and give me the love and encouraging words that I would need to hear. To Laiba Hussain, thank you for never failing to tell me exactly how it is, along with trying to make me laugh when I would feel low. Thank you to Sofia Azam for never letting me doubt the power of prayer and hard work, I look up to you more than you know. And to Gulnar Vasta, thank you for the advice and support that you give so freely and lovingly.

Last, but most definitely not least, I want to thank my incredible husband Abu Zer Saeed. It's hard to put into words how thankful I am for your support, especially during the end of this journey. Thank you for lifting me up when I needed it, for encouraging me to push myself, but most of all for just being there for me, particularly keeping me company during the writing period.

I would not have gotten through these 3-4 years without you all.

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List of Abbreviations

Central nervous system	CNS
Blood brain barrier	BBB
Glucuronoxylomannogalactan	GXMGal
Glucuronoxylomannan	GXM
Cerebrospinal fluid	CSF
cyclic AMP	cAMP
Protein kinase A	PKA
Protein kinase C	PKC
Deoxyribonucleic acid	DNA
Reactive oxygen species	ROS
Yeast extract peptone	YPD
Yeast nitrogen base	YNB
Foetal calf serum	FCS
Heat inactivated foetal calf serum	HI-FCS
Phosphate buffered saline	PBS
Minimum inhibitory concentration	MIC
Dimethyl sulfoxide	DMSO
Rotenone	R
Antimycin A	AA
Salicylhydroxamic acid	SHAM
Potassium cyanide	KCN
Sodium azide	SA
Rhodamine 123	Rho123
Calcofluor white	CFW
Host response to microbe analysis	HRMA _n
Gene ontology	GO
Propidium Iodide	PI
Chromosome	Chr
Tanzanian clinical isolate	TZ
Median fluorescence intensity	MFI
Complex	C
4',6-diamidino-2-phenylindole	DAPI
Titan inhibitor	TI
Retinoic acid	Ret
Metyrapone	Met
Nicardipine hydrochloride	Nic
Alternative oxidase pathway	AOX
Glucose-6-phosphate	G6P
Trehalose-6-phosphate	T6P
The National Centre for Replacement, Refinement, and Reduction of animals in research	NC3Rs
Artificial intelligence	AI
Titan inducing	YT

Chapter 1. Introduction

In the past few decades there has been an increase in occurrence of severe *Cryptococcus neoformans* infection in immunocompromised patients and, more recently, immunocompetent patients. For people living with HIV/AIDS, the most common fungal infection is *Cryptococcus neoformans*, which causes almost 220,000 cryptococcal meningitis cases each year worldwide with nearly 181,000 deaths. In countries that are limited in resources and healthcare access with high HIV prevalence, cryptococcal meningitis remains a major concern (Rajasingham et al., 2017).

Fungal infections are a growing global burden and yet they are still an underappreciated pathogen by public health officials (Rodrigues and Nosanchuk, 2020). It has been shown that over 300 million people have experienced a fungal infection with more than 1.3 million deaths, and the genera of species causing 90% of these deaths were: *Aspergillus*, *Candida* and *Cryptococcus* (Brown et al., 2012).

Aspergillus fumigatus is amongst the most ever-present airborne saprophytic fungi which evidence shows mortality surpassing 50% whilst causing 200,000 cases annually of aspergillosis (Brown et al., 2012, Garcia-Vidal et al., 2008, Guinea et al., 2010). Although many *Candida* species cause mortal diseases, *Candida albicans* leads in healthcare-associated infections and in spite of treatment, elicits mortality rates of between 40% to 60% (Wu et al., 2017). As mentioned earlier, *Cryptococcus neoformans* mainly affects those who are immunocompromised, causing an intense infection in the central nervous system (May et al., 2016). Interestingly, fungal pathogens not only have an effect on human health, but also on food through destruction to crops (Fisher et al., 2012). Fungal pathogens are also

threatening biodiversity by causing diseases in plants and animals leading to extinction, along with being part of the rise in pathogens caused by global warming (Fones et al., 2017).

Recognition of the threat that fungal pathogens impose on human health is necessary, especially when there is a lack of funding and knowledge behind the species and the diseases they cause. For many years, bacterial and viral diseases have been acknowledged as an important health issue for the public and yet aggressive mycoses have only just been recognised widely as a critical pathogen medically in the 1980s (Brachman, 2003, Nucci and Marr, 2005). However, there are insufficient number of antifungal drug classes that have been approved for human usage, with the last approval in 2002 (Pianalto and Alspaugh, 2016). Remarkably, it was found that research into cryptococcosis received less than 5% global funding whilst tuberculosis and malaria in total received 35.5%, even though mortality rates are not that far apart (Rodrigues, 2016, Almeida et al., 2019). The one-year mortality rate of cryptococcal infections was found to be up to 78% in HIV-associated patients and up to 42% in non-HIV-associated patients (Pasquier et al., 2018).

In this chapter, I will introduce *Cryptococcus* as an organism, outline the major aims and objectives of my thesis work, and then go into detail about relevant background necessary to contextualise these objectives.

1.1 *Cryptococcus* spp

Unlike the majority of opportunistic human fungal pathogens which are ascomycetes, *Cryptococcus* spp is a family of basidiomycetes yeast (Kwon-Chung et al., 2002).

Classification of this fungus has been refined through improved molecular methods over the years. For several years the fungus was classified as two different anamorph species; *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. They differ in ecology, pathology, biochemical and molecular characteristics, and morphological features. The two species also have serological differences; *C. neoformans* has A, D and AD serotypes while *C. gattii* has B and C serotypes (Kwon-Chung et al., 2002). However, more recently due to new information through whole-genome sequencing-based analyses, there is a proposal to split the two species up further. The study by Hagen *et al* proposes *C. neoformans* to be split into *C. neoformans* and *C. deneoformans*, while *C. gattii* splits in *C. gattii*, *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii*, and *C. decagattii* (Hagen et al., 2015). This is due to the different serotypes as *C. neoformans* serotype is A whilst *C. deneoformans* serotype is D. Furthermore *C. gattii* was also suggested to be divided into further sub-lineages that consist of serotypes B or C (Farrer et al., 2019, Montoya et al., 2021). Across the serotypes, serotype A is known to cause majority of cryptococcal infections (Montoya et al., 2021). Interestingly, a study has found that the manifestation of the disease can also differ depending on the serotype (Desnos-Ollivier et al., 2015).

The work described for the remainder of this thesis focuses on *C. neoformans*, serotype A, the major cause of human disease.

1.2 Cryptococcosis

Cryptococcosis is an invasive global mycosis caused by the pathogenic *Cryptococcus spp*, particularly *C. neoformans*. The disease is predominantly found in immunocompromised patients- with cancer chemotherapy patients, organ transplant recipients, and those with

HIV infection at risk with high mortality and morbidity (Brown et al., 2012). However, recently there was an outbreak of Cryptococcosis due to *C. gatti* found in immunocompetent patients in Canada and North America (May et al., 2016, MacDougall et al., 2007, Byrnes et al., 2009, Datta et al., 2009). For sub-Saharan African patients with HIV, the most common cause of fungal meningitis is this encapsulated yeast (Rajasingham et al., 2017).

Cryptococcosis is known to happen in humans and animals, however it is not known to transmit through human-human or human-animal. The beginning of human infection starts with inhalation of airborne fungal cells/spores (Botts and Hull, 2010) (figure 1a and figure 1b). Within the lungs the spores can cause a human pulmonary infection such as pneumonia in immunocompromised patients (figure 1c). If the host defence mechanism such as phagocytic cells fail to dispose of the yeast, this can lead to dissemination (figure 1d). *C. neoformans* are capable of disseminating through macrophages in the blood circulation to reach its final location, the central nervous system (CNS) (Casadevall and Perfect, 1998) (figure 1e). The encapsulated yeast can replicate in the macrophage due to protection from phagocytic environment. *Cryptococcus* is able to escape the macrophage through vomocytosis; the macrophages release the fungus without harming it and triggering inflammation leading to fungal meningitis (Alvarez and Casadevall, 2007, Ma et al., 2006).

In order for the pathogen to infect the brain and the membranes surrounding it, it needs to pass through the blood brain barrier (BBB). This is a barrier that separates the peripheral circulation and the CNS. In the case of *C. neoformans*, through blood circulation the yeast cells reach the BBB and reach the meninges by crossing the barrier to replicate and cause inflammation and subsequent meningoencephalitis (Chang et al., 2004). Macrophages

containing *C. neoformans* may also be able to deliver the yeast cells to the brain by crossing the BBB (Liu et al., 2012).

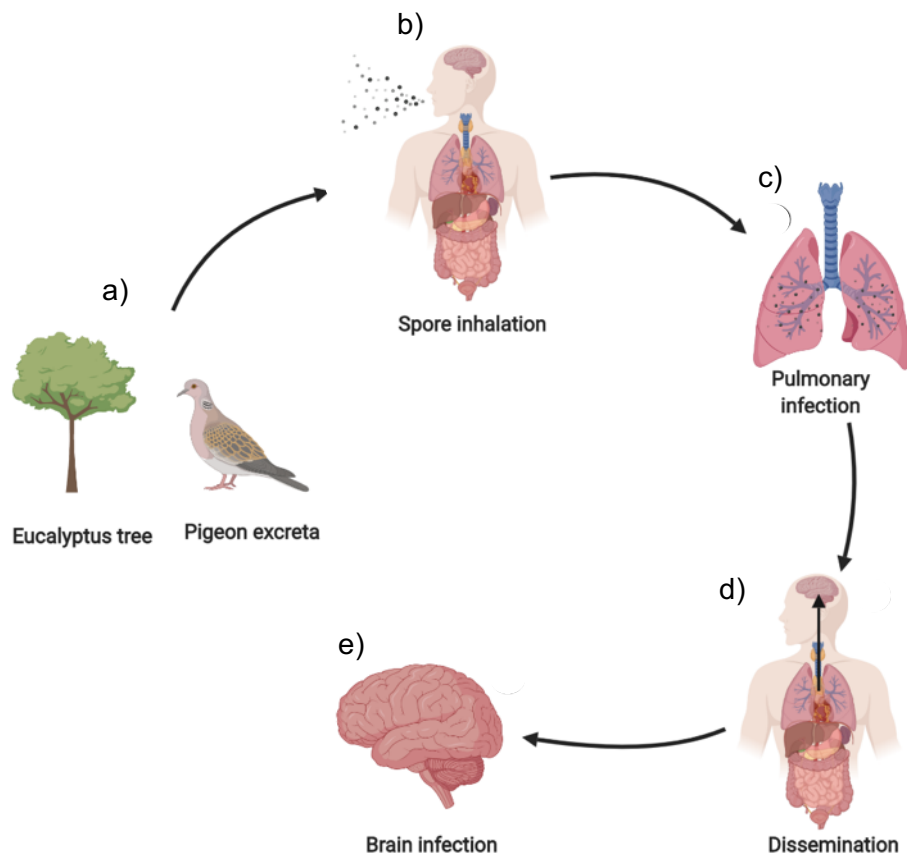


Figure 1. Infection cycle of *Cryptococcus neoformans*. A diagram depicting the steps leading to cryptococcosis. Step 1 shows *Cryptococcus* spores being released from pigeon excreta and eucalyptus tree. Step 2 shows the inhalation of the desiccate spores by humans, and within the lungs these cells can cause a pulmonary infection such as pneumonia depicted in step 3. Following this, the cells are able to disseminate to the brain causing fungal meningitis seen in step 4 and 5. This diagram was made on biorender.

While most *C. neoformans* patients may potentially develop meningitis, various studies have as of late featured cryptococcal pneumonia as an undervalued part of the infection (Scriven et al., 2018, Wong et al., 2007, Spec et al., 2017, Singh et al., 2007, Raval et al., 2017). Intriguingly, *C. neoformans* can settle in the host's respiratory tract whilst not causing harm to the host. The infection is asymptomatic at this point, and is either removed by the immune system or stays dormant in the host. Once the individual is

immunocompromised, a systemic infection occurs due to reactivation and dissemination of the yeast (Baker, 1976, Baker and Haugen, 1955, Garcia-Hermoso et al., 1999).

Serological studies have demonstrated that most people as young as 2 years old have a memory response to the fungus and are able to produce antibodies efficiently against this fungus, indicating that many individuals in their early childhood come across this pathogen (Goldman et al., 2001, Casadevall et al., 1998). It is unclear what effect this asymptomatic pulmonary *C. neoformans* infection has on the person. Interestingly, for patients who later become immunocompromised and develop the disease, these lifelong infections could dramatically affect the production of drug resistance. There is a possibility that before *Cryptococcus* emerges from latency, drug resistance occurs due to the exposure to antifungal drugs from treatment of other infections or from the environment during agriculture use during a human's lifetime whilst the pathogen is dormant in the host (Zafar et al., 2019, Smith et al., 2015, Stone et al., 2019).

1.3 *Cryptococcus neoformans* morphology and life cycle

Many pathogenic fungi have the ability to switch their morphologies in certain environmental conditions which plays a key role in their pathogenesis. Unlike *Candida albicans* which has the ability to change its morphology from yeast to pseudohyphae or hyphae, *C. neoformans* does not form hyphae during infection (Velagapudi et al., 2009). *C. neoformans* is a basidiomycete unicellular budding yeast which is 5-7 µm in diameter that is bound by a cell wall and plasma membrane. The cryptococcal cell wall includes glycosylated protein, chitin and glucan, and can be strengthened further by the presence of melanin (Doering, 2009). *C. neoformans* produces a unique capsule made up of two large polysaccharides;

glucuronoxylomannogalactan (GXMGal) and glucuronoxylomannan (GXM), which helps distinguish the fungus from others (O'Meara and Alspaugh, 2012).

1.3.1 Cryptococcus mating and spore formation

C. neoformans is heterothallic thus in order to produce sexual spores it requires two compatible mating types, in this case it is *MAT α* and *MATa* (Kwon-Chung et al., 2014, Kwon-Chung, 1976) (figure 2). *C. neoformans* grows asexually as budding yeast, but during the sexual cycle it is able to shift from yeast to hyphae (Sia et al., 2000, Kozubowski and Heitman, 2012). Although this is essentially a morphological change, *C. neoformans* is not a dimorphic fungus due to being predominantly found in the host and environment as yeast cells and not switching its morphology in response to temperature (Kozubowski and Heitman, 2012). The serotypes of *Cryptococcus* species differ in their mating capabilities, with *C. gattii* and serotype A (*C. neoformans*, the focus of this study) being strain specific while majority of serotype D (*C. deneoformans*) can mate (Fraser et al., 2003, Nielsen et al., 2003).

During bipolar mating, the two mating types α and **a** produce pheromone that triggers the fusion of the cells (figure 2a). This forms a dikaryotic hypha, and as the two parental nuclei are separate it begins filamentous growth, where the clamp cell and septum form to separate the two nuclei. During the formation of the basidium, the two opposite mating type nuclei fuse to become a diploid nucleus (figure 2b). This single diploid nucleus goes through a cycle of meiosis with four meiotic products produced after (figure 2c), these products undertake mitosis leading to budding at the basidium surface. This produces four chains of haploid basidiospores (figure 2d) (Kwon-Chung et al., 2014, Kwon-Chung, 1980,

Kozubowski and Heitman, 2012, Idnurm et al., 2005). Just like the yeast cells, these spores are capable of inducing infection (Giles et al., 2009).

C. neoformans sexual cycle does not just consist of this bipolar mating, they also undergo monokaryotic fruiting, which is unisexual production. This occurs when two of the same mating type α cells produce diploid nuclei from nuclear fusion or endoreduplication (figure 2e). This also produces a diploid monokaryotic hyphae which have clamp cells that are unfused. When the basidium has formed (figure 2f), meiosis takes place and the four chains of haploid basidiospores are produced (figure 2g) (Kozubowski and Heitman, 2012, Idnurm et al., 2005). These spores are morphologically alike to the spores produced from bipolar mating (Lin et al., 2005, Ni et al., 2013).

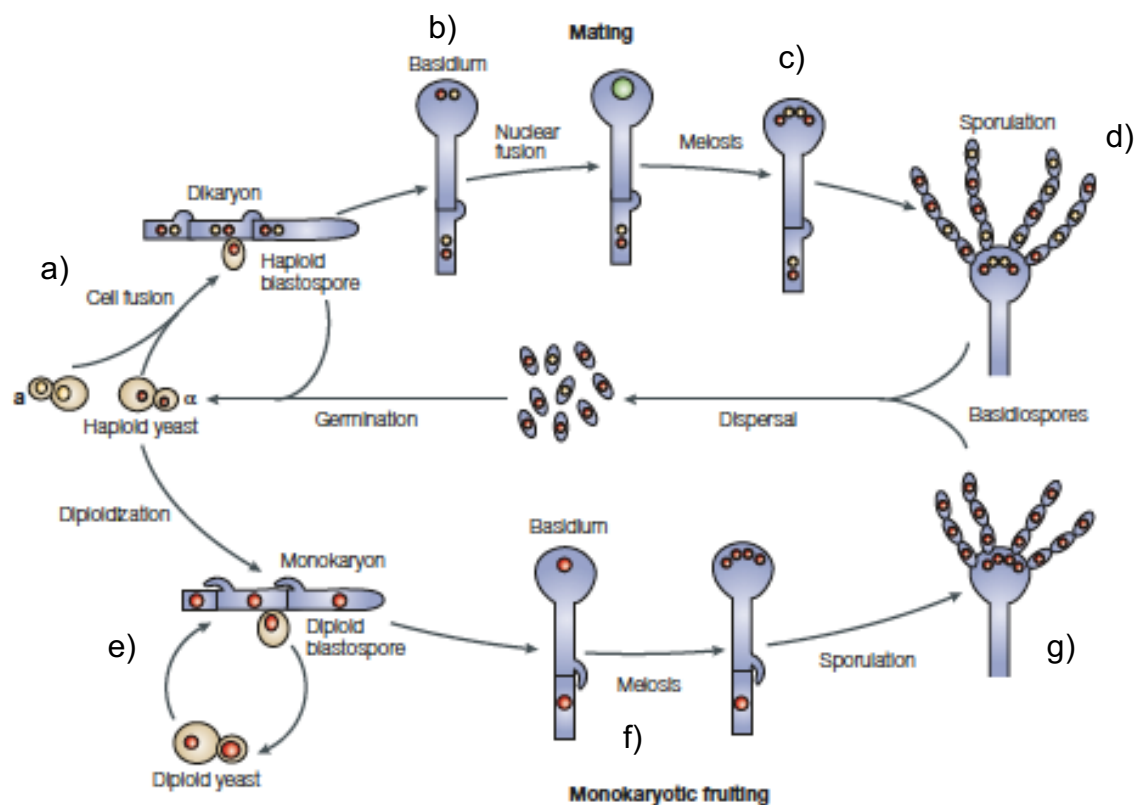


Figure 2 *C. neoformans* mating cycle. A diagram displaying the different forms of *C. neoformans* mating and their stages. There are two sexual cycles: mating (above) and monokaryotic fruiting (below). The mating stages include: a) cell fusion, b) basidium formation, c) production of 4 meiotic products, and d) sporulation. The monokaryotic fruiting stages include: e) diploid monokaryotic hyphae production, f) Basidium formation followed by meiosis, g) sporulation. Taken from Idnurm et al., 2005.

1.4 Titan cell

1.4.1 Titan cell morphology

After inhalation of the *C. neoformans* spores into the lungs or induced *in vitro*, a morphological transition takes place. Within the population, 10-20% of the cells change from a small yeast of 5-7 μm to a large titan cell of over 10-100 μm (Okagaki et al., 2010, Zaragoza et al., 2010). The yeast grows uniformly and undergoes rounds of endo-reduplication repeatedly, causing the formation of the highly polyploid, large titan cells. During cell division, the titan cells asymmetrically divide their DNA which instigates the production of

daughter cells that are small and aneuploid, haploid, or diploid (Dambuza et al., 2018, Zhou and Ballou, 2018).

In vivo titan cells have very distinct genomic and morphological differences in comparison to yeasts. The capsule of *C. neoformans* is a well-known virulence factor (discussed further below), and in titan cells it is structurally different having more cross-links and being a lot denser (Probert et al., 2019, Zaragoza et al., 2010). Depending upon the environment the capsule can change in structure and density such as having a denser crosslinked capsule can affect its permeability to host complement proteins (Garcia-Rodas et al., 2011, O'Meara and Alspaugh, 2012). Not only is the titan capsule different, but the titan cell wall is significantly thicker than seen in the yeast cell, and can reach up to 2-3 μm . Alongside this, titan cells maintain a large vacuole that may play a role in extracellular vesicle production (Zaragoza et al., 2010). A study had shown cell size to be in relation to increased DNA content (Okagaki et al., 2010). Consistent with this, titan cells are highly polyploid, with some cells containing 16-64 copies of the genome while producing smaller daughter cells that are aneuploid or diploid (Gerstein et al., 2015). However, more information is needed as to how daughter cell formation from titan cell differs from daughter cell formation from yeast.

1.4.2 Challenges to studying Titan cells and how 3Rs can help address this

There have been very limited studies on the molecular mechanisms that drive titanisation because they require mice (Okagaki et al., 2010). As titan cells are a complex phenotype that involves numerous genetic pathways, this can be challenging to investigate *in vivo*, and they are a heterogenous subpopulation, further complicating *in vivo* analysis. A previous study looking into titan cells required approximately 600 mice (Okagaki et al., 2010).

The National Centre for the Replacement, Refinement, and Reduction of animals in research (NC3Rs) focuses on the initiative to find new methods and technologies that will allow the use of animals in research to be reduced or completely avoided, whilst also improving the welfare of animals used in research. The 3Rs consist of replacement, refinement and reduction. Replacement includes methods and technologies that have been designed to replace the use of animals or completely avoid the use of them. Refinement applies to the methods that help improve the animal's welfare through reduction of suffering, pain or lasting harm whilst they are used in research, and Reduction refers to the methods used in experiments that lessen the number of animals used (Burden et al., 2015). *In vitro* titan cell assays would therefore allow us to replace animals. It would additionally allow us to deal with the other two major challenges presented by the complexity and heterogeneity of titan populations

At the start of this project, three *in vitro* assays were developed that can help address this challenge, all of which showed titan cells produced with similar characteristics to that seen *in vivo* (Dambuza et al 2018, Trevijano-Contador et al., 2018, Hommel et al., 2018) (figure 3). Our lab found that the induction of this morphological transition was by growing the cells in minimal media, yeast nitrogen base (YNB) medium, followed by growth in the presence of serum that contained the cell wall of bacteria (figure 3a). Another *in vitro* model, published by Trevijano-Contador et al also uses minimal media, sabouraud, but then adds sodium azide into the serum (Trevijano-Contador et al., 2018) (figure 3b). However, Hommel et al. *in vitro* model is slightly different as they grew their cells in yeast extract peptone dextrose (YPD) followed by continuous mixing under hypoxia (Hommel et al., 2018) (figure 3c).

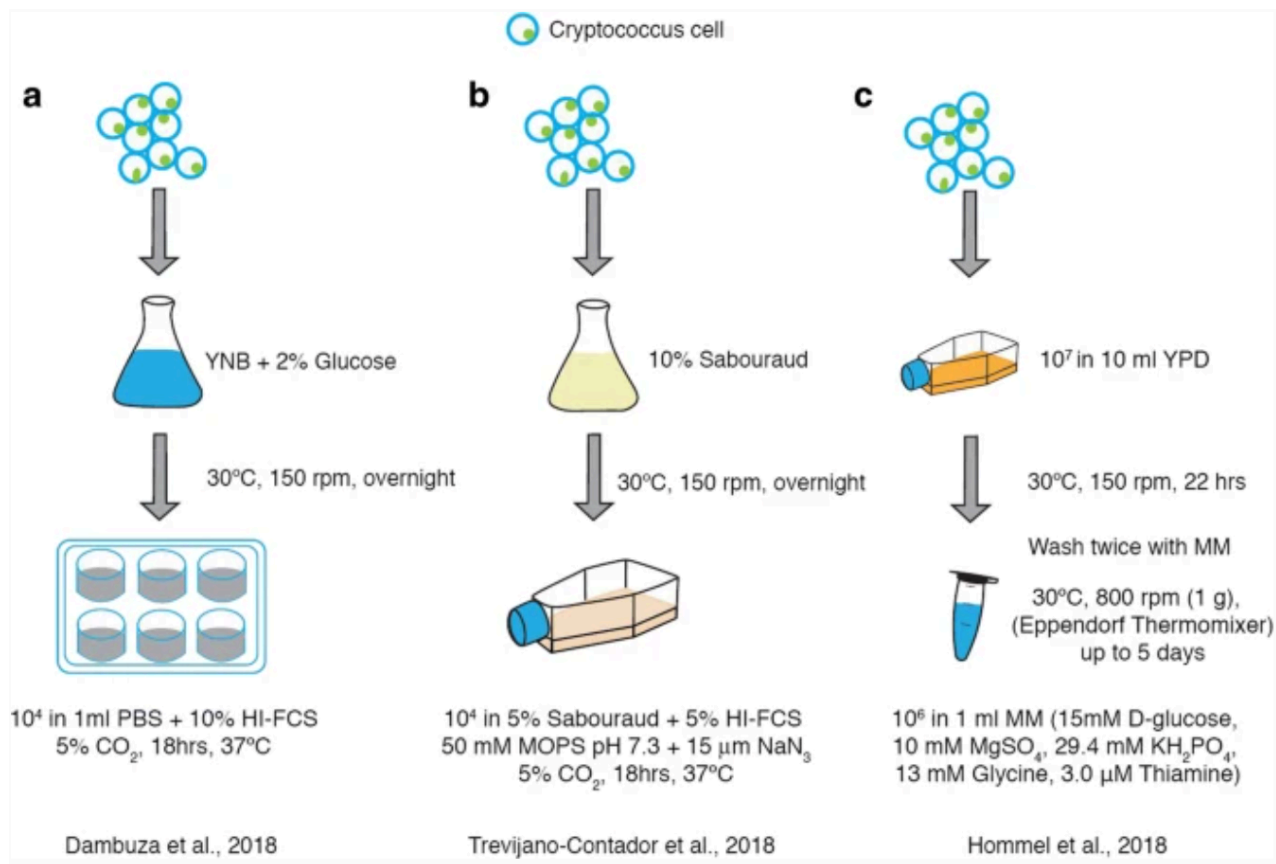


Figure 3. *C. neoformans in vitro* titan induction. A diagram displaying the different *in vitro* methods to induce the yeast-to-titan transition. Titan cells were induced through a) the use of minimal media as pre-growth after which cells were exposed to 10% heat-inactivated serum (Dambuza et al., 2018), b) the use of minimal media as pre-growth after which cells were exposed to sodium azide and 5% heat-inactivated serum (Trevijano-Contador et al., 2018), c) cells were grown under hypoxia (Hommel et al., 2018). Taken from Zhou and Ballou, 2018.

In vitro titan cells have been defined with 4 key conditions; the cell ploidy is more than 2 copies of the genome, the cell size is more than 10 μm, the cell wall and capsule are altered, and the majority of the intracellular space is comprised of a single large vacuole (figure 4). However, these conditions may need further fine-tuning due to emerging data needing to be taken to account. For example, the capsule is not needed for titan cells to form. Another key fact to consider is the conditions defined for titan cells have predominantly been found in the *C. neoformans* wild type strain H99, therefore more diverse strains are required across the

Cryptococcus sp to fully define the characteristics of this cell (Dambuza et al., 2018, Trevijano-Contador et al., 2018). For the *in vitro* model used in this thesis, Dambuza et al. found when bacterial cell wall peptidoglycan component muramyl dipeptide is present, there was an increase in ploidy and in cell size. In their study, this bacterial component proved the ability of a co-culture containing bacteria and bronchial lavage fluid is able to induce titanisation in host related environment (Dambuza et al., 2018). Hence, the use of the serum containing bacterial cell wall component in the *in vitro* model.

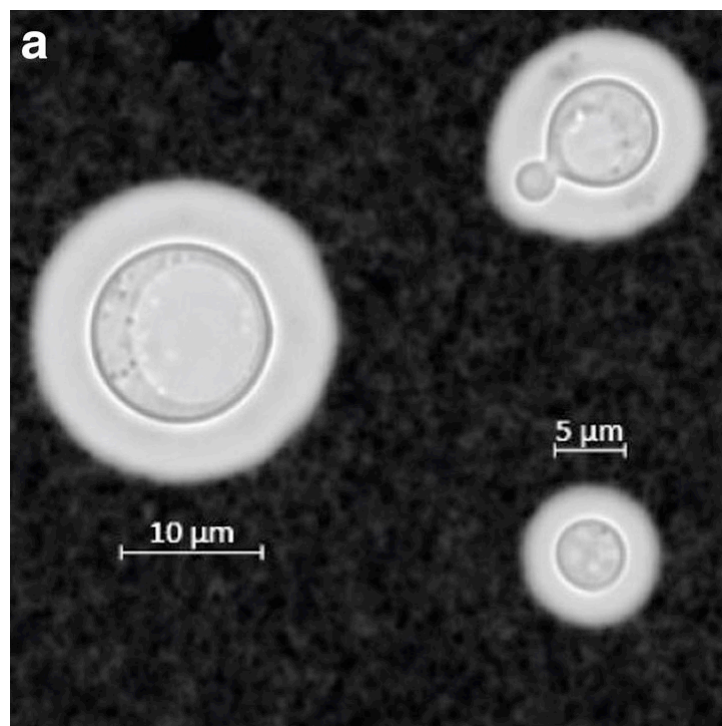


Figure 4. *In vitro* induced titan cells. Image taken from Zhou and Ballou showing the difference in morphology between *C. neoformans* yeast and Titan cells. The cells were grown following our *in vitro* titan inducing conditions. On the left the cell with a diameter of 10 μm was a titan cell whilst the bottom right, the cell with a diameter of 5 μm was a yeast cell (Zhou and Ballou, 2018).

1.4.3 Signaling pathway induction

Along with cell density and role of nutrients, studies have shown that *in vivo* titan cell induction is in part regulated by the core cyclic AMP (cAMP)/protein kinase A (PKA) pathway, which is also a central regulator of other known *Cryptococcus* virulence factors.

These pathways regulate capsule structure and melanin production, and mutants are avirulent when deficient in this pathway (Alspaugh et al., 2002, D'Souza et al., 2001). Additionally, initial studies revealed the pheromone pathway and mating type to be important in inducing titan cell production: mating type MAT α cells produce more Titan cells than MAT α , which indicated a specific response to alpha pheromone through Ste3a pheromone receptor (Okagaki et al., 2010). Further on, Okagaki et al found Gpr5, a g-protein coupled receptor, was needed for formation of titan cells, by signalling through G α protein Gpa1 and Ste3a (Okagaki et al., 2010). While alpha pheromone interacts with Ste3a, it is not known what ligand interacts with Gpr5, other than that it can be found in the pulmonary environment due to Gpr5-dependent titan cell formation in the lungs. The interaction of Ste3a and Gpr5 with Gpa1, a G α protein, triggers titan cell induction through adenylyl cyclase Cac1, producing cyclic AMP and releasing Pka1 from Pkr1-mediated negative regulation (Alspaugh et al., 2002, Xue et al., 2006) (figure 5). Using the new *in vitro* models, induction of titan cells through Cac1, Pka1 and Gpr5 was seen to be similar in morphology to that observed *in vivo* (Dambuza et al., 2018). Furthermore, the capsule regulating transcription factors Rim101, Gat201 and Usv101, which are also influenced by cAMP, have been found to play a role in titanisation (Okagaki et al., 2011, Gish et al., 2016, Dambuza et al., 2018). Despite this progress, there remains limited information about the molecular mechanisms of titan cell formation and their impact on disease.

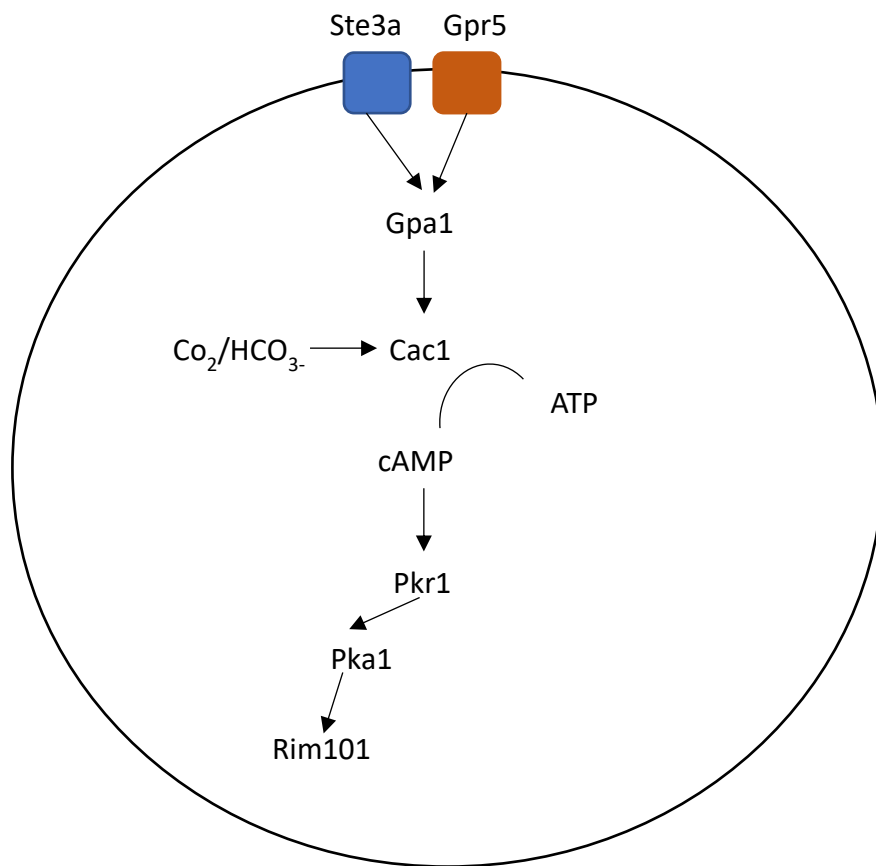


Figure 5. Induction of titan cell through cAMP/PKA pathway. A diagram depicting the pathway that has been described through the literature about how titan cell formation is induced. A lot of the literature focuses on the cAMP/PKA pathway. Ste3a pheromone receptor is activated through the alpha pheromone whilst the ligand that binds to the Gpr5 receptor is unknown. This leads to a cascade of events, producing cAMP and activating rim101.

1.5 Virulence factors

The agreed definition of virulence factors are agents that allow the pathogen to cause and maintain the disease in a host (Pirofski and Casadevall, 2015). In the case of *C. neoformans* there are well documented factors: urease production, polysaccharide capsule formation, growth at mammalian body temperature, and melanisation (Chang and Kwon-Chung, 1994, Petter et al., 2001, Salas et al., 1996).

1.5.1 Urease production

Urease has been identified as a virulence factor in other organisms such as *Coccidioides posadasii* and *Helicobacter pylori*, however the role of urease as a virulence factor in *C. neoformans* is still being studied (Mirbod-Donovan et al., 2006, Singh et al., 2013, Fu et al., 2018). Urease levels are now being used as a tool for diagnosing cryptococcosis due to increased levels of urease produced by *C. neoformans* being detectable in the blood of infected patients (Zimmer and Roberts, 1979, Zaragoza, 2019).

Urease is an enzyme produced by organisms when in an environment with limited nitrogen, and works by converting urea into ammonia and carbon dioxide. This ammonia is required as a nitrogen source in *C. neoformans* (Callahan et al., 2005). There is a possibility that urea is used by pathogens for nutritional purposes owing to its even distribution in humans, making it accessible as a nitrogen source (Rutherford, 2014).

Urease has been acknowledged as a virulence factor in more ways than one. It was found to be necessary for cryptococcal host response as urease encourages the production of nonprotective Th2 response along with recruitment of young dendritic cells to the site of infection (Osterholzer et al., 2009). Whilst infection takes place, in the microcapillary vessels sequestration of *Cryptococcus* yeast happens due to urease, which allows penetration to the brain through the BBB (as mentioned in section 1.1) (Cox et al., 2000, Olszewski et al., 2004). It has been hypothesised that *C. neoformans* adhesion is promoted by ammonia, either through enhanced adhesins expression or lethal force on BBB junction integrity which could play a role in the pathogens ability to cross this barrier (Olszewski et al., 2004). When

this enzyme is not present, at basic pH there is a defect in *C. neoformans* fitness (Fu et al., 2018).

1.5.2 Polysaccharide capsule formation

The cryptococcal cell wall consists of chitin, glucans, glycoprotein and chitosan, and anchors the cryptococcal capsule that surrounds it. The capsule is predominantly composed of two large polysaccharides; glucuronoxylomannogalactan (GXMGal) and glucuronoxylomannan (GXM), which are involved in virulence (Kumar et al., 2011). The synthesis of the capsule is still not fully understood, with some studies suggesting that the polysaccharides are synthesised in the Golgi (Yoneda and Doering, 2006). On the other hand, there is evidence suggesting the components of the polysaccharide are made in the endoplasmic reticulum before being transferred into small vesicles and transported to the Golgi (Rodrigues et al., 2007).

Intriguingly, the size of the capsule is dependent on the fungal response to environmental stress. Low iron levels, host CO₂ concentration, low glucose and nitrogen concentration, pH, and serum have been seen to induce capsule *in vitro* (Zaragoza et al., 2003).

The capsule is unique in its structure and has been used as a way for identification of *C. neoformans* in patient samples such as cerebrospinal fluid (CSF) (O'Meara and Alsbaugh, 2012). The capsule is not a necessity for the yeast to survive, as cells without capsule are capable of replicating and surviving *in vitro*, but acapsular cells are avirulent (Chang and Kwon-Chung, 1994, Chang and Kwon-Chung, 1998, Chang and Kwon-Chung, 1999, Zaragoza et al., 2009). This is due to the role that capsule plays in protecting yeast during

infection, such as avoiding phagocytic uptake and degradation by masking fungal binding sites from macrophage receptors (Cross and Bancroft, 1995, Chun et al., 2011). If the yeast is inside the macrophage, the capsule becomes an antioxidant defence against host reactive oxygen species (Zaragoza et al., 2008).

In order for *C. neoformans* to adjust to environmental stresses, activation of certain transduction pathways is essential. A study has shown that there is cross talk between transduction pathways that play roles in capsule regulation (Kwon-Chung et al., 2014). *C. neoformans* reacts to low iron, glucose and nitrogen by activating the cyclic AMP (cAMP)/PKA pathway, where Pka1 phosphorylation activates the Rim101 transcription factor (D'Souza et al., 2001). Pka1 negatively regulates mannoprotein Ova1, which allows enhanced production of capsule (Hu et al., 2007). In response to increased carbon dioxide (CO₂) levels in the host, *C. neoformans* activates the cAMP pathway. It does so by converting CO₂ into HCO₃⁻ through Can2 carbonic anhydrase, which is sensed by Cac1, stimulating cAMP production (Klengel et al., 2005). For the majority of fungi, pH sensing is required to tolerate environmental pH. *C. neoformans* has integrated this pathway the pH sensing pathway with the cAMP pathway, with activation of Rim101 transcription factor not only from Pka1 but also as a result of cleavage by Rim20, which is activated via alkaline pH (O'Meara et al., 2010). As pH is influenced by CO₂, this may also help signal neutral pH (Klengel et al., 2005).

1.5.3 Growth at body temperature

In order for a fungus to be able to infect the host, it must be able to grow and thrive in the host body temperature, 37°C (Boyce and Andrianopoulos, 2015), and the ability to withstand

host temperatures has been seen in *C. neoformans*. This is what distinguishes it from other species of *Cryptococcus* that are non-pathogenic (Petter et al., 2001). *C. neoformans* maintains yeast morphology at high temperatures, despite an impact on budding and growth (Nichols et al., 2007).. One factor that helps *C. neoformans* grow at host temperature of 37°C is the Ras pathway, which maintains *C. neoformans* cytoskeletal integrity at high temperatures. There was a theory that in order to regulate yeast morphology during stress, *C. neoformans* Ras1 works through the conserved Rho-GTPases Rac1 and Cdc42 (Nichols et al., 2007). Cdc42 plays an important role in eukaryotic polar growth as well as being a key element during pathogenic morphological transitions in yeasts. Price *et al* have shown that, in order to tolerate the high temperature, the yeast requires the Cdc42 pathway, Consistent with this, the *cdc42Δ* mutant was still able to form filaments and carry out mating, while *ras1Δ* mutant could not (Price et al., 2008). Interestingly, *C. neoformans* has two Cdc42 paralogs, Cdc42 and Cdc420, which are not seen in other species. This duplication supports environmental stress responses. For growth at 37°C, Cdc42 is needed, and in a cryptococcosis murine model, Cdc42 is necessary for virulence, while Cdc420 is not (Ballou et al., 2010). In contrast, Rac1 is needed for production of mating hyphae at 25°C and also influences cell ploidy in a Ras-dependent manner (Ballou et al., 2013).

1.5.4 Melanisation

The final well-established virulence factor of *C. neoformans* is melanin, a dark pigment found in the cell wall that in the presence of environmental stress helps safeguard the yeast (Nosanchuk and Casadevall, 2006). Melanin is able to forage oxygen radicals released by the host, inhibiting oxidative damage and allowing growth inside phagocytes (Rosas and Casadevall, 1997, Nosanchuk and Casadevall, 2006). It has also been found to protect *C.*

neoformans from environmental stresses such as antifungal compounds, reducing drug efficacy by binding to the fungicides (McDonald et al., 2012, Salas et al., 1996). Melanin has also been shown to play a role in helping *C. neoformans* to grow at low temperatures by enabling heat capture (Cordero et al., 2018).

In order to produce melanin, *C. neoformans* requires the substrate 3,4-dihydroxyphenylalanine (L-DOPA), an exogenous diphenolic compound (Eisenman et al., 2011). The synthesis of melanin from L-DOPA involves *LAC1* and *LAC2* genes which encode diphenol oxidases, but *LAC1* plays a larger role in the synthesis and in the virulence (Salas et al., 1996, Zhu and Williamson, 2004, Pukkila-Worley et al., 2005). Mutants that were unable to produce melanin had a substantial decrease in virulence (Salas et al., 1996).

As well as its protective role, melanin additionally alters the production of cytokines in the host thus, shielding the yeast from macrophages. This suggests a new mechanism whereby the pigment adds to virulence by changing the host immune system in response to the presence of melanin (Mednick et al., 2005, Liu et al., 1999). Another study also demonstrated the pigment's role in dissemination of *C. neoformans* through infection from the respiratory tract, showing that the yeast which produced melanin were not able to grow in the brain or lung, but were better able to disseminate from the lungs (Noverr et al., 2004).

1.5.5 Titan cell formation

Titan cells have many characteristics that indicate they could be classified as a virulence factor, due to promoting survival in the host and influencing disease through enhancing dissemination and virulence of *C. neoformans*. As titan cells are fairly large cells, they are

unable to be phagocytosed and therefore cannot be cleared by the immune system from the lungs. Titan cells are resistant to phagocytic killing and oxidative stress, and they have been shown to play a role reducing phagocytosis of the normal sized *C. neoformans* (Okagaki and Nielsen, 2012).

Cryptococcus infection starts in the lungs causing cryptococcal pneumonia, which is a forerunner to cryptococcal meningitis. In order for cryptococcal meningitis to occur, *C. neoformans* need to disseminate to the brain. Studies have found that dissemination from the lung occurs through paracellular crossing of lung epithelium, phagocytosis and transcytosis (Crabtree et al., 2012, Zhou and Ballou, 2018). As titan cells are large, they are unable to disseminate (Okagaki et al., 2010, Crabtree et al., 2012). However, the progeny of titan cells are able to survive in phagocytic cells due to being resistant to oxidative stresses, which would enhance intracellular dissemination (Okagaki et al., 2010). *C. neoformans* dissemination and survival due to titan cells increases virulence in murine model due to a decrease in phagocytosis (Crabtree et al., 2012).

In vivo studies have shown that titan cells play a role in the progression of the disease. In mice, mutants with defects in titanisation or isolates that are hypotitanising have altered dissemination (Crabtree et al., 2012, Dambuza et al., 2018). But other literature suggests titan cells may not be alone in enabling dissemination, as mutants which produce high-levels of titan cells, *usv101Δ* or *otc1Δ*, have been found to cause a reduction in dissemination (Dambuza et al., 2018, Crabtree et al., 2012). There could be a correlation between these two observations by keeping in mind phagocytosis of titan cells happens at low rates due to the large size, and phagocytes help traffic engulfed yeast out of lungs. Therefore, mutants

that produce high levels of titan cells are expected to be less phagocytosed and to disseminate less than those with low levels of titan cells (Dambuza et al., 2018).

The occurrence of these virulence factors during infection, contributes to the high disease burden and mortality. Which gives rise to the importance of developing new antifungals that are able to target these virulence factor to help fungal clearance. Therefore, I am going to discuss the antifungal therapies available to date and the knowledge behind the mechanisms of drug resistance across fungi.

1.6 Literature review- Antifungals: mode of action and resistance

1.6.1 Antifungals research is needed

There are insufficient number of antifungal drugs that have been approved for human usage, with the last approval in 2002 (Pianalto and Alspaugh, 2016). Remarkably, it was found that research into cryptococcosis received less than 5% global funding whilst tuberculosis and malaria in total received 35.5%, even though mortality rates are not that far apart (Rodrigues, 2016, Moran et al., 2015, Almeida et al., 2019). The one-year mortality rate of cryptococcal infections was found to be up to 78% in HIV-associated patients and up to 42% in non-HIV-associated patients (Pasquier et al., 2018).

The antifungal drug classes that have been approved and are offered comprise of: allylamines, azoles, echinocandins, flucytosine, and polyenes (Kathiravan et al., 2012, Robbins et al., 2017). Allylamines and azoles inhibit ergosterol biosynthesis of the fungal cell membrane (figure 6a), whilst echinocandins inhibit the biosynthesis of the fungal cell

wall component β -1,3-D-glucan (figure 6c). Flucytosine inhibits DNA synthesis and metabolism of pyrimidine, meanwhile polyenes cause cell lysis by binding to fungal ergosterol in the plasma membrane, leading to cell function disruption (figure 6b) (Cowen et al., 2014, Perfect, 2017, Robbins et al., 2017). There has been an increase in fungal strains with antimicrobial resistance, threatening the limited number of antifungals available (Fisher et al., 2022). Studies have documented resistance against echinocandins and azoles, but resistance to polyenes seem to be rare (Vincent et al., 2013). In order to develop new and improved antifungal treatments, further research is required into the mechanisms that drive resistance. This review will examine the mechanisms of action of antifungals (figure 6) and the fungal mechanisms of resistance.

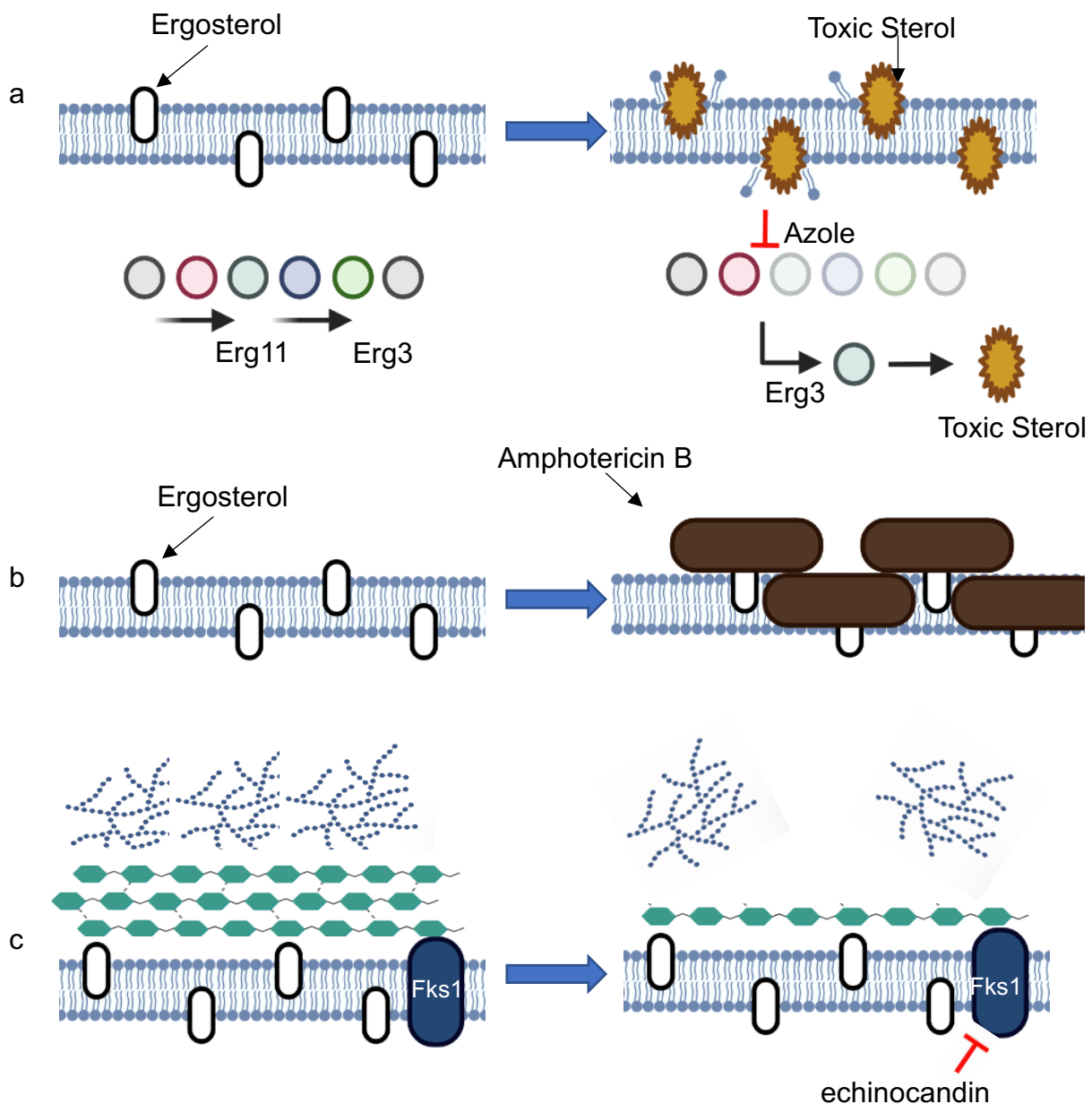


Figure 6. Different antifungals and their mode of action. a) Azoles function by targeting either *cyp51A* (*A. fumigatus*) or *ERG11* (*C. neoformans* and *C. albicans*) which encodes lanosterol demethylase which is the ergosterol biosynthetic enzyme. This inhibits the ergosterol biosynthesis pathway through inactivation of *ERG3* leading to the formation of toxic sterol and causing stress to the membrane. b) Polyenes function by binding to the plasma membrane ergosterol causing cell lysis. c) Echinocandins function by binding to *FKS1*, which encodes the (1,3)-B-D-glucan synthase, by acting as a non-competitive inhibitor which leads to stress to the cell wall and loss of integrity. This image is adapted from (Revie et al., 2018).

1.6.2 Azole mode of action

Before the development of azoles, the limited number of antifungal drugs available were known to have unfavourable effects in humans because they targeted fungal factors shared by humans, as a result of shared evolutionary history of eukaryotes. The development of azoles in the late 1960s changed this, with the discovery of ketoconazole, the less toxic derivatives of the imidazole group (Heeres et al., 1979, Drouhet and Dupont, 1980, Maertens, 2004). But the introduction of triazoles, such as itraconazole and fluconazole, did not take place until the late 1980s. These are now widely used as a prophylaxis as they presented a wide range of antifungal activity and are much safer to use than ketoconazole (Arndt et al., 1988, Brammer et al., 1990, Maertens, 2004). The differences between the two groups of azoles is the triazoles azole ring have 3 nitrogen atoms instead of 2 that the imidazole group has. However, all the azoles function in the same manner.

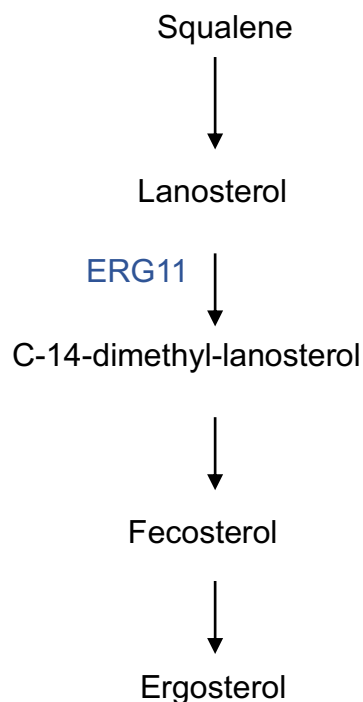


Figure 7. Ergosterol biosynthesis pathway. Azoles work by targeting the ergosterol biosynthesis pathway, specifically ERG11, a CP540 14 α -demethylase which helps convert lanosterol to ergosterol through removal of two methyl groups. This pathway was adapted from (Lv et al., 2016).

Ergosterol is the fungal cell membrane principal component, and operates as the membrane integrity, fluidity and asymmetry bioregulator (Nozawa and Morita, 1986, Ghannoum and Rice, 1999). Azoles' antifungal activity begins through inhibition of ergosterol biosynthesis by inhibiting 2 cytochrome P450 enzymes, *ERG11/CYP51* encoded lanosterol 14 α -demethylase and *CYP61* encoded Δ^{22} -desaturase (figure 7) (Kelly et al., 1997). By interrupting this synthesis pathway, 14 α -methyl sterol accumulation occurs which causes toxicity. This toxicity through sterol accumulation instigates modifications to the function of membrane bound enzymes, membrane permeability and stability (White et al., 2002, Zhang et al., 2010). In the ergosterol biosynthesis pathway, the lanosterol 14 α -methyl group is oxidatively removed by Erg11. The Erg11 protein contains a heme cofactor and in the active site encompasses a ferric iron moiety which is what the azole binds to. This blocks

lanosterol, which is the enzyme's natural substrate, from binding to the enzyme and in turn disrupts the pathway (Odds et al., 2003). The extent of the azole inhibitory effect in different species of fungi is determined by the interaction between Erg11 enzyme and the azole. What is also important to note here is the active site of Erg11 enzyme is different between mammalian and fungal cholesterol biosynthesis P450 lanosterol 14 α -demethylase (Koltin and Hitchcock, 1997, Francois et al., 2006).

Azoles are mainly known to inhibit the 14 α -demethylase but they have been shown to have other modes of actions. The imidazole group has been shown to inhibit biosynthesis of the lipid membrane and other membrane-bound enzymes (Francois et al., 2006). Another mode of action shown was induction of the biosynthesis of farnesol, a quorum-sensing molecule, which is activated when azoles such as miconazole, ketaconazole and fluconazole are incubated with *Candida* sp (Hornby and Nickerson, 2004).

Although these antifungals are used regularly, not including ketoconazole, they do have their limitations depending on the fungal species. For example, fluconazole is ineffective against aspergillus due to resistance but itraconazole is effective and is given as a first-line treatment (Leonardelli et al., 2016). On the other hand, itraconazole is not recommended as a first-line of treatment for other fungal infections due to its low bioavailability (Bae et al., 2011). Another major limitation is the increase in azole resistance across fungal pathogens, and this will be discussed in detail below.

1.6.3 Molecular mechanisms of azole resistance

1.6.3.1 Changes in Ergosterol Biosynthetic Enzyme

In *C. albicans* clinical isolates, one of the most frequent mechanism of azole resistance is the over-expression of *ERG11/CYP51*. This causes an increase in the target protein which compels the need for more azoles in order to inhibit ergosterol biosynthesis, diminishing susceptibility (Franz et al., 1998, Perea et al., 2001). One of the reasons for the over-expression of *ERG11* to occur is initiating mutations in the transcription factor Upc2. Upc2 is a zinc-cluster transcription factor that regulates gene expression of *ERG11* and other genes in the biosynthesis pathway. Studies have found in *C. albicans* clinical isolates that gain-of-function mutation of Upc2 increases expression in *ERG11* which further leads to fluconazole resistance (Silver et al., 2004, Flowers et al., 2012, Dunkel et al., 2008). Other fungal pathogens have also shown azole resistance through over expression of *ERG11* such as *C. tropicalis*, and *C. glabrata* (Jiang et al., 2013, Rogers et al., 2006). However, the reason for azole resistance and the mechanism of over expression are not known.

Another mechanism of azole resistance seen in *Candida* sp. is substitutions of amino acids that prevent the binding of the drug to the Erg11 enzyme. The more frequently seen substitutions in *C. albicans*, G464S and R467K, occurs near the heme binding site (Morio et al., 2010, Balashov et al., 2005). For *A. fumigatus*, the most common resistance mechanism to azoles seen is alterations in the target site of the Cyp51 enzyme, but during infection additional mutations can be gained (Howard et al., 2009). Similar to *C. albicans*, these alterations take place close to the heme-binding site, mainly in codons 54 and 220 which causes a change in Cyp51 (Xiao et al., 2004, Fraczek et al., 2011). Interestingly,

patients who have not had any azole therapy have shown environmentally derived azole resistance in *A. fumigatus* due to agricultural azole use (Verweij et al., 2013).

In *C. neoformans* *EGR11*, whilst certain clinical isolates and mutator strains have indicated other point mutations whose role in drug resistance is unknown, *ERG11* point mutations are rarely seen in clinical isolates (Rhodes et al., 2017, Stone et al., 2019, Arras et al., 2017, Boyce et al., 2017, Rodero et al., 2003, Sionov et al., 2012). Despite this, azole resistance is commonly observed (Xu et al., 2001). This suggests an alternative mode of azole resistance and this difference in drug resistance mechanisms between *C. neoformans* and other fungi is further discussed in chapter 3 introduction.

1.6.3.2 Drug efflux

In *C. albicans*, drug efflux has been shown to play more of a role in drug resistance than mutations in *ERG11* (Perea et al., 2001). Similarly, as *C. glabrata* is a haploid fungal pathogen it would seem more probable for azole resistance to occur due to *ERG11* point mutations but a rise in drug efflux is the common reason for resistance (Borst et al., 2005). There are two different systems of membrane associated drug efflux which regulate azole resistance in fungi: the major facilitator superfamily (MFS) and the ATP-binding cassette (ABC) superfamily.

The ABC superfamily consists of ATP-dependent transporter proteins which catalyses ATP hydrolysis. The transporter made of 2 cytoplasmic nucleotide-binding domains (NBD) and 2 transmembrane span (TMS) domains (Cowen et al., 2014). Across the genomes of fungal pathogens there are numerous different structures of the ABC transporter. Studies have

found that in clinical isolates shown to be azole resistant, the *Candida* sp. ABC transporters Cdr1 and Cdr2, along with Mdr1, are involved; increased expression of both *CDR1* and *CDR2*, which encode the pleiotropic drug resistance (PDR) class transporters, lowers accumulation of azoles and amplifies drug efflux (Coste et al., 2006). Mdr1 upregulation initiates azole resistance in *C. albicans* through efflux but also avoids intrinsic host defences through antimicrobial peptide efflux (Hampe et al., 2017).

In other fungal pathogens, ABC transporters also play a role in azole resistance. In *A. fumigatus*, during azole exposure there is an increase in expression of ABC transporter genes including *MDR1* and *MDR2*, along with increase in AtrF (Slaven et al., 2002, da Silva Ferreira et al., 2006). In *C. neoformans*, Afr1 has been shown to play a role, this is further discussed in Chapter 3 introduction.

1.6.3.3 Chromosomal abnormalities

Fungal pathogens undergo many evolutionary adaptations when faced with environmental stress, one of them being genome plasticity which plays a role in azole resistance (Selmecki et al., 2006). Chromosome-level changes are a form of genome plasticity, and this can be seen in resistant *C. albicans* such as increase in chromosome copy number through the formation of an isochromosome of chromosome 5 which contains *ERG11* (Selmecki et al., 2006). Aneuploidy is commonly seen in most azole resistant isolates, which gives rise to the hypothesis that azole exposure helps the formation of aneuploidies. This was seen in *C. albicans* where following azole exposure there was a formation of tetraploid cells, whilst *C. albicans* itself is diploid (Harrison et al., 2014). In addition, chromosome 3 and 6 copy

number increase were seen in *C. albicans* to help decrease azole susceptibility (Hirakawa et al., 2017).

Alterations in chromosomes have also been seen in *C. neoformans* and *C. glabrata* (Sionov et al., 2010, Poláková et al., 2009). In azole resistant *C. glabrata* isolates there have been formations of aneuploidies where resistance occurs by increasing *ERG11* copy number (Poláková et al., 2009, Marichal et al., 1997). In *C. neoformans*, formation of chromosome 1 disomy is commonly seen in azole resistant isolates (Sionov et al., 2010). Interestingly, titan cell formation causes a change in the ploidy of *C. neoformans* cells which in theory could also result in azole resistance. This will be further discussed in chapter 3 introduction.

1.6.3.4 Mitochondrial defects

There are some fungal species that are known to be 'petite', meaning that they can survive on partial or complete loss of mitochondrial DNA or partial loss (Ferrari et al., 2011). *C. glabrata* is one of them and this has allowed experimental work to be undertaken on the role of azole resistance due to mitochondrial dysfunction. In *C. glabrata* the petite mutant is not commonly seen in clinical isolates and does not show a defect in fitness, although avirulence has been seen in some petite mutants (Bouchara et al., 2000, Singh-Babak et al., 2012). Other fungal pathogens such as *C. neoformans* and *C. albicans* are known to be 'petite-negative' as they cannot survive if mitochondrial DNA is lost. Be that as it may, inhibition of the mitochondria using pharmacological compounds has been shown to increase *C. albicans* susceptibility to azoles, in contrast to *C. glabrata*, raising the possibility that fluconazole sensitivity and mitochondrial dysfunction are linked (Sun et al., 2013).

Further studies have also begun to look into the role of reactive oxygen species (ROS) in azole resistance. A study carried out by Kobayashi et al found in *C. albicans* intracellular production of ROS is induced by miconazole. They also showed fluconazole inducing ROS, however it was less than compared to miconazole (Kobayashi et al., 2002). Another study carried out by Francois et al found in *C. albicans* miconazole was able to increase a significant level of endogenous ROS. This gave rise to the theory that antifungal drugs that are able to induce ROS production are fungicidal. Miconazole which did not produce a significant rise in endogenous ROS levels in *C. albicans*, was shown to be fungistatic (Francois et al., 2006). This raises the possibility that azole induced ROS adds to ROS produced during mitochondrial dysfunction, leading to fungal cell death; however, the source of this azole-induced ROS remains unclear.

1.6.4 Echinocandin mode of action

Echinocandins are a recent class of antimycotics developed for fungal infections. Echinocandins are lipopeptides that are semisynthetic and consist of a lipid side chain (Hoffman and Walsh, 2011). The parental compound, echinocandin B, was first discovered in 1974, however it was not used as an antifungal due to its action being haemolytic and therefore toxic in humans (Balkovec et al., 2014). Following this, caspofungin was the next echinocandin drug to be revealed in 1989, and closely after micafungin in 1990 (Chen et al., 2011). Caspofungin was the first echinocandin to be approved by the FDA in 2001, accompanied by micafungin which was approved in 2005. Anidulafungin was discovered before the other two in 1970, however was not approved and introduced to the market until 2006 (Aguilar-Zapata et al., 2015). More recently, a new echinocandin called rezafungin is

currently in phase 3 clinical trials and is showing promising safety and efficacy, with promises that it will help with early discharge of patients (Ham et al., 2021).

What makes echinocandin different is that it selectively targets the cell wall of fungi. This is key as fungi are evolutionarily similar to humans, but echinocandins do not have an effect on mammals as they do not have cell walls, therefore they have less side effects compared to azoles (Balkovec et al., 2014). Echinocandins mode of action is acting as non-competitive inhibitors of the enzyme that is required for the synthesis of the fungal cell wall, β -(1,3)-D-glucan synthase. This enzyme consists of 2 subunits: a regulatory subunit Rho1p which is part of the Rho GTPase family, and the catalytic subunit Fks1p which is encoded by the genes *FKS1*, *FKS2*, and *FKS3* (Liu and Balasubramanian, 2001). β -(1,3)-D-glucan synthase polymerises UDP-glucose to form β -(1,3)-D-glucan, an essential factor in upholding the strength and integrity of the cell wall (Fesel and Zuccaro, 2016, Patil and Majumdar, 2017). By inhibiting the enzyme, echinocandin inhibits the synthesis of β -(1,3)-D-glucan, causing the cell wall to be exceedingly permeable which results in osmotic instability and cell lysis (Bartizal et al., 1997).

All of the echinocandin drugs are fungicidal against *Candida* sp. *Candida* cells when treated with an echinocandin become quite distorted and large with the expected cell membrane lysis (Nishiyama et al., 2002). For *Aspergillus* sp it is different, causing a fungistatic effect. When exposed to echinocandin, *Aspergillus* morphology is altered and the hyphal cell wall structure becomes disrupted and changes (Kurtz et al., 1994). Finally, the echinocandins are not effective against *Cryptococcus* species. This is probably due to fungal species having different proportions of glucan in their cell wall. As echinocandins have been shown

to be effective and safe, they are the frontline treatment, along with fluconazole, against candidemia (Pappas et al., 2009).

1.6.5 Molecular mechanisms of echinocandin resistance

1.6.5.1 Amino acid substitutions and Polymorphism

Resistance against echinocandin does not occur like azoles through multidrug transporters, but rather through amino acid substitutions of the β -(1,3)-D-glucan synthase highly conserved Fks subunits (Park et al., 2005). In *C. glabrata* the mutation most commonly takes place in regions of *FKS2* followed by *FKS1*, whilst in *C. albicans* it occurs only in *FKS1*, and these substitutions result in a decrease in sensitivity of the enzyme to echinocandin (Garcia-Effron et al., 2009, Johnson et al., 2011). Surprisingly, in *C. glabrata* *FKS2* resistance can be reversed as its expression is dependent on calcineurin, a serine/threonine phosphatase, and administering a calcineurin inhibitor FK506 helps decrease resistance (Katiyar et al., 2012). Strikingly, *C. albicans* Fks1 amino acid substitutions lessen the glucan biosynthesis catalytic reaction rate which results in altered morphology of the cell wall due to decreased fitness (Garcia-Effron et al., 2009, Ben-Ami et al., 2011). It has been shown that between patients, resistant strains are rarely transmitted and this could be due to the resistant strain decreased fitness, but also that most patients have infection that is the same strain as found in their gut (Ben-Ami et al., 2011). In addition, certain *Candida* sp have shown to display higher levels of resistance than others; *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (Garcia-Effron et al., 2008). A reason for this could be due to the naturally occurring Fks1 polymorphism across the species which could contribute to the reduction in susceptibility to echinocandins (Garcia-Effron et al., 2008).

1.6.5.2 Chitin synthesis

Fungi are able to acquire stress response factors that help with increasing tolerance to drugs such as echinocandins. One of these response factors is increasing chitin synthesis. In *C. albicans*, when exposed to echinocandin at low levels, chitin synthase gene expression is induced. High osmolarity glycerol (HOG), calcineurin and protein kinase C (PKC) signaling pathways synchronises this chitin increase (Munro et al., 2007). Chs2 and Chs8 are chitin synthases which when activated allow *C. albicans* to persist through high doses of echinocandin (Walker et al., 2008). In other *Candida* sp., such as in *C. tropicalis*, high levels of chitin as a response to exposure to echinocandin have also been seen (Walker et al., 2013). Similarly, the increase in chitin synthesis as a mechanism for resistance also takes place in *Aspergillus* sp. When the chitin synthase genes *CSMA* and *CSMB* in *A. fumigatus* are deleted it increases echinocandin susceptibility; however, this does not have an effect on the chitin content of the cell wall (Jiménez-Ortigosa et al., 2012).

1.6.5.3 Cell wall integrity

The molecular chaperone Hsp90, PKC, and calcineurin have been demonstrated to mediate the integrity of the fungal cell wall, and are crucial in chitin synthesis upregulation along with tolerance of echinocandin (Singh et al., 2009). Hypersusceptibility to echinocandins in *S. cerevisiae* occurs following loss of cell wall integrity encoding genes such as *PKC1* and *SLT2*, whilst exposure to the antifungal also induces PKC cell wall integrity genes (Markovich et al., 2004, Reinoso-Martín et al., 2003). Cell wall integrity due to echinocandin induced cell wall stress also takes place in *C. albicans*. Mkc1 which is *C. albicans* terminal MAPK of the PKC pathway, is stimulated by echinocandin exposure and

is necessary for tolerance (LaFayette et al., 2010). Alongside this, a study showed echinocandin is synergistic (interaction that produces a greater effect) with the Pkc1 kinase inhibitor against *C. albicans* (Sussman et al., 2004).

Interestingly, the only way to have echinocandins is as an intravenous formulation, and this can be quite limiting when used for less severe infections or as an oral agent (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3913534/>). There is a natural product isolated from *Hormonema carpetanum* that has the same mode of action of inhibiting β -(1,3)-D-glucan synthase but is structurally different and they are called enfumafungins (Schwartz et al., 2000, Pelaez et al., 2000 in notes). They are said to have a better oral bioavailability, with antifungal activity taking place more so *in vitro* than *in vivo*. A new form of enfumafungin called ibrexafungerp is the first oral therapy and has been found to be effective against *Candida* sp that are resistant to echinocandin (Davis et al., 2020).

Mechanisms of echinocandin resistance in *Cryptococcus* likewise are thought to be due to changes in cell wall integrity. A recent paper showed that exposure to echinocandins blocked expression of the Puf4 mRNA binding protein, which negatively regulates translation of *FKS1* mRNA and other mRNA encoding cell wall remodelling proteins (Kalem et al., 2021, Papon and Goldman, 2021).

1.7 Project aims

Previous research links aneuploidy with fluconazole resistance through two possible mechanisms: direct impact on nuclear division, as has been observed in *C. albicans*, and

indirect selection of heteroresistant aneuploid sub-populations, observed in murine models and patient isolates. Both studies focused on yeast-phase growth. However, during infection, *Cryptococcus* undergoes titanisation, forming highly polyploid Titan cells. The role of Titanisation in drug resistance has not yet been explored. This project will determine if titan cell formation influences niche adaptation such as drug resistance using the *in vitro* titanisation assay developed in Dr Ballou's lab. Alongside this, thanks to the development of our *in vitro* model I can successfully study titan cells without the use of mice, and allow for a better understanding of the mechanisms behind this morphological transition.

The aims of my project are:

1. To improve understanding of the mechanisms of titan cell formation through screening of chemical, genomic and genetic analyses
2. To test titan cell contribution to antimicrobial resistance (AMR)
3. To reinforce 3Rs objectives by showing the replacement of animal use by using the *in vitro* model.

Chapter 2. Materials and Methods

2.1 *C. neoformans* Strains and Media

The *C. neoformans* strains, in addition mutants from the Madhani library were used this in this study (table 1). All mutants and strains were stored at -80°C in water with 50% glycerol. The transcription factor deletion library plates (Jung et al., 2015), kinase deletion library plates (Lee et al., 2016), and the unpublished protein knockout library which was made freely available ahead of publication to the scientific community, we acknowledge the Madhani laboratory and NIH funding (R01AI100272) (available from the Fungal Genetics Stock Center in Kentucky, USA (www.fgsc.net), were cultured to use as source of mutant strain. The Tanzanian clinical isolate pairs were kindly given to us by Dr Neil Stone (Stone et al., 2019). To recover the strains, cells were streaked on yeast extract peptone (YPD) agar plates. This consisted of 1% yeast extract, 2% glucose, 2% bacto-peptone agar which was autoclaved and left to be set in sterile petri dishes. The strains were streaked and incubated at 30°C for 3 days before being stored at 4°C.

Table 1 *C. neoformans* strains. Table displaying the information about each strain used in this study. H99 was the wild type background in which all mutants were generated.

<i>C. neoformans</i> strain	Reference	Genotype
H99 (wild type)	(Perfect et al., 1980)	<i>H99S</i>
<i>cac1</i> Δ	(Madhani et al, unpublished)	<i>CAC1:NAT</i>
<i>tps1</i> Δ	(Madhani et al, unpublished)	<i>TPS1:NAT</i>
<i>tps2</i> Δ	(Madhani et al, unpublished)	<i>TPS2:NAT</i>
<i>pex13</i> Δ	(Madhani et al, unpublished)	<i>PEX13:NAT</i>
<i>pex14</i> Δ	(Madhani et al, unpublished)	<i>PEX14:NAT</i>
<i>aat1</i> Δ	(Madhani et al, unpublished)	<i>AAT1:NAT</i>
<i>nth1</i> Δ	(Madhani et al, unpublished)	<i>NTH1:NAT</i>

For regular cultures, cells were inoculated in 10 ml YPD media, which was the same as YPD agar except it was 2% bacto-peptone alone, overnight in a shaking incubator at 150rpm at 30°C.

When inducing titan cells, the cells were inoculated in 10 ml yeast nitrogen base (YNB) without amino acids (Sigma Y1250) with 2% glucose overnight in a shaking incubator at 150rpm and 30°C. Foetal calf serum (FCS) was acquired from Sigma and heat-inactivated (HI-FCS) by incubating for 30 minutes at 56°C before being filtered and stored at -20°C.

2.2. Titan Cell Induction Assay

To induce titan cells, cells were inoculated in YNB media as described in 2.1 overnight. Using a spectrophotometer at 600nm, 1 in 10 diluted cells absorbance was measured with PBS as a blank. Following this, the volume required for 10^4 cells/ml was calculated. 2 ml of 10% FCS in PBS was loaded per well in a 6 well plate with 10^4 cells/ml. The plate was incubated for 2-3 days at 37°C and 5% CO₂ unless otherwise indicated.

2.3 Drug sensitivity assay

2.3.1 Minimum inhibitory concentration assay

To assess the sensitivity of a strain to fluconazole, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) fluconazole sensitivity assay/ minimum inhibitory concentration (MIC) was carried out as described by clinical laboratory standards institute (CLSI) (<https://clsi.org>) with modifications as indicated below. After a regular culture

overnight of the strains, a spectrophotometer was used to measure the absorbance of 1 in 10 diluted cells. For yeast cells, the concentration needed per well was 10^6 cells/ml. If the cells were to be grown under titan inducing conditions, the number of cells per well was 10^4 cells/ml. In a 96-well plate, 64 $\mu\text{g/ml}$ fluconazole (sigma) in PBS was serially diluted by 2-fold in 100 μl YNB+ 2% glucose or PBS with 10% HI-FCS for titan induction, with the final concentrations in the wells after the cells were added was 32 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 2 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$ and DMSO as control. 100 μl of cells from each strain was added to its respective well, the final concentration of cells was 10^3 cells/ml. The plate was incubated at 30°C for 48 hours to induce yeast cells, or incubated the plate at 37°C and 5% CO_2 to induce titan cells before measuring the endpoint absorbance on FLUOstar spectrophotometer.

2.3.2 Growth curve assay

The role of cell size in fluconazole sensitivity over time was seen through a growth curve assay. Titan cells were induced as described in 2.2, following which using a 11 μm and 3 μm filter, the cells were separated and collected by size. The 11 μm was used to collect titan cells as they are defined as larger than 10 μm , while the 3 μm allowed collection of presumed titan daughter cells called titanides. These cells were then added to the MIC assay as described in 2.3. The growth absorbance was measured using FLUOstar for up to 64 hours, and was graphically displayed using GraphPad prism 8.

2.3.3 Checkerboard assay

When looking into the effect of the interaction of recently determined titanisation inhibiting (TI) drug with fluconazole (table 2), a checkerboard assay was carried out similar to Sun et

al (Sun et al., 2009). Following an overnight culture, the number of cells needed, 10^6 cells/ml for yeast and 10^4 cells/ml for titan inducing, was prepared. The stock dilutions of drugs were made at 4 times the preferred concentration. For each 96-well plate, 50 μ l of the stock fluconazole was serially diluted horizontally to column 11, while 50 μ l of the TI drugs were serially diluted vertically to row G. Column 12 and row H consisted of only the TI and fluconazole respectively. The final concentration of fluconazole in the wells after the addition of 100 μ l of cells was between 256- 0.25 μ g/ml, and 15- 0.23 μ M for the titanisation inhibiting drugs. The plates were then grown in 30°C to induce yeast cells or at 37°C and 5% CO₂ to induce titan cells for 48 hours. The absorbance was measured at 24hrs and 48hrs on FLUOstar.

Studies use checkerboard assays to calculate the fractional inhibitory concentration (FIC) index value, but this is usually based on inhibition of growth resulting from both drugs (Orhan et al., 2005). In this study however, the TI drugs do not inhibit growth and therefore FIC index value could not be determined. Instead the MIC was determined through changes in absorbance similar to 2.3.1.

Table 2. Drugs used in assay. Table displaying the information about each drug used and its role.

Name of Drug	Role in <i>C. neoformans</i>	Fungicidal or Fungistatic	Final concentration	Dissolved in	Company
Fluconazole	Targets the ergosterol biosynthesis pathway	Fungistatic	Either 256µg/ml or 64µg/ml	DMSO	Sigma
Retinoic acid	Inhibits titanisation	Neither	15 µM	DMSO	Sigma
Metyrapone	Inhibits titanisation	Neither	15 µM	DMSO	Sigma
Nicardipine hydrochloride	Inhibits titanisation	Neither	15 µM	DMSO	Sigma

2.4 Mitochondria assay

2.4.1 Mitochondrial inhibitors

To test the role of mitochondria in titanisation, we used certain metabolic compounds (table 3) that have been found to obstruct certain parts of the electron transport chain. This protocol was based on Trevijano-Contador *et al* study using the same compounds (Trevijano-Contador et al., 2017).

Table 3. Mitochondrial inhibitors and their role. Table displaying the information about each mitochondrial inhibitor used and its role.

Name of compound	Target	Stock concentration	Final concentration	Company
Rotenone	Inhibits complex I	1mM in DMSO	1 µM	Sigma-Aldrich
Antimycin A	Inhibits complex III	500mM in ethanol	0.11 µM	Sigma-Aldrich
Potassium cyanide	Inhibits complex IV	80mM in distilled water	1 mM	Sigma-Aldrich
Sodium azide	Inhibits complex IV	150mM in distilled water	0.25 mM	Sigma-Aldrich
Salicylhydroxamic acid	Inhibits alternative cytochrome oxidative pathway	160mM in ethanol	0.5 mM	Sigma-Aldrich

H99 and other mutant strains were grown in titan conditions as described in section 2.2, with the inhibitors at the final concentration stated in table 2, for 48 hours. These cells were then taken for flow cytometry (section 2.6) and microscopy (section 2.7).

2.4.2 Mitochondrial membrane potential

In order to measure mitochondrial potential, the dye Rhodamine 123 (Invitrogen) was used followed by measurement through flow cytometry. After cells had been cultured (section 2.2), they were collected by centrifugation at 4000rpm for 5 minutes. Next the cells were washed with PBS and spun again before discarding the supernatant. The cells were resuspended in flow cytometry buffer which consisted of PBS with 10% FCS. Rhodamine 123 was added at a final concentration of 20 µg/ml, and cells were incubated at 30°C for 30 minutes before being measured by flow cytometry. The data obtained were analysed using FlowJo v.10.1. The gating strategy FSC-H vs FSC-A was used to help exclude clumps and doublets.

2.5 Deletion mutant library screen

2.5.1 Plate culture

The original library plates were replicated in 200 µl YPD media containing 96-well plates using a sterilised metal replica plater (Sigma-Aldrich). These plates were cultured in a non-shaking incubator at 30°C for 48 hours. Following this, 2 µl of the cultured cells were transferred into 200 µl YNB media containing 96-well plates and placed into the non-shaking incubator at 30°C overnight. The cells were then transferred into 96 well plates consisting of 200µl PBS + 10% HI-FCS for 24 hours for titan induction (section 2.2). Fixation of the cells

was carried out using methanol with a final concentration of 33% in the wells for 5 minutes before being centrifuged at 1000rpm for 3 minutes. Following the removal of the supernatant, cells were resuspended in a stock of PBS containing 0.1 µg/ml calcofluor white (CFW) (Sigma-Aldrich) and 0.01x Sybr green (ThermoFisher).

2.5.2 Plate imaging

Robert Evans (University of Birmingham) carried out the high-throughput imaging of the deletion mutant library plates. Images were taken on a Zeiss cell discover 7 fluorescence microscope. 3 images were taken from each well in the brightfield channel, the calcofluor 405nm channel and the Sybr green 488nm channel.

2.5.3 *In silico* analysis

Daniel Fisch (University of Birmingham) carried out the high-throughput *in silico* analysis of mutant library screen using a programme he designed called host response to microbe analysis (HRMAN) (Fisch et al., 2019). 5 different methods were used on HRMAN (area, percentile, k-means, artificial intelligence, circularity) to categorise the cell, and HRMAN produced statistical z-score for each method. A threshold was determined where strains with a z-score <-2 were hypotitanising and >+2 were hypertitanising.

2.5.4 Gene Ontology Enrichment

Gene ontology (GO) analysis was performed using the FungiDB platform and the algorithm established by (Ashburner et al., 2000, Amos et al., 2021). This created a list of GO terms with reference to *C. neoformans* H99, and categorised the list based on 3 ontologies:

biological process, cellular component and molecular function. REVIGO was used in order to summarise the long list of GO terms (Supek et al., 2011).

2.5.5 RNA sequencing

RNAseq was performed on samples grown overnight in YNB (0h) or inoculated at either OD600 0.1 or 0.001 into 10% FCS in 1xPBS and incubated at 37°C, 5% CO₂ for 5, 7, 14, and 21 hours, with 21 hours filtered by size (>11 µm or <3 µm). Samples were fixed using 30% methanol on ice, lyophilised overnight, ground using a sterile mortar and pestle, and then RNA extracted using the Trizol extraction method. RNA quality was checked using bioanalyser and required to be RIN>6 (<https://cshprotocols.cshlp.org/content/2010/6/pdb.prot5439>). cDNA was prepared by Lexogen using oligo-dT priming and dual indexing, and resulting samples were sequenced using 3' targeted sequencing technology (QuantSeq) 3' ends of the read. The resulting reads were trimmed of their poly(A) tails using cutadapt, aligned to the H99 genome using hisat2, sorted and indexed the alignment files using samtools, and counted (transcripts per million) using featureCountsaligned (Edward Wallace, University of Edinburgh). The resulting dataset can be viewed here: <https://bifx-rta.bio.ed.ac.uk:3838/users/ewallac2/CryptoYTNNov2020/>.

2.6 Flow Cytometry

All data were collected using an NxT Attune flow cytometer with the following specifications: blue (BL-1, BL-2, BL-3) which has a wavelength of 405nm, violet (VL-1, VL-2, VL-3) which has a wavelength of 488nm, and yellow (YL-1, YL-2, YL-3) which has a wavelength of 561nm.

2.6.1 Prepping YPD culture control

When prepping YPD cultures to use as a control in flow cytometry, H99 was inoculated in YPD media as mentioned in 2.1 and left overnight in the shaking incubator. 1ml of YPD inoculate was added into 9ml of fresh YPD media and placed in the same shaking incubator for 3 hours before being stained.

2.6.2 DAPI

Cells were induced for Titan cells as described in 2.2. 1ml of the overnight cultures (section 2.1) and Titan cell cultures were centrifuged at 13,000rpm followed by a wash with PBS. The cells were then fixed for 10 minutes with methanol to a final concentration of 50%. Cells were spun again at 4,000rpm, followed by removal of the supernatant, and the pellet was resuspended in PBS. Cells were counted using a haemocytometer to determine the volume of cells required for 1×10^6 cells/ml. The required volume was added to flow cytometry buffer, consisting of PBS with 10% FCS, to make a total volume of 500 μ l and the DNA was stained with 0.3 μ l of DAPI which was analysed by flow cytometry to measure DNA content. Cells were analysed using Attune NxT flow cytometer on the VL-1 channel, and for each sample approximately 10,000 events were measured.

Table 4. Flow cytometry buffer concentrations. Table displaying the information about each reagent and its concentration in the buffer.

Reagent	Initial concentration	Final concentration	Company
PBS	1x	1x	Merck Life Science
EDTA	5mM	0.5mM	Sigma-Aldrich
HI-FCS	100%	0.5%	Sigma-Aldrich
Triton X-100	0.1%	0.05%	Sigma-Aldrich

2.6.3 Propidium Iodide

Similar to DAPI, the cells were induced for titan cells as described in 2.2. 1ml of titan cells and overnight culture (section 2.6.1) was centrifuged at 4000rpm for 5 minutes after which the supernatant was removed. The cells were then resuspended in 150 µl cold PBS, followed by drop-wise addition of 100% 350 µl cold ethanol (final concentration 70%) and mixed by gentle pipetting. Cells were incubated on ice for 1 hour and then washed with twice with PBS. Resuspension of the cells was carried out with a propidium iodide (PI) staining solution (table 5). Cells were analysed using Attune NxT flow cytometer on the YL-2 channel, and for each sample approximately 10,000 events were analysed.

Table 5. PI staining solution concentration. Table displaying the information about each reagent and its concentration in the stain solution.

Reagent	Initial concentration	Final concentration	Company
Sodium citrate	50mM	3.8 mM	Sigma-Aldrich
PI	1mg/ml	50 µg/ml	Sigma-Aldrich
PBS	1x	1x	Merck Life Science
RNAse	20mg/ml	0.5 µg/ml	Qiagen

2.6.4 Superoxide

Likewise, when staining for superoxide, cells were induced for titan cells as mentioned in section 2.2. Centrifugation of the cells was carried out at 4000rpm following which the supernatant was removed. Cells were washed with PBS and 100 µl flow cytometry buffer (table 4) was added. To stain the cells for measuring superoxide, 0.3 µl of superoxide detection reagent (Enzo life sciences) was added to each sample. Cells were analysed using Attune NxT flow cytometer on the YL-2 channel, and for each sample approximately 10,000 cells were obtained.

2.6.5 Flow cytometry analysis

The data gained from flow cytometry was analysed using FlowJo v.10.1. In order to gate a heterogenous population like titan culture, it is important to try to distinguish the population. Here this was done by plotting the data as forward scatter height (FSC-H) vs forward scatter area (FSC-A), with the forward scatter representing an estimated size of the cells (figure 8a). Following this, the cells have to be selected through gating. This was done by creating a gate using the polygon gate tool, and to draw the gate around the subset population so as to exclude the clumps and doublets (figure 8a). The doublets and clumps were identified as those whose area was increasing but not height and therefore were not included in the gate. This same gate was then added to all the other samples from that experiment. The selected population was displayed by clicking on the gate, following which the axis changed. On the y-axis, it was always histogram which showed the cell count, and on the x-axis depending on the experiment it was the channel that the dye had been measured on (figure 8b). All axis was set to log.

When analysing the second population for certain experiments, I did it by gating the last approximately 2.5% of the population on the graph (figure 8c). This is because statistically there should be a 95% confidence range, and therefore on either side of this range is 2.5%. This 2.5% was gated on cells that were untreated. When this gate was created I took the percentage of cells calculated by the programme, and displayed this data graphically using GraphPad Prism 9.

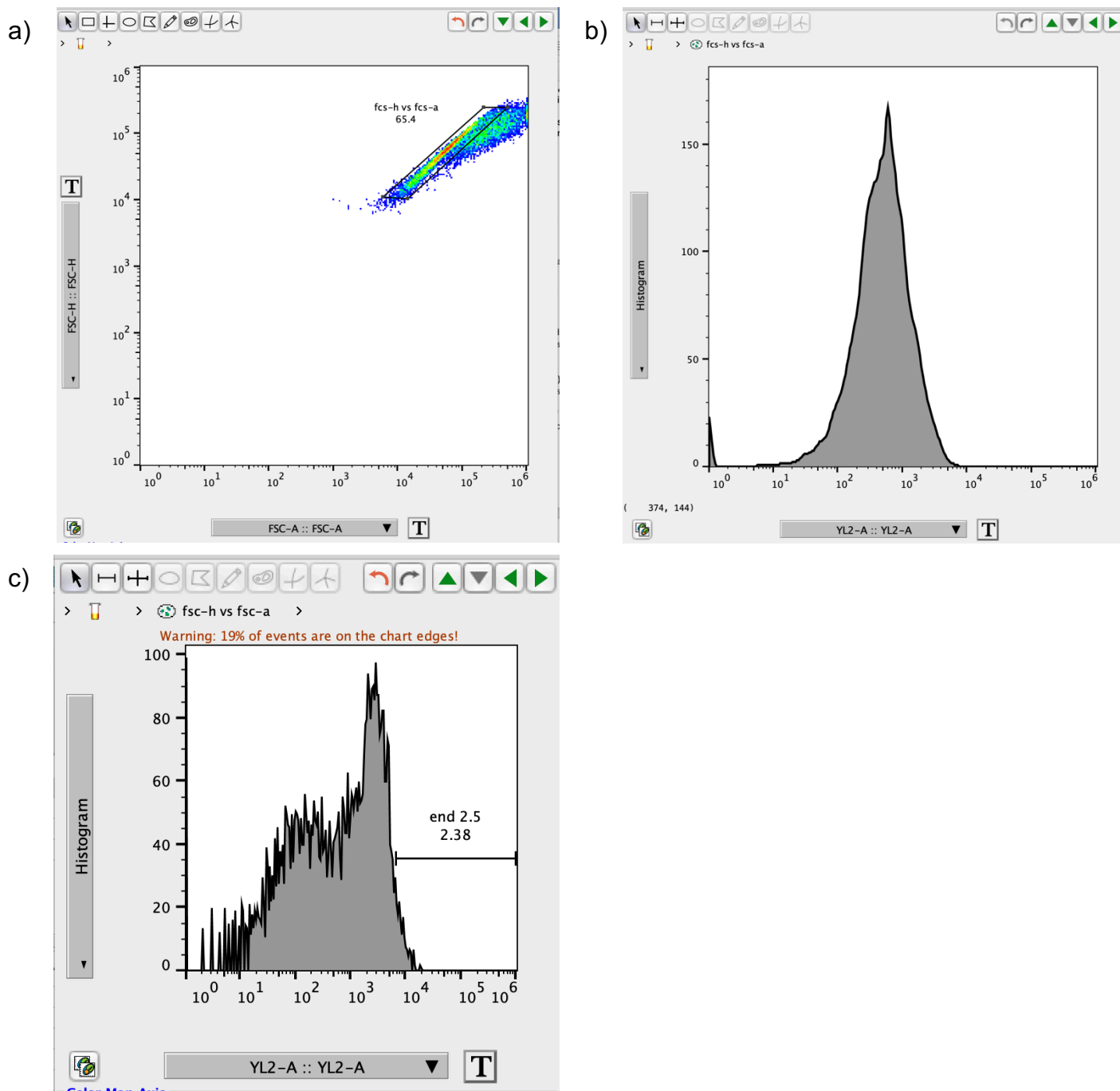


Figure 8. Screenshots of gating strategy used on FlowJo. The software FlowJo was used to analyse the flow cytometry data. a) FSC-H vs FSC-A gating strategy was used to remove doublets and clumps from the data set. b) data was plotted graphically using the channel dye as the x axis. c) in order to differentiate the large population, data was gated at the last 2.5%.

2.7 Microscopy

Cells were imaged using Zeiss Axio Imager with the following specifications: Zeiss inverted live imaging with apotome and with perfect focus.

To visually see if Titan cells were present in the cultures after inducing them for 3 days, microscopy was carried out. 1ml of the Titan cultures were spun at 5,000rpm for 2 minutes. After removing the supernatant, culture was washed with PBS once before adding 5 µg/ml calcofluor white (CFW) to stain for total chitin, and sybr green (invitrogen) which stains nucleic acid. It is important to note that sybr green is known to stain not only DNA but there is a possibility it stains RNA. The cells were imaged using the Zeiss Axio Imager. When measuring the size of the cell, the diameter was measured using FIJI, with a scale bar set to 10 µM. Here 5 images were acquired for each sample for one independent run, counting a maximum of 300 cells per image.

2.8 Statistics

All statistical analyses were carried out on GraphPad Prism 8. The data set was checked for normality, and where data sets were not normal a Mann-Whitney or Kolmogorov-Smirnov for t-test, and Kruskal-Wallis analysis was used for one-way anova. For multiple comparisons, Dunn's was used. For all data, $p < 0.05$ was taken as significant.

Declaration

This chapter is adapted from an existing paper in which Hanna Zafar is the first author, and Dr. Sophie Altamirano, Dr Kirstein Nielson, and Dr Elizabeth Ballou are the corresponding authors. This chapter only contains works that are conducted by Hanna Zafar. This paper is currently available on <https://www.sciencedirect.com/science/article/pii/S1369527418301097?via%3DiHub> .

‘A titanic drug resistance threat in *Cryptococcus neoformans*’

Signature:



Date: 16 Jan 2022

Signature:



Date: 16 Jan 2022

Chapter 3. Investigating the Role of Titan Cells in Drug Resistance using Clinical and Laboratory isolates

3.1 Introduction

Part of this section has been adapted from a review article published; **Zafar, H.** et al (2019) A titanic drug resistance threat in *Cryptococcus neoformans*. Current Opinion in Microbiology **52**: 158-164

The growing degree of resistance to antimicrobials is a reason for concern worldwide, especially for fungal infections with limited treatment options. Not only is the health of the global population affected by antifungal resistance, it has also had an impact on the cost of healthcare and gross domestic product (GDP) (O'Niell, 2016, Benedict et al., 2018). Advancement in *C. neoformans* drug resistance could devastatingly affect human wellbeing, however studies have just started to delve deeper into the complicated mechanisms that drive resistance to antifungals (Semighini et al., 2011, Rhodes et al., 2017, Altamirano et al., 2017, Chang et al., 2018)

Titan cells have also been found to possibly play a role in antifungal drug resistance. When treated with fluconazole, titan cells produced aneuploid daughter cells that were resistant (Gerstein et al., 2015). Further work is needed to confirm the mechanism underpinning this.

3.1.1 Heritable fluconazole resistance in *Cryptococcus neoformans*

Even with the most innovative treatment, cryptococcal meningoencephalitis is lethal especially in immunocompromised patients. Guidelines from the World Health Organisation (WHO) recommend amphotericin B in combination with flucytosine for one week, followed by 8 weeks consolidation therapy using fluconazole (WHO, 2018). Up to 60% of meningitis patients have been shown to relapse (Perfect et al., 2010, Bicanic et al., 2006). Certain therapies using azoles as long-term therapies can generate emergence of drug resistance (Bicanic et al., 2006).

As discussed in section 1.6.2, fluconazole is an azole antifungal which is commonly used against fungal infections. Azoles inhibit ergosterol biosynthesis by targeting two cytochrome P450 enzymes $\Delta 22$ (Erg3) and lanosterol 14 α -demethylation (Erg11) (Kelly et al., 1997). By inhibiting these enzymes, it interrupts ergosterol biosynthesis, whilst preventing the integrity of the membrane from being disturbed, and causing the accumulation of the sterol intermediates that are toxic (Zhang et al., 2010).

Azole resistance mechanisms can be categorised into two groups: transient and heritable. When fluconazole resistance is genetically transmissible from parent to daughter cells it is deemed as heritable, and this has been studied at length in *Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus* sp. The most common form is point mutations, particularly in the *ERG11/CYP51* resulting in a surge of protein levels of Erg11, or a rise in gene copy of multidrug transporters due to gene duplications (Lupetti et al., 2002, White et al., 2002, Perea et al., 2001, Xiang et al., 2013, Mann et al., 2003). In *C. neoformans* *EGR11* point mutations are rarely seen in clinical isolates. Whilst certain clinical isolates and mutator

strains do show evidence of other point mutations, their role in drug resistance is unknown (Rhodes et al., 2017, Stone et al., 2019, Arras et al., 2017, Boyce et al., 2017, Rodero et al., 2003, Sionov et al., 2012).

3.1.2 Transient fluconazole resistance in *Cryptococcus neoformans*

There have been studies carried out *in vitro* and *in vivo* in *C. neoformans* identifying intrinsic heteroresistance - this is a subpopulation of cells which have transient resistance and can grow in increasing concentration of fluconazole, but once the drug is removed they lose this resistance (Xu et al., 2001, Sionov et al., 2009).

Further studies demonstrated the role of aneuploidy in heteroresistant *C. neoformans* cells, with chromosome (chr) 1, 4, 10 and 14 seen predominantly disomic in the subpopulation that were fluconazole heteroresistant (Sionov et al., 2010, Ngamskulrungrroj et al., 2012, Stone et al., 2019). In fluconazole resistant strains, the most frequent disomic chromosome seen was chr1. There are well documented fluconazole resistant genes present on chr1; specifically, *ERG11*, which is a target of fluconazole and also plays a role in the production of ergosterol, and *AFR1*, an ATP-binding cassette transporter. Disomy of chr1 causing an increase in expression of these genes has been shown to contribute in *C. neoformans* drug resistance (Sionov et al., 2010).

Following chr1, the second most frequent disomic chromosome has been shown to be chr4 in fluconazole resistant *C. neoformans* strains. According to the literature, chr4 does not encode any genes related to efflux pumps or biosynthesis of ergosterol. A study by Ngamskulrungrroj *et al* focused on genes in chr4 that may be involved in drug resistance and

disomy. They identified three genes, *SEY1*, *GLO3*, and *GCS2*, known to be associated in integrity maintenance of the endoplasmic reticulum, which were significant in aneuploidy formation during fluconazole stress. Whilst these genes were disrupted, chr4 disomy frequency decreased and sensitivity to fluconazole increased (Ngamskulrungrroj et al., 2012).

The process of disomic formation in *C. neoformans* due to fluconazole stress remains unclear, however *in vitro* studies have proposed possibilities and theories regarding this. *C. albicans* is a diploid yeast which, in the presence of fluconazole, acquires aneuploidy as a result of incomplete cytokinesis that forms intermediate tetraploid multimeras (Harrison et al., 2014). Interestingly, Altamirano et al. found a similar mechanism in *C. neoformans*, with fluconazole having a direct impact on nuclear division in yeast cells which caused production of multinucleate, inadequately separate cells (figure 9b) (Altamirano et al., 2017, Selmecki et al., 2009). As an alternative mechanism, Chang et al revealed resistance may begin in *C. neoformans* cells that are euploid and uninucleate, and fluconazole instigates chromosome mis-segregation in these originating cells which leads to resistance (figure 9a) (Chang et al., 2018). Collectively, it seems that drug resistance is induced by fluconazole indirectly as a consequence of genome plasticity or directly as a result of blocking cytokinesis (Zafar et al., 2019).

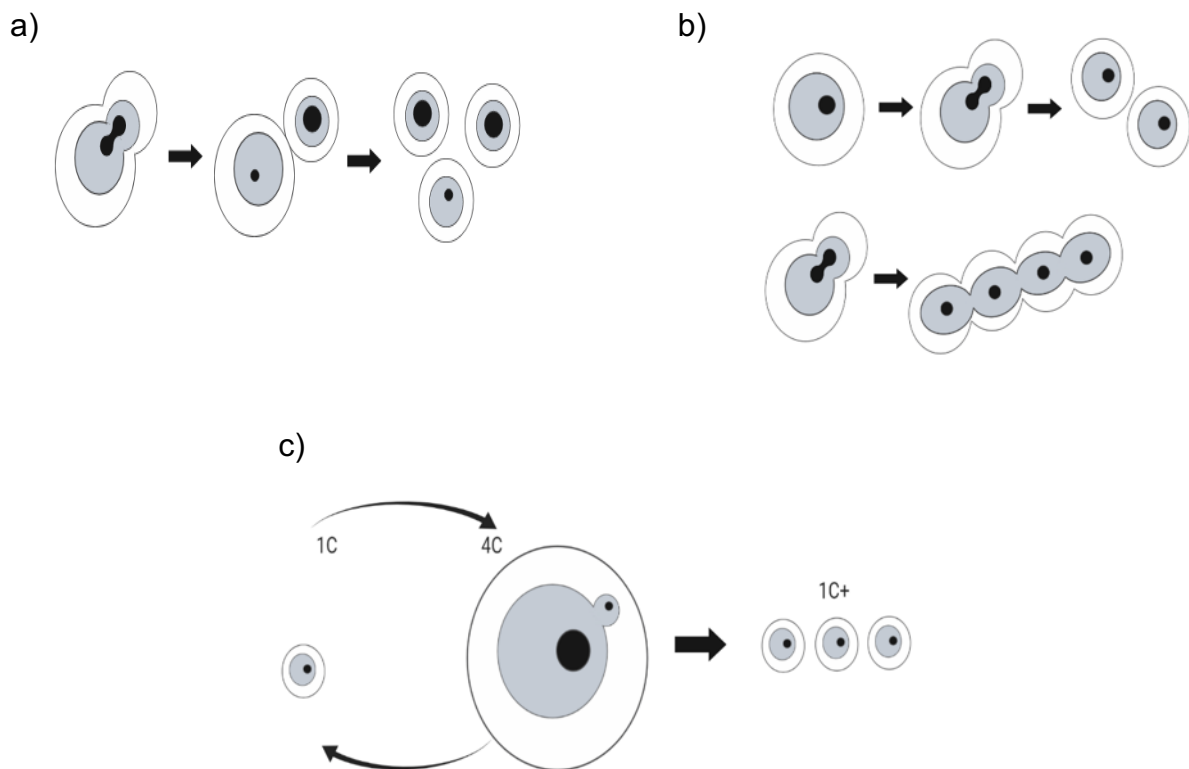


Figure 9. Possible mechanisms of *C. neoformans* drug resistance. Diagrams depicting the different possible mechanisms of resistance from the literature and a potential new mechanism. a) Nuclear missegregation interpreted from Chang et al., 2018, b) Nuclear damage interpreted from Altamirano et al., 2017, c) Titan cell formation.

3.1.3 Titan cell role in drug resistance

Despite the possibility that fluconazole directly induces aneuploidy (Altamirano et al., 2017, Selmecki et al., 2009, Harrison et al., 2014), and the capacity of cells to become polyploid during the yeast-to-titan switch, the role of titanisation in drug resistance has yet to be looked into. Daughter progeny of titan cells are aneuploid and show enhanced fluconazole resistance yet are seemingly able to adjust to the environment of the host (figure 9c) (Gerstein et al., 2015). Titan cells have been identified as polyploid cells, comprising of more than one copy of the genome, while *Cryptococcus* yeast cells are known to be haploid, having a single copy of the genome (Okagaki et al., 2010, Zaragoza et al., 2010, Dambuza

et al., 2018, Alanio et al., 2015). Interestingly, when producing daughter cells, titan cells undertake a reduction in the genome copy and generate daughter cells that are haploid, diploid or aneuploid (Okagaki et al., 2010, Gerstein et al., 2015, Dambuza et al., 2018). A study carried out by Gerstein et al demonstrated how the titan cell progeny were more resistant to nitrosative and oxidative stresses, and are also more resistant to fluconazole *in vitro* (Gerstein et al., 2015). They also showed when the titan cells were subjected to fluconazole, the daughter cells were found to be diploid and aneuploid (Gerstein et al., 2015). This prompts the question as to whether the formation of titan cells is a drug resistance mechanism by reason of aneuploidy (Zafar et al., 2019).

Apart from the production of daughter cells that are aneuploid, it is also possible that titan cells could also contribute to the increase in drug resistance due to its unique morphology. The cell wall of a *C. neoformans* yeast is 150-200nm thick, contrary to titan cells which are up to 2-3µm (Zaragoza and Nielsen, 2013, Wiesner et al., 2015). When comparing the composition of the cell wall in titan cells with that of yeast cells, a study found that on the surface there was a potential mannan layer along with an increase in chitin and a decrease in glucan (Mukaremera et al., 2018). Moreover, *C. neoformans* are well known for their distinctive polysaccharide capsule that encompasses the cell, allowing adaptation to the host environment by being a protective outer layer. This capsule in titan cells is structurally different than that of yeast cells, with the presence of more cross-links that make it considerably denser (Zaragoza et al., 2010, Mukaremera et al., 2018). To date, no study has directly associated enhanced drug resistance with alterations in the surface of a titan cell, but there are theories as to how changes to *C. neoformans* could be obstacles in

antifungal treatment and should be considered as such (Grossman and Casadevall, 2017, Cordoba et al., 2011, Feldmesser et al., 2001, Okagaki et al., 2010).

3.2 Results

3.2.1 Clinical isolates are capable of forming titan cells, but this capacity can be influenced by exposure to fluconazole

To better understand the link between titan cell formation and drug resistance, I screened a collection of 20 paired clinical isolates previously shown to be competent for heteroresistance to fluconazole (Stone et al., 2019). I determined their capacity to form titan cells before and after exposure to fluconazole.

Before looking into the role of titanisation in drug resistance, I set out to see if there was a difference in titan cell formation in clinical isolates that were known to be resistant to fluconazole using this *in vitro* assay. The capacity to form titan cells is defined as the percentage of cells with DNA content 4C or higher and cells larger than 10 µm diameter.

When screening for this, Tanzanian HIV-associated clinical isolates were selected. Stone *et al.* retrieved these isolates from the cerebral spinal fluid of infected patients. These clinical isolates were paired: half of the clinical isolates were those directly from the patient sample (Stone et al., 2019), labelled 'parent' throughout this report. The other half were obtained after Stone *et al.* took the respective parent colonies and carried out an Etest to see fluconazole heteroresistant colonies (Stone et al., 2019), which were selected for further

analysis. They are labelled 'Fluc^R' and 'R' after the clinical isolate name throughout this report.

I hypothesised that the clinical isolates would be capable of forming titan cells. In order to test the variety of titanisation capacities in these paired samples, they were tested in two ways: by flow cytometry to measure DNA content and cell size, and microscopy to visualise the cells. Following the screen in triplicate using both approaches, isolates were analysed for cell size and ploidy, and a variety of patterns were observed. Below I will describe the different types of findings.

To visually see whether there were any changes in cell size in the isolates following induction of Titan cells, microscopy was carried out. As seen in figure 10a, 2 of the Fluc^R clinical isolates were able to form large cells compared to their paired parent clinical isolate (figure 10a). I then looked into the DNA content of these cells as titan cells are known to be polyploid. This particular clinical isolate Fluc^R had an increased DNA content as the median fluorescence intensity was higher, suggesting that they had become polyploid compared to the parental isolate (figure 10b). However interestingly the size of the isolate pair was the same (figure 10c), but when looking into the relationship between the size and DNA content of the cells we could see that the large cells had a higher DNA content regardless of whether they were fluconazole resistant or not (figure 10d).

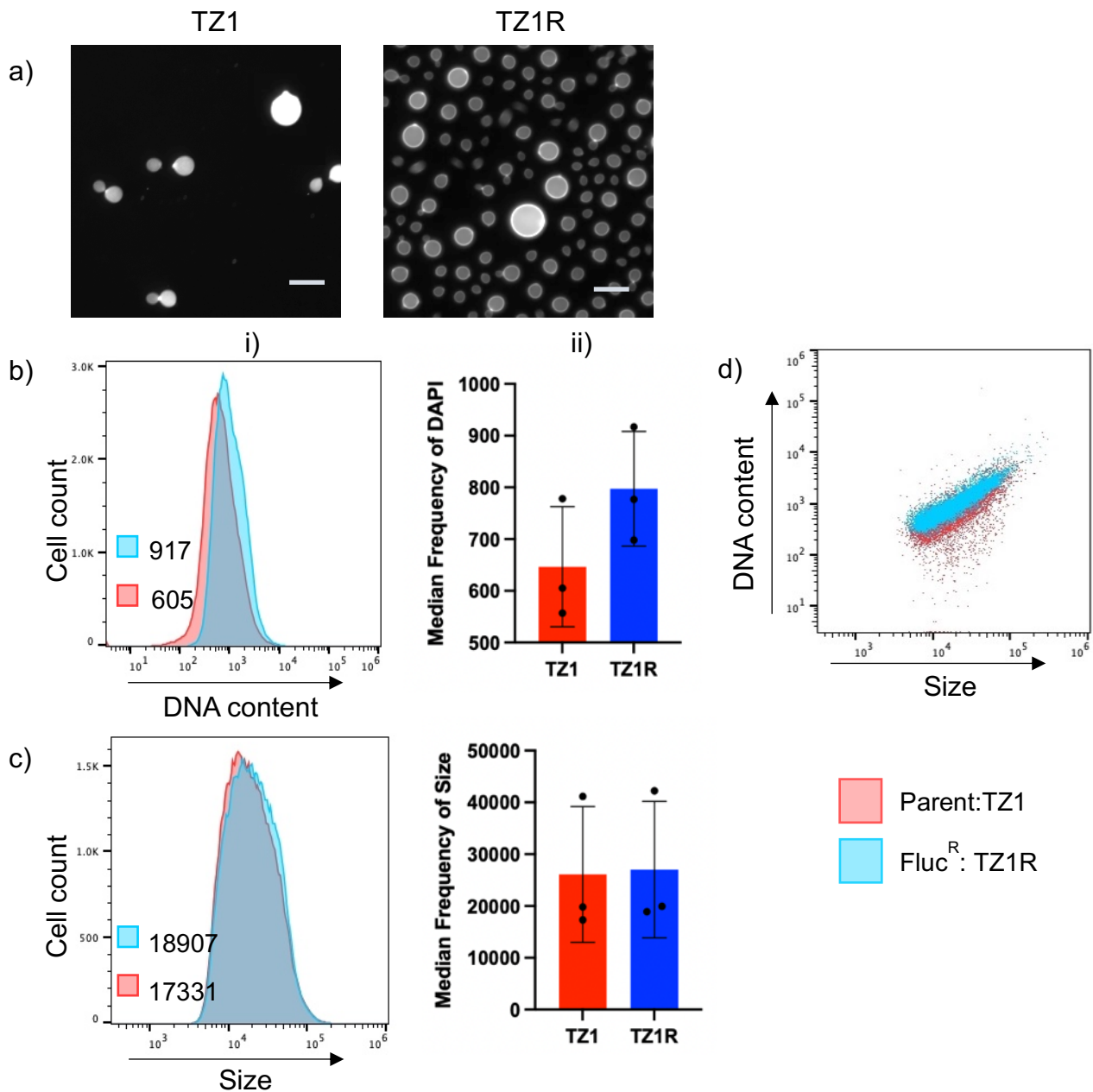


Figure 10. Representative of certain isolates where Fluc^R was able to form titan cells. H99 and clinical isolates were grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. a) Microscopy images of clinical isolates representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μm . Cells were stained with DAPI for DNA content. b)i) Data is representative of 3 biological repeats and the median fluorescence of DAPI for the representative is on the histogram generated from FlowJo. bii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. c)i) Data is representative of 3 biological repeats and the median fluorescence of size for the representative is on the histogram generated from FlowJo. cii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. d) scatter plot depicting DNA content vs size of the representative data. Data is representative of 3 biological repeats.

When inducing titan cells, one of the paired clinical isolates would form a new morphological shape. In the Fluc^R isolate, the cells seemed elongated and rod shaped which is rarely seen in *C. neoformans* (Fernandes et al., 2018), while the parent isolate formed chains (figure 11a). When looking at the DNA content of these cells, the Fluc^R isolate had a slight increase in DNA content compared to the parent isolate indicated by the increase in median fluorescence of DAPI (figure 11b). But the cell size both isolates were relatively the same (figure 11c). Similarly, the data here also confirmed that the larger cells were the ones with a higher DNA content (figure 11d).

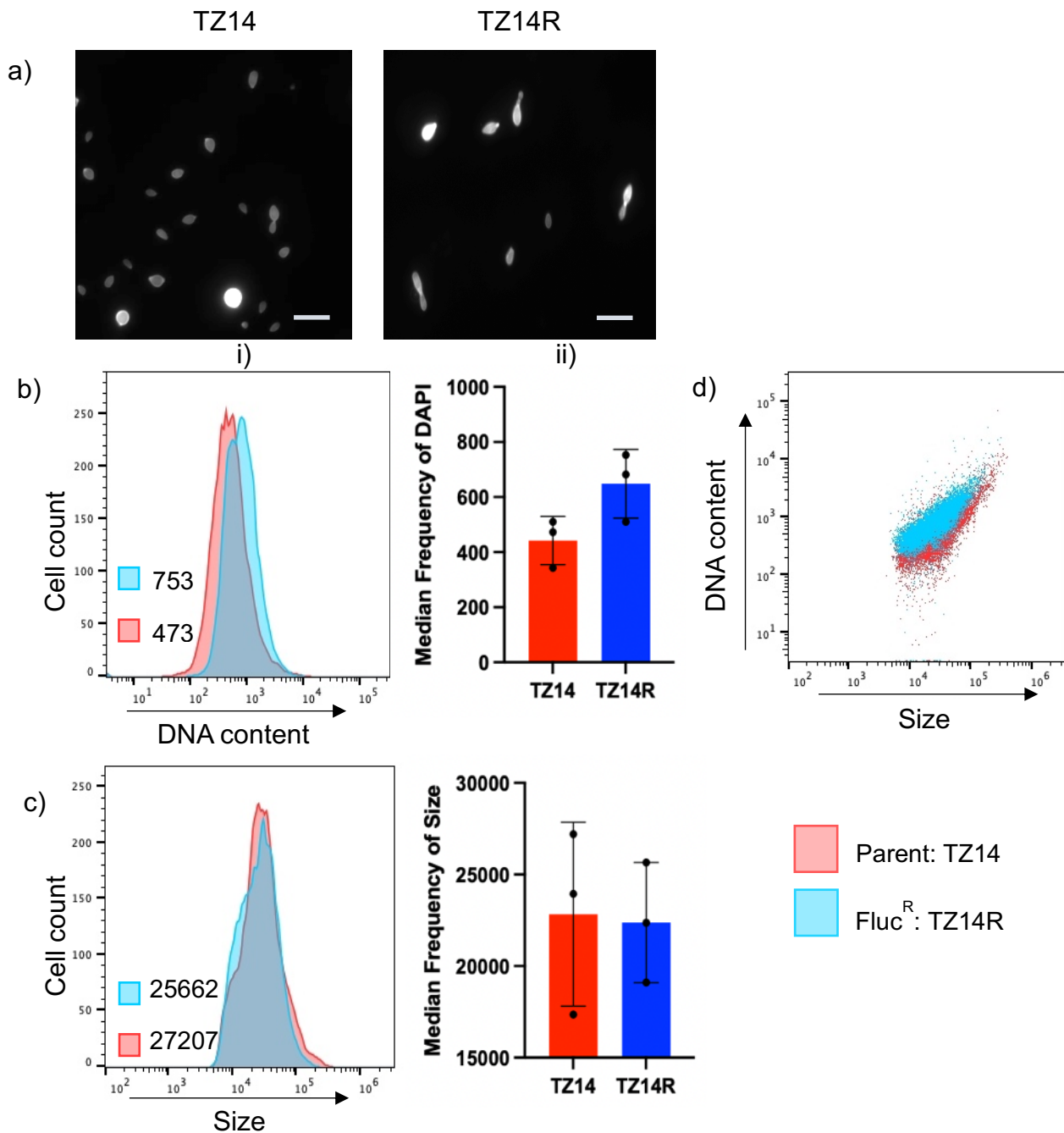


Figure 11. Representative of certain clinical isolates where paired isolates form different cell morphology. H99 and clinical isolates were grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. A) Microscopy images of clinical isolates representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μ m. Cells were stained with DAPI for DNA content. b)i) Data is representative of 3 biological repeats and the median fluorescence of DAPI for the representative is on the histogram generated from FlowJo. bii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. c)i) Data is representative of 3 biological repeats and the median fluorescence of size for the representative is on the histogram generated from FlowJo. cii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. d) scatter plot depicting DNA content vs size of the representative data. Data is representative of 3 biological repeats.

Intriguingly, while there were 2 clinical isolate pairs where Fluc^R formed Titan cells (figure 10a), there were some clinical isolates pairs where parent isolate as well as the Fluc^R isolate produced large cells (figure 12a). There was a large increase in DNA content in the parent isolate compared to the paired Fluc^R isolate (figure 12b) and when looking at the size of the cells through flow cytometry, the median fluorescence of the size was higher in the parent isolate than in the Fluc^R isolate pair (figure 12c). The link between the two was confirmed when using the scatter plot which shows how the parent isolate's large cells were the ones that had the higher DNA content (figure 12d).

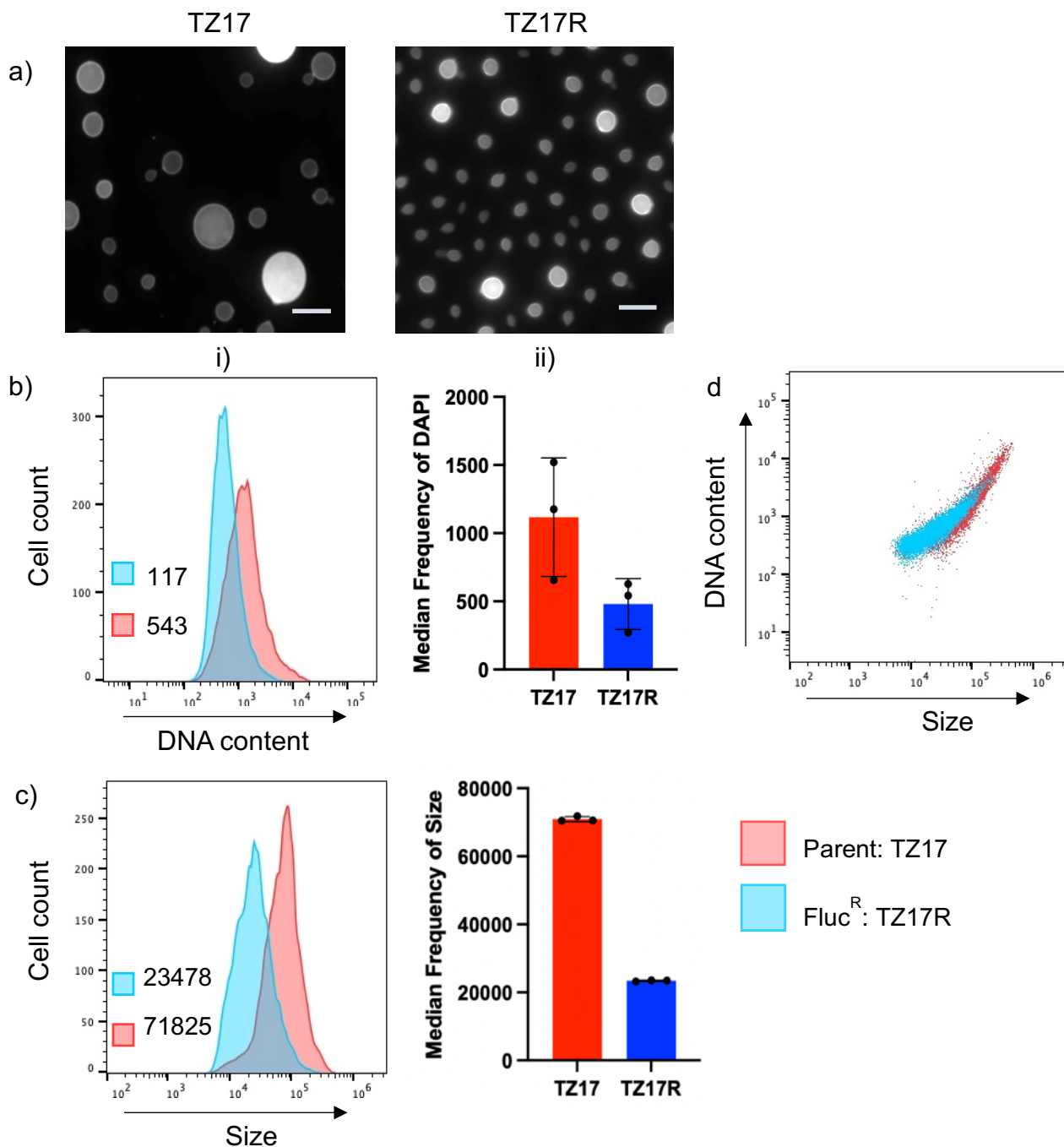


Figure 12. Representative of certain clinical isolates where parent isolates were able to form titan cells. H99 and clinical isolates were grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. A) Microscopy images of clinical isolates representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μ m. Cells were stained with DAPI for DNA content. b)i) Data is representative of 3 biological repeats and the median fluorescence of DAPI for the representative is on the histogram generated from FlowJo. bii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. c)i) Data is representative of 3 biological repeats and the median fluorescence of size for the representative is on the histogram generated from FlowJo. cii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. d) scatter plot depicting DNA content vs size of the representative data. Data is representative of 3 biological repeats.

Finally, 15 of the paired clinical isolates were unable to form titan cells, following titan cell induction, and remained in the yeast morphology (figure 13a) (table 7). The paired clinical isolates also had the same DNA content and size, with no characteristic stepwise increase in DNA content, further confirming the absence of titan cells (figure 13b and c).

Together, these data demonstrate that 1) the capacity to form titan cells is not required for the emergence of heteroresistance and 2) exposure to fluconazole can be associated with gain or loss of the capacity to form titan cells.

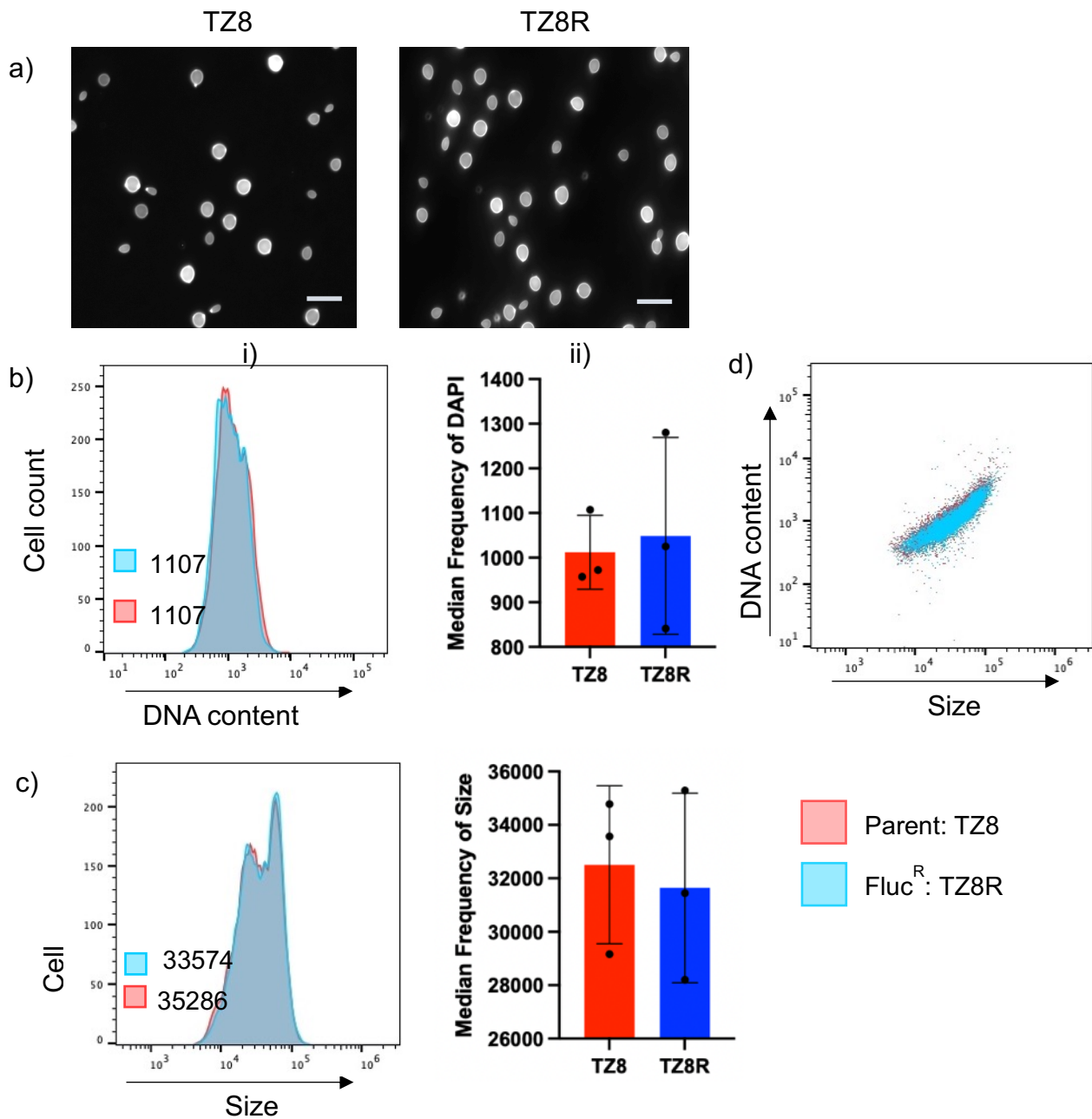


Figure 13. Representative of certain clinical isolates where paired clinical isolates formed yeast. H99 and clinical isolates were grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. A) Microscopy images of clinical isolates representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μ m. Cells were stained with DAPI for DNA content. b)i) Data is representative of 3 biological repeats and the median fluorescence of DAPI for the representative is on the histogram generated from FlowJo. bii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. c)i) Data is representative of 3 biological repeats and the median fluorescence of size for the representative is on the histogram generated from FlowJo. cii) The median fluorescence intensity was taken from the 3 biological repeats and plotted using GraphPad. d) scatter plot depicting DNA content vs size of the representative data. Data is representative of 3 biological repeats.

3.2.2 Fluconazole resistance is transient, even in strains that gain the capacity to form titan cells following fluconazole exposure

After seeing if the Tanzanian HIV-associated clinical isolates said to be fluconazole resistant can produce Titan cells (figure 10), I decided to look into whether these clinical isolates were still resistant to fluconazole due to literature showing that resistance could be lost (Sionov et al., 2009), and began to look into the role of titan cells in anti-fungal resistance.

As mentioned before, the Fluc^R isolate from the paired clinical isolate were confirmed to be resistant by Stone et al (Stone et al., 2019) due to colonies growing on the Etest zone of clearance directly after isolation from the patient. These isolates were subsequently frozen down into glycerol stocks and stored at -70°C. To determine if these isolates were still fluconazole resistant compared to their respective parent isolates, a fluconazole sensitivity assay/ minimum inhibitory concentration (MIC) was carried out and analysed. To align with conditions tested by Stone et al., this assay was performed on the yeast-phase, not titan-induced, cells. Table 6 summarises the results of this analysis. No isolate was more resistant to fluconazole than their parent with an average MIC of 8 µg/ml (table 6). Interestingly, there were some paired isolates that were hyper-sensitive to fluconazole, with growth inhibited at very low concentrations of fluconazole (table 6).

Table 6. MIC values for clinical isolates. MIC values for all 20 paired Tanzanian HIV- associated clinical isolates. Data is mode of 3 biological repeats.

Parental	Fluconazole MIC (µg/ml)	Fluc ^R	Fluconazole MIC (µg/ml)
TZ1	8	TZ1R	8
TZ2	8	TZ2R	8
TZ3	8	TZ3R	4
TZ6	0.5	TZ6R	0.5
TZ7	8	TZ7R	8
TZ8	8	TZ8R	8
TZ9	8	TZ9R	8
TZ10	8	TZ10R	8
TZ11	8	TZ11R	8
TZ13	8	TZ13R	8
TZ14	0.5	TZ14R	0.5
TZ15	8	TZ15R	8
TZ16	8	TZ16R	8
TZ17	8	TZ17R	8
TZ18	8	TZ18R	8
TZ19	8	TZ19R	8
TZ21	8	TZ21R	8
TZ22	8	TZ22R	4
TZ23	0	TZ23R	8
TZ24	8	TZ24R	8
Wild type H99			8

Table 7. Summary table of clinical isolates. Overall table of all 20 paired Tanzanian HIV-associated clinical isolates, their ability to form titan cells and whether they are still resistant or not.

Parental	Fluc ^R	Ability to form titan cell	Increase DNA content	Change in Fluc ^R
TZ1	TZ1R	Fluc ^R	Fluc ^R	No change
TZ2	TZ2R	Neither	Parental	No change
TZ3	TZ3R	Fluc ^R	Fluc ^R	Fluc ^R decreased
TZ6	TZ6R	Neither	No difference	No change
TZ7	TZ7R	Neither	No difference	No change
TZ8	TZ8R	Neither	No difference	No change
TZ9	TZ9R	Neither	No difference	No change
TZ10	TZ10R	Neither	Parental	No change
TZ11	TZ11R	Neither	No difference	No change
TZ13	TZ13R	Neither	Parental	No change
TZ14	TZ14R	Neither	Fluc ^R	No change
TZ15	TZ15R	Neither	No difference	No change
TZ16	TZ16R	Present in both, more so in parental	Parental	No change
TZ17	TZ17R	Present in both, more so in parental	Parental	No change
TZ18	TZ18R	Neither	No difference	No change
TZ19	TZ19R	Neither	No difference	No change
TZ21	TZ21R	Neither	No difference	No change
TZ22	TZ22R	Neither	Did not grow	Fluc ^R decreased
TZ23	TZ23R	Neither	Parental did not grow	Parental did not grow
TZ24	TZ24R	Neither	No difference	No change

3.2.3 Titan cells are not intrinsically drug resistant

Previous studies have demonstrated between 25-30% of *C. neoformans* clinical isolates were resistant to fluconazole before treatment was given (Smith et al., 2015, Stone et al.,

2019). This gives rise to the possibility that there may be another mechanism influencing drug resistance. There was no correlation between titan cell and the occurrence of drug resistance in the history of a given isolate. I hypothesise titanisation as a possible mechanism of drug resistance due to aneuploidy.

I hypothesised that, if the presence of titan cells contributes to drug resistance, strains grown in titanising conditions would have a higher MIC than those grown in yeast-phase conditions. Titan induction leads to the generation of a mixed population formed of titans, yeast, and daughter cells (figure 14).

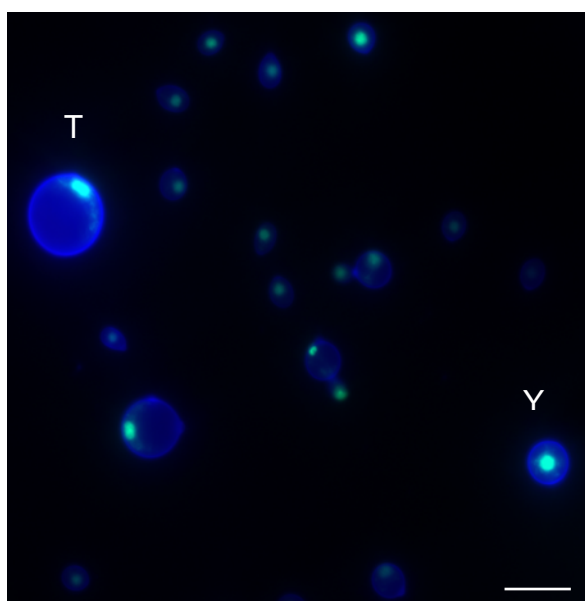


Figure 14. Titan induced culture. H99 was grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. Microscopy images of clinical isolates representative of 5 technical repeats. Cells were stained for chitin with CFW and nucleic acid with Sybr Green, scale bar of 10 μm . Image labelled T for titan and Y for yeast.

The results of this analysis are summarised in table 8: there were no major changes in the MIC following titan induction assay compared to cells grown under rich media conditions. There were two isolates that seemed more sensitive to fluconazole, clinical isolates TZ3R

and TZ22R, with an MIC of 4 µg/ml (table 8). Broadly, the *in vitro* approach used here suggests that growing strains in titan inducing conditions, which might reflect more similarly the host conditions, does not increase fluconazole resistance.

Table 8 MIC values for titan induced clinical isolates. MIC values for selected paired clinical isolates known to form Titan cells. Data is mode of duplicate technical repeats and triplicate biological repeats.

Tanzanian clinical isolates		Fluconazole MIC Yeast conditions(µg/ml)	Fluconazole MIC Titan conditions(µg/ml)
Parent	TZ1	8	8
Parent	TZ16 (forms titans)	8	4
Wild type	H99 (forms titans)	8	8

I asked whether in the population drug resistance is being driven by the larger cells or by the smaller cells. If titan cells are more resistant to fluconazole then large cells should be able to grow better in the presence of MIC level drug. To test this, H99 was grown in titanising conditions and cells were separated using 11 µm and 3 µm filters, followed by growth in the presence of differing concentrations of fluconazole.

I hypothesised that if titan cells are more resistant to fluconazole, they would be able to proliferate in MIC of 8 µg/ml and supraMIC concentrations of fluconazole. This was because if titan cells are drug resistant they should be able to grow in levels of fluconazole that inhibits yeast growth (MIC). However, after 65h of exposure to supraMIC concentrations of

fluconazole, the large cells did not grow (figure 15). There were slight fluctuations in absorbance for cells grown in 16 $\mu\text{g/ml}$ after 35 h, but not a substantial change (figure 15).

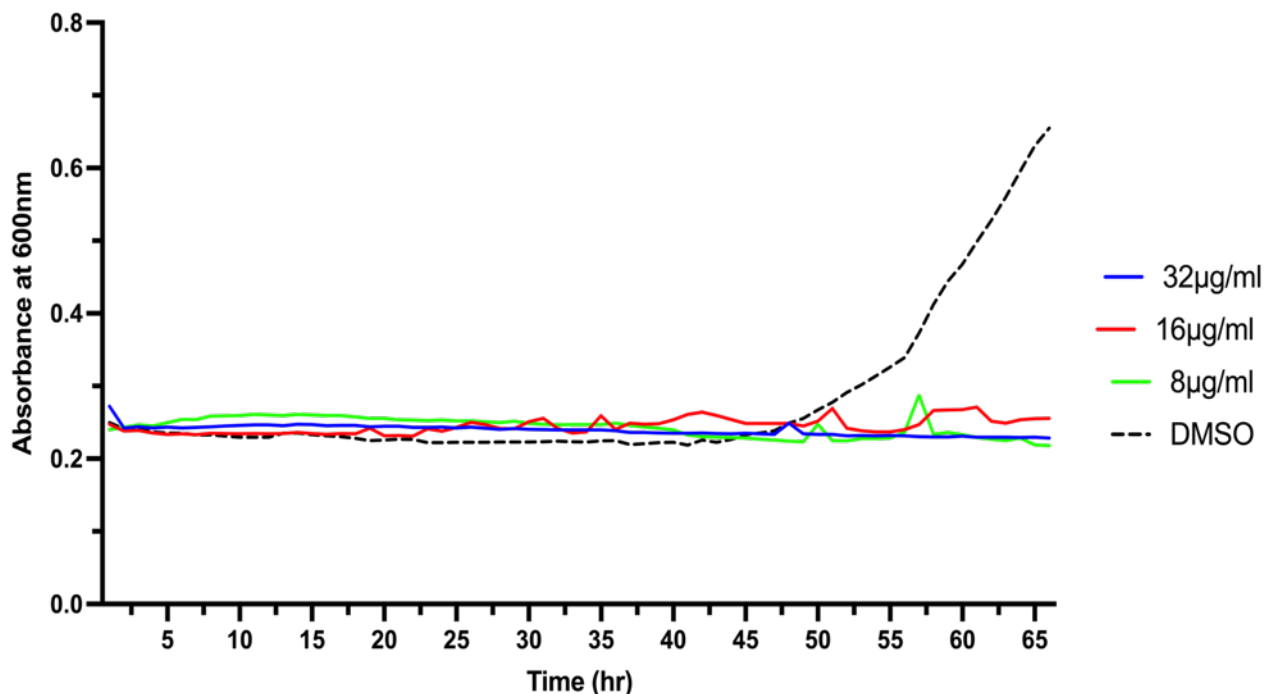


Figure 15. Growth curve of titan cells in differing concentrations of fluconazole. H99 was grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. Titan cells were collected using 11 μm filter and grown at 32 $\mu\text{g/ml}$ fluconazole, 16 $\mu\text{g/ml}$ fluconazole, 8 $\mu\text{g/ml}$ fluconazole and DMSO (control) for 65h. Data is representative of 2 biological repeats.

A previous study showed an increase in fluconazole resistance in titan daughter cells *in vitro* after the parental titan cells were exposed to fluconazole (Gerstein et al., 2015). In the literature, titan daughter cells have been defined as cells that are smaller than 3 μm (Dambuza et al., 2018), therefore we used the 3 μm filter to obtain and separate them from the culture. For cells less than 3 μm , through the use of a greater range of drug concentrations, there was a dose dependent decrease in growth, with cell growth observed up to 4 $\mu\text{g/ml}$ (figure 16). Until 10 h, cell growth was seen up to 2 $\mu\text{g/ml}$ fluconazole, following this however, 0.5 $\mu\text{g/ml}$ fluconazole treated cells had a higher increase in growth compared to the other concentrations. No growth was seen in concentrations of fluconazole higher

than the MIC of 8 $\mu\text{g/ml}$ up until 35 hrs, following which a very slight increase was observed in 8 $\mu\text{g/ml}$ and 16 $\mu\text{g/ml}$ of fluconazole (figure 16).

Together, these data suggest that both large titan cells and small daughter cells proliferate poorly in supraMIC concentration of fluconazole.

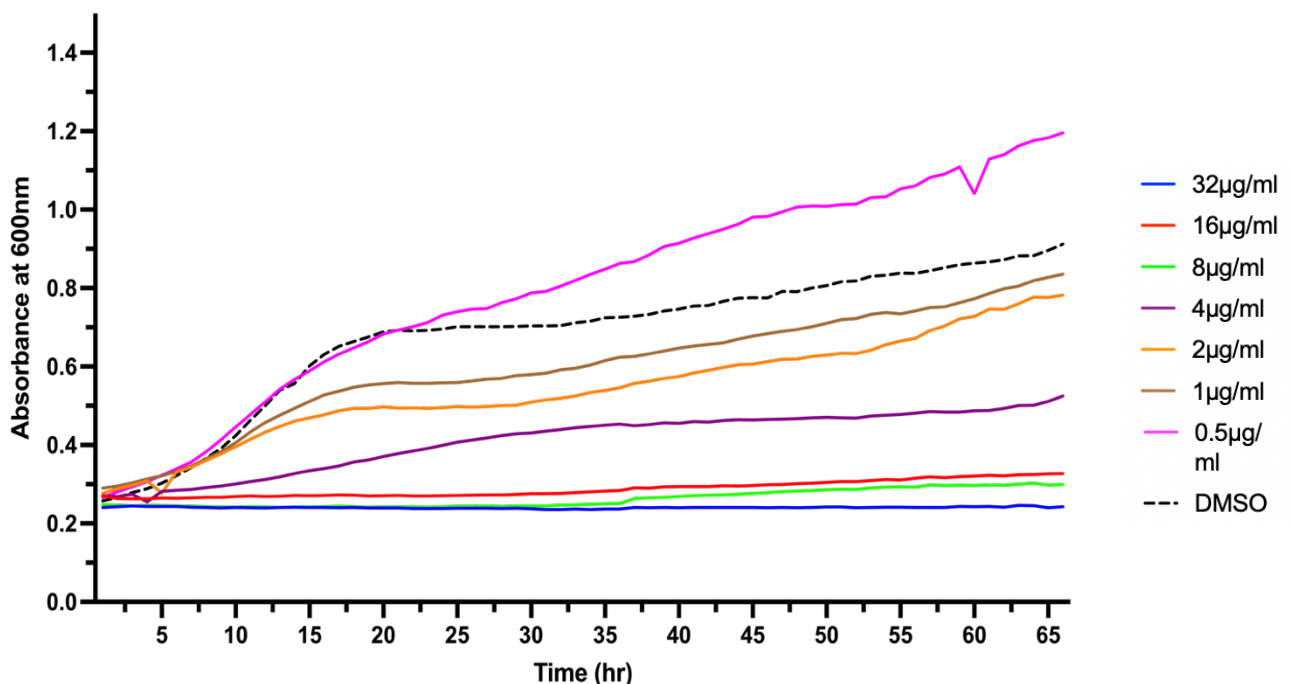


Figure 16. Growth curve of titan daughter cells in differing concentrations of fluconazole. H99 was grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS. ‘Daughter cells’ were collected using 3 μm filter and grown in different concentrations of fluconazole for 65hrs. Data is representative of 2 biological repeats.

3.2.4 Fluconazole does not inhibit the yeast-to-titan transition

As fluconazole is a fungistatic antifungal, and cultures that have already stimulated to form titan cells proliferate poorly at supraMIC, we asked whether fluconazole can prevent the formation of titan cells.

To see this, H99 was grown under titan inducing conditions without fluconazole, with 2 µg/ml fluconazole as a below MIC concentration chosen, and with 8 µg/ml fluconazole which is the MIC (figure 17). Due to the fungistatic nature of the drug mentioned earlier, the cells were unable to grow well at 8 µg/ml which is the known clinical MIC, but there was limited growth at 2 µg/ml (figure 17). However, those that could grow were able to become titan cells when grown in titan inducing conditions (figure 17). This indicates that fluconazole does not block titanisation, but it does inhibit growth of the cells.

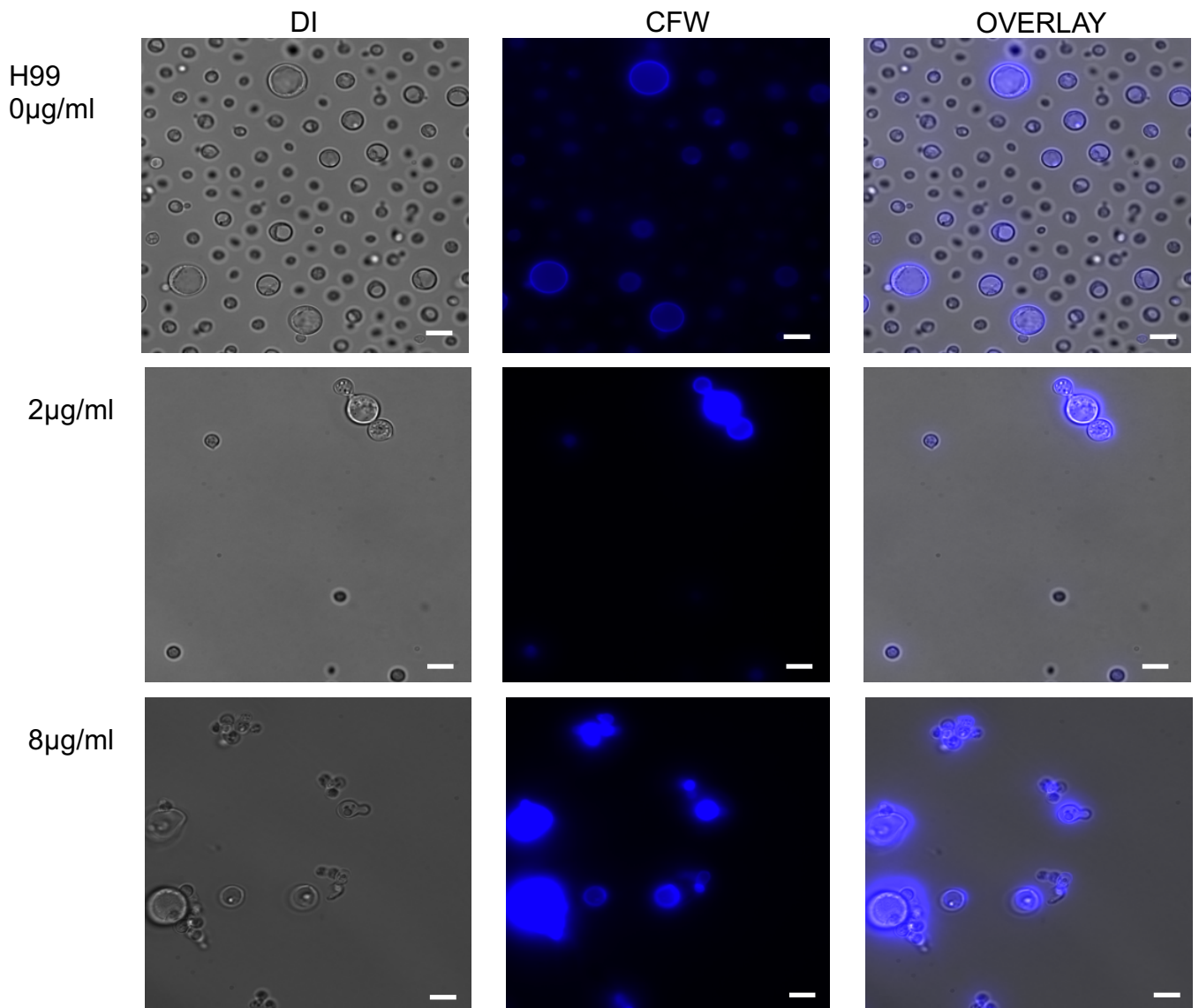


Figure 17. Fluconazole does not inhibit titanisation H99 was grown in YNB overnight, followed by induction of titan cells in 10% HI-FCS with PBS, with and without differing concentration of fluconazole, for 2 days at 37°C and 5% CO₂. H99 with no drug, H99 with 2 µg/ml fluconazole, and H99 with 8 µg/ml fluconazole. Data is representative of 5 technical repeats and 3 biological repeats. Cells were stained for chitin with CFW, scale bar of 10 µm.

3.2.5 Drug resistance is associated with increased DNA content

As mentioned earlier, a study carried out by Chang et al revealed that chromosome missegregation leads to the formation of aneuploidy which drives fluconazole resistance (Chang et al., 2018). On the other hand, this was done using YPD grown cells on solid agar

which are not host relevant conditions. One of the features of titan cell formation is increased DNA content. So, I asked whether DNA content itself was affected by fluconazole treatment, in non titanising and titanising conditions similar to that in the host.

In order to test this, two cultures were set up, one with yeast grown cells (figure 18a) and one with titan cells (figure 18b), and each culture was treated with either 2 or 8 µg/ml fluconazole. As a positive control, cells were treated with DPTA nonoate, a nitric oxide donor drug shown to increase in DNA content (Zhou, unpublished data). Interestingly in the yeast culture, neither 2 µg/ml nor 8 µg/ml of fluconazole affected the DNA content of the cells, as can be seen from the median fluorescence intensity (MFI) of DAPI (figure 18a). However, in the titan culture the overall DNA content increases in a dose dependent manner (figure 15b). In view of this, fluconazole treatment does alter DNA content when *C. neoformans* are grown under titan inducing conditions

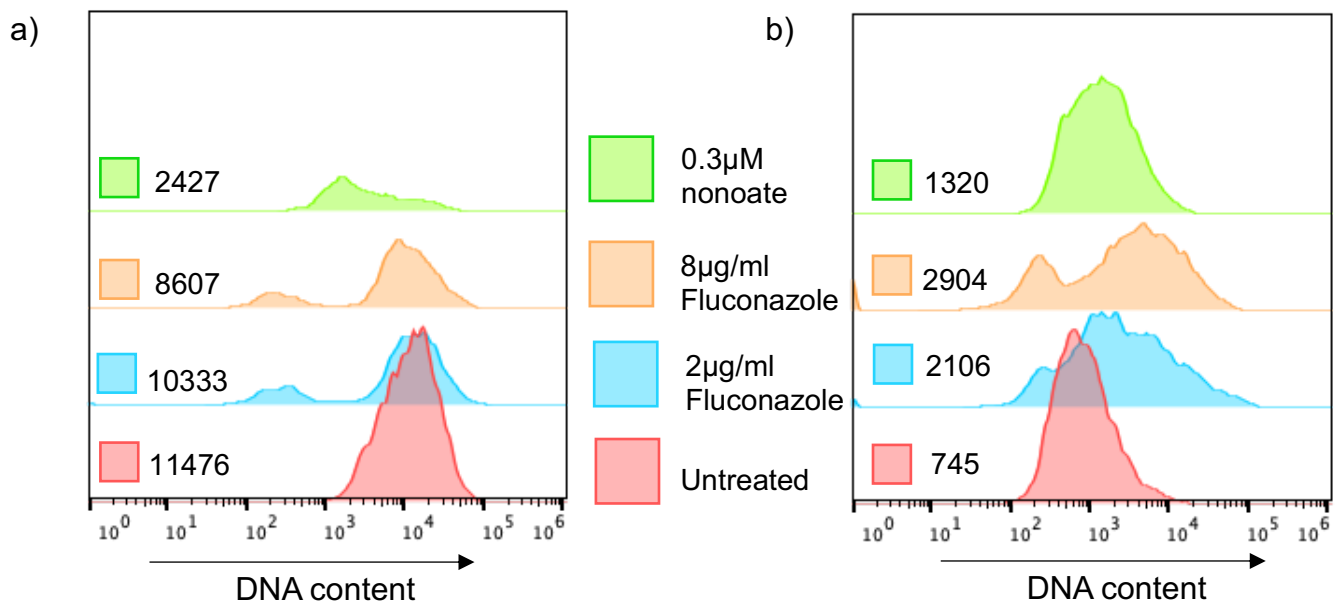


Figure 18. Fluconazole alters DNA when cells are titanised. H99 was grown in YNB overnight. a) cells were grown in yeast conditions of YNB + 2% glucose for 2 days at 30°C, b) cells were grown in titanising conditions of 10% HI-FCS with PBS for 2 days at 37°C and 5% CO₂ with and without differing concentration of fluconazole and nonoate was used as a control. Cells were stained with DAPI and measured by flow cytometry. The median frequency intensity of DAPI is on each graph and was calculated using flowjo, the histograms were also generated using flowjo. Data is representative of 2 biological repeats.

3.2.6 DNA content increase is not dependent on cell size increase

As this data suggested that the increase in DNA content only occurred after the cells had undergone titanisation, I asked whether genes required for titanisation are also required for DNA content increase. I decided to focus on the cAMP pathway because its role in titanisation has been well documented in the literature. One important part of the pathway is the conversion of ATP into cAMP by adenylyl cyclase (Cac1)(Choi et al., 2015). Therefore, we used a *cac1Δ* which inhibits the pathway and blocks titanisation, to see if fluconazole treatment could exert an effect on the DNA content in this strain.

Titanisation inhibition was confirmed through microscopy as seen in figure 12a when *cac1Δ* was grown in titan inducing conditions but the cells did not increase in size. In the presence of 2 µg/ml fluconazole, the size of *cac1Δ* cells were the same for both yeast conditions and titan conditions (figure 19a). That said, there was an increase in fluorescence of sybrgreen which stains for nucleic acids (figure 19a). This however cannot be used as a measurement for DNA content as sybrgreen is known to stain DNA and sometimes RNA. Interestingly, fluconazole still caused an increase in DNA content in *cac1Δ* after titan induction, more so at 2 µg/ml fluconazole (figure 19b). Also, to note, the addition of nonoate no longer increases DNA content in the *cac1Δ* grown under titan conditions. Overall this data indicated that cAMP pathway, which is required for titanisation, does not impair the increase of DNA content when cells are titan induced in the presence of fluconazole.

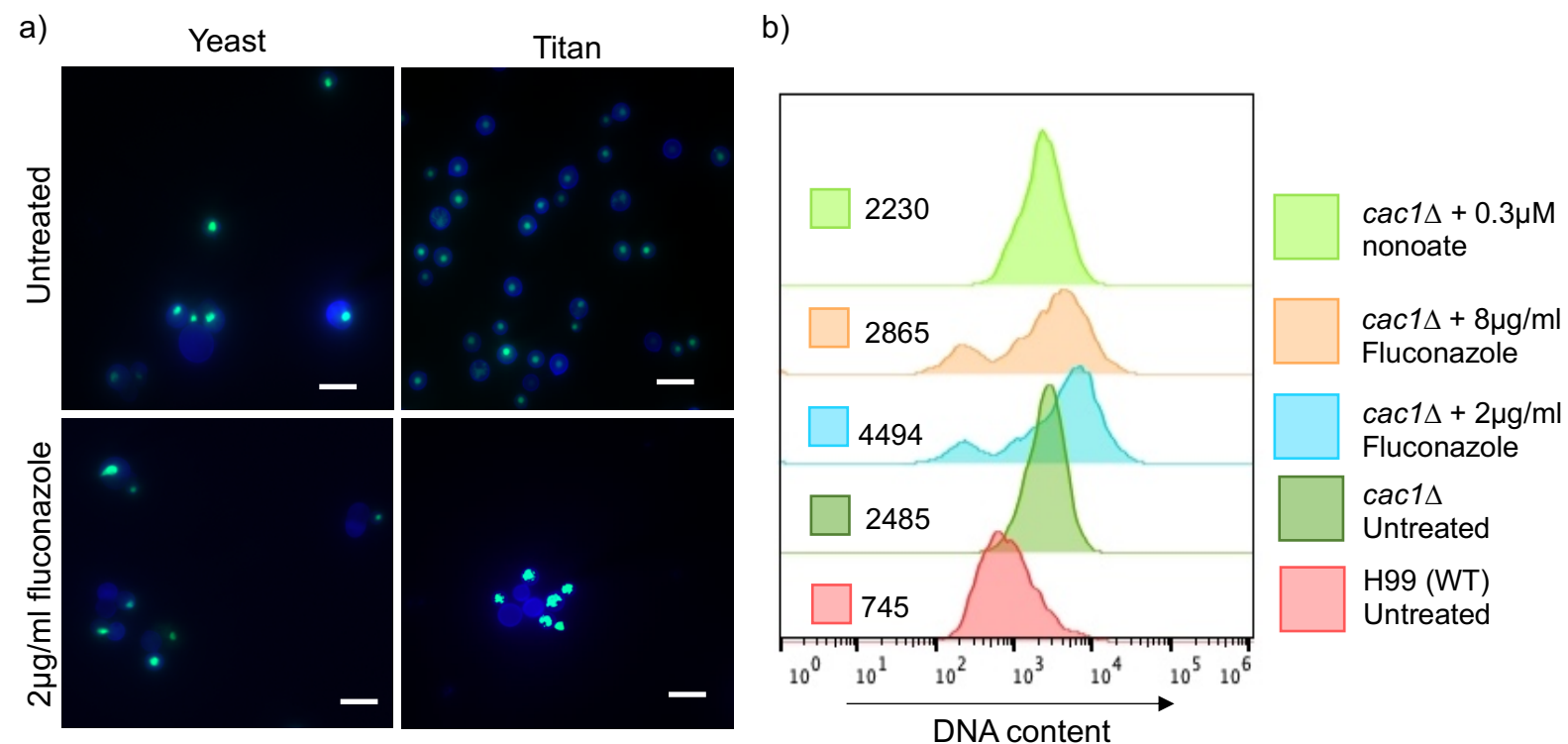


Figure 19. cAMP pathway alone does not alter DNA content. *cac1Δ* was grown in YNB overnight. a) microscopy images of *cac1Δ* after growth in yeast conditions and titan inducing conditions, with and without 2 μg/ml fluconazole. Cells were stained for chitin with CFW and sybr green for nuclei, scale bar represents 10 μm. The images are representative of 5 technical repeats. b) cells were grown in in titanising conditions of 10% HI-FCS with PBS for 2 days at 37 °C and 5% CO₂ with and without differing concentration of fluconazole and nonoate was used as a control. Cells were stained with DAPI and measured by flow cytometry. The median frequency of DAPI is on the graph and was calculated using flowjo, the histogram was generated using flowjo. Data is representative of 2 biological repeats.

3.2.7 DNA content during drug resistance can be mediated via cAMP-dependent and cAMP-independent mechanisms.

Elevated levels of reactive oxygen species (ROS) have been found to mediate oxidative stress which is linked to chromosomal instability in turn causing damage to DNA (Peng et al., 2018). ROS carry unpaired electrons that are highly reactive and unstable known as free radicals (Winterbourn, 2008). In *C. albicans*, and fairly recently in *C. neoformans*, an increase in ROS has been seen following treatment with fluconazole (Wang et al., 2009,

Mahl et al., 2015, Peng et al., 2018). Data in the literature suggests that there is a role for mitochondria in titanisation and some role for fluconazole in mitochondrial perturbation (Zhou et al., unpublished) (Brun et al., 2004, Bouchara et al., 2000). I hypothesize that fluconazole targets the mitochondria in *C. neoformans* and increases ROS levels, and that this in turn increases DNA content.

To test this hypothesis, I asked whether 1) ROS levels increase when the cells are grown under titan inducing conditions with fluconazole, and 2) if the mitochondrial potential increases. In a previous study, mitochondrial potential has been used as a way to measure mitochondrial functionality using a fluorescent probe called Rhodamine 123 (Trevijano-Contador et al., 2017). When grown under titan conditions, similar to those presented in figure 18, cells treated with fluconazole exhibit increased levels of superoxide compared to untreated cells, (figure 20ai). Cells treated with 8 µg/ml fluconazole have higher fluorescence of superoxide dye than those treated with 2 µg/ml fluconazole, indicating higher levels of superoxide (figure 20aii). Comparably, fluconazole causes higher levels of mitochondrial potential, particularly 8 µg/ml fluconazole (figure 20ci and ii). Although there was not a statistically significant increase in superoxide upon fluconazole treatment for all conditions (2 µg/ml), there is an overall trend towards increased superoxide associated with increased fluconazole (8 µg/ml). This is consistent with a model in which fluconazole acts by inducing ROS stress.

Interestingly when looking at the flow cytometry data more clearly, for some of the data there were 2 population peaks present (figure 20a and c). I assumed that these 2nd population were those that were of a larger cell size as they were at the right end of the peak. Therefore,

using the gating strategy I mentioned in section 2.6.5 for this second population, I took the data from the 3 biological repeats and calculated the percentage of cells that had a high MFI. It further supported the data showing that a higher percentage of the 2nd population of cells treated with 2 µg/ml and 8 µg/ml fluconazole had increased levels of superoxide compared to those that were untreated, with the latter being the highest level (figure 20b). Similarly, for mitochondrial potential, when looking at the 2nd population of the data, fluconazole treated cells with this 2nd population seemed to also cause high levels of mitochondrial potential (figure 20d)

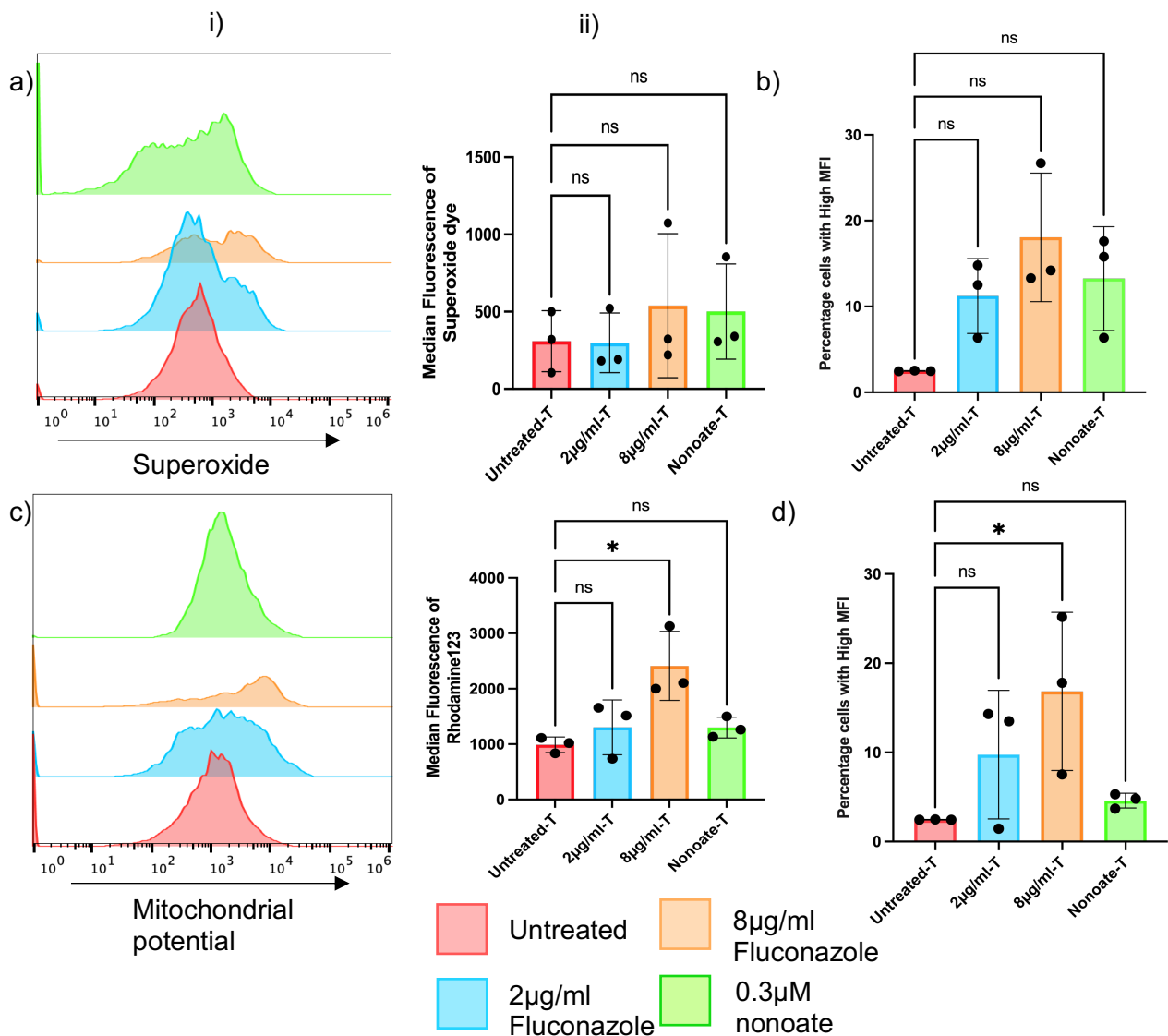


Figure 20. Fluconazole increases superoxide levels and mitochondrial potential. H99 was grown in YNB overnight, followed by induction of titan cells in 10% HI-FCS with PBS, with and without differing concentration of fluconazole and nonoate (control), for 2 days at 37°C and 5% CO₂. Cells were stained with a) superoxide dye and c) rhodamine 123 before being measured by flow cytometry. Data is a representative of 3 biological repeats and the histograms were created using FlowJo (i). The median fluorescent intensity of each concentration was taken from the histograms on FlowJo and plotted using GraphPad (ii). The second population from the b) superoxide stained cells and d) rhodamine 123 stained cells, was gated and the percentage of cells was plotted. Data was the mean ± SD, ns= not significant and *= p<0.05 (Kruskall-Wallis with Dunn's multiple comparisons test).

After confirming the increase in ROS levels and the activation of the mitochondria, I wanted to see if the mitochondria were playing a role in the increase in DNA content seen in figure 18. As *C. neoformans* is a petite negative fungus meaning it cannot survive without the

mitochondria, it is not possible to completely remove the mitochondria to test this hypothesis. Instead, inhibitors of the electron transport chain complexes found in the mitochondria were used (figure 21).

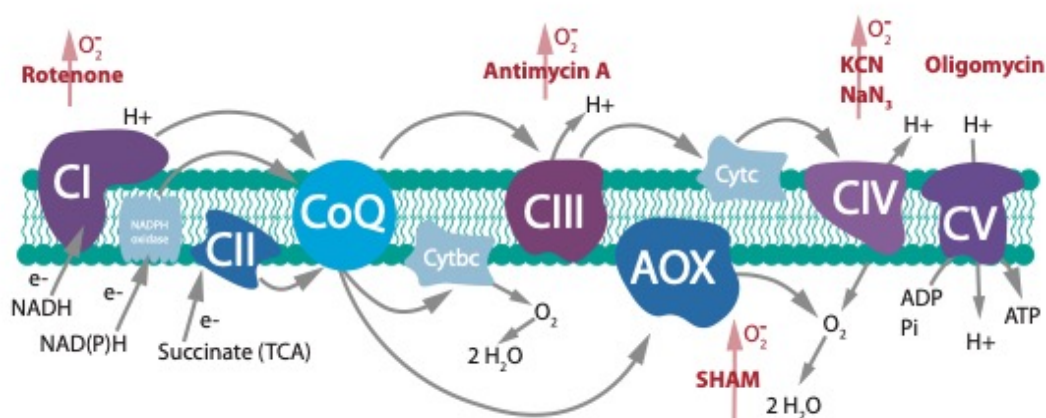


Figure 21. The elements of the electron transport chain in the mitochondria, and the impact of the chemicals that inhibit their activity. The mitochondrial membrane is shown in green, and Complex (C) I, II, III, IV, and V are represented throughout the matrix. The flow of electrons is presented in grey, while the action of six different mitochondrial inhibitors is paired with its predicted target and the expected impact on reactive oxygen (red). AOX is the Alternative Oxidase, a fungal specific enzyme that compensates for inactivation of Complex III. (Adapted from Trevijano-Contador et al., 2017).

There were 5 inhibitors used: rotenone (R), antimycin A (AA), potassium cyanide (KCN), sodium azide (SA), and salicylhydroxamic acid (SHAM). Complex I is inhibited by R, complex III is inhibited by AA, complex IV is inhibited by both KCN and SA, whilst the alternative cytochrome oxidative pathway is inhibited by SHAM (figure 14). By inhibiting certain parts of the mitochondrial electron transport chain, it would show what part of the chain fluconazole is interacting with and whether this makes a difference to the DNA content.

When looking at the mitochondrial inhibitors effect on DNA content alone, inhibition of either complex I (via R) or complex III (via AA) had the most substantial impact (figure 22ai and

ei). Inhibition of complex IV with KCN did not increase DNA content (figure 22bi), but DNA content was somewhat increased when complex IV was inhibited with SA (figure 22di). These compounds target different aspects of complex IV, which may explain their differential influence on DNA content. Finally, inhibition of the alternative oxidase via SHAM had no apparent impact on DNA content (figure 22ci). The alternative oxidase acts in a parallel pathway to complex III, so may not play an essential role in the absence of complex III inhibition. What is interesting to note is the size of the cells after their treatment with the mitochondrial inhibitors: they all had an increase in size in comparison to H99 alone (figure 22ii), except SHAM (figure 22cii). Alongside this, it further confirms that an increase in cell size also is correlated with an increase in DNA content (figure 22ii). In this data set, when some complexes were inhibited I saw 2 populations of cells. Looking at these in more detail after gating them (section 2.6.5), it was clear that when complex I and complex III is inhibited through R and AA respectively, there was a higher percentage of the 2nd population of cells that produced had higher DNA content (figure 22f). Overall, this implies 1) that mitochondrial inhibitors do not seem to block titanisation; 2) that perturbing different aspects of the mitochondrion differentially impacts DNA content, and 3) that perturbing the mitochondrion in any way has an impact on cell size.

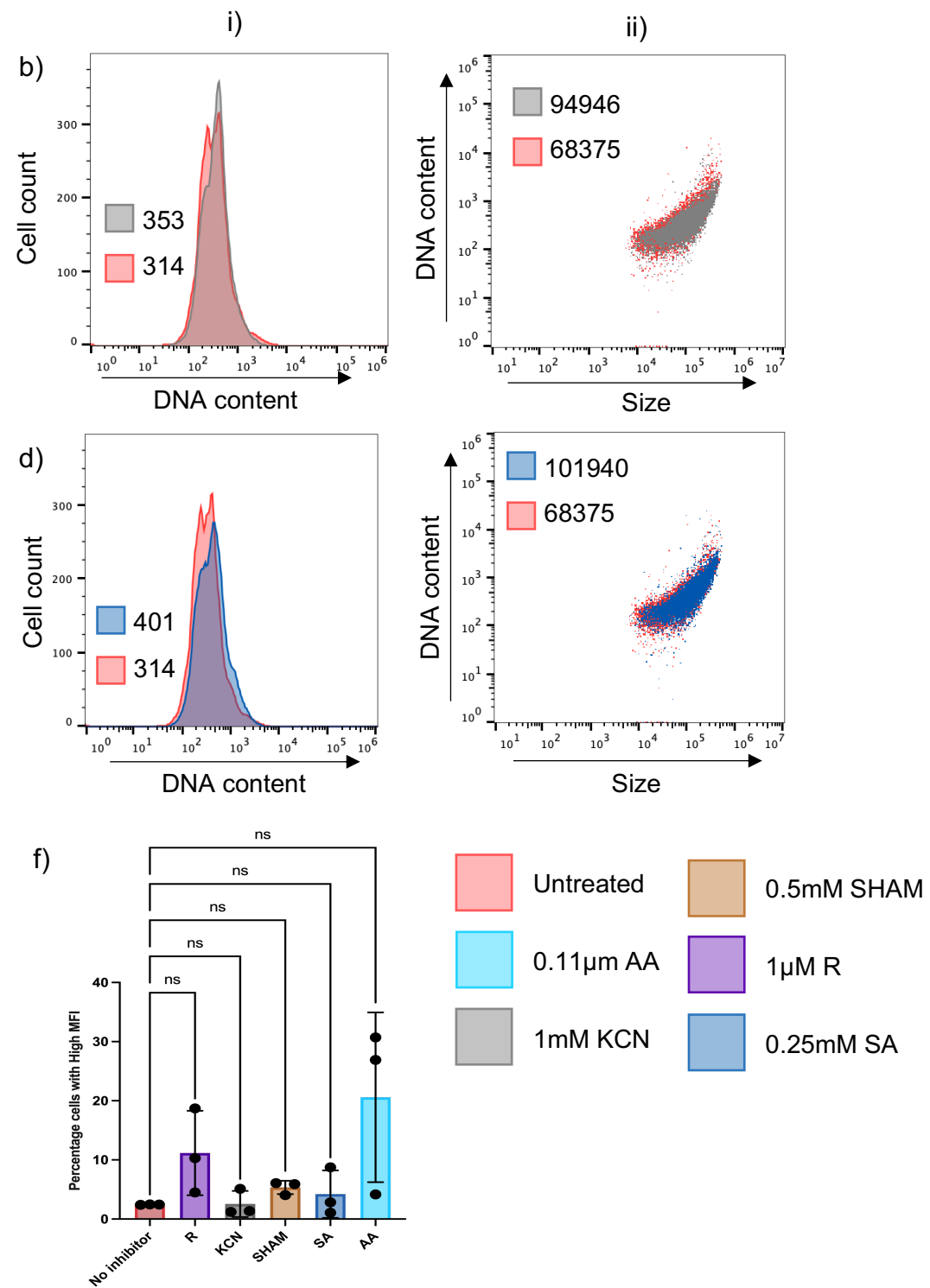
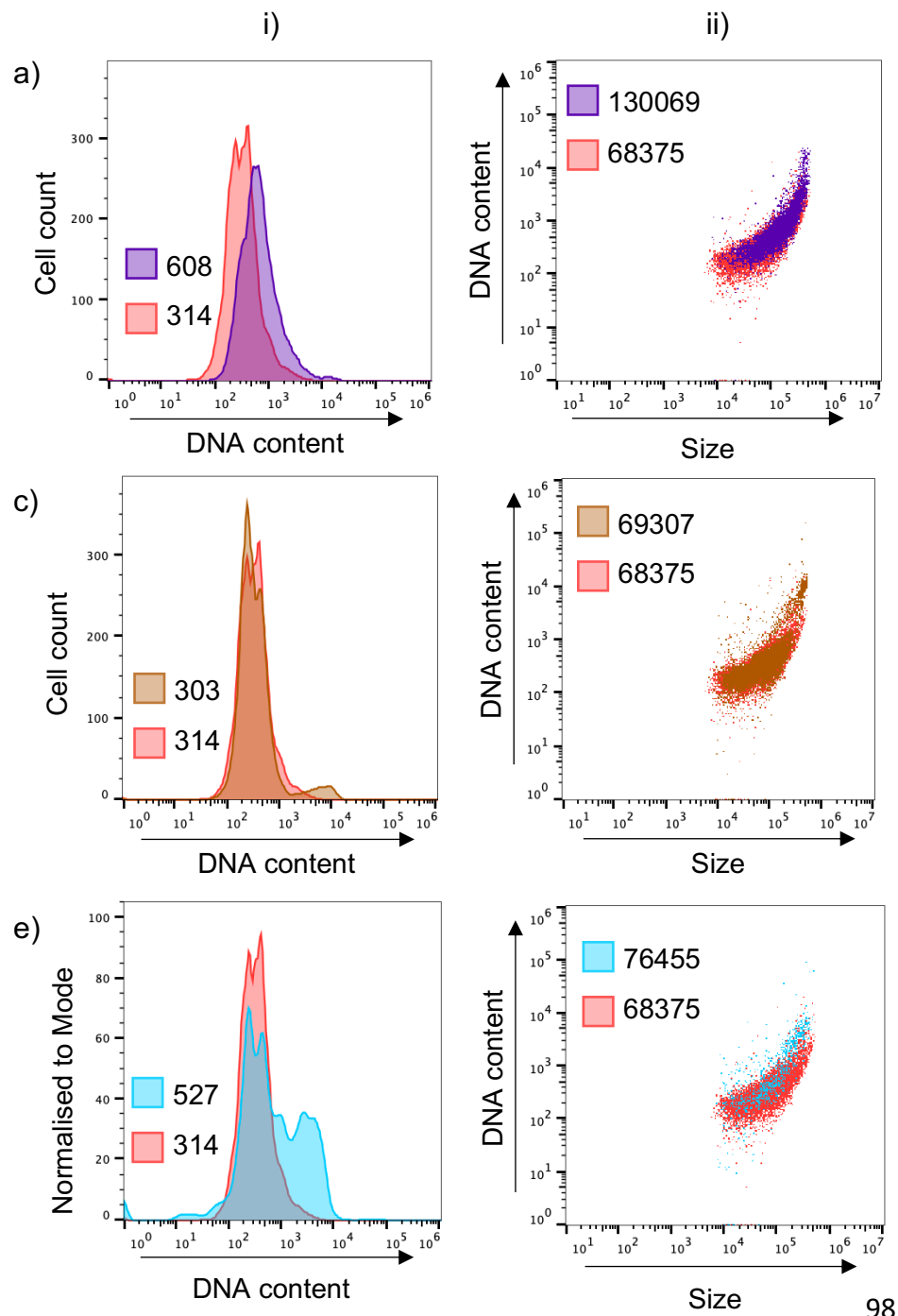


Figure 22. Certain mitochondrial inhibitors increase cell size and DNA content when grown in titan inducing conditions. H99 was grown in YNB overnight, followed by induction of titan cells in 10% HI-FCS with PBS, with and without specific concentration of the mitochondrial inhibitors, for 2 days at 37°C and 5% CO₂. a) H99 with complex I inhibitor R, b) H99 with complex IV inhibitor KCN, c) H99 with alternative oxidase pathway inhibitor SHAM, d) H99 with complex IV inhibitor SA, and e) H99 with complex III inhibitor AA. Cells were stained with DAPI before being measured by flow cytometry. i) histogram showing DNA content of the cells with medium fluorescent intensity of DAPI of the representative data. ii) scatter plot depicting DNA content vs size with the medium fluorescent intensity of size of the representative data. f) the second population from these histograms were gated and the percentage of cells was plotted. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, ns means non-significant (Kruskall-Wallis with Dunn's multiple comparisons test).

Following this, I then added fluconazole to see if there were any further effects on the DNA content when mitochondrial inhibitors were present, which would indicate the role of the mitochondria in the increase in DNA content seen in figure 18. When 2 μ g/ml fluconazole was added, there was a further increase in DNA content when the alternative pathway and complex III were inhibited via SHAM and AA respectively (figure 23c and e). However, no change in DNA content was seen during inhibition of complex I through R (figure 23a) and complex IV through KCN and SA (figure 23b and d), indicating that these complexes may not be playing a role at this concentration of fluconazole. On the other hand, the addition of 8 μ g/ml fluconazole produced differing results. There was an increase in DNA content when 8 μ g/ml fluconazole was added (figure 23) in comparison to the inhibitors alone (figure 22). Yet, when combined, perturbing the mitochondria seemed to cause a decrease in the medium fluorescence of DAPI which indicates a decrease in DNA content (figure 23ii). This was seen more so when complex I was inhibited by R (figure 23aii), complex III was inhibited by AA (figure 23eii), and complex IV was inhibited by SA (figure 23dii). But the most striking result was seen when the alternative oxidase pathway was inhibited by SHAM (figure 23cii). However, the DNA content of the perturbation of the mitochondria along with MIC levels of fluconazole (8 μ g/ml) was still higher than that of untreated cells, suggesting that the

mitochondria alone does not play a role in this increase in DNA content seen in the presence of fluconazole in titan inducing conditions.

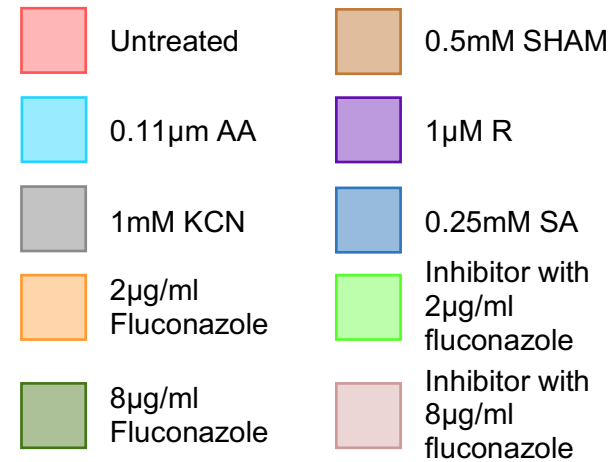
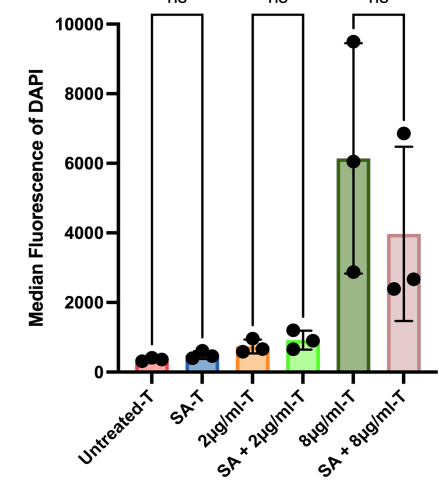
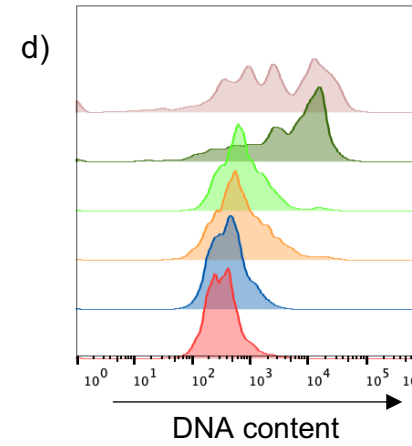
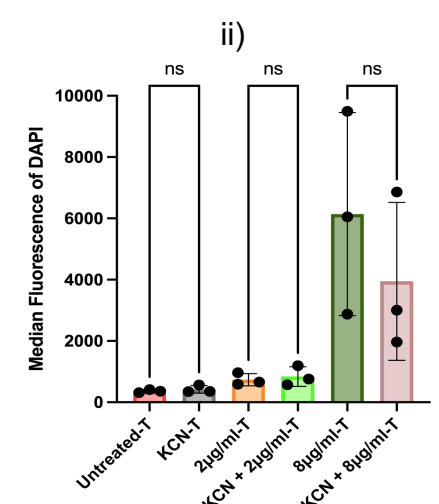
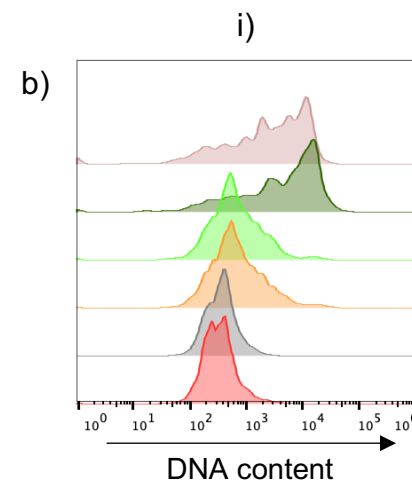
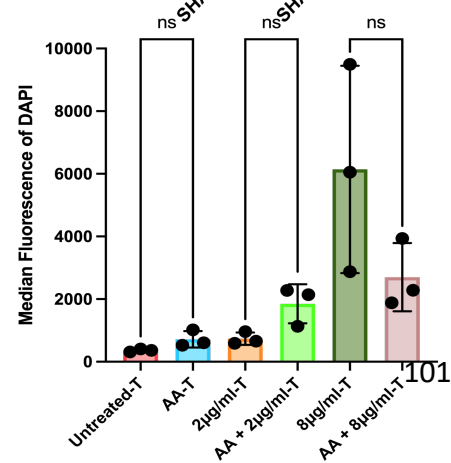
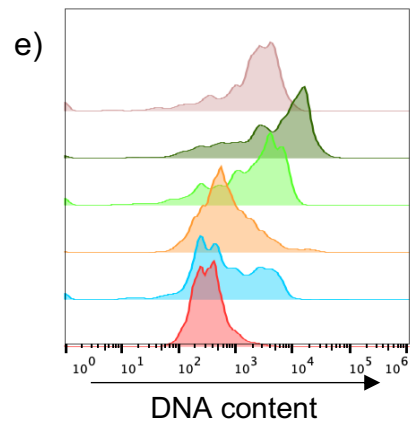
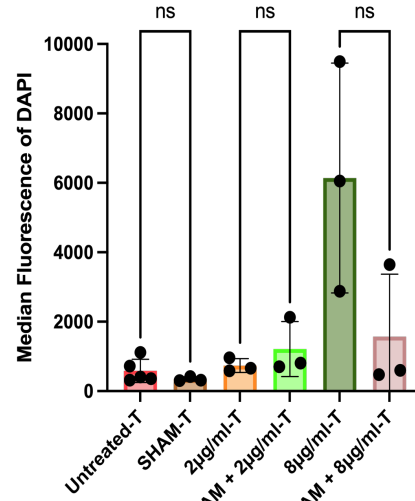
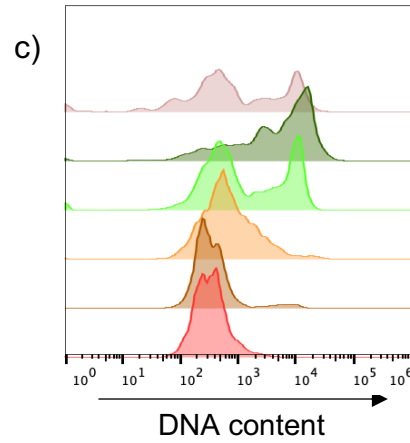
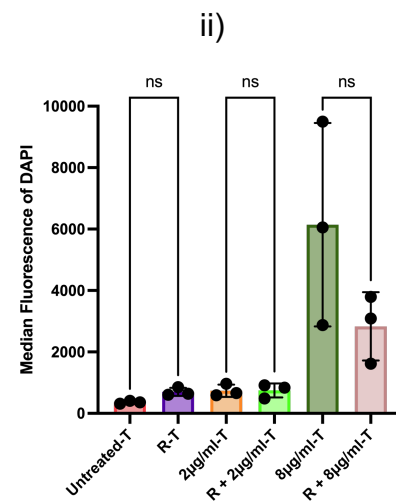
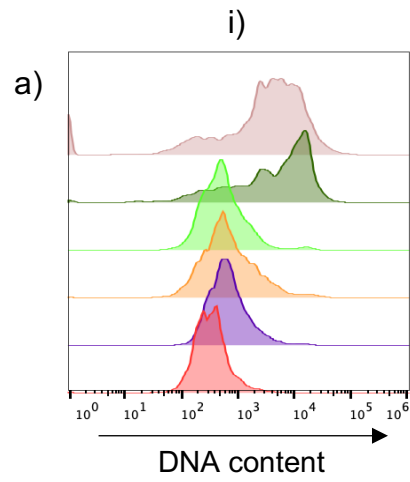


Figure 23. Inhibiting the mitochondria causes a decrease in DNA content in the presence of 8 µg/ml fluconazole. H99 was grown in YNB overnight, followed by induction of titan cells in 10% HI-FCS with PBS, with and without specific concentration of the mitochondrial inhibitors and 2 different concentrations of fluconazole, for 2 days at 37°C and 5% CO₂. a) H99 with complex I inhibitor R, b) H99 with complex IV inhibitor KCN, c) H99 with alternative oxidase pathway inhibitor SHAM, d) H99 with complex IV inhibitor SA, and e) H99 with complex III inhibitor AA, with and without fluconazole. Cells were stained with DAPI before being measured by flow cytometry. i) histogram showing DNA content of the cells with medium fluorescent intensity of DAPI of the representative data produced on FlowJo. ii) The median fluorescent intensity of each concentration was taken from the histograms on FlowJo and plotted using GraphPad. Data is representative of 3 biological repeats. Data was the mean ± SD, ns means non-significant (Kruskall-Wallis with Dunn's multiple comparisons test).

As the previous data had shown that there was an increase in superoxide levels and mitochondrial potential when the cells were treated with fluconazole (figure 20), we hypothesized that there would be an increase in superoxide levels in the presence of mitochondrial inhibitors that block the capacity of each complex to detoxify superoxide.

To test this hypothesis H99 was treated with mitochondrial inhibitors and fluconazole before being stained with superoxide dye. Intriguingly, inhibition of complex I via R and complex IV with SA had the most noticeable increase in superoxide levels in comparison to untreated cells (figure 24ai and di). As there was a noticeable increase in DNA content when complex I was inhibited, this could be due to the increase in ROS levels seen also when complex I was inhibited. When the other complexes were inhibited, there was only a slight increase seen in superoxide levels (figure 24bi, ci and ei). When looking at the cell size vs superoxide in the presence of the inhibitors, there is a correlation that larger cells have higher levels of superoxide, more noticeable when complex I and complex IV are inhibited (figure 24aii and dii). However, when looking more clearly at the scatter plots, there are population of cells that are big but have lower levels of superoxide (figure 24ii). This implies that not all large cells have high levels of superoxide.

For superoxide, when the complexes were inhibited there were 2 population of cells (figure 24f) similar to that seen when measuring the DNA content mentioned earlier (figure 22f). The second population produced when complex I is inhibited via R and complex IV is inhibited via SA had significantly high levels of superoxide in comparison to H99 cells untreated (figure 24f). Interestingly when looking at the perturbation of complex III via AA, this also produced high levels of superoxide (figure 24f), although this was not clear when looking at the flow cytometry figure 24e).

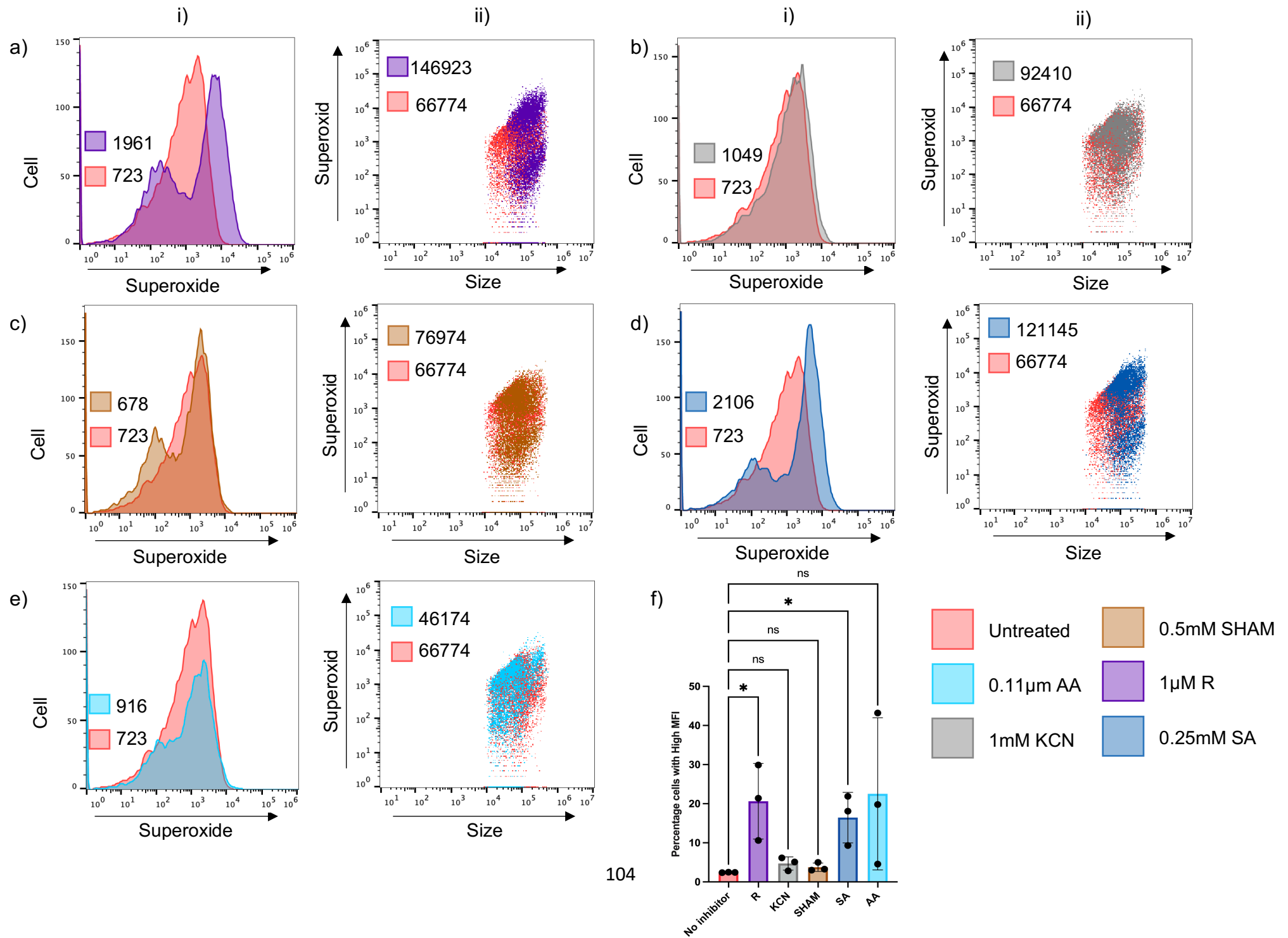


Figure 24. Inhibiting certain mitochondrial complexes increase superoxide levels. H99 was grown in YNB overnight, followed by induction of titan cells in 10% HI-FCS with PBS, with and without specific concentration of the mitochondrial inhibitors, for 2 days at 37°C and 5% CO₂. a) H99 with complex I inhibitor R, b) H99 with complex IV inhibitor KCN, c) H99 with alternative oxidase pathway inhibitor SHAM, d) H99 with complex IV inhibitor SA, and e) H99 with complex III inhibitor AA. Cells were stained with DAPI before being measured by flow cytometry. i) histogram showing DNA content of the cells with medium fluorescent intensity of DAPI of the representative data. ii) scatter plot depicting DNA content vs size with the medium fluorescent intensity of size of the representative data. f) the second population from these histograms were gated and the percentage of cells was plotted. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, ns= not significant and *= p<0.05 (Kruskall-Wallis with Dunn's multiple comparisons test).

Subsequently, we then added fluconazole to see if there were any further effects on the level of superoxide when inhibiting mitochondrial complexes. In the presence of 2 μ g/ml fluconazole, there was an increase in medium fluorescence of the superoxide dye across all inhibited complexes compared to 2 μ g/ml fluconazole alone, suggesting a further increase in superoxide levels. This was more apparent when complex I was inhibited by R (figure 25a), and complex IV inhibition via SA (figure 25d). This indicates that the increase in superoxide seen at 2 μ g/ml fluconazole may be coming from somewhere else. Interestingly, there was a variation of superoxide levels for 8 μ g/ml fluconazole depending on the complex inhibited. Inhibition of complex I by R, complex III by AA, and complex IV by SA caused a further increase in the median fluorescence of superoxide dye which indicates an increase in superoxide levels (figure 25aii, dii, and eii). Conversely, inhibition of complex IV through KCN did not cause any change in the superoxide levels in combination with 8 μ g/ml fluconazole (figure 25b). Intriguingly, when the alternative oxidase pathway was inhibited by SHAM there was a decrease in the median fluorescence of superoxide dye which indicates a decrease in superoxide levels. This suggests that the alternative pathway may play a partial role to the increase in superoxide levels seen when H99 is grown with 8 μ g/ml fluconazole alone.

Overall, it is clear that the mitochondria plays a role in the increase of DNA content and superoxide levels when cells are grown in the presence of fluconazole. The alternative oxidase pathway seems to play a role in both the increase in DNA content and superoxide levels of MIC concentration of fluconazole. Whilst the other complexes seem to have differed results such complex I seems to play a role in the increase in DNA content but not in the increase in superoxide. This suggests that these two conditions may not always be correlated.

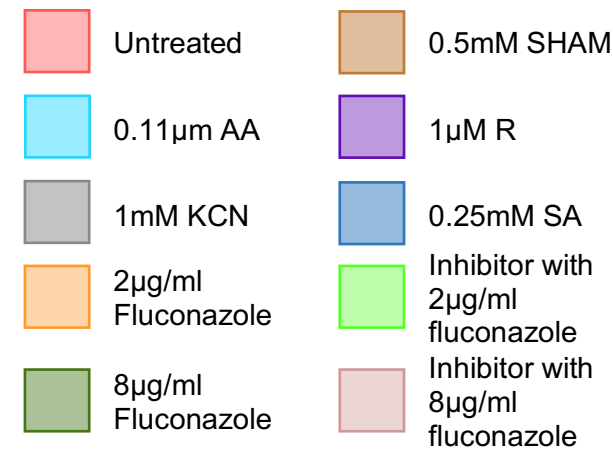
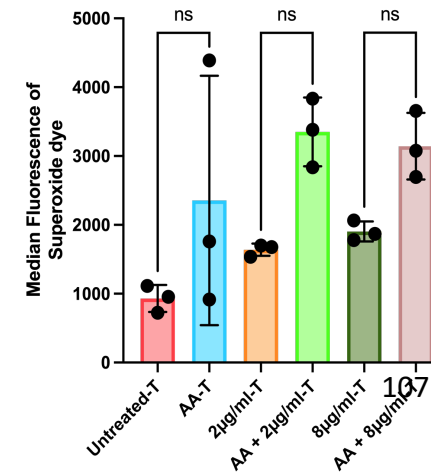
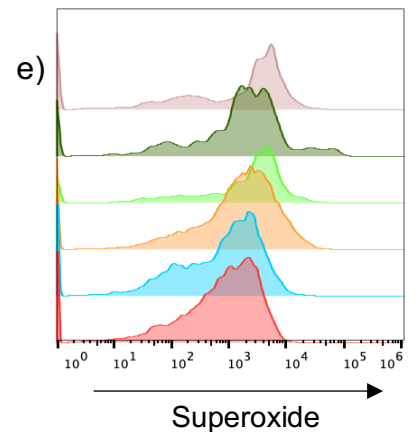
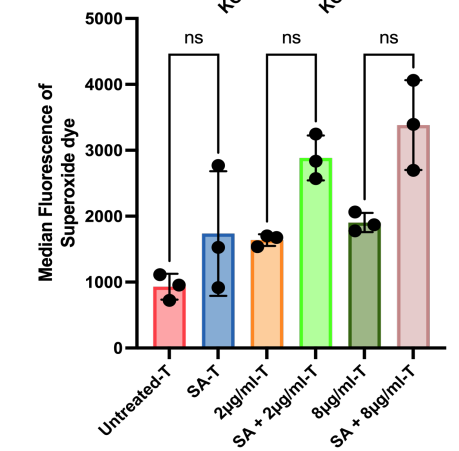
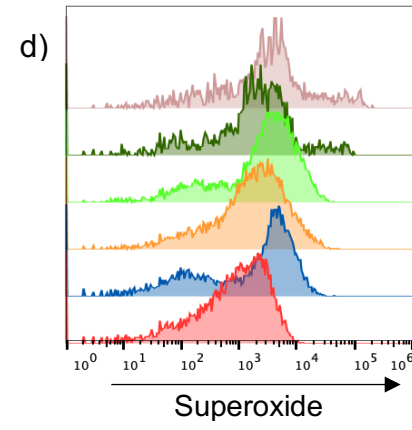
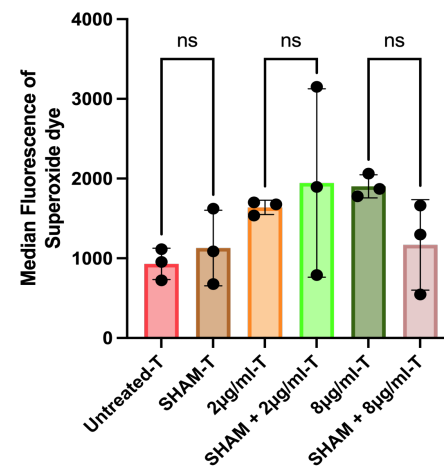
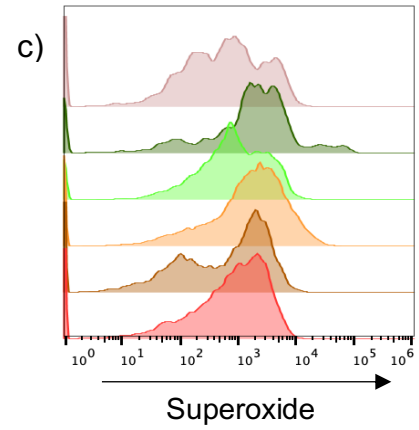
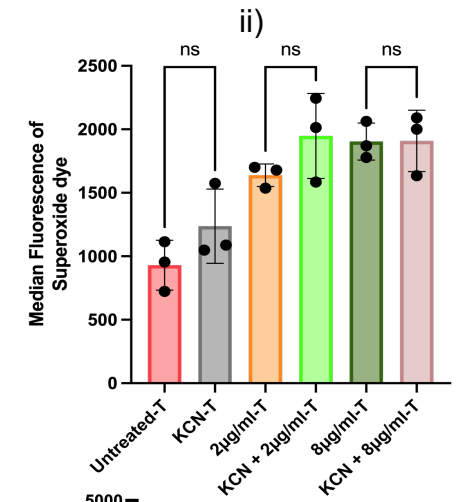
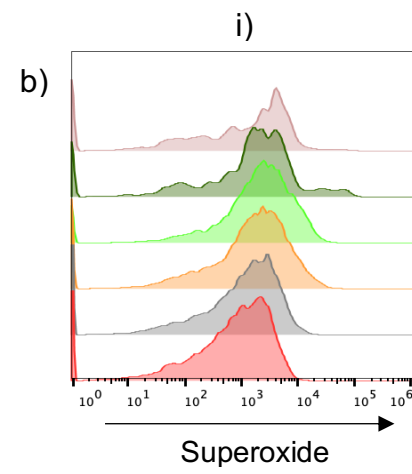
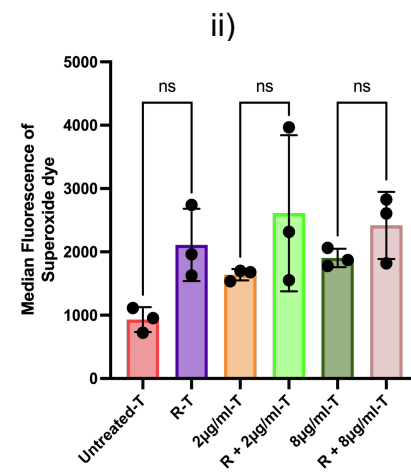
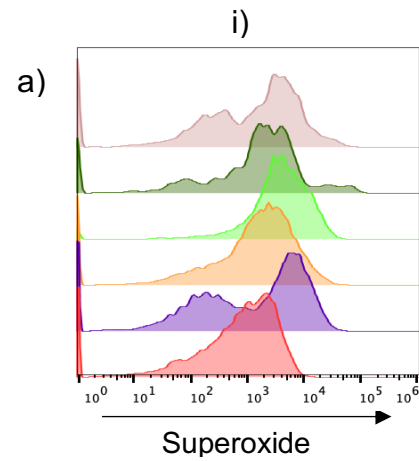


Figure 25. Varying levels of superoxide caused by inhibiting the mitochondria at MIC concentration of fluconazole. H99 was grown in YNB overnight, followed by induction of titan cells in 10% HI-FCS with PBS, with and without specific concentration of the mitochondrial inhibitors and 2 different concentrations of fluconazole, for 2 days at 37°C and 5% CO₂. a) H99 with complex I inhibitor R, b) H99 with complex IV inhibitor KCN, c) H99 with alternative oxidase pathway inhibitor SHAM, d) H99 with complex IV inhibitor SA, and e) H99 with complex III inhibitor AA, with and without fluconazole. Cells were stained with DAPI before being measured by flow cytometry. i) histogram showing DNA content of the cells with medium fluorescent intensity of DAPI of the representative data produced on FlowJo. ii) The median fluorescent intensity of each concentration was taken from the histograms on FlowJo and plotted using GraphPad. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, ns means non-significant (Kruskall-Wallis with Dunn's multiple comparisons test).

3.2.8 Titan inhibitors successfully block titanisation but they increase *C. neoformans* DNA content

Going back to our aim, I next looked into the mechanism of titanisation and how this may play a role in fluconazole resistance. If titan cells are an important part of fluconazole resistance, then the molecular mechanism that drives titanisation would also be important. I hypothesized that chemically blocking titanisation might influence fluconazole resistance. Previously members of the lab carried out a drug screen using the Prestwick library to identify FDA approved drugs that are able to inhibit the yeast to titan transition. We found 3 drugs not currently used as antifungals that were able to reduce titanisation: retinoic acid, metyrapone, and nifedipine hydrochloride.

Retinoic acid is an agent that derives from vitamin A (retinol) and is necessary for differentiation, organogenesis and cell growth in humans as well as a well-known anti-oxidant (Kam et al., 2012, Ahlemeyer et al., 2001). Metyrapone is a drug used for treatment of Cushing's disease hypercortisolism in humans by inhibiting the 11 β -hydroxylase (Sonino et al., 1981), however, its mode of action in fungi is not known. By using these drugs in combination with fluconazole we would predict to see a decrease in MIC if titanisation is important for drug resistance.

First, I quantified their ability to inhibit titan cell formation using microscopy and flow cytometry, as described previously. Microscopy data for three independent biological replicates are aggregated in figure 26a. While a reduction in the frequency and maximum size was observed in the presence of all three inhibitors, cells larger than 10 μm in diameter were occasionally observed (figure 26a).

It is important to note that none of these titan inhibitor drugs are fungicidal or fungistatic. When measuring the diameter of the cells, H99 when treated with retinoic acid produced a population less than 10 μm in diameter compared to H99 alone, although there were cells above this border (figure 26b). A similar pattern was observed when cells were treated with metyrapone and nicardipine, where metyrapone treated H99 cell diameter was below 10 μm , but likewise there was a small population of cells that were above the 10 μm border (figure 26c and d). Although the few instances of larger cells are less frequent and smaller than the untreated cells, there are titan cells present in the presence of all drugs. This indicates that these drugs may not necessarily completely block titanisation.

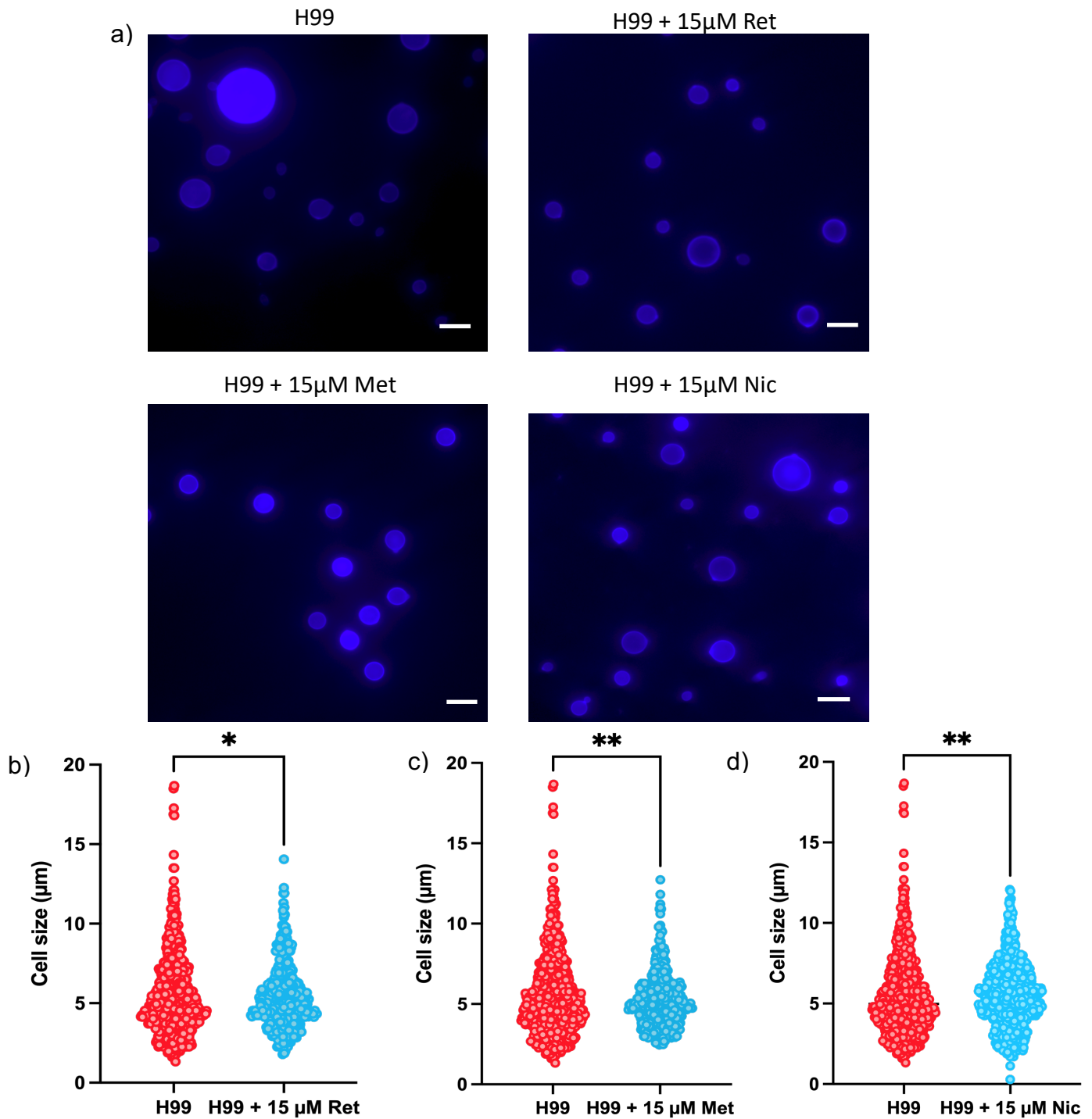


Figure 26. The titan inhibitor drugs block titanisation although few titan cells can be seen. H99 was grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS with and without 15 μ M retinoic acid for 2 days. a) microscopy images of H99 alone and H99 with 15 μ M ret, 15 μ M met and 15 μ M nic, representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μ m. b) Size of H99 and H99 with 15 μ M ret measured from microscopy images. c) Size of H99 and H99 with 15 μ M met measured from microscopy images. d) Size of H99 and H99 with 15 μ M nic measured from microscopy images. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, * = $p < 0.05$, ** = $p < 0.005$ (Kolmogorov-Smirnov test).

Following this I looked into whether these drugs affected the DNA content or superoxide levels of the cells. Interestingly, although titanisation is reduced, there seems to be a dose-dependent increase in DNA content when cells are grown with retinoic acid (figure 27ai). However, there was a decrease in superoxide levels when cells were exposed to retinoic acid at the highest concentration, lower than that of superoxide in H99 alone (figure 27aii and aiii). This demonstrates that retinoic acid is able to inhibit titanisation but somehow causes an increase in DNA content that is seemingly not caused by superoxide. Intriguingly, although metyrapone was able to inhibit titanisation, the cells treated with metyrapone also had an increase in DNA content in a dose-dependent manner (figure 27bi). On the other hand, the superoxide levels of metyrapone treated H99 were lower than that of H99 alone (figure 27bii and biii). Similar to retinoic acid, this demonstrates that metyrapone causes an increase in DNA content and a decrease in superoxide levels and yet it is able to reduce titanisation. Unlike the other titan inhibitor drugs, nicardipine hydrochloride did not affect the DNA content of H99 (figure 27ci). However similar to the other drugs, nicardipine caused a decrease in superoxide levels in the cells (figure 27cii and ciii).

When looking at the DNA content of the cells treated with the higher concentration of the TI drugs, there was 2 peaks indicating 2 populations (figure 27i), particularly when treated with retinoic acid (figure 27ai). When looking into these 2 population, I found that cells treated with 15 μ M retinoic acid had a large percentage of cells with high DNA content compared to the other TI drugs (figure 27d).

Together, these findings suggest that while increased superoxide may be associated with increased DNA content, it is not required: retinoic acid, metyrapone impair the formation of

large cells and superoxide accumulation, but still cause an increase in DNA content independent of superoxide. This is consistent with our finding that fluconazole treated cAMP pathway mutants that fail to form large cells still exhibit increased DNA content and further supports our hypothesis that pathways other than the mitochondrion or cAMP/PKA pathway can play a role in the induction of titanisation.

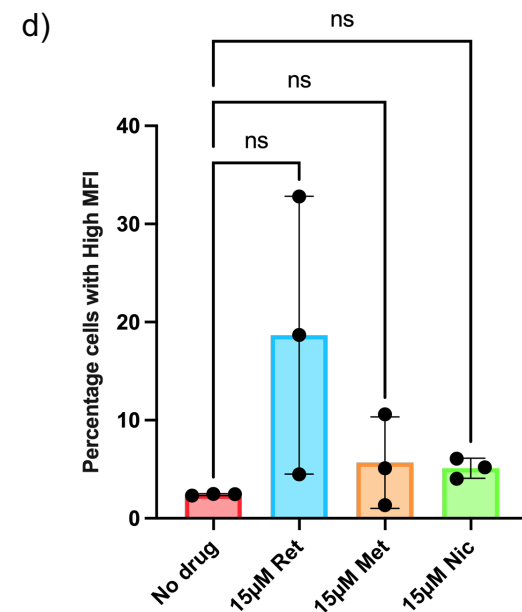
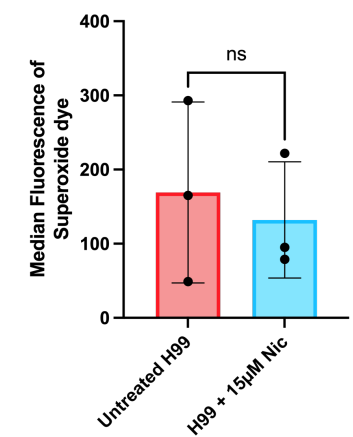
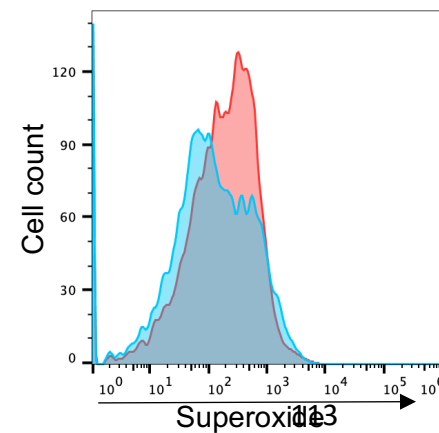
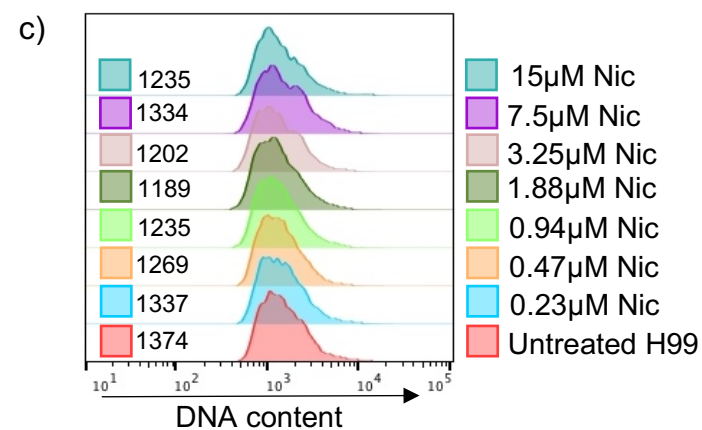
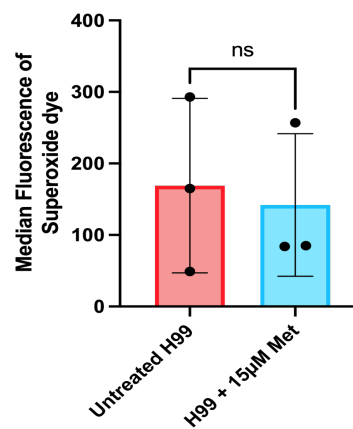
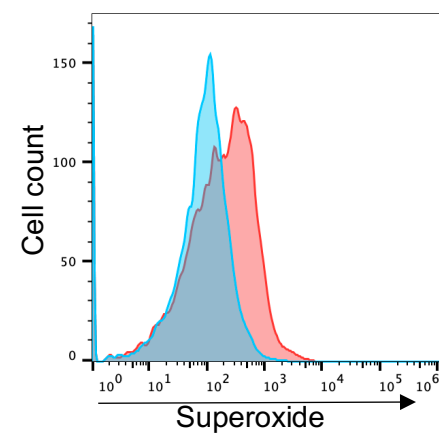
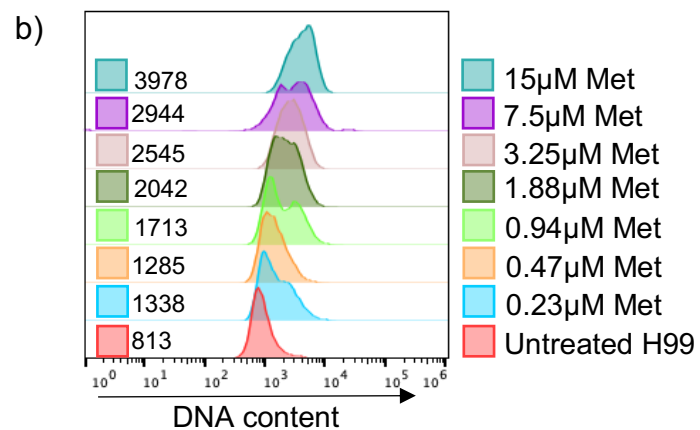
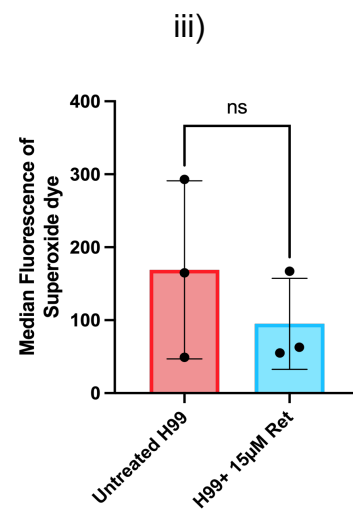
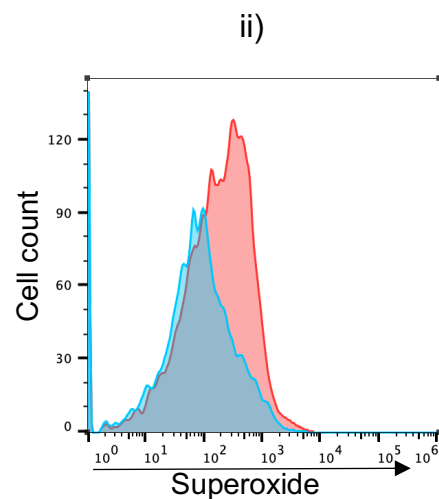
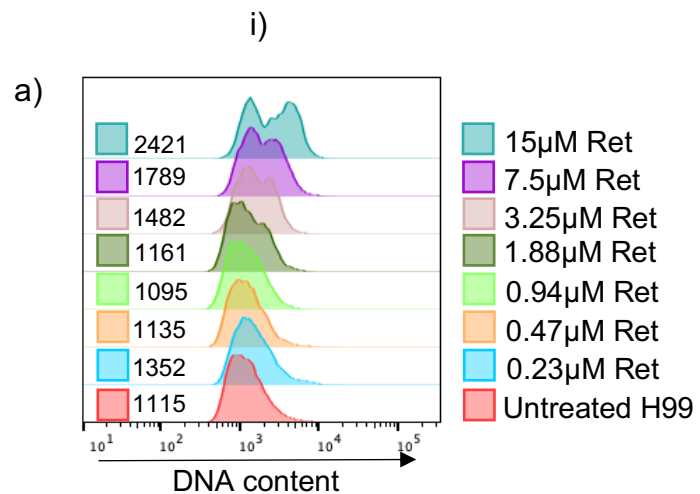


Figure 27. All titan inhibitors cause an increase in DNA content and a decrease in superoxide levels in titan inducing conditions. H99 was grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS with and without 15 μ M metyrapone for 2 days. a) H99 with retinoic acid, b) H99 with metyrapone, and c) H99 with nicardipine hydrochloride. i) Cells were stained with DAPI and measured by flow cytometry. Data is representative of 3 biological repeats. On the histogram showing DNA content of the cells has the data of median fluorescent intensity of DAPI of the representative data produced on FlowJo. ii) Cells were stained with superoxide dye and measured by flow cytometry. Data is representative of 3 biological repeats. iii) The median fluorescent intensity of superoxide dye of the drug was taken from the histograms on FlowJo and plotted using GraphPad. d) the second population from these histograms were gated and the percentage of cells was plotted. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, ns= not significant (Mann-Whitney test (ii) and Kruskal-Wallis with Dunn's multiple comparisons test (d)).

3.2.9 Titan inhibitors are not synergistic with fluconazole in *C. neoformans*

After we confirmed these drugs may be able to reduce titanisation, I hypothesized that by using these drugs in combination with fluconazole there would be a decrease in MIC if titanisation is important for drug resistance.

To test this, a checkerboard assay was carried out to measure the effect on growth of *C. neoformans* through combination of the 3 titan inhibitors (TIs) with fluconazole. Using a 96 well plate, fluconazole was serially diluted across the columns from 256 μ g/ml to 0.25 μ g/ml, while TIs was serially diluted down the rows from 15 μ M to 0.23 μ M. H99 was grown in yeast conditions so as to see the effects of the drug combination on the originating cells. Classically, checkerboard assays are used to measure whether drugs are synergistic, but this requires determination of MIC for both drugs independently. This was not possible for the TIs, which do not block yeast phase growth (Wambaugh et al., 2020). The cells grew well even at high TI concentrations (15 μ M) as seen in column 12 (figure 28). Instead, I carried out a bliss independence assay which recognises whether the drugs heighten fluconazole action (Tang et al., 2015). The MIC of fluconazole alone was 4 μ g/ml as seen in row H (figure 25). If the

drugs are synergistic we would expect a decrease in fluconazole MIC as there would be an increase in the growth inhibition. Intriguingly, when cells were treated with retinoic acid, metyrapone and nicardipine hydrochloride with fluconazole, the MIC of fluconazole increases to 16 µg/ml (figure 28). This instead suggests that the TIs are antagonistic with fluconazole.

a)

		Fluc												
		256µg/ml	128µg/ml	64µg/ml	32µg/ml	16µg/ml	8µg/ml	4µg/ml	2µg/ml	1µg/ml	0.5µg/ml	0.25µg/ml	0µg/ml	
		1	2	3	4	5	6	7	8	9	10	11	12	
Ret	15µM	A	0.189	0.117	0.207	0.296	0.216	0.32	0.548	0.786	0.877	0.903	0.956	0.857
	7.5µM	B	0.161	0.157	0.133	0.188	0.205	0.261	0.338	0.723	0.856	0.808	0.866	0.834
	3.75µM	C	0.248	0.21	0.265	0.191	0.147	0.218	0.336	0.688	0.829	0.804	0.783	0.909
	1.875µM	D	0.168	0.146	0.192	0.149	0.237	0.256	0.269	0.592	0.763	0.801	0.786	0.809
	0.9375µM	E	0.119	0.168	0.174	0.204	0.169	0.167	0.317	0.489	0.672	0.777	0.829	0.861
	0.46875µM	F	0.182	0.183	0.24	0.164	0.105	0.188	0.307	0.43	0.751	0.816	0.878	0.922
	0.234375µM	G	0.234	0.204	0.163	0.172	0.177	0.229	0.249	0.414	0.782	0.826	0.848	0.976
	0uM	H	0.202	0.185	0.14	0.23	0.161	0.14	0.267	0.364	0.73	0.769	0.903	1.008

b)

			Fluc											
			256µg/ml	128µg/ml	64µg/ml	32µg/ml	16µg/ml	8µg/ml	4µg/ml	2µg/ml	1µg/ml	0.5µg/ml	0.25µg/ml	0µg/ml
			1	2	3	4	5	6	7	8	9	10	11	12
Met	15µM	A	0.13	0.143	0.165	0.196	0.247	0.326	0.601	0.898	0.88	0.951	0.948	0.884
	7.5µM	B	0.207	0.118	0.165	0.222	0.171	0.209	0.324	0.656	0.801	0.791	0.859	0.91
	3.75µM	C	0.189	0.163	0.167	0.191	0.171	0.204	0.285	0.669	0.756	0.781	0.865	0.865
	1.875µM	D	0.136	0.15	0.174	0.133	0.165	0.168	0.275	0.48	0.712	0.779	0.856	0.816
	0.9375µM	E	0.143	0.146	0.163	0.181	0.161	0.152	0.23	0.399	0.65	0.795	0.833	0.913
	0.46875µM	F	0.25	0.141	0.18	0.144	0.114	0.193	0.246	0.37	0.626	0.768	0.768	0.891
	0.234375µM	G	0.23	0.184	0.135	0.138	0.145	0.136	0.176	0.217	0.494	0.756	0.773	0.909
	0uM	H	0.212	0.28	0.189	0.155	0.134	0.15	0.15	0.282	0.473	0.732	0.657	0.898

c)

		Fluc												
		256µg/ml	128µg/ml	64µg/ml	32µg/ml	16µg/ml	8µg/ml	4µg/ml	2µg/ml	1µg/ml	0.5µg/ml	0.25µg/ml	0µg/ml	
		1	2	3	4	5	6	7	8	9	10	11	12	
Nic	15µM	A	0.102	0.099	0.113	0.122	0.168	0.397	0.463	0.625	0.681	0.75	0.783	0.836
	7.5µM	B	0.046	0.085	0.074	0.118	0.097	0.196	0.402	0.566	0.833	0.755	0.834	0.879
	3.75µM	C	0.213	0.136	0.137	0.1	0.075	0.161	0.256	0.497	0.697	0.742	0.839	0.783
	1.875µM	D	0.157	0.161	0.133	0.144	0.126	0.137	0.27	0.26	0.507	0.627	0.734	0.86
	0.9375µM	E	0.132	0.132	0.155	0.103	0.149	0.107	0.175	0.282	0.6	0.673	0.789	0.842
	0.46875µM	F	0.141	0.207	0.221	0.171	0.158	0.155	0.184	0.346	0.586	0.745	0.795	0.865
	0.234375µM	G	0.159	0.154	0.123	0.185	0.133	0.171	0.214	0.282	0.535	0.684	0.785	0.867
	0uM	H	0.225	0.138	0.185	0.119	0.185	0.149	0.216	0.363	0.584	0.815	0.75	0.861

Figure 28. Titan inhibitors are antagonistic with fluconazole. H99 was grown in YNB overnight, followed by seeding in 96 well plate in YNB + 2% glucose for 48 hours. Checkerboard assay of a) retinoic acid and fluconazole, b) metyrapone and fluconazole, c) nicardipine hydrochloride and fluconazole. Data is representative of 3 biological repeats. Blue shading represents cell growth.

As this checkerboard assay was carried in yeast conditions (figure 28), I next wanted to see how the drug combination with fluconazole works following titan induction but focusing on the DNA content and superoxide levels. This was because there were some difficulties in measuring absorbance via plate reader for titan induced population, which are heterogenous. The DNA content of cells treated with the TIs at different concentrations was measured, along with the combination of 8 µg/ml fluconazole as that is the clinical MIC. Cell size was also measured to see if the TIs were still able to inhibit titanisation in the presence of fluconazole.

Earlier data in this study confirmed that fluconazole does not inhibit titanisation, whilst retinoic acid causes a reduction. When combining the two together, retinoic acid is still able to inhibit titanisation in the presence of fluconazole (figure 29a and b). There was a significant difference in cell size diameter between H99 with 8 µg/ml fluconazole and H99 with both 8 µg/ml fluconazole and 15 µM retinoic acid (figure 29b), indicating that retinoic acid helps block titanisation in fluconazole treated cells. Retinoic acid treated cells can be seen to have a higher DNA content than H99 alone, however there was a larger shift in DNA content when fluconazole was added in combination (figure 29c). Alongside this, retinoic acid treated cells have been seen to have a decreased superoxide level in comparison to H99 alone, but when combined with fluconazole there was an increase in superoxide level. However, this is almost the same level as only fluconazole treated H99 (figure 29d). When looking at the superoxide levels in figure 29d, I noticed there were 2 population of cells, particularly when fluconazole

was present. When I looked into this further I found a high percentage of this 2nd population had much higher superoxide levels when treated with fluconazole than those without (figure 29e).

This suggests that although adding fluconazole does not stop retinoic acid from blocking titanisation, it does cause an increase in DNA content and superoxide levels which could be the reason for the antagonistic relationship.

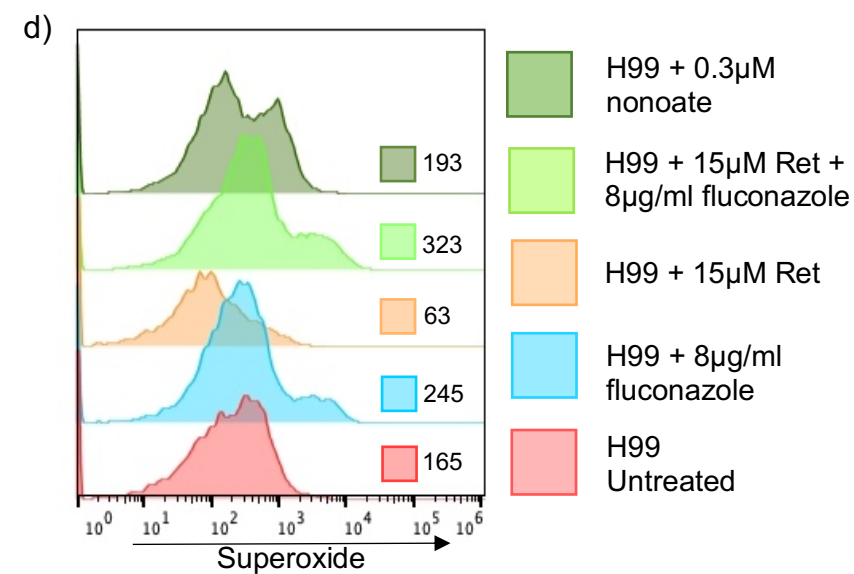
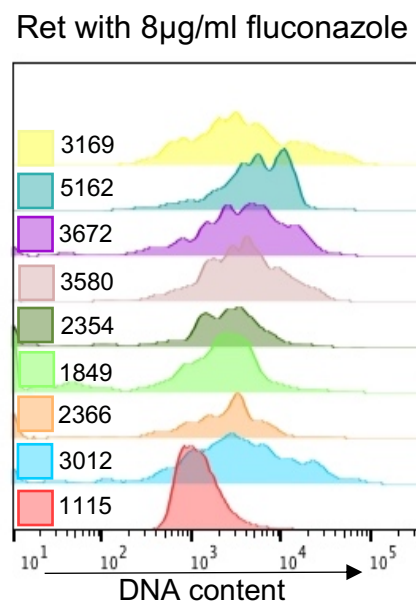
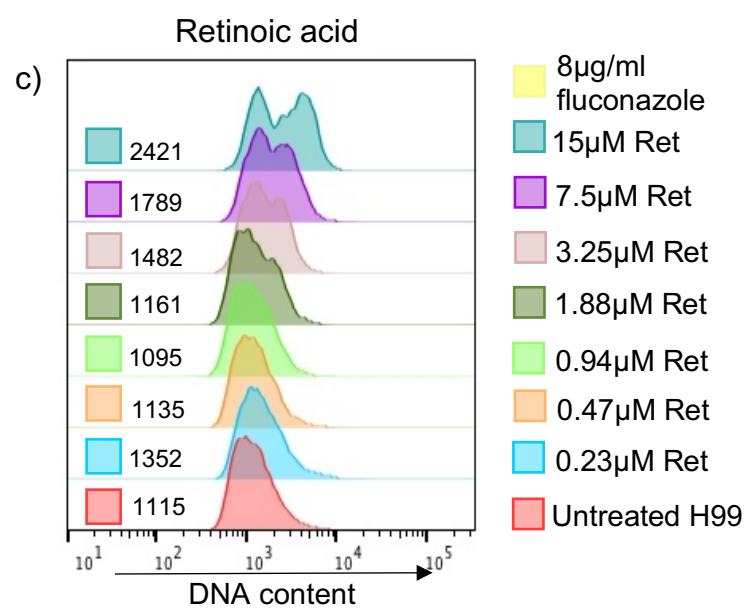
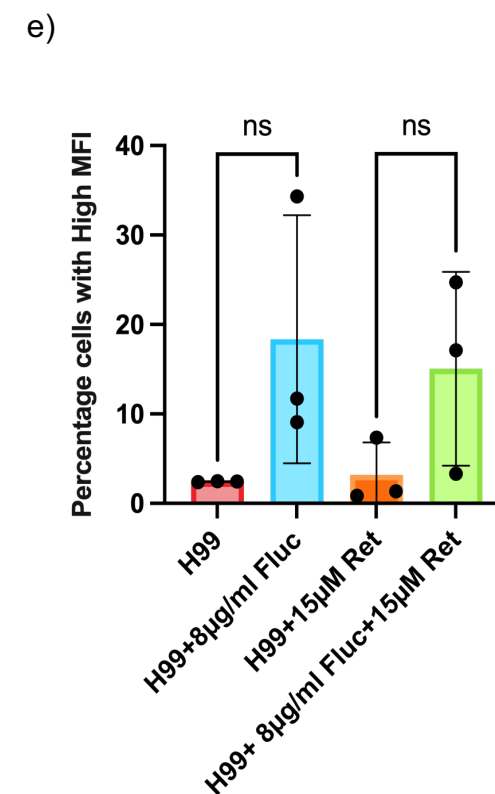
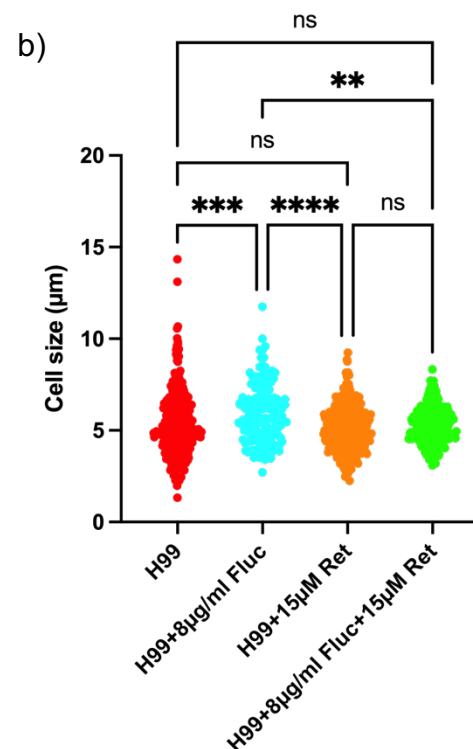
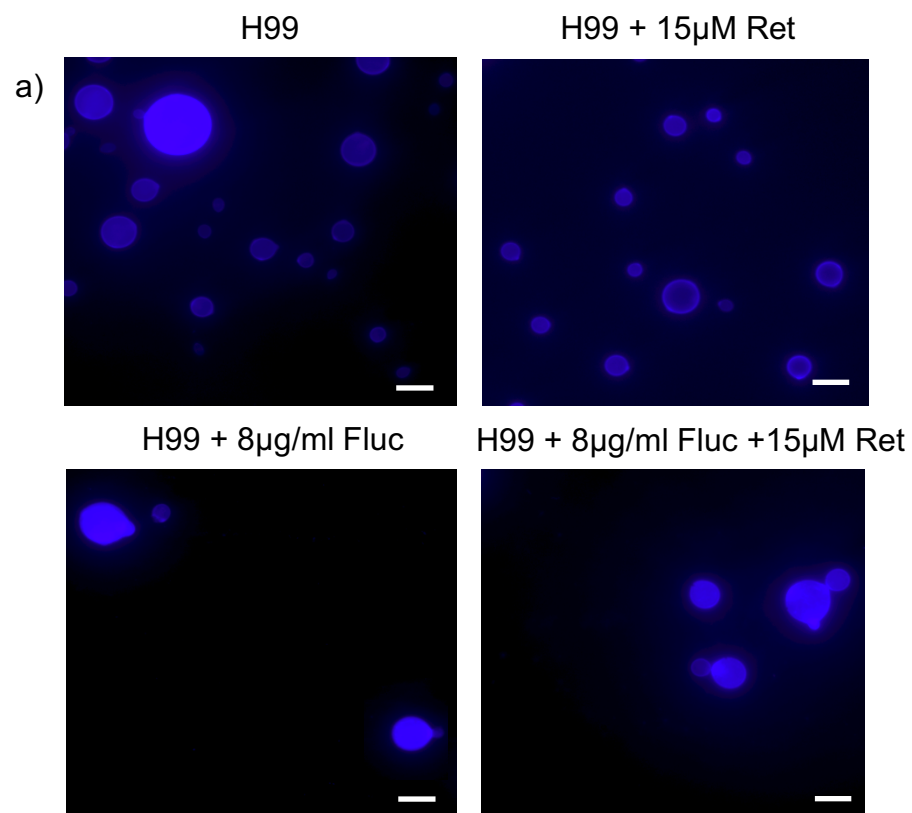


Figure 29. Fluconazole causes an increase in DNA content and superoxide levels in cells treated with retinoic acid following titan induction. H99 was grown in YNB overnight, followed by induction of Titan cells in 10% HI-FCS with PBS with and without metyrapone and fluconazole for 2 days. a) microscopy images of H99 alone, H99 with 15 μ M met, H99 with 8 μ g/ml fluconazole, and H99 with 8 μ g/ml fluconazole and 15 μ M met. Representative of 5 technical repeats. Cells were stained for chitin with CFW, with scale bar of 10 μ m. b) Cell size measurements from a). c) Cells were stained with DAPI and measured by flow cytometry. The median fluorescence intensity of DAPI is shown on the histogram and data is representative of 3 biological repeats. d) Cells were stained with superoxide dye and measured by flow cytometry. The median fluorescence intensity of superoxide dye is shown on the histogram. e) the second population from these histograms were gated and the percentage of cells was plotted. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, **= $p < 0.005$, ***= $p < 0.0005$, ****= $p < 0.0001$ (Kruskall-Wallis with Dunn's multiple comparisons test).

Following titan induction, the combination of fluconazole and metyrapone does not deter metyrapone's ability to block titanisation (figure 30a), similar to that of retinoic acid. There was also a significant size difference between H99 with 8 μ g/ml fluconazole and H99 with both 8 μ g/ml fluconazole and 15 μ M metyrapone again indicating that metyrapone also blocks titanisation in fluconazole treated cells (figure 30b). Previously I showed that metyrapone causes a dose dependent increase in DNA content of H99 cells (seen in figure 30c also), but when fluconazole was added a further increase was seen (figure 30c). What was also interesting here was that fluconazole with metyrapone had a higher DNA content than fluconazole treated H99 alone (figure 30c). Similarly, compared to H99 alone, metyrapone treated cells have lower levels of superoxide but in combination with fluconazole there was an upturn in levels of superoxide. The superoxide level in the combined drugs treated cells was similar to that of only fluconazole treated H99 (figure 30d). Similar to figure 29e, fluconazole treated cells had 2 populations of cells, including when treated with metyrapone. When I looked into this further I found a high percentage of this 2nd population had much higher superoxide levels when treated with fluconazole than those without (figure 30e).

Like retinoic acid, the data here shows fluconazole caused an increase in DNA content and superoxide levels even though titanisation is still blocked.

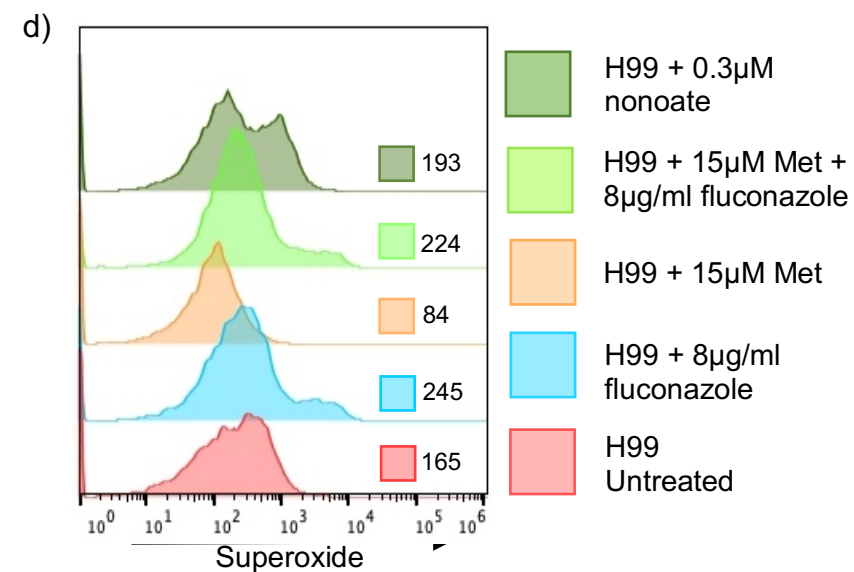
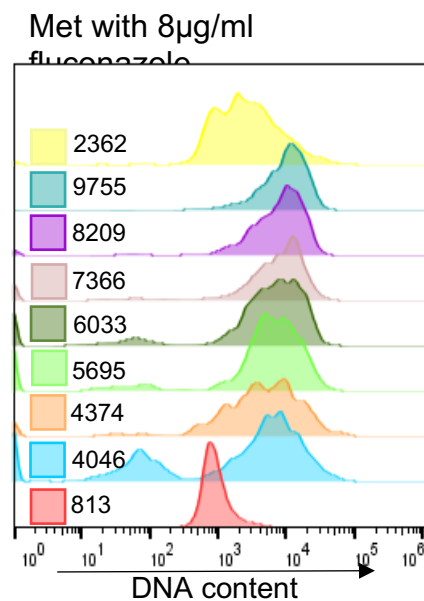
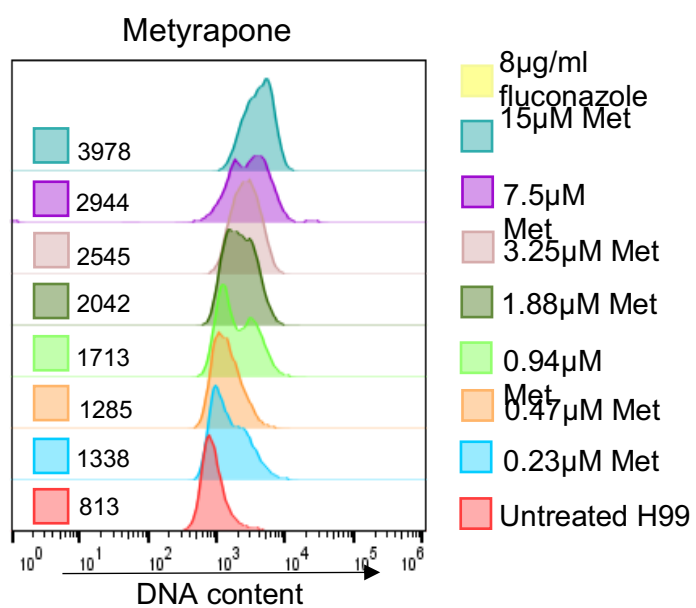
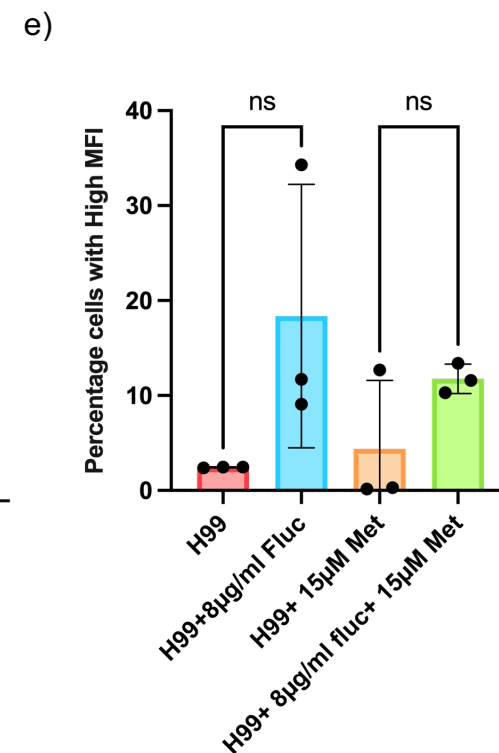
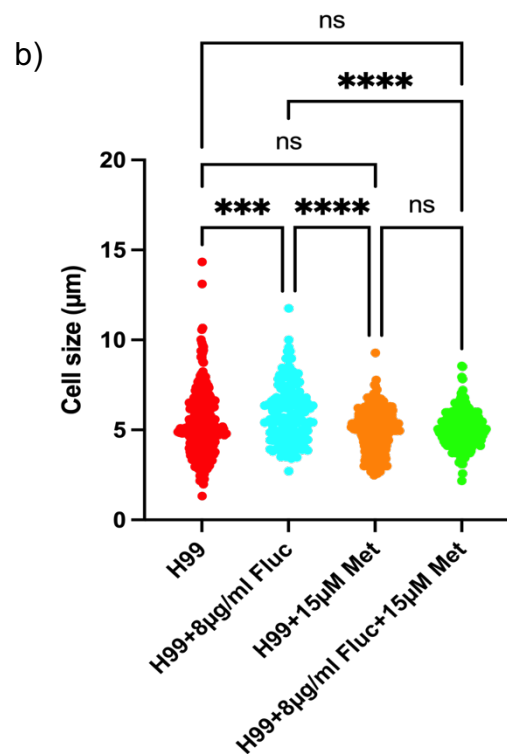
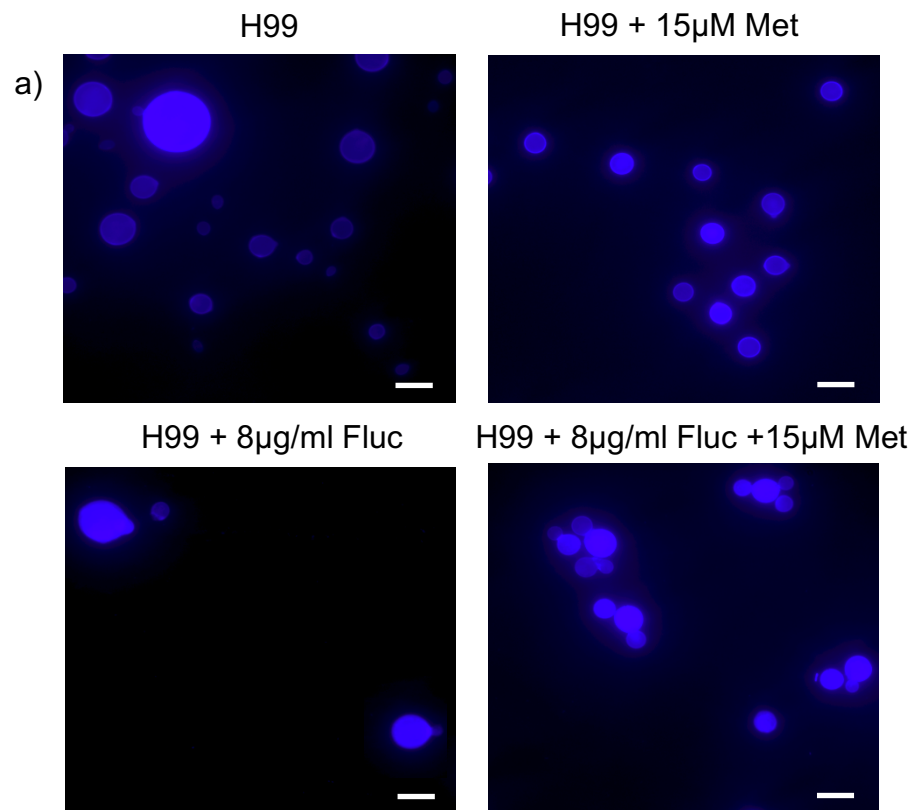


Figure 30. Fluconazole causes an increase in DNA content and superoxide levels in cells treated with metyrapone following titan induction. H99 was grown in YNB overnight, followed by induction of Titan cells in 10% HI-FCS with PBS with and without metyrapone and fluconazole for 2 days. a) microscopy images of H99 alone, H99 with 15 μ M met, H99 with 8 μ g/ml fluconazole, and H99 with 8 μ g/ml fluconazole and 15 μ M met. Representative of 5 technical repeats. Cells were stained for chitin with CFW, with scale bar of 10 μ m. b) Cell size measurements from a). c) Cells were stained with DAPI and measured by flow cytometry. The median fluorescence intensity of DAPI is shown on the histogram and data is representative of 3 biological repeats. d) Cells were stained with superoxide dye and measured by flow cytometry. The median fluorescence intensity of superoxide dye is shown on the histogram. e) the second population from these histograms were gated and the percentage of cells was plotted. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, **= $p < 0.005$, ***= $p < 0.0005$, ****= $p < 0.0001$ (Kruskal-Wallis with Dunn's multiple comparisons test).

The final TI, nicardipine hydrochloride, was also tested with fluconazole under titan inducing conditions to see if there were any changes in DNA content or superoxide levels. Like the other two TI drugs, nicardipine hydrochloride was still able to prevent titan cell formation even in the presence of fluconazole (figure 31a). Although nicardipine hydrochloride does block formation of titan cell, there were some that were quite close to be 10 μ m in diameter, but when cells were grown with both fluconazole and nicardipine hydrochloride, majority of the cells were significantly smaller (figure 31b). Nicardipine hydrochloride has been shown to cause an increase in DNA content that is dose dependent, but in the presence of fluconazole there is a dramatic rise in DNA content (figure 31c). One of the differences of nicardipine hydrochloride seen here compared to the other TI drugs is that it actually has slightly higher levels of superoxide than that of H99 alone. This increases further when combined with fluconazole, although the levels are similar to that of H99 with fluconazole alone (figure 31d). Like the other two TI drugs, the 2 population of cells was also seen here, particularly when fluconazole was present. Parallel to figure 29e and 30e, fluconazole treated cells had the higher percentage of the 2nd population with higher levels of superoxide (figure 31e). This adds to the evidence that fluconazole has an effect on the superoxide level.

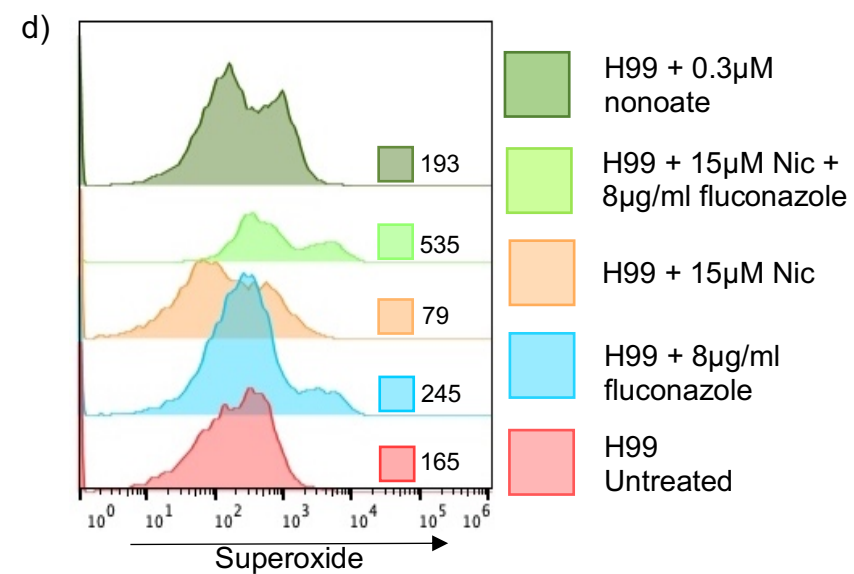
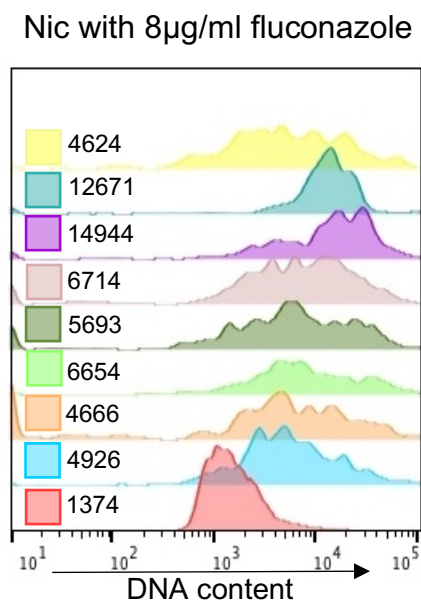
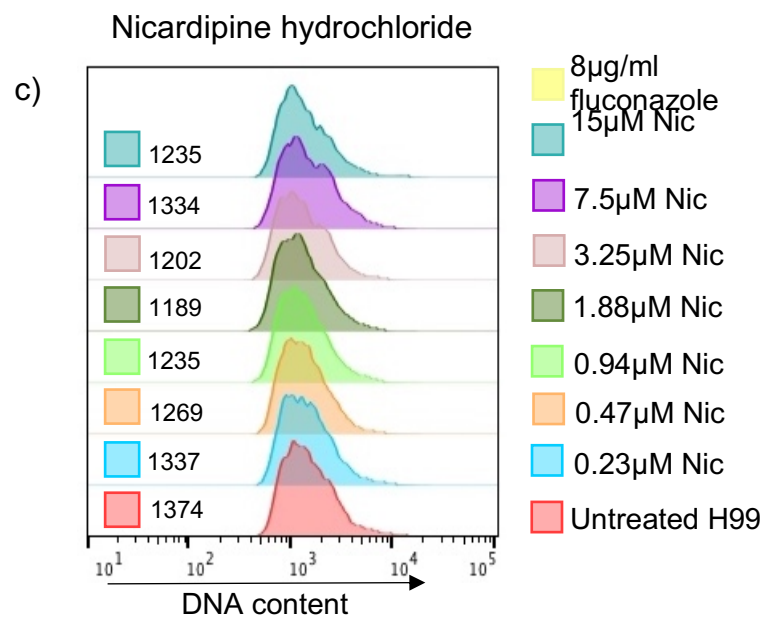
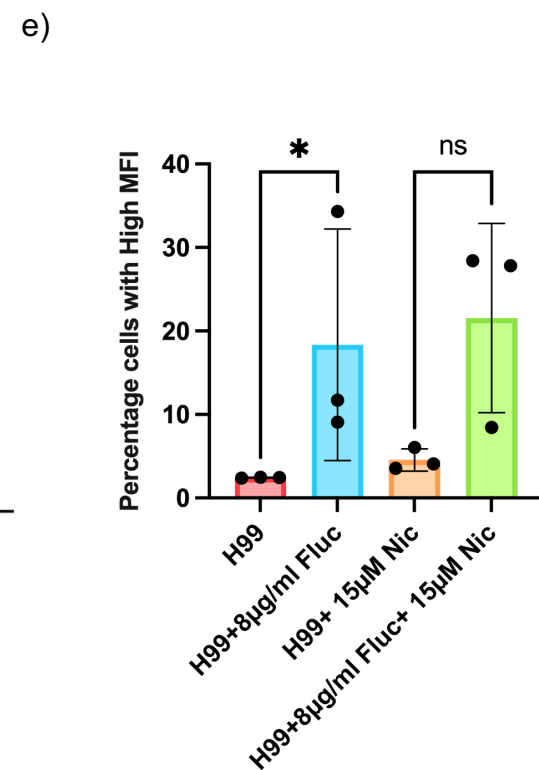
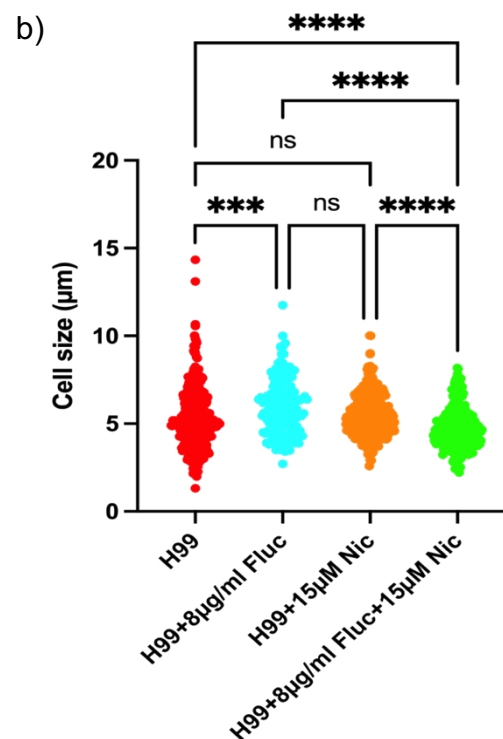
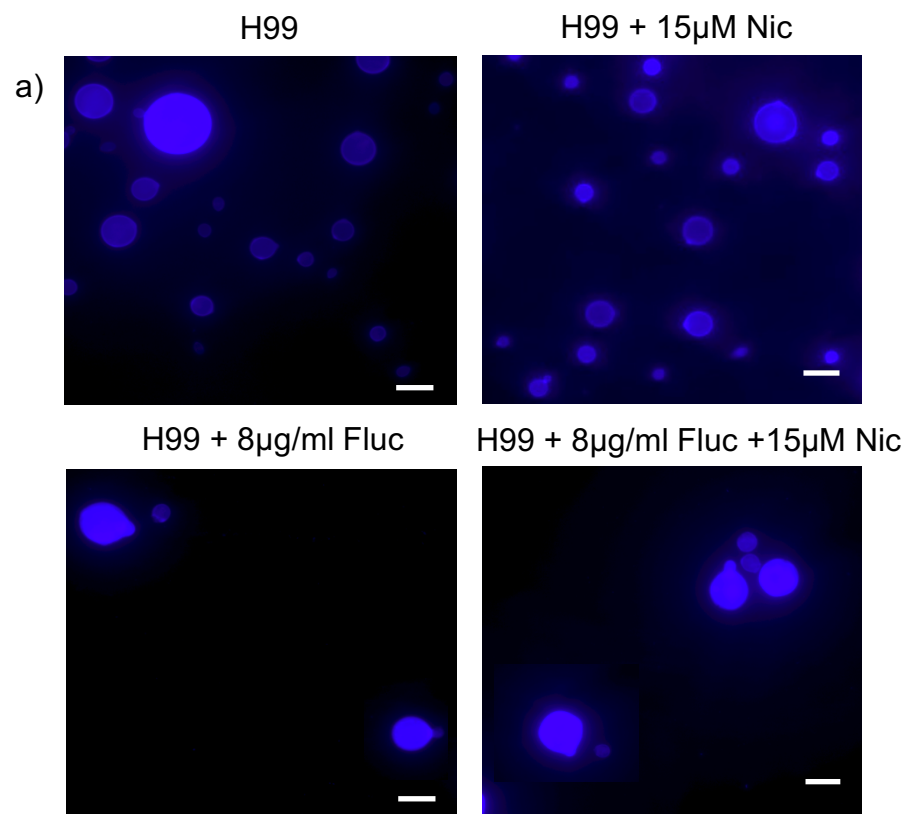


Figure 31. Fluconazole causes an increase in DNA content and superoxide levels in cells treated with nicardipine hydrochloride following titan induction. H99 was grown in YNB overnight, followed by induction of Titan cells in 10% HI-FCS with PBS with and without nicardipine hydrochloride and fluconazole for 2 days. a) microscopy images of H99 alone, H99 with 15 μ M nic, H99 with 8 μ g/ml fluconazole, and H99 with 8 μ g/ml fluconazole and 15 μ M nic. Representative of 5 technical repeats. Cells were stained for chitin with CFW, with scale bar of 10 μ m. b) Cell size measurements from a). c) Cells were stained with DAPI and measured by flow cytometry. The median fluorescence intensity of DAPI is shown on the histogram and data is representative of 3 biological repeats. d) Cells were stained with superoxide dye and measured by flow cytometry. The median fluorescence intensity of superoxide dye is shown on the histogram and data is representative of 3 biological repeats. e) the second population from these histograms were gated and the percentage of cells was plotted. Data is representative of 3 biological repeats. Data was the mean $\pm$ SD, **= $p < 0.005$, ***= $p < 0.0005$, ****= $p < 0.0001$ (Kruskall-Wallis with Dunn's multiple comparisons test).

Overall, the TI drugs were antagonistic with fluconazole in yeast and titan inducing conditions, even though titan cell formation was blocked. Together these findings are consistent with a model in which fluconazole can increase DNA content and therefore cause drug resistance independent of the yeast-to-titan transition.

3.3 Discussion

In this chapter, I investigated the link between titan cell formation and drug resistance. I examined 10 clinical isolates that had been exposed to fluconazole *in vitro* and paired parent heteroresistant colonies collected (denoted parent and Fluc^R). I determined the capacity of these pairs to form titan cells. Stone et al. previously demonstrated that some post-treatment Fluc^R clinical isolates were aneuploid compared to their parent isolate, suggesting that fluconazole exposure can cause aneuploidy, however the role of titan cell formation in this process was not investigated. To address whether titan cell formation is a cause of this aneuploidy and gain a better understanding of the molecular mechanisms connecting titan cell formation, aneuploidy, and drug resistance, I performed a series of experiments. I demonstrated that clinical isolates are capable to form titan cells but this capacity can be

influenced by exposure to fluconazole either positively or negatively. In addition, fluconazole resistance is transient even in strains that have the ability to form titan cells following fluconazole resistance. I found titan cells are not intrinsically drug resistant, and that fluconazole itself does not inhibit the yeast-to-titan transition, however drug resistance is associated with increased DNA content. But DNA content increase is not dependent on cell size increase, but it could be influenced by the mitochondria and an increase in superoxide levels. Titan inhibitor drugs successfully reduce titanisation but they increase DNA content and are not synergistic with fluconazole.

3.3.1 Does fluconazole treatment affect the ability of a cell to form titans?

When testing whether fluconazole heteroresistance alters the capacity of clinical isolates to form titan cells when induced *in vitro*, I compared parent and Fluc^R isolates and observed differences in the ability for these isolates to form Titan cells. Overall, I observed three different patterns of change, which can be organized into three groups, typified by isolate pairs TZ1/TZ1R, TZ17/TZ17R, and TZ8/TZ8R.

In figure 10a, the microscopy data shows that there were titan cells present in the Fluc^R isolate, as well as the parent isolate. However, when analysing the DNA content, there was a slight increase in DNA content of the cells, but this was not the stepwise increase that is usually seen in Titan cells (figure 10b). When measuring the size of the cells through flow cytometry, there was a slight difference in cell size between the paired isolates (figure 10c). This first patterns of change show that both parent and Fluc^R paired isolates are capable of forming titan cells, with the Fluc^R having more in their population.

As well as Fluc^R having altered DNA content, there were certain clinical isolate pairs in which the parent isolate had altered DNA content and cell size (figure 12). The microscopy data showed titan cells seen in both the parent and Fluc^R isolate (figure 12a). This was further confirmed after analysing the DNA content, which showed the parent isolate having a dramatic increase in DNA content compared to the Fluc^R isolate (figure 8c). Many of the clinical isolate pairs, both parent and Fluc^R, were unable to form Titan cells and stayed as yeast with no difference in DNA content (figure 12).

This indicates that fluconazole treated clinical isolates can have altered DNA content compared to their respective parent isolate, and that the capacity of clinical isolates to form titan cells can be gained or lost following drug exposure. Together, these findings imply that fluconazole alone is not the main stress to induce titan cell formation: titan cells can form in the absence of fluconazole treatment, and strains that haven't been exposed to fluconazole can form titan cells at similar rates to those that have previously been exposed.

3.3.2 Does the yeast-to-titan transition influence transient drug resistance?

Throughout the literature, there have been two main mechanisms of drug resistance discussed; heritable and transient. For *Cryptococcus neoformans* there are two mechanisms, as certain studies have shown heritable resistance such as point mutations (Boyce et al., 2017, Chow et al., 2012, Magditch et al., 2012), while others have shown transient resistance through formation of multimeras or chromosome missegregation (Altamirano et al., 2017, Chang et al., 2018). However, throughout these studies, there have not been any studies correlating titanisation with drug resistance. I hypothesised that titanisation is a mechanism of drug resistance in *C. neoformans*.

There is no clear clinical *in vitro* MIC breakpoint in the literature for *Cryptococcus*, but the majority seem to agree that over 8mg/l indicates fluconazole resistance, particularly in relapse patients (Stone et al., 2019, Bongomin et al., 2018, Pfaller et al., 2011). Consistent with transient resistance, when the clinical isolates were tested for fluconazole MIC during yeast phase growth, none were fluconazole resistant, even the "Fluc^R" isolates previously isolated as hetero-resistant colonies. Instead, all isolates exhibited an MIC of 8 µg/ml (table 6). This loss of resistance is characteristic of genome plasticity, which is the aptitude of an organism to tolerate changes in genome size and overall organisation. Sionov et al showed that heteroresistant colonies that are aneuploid rapidly lose that aneuploidy when fluconazole stress is removed, and Gerstein et al showed similar plasticity (Sionov et al., 2010). Therefore, the gene amplification required for drug resistance may have been lost in these clinical isolates during their passage following recovery from frozen stocks. However, how these changes occur and how they are tolerated remains unclear. I tested the hypothesis that titan cell formation may be a mechanism for the generation of the genomic plasticity underlying hetero-resistance and aneuploidy leading to drug resistance.

To see if the switch to titan-phase growth contributes to drug resistance we used the clinical isolates that were known to form titan cells, and tested them for fluconazole resistance during yeast and titan phase growth (table 8). During yeast phase growth, the clinical isolates did not show any increased resistance to fluconazole, with a MIC of 8 µg/ml. After inducing titan cells, the MIC of one of the clinical isolates decreased from 8 µg/ml to 4 µg/ml (table 8), indicative of a higher sensitivity to the drug. This may be explained by the heterogenous population present following titan induction: small titan daughter cells that

comprise a large proportion of the population have thin cell walls compared to yeast and titan cells (Dambuza et al., 2018), which may make them more sensitive to fluconazole, and decrease the overall MIC of the culture. Consistent with titan daughter cells causing decreased overall resistance to fluconazole, filtered titan daughter cells were unable to proliferate in concentrations of fluconazole 8 µg/ml and above (figure 16).

Interestingly there was a slight increase in growth of these titan daughter cells filtered cultures in 8 µg/ml and 16 µg/ml fluconazole after 35 hours. This may be due to the fact that fluconazole half-life is 31.6 ± 4.9 hr (Debruyne and Ryckelynck, 1993), and therefore at this point the drug concentration and activity has halved. An alternative explanation may be found in a study carried out by Gerstein et al showing that titan cells treated with fluconazole produced daughter cells resistant to the anti-fungal (Gerstein et al., 2015). Although this was not seen in figure 16 where the daughter cells/titanides were unable to grow in these high concentrations of fluconazole, this could be due to the fact that the experimental design approached by them was different. At the point of inducing H99 to form titan cells, the cells were not treated with fluconazole, they were only treated after separating them by size whilst Gerstein et al treated the cells with fluconazole before separating them by size (Gerstein et al., 2015). After obtaining the titan cells, the cells were grown in supraMIC concentration of fluconazole only as the yeast cells and titan daughter cells could only grow at 4 µg/ml and below.

The large cells were not able to proliferate in supraMIC concentrations of fluconazole (figure 15). This was surprising as titan cells are known to have a thick cell wall and a dense capsule, and these characteristics have shown an increase in resistance to fluconazole in yeast (Vitale et al., 2012, Bouklas et al., 2013). On the other hand, fluconazole resistance

has been seen more so in acapsular mutants compared to encapsulated wild type (Zaragoza et al., 2008). A possible reason for this could be that fluconazole is known to be hydrophilic, which aids in the cell uptake due to polysaccharide capsule also being hydrophilic (Casadevall et al., 2019). Thus, this could be why the titan cells did not proliferate in high levels of fluconazole.

Overall, these data suggest that the yeast to titan switch alone does not influence drug resistance: when cells were induced to form titan cells prior to drug exposure and then incubated in fluconazole, I saw a fungistatic impact on titan cells at MIC and an increased sensitivity for small cells. However, the increased MIC over time raised the possibility that growth of titan-induced cells in fluconazole may lead to increased resistance.

3.3.3 Does fluconazole influence DNA content during titan-phase growth?

In the literature, the role of aneuploidy in fluconazole resistance has been discussed in detail, particularly how fluconazole influences the formation of aneuploidy of specific chromosomes in *C. neoformans* (Sionov et al., 2010, Ngamskulrungroj et al., 2012, Stone et al., 2019). Given the capacity of titan cells to be polyploid, I wanted to see if fluconazole directly influenced changes in DNA content following titan induction of H99. Consistent with this, I observed that DNA content increased in a dose dependent manner when titan cultures were induced in the presence of fluconazole (figure 18b), while there was no detectable change in the DNA content when the cells were grown in yeast conditions in the presence of fluconazole (figure 18a). It is important to note that there were higher levels of DAPI fluorescence when the untreated H99 cells were grown as yeasts than titans. The reason for this was that the cells were grown in YNB + 2% glucose which is a minimal media that

can cause stress to the cells, leading to this high fluorescence (figure 18a). The data here suggests that the fluconazole treatment does alter DNA content when *C. neoformans* are grown under titan inducing conditions, which more closely mimic the host environment. Together, this suggests that while Titan cell growth itself does not necessarily lead to drug resistance, fluconazole does impact genome plasticity and polyploidy, which in turn contribute to increased resistance.

3.3.4 What are the molecular mechanisms by which fluconazole influences DNA content during titan-phase growth?

As this increase in DNA content was seen only in titan inducing conditions, we asked whether the cAMP pathway, a central regulatory pathway required for titanisation, is required for fluconazole-induced DNA content changes. The *cac1Δ* mutant lacks adenylyl cyclase activity, which blocks formation of titan cells. The addition of 2 µg/ml fluconazole did not restore the large cell phenotype (figure 19a), confirming the mutants' role in inhibiting titanisation, however fluconazole treatment still increased the DNA content of *cac1Δ* (figure 19b), albeit to a lesser extent in comparison to the wild type (figure 16b). Therefore, it appears that fluconazole increases DNA content independent of the cAMP pathway. Moreover, this data suggests that the mechanisms that regulate DNA content changes during titanisation may be separable from cell size changes to some degree.

The role of the cAMP signalling pathway in azole resistance is an area of ongoing study but there is evidence that suggests that the cAMP-PKA pathway is involved. In *C. albicans* the removal of *CDC35*, which encodes adenylyl cyclase, caused sensitivity to azole (Jain et al., 2003). However, here there was no evidence of *C. neoformans* *cac1Δ* being more sensitive

to fluconazole. The data also suggests that there may be another signalling pathway that is causing this increase in DNA content alongside the cAMP pathway. Changes to the chromosome in *C. neoformans* have been linked to high levels of ROS, and ROS has been shown to cause DNA damage (Limoli and Giedzinski, 2003, Degtyareva et al., 2008). Other work in our lab has shown that this is associated with increased ploidy (Zhou, unpublished work). Treatment with fluconazole has been identified to cause high levels of ROS which could in turn lead to chromosomal instability (Peng et al., 2018).

3.3.5 Does endogenous ROS influence DNA content?

It is therefore possible that the pathway producing ROS could be causing this DNA content increase when fluconazole is present. I set out to test if fluconazole does cause an increase in ROS levels in *C. neoformans* and whether this increase in ROS is coming from the mitochondria. Following titan induction, H99 treated with fluconazole did cause an increase in ROS levels in a dose dependent manner (figure 20a). As mentioned earlier, increase in ROS levels due to fluconazole have been seen in *Candida* sp such as *C. glabrata*. In *C. glabrata*, the increase level in ROS does not differ in resistant and susceptible strains, neither does the response to the oxidative stress. Interestingly, oxidative damage occurs only in *C. glabrata* DNA during fluconazole treatment (Mahl et al., 2015). Parallel to this, transcription changes in *C. neoformans* genes associated with chromosome segregation and DNA repair has been seen during fluconazole treatment along with DNA damage in the presence of redox-active metals complex with fluconazole due to increase in ROS levels (Peng et al., 2018). It is therefore possible that this increase in DNA content also be due to DNA damage caused by fluconazole induced increase in ROS. However future studies would be need to further confirm this.

3.3.6 How does the mitochondrion influence titan cell formation?

Once it was confirmed that fluconazole does induce an upturn in ROS levels in *C. neoformans* after titan inducing conditions, we wanted to find out the origin of this ROS. Endogenous ROS is mainly produced in the mitochondria in fungi (Kobayashi et al., 2002, Lamarre et al., 2001). One way to confirm whether the increase in ROS here was due to an increase mitochondrial activity was to look at the functionality of the mitochondria in the presence of fluconazole. To do this we used the fluorescent probe rhodamine 123. The build-up of this probe within mitochondria indicated the mitochondrial potential which is a correlate of mitochondrial activity. When H99 was grown with fluconazole during titan inducing conditions, there was an increase in mitochondrial potential, and more so when 8 µg/ml fluconazole was added (figure 20b). This suggests that there was an increase in mitochondrial activity during fluconazole treatment and this dose dependent increase in activity is associated with increased ROS levels (figure 20a).

Across eukaryotes mitochondria are vital organelles required for certain pathways such as production of ATP, β - oxidation and the detoxification of ROS that accumulates during these processes (Chang and Doering, 2018). Removing mitochondrial DNA is a key tool when studying mitochondrial function, as has been done in *S. cerevisiae* (Lasserre et al., 2015). However, although it has been studied considerably in *S. cerevisiae* as a model, the mitochondria in human fungal pathogens is still a relatively understudied organelle. The reason for this is that most fungi, such as *C. albicans* and *C. neoformans*, are petite negative, which means that without functional mitochondria they are not able to survive (Shingu-Vazquez and Traven, 2011).

In eukaryotes, mitochondrial respiration is essential for producing ATP through the electron transport chain as a source of energy. The classical pathway in animal and human respiration is achieved via as a relay between 5 protein complexes (CI, CII, CIII, CIV, CV, Figure 32) across the inner mitochondrial membrane, where electron transfer occurs. However, in fungi, in addition to the classical pathway, there are other pathways for electron transfer (figure 32). Alongside the transferring of electron from NADH to complex CI, there is also a complex called NAD(P)H oxidase that does not add protons to the mitochondrial intermembrane space, but it is rotenone insensitive which allows the fungi to have another alternative pathway (Kerscher, 2000). This pathway can compensate for loss of Complex CI activity (rotenone insensitive). There is also the alternative oxidase (AOX) pathway which accepts electrons from the coenzyme ubiquinone (CoQ) and through this pathway produces $\frac{1}{2} O_2$ (Vanlerberghe, 2013). In this way, AOX can compensate for loss of CIII and CIV superoxide detoxification activity (Kerscher, 2000, Ruy et al., 2006, Akhter et al., 2003, Magnani et al., 2008). Lastly, the final alternate pathway that fungi use is the parallel pathway which involves the cytochrome c proteins (Cytbc) (Alonso-Monge et al., 2009, Milani et al., 2001) (figure 32).

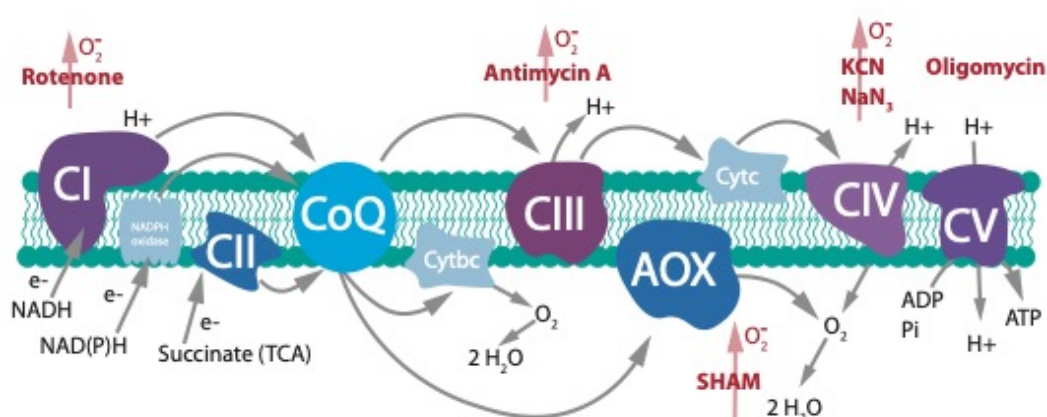


Figure 32. The elements of the electron transport chain in the mitochondria, and the impact of chemicals that inhibit their activity. The mitochondrial membrane is shown in green, and Complex (C) I, II, III, IV, and V are represented throughout the matrix. The flow of electrons is presented in grey, while the action of six different mitochondrial inhibitors is paired with its predicted target and the expected impact on reactive oxygen (red). AOX is the Alternative Oxidase, a fungal specific enzyme that compensates for inactivation of Complex III. This image was adapted from Trevijano-Contador et al., 2017.

To further investigate the source of ROS produced during fluconazole treatment, we used mitochondrial inhibitors that target different parts of the electron transport chain. There were 5 inhibitors used: antimycin A (AA), potassium cyanide (KCN), salicylhydroxamic acid (SHAM), rotenone (R), and sodium azide (SA). R inhibits complex I, AA inhibits complex III, KCN and SA both inhibit complex IV, and SHAM inhibits the alternative cytochrome oxidative pathway (AOX) (Figure 32). Inhibiting each complex in turn during titan induction and during exposure to fluconazole allowed us to determine what part of the electron transport chain fluconazole interacts with and whether this impacts DNA content and ROS levels.

To date, there are no studies on how these mitochondrial inhibitors impact titan induction in wild type *C. neoformans* (H99). Here, I found that treating H99 with different mitochondrial inhibitors differentially impacted DNA content and ROS. Inhibition of either complex I (via R) or complex III (via AA) had the most substantial impact on H99 DNA content along with cell size (figure 22a and e). Similarly inhibiting complex I with R also caused a noticeable increase in superoxide, as did the inhibition of complex IV through SA (figure 24a and d). Interestingly, when looking at the relationship between superoxide and cell size, there was not a clear correlation like the one seen with DNA content and cell size. Here I saw cells that were relatively the same size with differing levels of superoxide (figure 24ii). This could be due to the fact that high levels of superoxide may have caused harm to the cell and its growth cycle, therefore it no longer could increase in size. Interestingly though, some of our

previous data has suggested that increase in superoxide levels plays a role in titan cell formation (Zhou, unpublished), though this was not seen in this data set.

Intriguingly, the size of the cells as measured by flow cytometry increased when they were treated with any of the mitochondrial inhibitors (figure 22ii). There is evidence to suggest that mitochondrial activity plays a role in the yeast-titan transition: For example, one *in vitro* induction protocol uses sodium azide (SA), suggesting that inhibition of mitochondrial function does produce a large population of titan cells (Trevijano-Contador et al., 2018). Similarly, we also see large cells being produced in the presence of SA, further confirming this phenomenon. However, further experiments are required to understand the role of mitochondria in titan cell induction, including more detailed microscopic characterization of key cell changes. But what is important to note is that this further confirms that there is more than one mechanism of induction of titan cells other than just the cAMP pathway shown in the literature.

3.3.7 How does the mitochondrion influence fluconazole-induced increases in DNA content?

Following this, when looking at what effect these mitochondrial inhibitors have on fluconazole, there were differing results. One of the key results seen was the effect of the mitochondrial inhibitors on cells treated with MIC levels of fluconazole (8 µg/ml). Inhibition of each mitochondrial complex caused a decrease in the DNA content of the cell treated with 8 µg/ml fluconazole, compared to 8 µg/ml fluconazole alone. This was seen more so when complex I was inhibited by R (figure 23aiii), complex III was inhibited by AA (figure 23eii), and complex IV was inhibited by SA (figure 23dii). But the most striking result was seen when the alternative oxidase pathway was inhibited by SHAM (figure 23cii). What is

important to note here is that the DNA content of the perturbation of the mitochondria along with 8 µg/ml fluconazole was still higher than that of untreated cells. This suggests that although the mitochondria does seem to play a role in the increase in DNA content seen when cells are grown in titan inducing condition in the presence of fluconazole, it alone does not affect the DNA content completely. As we had shown earlier that cAMP also seems to play a role in the increase in DNA content, but there was also still a slight increase in DNA content. It is possible that the cAMP pathway and mitochondria are working together in causing this increase in DNA content.

Alongside this, when looking at the effect of superoxide levels of these cells treated with fluconazole and the mitochondrial inhibitors, there were differing results here too. We had hypothesised that the increase in DNA content in fluconazole treated cells is due to the high level of superoxide level produced by the mitochondria. And keeping in mind the perturbation of certain complexes that did cause an effect on the DNA content, we hypothesised that this would be parallel with superoxide levels too. However, here we saw that although inhibition of R caused a decrease in DNA content in 8 µg/ml fluconazole treated cells, there was an increase in DNA content (figure 25a). But when the alternative oxidase pathway was inhibited via SHAM there was a decrease in superoxide levels (figure 25c). This suggests that the alternative pathway may play a partial role to the increase in superoxide levels seen when H99 is grown with 8 µg/ml fluconazole alone.

There is limited information in the literature about the effects of mitochondrial inhibitors and fluconazole on *C. neoformans* in terms of superoxide levels and DNA content. However, one study looked at whether fluconazole and the Complex I inhibitor R were synergistic with

one another in *C. neoformans* in order to find a new way to treat systemic infections. Here they found that R was antagonistic with fluconazole, meaning fluconazole had reduced efficacy as a drug when combined with R (Wambaugh et al., 2020). This coincides with our data as R with fluconazole increases in DNA content and superoxide levels which could make it more lethal for the cells. On the other hand, another study observed increased *C. albicans* sensitivity to fluconazole in the presence of mitochondrial inhibitors. They found the mitochondria electron transport chain complex III inhibitor AA and the complex I inhibitor R were able to increase fluconazole susceptibility (Sun et al., 2013). This was not seen in our study with *C. neoformans* as all inhibitors seem to be either antagonistic or did not interact with fluconazole. Our data shows that perturbations of the mitochondria cause an increase in cell size and in superoxide, with and without fluconazole depending on the complex inhibited.

Therefore, these data together suggest that the where fluconazole effects take place is the alternative cytochrome oxidative pathway, and that perturbing either Complex I or Complex III can influence fluconazole sensitivity. Moreover, Complex I and complex III is a major regulator of DNA content and cell size, and this is further impacted by fluconazole exposure.

3.3.8 Does blocking titanisation reduce fluconazole resistance?

Overall, these data suggest that while the presence of titan cells *per se* does not lead to fluconazole resistance, and the capacity to form titan cells is not required for fluconazole resistance, there is a correlation between the mitochondrial signals that trigger both titan cell formation and fluconazole-mediated DNA increase. I therefore asked whether drugs that block the yeast-to-titan transition might likewise reduce fluconazole resistance.

The molecular mechanisms that drive titanisation are still being studied, especially now that there are methods that allow this process to be reproduced *in vitro*. With the original hypothesis in mind, I looked into whether some of the molecular mechanisms that drive titanisation exert some effect in fluconazole resistance, and if titan cells do play a role in the high prevalence of drug resistance observed in *C. neoformans*. There are 3 drugs, which are not antifungals, that have been identified in our lab as being able to inhibit the yeast to titan transition: retinoic acid, metyrapone and nicardipine hydrochloride.

Retinoic acid is an agent that derives from vitamin A (retinol) and is necessary for differentiation, organogenesis and cell growth in humans as well as a well-known anti-oxidant (Kam et al., 2012, Ahlemeyer et al., 2001). As this drug is not an antifungal, we first looked into what effect it has on *C. neoformans* following growth in titan inducing conditions. It is clear that retinoic acid was able to successfully reduce this morphological transition, where the cells that were grown under this drug were smaller than 10 µm in diameter (figure 26a and b). Interestingly, when measuring the DNA content of these cells there was a dose-dependent increase even though the cells did not increase in size (figure 27ai), and superoxide levels were lower than that of H99 alone (figure 27aii and iii). This confirms that retinoic acid does inhibit titanisation, but seems to increase DNA content. Vitamin A has been known to have antioxidant properties, but retinoic acid has less antioxidant properties compared to the other retinoids (Palace et al., 1999, Estornell et al., 2000). Previous study in our lab has shown how increase in ROS can cause an increase in DNA damage leading to this increase in DNA content and titan cell production (Zhou et al., unpublished).

Therefore, it is possible that retinoic acid is inhibiting titanisation by scavenging ROS.

It was previously shown in yeast-phase cultures that the combination treatment of fluconazole with ascorbic acid, another well-known antioxidant, restores growth after fluconazole induced inhibition (Peng et al., 2018). Consistent with retinoic acid scavenging ROS, retinoic acid was antagonistic with fluconazole (figure 28a). This was observed both when cells were grown in yeast culture, with fluconazole MIC increasing from 4 µg/ml to 16 µg/ml (figure 28a). I could not do this in titan inducing conditions as there were some difficulties in measuring absorbance on the plate reader when the culture contained titan cells. Interestingly, while the superoxide levels of cells treated with retinoic acid was reduced relative to untreated cells, retinoic acid did not block ROS accumulation when cells were also treated with fluconazole (Figure 29d). Instead, ROS levels for combination treated cells were similar to that of cells treated with fluconazole alone (figure 29d). A previous study showed that fluconazole elevates ROS levels, thereby inhibiting *C. neoformans* growth, and this could be recovered by antioxidants such as retinoic acid (Dbouk et al., 2019). Overall this suggests that retinoic acid reduces titanisation by reducing superoxide levels however it does not affect superoxide levels of fluconazole treated cells.

When the cells were grown in titan inducing conditions, the DNA content of the cells increased slightly as the concentration of retinoic acid increased (figure 29c), and when used in combination with fluconazole there was a further increase, although not as high as in fluconazole treated cells alone (figure 29c teal vs yellow). Overall, it does seem likely that in our study retinoic acid may be rescuing the growth inhibition of fluconazole by reducing fluconazole-associated ROS, therefore increasing the MIC, however retinoic acid alone is not sufficient to block DNA content increases.

Metyrapone is a drug used for treatment of Cushing's disease hypercortisolism in humans by inhibiting the 11 β -hydroxylase (Sonino et al., 1981), however, its mode of action in fungi is not known. Parallel to retinoic acid, metyrapone also successfully reduced titanisation as the cells were similar size to that of yeast cells (figure 22a and c). Also similar to retinoic acid, there was a dose-dependent increase in DNA content when metyrapone was present (figure 27bi) but a decrease in superoxide levels (figure 27bii). As both retinoic acid and metyrapone have successfully inhibited titanisation yet there is an increase in DNA content, it implies that the two may not necessarily go hand in hand. When discussing characteristics of titan cells, an increase in DNA content is one of them. In this case, it seems that all titan cells have a high DNA content, but not all cells that have a high DNA content are titan cells.

When H99 cells were grown in yeast culture, metyrapone acted antagonistically with fluconazole with the MIC increasing from 4 μ g/ml to 16 μ g/ml (figure 28b). When the cells, after titan induction, were treated with metyrapone alone and in combination with 8 μ g/ml fluconazole, there was a dose dependent increase in DNA content (figure 30c). Previously we showed that metyrapone decreases levels of superoxide in H99, however when in combination with fluconazole, there is an increase in superoxide level, matching that of fluconazole treated cells alone (figure 30d). What is important to note here is that although there was an increase in DNA content and superoxide levels in the presence of fluconazole, there was no increase in cell size (figure 30b). Metyrapone has been shown to inhibit cytochrome p450 activity in humans, specifically CYP11B1 which is located in the mitochondrial inner membrane (Shumyantseva et al., 2011, Cauet et al., 2001). It has been demonstrated that expression of genes in the ROS pathway decreased in the presence of metyrapone, especially those expressing subunits of mitochondrial complex 1 NADH

dehydrogenase. This complex is known to produce the most ROS in the mitochondria, signifying that metyrapone plays a role in reducing cell death due to ROS (Hellmich et al., 2013, Murphy, 2009). All the studies carried out on metyrapone have been performed on humans, not fungi. That said, it seems it inhibits cytochrome p450 enzyme which would reduce ROS but also increases DNA content. In addition, this increase in DNA content does not seem to be due to DNA damage as the drug is non-toxic. Interestingly, fluconazole also inhibits cytochrome p450, specifically Cyp51. the lanosterol 14 α -demethylase (Lepesheva and Waterman, 2011, Sagatova et al., 2015). It is therefore still unclear what is causing this increase in cells treated with metyrapone DNA content and increase in superoxide level in the presence of fluconazole. It is possible that metyrapone is acting similarly to retinoic acid as an anti-oxidant but does not decrease fluconazole superoxide levels.

The final TI drug investigated in this study was nicardipine hydrochloride, which is a dihydropyridine agent that blocks calcium channels and is a treatment for angina and hypertension (Sorkin and Clissold, 1987). This drug, like the others, is only known to be used and studied in humans, and has not been used on fungi to date. We found that nicardipine hydrochloride also reduces titan cell formation successfully, comparable to the other TI drugs, although there were some that were close to the 10 μ m diameter border (figure 26a and d). Similar with the other TI drugs, nicardipine hydrochloride also caused an increase in DNA content in a dose-dependent manner (figure 23ci) but it seems slight in comparison to the other TI drugs. Interestingly, nicardipine hydrochloride is the only TI drug that has the same level of superoxide as untreated H99 (figure 27cii and iii). The mode of action of nicardipine hydrochloride is not known in fungi however it blocks calcium channels in humans. Nevertheless, calcium channels such as the Cch1-Mid1 calcium (CMC) do play

important roles in the tolerance of external stresses such as oxidative stress. Regulation of calcium levels is key in fungi as inadequate control of calcium can lead to damage to the cell which then causes cell death (Ermak and Davies, 2002). A study found the CMC in *C. neoformans* has a role in lessening oxidative stress (Vu et al., 2015). It is possible that nicardipine hydrochloride is blocking key calcium channels, which in turn is causing an increase in superoxide and oxidative stress. However, this does not explain how nicardipine hydrochloride is able to inhibit titanisation.

Parallel to the other TI drugs, nicardipine hydrochloride also acts antagonistically with fluconazole (figure 28c). Alongside this there was a dramatic increase in DNA content when H99 was grown in titan inducing conditions with nicardipine hydrochloride in combination with 8 µg/ml fluconazole (figure 31c). Similarly, when fluconazole was present, nicardipine hydrochloride superoxide levels increased further although they did not surpass the levels of fluconazole treated cells alone (figure 31d). It is not known why nicardipine hydrochloride is antagonistic with fluconazole, however as mentioned previously calcium channels play a role in *C. neoformans* in tolerating stress and blocking them with nicardipine could cause an increase in oxidative stress. Fluconazole is also known to cause an increase in superoxide in cells and therefore this accumulation could lead to DNA damage which may be the cause for the dramatic increase in DNA content. Nevertheless, more experimental work will be required to further look into the roles of these TI drugs and their mode of action. Yet, the data here has further confirmed that titan cells do not play a role in antifungal resistance.

3.3.8 Future work and improvements

Whilst this work is relatively new, particularly the molecular mechanisms of titanisation, it is also building upon the literature so it is possible to compare methodology and results. However, there are many things that are relatively new to the world of *Cryptococcus neoformans* and therefore further work would be needed to be carried out in the future.

This study found that fluconazole can have a negative or positive impact on the ability for a clinical isolate to form titan cells. However, like most studies the methodology used can be slightly limiting. Throughout this thesis I used flow cytometry and microscopy as my main forms of obtaining data, but when looking at the effect fluconazole had on the capacity for clinical isolates to form titan cell there was a difference in the data between flow cytometry and microscopy. In the microscopy images it was obvious that there were more cells present that were large in size in the Fluc^R isolate. However, in the flow cytometry data it showed that the parent and Fluc^R have similar cell size. This may be due to the sample size, as more cells are counted through flow cytometry than that of microscopy. Therefore, future work would carry out more microscopy analysis, not just in this study but all, so as to generate more sample which may bridge the differences in the methods.

To date, the use of mitochondrial inhibitors to look into the role of the mitochondria in induction of titan cells has not been studied elsewhere. Here I found that complex I, complex III and the alternative cytochrome oxidase pathway plays a role in the induction of titan cells. However due to Covid-19 and time constraints most of the work was done through flow

cytometry. Future work would focus on looking at the effects of these inhibitors using microscopy so as to confirm the flow cytometry data. It would also be interesting to look at the effect these inhibitors have on the capsule after titan induction as a study carried out by Trevijano-Contador et al. found that the mitochondria plays a role in the formation of capsule in yeast conditions (Trevijano-Contador et al., 2017).

One of the main findings in this chapter is the titan inhibitor drugs. These drugs are not antifungals and are mainly used on humans so their mode of action in *C. neoformans* is not known. I found that these drugs are able to reduce titanisation however they would cause an increase in DNA content, whilst lowering the superoxide levels. However, we do not know why this is the case and what these drugs are targeting. It would be interesting to look into the molecular mechanisms of the drugs and how it is capable of blocking this yeast to titan transition. This could be done through a high through-put screen.

Future work will address these challenges, and place these findings in a more clinically relevant context by 1) investigating how genetic changes in the clinical isolate's pairs impact the capacity to form titan cells (chapter 4) and 2) taking a high-throughput approach to identifying genetic regulators of the yeast-to-titan switch (chapter 5).

Chapter 4. SNPs in Clinical Isolates Identify New Pathways that Play a Role in Induction of Titan Cell

This chapter includes work done by a summer student, Bryan Chang. He has been credited where his work is presented.

4.1 Introduction

Previously, Titan cells could only be seen *in vivo* with no suitable *in vitro* model available, making it difficult to understand what induces titan formation (Okagaki et al., 2010, Zaragoza and Nielsen, 2013). One of the key findings from *in vivo* studies was the regulation of titanisation by the core cyclic AMP (cAMP)/protein kinase A (PKA) pathway (Alspaugh et al., 2002, D'Souza et al., 2001). The data presented in chapter 3 suggest that multiple other pathways also regulate the yeast-to-titan transition. However, it was difficult to understand the molecular mechanisms that drive titanisation using *in vivo* models, and knowledge behind this is quite limited. But now there are 3 reproducible *in vitro* models that show similar characteristics of titan cells, allowing for better understanding of the mechanism (Dambuza et al., 2018, Trevijano-Contador et al., 2018, Hommel et al., 2018).

In chapter 3, I demonstrated using paired naive and heteroresistant clinical isolates that exposure to fluconazole can impart or remove the capacity to form titan cells. Sequencing of these isolates revealed numerous SNPs, for example there was a range of SNPs per day and per patient, from 1 to 842 (Stone et al., 2019). I screened through these clinical isolates for their ability to titanise before and after exposure to fluconazole. Across the paired clinical isolates there was wide but stable phenotypic variation where the FlucR member of the isolate pair had lost or gained the ability to form titan cells. This stable phenotype created

two related hypotheses: first, loss of titan cell formation would be evident in the single nucleotide polymorphisms, resulting from exposure to fluconazole, in genome sequences. Conversely, a gained capacity for an isolate to form titan cells would also be linked to detectable genetic variations.

By grouping sequenced isolates by phenotype and examining shared genetic variation, in this study we identified the trehalose biosynthesis pathway as a major mediator of titan cell formation. There are many different pathways for biosynthesis of trehalose, but here we focus on the first pathway based of model yeast *S. cerevisiae* (figure 33). This involves two key enzymes: trehalose-6-phosphate synthase (Tps1), which converts glucose-6-phosphate (G6P) and UDP-glucose into UDP and trehalose-6-phosphate. The next key enzyme in this pathway is trehalose-6-phosphate phosphatase (Tps2), which converts T6P into free organic phosphate and trehalose (Thammahong et al., 2017, Cabib and Leloir, 1958).

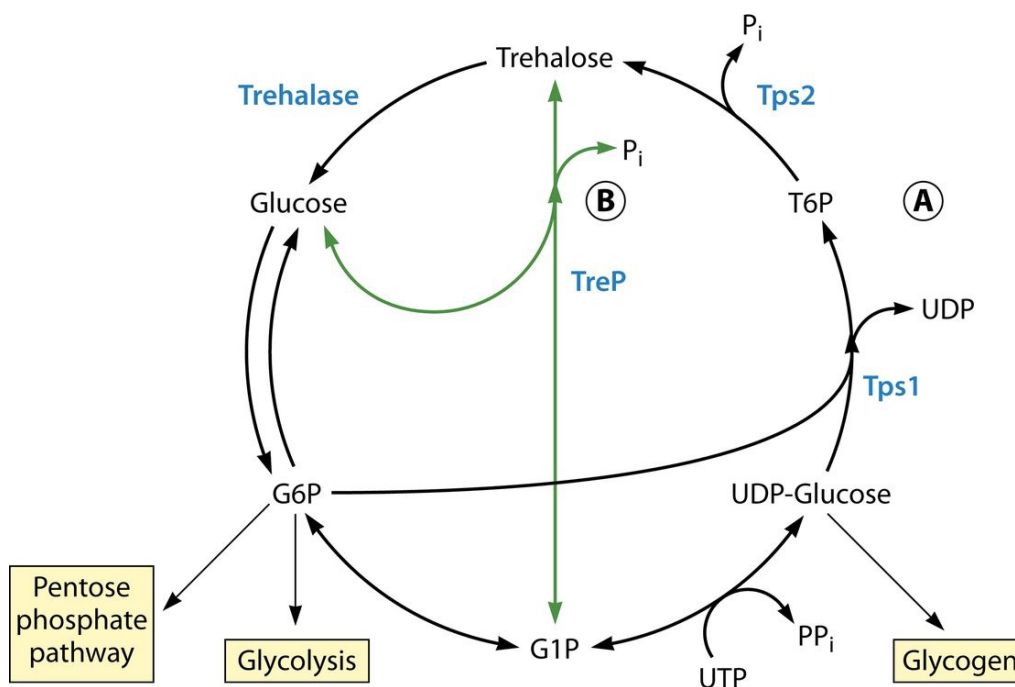


Figure 33. Schematic diagram of one of the trehalose biosynthesis pathway. The first pathway labelled A is the Tps1 and Tps2 pathway. Tps1 converts glucose-6-phosphate (G6P) and UDP-glucose into UDP and trehalose-6-phosphate. Following this Tps2 converts T6P into free organic phosphate and trehalose. This image was taken from Thammahong et al., 2017.

To further investigate the role of trehalose biosynthesis on titan cell formation, I analysed *tps1Δ* and *tps2Δ* mutants deficient in trehalose biosynthesis for their capacity to form titan cells. The results of this analysis are discussed below.

4.2 Results

4.2.1 Bioinformatic analysis reveals SNPs associated with gain or loss of the capacity to form titan cells

To test these hypotheses, Bryan Chang (a summer student) accessed the publicly available whole genome sequences of the phenotyped paired clinical isolates mentioned in chapter 3 through the EBI database. Following this, he used galaxy workflow/veupathdb to carry out variant calling <https://veupathdb.globusgenomics.org/>: each isolate was compared to the

H99 reference genome and single nucleotide polymorphisms were identified. The bcftools pipeline was used to carry out a pairwise analysis of the SNP differences between sampled pairs for those which exhibited increased/decreased titanisation in resistant samples. Further analysis within the clinical isolate pairs, Bryan identified many SNPs. The number of SNPs in genes ranged from 10 in some pairs to 100s in others. FungiDB was utilised to find common genes in increased titanisation and reduced titanisation samples. Finally, as a primary functional screen to test whether genes with SNPs between parent and FlucR isolates might influence the capacity to form titan cells, genes identified in this way were compared to an existing high throughput dataset for genes influencing titan cell formation (Drake and Ballou, unpublished data). This yielded a small number of genes (table 9 and 10) with a potential role in titan cell formation.

Table 9. Genes found in fluconazole resistant isolates that were able to titanise. Genes with SNPs linked to titan cell phenotypes. Qualitative analysis of size and labeled as S is small cells, M is medium cells, L is large cells. Biological function information from FungiDB. This table was generated by Tom Drake (3 biological repeats) and Brian Chang (biological function).

Genes	Repeat1	Repeat2	Repeat3	Biological function
CNAG_01126	S/M	M	M	guanine nucleotide exchange protein for ADP-robosylation factor (ARF); regulate vessicle trafficking, actin remodelling
CNAG_02964	S/M	L	M	Hypothetical protein; deletion may impact anaphase promoting complex 8 (CNAG_02963)
CNAG_03670	S	M	M	IRE protein kinase
CNAG_04992	S	S	S	hypothetical protein with SNARE associated golgi domain
CNAG_05181	S	L	L	White collar 1
CNAG_05292	S	S	M	alpha,alpha-trehalose-phosphate synthase (UDP-forming)
CNAG_05520	S	M	M	hypothetical protein

CNAG_06896	S	M	L	cytoplasmic protein
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Table 10. Genes found in resistant titanising and non titanising isolates. Genes identified in strains able to titanise and strains unable to titanise. Qualitative analysis of size and labeled as S is small cells, M is medium cells, L is large cells, O is odd cells and C? is possible contamination. Biological function information from FungiDB. This table was generated by Tom Drake (3 biological repeats) and Brian Chang (biological function).

Genes	Repeat1	Repeat2	Repeat3	Biological function
CNAG_03168	S	S	S	sulfite reductase (NADPH) flavoprotein alpha-component
CNAG_03765	O	M	M	trehalose-phosphatase
CNAG_04108	O	L	L	pyruvate dehydrogenase kinase
CNAG_04283	S	S	S	integral membrane protein
CNAG_07435	S	S	S	CCAAT-binding transcription factor activator, Hap2
CNAG_07937	C?	L	L	O-acetyltransferase

He first found genes that varied in fluconazole resistant isolates that could titanise (table 9), then he found genes that were found in resistant titanising and non titanising isolates (table 10). Notably, we found CNAG_05292 and CNAG_03765 to encode enzymes involved in the trehalose biosynthesis pathway.

4.2.2 Tps mutants are able to form titan cells and the trehalose pathway may play a role in titanisation

Trehalose is a nonreducing disaccharide which consists of an α,α -1, 1-glycosidic linkage between two glucose subunits (Thammahong et al., 2017). In yeasts this non-reducing sugar has been stated to play a role in stabilising the membrane under many different stresses such as osmotic or oxidative stress, hypoxia and temperature (Chen and Haddad, 2004). It is formed by the trehalose biosynthesis pathway.

CNAG_05292 encodes one of the main enzymes of the trehalose biosynthesis pathway, the trehalose-6-phosphate synthase (Tps1). In fungi, this enzyme converts glucose-6-phosphate (G6P) and UDP-glucose into UDP and trehalose-6-phosphate (Thammahong et al., 2017, Cabib and Leloir, 1958). SNPs in Tps1 were associated with titanising samples (table 9). CNAG_03765 encodes for the other main enzyme of this pathway, the trehalose-6-phosphate phosphatase (Tps2). This enzyme converts T6P into free organic phosphate and trehalose (Thammahong et al., 2017, Cabib and Leloir, 1958). SNPs in Tps2 were associated with fluconazole exposure.

I examined gene knockout mutants of these enzymes to see how what role they played in titanisation. One of the conditions of induction of titan cells is growing the cells at 37°C. When trying to grow the tps mutants in titan inducing conditions we noticed that the cells failed to proliferate at 37°C.

As trehalose has been shown to play a protective role against certain stresses such as temperature, we hypothesised that the mutants were thermosensitive. This was tested by growing the cells in YPD and then made spot plates where it can be seen that the tps mutants, particularly *tps2Δ* grew better at 30°C than at 37°C (figure 34).

Thereafter It was important to ensure that this change in temperature did not affect the way the cells grew under titan inducing conditions. While these conditions reflect the human body, and the temperature for growth was usually 37°C, H99 is able to successfully form

titan cells at 30°C (figure 34). I therefore adopted this variation in the protocol to determine the role of trehalose in titan cell formation independent of its role in thermotolerance.

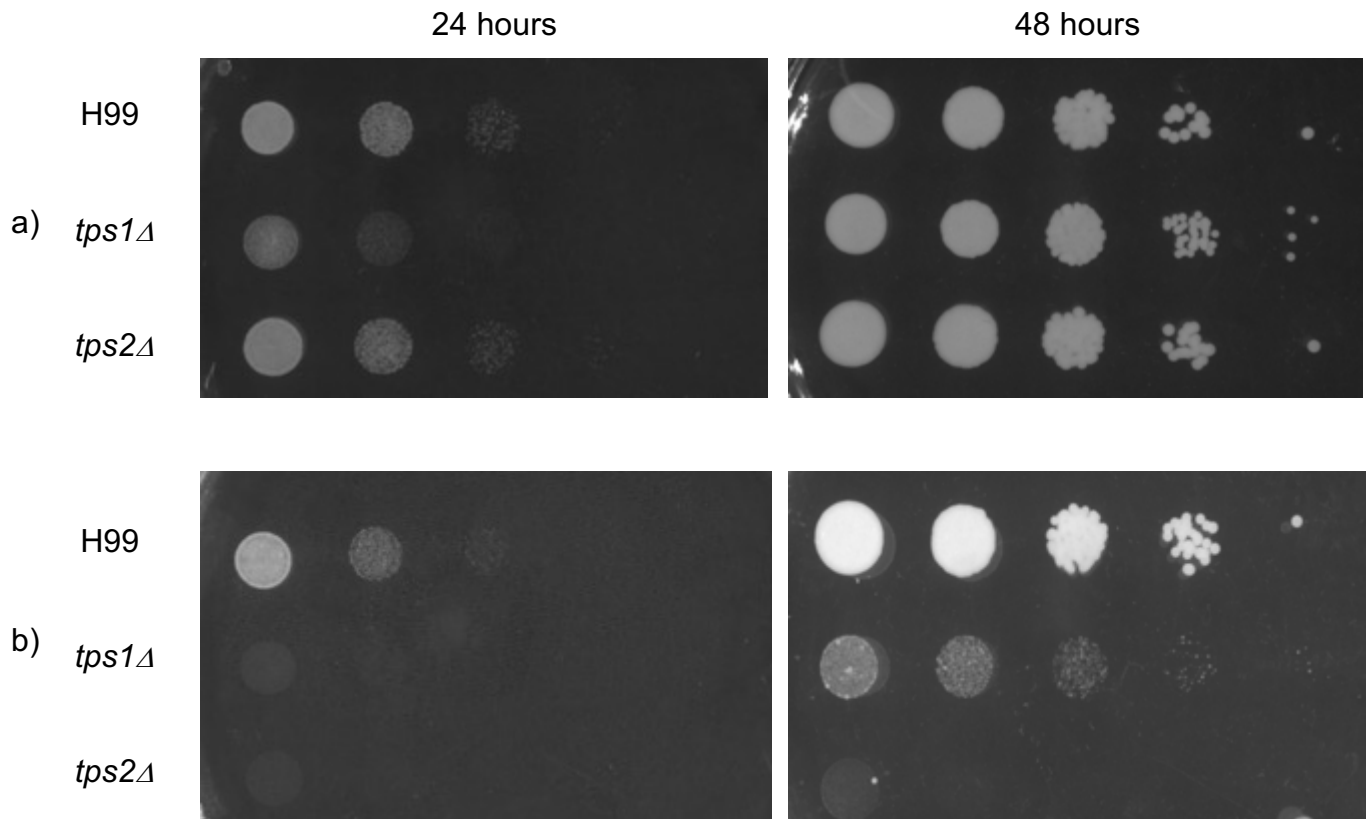


Figure 34. Trehalose mutants are thermosensitive. H99, *tps1Δ* and *tps2Δ* were grown overnight in YPD followed by spotting them on a plate and leaving them in the standing incubator for 2 time points: 24 and 48 hours. a) *tps* mutants and H99 grown at 30°C, b) *tps* mutants and H99 grown at 37°C.

I next set out to see if deleting these genes had any effect on titanisation at 30°C, 5%CO₂. Microscopy images show *tps1Δ* was able to form titan cells (figure 35a). Fascinatingly, *tps1Δ* produced significantly far larger population of cells that were large than that of H99 (figure 35b). In contrast, *tps2Δ* did not produce as many cells as *tps1Δ*, and those that were present and measured mainly reached the 10 μm border but did not exceed it (figure 35). Examination of the cells stained with sybr green seemed to suggest increased fluorescence

in *tps1Δ*, which could indicate high DNA content, however this cannot be used as a qualitative result due to sybr green staining for both DNA and RNA (figure 35a). Therefore, following this, flow cytometry was carried out to measure the DNA content of the mutants.

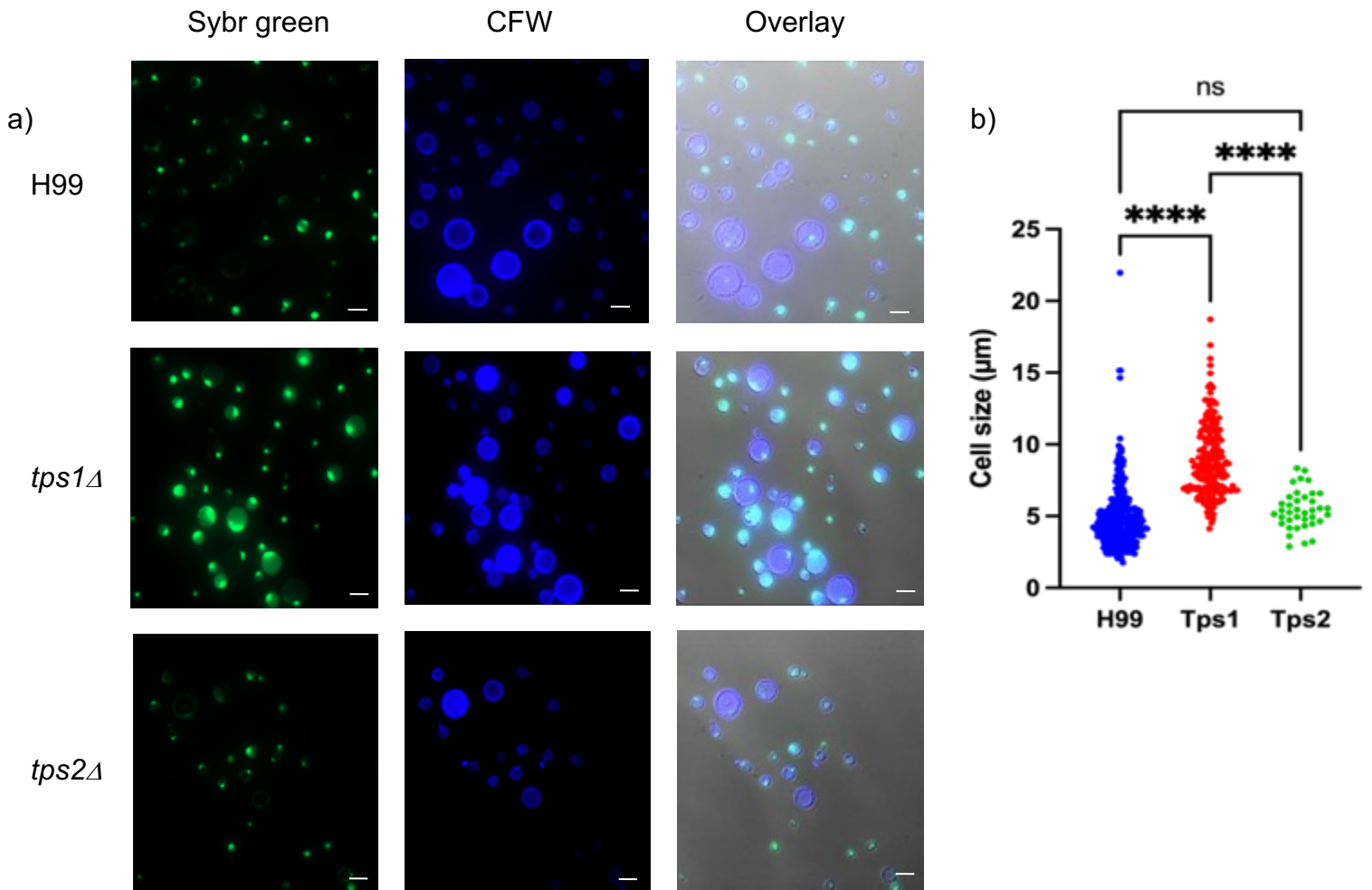


Figure 35. H99 and *tps1Δ* were able to form titan cells at 30°C. H99, *tps1Δ* and *tps2Δ* were grown overnight in YNB, followed by induction of titan cells in 10% HI-FCS with PBS for 2 days at 30°Cw with 5% CO₂. a) microscopy images of H99, *tps1Δ* and *tps2Δ*, representative of 5 technical repeats. Cells were stained for chitin with CFW and DNA with sybr green, with scale bar of 10 μm. b) cell size diameter measurements from the microscopy images from 3 biological repeats. Data was the mean ± SD, ****= p<0.0001 (Kruskal-Wallis with Dunn's multiple comparisons test). Data is representative of 3 biological repeats.

As increase in DNA and superoxide levels seem to be part of the induction of titan cells in the literature, I hypothesized that *tps1*Δ plays a role in causes titan cell formation through increased ROS and increased DNA content. *tps1*Δ and *tps2*Δ had higher DNA content than that of H99, although the median fluorescence of DAPI is lower in *tps2*Δ than *tps1*Δ (figure 36ai). Alongside this there were more cells that were larger in size in *tps1*Δ than in *tps2*Δ (figure 33aii). When looking at the superoxide levels of these tps mutants, similar to the DNA content, it was higher in *tps1*Δ than *tps2*Δ, but both were higher than H99 (figure 36bi).

Interestingly when looking at the cell size of these mutants through flow cytometry, we found *tps1*Δ had larger cells than H99 and *tps2*Δ, although the latter also had larger cells than H99 (figure 36bii). What was also interesting here was the relationship between DNA content and superoxide with the size of the cell. It is clear that there was a correlation between DNA content and cell size (figure 36aiii), but with superoxide this is not always the case (figure 36biii). Looking at *tps1*Δ superoxide vs cell size, the cell size of part of the population stays the same but the levels of superoxide are different (figure 36biii). The data here indicates tps mutants were able to successfully induce titan cells, and could potentially be through this increase in DNA content and superoxide levels particularly in *tps1*Δ.

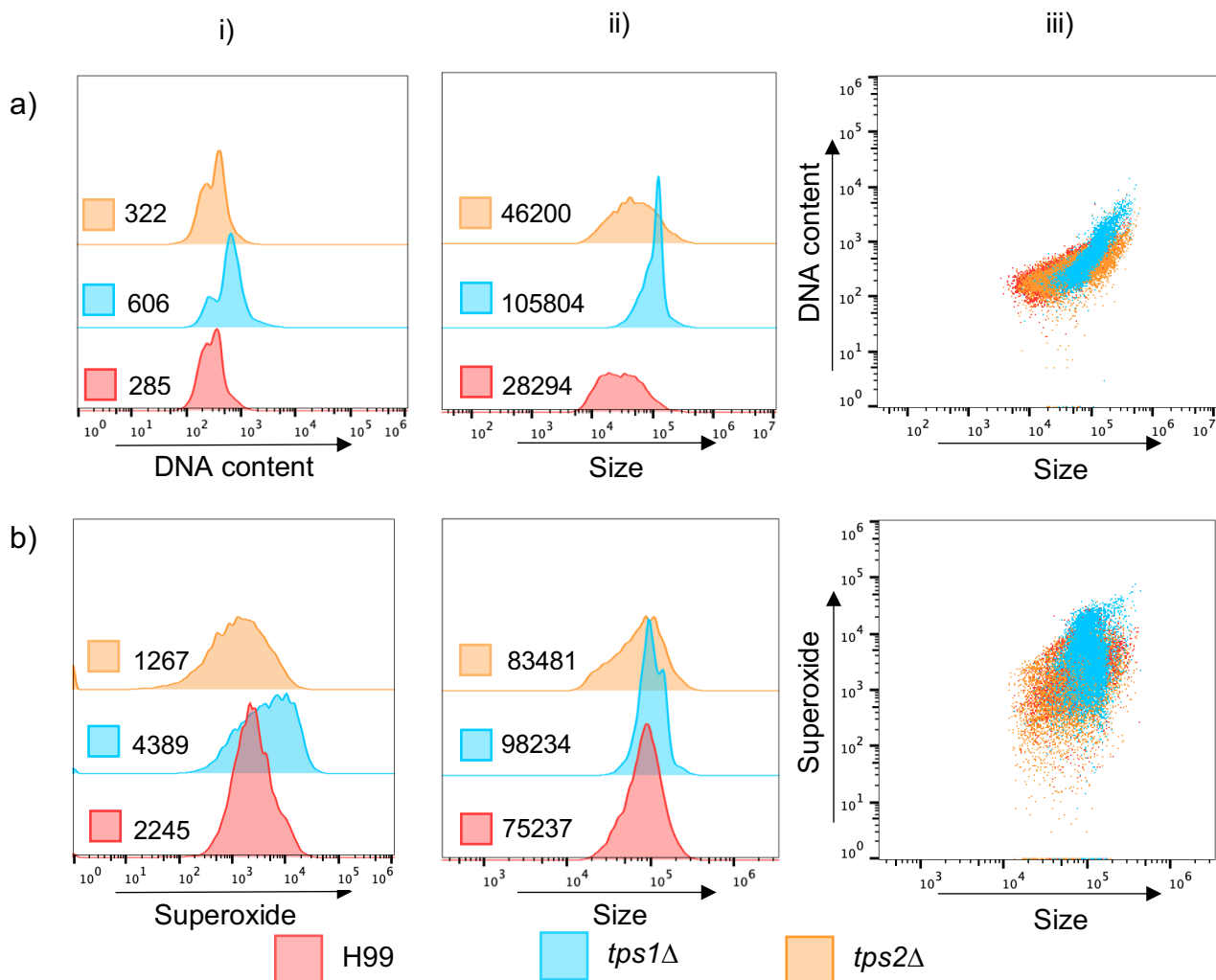


Figure 36. Tps mutants have high DNA content and superoxide after titan induction. H99, *tps1Δ* and *tps2Δ* were grown overnight in YNB, followed by induction of titan cells in 10% HI-FCS with PBS for 2 days at 30°C with 5% CO₂. a) cells were stained with DAPI and b) superoxide dye, and measured by flow cytometry. i) median fluorescence of DAPI for the representative data is on the histogram and labelled accordingly. ii) median fluorescence of size for the representative data is on the histogram and labelled accordingly. iii) scatter plot of dye vs size. Data is representative of 3 biological repeats.

As we had previously found there are TI drugs that are able to chemically block titanisation, here one of the drugs metyrapone, was used to see if it could inhibit the titan cell formation in the *tps* mutants. As shown before metyrapone was able to inhibit titanisation in H99 (figure 37a). Intriguingly, metyrapone was not able to completely inhibit titan cell formation in *tps1Δ*, but instead caused the cells to either clump together or were unable to separate (figure 37b). When measuring the size of the cells, there were a few above the 10 μ m diameter, however

when comparing it to *tps1* Δ alone there was an inhibition of much larger cells (figure 37d).

In *tps2* Δ , there were no titan cells to be seen through microscopy (figure 37d).

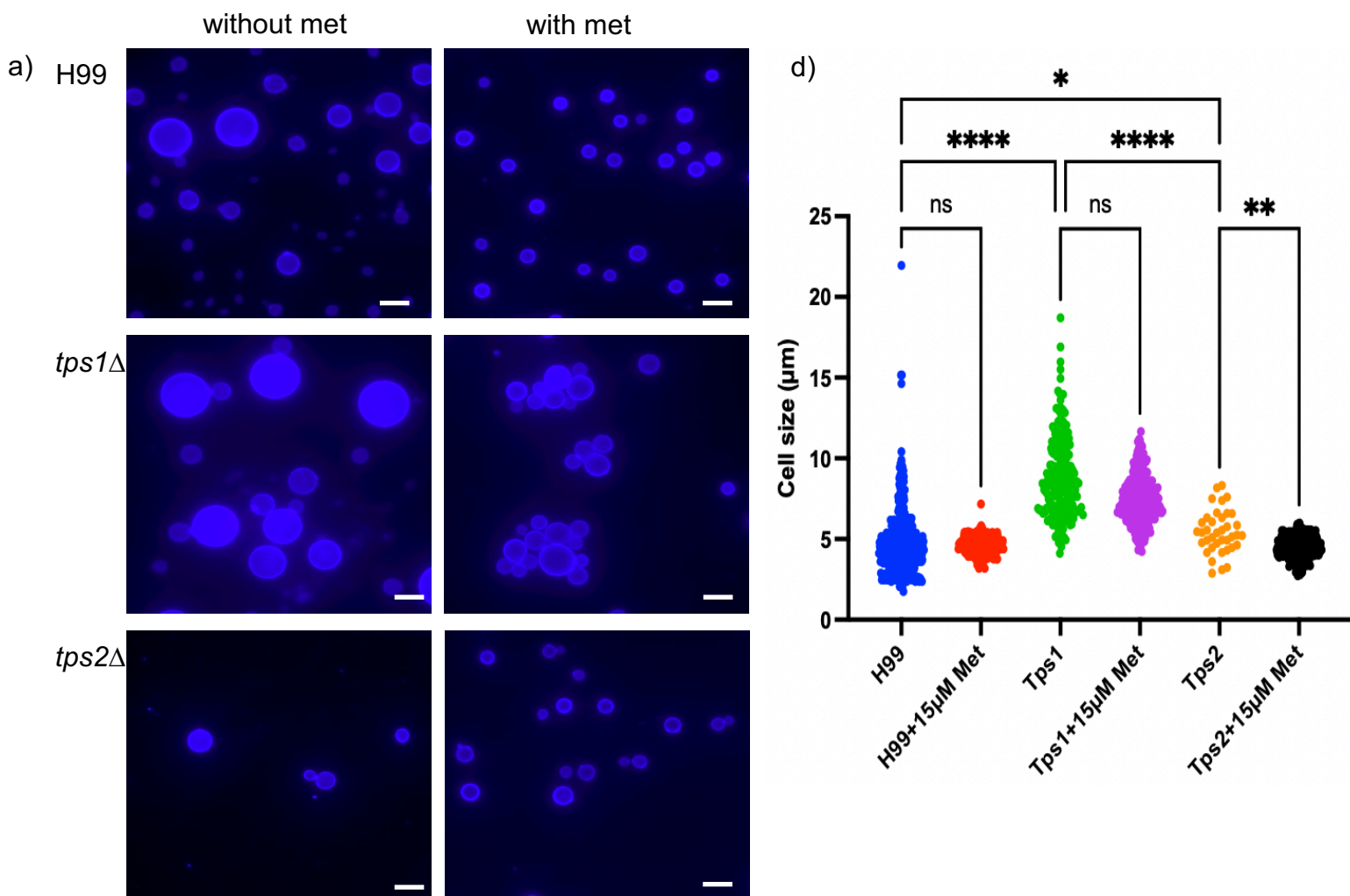


Figure 37. Metyrapone was unable to block titanisation in *tps1* Δ but was able to in *tps2* Δ and H99. a) microscopy images of H99, *tps1* Δ and *tps2* Δ after they were grown overnight in YNB, followed by induction of titan cells in 10% HI-FCS with PBS for 2 days at 30°C with 5% CO₂. Cells were stained for chitin with CFW with scale bar of 10 μ m. A representative of 5 technical repeats. d) cell size diameter measurements from the microscopy images. Data was the mean $\pm$ SD, *= p <0.05, **= p <0.005, ****= p <0.0001 (Kruskall-Wallis with Dunn's multiple comparisons test). Data is representative of 2 biological repeats.

The TI drug alone has been found to decrease superoxide levels in H99 whilst increasing DNA content. So, I set out to see whether metyrapone has an effect on the DNA content on the *tps* mutants.

Similar to what was seen before, when metyrapone was added H99 has a slight increase in DNA content but a decrease in superoxide levels (figure 38a). However, in *tps1Δ*, in the presence of metyrapone there was an upsurge in DNA content compared to *tps1Δ* alone. The levels of superoxide of *tps1Δ* looks like it increased in the presence of metyrapone, however when looking at the median fluorescence of the dye, it looks like there was a slight decrease (figure 38b). *tps2Δ* had parallel patterns to that of H99, where the DNA content increased slightly and the superoxide levels also decreased slightly due to the addition of metyrapone (figure 38c).

Overall it seems that the trehalose pathway is associated with titanisation, with Tps1 being a negative regulator. However, judging from the previous results from chapter 3 and the list of genes given in table 4 and 5, the trehalose pathway alone is not the main contributor for titanisation. There are other proteins that are also negative regulators of titanisation, such as Rim101, and some that are positive regulators, such as Cac1. The trehalose pathway plays a role in this morphological transition in *C. neoformans*, but it is possible it does this along with the other pathways.

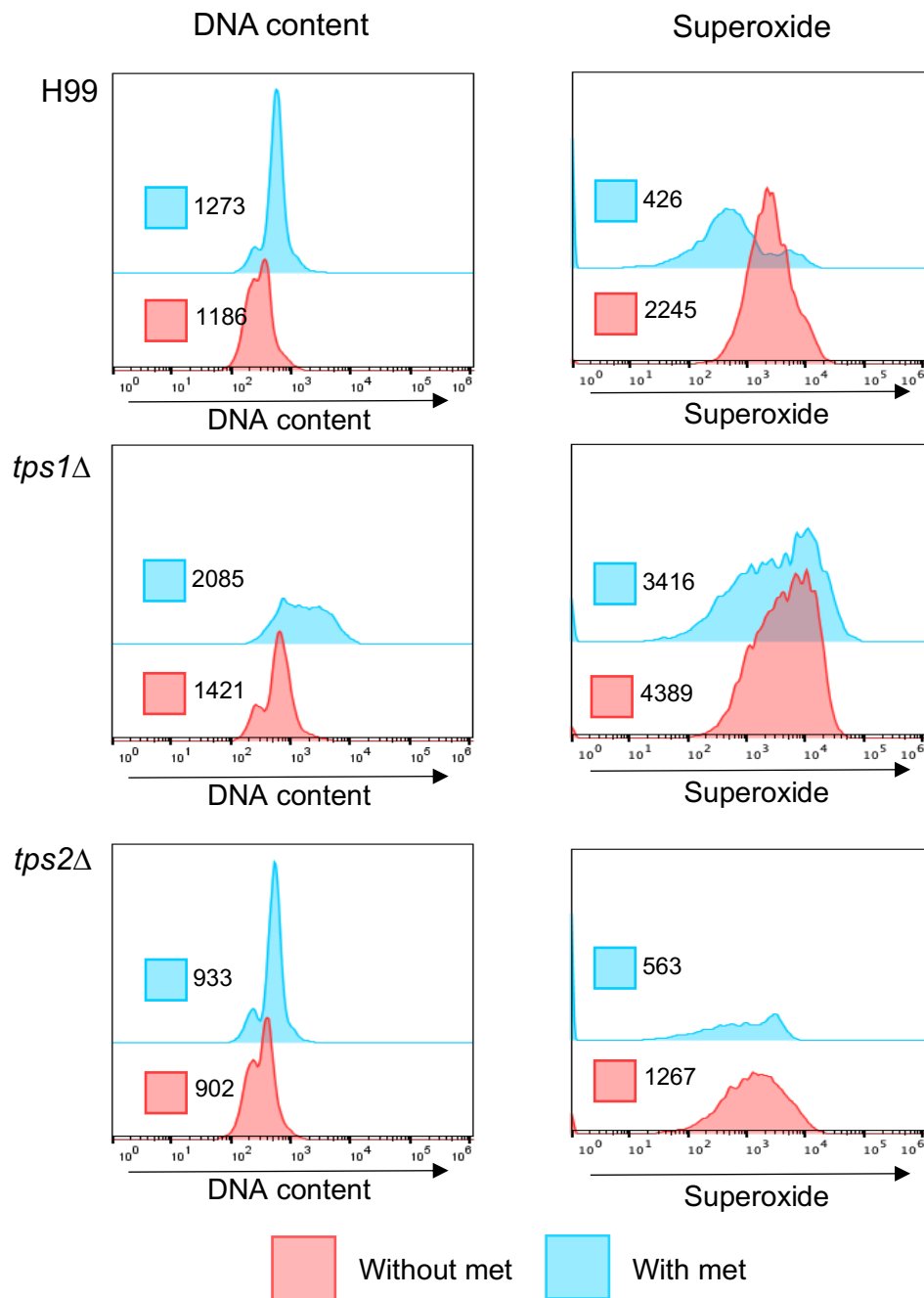


Figure 38. Metyrapone causes an increase in DNA content and a decrease in superoxide in *tps1*Δ superoxide. H99, *tps1*Δ and *tps2*Δ were grown overnight in YNB, followed by induction of titan cells in 10% HI-FCS with PBS for 2 days at 30°C with 5% CO₂. Cells were stained with either DAPI or superoxide dye and measured by flow cytometry. Red represents the cells without metyrapone and blue represents the cells with 15 μM metyrapone. The median fluorescence of the dyes for the representative data is on the histogram and labelled accordingly. Data is representative of 3 biological repeats.

4.3 Discussion

I earlier screened through clinical isolates for their ability to form titan cells before and after exposure to fluconazole. Throughout the paired clinical isolates there was a wide but stable phenotypic variation. The isolate pairs had either increased their ability to form titan cells or had lost it (section 3.2.1). We therefore hypothesised that genetic variations detected in these clinical isolates will show the capacity for the given isolate to form titan cells. Bryan Chang, our former summer student, went through the publicly available whole genome sequences of these isolates and carried out different bioinformatic analysis to create a list of common genes in increased titanisation and reduced titanisation samples (table 9 and 10). In this list he found genes that encode enzymes involved in the trehalose biosynthesis pathway as having a positive and negative association with the ability to form titan cells.

Trehalose is a nonreducing disaccharide which consists of an α,α -1, 1-glycosidic linkage between two glucose subunits (Thammahong et al., 2017). It can form hydrogen bonds with proteins to replace water, thereby protecting proteins from temperature perturbation (Colaço and Roser, 1994, Thammahong et al., 2017). It is also able to act as a free radical scavenger, protecting cells from oxidative damage (Benaroudj et al., 2001). This nonreducing disaccharide sugar has not been found in mammals but has been found in a variety of bacteria, plants, algae and fungi (Elbein et al., 2003). In many fungi, the trehalose biosynthesis pathway has been found to play a role in viable spore production, morphogenesis and virulence (Gancedo and Flores, 2004).

In the list of genes with SNPs linked to titan cell phenotypes, Tps1 was found in titanising samples (table 9), whilst Tps2 was found in both titanising and non titanising clinical isolates (table 10). After obtaining this information we hypothesised that trehalose synthesis is required for the yeast to titan transition. To see if the mutants of these enzymes were able to form titan cells, we grew them under the titan inducing conditions of 37°C with 5% CO₂ for 2 days. However, the tps mutants did not grow well in these temperatures. Trehalose has been shown to play a role in helping fungi grow in high temperatures (De Virgilio et al., 1993). Another study has also found both *tps1Δ* and *tps2Δ* in *C. neoformans* to be sensitive to high temperatures and to have a growth defect at that temperature (Petzold et al., 2006). As the mutants were able to grow at 30°C and not at 37°C, similar to that in the literature, it confirms trehalose plays a role in thermotolerance in *C. neoformans* as (figure 34). Following this I changed the titan inducing conditions to 30°C whilst studying the tps mutants.

Interestingly, *tps1Δ* was able to produce significantly large cells above 10 μm in diameter, more so than H99 (figure 35). The large cells in *tps1Δ* had multiple daughter cells still attached indicating that there may be some kind of cytokinesis defect. In addition, daughter cells were oriented at right angles to the mothers, possibly indicating a bud site selection defect. SNPs were seen in Tps1 in the clinical isolates that were able to titanise (table 9), so this data increases my confidence in the bioinformatics analysis approach carried out as the *tps1Δ* was able to titanise.

Although *tps2Δ* should be able to grow well at 30°C, this was not seen following incubation for 2 days, and cells that did grow were less than 10 μm in diameter (figure 35). But, in the

flow cytometry data *tps2Δ* cells were larger than that of H99, suggesting there may have been a problem when imaging the cells and the transferring of cells onto the slide.

Another characteristic of a titan cell that we have discussed is the increase in DNA content. I carried out flow cytometry on the mutants to see if there were any changes in their DNA content, along with superoxide levels. Both *tps* mutants had higher levels of DNA content than H99, though there seemed to be a higher population of large cells in *tps2Δ* than in *tps1Δ* (figure 36a). When looking at the superoxide levels, *tps1Δ* had an increase in superoxide levels compared to H99, whilst *tps2Δ* had lower superoxide level (figure 36b). This suggests that the increase in DNA content seen in the *tps* mutants may be due to upturn in superoxide levels.

The trehalose biosynthesis pathway influences multiple aspects of cell stress resistance and growth via interaction with multiple up and downstream pathways. A study carried out found the phenotype of *tps1Δ* had an increase in susceptibility to oxidative stress (Petzold et al., 2006). This parallels a study carried out in *S. cerevisiae* which found trehalose necessary to protect the cells from oxidative damage by acting as a free radical scavenger (Benaroudj et al., 2001). So, inhibiting the first part of this pathway may cause a build-up of ROS due to a lack of trehalose. However, the molecular mechanism behind trehalose detoxification of superoxide is unknown. The data here show that the trehalose biosynthesis pathway does play a role in regulating titanisation.

I have previously shown that there are drugs which are not antifungal that are able to inhibit the yeast to titan transition, known as TI drugs. To test for interaction of these inhibitors with

the trehalose biosynthesis pathway, H99 and the *tps* mutants were tested for titan cell induction in the presence and absence of metyrapone. Similar to the superoxide level results, *tps2* Δ had alike results to H99, with metyrapone successfully able to inhibit titanisation in both (figure 37a, c and d). Likewise, the DNA content increased in both H99 and *tps2* Δ in the presence of metyrapone whilst the superoxide levels stayed the same (figure 38a and c). This shows that metyrapone does not interact with Tps2 in the trehalose biosynthesis pathway. In the future, studies would also look into the role of the trehalose biosynthesis pathway in antifungal susceptibility.

Conversely, Tps1 does seem to play a role in this interaction. The data suggests that Tps1 and metyrapone may act similarly to regulate titan cell formation, with metyrapone able to compensate for loss of trehalose biosynthesis activity (figure 37b and d). *tps1* Δ when grown in titan inducing conditions were shown to have a cytokinesis defect (figure 34), however in the presence of metyrapone the cells seem to clump together. This may be due to the absence of capsule as that is usually what this phenotype indicates but further investigation would be required to find this out. Furthermore, the data suggests that trehalose biosynthesis influences DNA content independent of cell size, while metyrapone can block cell size increase, but not DNA content increases.

All in all, it seems that the trehalose metabolism is linked, directly or indirectly, to titanisation. However, we showed that this pathway was discovered as potentially playing a role due to being present as a SNP in isolates that were capable of forming titan cells and those that could not. This then asks the question of whether changes in this biosynthesis pathway explain the phenotypic differences seen in the sets of clinical isolates analysed. Looking at

the data as a whole in this chapter and the information about titan induction in the literature, trehalose plays a role in the ability to form titan cells but it is not the only pathway to play a role. We have shown that superoxide and the mitochondria are also potentially involved in inducing titan cells, whilst studies have confirmed the role of cAMP pathway. There are so many more pathways that could potentially play a role in the changes seen and therefore more research is needed to look into the other genes and see if there are connections between all the given pathways.

Chapter 5: What are the genes that influence titanisation? Screening of mutant libraries.

This chapter contains work done by collaborators: Robert Evans (Frickel lab, University of Birmingham) and Daniel Fisch (Frickel lab, University of Birmingham). They have been credited where due.

5.1 Introduction

5.1.1 3Rs research and the use of *in vitro* models for high throughput screens

Previously, due to a lack of *in vitro* models for titan cell induction, the majority of our understanding about titan cells has come from *in vivo* studies (Crabtree et al., 2012, Okagaki et al., 2010). One of the key findings from *in vivo* studies was the regulation of titanisation by the core cyclic AMP (cAMP)/protein kinase A (PKA) pathway (Alspaugh et al., 2002, D'Souza et al., 2001, Okagaki et al., 2011). However, it was difficult to understand the molecular mechanisms that drive titanisation using *in vivo* models because of the demanding and time-consuming experimental process. When thinking about all the optimisation that would be required, followed by the actual experiment and the repeats needed it would require many mice, with the possibility of more if certain experiments do not work, it was difficult to go into detail about the molecular mechanisms. But now there are 3 reproducible *in vitro* models that show similar characteristics of titan cells, allowing for better understanding of the mechanism (Dambuza et al., 2018, Trevijano-Contador et al., 2018, Hommel et al., 2018).

In this study, we successfully replaced the use of mice completely through the use of our *in vitro* model. Titans are transient and primarily observed in the lung (Okagaki and Nielsen, 2012). Before access to these *in vitro* models, analysis involved infecting large numbers of mice through inhalation of *C. neoformans* yeast (Okagaki and Nielsen, 2012). This would typically consist of 10 mice per group. Endpoint analysis through bronchial lavage (BAL) would occur following culling after 7 days. Titan cells as a research topic has increased over the years which in turn meant more mice were needed to carry out experiments. This can become quite time consuming and costly, especially for large screens or complex experiments. An example of this is that a single titanisation study in a lab screened more than 60 mutants and reportedly used over 600 mice to obtain the data (Okagaki et al., 2011).

Genome scale analyses of *C. neoformans* have enhanced the knowledge of genes that code for certain proteins and their functions (Janbon et al., 2014, Stone et al., 2019). Genetic studies have further advanced our knowledge of *C. neoformans* through the generation of knockout libraries and mutant strains, which have also helped in expanding the understanding of cell signalling pathways (Lee et al., 2016, Jung et al., 2015, Liu et al., 2008). High throughput screening has become widely used in industry particularly for drug discovery, however it is also becoming popular within researchers in academia, including for fungal pathogens (Samantaray et al., 2016, Liu et al., 2017). This screening method can be used for various kinds of libraries and is fast becoming a preferred technique due to how quickly experiments are carried out, particularly when there are large number of samples, through automation (Szymański et al., 2012).

5.1.2 Use of mutant libraries to identify regulatory networks

For several decades, studies have been undertaken to better understand *C. neoformans* pathogenicity and its mechanisms. Recent large-scale genetic analyses have provided insight into the overall system that *C. neoformans* requires to function, providing insight into conserved regulators of cellular growth and pathogenesis such as transcription factors and kinases (Jung et al., 2015, Lee et al., 2016).

Following the activation of a signal cascade, the expression of effector proteins is either suppressed or activated through binding of a transcription factor to target gene promoters (Janbon et al., 2014, Jung et al., 2015). Jung *et al* assembled a gene deletion library consisting of 155 predicted *C. neoformans* transcription factors through homologous recombination and replacement of the open reading frame using a drug selectable marker (Wilson et al., 2008, Jung et al., 2015). This mutant library was created to further increase the understanding of *C. neoformans* transcription factors and their phenotypic traits, but also to detect further pathways that may be targets of potential cryptococcosis treatments. They were tested for different phenotypic traits *in vitro*, along with a virulence test in an insect model at a large scale (Jung et al., 2015). Because of the lack of an *in vitro* system, there has been limited progress identifying transcription factors that regulate titanisation. Efforts have instead required gene by gene analysis. For example. Rim101, a cAMP/PKA pathway target, was shown to impact production of capsule and titanisation (Okagaki et al., 2011). Pdr802, a transcription factor required for virulence, was shown to be a negative regulator for titanisation (Reuwsaat et al., 2021). However, there is little else known about the transcriptional networks that regulate this complex phenotype.

Kinases play a crucial part in signalling pathways by converting environmental signals into chemical signals that can eventually activate transcription factors and other effector pathways (Cohen, 2000). To provide insight into *Cryptococcus* kinases, Lee *et al* assembled a gene deletion library consisting of 129 presumed kinases inactivated through homologous recombination with a selectable drug marker (Lee et al., 2016). This mutant library was created to further increase the understanding of *C. neoformans* kinases related to virulence and their phenotypic traits (Lee et al., 2016). (Jung et al., 2015). Similar to the transcription factor library, these mutants were tested for different phenotypic traits *in vitro*, along with a virulence test in an insect model at a large scale. However, due to the lack of an *in vitro* system, titanisation was not included as a measure, despite evidence that individual kinases are key regulators. For example, the kinase Pka1, a target of cAMP signaling cascade, is known to play a role in titanisation (Choi et al., 2012). Therefore, other kinases may also play a similar role but this has yet to be investigated.

Finally, another publicly available gene deletion library is the Madhani whole genome collection. This library has been released in batches by the Professor Hiten Madhani lab at University of California, San Francisco, over the last 8 years but is not yet published. However, to construct this library, the Madhani lab took a similar approach to a deletion collection they published in 2008 (Liu et al., 2008). In this collection the Madhani lab created a set of 14 plates consisting of 1201 targeted gene knockout strains that were signature tagged (Liu et al., 2008). Signature tagged mutagenesis screening is an effective method that multiple strains can be tested in one mouse, allowing speedy analysis of several strains. This is done by adding a signature tag which is a unique DNA-based barcode, to the mutant's DNA. This allows strains to be detected through qPCR using specific primers to

the signature tag (Chun and Madhani, 2010). This library was created to screen for new virulence mechanisms other than capsule and melanisation, which are the main virulence mechanisms discussed in the literature (Liu et al., 2008). Following this, the Madhani lab have created an additional 29 plates of knockout strains that are not yet published but are available from the Fungal Genetics Stock Center in Kentucky, USA (<https://fgsc.net>).

Previous lack of *in vitro* titanisation protocols, none of these collections have been assayed for titan cell formation during their initial validation by their creators. However, a previous student in the Ballou group, Tom Drake, screened the first 21 plates in the Madhani collection for titan cell formation using a qualitative scoring system (small, medium, and large cells). This data was used in Chapter 4 as a first pass to understand the potential contribution of SNPs in the clinical isolates to titan cell formation. This initial screen, which successfully identified the trehalose biosynthesis pathway as a key regulator of titan cell formation, validates the use of cell size as a high through approach for titan cell regulators. In this chapter, the libraries discussed have been used to carry out a high-throughput screen to identify genes that may be involved in regulating titanisation. It was also used to highlight pathways and proteins that require additional investigation into their role in titanisation.

5.2 Results

As previously mentioned in chapter 3, one of the hypotheses was that the capacity of *C. neoformans* to form titan cells may be one mechanism underlying heteroresistance to antifungals. While I showed this is not the case, these studies highlighted the lack of information regarding the molecular mechanisms that drive titanisation. I showed that certain

FDA-approved drugs, that are not anti-fungal, are able to inhibit the yeast to titan transition, however the mechanism behind their mode of action is unknown. Hence, in order to learn about how titanisation works, I decided to investigate the genes that are required for titanisation.

5.2.1 Setting up a high throughput mutant screen

To do this, a high-throughput screen of the deletion libraries was carried out to detect previously unknown regulators involved in titanisation in *C. neoformans*. There were three deletion libraries used. One was a transcription factor deletion library from Jung *et al.* which consisted of 155 predicted transcription factors across 322 signature tagged gene deletion strains (Jung et al., 2015). Another was a protein kinase deletion library from Lee *et al.* which consisted of 129 predicted kinases across 264 signature tagged deletion strains (Lee et al., 2016). In both the transcription factor and the kinase collections contain multiple, independently tagged replicates of the same gene. The final library used was from the Madhani lab which covers 43x96 genes that were deleted through replacement of the predicted coding region with a selectable marker (<https://fgsc.net>). There is some overlap between the Madhani collection and the other two libraries, enabling internal validation of findings.

To complete the screen, we decided to fluorescently label cell walls and DNA to enable automated analysis of cell size and DNA content using an AI algorithm developed by our collaborators in the Frickle lab at the University of Birmingham. Before undertaking the screen, optimisation of certain conditions was required. H99 wild type cells were first stained with calcofluor white (CFW) or hoerch cell wall staining, alongside DAPI or SybR green for

DNA staining. After inspecting stained cells under the microscope, it was clear that CFW and sybr green provided the best combined signal profile. DAPI cannot be used alongside CFW as both require the blue channel (data not shown).

Following this optimisation, each mutant in the library was systematically induced to form titan cells using the titan induction protocol using a 96 well flat bottom plate format by growth in YPD media at 37°C for 48 hr, then YNB media at 37°C for 24h, followed by PBS and HI-FCS and incubation at 37°C for 24h (figure 39a and b). Cells were fixed and stained, and the plates were imaged in a high-throughput manner by Robert Evans using a Zeiss cell discover 7 fluorescence microscope (Frickel lab, University of Birmingham) (figure 39c). Triplicate images were taking for each well and each condition. These images were given to Daniel Fisch (Frickel lab, University of Birmingham) to analyse them using a high-throughput computer program called host response to microbe analysis (HRMAN) (figure 39d).

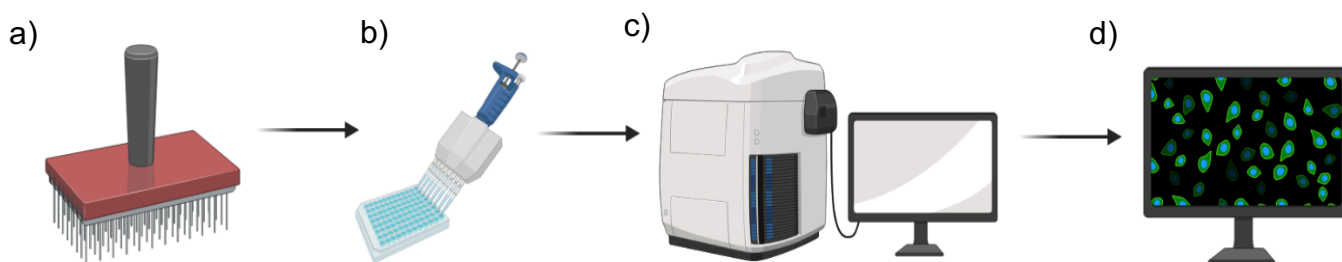


Figure 39. Schematic diagram of the screen of the mutant library. This mutant library was carried out in collaboration with the Frickle lab. a) a replicator was used to transfer the cells from the original library plate to another 96 well plate consisting of YPD media. b) Once the cells grew they were transferred into titan cell inducing conditions. c) Following this the cells were fixed before being given to Robert Evans to image the cells in a high throughput manner using a Zeiss cell discover 7 fluorescence microscope. d) After all the cells were imaged, the images were given to Daniel Fisch to analyse using a high-throughput computer program.

5.2.2 *In silico* analysis of the screen

HRMAN is a program, produced by the Frickel lab, which is able to carry out image analysis on single cells in a high throughput manner. It integrates neural networks with machine learning to differentiate host cells from pathogens (Fisch et al., 2019). However, in this study, HRMAN was programmed to classify cells from the screen as either yeasts or titans. It works by loading images onto the software, following which a plate map is provided and the program groups the data by parameters that the users have specified (Fisch et al., 2019).

Daniel took the images from the screen and processed them through HRMAN which showed that 87.4% of the wells in the screen had cells that were visible and could be analysed (data not shown). He then identified titan cells within the mutant strains through 5 different methods, one of which was circularity which was deemed not a good representation, all producing a statistical z score and created graphs (figure 40).

One of the methods was unsupervised k-means clustering. This is where the user gives the algorithm parameters of the objects that were detected. In this screen the parameters given were area, circularity, complexity, and extended diameter. Daniel then grouped the ranges of values into two clusters, which were based on the area and diameter parameter given. The program would then classify individual cells into the two clusters, which was later labelled as 'titans' and 'non-titans', following which the z-score was determined (figure 40a), which shows how the rate of titanisation in the mutants differs from the overall population mean.

The other method that Daniel used was classifying the cells based on artificial intelligence (AI) that they had trained. They created a neural network that looked at cropped images of the individual fungi and then classified it as to whether it was a titan cell or not. The training of the AI was 96.1% accurate (data not shown), and the z-score was produced (figure 40b).

Previous studies have agreed that on average 10% of *C. neoformans* population consists of titan cells (Okagaki et al., 2010). Daniel used this information and assumed that the overall rate of 10% titanisation across the whole screen would be constant, with this method being based on the percentile rate of titanisation. He took all the mutants across the screen and set the program to show the top 10% of the largest cells are titan cells, whilst the rest are not. Likewise, a z-score was determined for each mutant screen based on this parameter (figure 40c). Alongside this, the other methods he used and produced z-scores for were area and circularity. Circularity is not shown here as the method was decided not to be a good measure for titanisation due to the fact that its measurement focuses on the shape of the cell. As titan and yeast cells are of a similar shape, their size is different, this measurement did not give enough information and was not used.

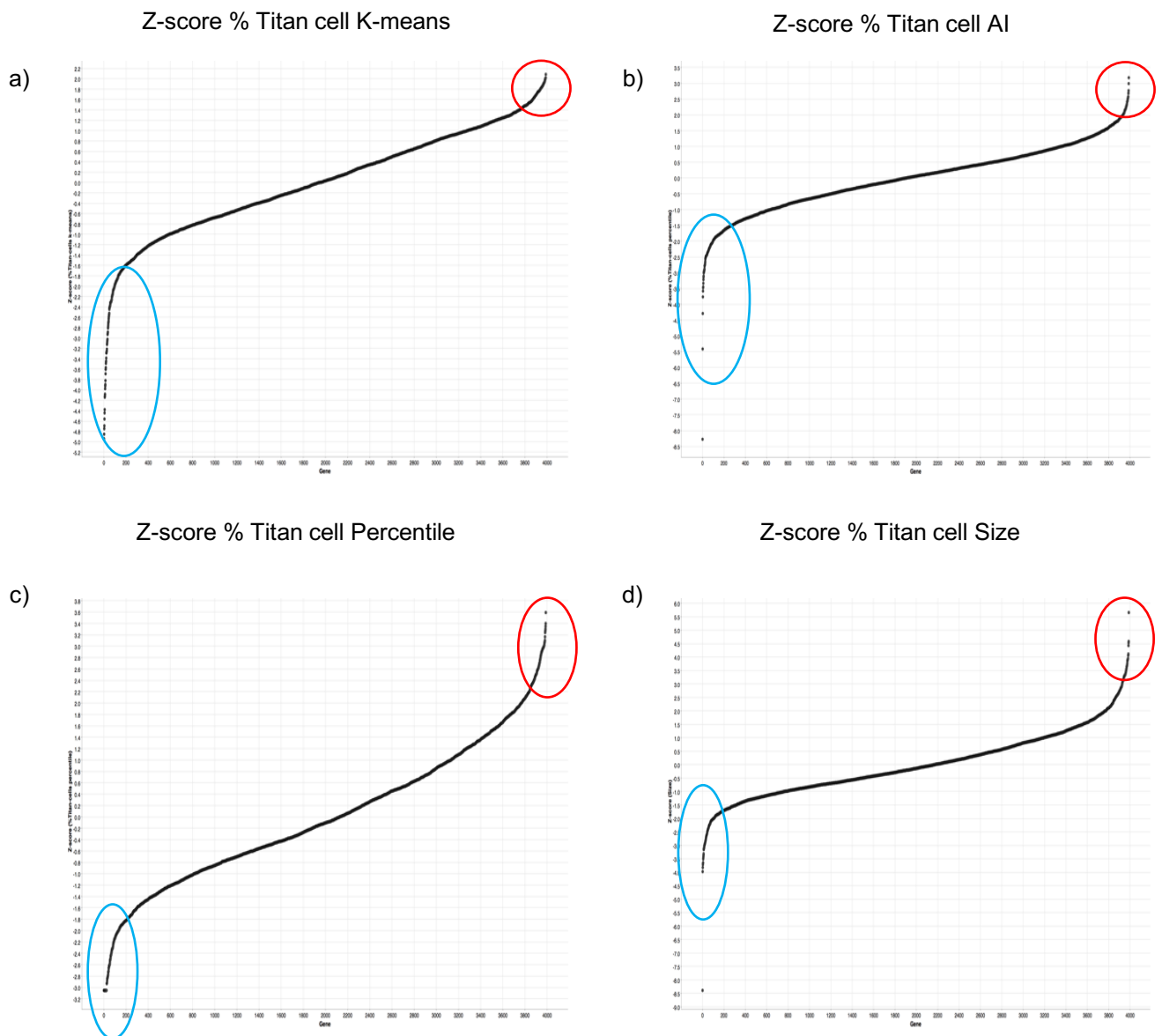


Figure 40. Deletion library high-throughput screen Z-scores. Rate of titanisation was determined by the allocation of z-scores for each strain. 4 out of 5 methods are shown here, a) K-means clustering, b) artificial intelligence (AI) c) percentage titan cells, and d) size. The red circle indicates where we would predict a z score of >+2 to be, and the blue circle indicates where we would predict a z score of <-2 to be. The data and these graphs were generated on HRMAN by Daniel Fisch.

5.2.3 Prioritising hits

In order to prioritise the hits from this screen, the list Daniel had provided of all the genes was shortlisted to those that have a z score of more than (>) +2 and less than (<) -2. This

was done for each method. The reason for this was due to the fact that although a z-score of ± 3 would have been better as it is equivalent to $p=0.05$, the dataset was too limiting and this may have caused type 2 errors (false negatives). Whilst a z-score of ± 2 is equivalent to $p=0.1$ and may give room to false positives, it was satisfactory for this study and the mutant strains that were of interest were going further validated independently to confirm the screen.

Table 11 shows the number of genes that were the top hit. A Venn diagram was also generated to show how much overlap there was between the groups. Interestingly, there was more overlap in the groups that were hypotitanising than in the groups that were hypertitanising, with one gene present in the lists from all methods used but this was a hypothetical protein (figure 41). There was little overlap for hypotitanising lists, except between percentile and area. Following the Venn diagram, I wanted to see how the distribution of genes in each method differs from random chance. This was based on the number of overlaps seen in figure 41, and all the overlaps were significant except (table 12).

Table 11. Z-scores of the measures. Number of top hits of genes based on z-score of >2 (hypertitanising) and <-2 (hypotitanising).

Z-score measure	>2	<-2
Area	220	106
Percentile	221	132
K-means	5	98
AI	54	98
Circularity	1	153

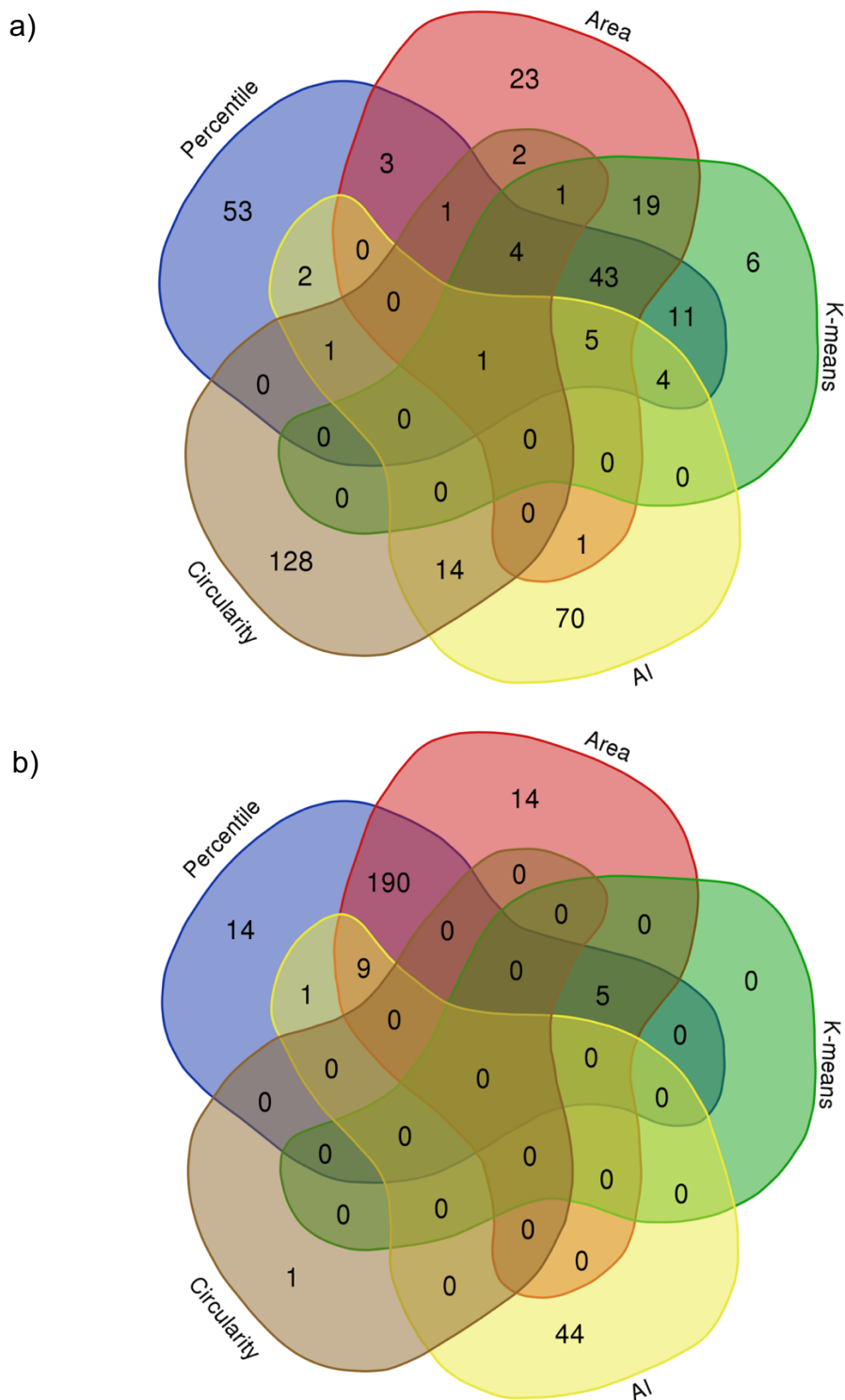


Figure 41. Venn diagram of top gene hits from high through-put screen, showing genes that were identified by 1, 2, 3, 4, or all 5 methods. This was split into the 5 different methods of measuring the rate of titanisation. a) the genes that had a z-score of <-2 and categorised as hypotitanising, b) the genes that had a z-score of >2 and categorised as hypertitanising. Diagram generated on <https://bioinformatics.psb.ugent.be/webtools/Venn/> based on the gene list shown in appendix 4 and 5.

Table 12. Statistical significance of the overlap between 2 z-score measures. A formula from http://nemates.org/MA/progs/overlap_stats.html was used to compare 2 groups of z-score measures and to find the probability of finding genes that overlap in the two groups. These were taken from the number of top hits of genes based on z-score of >2 (hypertitanising) and <-2 (hypotitanising) seen in table 11.

	Random chance for >2 (p=)	Random chance for <2 (p=)
Area vs Percentile	< 0x10 ⁺⁰⁰	< 2.357x10 ⁻⁷⁴
Area vs K-means	< 3.152x10 ⁻⁰⁸	< 1.353x10 ⁻¹²⁴
Area vs AI	< 4.407x10 ⁻⁰⁵	< 7.276x10 ⁻⁰⁴
Area vs Circularity	< 0.032	< 5.377x10 ⁻⁰⁴
AI vs Percentile	< 6.447x10 ⁻⁰⁶	< 3.442x10 ⁻⁰⁸
AI vs K-means	< 0.039	< 1.047x10 ⁻⁰⁶
AI vs Circularity	< 0.008	< 3.089x10 ⁻¹⁰
Circularity vs Percentile	< 0.032	< 0.027
Circularity vs K-means	< 7.246x10 ⁻⁰⁴	< 0.021
K- means vs Percentile	< 3.225x10 ⁻⁰⁸	< 5.794x10 ⁻⁶⁹

Following this, the list was filtered to remove duplicates and in total 613 unique hits were obtained. 344 knockout mutants were found to be hypotitanising whilst 269 were hypertitanising in at least 1 or more of the HRMAN titanisation rate methods (appendix 4 and 5).

The 613 genes were then taken forward to a gene ontology (GO) term analysis using the online FungiDB software, which provides a list of GO terms with reference to the entire *C. neoformans* H99. Each of the 613 genes, was categorised based on 3 ontologies: biological process, which is an open description of what the gene may do; cellular component, which is the location of things in the cell; and molecular function, which describes the gene's action in the cell. We then performed a GO term enrichment analysis for the 613 genes to identify particular GO terms associated with the titanisation phenotypes.

Measuring GO term enrichment allows a statistical analysis of the predicted annotations of a given list of genes. GO Enrichment Odds Ratio was selected as the most useful output because it gives a ratio based on the chance that observed genes with a specific GO term could be identified in the screen accidentally. Using this, the list was further sorted to allow those with the highest ratio to be at top which would be predicted to be the least likely to occur by random chance, and also as one of the most interesting genes to look at. This was the case for the biological process (appendix 1), cellular component (appendix 2), and molecular function (appendix 3).

To summarise this long list of GO terms, REVIGO was used. This is an online server which helps visualise the list by grouping them together semantically based on the GO term similarity in a graphical format, regardless of whether they have a hypo or hyper titanising phenotype (Supek *et al.*, 2011). This analysis was carried out on the biological process list (figure 42a) and the cellular component list (figure 42b).

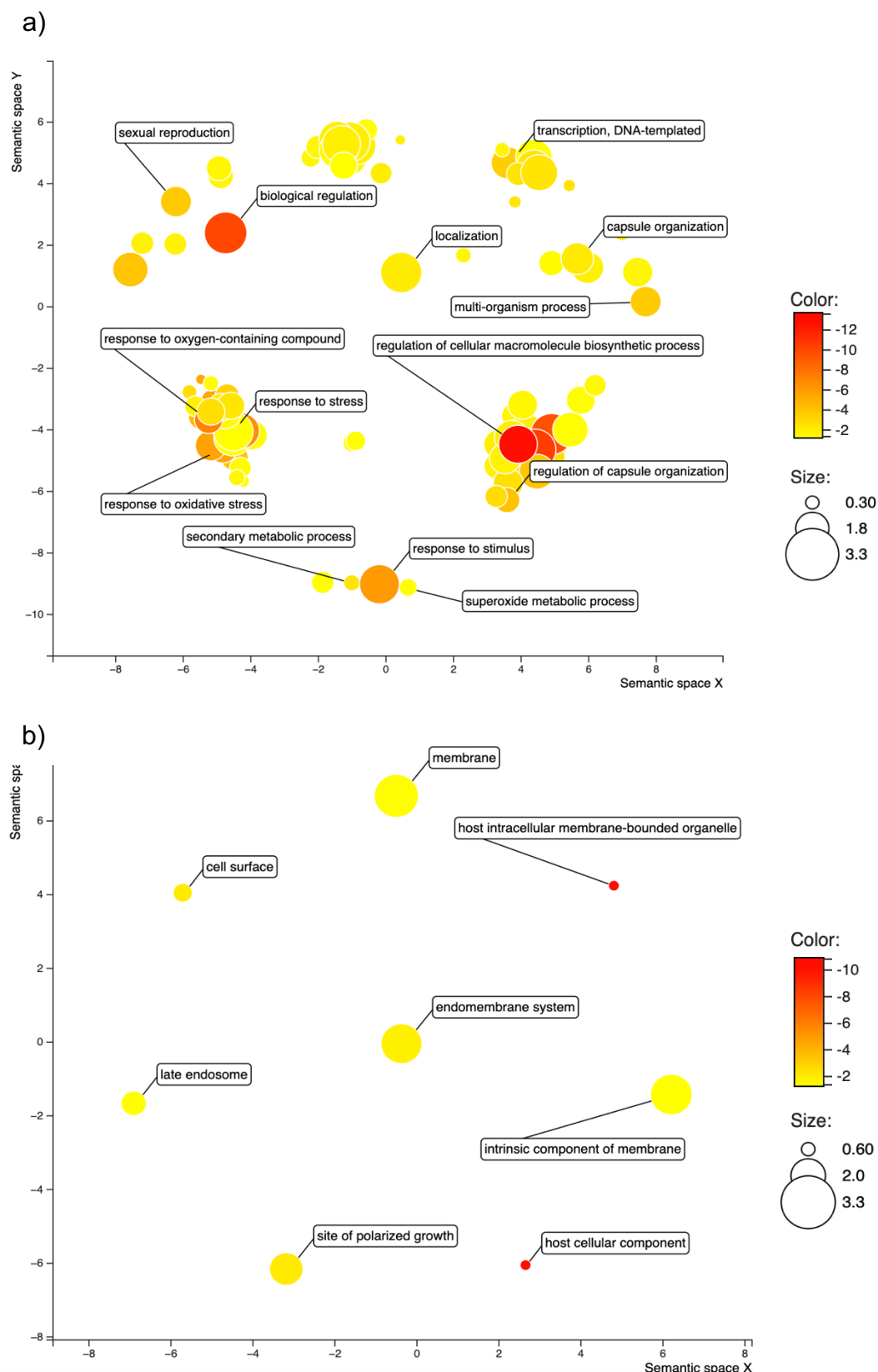


Figure 42. Revigo plots of GO term enrichment list. a) biological process enrichment list, b) cellular component enrichment list. The circle symbolises a gene from the shortlist that has an enriched GO term. The colour represents the GO term enrichment p value whilst the size represents the number of genes with that GO term in the list of identified genes.

So far all of the work done has been *in silico*, and therefore it is important to validate that this mutant screen has worked. To do this, we first looked for genes that are known to play a role in titanisation in the GO term enrichment shortlist. Whilst going through the list one gene that came up was *RIM101*. Rim101 is a capsule inducing transcription factor which has been shown to control virulence factor production, but is also necessary for titanisation (Okagaki et al., 2011, Gish et al., 2016, Hommel et al., 2018). Rim101 GO terms include response to lithium ion, chitin localisation, and polysaccharide localisation (appendix). In the mutant screen, HRMAN detected the *rim101Δ* in 3 out of 5 of the rates of titanisation methods, all showing it to be hypotitanising. This confirms the data from the screen reflects what has been shown in previous studies, increasing confidence that other, previously undescribed genes identified in the screen will also be bona fide titan regulators.

Using the GO term enrichment list, the genes in the mutant screen that are highly implicated based on the odds ratio were investigated further as to their role in titanisation. From the biological process ontology (appendix 1), two genes came up with odds ratio of infinity: peroxin 13 (*PEX13*) and peroxin 14 (*PEX14*). Their GO terms were protein import into peroxisome matrix, docking, and peroxisomal membrane transport. Not a lot is known about the role of peroxin in *C. neoformans* particularly Pex13 and Pex14, whose function has not yet been investigated. But peroxins are present in plants, mammals and yeast and are known to be necessary proteins that are required for the peroxisome assembly (Crookes and Olsen, 1999). A peroxisome is found in all eukaryotic cells in the cytoplasm, and is a single membrane-bound organelle. They have been found to be involved in reactive oxygen species conversion and metabolism of lipids (Idnurm et al., 2007). They have been seen as vital in microbial virulence, such as in *Magnaporthe oryzae* and *Colletotrichum lagenarium*

(Wang et al., 2013, Kimura et al., 2001). In the mutant screen, *pex13Δ* was shown to be hypertitanising in 2 out of 5 rates of titanisation methods. Whilst *PEX14Δ* was shown to be hypotitanising in 1 out of 5 rates of titanisation methods. These mutants were taken for further investigation shown in section 5.2.4.

In the molecular function ontology GO term enrichment list (appendix 3), another two genes also had odds ratio of infinity: alpha-alpha-trehalase (Aat1) and Nth1. Their GO terms were trehalase activity and alpha-alpha-trehalase activity. Interestingly, our previous data has shown that the trehalose pathway does play a role in titanisation, where both *tps1Δ* and *tps2Δ* produced more titan cells (figure 32). *NTH1*, neutral trehalase, is known to encode the enzyme trehalase which converts trehalose to glucose (Petzold et al., 2006). In the mutant screen, Aat1 gene was hypotitanising in 1 out of the 5 rates of titanisation methods. Nth1 was hypertitanising in 2 out of 5 of the titanisation methods. These mutants were taken for further investigation shown in section 5.2.4.

5.2.4 Validation of hits

In order to validate the computationally predicted hits identified in the previous section, I first repeated the phenotypic analysis of a limited number of mutants. This was done through fluorescence microscopy, and was only carried out as one biological repeat owing to time constraints due to covid restrictions. Flow cytometry was also not carried out due to time constraints.

As mentioned earlier, computational analysis said Pex13 and Pex14 have differing titan inducing capabilities, with *pex13Δ* predicted to be hypertitanising and *pex14Δ* to be hypotitanising. To confirm this, we did qualitative analysis to see if titan cells were present

in differing conditions. In figure 43, *pex13* Δ was able to form large cells that could be titan cells in low density cultures, which is also the titan inducing culture. However, in high density cultures, which is the capsule inducing conditions, they mainly seem to form yeast with the occasional large cell present (figure 43b). For *pex14* Δ , there were very few large cells present in the low-density culture and the majority seem to be yeast cells. But interestingly, in the high-density culture there were slightly larger cells and they seem to cluster together. Alongside this, *pex14* Δ also had a different morphology with being slightly longer and oval in shape (figure 43c). This confirmed the computational analysis findings, and further confirmed our confidence in the success of the screen.

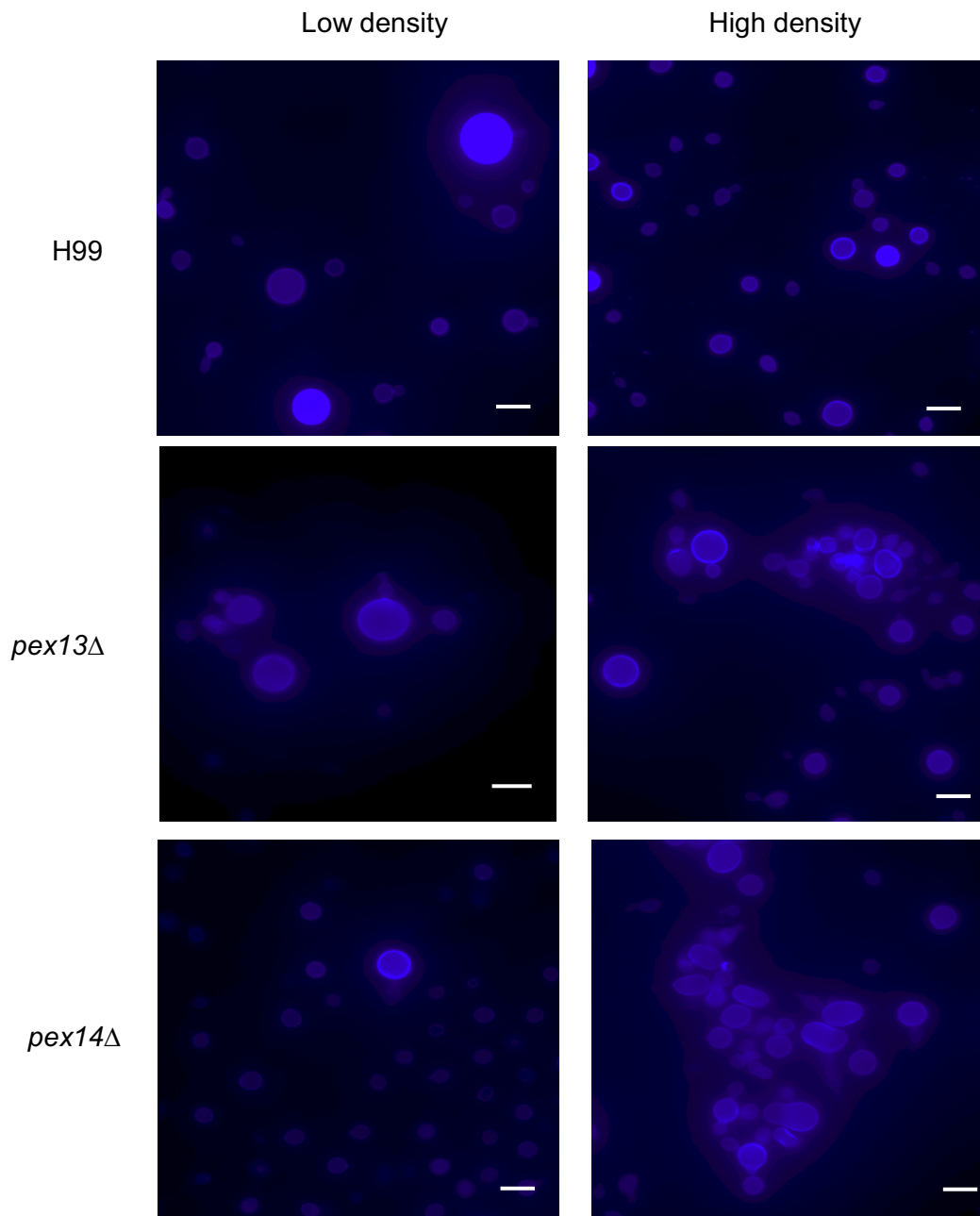


Figure 43. Pex13 and Pex14 phenotype confirm the computational analysis. H99, *pex13*Δ and *pex14*Δ were grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS in two different densities, high (OD= 0.1) and low (OD= 0.001). Microscopy images of H99 and the mutant's representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μm.

The next set of genes that were of interest through the computational analysis were *AAT1* and *NTH1*, which were predicted to be hypotitanising and hypertitanising respectively.

As I did not know the official name of Aat1 or its similarities with Nth1, I decided to compare the amino acid sequence of the two trehalases. I used an online server called Clustal Omega, and the results indicated that the two were not similar (figure 44a). Furthermore, when looking at the percentage identity matrix, which measures the number of identical amino acids in relation to the alignment length, there was 23.57% which suggests that they are not orthologous genes (figure 44b).

When looking at the phenotype of these mutants during different conditions, *aat1*Δ did not produce large cells in titan inducing condition (low density), likewise in the capsule inducing conditions (high density), although the odd large cell was seen (figure 45b). On the other hand, *nth1*Δ mutant produced large cells in low density conditions that could potentially be titan cells (figure 45c), similar to that of the wild type (figure 45a). During high density condition the cells were slightly smaller (figure 45c). This confirms the computational predictions as it was predicted that Aat1 mutant would be hypotitanising whilst Nth1 mutant would be hypertitanising.

a)

Aat1	-----	0
Nth1	MSSQPLEKAKLPVPKLLFIENDVPPSTTPSTATTPTDQQQDPFAQAGDKAELLTRALHE	60
Aat1	-----MSRLLSWAAFS-----	11
Nth1	SQAPTQPMSPNGVPRLGKLGQLETRLPDINAEPNEYGGAQVTSRRATFSNVGVEGEFK	120
Aat1	-----LLSLTAAQNTQTTSFSFSP	31
Nth1	RRPMQMGGEKISRNRRLSHDESTPSAPRRYLIDVEETMRVLVEQEDTDNNFQISIIDSGP	180
Aat1	TPVSTSVFSPPTAPLNSTVPGQGIYPPIQALCAGGANVPFCPGVLLQDVQLSGIFSFSKTF	91
Nth1	KLLSLGTASSNA--HKTFDIRGT-----YMLSNLLQELALARDYGRKRIV	223
Aat1	V-----DKPTAKTL-NETLSAWEALGDNVTVGQVETFEVQYFKGEGLELSQIQLENF--	142
Nth1	LDEARLAENPVDRLSRMKNSFWNSLTRID-----AEGLEIACADPKRNSQ	270
Aat1	-----VEDP-----AIL-DNIADPVFRAWVKVNGYWTLLA	172
Nth1	HVRARIYIPHGEDEMAEHYQIAREKPNLKLEVDILPKCEDSP---AFVKTLNDKPGILA	327
Aat1	RETNSALCNGDCESSLIPLNHTIIVPGGRYREIYYWDSFWVLEGLLKSELYDAWDLQ	232
Nth1	LAMDKKVDANGKTHL---EALPFVVPGARFNEKYGWDSYFMALGLLDVGRIDLAKSIVE	383
Aat1	NFMDIVDVGYLPNGQRKYLLNRSQPPVFQMDIAYIKTT-----NNITLLERALPVAS	286
Nth1	HCIFELIKYYNKVLNGNRSYLCRSQPPFLTFALQIYNQLDRSKPAENKEWLKRTIQAAI	443
Aat1	A-ELEWANNRTSNFTSPFTNKSRIIARYSVTNSAPRPEGYVEDFETVM-----GAS-	337
Nth1	KEYHRYWMQEPPLDVV-----TGLSRYRPDGLGIPPETEASHFTHLPYQKIGISV	496
Aat1	-----PALNETEQAEELYSELATGAESGWDYSSR---WCEQPLLNTDNNPALRT--	383
Nth1	NEYIDGYNDGTIKEPELDEYFLHDPGVRESGHDTSYRLDRKCGDL--ATVDLNSLLKYE	554
Aat1	LKIKSIIPVDLLSLMAGDHALLANMYELYN-----STGGGESGNEKKAKRNGSGDDSD	438
Nth1	FDIASAIQV---AFDDELILEEFELSPWPITAEAFKAGATYELSTK-----TPQ	601
Aat1	ASKIAYHRQMAQEFSDSILDLCWDPEKSWFYDFNMTSNSRSNIFHPGGAWPLWQNIPTSE	498
Nth1	TSRHWF--EAAKRRKATMDELWNQDQGMFYDYDTKLKQARYSVTSLWPLWAGSASEE	659
Aat1	IAGNESAAFSVSGFRFLGHYSV---PSVATLLFTGLNWDFPNAPPHAYTSIKAFET	555
Nth1	QALKLV--RNALPKFEVAGGLVSGTEESRGVISLDRPNRQWDYPYGPWPHQIMAWVGLER	717
Aat1	LGRMLPNAAVLSNLTIPFDSVTQNQLGLSESELQFPQSTIGNVSLNTETSQDKPWPLAL	615
Nth1	YGFVDDASRLA-----YRWIYMM	735
Aat1	SIEFANRYLGAFCWSYSTGGEISGLLTQLPLSDLNATGTYTSEQSGVMFEKFNVTDTDA	675
Nth1	TLSFM-----DFNGIVPEKFDVVELS-----HMVDAEYGN	765
Aat1	AGGGGEYTVQVGFGTNGVALWAAGEYGYI--PAPTCP-LIPIIEVNAT---SSSNS--	727
Nth1	QGTDFRYVPREGFGWMNAAYQIGLQCLSTGMRRAAACVPPWVFFNLPAPDFTSARKRRA	825
Aat1	---SD-----SSVYNSTDEGPTASDNTTSTSLFVGYRIPRE-----	762
Nth1	EREARDAAEAASGHGGVPKNEVHNEPPSLEQA--IASLKLELTAPYSKSVNK	875

b)

1: Aat1	100.00	23.52
2: Nth1	23.52	100.00

Figure 44. Aat1 and Nth1 are not similar in amino acid sequences. The online programme Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to generate this data. a) Alignment of the two trehalases. An “*” means that there is a fully conserved residue, the “.” means that there is a conservation of similar properties between the two group, and the “:” means that there is a conservation of weakly similar properties between the two groups. b) Percentage identity matrix shows the number of identical amino acids concerning the alignment length.

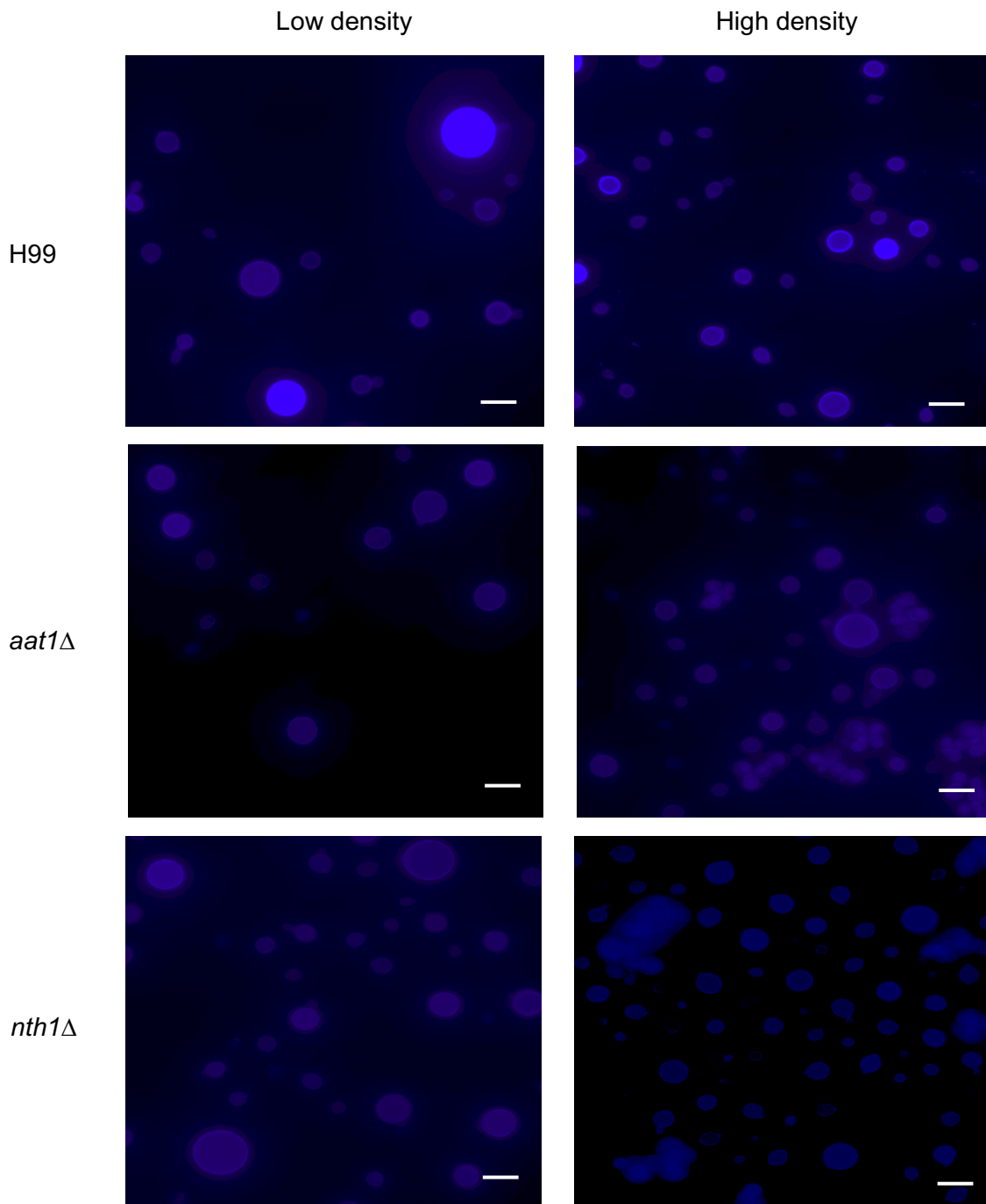


Figure 45. Aat1 and Nth1 have slightly different phenotypes to those in the computational analysis. H99, *aat1Δ* and *nth1Δ* were grown in YNB overnight followed by induction of Titan cells in 10% HI-FCS with PBS in two different densities, high (OD= 0.1) and low (OD= 0.001). Microscopy images of H99 and the mutants representative of 5 technical repeats. Cells were stained for chitin with CFW, scale bar of 10 μ m.

Following the effects of titanisation *in vitro* of the mutant strains, we used an RNA dataset, created by Diana Tamayo at University of Birmingham, to compare the mRNA expression of these genes in different growth conditions over time. A common technique used to identify regulators of virulence phenotypes is to perform RNAseq and generate expression profiles of differential expressed genes in inducing and non-inducing conditions. Therefore, we might expect to see differences in expression patterns of our top hit genes in an RNAseq analysis that compares titan-inducing (low density) and capsule-inducing (high density) conditions. Briefly, Diana measured expression of genes in wild type (H99) cells grown either in YNB or in capsule or titan inducing (YT) conditions. Capsule and YT are different only in the density of cells used in the starting culture. For both conditions, cells were grown overnight in YNB, then inoculated at OD600= 0.1 (capsule) or OD600= 0.001 (YT) into 10% FCS in PBS and incubated at 37°C, 5% CO₂.

Rim101 is a well-studied transcription factor in *C. neoformans*, and its role in titan cell formation has also been accounted for (Okagaki et al., 2011). I queried the RNAseq database and found that when cells were transferred from YNB to capsule or titan inducing conditions, the transcription of *RIM101* increased from 100 to 500 tpm (figure 46a). When the cells were grown in titan inducing conditions, *RIM101* transcript levels remain relatively stable across the time course and between conditions. Interestingly, when the cells were filtered based on size at this time point, the large cells in the population had a slight increase in expression of *RIM101* in comparison to the small cells in the population. In capsule growth conditions the expression was almost the same across the time points (figure 46a). Interestingly, when the data was normalised to actin and the fold change was determined, *RIM101* had a higher fold change in capsule inducing conditions for 14h (figure 46b).

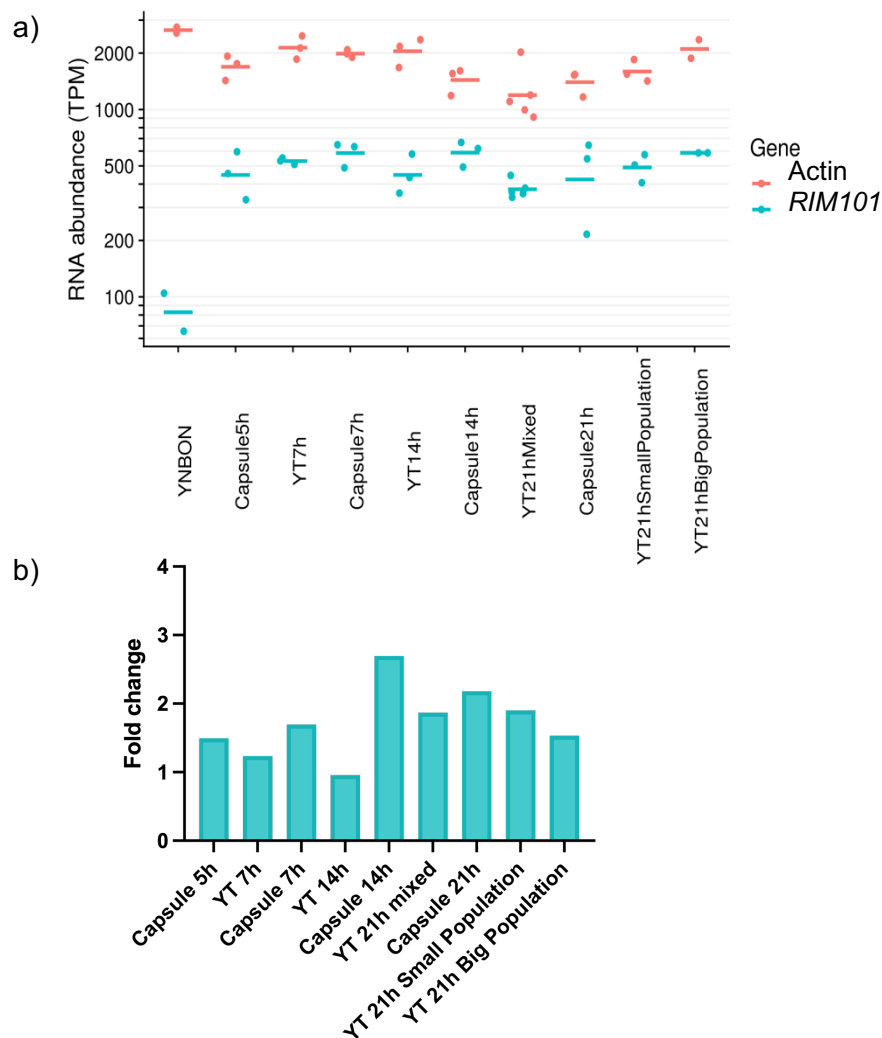


Figure 46. Rim101 transcriptional expression using RNAseq database. a) Cells were grown in YNB media overnight (YNBON), capsule inducing conditions (high density, 5% CO₂, 37°C) at 3 different time points and titan inducing conditions (YT) (low density, 5% CO₂, 37°C) at 3 different time points. The last titan inducing condition time point was also separated out based on population size, big and small. Transcripts per million (TPM) was used as the measurement for RNA abundance. Control gene actin is in red and RIM101 is in green. Database created by Tamayo et al. unpublished, University of Birmingham and Edinburgh university. b) taking the raw data from this RNA seq, I normalised the *RIM101* data to actin followed by calculating the fold change in each of the conditions.

Next, I examined the expression of peroxin 13 and peroxin 14, where transcript levels for *PEX13* and *PEX14* remain the same across the experimental conditions, with relatively low abundance throughout (figure 47a). During titan inducing conditions, peroxin 13 transcription level was lower than that of peroxin 14, except when the cells were filtered for size at 21

hours. What was also noticeable was the very low level of transcripts per million in comparison to actin (figure 47a). Likewise, when the data was normalised to actin, *PEX13* and *PEX14* fold change was low, with *PEX13* having no fold change in some conditions (figure 47b and c).

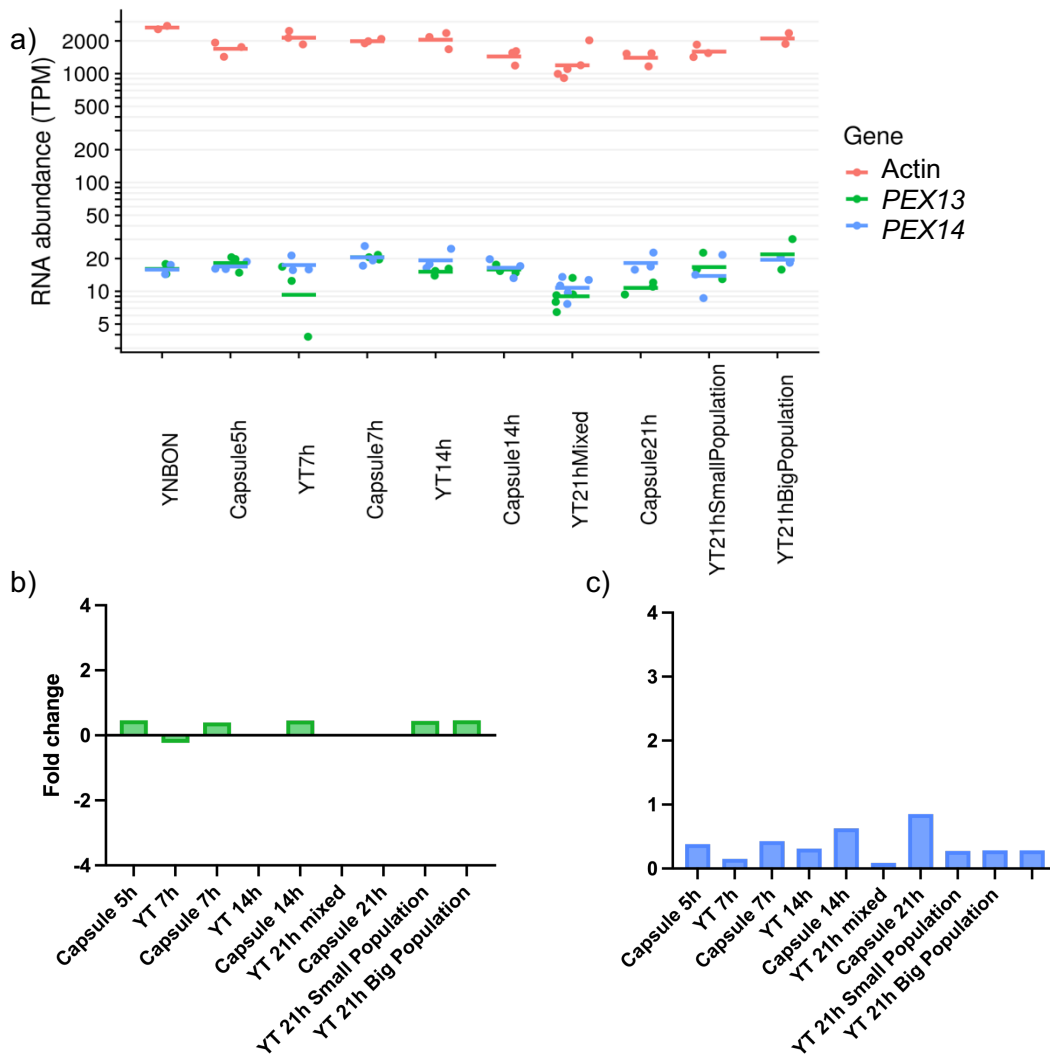


Figure 47. Comparison of peroxin 13 and peroxin 14 transcriptional expression using RNAseq database. a) Cells were grown in YNB media overnight (YNBON), capsule inducing conditions (high density, 5% CO₂, 37°C) at 3 different time points and titan inducing conditions (YT) (low density, 5% CO₂, 37°C) at 3 different time points. The last titan inducing condition time point was also separated out based on population size, big and small. Transcripts per million (TPM) was used as the measurement for RNA abundance. Control gene actin is in red, *PEX13* is in green, and *PEX14* is in blue. Database created by Tamayo et al. unpublished. Taking the raw data from this RNA seq, I normalised the b) *PEX13* and c) *PEX14* data to actin followed by calculating the fold change in each of the conditions.

Similar to that of peroxin, the rate of transcripts per million for *AAT1* and *NTH1* were much lower than that of actin. But there were differences between *AAT1* and *NTH1*, with the latter expressing more transcripts in each condition. For *NTH1* (blue), the rate of transcripts was higher in non titanising conditions, but was consistent in capsule growth conditions. For titan inducing conditions, the expression of transcripts was lower than that of capsule but was

consistent, except when the cells were separated based on size. *AAT1* (green) also had a similar pattern in that the transcripts per million were higher in capsule growth conditions than in titan inducing growth conditions (figure 48a). Intriguingly, when looking at the fold change following normalisation of the data to actin, *NTH1* had some negative fold change or no fold change at all, whilst *AAT1* only had a negative fold change when the cells were grown in titan inducing conditions (figure 48b and c).

Together these findings highlight that the HRMAN assisted high throughput screen was able to identify genes that would not be identified using transcriptional analysis.

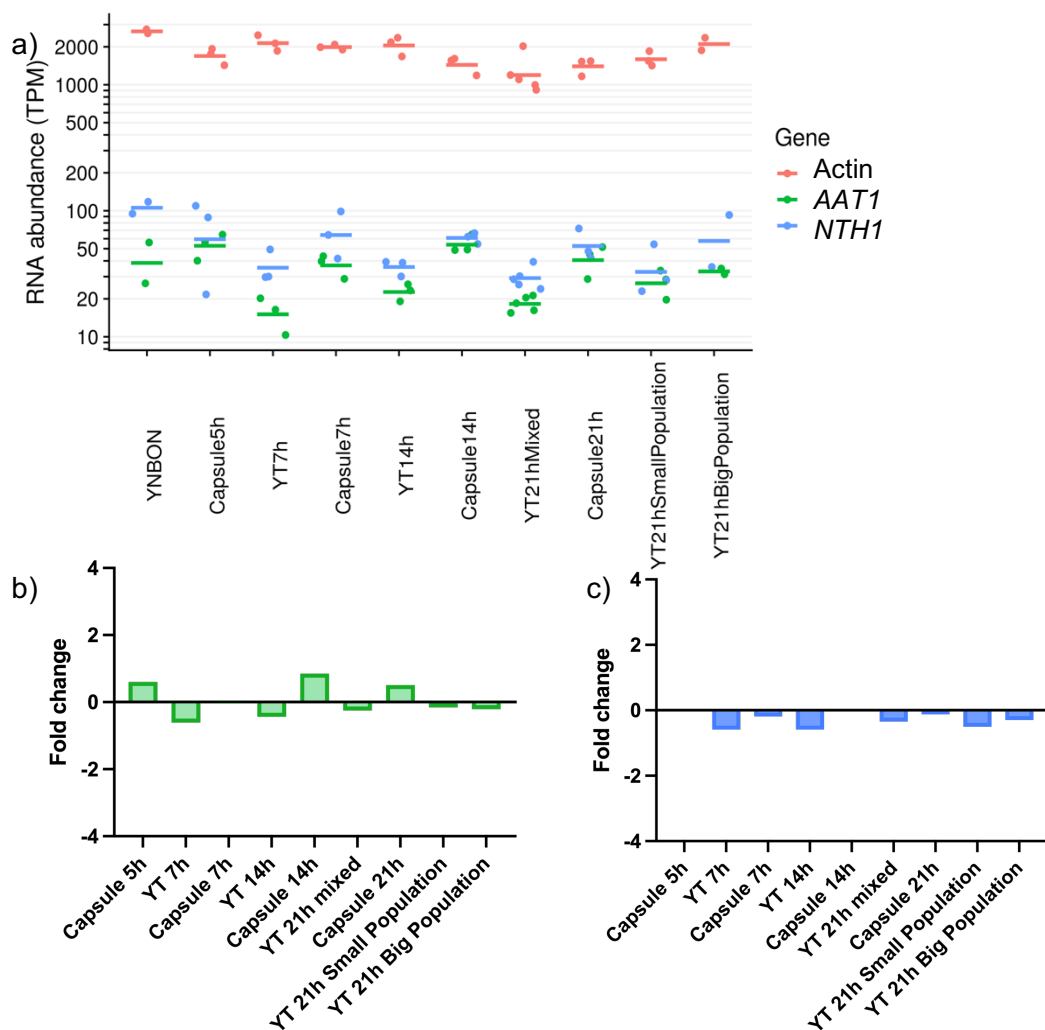


Figure 48. Comparison of Aat1 and Nth1 transcriptional expression using RNAseq database. Cells were grown in YNB media overnight (YNBON), capsule inducing conditions (high density, 5% CO₂, 37°C) at 3 different time points and titan inducing conditions (YT) (low density, 5% CO₂, 37°C) at 3 different time points. The last titan inducing condition time point was also separated out based on population size, big and small. Transcripts per million (TPM) was used as the measurement for RNA abundance. Control gene actin is in red, alpha alpha-trehalase is in green, and NTH1 is in blue. Database created by Tamayo et al. unpublished. Taking the raw data from this RNA seq, I normalised the b) *PEX13* and c) *PEX14* data to actin followed by calculating the fold change in each of the conditions.

5.3 Discussion

5.3.1 Successfully carried out a high throughput screen using *in vitro* culture and *in silico* analysis

There have been very limited studies on the molecular mechanisms that drive titanisation due to no *in vitro* model being available. Most knowledge in titan cells have been obtained from the characterisation of cells isolated from infected mice (Okagaki et al., 2010). However, there are now *in vitro* models available and, in this study, one model was used to successfully carry out a high throughput screen of three deletion mutant libraries. This screen has provided a large amount of data regarding the induction of titan cells, which if there were no *in vitro* models available would have required the use of approximately 1200 mice.

Whilst culturing the mutant strains, they were all grown under titan inducing conditions (figure 39). This could possibly mean that strains which are temperature sensitive, such as *tps1Δ* and *tps2Δ* (figure 34), may not have formed titan cells in this screen. And this is the case as we have data that confirms titanisation in *tps* mutants, but at a lower temperature (figure 35). But it was important to make sure that the screen conditions remained consistent throughout, which means the same temperature for all strains.

This high throughput analysis generated a large amount of data, with over 43,200 images across three channels, necessitating a high throughput analysis pipeline. Collaborating with Daniel Fisch from the Frickel lab in University of Birmingham allowed us to use an *in silico* programme to analyse the data (figure 39).

HRMAN successfully analysed 4,193 wells images which was 87.4% of the screen. This allowed for a widespread dataset of mutant phenotypes which were not known in the literature. However, it should be pointed out that 12.6% of the wells were excluded as either contaminated or out of focus. This means there was a chance that an important mutant strain which may have been significant to the induction of titan cells was possibly excluded. Despite this, the screen allowed us to generate a large list of mutants which may play a role in titanisation. Future work will repeat the excluded wells to limit the possibility that key genes will be missed from the overall analysis.

5.3.2 Bioinformatics tools allowed further analysis of this screen

A shortlist was generated with the mutant strains that had a z score of ± 2 (table 11), following which a Venn diagram was produced to visually show the genes identified by each method (figure 41). Interestingly, more genes were identified by multiple methods in the hypotitanising category than in the hypertitanising category. There was also a gene with a z-score of <-2 , that was present in every method. CNAG_05010, which encodes a hypothetical protein of unknown function (figure 41a). While the 5 methods in general identified the same number of hypotitanising strains, the circularity and k-means methods both identified very small numbers of hypertitanising strains. In the circularity method for genes with a z-score of >2 , there was only 1 gene present and this gene did not overlap with any of the other categories (figure 41b). This suggests that circularity was not a good measure for hypertitanisation.

What is important to note here is that although we stained the cells with CFW and sybr green, the automated analysis was unable to resolve data in the images in the green channel. Therefore, all the methods of measurements mentioned using HRMAN have mainly focused on cell size. Studies have shown the main characteristics of a titan cell *in vivo* are their large size, high DNA content, thick cell wall and dense capsule (Okagaki et al., 2010, Zaragoza et al., 2010). Just focusing on the cell size for this screen may be limiting the search for titan cell regulators. However, as mentioned in the previous chapter, we have shown all titan cells have a high DNA content but not all cells with a high DNA content are titan cells. In this screen, the genes that have been shown to surpass the threshold were taken for further validation and future work would include using flow cytometry to measure the DNA content of the cells.

The genes that surpassed the set threshold were then chosen for GO term analysis, to find GO term clusters associated with a given phenotype, using FungiDB followed by visualisation using Revigo using a medium stringency filter so as to not remove any non-redundant GO terms, and *Saccharomyces cerevisiae* was chosen as the reference species. Although there are similarities between *S. cerevisiae* and *C. neoformans* which would allow this analysis to be fungi-specific, there are also many differences between the two evolutionarily distant species, which may lead to misattributed functions. Revigo's axis are arbitrary and the graph itself could possibly be quite non-linear, but the concept is that GO terms which are similar semantically are close to each other on the graph (Supek et al., 2011). In this study, there were more GO term clusters in the biological process ontology (figure 42a) than in the cellular component ontology (figure 42b), which is a more specific and limiting descriptor.

5.3.3 *rim101Δ* is hypotitanising in this screen, reflecting what the literature shows and confirming the success of the screen

As most of the analysis of this screen has been done *in silico*, a way to validate the screen was to find genes in the GO term enrichment list that are known to play a particular role in titanisation. *RIM101* was found to have a relatively high odds ratio and the *rim101Δ* mutant was also seen in 3 out of 5 of the analysis methods as hypotitanising. Rim101, a transcription factor downstream of the PKA pathway, has been shown to play a role in virulence factors of *C. neoformans*. In particular, cell wall gene expression is controlled by the rim pathway and, once activated, allows the cell to adapt to the environmental change in the host (Okagaki et al., 2011, Hommel et al., 2018, Trevijano-Contador et al., 2020). The *rim101Δ* mutant has been seen to be defective in the formation of titan cells and this could be due to the cell wall formation and capsule defect. In this study, Rim101 was a good positive control as it was found in the z-score cutoff point in 3 out of 5 of the analysis methods. This gave an increased confidence that the screen could identify hypotitanising strains along with the *in silico* analysis, all whilst being carried out *in vitro*.

When looking into the role of Rim101 in titanisation, we looked into the number of transcripts produced in different conditions using an RNA sequencing database. The transcript count for Rim101 was low when the cells were grown in YNB overnight, but relatively high in all other conditions. However, there were no large differences in transcription of Rim101 in titan inducing condition and capsule inducing conditions, nor were there differences between the timepoints of the conditions (figure 46). Rim101 is a highly conserved transcription factor in many other fungal pathogens, and in *C. neoformans* the protein is modified and processed to allow adaptation in environmental changes and host stress (O'Meara et al., 2014). These

protein-level changes will not be reflected at the transcriptional level. This suggests that approaches that rely only on transcriptional profiling to identify regulators of titan cell formation will miss important regulators that are post-transcriptionally regulated and further reinforces the benefits of our high throughput screening approach.

5.3.4 Peroxins may also play a role in titanisation

Whilst looking at the GO term enrichment list, we found genes that are highly implicated based on odds ratio but do not seem to have a direct role in titanisation. In the biological process ontology list (appendix 1), there were genes with an odds ratio of infinity: *PEX13* and *PEX14*. *pex13Δ* was seen to be hypertitanising in 2 out of the 5 methods of rates of titanisation, whilst *pex14Δ* was seen to be hypotitanising in 1 out of the 5 methods of rates of titanisation.

PEX genes are known to encode peroxin proteins, which are involved in the importation of peroxisomal membrane proteins (PMPs) along with matrix proteins from the cytosol to the peroxisome (Lazarow and Fujiki, 1985, Wang et al., 2019). Peroxisomes are single membrane-bound organelles that are found throughout eukaryotic cells and are where essential metabolic processes such as removal of ROS and β -oxidation of fatty acids take place (Lazarow and Fujiki, 1985, Wang et al., 2019). In the literature, peroxisomes have been demonstrated to play a role in virulence in some fungal pathogens. For example, in *Magnaporthe oryzae*, a rice plant fungus, Pex11 and Pex1 are necessary for pathogenicity and virulence (Wang et al., 2015, Deng et al., 2016).

There are few studies which have looked into the role of peroxins in *C.neoformans* (Idnurm et al., 2007). But none have specifically looked into the role of *PEX13* and *PEX14*. In filamentous fungi, Pex13 and Pex14 are responsible for forming the receptor-docking complex (Lazarow, 2003). However, a study carried out by Fujihara *et al* showed deletion of *Collectotrichum orbiculare PEX13* caused melanisation defectiveness and the inability to infiltrate host plants (Fujihara et al., 2010). Similar was seen in *M. oryzae*, where deletion of *PEX13* caused defects in morphogenesis, and pathogenicity was dramatically reduced (Wang *et al.*, 2019). Interestingly, *pex13Δ* in this screen caused increased titan cell formation (figure 43b), which may suggest that it does not play a role in virulence, contrast to other fungal pathogens. A study carried out by Wang *et al* demonstrated the role of Pex14 was less than that of Pex13 in *M. oryzae* in fungal development and import of peroxisomal proteins (Wang et al., 2019). Intriguingly, they found the defects in development of *pex14Δ* in *M. oryzae* to be moderate (Wang et al., 2019). Similarly, *pex14Δ* was only seen to be hypotitanising in 1 out of 5 of the rates of titanisation, suggesting it is not a strong phenotype, and may not be completely necessary for titanisation. But when we carried out microscopy to better understand the pex mutant phenotypes, there were mainly yeast/small cells seen (figure 43c), compared to the wild type (figure 43a). Although the hypotitanising trait of *pex14Δ* was seen in only one of the computational methods, it seems accurate and gives us confidence in the screen data.

5.3.5 Further evidence shows the trehalose pathway plays a role in titanisation

In the molecular function ontology GO term enrichment list, 2 genes from a familiar pathway were also found to have infinity odds ratio: alpha alpha-trehalase and Nth1. These genes are part of the trehalose synthesis pathway, and we have previously shown other genes in this pathway to play a role in titanisation (chapter 4). The alpha-alpha-trehalase does not

have an official name but in this study, we have named it Aat1, whilst Nth1 is also known to be an alpha-alpha-trehalase. When looking at the protein sequence to see if there were any similarities between the two, there was 23.57% (figure 44b), which suggests they are not orthologous genes and may perform distinct functions in trehalase biosynthesis.

As previously mentioned, trehalose is a disaccharide, and its biosynthesis is closely associated with spore production, virulence and morphogenesis (Botts et al., 2014). Nth1, also known as neutral trehalase, is the enzyme required to convert the used trehalose into glucose in the cytosol (Nwaka et al., 1995, Petzold et al., 2006). Therefore, when Nth1 is not present, there is an increase in trehalose in the cytosol.

Interestingly, in this screen *nth1Δ* was hypertitanising in 2 out of 5 of the titanisation methods. We further confirmed this when looking at the *nth1Δ* mutant under the microscope and found it was hypertitanising. This suggests that accumulating trehalose may be a negative regulator of titanisation. The trehalose pathway has been studied extensively across many fungal pathogens, such as *S. cerevisiae* and *C. gattii*, and all have found *nth1Δ* is dispensable for virulence (Nwaka et al., 1995, Ngamskulrungraj et al., 2009). Similarly, this was also seen in *C. neoformans*, where *nth1Δ* produced phenotypes similar to that of H99 such as melanin production and capsule, key virulence factors, however despite this the mutant was deficient for virulence and failed to undergo sexual development during mating (Petzold et al., 2006, Botts et al., 2014). Whilst the virulence of *nth1Δ* was not studied here, if the mutant has reduced virulence this could be due to the hypertitanisation. Studies have found that strains known to hypertitanise do not disseminate and cause disease well such as Usv101 and Pdr80.

When looking at the transcript level of the gene, *NTH1* transcripts per million were lower than that of actin, but higher than *AAT1* (figure 48). A study carried out in *C. gattii* found when cells were grown in mating medium or rich medium, *NTH1* is highly expressed (Ngamskulrungrroj et al., 2009). Another study carried out in *C. neoformans* found *NTH1* expression was consistent in different medias and sexual conditions (Botts et al., 2014). Future work will examine the relative levels of *NTH1* mRNA under mating, rich medium, and titan inducing conditions.

5.3.6 Future work and improvements

Due to covid-19 and the impact it had on being able to work in the lab, this project could only go this far. This study demonstrated the ability to screen a deletion mutant library successfully *in vitro*. However, one improvement could be to quantify DNA content changes for the library under titan inducing conditions. This could be achieved either by optimizing sybr green nucleic acid stain or through a high throughput flow cytometry approach.

Following the finding of the genes from the ontology list, as a consequence of lockdown and the time limitations, validation of these hits was carried out through microscopy and using the RNA seq database. For future studies it would be interesting to carry out flow cytometry on these mutants after they have been grown in titan conditions, using DAPI to look at the DNA content, but also using a superoxide dye to see if there are differing levels of superoxide. Peroxisomes have been found to consist of antioxidants to regulate the accumulation of ROS (Sandalio and Romero-Puertas, 2015). Therefore, perhaps the deletion of *PEX13* may cause an increase in ROS levels which lead to the hypertitanisation seen in the HRMA analysis and in microscopy.

In this screen, alpha alpha-trehalase was also found to play a role in titanisation, as its mutant was shown to hypotitanise. There is not a specific name for this alpha-alpha-trehalase gene, and the function for it is also unknown in the literature. This made it challenging to look into further, particularly due to the limitation in time caused by covid-19. However, for future studies it would be interesting look into this gene further, how closely linked to Nth1 it is and what role it plays in the trehalose biosynthesis pathway. Comparing this gene with other known alpha-alpha-trehalase in other fungal pathogens would also be a way to finding out what this gene represents.

Chapter 6: Summary

Through the use of our *in vitro* model, this thesis has helped create a foundation for our understanding of the yeast-to-titan morphological transition that takes place in *Cryptococcus neoformans*.

I was able to identify that although aneuploidy has been found to play a role in fluconazole resistance in *C. neoformans*, titan cells which are polyploid do not directly influence fluconazole resistance. However, fluconazole does impact genome plasticity and polyploidy, which contributes to increased resistance. The pathway that causes this increase in genome plasticity is still unknown, but from the data in this thesis I found that independent of the cAMP pathway, a major regulator of the yeast-to-titan transition. As shown in Figure 49a, the addition of fluconazole in the absence of a functional cAMP pathway (*cac1Δ* mutant) still increased DNA content. A similar effect was not observed when *cac1Δ* cells were exposed to RNS stress (NO⁻). When looking at what could be causing this polyploidy, I found that the mitochondria may be playing a role in this yeast-to-titan transition (figure 49b), as well as interacting with fluconazole through AOX that may be facilitating this genome plasticity through increase in superoxide levels (figure 49c). This finding is important because it challenges the literature suggesting that titan cells are a path to fluconazole resistance. I showed that strains that are fundamentally incapable of making titan cells can still increase DNA in response to fluconazole. That tells us that aneuploidy leading to fluconazole resistance in *C. neoformans* is being caused by something other than the pathways that regulate titan cell formation, and that the mitochondria is involved in both mechanisms.

While investigating the role of titan cells in antifungal resistance, I examined inhibiting this morphological phenomenon using drugs that are not fungicidal or fungistatic. These drugs -retinoic acid, metyrapone and nicardipine hydrochloride- are able to reduce the amount of titan cells formed in the population, whilst also reducing superoxide levels in the cell, but they do cause an increase in DNA content via an unknown mechanism (figure 49d). Future studies would investigate the molecular mechanisms behind this. Despite this, my data does reveal a repetitive pattern that although all cells that are titan cells have a high DNA content, not all cells that have a high DNA content are large. This is something that I feel is necessary to keep in mind when defining what a titan cell is, particularly when grown using *in vitro* methods.

I showed throughout the chapters that there are many different pathways that initiate titanisation. In the literature thus far, much of the focus has been on the role of the cAMP pathway (figure 49a). And whilst my data also confirmed the role of the cAMP pathway, I showed by chemically inhibiting certain complexes of the electron transport chain, that the mitochondria are also major regulators of the transition (figure 49b). Inhibiting most of the complexes, except AOX, seemed to cause an increase in cell size, with complex I and III being the major regulators of cell size and DNA content. These data reveal a major new mechanism by which the fungal cell can regulate its size and ploidy and open new avenues for future research.

Finally, multiple threads of work in this project identified the trehalose biosynthesis pathway as a key regulator of cell size and ploidy. First, whilst investigating how fluconazole may influence the capacity of a cell to become a titan cell, I found that

the trehalose biosynthesis pathway influences the yeast-to-titan transition: inhibition of Tps1 induced titanisation (figure 49e). In addition to this, through my high-throughput screen I found another part of the trehalose pathway, Nth1, also plays a role in titanisation. Similar to Tps1, the inhibition of Nth1 also produced large cells (figure 49e). It is possible that all these pathways are cross-linked in a way that shows not one pathway plays an essential role in the yeast-to-titan transition.

Further experiments are required to look into the molecular mechanism by which trehalose impacts titan cell formation, but these findings give further scope into drug discovery. The trehalose biosynthesis pathway is not found in humans, which is key in finding antifungal because fungi are eukaryotes which share metabolic pathways with humans (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5485801/>). To be able to target a fungal metabolic pathway that is unique is key to reducing antifungal toxicity. Therefore, there is potential that blocking this pathway through inhibitors may block titan cell formation, which in turn may be a therapeutic potential as it would not harm humans. Importantly, my work showing that chemical inhibition of titan cell formation can be antagonistic with frontline antifungals urges caution when identify potential drug targets. Specifically, when I combined fluconazole with the drugs that blocked titan cell formation mentioned earlier, retinoic acid, metyrapone and nicardipine hydrochloride, it was antagonistic. However, loss of trehalose biosynthesis function both blocks titan cell formation and induces thermo-sensitivity (failure to grow at 37°C), which makes it a promising target for future investigation. Therefore, more research would need to be carried out to the role of trehalose biosynthesis pathway and its potential as a drug target.

The experiments in this thesis were all carried out using our *in vitro* model, which allowed me to successfully see titan cells without the need for mice. It also uses less materials, making it a model that all are able to use. As one of my aims was to reinforce the 3Rs objectives, I was able to show that through the replacement of animal use, particularly when carrying out my large high-throughput screen. Here I was able to screen over 4000 genes, and without this *in vitro* model I would have needed to use approximately 1200 mice. The data from this screen will be a major advantage for future investigations, and would be extremely beneficial in the fungal community.

I hope that this thesis will be a foundation to be further built upon through additional investigations into the mechanism of titanisation through the role of the mitochondria and the trehalose biosynthesis pathway, and also into the drug discovery potential.

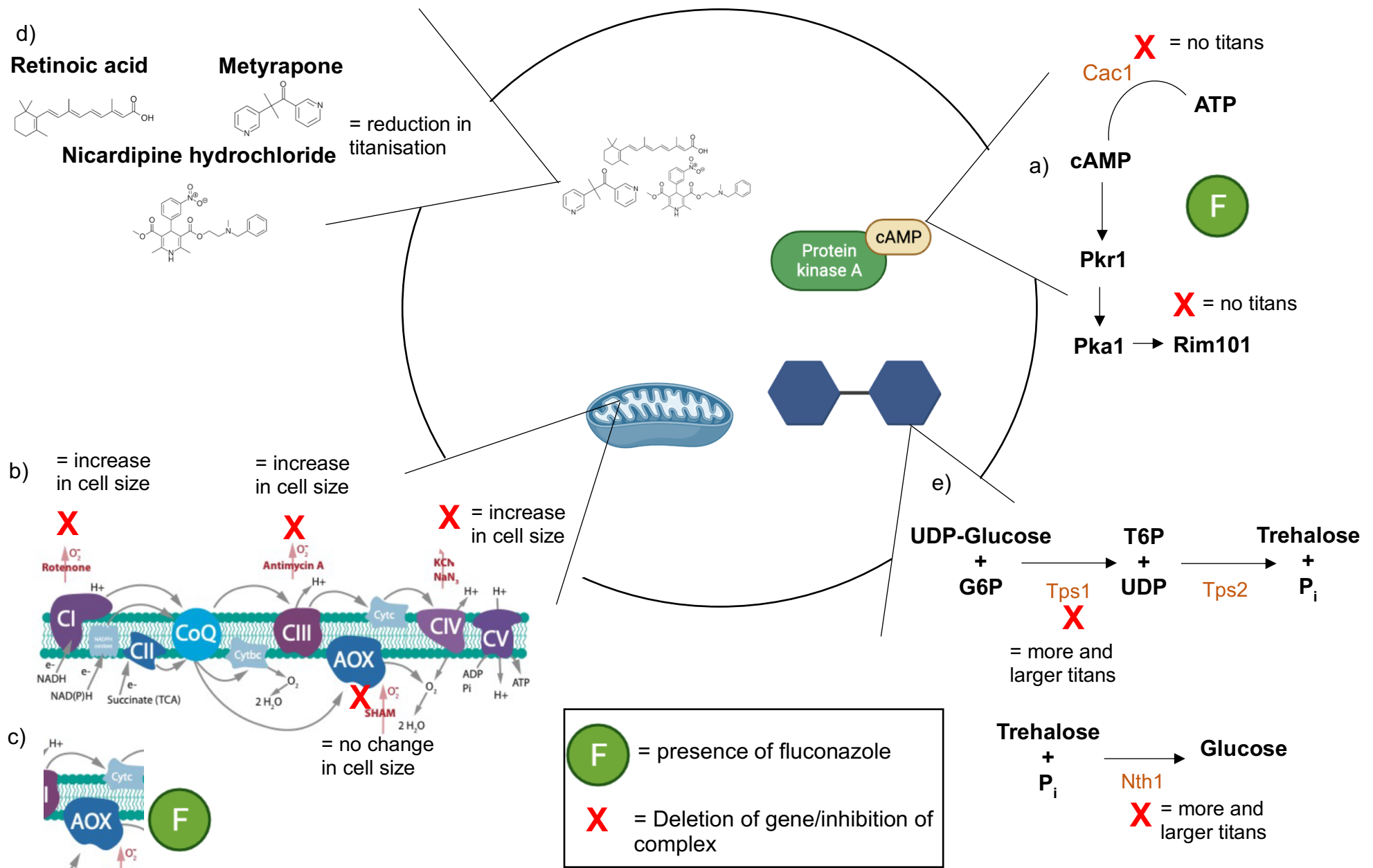


Figure 49. A model diagram of my thesis. Here I depict what I found through the data in this thesis. a) the cAMP pathway plays a role in titanisation and fluconazole seems to partially interact with this pathway to cause the increase in DNA content seen. b) the mitochondria plays a role in the morphological transition, which has been suggested and this data further confirms it. c) fluconazole was found to interact with AOX. d) TI drugs cause a reduction in titanisation, however the reason behind this is not known. e) different parts of the trehalose biosynthesis pathway play a role in the yeast-to-titan transition.

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Appendix

Appendix 1. List of GO terms generated from FungiDB based on the biological process ontology.

ID	Name	Bgd count	Result count	Result gene list	Pct of bgd	Fold enrichment	Odds ratio	P-value	Benjamini	Bonferroni
GO:0016558	protein import into peroxisome matrix	2	2	CNAG_02728,CNAG_06024,	100	11.69	inf	0.007305839656	0.09329452483	1
GO:0016560	protein import into peroxisome matrix, docking	2	2	CNAG_02728,CNAG_06024,	100	11.69	inf	0.007305839656	0.09329452483	1
GO:0015919	peroxisomal membrane transport	2	2	CNAG_02728,CNAG_06024,	100	11.69	inf	0.007305839656	0.09329452483	1
GO:0097549	chromatin organization involved in negative regulation of transcription	3	2	CNAG_01882,CNAG_02922,	66.7	7.79	21.47	0.02067251366	0.1844624296	1
GO:0040029	regulation of gene expression, epigenetic	3	2	CNAG_01882,CNAG_02922,	66.7	7.79	21.47	0.02067251366	0.1844624296	1
GO:0034401	chromatin organization involved in regulation of transcription	3	2	CNAG_01882,CNAG_02922,	66.7	7.79	21.47	0.02067251366	0.1844624296	1
GO:0055129	L-proline biosynthetic process	3	2	CNAG_04990,CNAG_05134,	66.7	7.79	21.47	0.02067251366	0.1844624296	1
GO:0006342	chromatin silencing	3	2	CNAG_01882,CNAG_02922,	66.7	7.79	21.47	0.02067251366	0.1844624296	1
GO:0045814	negative regulation of gene expression, epigenetic	3	2	CNAG_01882,CNAG_02922,	66.7	7.79	21.47	0.02067251366	0.1844624296	1

GO:0010226	response to lithium ion	7	4	CNAG_00396,CNAG_01208,CNAG_01720,CNAG_05431,	57.1	6.68	14.37	0.00150127185	0.02843225054	1
GO:0006033	chitin localization	6	3	CNAG_00396,CNAG_03582,CNAG_05431,	50	5.84	10.75	0.01022608773	0.1143350532	1
GO:00033037	polysaccharide localization	6	3	CNAG_00396,CNAG_03582,CNAG_05431,	50	5.84	10.75	0.01022608773	0.1143350532	1
GO:00043574	peroxisomal transport	4	2	CNAG_02728,CNAG_06024,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:0006801	superoxide metabolic process	4	2	CNAG_01019,CNAG_02292,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:0005991	trehalose metabolic process	4	2	CNAG_03525,CNAG_03785,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:00072663	establishment of protein localization to peroxisome	4	2	CNAG_02728,CNAG_06024,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:0005984	disaccharide metabolic process	4	2	CNAG_03525,CNAG_03785,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:0006625	protein targeting to peroxisome	4	2	CNAG_02728,CNAG_06024,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:00019932	second-messenger-mediated signaling	4	2	CNAG_00156,CNAG_01557,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:0006561	proline biosynthetic process	4	2	CNAG_04990,CNAG_05134,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:00010286	heat acclimation	4	2	CNAG_03115,CNAG_07924,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:00072662	protein localization to peroxisome	4	2	CNAG_02728,CNAG_06024,	50	5.84	10.73	0.03901380597	0.2567717159	1
GO:00019722	calcium-mediated signaling	4	2	CNAG_00156,CNAG_01557,	50	5.84	10.73	0.03901380597	0.2567717159	1

GO:0010446	response to alkaline pH	8	3	CNAG_01720,CNAG_03582,CNAG_05431,	37.5	4.38	6.45	0.025 15120 631	0.2121847 223	1
GO:0009991	response to extracellular stimulus	9	3	CNAG_02775,CNAG_04293,CNAG_05431,	33.3	3.9	5.37	0.035 37540 906	0.2567717 159	1
GO:0031667	response to nutrient levels	9	3	CNAG_02775,CNAG_04293,CNAG_05431,	33.3	3.9	5.37	0.035 37540 906	0.2567717 159	1
GO:0042594	response to starvation	9	3	CNAG_02775,CNAG_04293,CNAG_05431,	33.3	3.9	5.37	0.035 37540 906	0.2567717 159	1
GO:0009268	response to pH	9	3	CNAG_01720,CNAG_03582,CNAG_05431,	33.3	3.9	5.37	0.035 37540 906	0.2567717 159	1
GO:0007031	peroxisome organization	9	3	CNAG_02096,CNAG_02728,CNAG_06024,	33.3	3.9	5.37	0.035 37540 906	0.2567717 159	1
GO:0008213	protein alkylation	13	4	CNAG_01592,CNAG_01882,CNAG_05404,CNAG_06235,	30.8	3.6	4.78	0.020 26089 244	0.1844624 296	1
GO:0006479	protein methylation	13	4	CNAG_01592,CNAG_01882,CNAG_05404,CNAG_06235,	30.8	3.6	4.78	0.020 26089 244	0.1844624 296	1
GO:00071806	protein transmembrane transport	10	3	CNAG_01647,CNAG_02728,CNAG_06024,	30	3.51	4.6	0.047 40178 117	0.2952272 008	1
GO:00065002	intracellular protein transmembrane transport	10	3	CNAG_01647,CNAG_02728,CNAG_06024,	30	3.51	4.6	0.047 40178 117	0.2952272 008	1
GO:1903506	regulation of nucleic acid-templated transcription	236	58	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	24.6	2.87	3.86	2.58E-14	7.97E-12	2.39E-11
GO:0006355	regulation of transcription, DNA-templated	236	58	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_	24.6	2.87	3.86	2.58E-14	7.97E-12	2.39E-11

				03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,							
GO:2001141	regulation of RNA biosynthetic process	236	58	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	24.6	2.87	3.86	2.58E-14	7.97E-12	2.39E-11	
GO:0019219	regulation of nucleobase-containing compound metabolic process	240	58	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	24.2	2.82	3.77	5.73E-14	9.33E-12	5.31E-11	
GO:0051252	regulation of RNA metabolic process	240	58	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	24.2	2.82	3.77	5.73E-14	9.33E-12	5.31E-11	
GO:0010556	regulation of macromolecule biosynthetic process	254	60	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	23.6	2.76	3.67	6.03E-14	9.33E-12	5.60E-11	
GO:2000112	regulation of cellular macromolecule biosynthetic process	251	59	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,C	23.5	2.75	3.64	1.27E-13	1.18E-11	1.18E-10	

				NAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,							
GO:0031327	negative regulation of cellular biosynthetic process	32	8	CNAG_00791,CNAG_00828,CNAG_01882,CNAG_02922,CNAG_03902,CNAG_04090,CNAG_04878,CNAG_06632,	25	2.92	3.61	0.004505013568	0.06532269674	1	
GO:0009890	negative regulation of biosynthetic process	32	8	CNAG_00791,CNAG_00828,CNAG_01882,CNAG_02922,CNAG_03902,CNAG_04090,CNAG_04878,CNAG_06632,	25	2.92	3.61	0.004505013568	0.06532269674	1	
GO:0048022	negative regulation of melanin biosynthetic process	24	6	CNAG_00791,CNAG_00828,CNAG_03902,CNAG_04090,CNAG_04878,CNAG_06632,	25	2.92	3.6	0.01348021499	0.1421549944	1	
GO:1900377	negative regulation of secondary metabolite biosynthetic process	24	6	CNAG_00791,CNAG_00828,CNAG_03902,CNAG_04090,CNAG_04878,CNAG_06632,	25	2.92	3.6	0.01348021499	0.1421549944	1	
GO:0051171	regulation of nitrogen compound metabolic process	272	62	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06553,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	22.8	2.66	3.51	1.26E-13	1.18E-11	1.17E-10	
GO:0080090	regulation of primary metabolic process	273	62	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06553,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	22.7	2.65	3.49	1.51E-13	1.27E-11	1.40E-10	
GO:0010468	regulation of gene expression	273	60	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CN	22	2.57	3.33	1.81E-12	1.27E-10	1.68E-09	

				AG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,						
GO:0033194	response to hydroperoxide	109	25	CNAG_00460,CNAG_00554,CNAG_00830,CNAG_00896,CNAG_01883,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_03116,CNAG_03710,CNAG_04090,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05965,CNAG_06552,CNAG_06553,CNAG_07924,	22.9	2.68	3.31	3.25E-06	0.0001118527639	0.003020024625
GO:0033195	response to alkyl hydroperoxide	109	25	CNAG_00460,CNAG_00554,CNAG_00830,CNAG_00896,CNAG_01883,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_03116,CNAG_03710,CNAG_04090,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05965,CNAG_06552,CNAG_06553,CNAG_07924,	22.9	2.68	3.31	3.25E-06	0.0001118527639	0.003020024625
GO:0072735	response to tert-butyl hydroperoxide	109	25	CNAG_00460,CNAG_00554,CNAG_00830,CNAG_00896,CNAG_01883,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_03116,CNAG_03710,CNAG_04090,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05965,CNAG_06552,CNAG_06553,CNAG_07924,	22.9	2.68	3.31	3.25E-06	0.0001118527639	0.003020024625
GO:0051701	interaction with host	30	7	CNAG_04878,CNAG_05431,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_07435,	23.3	2.73	3.29	0.01150456787	0.1256028116	1
GO:0009889	regulation of biosynthetic process	330	70	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06568,CNAG_06632,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	21.2	2.48	3.23	1.25E-13	1.18E-11	1.16E-10
GO:0031326	regulation of cellular biosynthetic process	330	70	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06568,CNAG_06632,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	21.2	2.48	3.23	1.25E-13	1.18E-11	1.16E-10

GO:0060255	regulation of macromolecule metabolic process	296	63	CNAG_00156,CNAG_00192,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06553,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	21.3	2.49	3.21	2.16E-12	1.34E-10	2.00E-09
GO:0031324	negative regulation of cellular metabolic process	35	8	CNAG_00791,CNAG_00828,CNAG_01882,CNAG_02922,CNAG_03902,CNAG_04090,CNAG_04878,CNAG_06632,	22.9	2.67	3.21	0.00803324263	0.0968172067	1
GO:0031323	regulation of cellular metabolic process	347	72	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06568,CNAG_06632,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	20.7	2.43	3.15	1.72E-13	1.33E-11	1.60E-10
GO:0044403	symbiotic process	31	7	CNAG_04878,CNAG_05431,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_07435,	22.6	2.64	3.15	0.01379692496	0.1422616263	1
GO:0019222	regulation of metabolic process	371	73	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06568,CNAG_06632,CNAG_06762,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	19.7	2.3	2.94	1.91E-12	1.27E-10	1.77E-09
GO:0052126	movement in host environment	28	6	CNAG_04878,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_07435,	21.4	2.5	2.94	0.02822372133	0.2276167347	1
GO:0044409	entry into host	28	6	CNAG_04878,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_07435,	21.4	2.5	2.94	0.02822372133	0.2276167347	1

GO:1901914	negative regulation of capsule organization	67	14	CNAG_02322,CNAG_02487,CNAG_03115,CNAG_03116,CNAG_03183,CNAG_03914,CNAG_04090,CNAG_04353,CNAG_04678,CNAG_04878,CNAG_05538,CNAG_06632,CNAG_06751,CNAG_07901,	20.9	2.44	2.88	0.001304495021	0.02575683786	1
GO:1901913	regulation of capsule organization	107	22	CNAG_00396,CNAG_01704,CNAG_01864,CNAG_02322,CNAG_02487,CNAG_03012,CNAG_03013,CNAG_03115,CNAG_03116,CNAG_03183,CNAG_03914,CNAG_04090,CNAG_04353,CNAG_04678,CNAG_04878,CNAG_05155,CNAG_05216,CNAG_05538,CNAG_05965,CNAG_06632,CNAG_06751,CNAG_07901,	20.6	2.4	2.86	7.70E-05	0.0023806444	0.071419332
GO:0016569	covalent chromatin modification	24	5	CNAG_01587,CNAG_01882,CNAG_02020,CNAG_05404,CNAG_05429,	20.8	2.43	2.83	0.048913186	0.3006055405	1
GO:0016570	Histone modification	24	5	CNAG_01587,CNAG_01882,CNAG_02020,CNAG_05404,CNAG_05429,	20.8	2.43	2.83	0.048913186	0.3006055405	1
GO:0044419	interspecies interaction between organisms	34	7	CNAG_04878,CNAG_05431,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_07435,	20.6	2.41	2.8	0.02260806236	0.1979271875	1
GO:0051129	negative regulation of cellular component organization	77	15	CNAG_01638,CNAG_02322,CNAG_02487,CNAG_03115,CNAG_03116,CNAG_03183,CNAG_03914,CNAG_04090,CNAG_04353,CNAG_04678,CNAG_04878,CNAG_05538,CNAG_06632,CNAG_06751,CNAG_07901,	19.5	2.28	2.64	0.00187557712	0.03412814837	1
GO:0048523	negative regulation of cellular process	109	21	CNAG_00791,CNAG_00828,CNAG_01638,CNAG_01882,CNAG_02322,CNAG_02480,CNAG_02487,CNAG_02922,CNAG_03115,CNAG_03116,CNAG_03183,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04353,CNAG_04678,CNAG_04878,CNAG_05538,CNAG_06632,CNAG_06751,CNAG_07901,	19.3	2.25	2.63	0.0002919720894	0.006451192833	0.270950099
GO:1901700	response to oxygen-containing compound	278	51	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01209,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_01883,CNAG_02066,CNAG_02241,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03115,CNAG_03116,CNAG_03401,CNAG_03710,CNAG_04090,CNAG_04268,CNAG_04353,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05538,CNAG_05642,CNAG_05965,CNAG_06327,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06762,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07924,	18.3	2.14	2.59	7.16E-08	3.50E-06	6.64E-05
GO:0072737	response to diamide	111	21	CNAG_00396,CNAG_00554,CNAG_00791,CNAG_00830,CNAG_00896,CNAG_02066,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03115,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05538,CNAG_05965,CNAG_06751,CNAG_07435,	18.9	2.21	2.57	0.000378662252	0.00787492762	0.3513985698
GO:0051128	regulation of cellular component organization	133	25	CNAG_00156,CNAG_00396,CNAG_01638,CNAG_01704,CNAG_01864,CNAG_02322,CNAG_02487,CNAG_03012,CNAG_03013,CNAG_03115,CNAG_03116,CNAG_03183,CNAG_03914,CNAG_04090,CNAG_04293,CNAG_04353,CNAG_04678,CNAG_04878,CNAG_05155,CNAG_05216,CNAG_05538,CNAG_05965,CNAG_06632,CNAG_06751,CNAG_07901,	18.8	2.2	2.56	0.0001193293286	0.003256988734	0.110737617
GO:0006979	response to oxidative stress	220	40	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00654,CNAG_00791,CNAG_00830,CNAG_00896,CNAG_01454,CNAG_01850,CNAG_01883,CNAG_02066,CNAG_02241,CN	18.2	2.12	2.51	2.59E-06	0.0001001324161	0.002403177985

				AG_02364,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03115,CNAG_03116,CNAG_03710,CNAG_04090,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05965,CNAG_06327,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06762,CNAG_07464,CNAG_07924,						
GO:000003	reproduction	147	27	CNAG_00396,CNAG_00791,CNAG_00896,CNAG_01209,CNAG_01647,CNAG_01850,CNAG_01883,CNAG_02490,CNAG_02678,CNAG_03116,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_04758,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05301,CNAG_05642,CNAG_05866,CNAG_06203,CNAG_06552,CNAG_06553,CNAG_06814,CNAG_07435,CNAG_07679,CNAG_07924,	18.4	2.15	2.5	9.68E-05	0.002723323783	0.08986968483
GO:0022414	reproductive process	147	27	CNAG_00396,CNAG_00791,CNAG_00896,CNAG_01209,CNAG_01647,CNAG_01850,CNAG_01883,CNAG_02490,CNAG_02678,CNAG_03116,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_04758,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05301,CNAG_05642,CNAG_05866,CNAG_06203,CNAG_06552,CNAG_06553,CNAG_06814,CNAG_07435,CNAG_07679,CNAG_07924,	18.4	2.15	2.5	9.68E-05	0.002723323783	0.08986968483
GO:0019953	sexual reproduction	136	25	CNAG_00396,CNAG_00791,CNAG_00896,CNAG_01209,CNAG_01647,CNAG_01850,CNAG_01883,CNAG_03116,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_04758,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05301,CNAG_05642,CNAG_05866,CNAG_06203,CNAG_06552,CNAG_06553,CNAG_06814,CNAG_07435,CNAG_07679,CNAG_07924,	18.4	2.15	2.49	0.0001729141939	0.00422274663	0.1604643719
GO:0051704	multi-organism process	136	25	CNAG_00396,CNAG_00791,CNAG_00896,CNAG_01209,CNAG_01647,CNAG_01850,CNAG_01883,CNAG_03116,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_04758,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05301,CNAG_05642,CNAG_05866,CNAG_06203,CNAG_06552,CNAG_06553,CNAG_06814,CNAG_07435,CNAG_07679,CNAG_07924,	18.4	2.15	2.49	0.0001729141939	0.00422274663	0.1604643719
GO:0044703	multi-organism reproductive process	136	25	CNAG_00396,CNAG_00791,CNAG_00896,CNAG_01209,CNAG_01647,CNAG_01850,CNAG_01883,CNAG_03116,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_04758,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05301,CNAG_05642,CNAG_05866,CNAG_06203,CNAG_06552,CNAG_06553,CNAG_06814,CNAG_07435,CNAG_07679,CNAG_07924,	18.4	2.15	2.49	0.0001729141939	0.00422274663	0.1604643719
GO:0009651	response to salt stress	136	25	CNAG_00156,CNAG_00554,CNAG_00668,CNAG_00896,CNAG_01704,CNAG_01883,CNAG_02066,CNAG_02364,CNAG_02487,CNAG_02526,CNAG_03115,CNAG_03116,CNAG_04630,CNAG_04678,CNAG_04841,CNAG_05155,CNAG_05170,CNAG_05380,CNAG_05431,CNAG_05965,CNAG_06339,CNAG_06553,CNAG_06751,CNAG_07435,CNAG_07464,	18.4	2.15	2.49	0.0001729141939	0.00422274663	0.1604643719
GO:0006970	response to osmotic stress	154	28	CNAG_00039,CNAG_00156,CNAG_00396,CNAG_00554,CNAG_00668,CNAG_00896,CNAG_01704,CNAG_01720,CNAG_01883,CNAG_02066,CNAG_02364,CNAG_02487,CNAG_02526,CNAG_03115,CNAG_03116,CNAG_04630,CNAG_04678,CNAG_04841,CNAG_05155,CNAG_05170,CNAG_05380,CNAG_05431,CNAG_05965,CNAG_06339,CNAG_06553,CNAG_06751,CNAG_07435,CNAG_07464,	18.2	2.12	2.47	8.67E-05	0.002595507974	0.08046074718
GO:0050794	regulation of cellular process	528	89	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01557,CNAG_01638,CNAG_01704,CNAG_01850,CNAG_01864,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03605,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04293,CNAG_04353,CNAG_04505,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05216,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05538,CNAG_05642,CNAG_05643,	16.9	1.97	2.46	4.61E-11	2.67E-09	4.28E-08

				_05687,CNAG_05838,CNAG_05965,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06568,CNAG_06632,CNAG_06751,CNAG_06762,CNAG_06772,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,						
GO:0007618	matings	126	23	CNAG_00396,CNAG_00791,CNAG_00896,CNAG_01209,CNAG_01647,CNAG_01850,CNAG_01883,CNAG_03116,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_04758,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05301,CNAG_05642,CNAG_06203,CNAG_06552,CNAG_06553,CNAG_06814,CNAG_07435,CNAG_07924,	18.3	2.13	2.46	0.0003481395992	0.007513338327	0.3230735481
GO:0042493	response to drug	54	10	CNAG_00396,CNAG_01208,CNAG_01720,CNAG_01850,CNAG_04090,CNAG_04505,CNAG_04678,CNAG_05431,CNAG_06327,CNAG_07464,	18.5	2.16	2.46	0.01475284059	0.1504465502	1
GO:000302	response to reactive oxygen species	132	24	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00791,CNAG_01454,CNAG_01850,CNAG_02066,CNAG_02241,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03115,CNAG_04630,CNAG_04678,CNAG_05019,CNAG_05200,CNAG_05431,CNAG_06327,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06762,CNAG_07464,	18.2	2.12	2.45	0.0002765753046	0.006260045918	0.2566618827
GO:0009628	response to abiotic stimulus	251	44	CNAG_00039,CNAG_00156,CNAG_00396,CNAG_00554,CNAG_00668,CNAG_00691,CNAG_00896,CNAG_01272,CNAG_01704,CNAG_01720,CNAG_01850,CNAG_01883,CNAG_02066,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_02602,CNAG_03115,CNAG_03116,CNAG_03316,CNAG_03582,CNAG_04090,CNAG_04268,CNAG_04630,CNAG_04678,CNAG_04681,CNAG_04737,CNAG_04841,CNAG_04878,CNAG_05155,CNAG_05170,CNAG_05380,CNAG_05431,CNAG_05654,CNAG_05915,CNAG_05965,CNAG_06339,CNAG_06553,CNAG_06632,CNAG_06751,CNAG_07435,CNAG_07464,CNAG_07924,	17.5	2.05	2.41	2.23E-06	8.99E-05	0.002067658141
GO:0006351	transcription, DNA-templated	147	26	CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01587,CNAG_01972,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03183,CNAG_03431,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04841,CNAG_05049,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06871,CNAG_07011,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	17.7	2.07	2.38	0.0002460370133	0.00570805871	0.2283223484
GO:00097659	nucleic acid-templated transcription	147	26	CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01587,CNAG_01972,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03183,CNAG_03431,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04841,CNAG_05049,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06871,CNAG_07011,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	17.7	2.07	2.38	0.0002460370133	0.00570805871	0.2283223484
GO:0042438	melanin biosynthetic process	95	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	17.9	2.09	2.38	0.002517423128	0.04247579386	1
GO:0006582	melanin metabolic process	95	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	17.9	2.09	2.38	0.002517423128	0.04247579386	1
GO:0044550	secondary metabolite biosynthetic process	95	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	17.9	2.09	2.38	0.002517423128	0.04247579386	1
GO:0046189	phenol-containing compound biosynthetic process	95	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	17.9	2.09	2.38	0.002517423128	0.04247579386	1

GO:0042542	response to hydrogen peroxide	130	23	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00791,CNAG_01454,CNAG_01850,CNAG_02066,CNAG_02241,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03115,CNAG_04630,CNAG_04678,CNAG_05019,CNAG_05200,CNAG_05431,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06762,CNAG_07464,	17.7	2.07	2.37	0.0005543451301	0.01118331045	0.5144322808
GO:0050789	regulation of biological process	554	90	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01557,CNAG_01638,CNAG_01704,CNAG_01850,CNAG_01864,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03431,CNAG_03605,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04293,CNAG_04353,CNAG_04505,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05216,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05524,CNAG_05538,CNAG_05642,CNAG_05687,CNAG_05838,CNAG_05965,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06568,CNAG_06632,CNAG_06751,CNAG_06762,CNAG_06772,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	16.2	1.9	2.35	2.76E-10	1.42E-08	2.56E-07
GO:0018958	phenol-containing compound metabolic process	96	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	17.7	2.07	2.35	0.002824426778	0.04571848751	1
GO:0019748	secondary metabolic process	96	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	17.7	2.07	2.35	0.002824426778	0.04571848751	1
GO:1904576	response to tunicamycin	108	19	CNAG_00156,CNAG_00396,CNAG_00791,CNAG_01454,CNAG_02490,CNAG_03116,CNAG_03902,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05380,CNAG_05431,CNAG_05538,CNAG_06632,CNAG_07435,CNAG_07464,	17.6	2.06	2.34	0.001767324024	0.03280153389	1
GO:0010035	response to inorganic substance	210	36	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00791,CNAG_01208,CNAG_01209,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_02066,CNAG_02241,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03902,CNAG_04090,CNAG_04353,CNAG_04505,CNAG_04630,CNAG_04678,CNAG_05019,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05431,CNAG_05965,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06762,CNAG_07317,CNAG_07464,	17.1	2	2.32	3.23E-05	0.001072141048	0.03001994935
GO:0065007	biological regulation	617	98	CNAG_00156,CNAG_00192,CNAG_00396,CNAG_00514,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01209,CNAG_01318,CNAG_01454,CNAG_01487,CNAG_01557,CNAG_01638,CNAG_01704,CNAG_01850,CNAG_01864,CNAG_01882,CNAG_01883,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02922,CNAG_03012,CNAG_03013,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03431,CNAG_03605,CNAG_03637,CNAG_03710,CNAG_03769,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04108,CNAG_04184,CNAG_04293,CNAG_04353,CNAG_04505,CNAG_04534,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05004,CNAG_05010,CNAG_05019,CNAG_05049,CNAG_05155,CNAG_05170,CNAG_05216,CNAG_05333,CNAG_05380,CNAG_05429,CNAG_05431,CNAG_05524,CNAG_05538,CNAG_05642,C	15.9	1.86	2.31	1.33E-10	7.26E-09	1.23E-07

				NAG_05687,CNAG_05838,CNAG_05965,CNAG_06327,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06568,CNAG_06632,CNAG_06751,CNAG_06762,CNAG_06772,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07566,CNAG_07593,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,						
GO:0032774	RNA biosynthetic process	151	26	CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01587,CNAG_01972,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03183,CNAG_03431,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04841,CNAG_05049,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06871,CNAG_07011,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	17.2	2.01	2.3	0.0003818661023	0.00787492762	0.3543717429
GO:0048519	negative regulation of biological process	130	22	CNAG_00791,CNAG_00828,CNAG_01638,CNAG_01882,CNAG_02322,CNAG_02480,CNAG_02487,CNAG_02922,CNAG_03115,CNAG_03116,CNAG_03183,CNAG_03345,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04353,CNAG_04678,CNAG_04878,CNAG_05538,CNAG_06632,CNAG_06751,CNAG_07901,	16.9	1.98	2.24	0.001343405223	0.02597250099	1
GO:0042221	response to chemical	407	65	CNAG_00039,CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00654,CNAG_00668,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01208,CNAG_01209,CNAG_01454,CNAG_01611,CNAG_01704,CNAG_01720,CNAG_01850,CNAG_01883,CNAG_02066,CNAG_02241,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03401,CNAG_03710,CNAG_03902,CNAG_04090,CNAG_04268,CNAG_04353,CNAG_04505,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_04807,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05538,CNAG_05642,CNAG_05965,CNAG_06327,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06751,CNAG_06762,CNAG_06814,CNAG_07317,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07924,	16	1.87	2.21	2.56E-07	1.19E-05	0.0002377947777
GO:0045230	capsule organization	107	18	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_01720,CNAG_02241,CNAG_03582,CNAG_03902,CNAG_04090,CNAG_04184,CNAG_04353,CNAG_04505,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_05817,CNAG_06762,CNAG_07464,CNAG_07924,	16.8	1.97	2.21	0.003835535879	0.05740931123	1
GO:1900376	regulation of secondary metabolite biosynthetic process	89	15	CNAG_00396,CNAG_00791,CNAG_00828,CNAG_02490,CNAG_03012,CNAG_03013,CNAG_03902,CNAG_04090,CNAG_04108,CNAG_04678,CNAG_04878,CNAG_05155,CNAG_06552,CNAG_06568,CNAG_06632,	16.9	1.97	2.21	0.007839093093	0.0957194525	1
GO:0043455	regulation of secondary metabolic process	89	15	CNAG_00396,CNAG_00791,CNAG_00828,CNAG_02490,CNAG_03012,CNAG_03013,CNAG_03902,CNAG_04090,CNAG_04108,CNAG_04678,CNAG_04878,CNAG_05155,CNAG_06552,CNAG_06568,CNAG_06632,	16.9	1.97	2.21	0.007839093093	0.0957194525	1
GO:0048021	regulation of melanin biosynthetic process	89	15	CNAG_00396,CNAG_00791,CNAG_00828,CNAG_02490,CNAG_03012,CNAG_03013,CNAG_03902,CNAG_04090,CNAG_04108,CNAG_04678,CNAG_04878,CNAG_05155,CNAG_06552,CNAG_06568,CNAG_06632,	16.9	1.97	2.21	0.007839093093	0.0957194525	1
GO:0010038	response to metal ion	104	17	CNAG_00396,CNAG_00554,CNAG_01208,CNAG_01209,CNAG_01720,CNAG_02487,CNAG_02490,CNAG_03018,CNAG_03902,CNAG_04090,CNAG_04353,CNAG_04505,CNAG_05155,CNAG_05216,CNAG_05431,CNAG_06762,CNAG_07317,	16.3	1.91	2.13	0.006581343938	0.08981598786	1
GO:0010033	response to organic substance	278	44	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01209,CNAG_01720,CNAG_01850,CNAG_01883,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_03116,CNAG_03401,CNAG_03710,CNAG_04090,CNAG_04268,CNAG_04353,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_04878,CNAG_05019,CNAG_	15.8	1.85	2.12	3.36E-05	0.001076023965	0.03120469499

				05155,CNAG_05170,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05431,CNAG_05538,CNAG_05642,CNAG_05965,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_07317,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07924,						
GO:0072736	cellular response to tert-butyl hydroperoxide	61	10	CNAG_00554,CNAG_02487,CNAG_02490,CNAG_04678,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05965,CNAG_06552,CNAG_06553,	16.4	1.92	2.12	0.03258578466	0.2478656407	1
GO:0071447	cellular response to hydroperoxide	61	10	CNAG_00554,CNAG_02487,CNAG_02490,CNAG_04678,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05965,CNAG_06552,CNAG_06553,	16.4	1.92	2.12	0.03258578466	0.2478656407	1
GO:0071448	cellular response to alkyl hydroperoxide	61	10	CNAG_00554,CNAG_02487,CNAG_02490,CNAG_04678,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05965,CNAG_06552,CNAG_06553,	16.4	1.92	2.12	0.03258578466	0.2478656407	1
GO:0009892	negative regulation of metabolic process	55	9	CNAG_00791,CNAG_00828,CNAG_01882,CNAG_02922,CNAG_03345,CNAG_03902,CNAG_04090,CNAG_04878,CNAG_06632,	16.4	1.91	2.11	0.04188940561	0.2692930968	1
GO:0006950	response to stress	467	71	CNAG_00039,CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00566,CNAG_00654,CNAG_00668,CNAG_00691,CNAG_00791,CNAG_00830,CNAG_00896,CNAG_01272,CNAG_01454,CNAG_01464,CNAG_01704,CNAG_01720,CNAG_01850,CNAG_01882,CNAG_01883,CNAG_02020,CNAG_02066,CNAG_02241,CNAG_02364,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_02555,CNAG_02602,CNAG_02775,CNAG_03115,CNAG_03116,CNAG_03316,CNAG_03449,CNAG_03637,CNAG_03710,CNAG_04090,CNAG_04268,CNAG_04293,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_04681,CNAG_04737,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05177,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05429,CNAG_05431,CNAG_05654,CNAG_05915,CNAG_05965,CNAG_05977,CNAG_06327,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06751,CNAG_06762,CNAG_06793,CNAG_07435,CNAG_07464,CNAG_07924,	15.2	1.78	2.09	4.81E-07	2.13E-05	0.0004467699239
GO:0036245	cellular response to menadione	68	11	CNAG_00896,CNAG_03710,CNAG_04678,CNAG_05155,CNAG_05216,CNAG_05380,CNAG_05538,CNAG_05642,CNAG_06339,CNAG_06553,CNAG_07435,	16.2	1.89	2.09	0.02809163117	0.2276167347	1
GO:1901617	organic hydroxy compound biosynthetic process	119	19	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_02834,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05200,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	16	1.87	2.08	0.005448635093	0.07661111161	1
GO:0046686	response to cadmium ion	88	14	CNAG_00396,CNAG_00554,CNAG_01209,CNAG_02487,CNAG_02490,CNAG_03018,CNAG_03902,CNAG_04090,CNAG_04353,CNAG_04505,CNAG_05155,CNAG_05216,CNAG_06762,CNAG_07317,	15.9	1.86	2.06	0.01637125986	0.1633605285	1
GO:1901655	cellular response to ketone	69	11	CNAG_00896,CNAG_03710,CNAG_04678,CNAG_05155,CNAG_05216,CNAG_05380,CNAG_05538,CNAG_05642,CNAG_06339,CNAG_06553,CNAG_07435,	15.9	1.86	2.05	0.03095293266	0.2442325279	1
GO:1902074	response to salt	156	24	CNAG_00039,CNAG_00156,CNAG_00554,CNAG_01209,CNAG_01611,CNAG_02241,CNAG_02487,CNAG_03018,CNAG_03710,CNAG_03902,CNAG_04353,CNAG_04678,CNAG_04807,CN	15.4	1.8	2	0.003139205136	0.04855303944	1

				AG_04878,CNAG_05019,CNAG_05170,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_06814,CNAG_07317,CNAG_07435,CNAG_07464,						
GO:0072706	response to sodium dodecyl sulfate	156	24	CNAG_00039,CNAG_00156,CNAG_00554,CNAG_01209,CNAG_01611,CNAG_02241,CNAG_02487,CNAG_03018,CNAG_03710,CNAG_03902,CNAG_04353,CNAG_04678,CNAG_04807,CNAG_04878,CNAG_05019,CNAG_05170,CNAG_05538,CNAG_06552,CNAG_06553,CNAG_06751,CNAG_06814,CNAG_07317,CNAG_07435,CNAG_07464,	15.4	1.8	2	0.003139205136	0.04855303944	1
GO:1905181	regulation of urease activity	84	13	CNAG_00396,CNAG_01209,CNAG_01704,CNAG_03914,CNAG_04090,CNAG_04678,CNAG_04878,CNAG_05010,CNAG_05431,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06814,	15.5	1.81	1.99	0.02504042688	0.2121847223	1
GO:0072720	response to dithiothreitol	131	20	CNAG_00554,CNAG_00791,CNAG_00828,CNAG_01209,CNAG_01850,CNAG_02487,CNAG_02490,CNAG_03401,CNAG_04268,CNAG_04353,CNAG_04630,CNAG_04678,CNAG_05155,CNAG_05170,CNAG_05216,CNAG_06483,CNAG_06553,CNAG_07435,CNAG_07464,CNAG_07593,	15.3	1.78	1.97	0.007338901199	0.09329452483	1
GO:0046148	pigment biosynthetic process	111	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	15.3	1.79	1.97	0.01252414706	0.1351442845	1
GO:0042440	pigment metabolic process	112	17	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	15.2	1.77	1.95	0.01363999968	0.1422238169	1
GO:1901654	response to ketone	72	11	CNAG_00896,CNAG_03710,CNAG_04678,CNAG_05155,CNAG_05216,CNAG_05380,CNAG_05538,CNAG_05642,CNAG_06339,CNAG_06553,CNAG_07435,	15.3	1.79	1.95	0.04077374978	0.266465069	1
GO:0045229	external encapsulating structure organization	127	19	CNAG_00156,CNAG_00396,CNAG_00460,CNAG_01720,CNAG_01758,CNAG_02241,CNAG_03582,CNAG_03902,CNAG_04090,CNAG_04184,CNAG_04353,CNAG_04505,CNAG_04863,CNAG_05081,CNAG_05431,CNAG_05817,CNAG_06762,CNAG_07464,CNAG_07924,	15	1.75	1.92	0.01095027273	0.1209744416	1
GO:0050896	response to stimulus	651	90	CNAG_00039,CNAG_00156,CNAG_00396,CNAG_00460,CNAG_00554,CNAG_00566,CNAG_00654,CNAG_00668,CNAG_00691,CNAG_00791,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01208,CNAG_01209,CNAG_01272,CNAG_01454,CNAG_01464,CNAG_01557,CNAG_01611,CNAG_01704,CNAG_01720,CNAG_01850,CNAG_01882,CNAG_01883,CNAG_02020,CNAG_02066,CNAG_02241,CNAG_02364,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_02555,CNAG_02602,CNAG_02775,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03316,CNAG_03401,CNAG_03449,CNAG_03582,CNAG_03637,CNAG_03710,CNAG_03902,CNAG_04090,CNAG_04268,CNAG_04293,CNAG_04353,CNAG_04505,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_04681,CNAG_04737,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05177,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05429,CNAG_05431,CNAG_05538,CNAG_05642,CNAG_05654,CNAG_05838,CNAG_05915,CNAG_05965,CNAG_05977,CNAG_06327,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06751,CNAG_06762,CNAG_06793,CNAG_06814,CNAG_07317,CNAG_07435,CNAG_07464,CNAG_07593,CNAG_07924,	13.8	1.62	1.9	9.30E-07	3.92E-05	0.0008634009914
GO:1901698	response to nitrogen compound	163	24	CNAG_00396,CNAG_00554,CNAG_00791,CNAG_00830,CNAG_00896,CNAG_02066,CNAG_02364,CNAG_02487,CNAG_02490,CNAG_02555,CNAG_03115,CNAG_03116,CNAG_04630,CNAG_04678,CNAG_04807,CNAG_05019,CNAG_05155,CNAG_05170,CNAG_05216,CNAG_05538,CNAG_05965,CNAG_06751,CNAG_07435,CNAG_07464,	14.7	1.72	1.89	0.005567670088	0.0771163857	1

GO:1901615	organic hydroxy compound metabolic process	133	19	CNAG_00396,CNAG_01173,CNAG_01454,CNAG_01720,CNAG_01850,CNAG_02834,CNAG_03345,CNAG_03582,CNAG_04090,CNAG_04637,CNAG_04807,CNAG_04863,CNAG_05081,CNAG_05200,CNAG_05431,CNAG_06483,CNAG_06552,CNAG_07435,CNAG_07464,	14.3	1.67	1.82	0.01745692916	0.1723407474	1
GO:0051336	regulation of hydrolase activity	98	14	CNAG_00396,CNAG_01209,CNAG_01704,CNAG_03914,CNAG_04090,CNAG_04678,CNAG_04878,CNAG_05010,CNAG_05431,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06814,CNAG_07566,	14.3	1.67	1.81	0.03804726496	0.2567717159	1
GO:0048518	positive regulation of biological process	121	17	CNAG_00156,CNAG_00396,CNAG_01557,CNAG_01704,CNAG_01864,CNAG_02490,CNAG_03013,CNAG_04108,CNAG_04678,CNAG_05004,CNAG_05155,CNAG_05216,CNAG_05965,CNAG_06552,CNAG_06568,CNAG_06772,CNAG_07924,	14	1.64	1.78	0.02752867346	0.2276167347	1
GO:0048522	positive regulation of cellular process	121	17	CNAG_00156,CNAG_00396,CNAG_01557,CNAG_01704,CNAG_01864,CNAG_02490,CNAG_03013,CNAG_04108,CNAG_04678,CNAG_05004,CNAG_05155,CNAG_05216,CNAG_05965,CNAG_06552,CNAG_06568,CNAG_06772,CNAG_07924,	14	1.64	1.78	0.02752867346	0.2276167347	1
GO:0065009	regulation of molecular function	107	15	CNAG_00396,CNAG_01209,CNAG_01704,CNAG_03914,CNAG_04090,CNAG_04678,CNAG_04878,CNAG_05010,CNAG_05431,CNAG_05524,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06814,CNAG_07566,	14	1.64	1.77	0.03765505863	0.2567717159	1
GO:0050790	regulation of catalytic activity	107	15	CNAG_00396,CNAG_01209,CNAG_01704,CNAG_03914,CNAG_04090,CNAG_04678,CNAG_04878,CNAG_05010,CNAG_05431,CNAG_05524,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06814,CNAG_07566,	14	1.64	1.77	0.03765505863	0.2567717159	1
GO:0071705	nitrogen compound transport	208	28	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03587,CNAG_03651,CNAG_04293,CNAG_04556,CNAG_04758,CNAG_04863,CNAG_06024,CNAG_06738,CNAG_06753,CNAG_06772,CNAG_07322,CNAG_07685,	13.5	1.57	1.71	0.009997460435	0.1143350532	1
GO:0033036	macromolecule localization	225	30	CNAG_00396,CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03582,CNAG_03587,CNAG_03651,CNAG_04151,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_05431,CNAG_06024,CNAG_06738,CNAG_06753,CNAG_06772,CNAG_07322,	13.3	1.56	1.69	0.008925389213	0.1035345149	1
GO:0015031	protein transport	191	25	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03587,CNAG_03651,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_06024,CNAG_06738,CNAG_06772,CNAG_07322,	13.1	1.53	1.65	0.02007667762	0.1844624296	1
GO:0015833	peptide transport	191	25	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03587,CNAG_03651,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_06024,CNAG_06738,CNAG_06772,CNAG_07322,	13.1	1.53	1.65	0.02007667762	0.1844624296	1
GO:0042886	amide transport	192	25	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03587,CNAG_03651,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_06024,CNAG_06738,CNAG_06772,CNAG_07322,	13	1.52	1.64	0.02130028183	0.1882539194	1

GO:0071310	cellular response to organic substance	185	24	CNAG_00156,CNAG_00554,CNAG_00896,CNAG_01209,CNAG_01850,CNAG_02487,CNAG_02490,CNAG_03710,CNAG_04268,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05538,CNAG_05642,CNAG_05965,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_07317,CNAG_07435,	13	1.52	1.63	0.02473639006	0.2121847223	1
GO:0045184	establishment of protein localization	194	25	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03587,CNAG_03651,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_06024,CNAG_06738,CNAG_06772,CNAG_07322,	12.9	1.51	1.62	0.02392192567	0.2074724021	1
GO:0019438	aromatic compound biosynthetic process	380	48	CNAG_00396,CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01587,CNAG_01720,CNAG_01850,CNAG_01972,CNAG_02285,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03183,CNAG_03320,CNAG_03345,CNAG_03431,CNAG_03582,CNAG_04090,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04637,CNAG_04807,CNAG_04841,CNAG_04863,CNAG_04961,CNAG_05049,CNAG_05081,CNAG_05200,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05431,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06871,CNAG_07011,CNAG_07435,CNAG_07464,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	12.6	1.48	1.61	0.003229066801	0.04912416379	1
GO:1901362	organic cyclic compound biosynthetic process	407	51	CNAG_00396,CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01587,CNAG_01720,CNAG_01850,CNAG_01972,CNAG_02285,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_02834,CNAG_03018,CNAG_03183,CNAG_03320,CNAG_03345,CNAG_03431,CNAG_03582,CNAG_04090,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04637,CNAG_04807,CNAG_04841,CNAG_04863,CNAG_04961,CNAG_04990,CNAG_05049,CNAG_05081,CNAG_05134,CNAG_05200,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05431,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06552,CNAG_06871,CNAG_07011,CNAG_07435,CNAG_07464,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	12.5	1.46	1.6	0.002857405469	0.04571848751	1
GO:0034654	nucleobase-containing compound biosynthetic process	245	31	CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01587,CNAG_01972,CNAG_02285,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03183,CNAG_03320,CNAG_03431,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04841,CNAG_04961,CNAG_05049,CNAG_05081,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06871,CNAG_07011,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	12.7	1.48	1.59	0.01631480449	0.1633605285	1
GO:1901701	cellular response to oxygen-containing compound	189	24	CNAG_00396,CNAG_00554,CNAG_00896,CNAG_01209,CNAG_01850,CNAG_02487,CNAG_02490,CNAG_03710,CNAG_04268,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05538,CNAG_05642,CNAG_05965,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_07435,	12.7	1.48	1.59	0.0310554292	0.2442325279	1
GO:0008104	protein localization	199	25	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03587,CNAG_03651,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_06024,CNAG_06738,CNAG_06772,CNAG_07322,	12.6	1.47	1.57	0.031572254	0.2462105186	1
GO:0055085	transmembrane transport	350	43	CNAG_00433,CNAG_00529,CNAG_00540,CNAG_00574,CNAG_00749,CNAG_01208,CNAG_01318,CNAG_01384,CNAG_01472,CNAG_01575,CNAG_01647,CNAG_01704,CNAG_01960,CNAG_02010,CNAG_02083,CNAG_02254,CNAG_02728,CNAG_02733,CNAG_02806,CNAG_03013,CNAG_03426,CNAG_03565,CNAG_03605,CNAG_03909,CNAG_04758,CNAG_04982,CNAG_05130,CNAG_05300,CNAG_05340,CNAG_05381,CNAG_05616,CNAG_05817,CNAG_06024,CNAG_06323,CNAG_06561,CNAG_06776,CNAG_06875,CNAG_07387,CNAG_07538,CNAG_07647,CNAG_07693,CNAG_07695,CNAG_07751,	12.3	1.44	1.55	0.008587220226	0.1008726629	1

GO:0071702	organic substance transport	246	30	CNAG_00411,CNAG_00971,CNAG_01414,CNAG_01610,CNAG_01647,CNAG_01707,CNAG_01720,CNAG_02113,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02741,CNAG_02761,CNAG_02989,CNAG_03013,CNAG_03426,CNAG_03587,CNAG_03651,CNAG_04151,CNAG_04293,CNAG_04556,CNAG_04863,CNAG_05817,CNAG_06024,CNAG_06738,CNAG_06753,CNAG_06772,CNAG_07322,CNAG_07685,	12.2	1.43	1.52	0.02845209183	0.2276167347	1
GO:0070887	cellular response to chemical stimulus	244	29	CNAG_00156,CNAG_00396,CNAG_00554,CNAG_00668,CNAG_00896,CNAG_01209,CNAG_01704,CNAG_01850,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_03710,CNAG_04268,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_05155,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05538,CNAG_05642,CNAG_05965,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_07317,CNAG_07435,	11.9	1.39	1.47	0.04147802526	0.26917208	1
GO:0051179	localization	674	76	CNAG_00396,CNAG_00411,CNAG_00433,CNAG_00529,CNAG_00540,CNAG_00574,CNAG_00749,CNAG_00971,CNAG_01175,CNAG_01208,CNAG_01318,CNAG_01384,CNAG_01414,CNAG_01472,CNAG_01575,CNAG_01610,CNAG_01647,CNAG_01704,CNAG_01707,CNAG_01720,CNAG_01960,CNAG_02010,CNAG_02083,CNAG_02113,CNAG_02254,CNAG_02292,CNAG_02373,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02733,CNAG_02741,CNAG_02761,CNAG_02775,CNAG_02806,CNAG_02989,CNAG_03013,CNAG_03426,CNAG_03565,CNAG_03582,CNAG_03587,CNAG_03605,CNAG_03651,CNAG_03909,CNAG_04151,CNAG_04293,CNAG_04311,CNAG_04556,CNAG_04758,CNAG_04863,CNAG_04982,CNAG_05130,CNAG_05300,CNAG_05340,CNAG_05381,CNAG_05431,CNAG_05616,CNAG_05817,CNAG_06024,CNAG_06323,CNAG_06381,CNAG_06561,CNAG_06738,CNAG_06753,CNAG_06772,CNAG_06776,CNAG_06875,CNAG_07322,CNAG_07387,CNAG_07538,CNAG_07647,CNAG_07685,CNAG_07693,CNAG_07695,CNAG_07751,	11.3	1.32	1.43	0.005278744788	0.07536423328	1
GO:0006810	transport	650	73	CNAG_00411,CNAG_00433,CNAG_00529,CNAG_00540,CNAG_00574,CNAG_00749,CNAG_00971,CNAG_01175,CNAG_01208,CNAG_01318,CNAG_01384,CNAG_01414,CNAG_01472,CNAG_01575,CNAG_01610,CNAG_01647,CNAG_01704,CNAG_01707,CNAG_01720,CNAG_01960,CNAG_02010,CNAG_02083,CNAG_02113,CNAG_02254,CNAG_02292,CNAG_02373,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02733,CNAG_02741,CNAG_02761,CNAG_02775,CNAG_02806,CNAG_02989,CNAG_03013,CNAG_03426,CNAG_03565,CNAG_03587,CNAG_03605,CNAG_03651,CNAG_03909,CNAG_04151,CNAG_04293,CNAG_04311,CNAG_04556,CNAG_04758,CNAG_04863,CNAG_04982,CNAG_05130,CNAG_05300,CNAG_05340,CNAG_05381,CNAG_05616,CNAG_05817,CNAG_06024,CNAG_06323,CNAG_06381,CNAG_06561,CNAG_06738,CNAG_06753,CNAG_06772,CNAG_06776,CNAG_06875,CNAG_07322,CNAG_07387,CNAG_07538,CNAG_07647,CNAG_07685,CNAG_07693,CNAG_07695,CNAG_07751,	11.2	1.31	1.42	0.006934267209	0.09326086913	1
GO:0018130	heterocycle biosynthetic process	296	34	CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01587,CNAG_01972,CNAG_02285,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03183,CNAG_03320,CNAG_03431,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04841,CNAG_04961,CNAG_04990,CNAG_05049,CNAG_05081,CNAG_05134,CNAG_05200,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06871,CNAG_07011,CNAG_07567,CNAG_07679,CNAG_07867,CNAG_07901,	11.5	1.34	1.42	0.04421148843	0.2791038181	1
GO:0051234	establishment of localization	655	73	CNAG_00411,CNAG_00433,CNAG_00529,CNAG_00540,CNAG_00574,CNAG_00749,CNAG_00971,CNAG_01175,CNAG_01208,CNAG_01318,CNAG_01384,CNAG_01414,CNAG_01472,CNAG_01575,CNAG_01610,CNAG_01647,CNAG_01704,CNAG_01707,CNAG_01720,CNAG_01960,CNAG_02010,CNAG_02083,CNAG_02113,CNAG_02254,CNAG_02292,CNAG_02373,CNAG_02468,CNAG_02676,CNAG_02728,CNAG_02730,CNAG_02733,CNAG_02741,CNAG_02761,CNAG_02775,CNAG_02806,CNAG_02989,CNAG_03013,CNAG_03426,CNAG_03565,CNAG_03587,CNAG_03605,CNAG_03651,CNAG_03909,CNAG_04151,CNAG_04293,CNAG_04311,CNAG_04556,CNAG_04758,CNAG_04863,CNAG_04982,CNAG_05130,CNAG_05300,CNAG_05340,C	11.1	1.3	1.41	0.008345828757	0.09929396265	1

				NAG_05381,CNAG_05616,CNAG_05817,CNAG_06024,CNAG_06323,CNAG_06381,CNAG_06561,CNAG_06738,CNAG_06753,CNAG_06772,CNAG_06776,CNAG_06875,CNAG_07322,CNAG_07387,CNAG_07538,CNAG_07647,CNAG_07685,CNAG_07693,CNAG_07695,CNAG_07751,							
GO:0033554	cellular response to stress	336	38	CNAG_00156,CNAG_00396,CNAG_00554,CNAG_00566,CNAG_00668,CNAG_00691,CNAG_01272,CNAG_01704,CNAG_01850,CNAG_01882,CNAG_02020,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_02602,CNAG_02775,CNAG_03316,CNAG_03449,CNAG_03637,CNAG_04268,CNAG_04630,CNAG_04678,CNAG_04681,CNAG_04737,CNAG_05155,CNAG_05177,CNAG_05200,CNAG_05216,CNAG_05429,CNAG_05654,CNAG_05915,CNAG_05965,CNAG_05977,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06793,	11.3	1.32	1.4	0.04257673301	0.2706247139		1
GO:0051716	cellular response to stimulus	471	51	CNAG_00156,CNAG_00396,CNAG_00554,CNAG_00566,CNAG_00668,CNAG_00691,CNAG_00896,CNAG_01209,CNAG_01272,CNAG_01557,CNAG_01704,CNAG_01850,CNAG_01882,CNAG_02020,CNAG_02480,CNAG_02487,CNAG_02490,CNAG_02526,CNAG_02602,CNAG_02775,CNAG_03316,CNAG_03449,CNAG_03637,CNAG_03710,CNAG_04268,CNAG_04505,CNAG_04630,CNAG_04631,CNAG_04678,CNAG_04681,CNAG_04737,CNAG_05155,CNAG_05177,CNAG_05200,CNAG_05216,CNAG_05380,CNAG_05429,CNAG_05538,CNAG_05642,CNAG_05654,CNAG_05838,CNAG_05915,CNAG_05965,CNAG_05977,CNAG_06339,CNAG_06552,CNAG_06553,CNAG_06632,CNAG_06793,CNAG_07317,CNAG_07435,	10.8	1.27	1.34	0.04207704638	0.2692930968		1

Appendix 2. List of GO terms generated from FungiDB based on the cellular component ontology.

ID	Name	Bgd count	Result count	Result gene list	Pct of bgd	Fold enrichment	Odds ratio	P-value	Benjamini	Bonferroni
GO:0030427	site of polarized growth	2	2	CNAG_05866,CNAG_06738,	100	11.69	inf	0.007305839656	0.1636508083	1
GO:0009986	cell surface	2	2	CNAG_02775,CNAG_03488,	100	11.69	inf	0.007305839656	0.1636508083	1
GO:0005770	late endosome	4	2	CNAG_00971,CNAG_04293,	50	5.84	10.73	0.03901380597	0.4166956765	1
GO:0033643	host cell part	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09
GO:0033648	host intracellular membrane-bounded organelle	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09

				04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,							
GO:0018995	host cellular component	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09	
GO:0033647	host intracellular organelle	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09	
GO:0042025	host cell nucleus	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09	
GO:0043656	host intracellular region	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09	
GO:0033646	host intracellular part	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09	
GO:0043657	host cell	148	40	CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01648,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03449,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04575,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,	27	3.16	4.26	1.50E-11	4.20E-10	3.36E-09	

GO:0012505	endomembrane system	231	30	CNAG_00411,CNAG_00461,CNAG_00971,CNAG_01175,CNAG_01298,CNAG_01378,CNAG_01414,CNAG_01592,CNAG_01647,CNAG_01704,CNAG_01720,CNAG_02468,CNAG_02487,CNAG_02715,CNAG_02761,CNAG_03079,CNAG_03426,CNAG_03587,CNAG_04293,CNAG_04488,CNAG_04556,CNAG_04863,CNAG_04949,CNAG_05301,CNAG_05781,CNAG_05817,CNAG_06548,CNAG_06654,CNAG_06772,CNAG_07322,	13	1.52	1.64	0.01277725627	0.2385087837	1
GO:0044464	obsolete cell part	541	61	CNAG_00156,CNAG_00299,CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01550,CNAG_01638,CNAG_01648,CNAG_01882,CNAG_01972,CNAG_02408,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_02678,CNAG_02775,CNAG_03018,CNAG_03075,CNAG_03116,CNAG_03183,CNAG_03282,CNAG_03431,CNAG_03449,CNAG_03565,CNAG_03637,CNAG_04184,CNAG_04208,CNAG_04450,CNAG_04518,CNAG_04534,CNAG_04674,CNAG_04841,CNAG_04985,CNAG_05049,CNAG_05225,CNAG_05333,CNAG_05373,CNAG_05380,CNAG_05404,CNAG_05429,CNAG_05642,CNAG_05654,CNAG_05866,CNAG_06203,CNAG_06339,CNAG_06357,CNAG_06440,CNAG_06793,CNAG_06814,CNAG_06871,CNAG_06925,CNAG_07011,CNAG_07434,CNAG_07435,CNAG_07679,CNAG_07867,CNAG_07901,CNAG_07924,	11.3	1.32	1.42	0.01245602239	0.2385087837	1
GO:0016020	membrane	1542	149	CNAG_00064,CNAG_00096,CNAG_00150,CNAG_00331,CNAG_00433,CNAG_00453,CNAG_00476,CNAG_00517,CNAG_00529,CNAG_00540,CNAG_00554,CNAG_00574,CNAG_00664,CNAG_00668,CNAG_00691,CNAG_00744,CNAG_00749,CNAG_00971,CNAG_01043,CNAG_01073,CNAG_01175,CNAG_01180,CNAG_01208,CNAG_01228,CNAG_01283,CNAG_01298,CNAG_01318,CNAG_01378,CNAG_01384,CNAG_01414,CNAG_01452,CNAG_01472,CNAG_01473,CNAG_01475,CNAG_01575,CNAG_01588,CNAG_01592,CNAG_01610,CNAG_01611,CNAG_01647,CNAG_01691,CNAG_01702,CNAG_01704,CNAG_01707,CNAG_01713,CNAG_01761,CNAG_01850,CNAG_01864,CNAG_01930,CNAG_01960,CNAG_02010,CNAG_02026,CNAG_02081,CNAG_02083,CNAG_02096,CNAG_02113,CNAG_02190,CNAG_02223,CNAG_02254,CNAG_02297,CNAG_02468,CNAG_02487,CNAG_02688,CNAG_02715,CNAG_02728,CNAG_02733,CNAG_02747,CNAG_02753,CNAG_02761,CNAG_02775,CNAG_02806,CNAG_02834,CNAG_02848,CNAG_02955,CNAG_02975,CNAG_03013,CNAG_03079,CNAG_03391,CNAG_03426,CNAG_03488,CNAG_03565,CNAG_03587,CNAG_03605,CNAG_03651,CNAG_03735,CNAG_03824,CNAG_03858,CNAG_03909,CNAG_04126,CNAG_04293,CNAG_04363,CNAG_04386,CNAG_04488,CNAG_04505,CNAG_04534,CNAG_04556,CNAG_04651,CNAG_04681,CNAG_04752,CNAG_04758,CNAG_04863,CNAG_04911,CNAG_04943,CNAG_04949,CNAG_04956,CNAG_04982,CNAG_05004,CNAG_05130,CNAG_05227,CNAG_05300,CNAG_05301,CNAG_05340,CNAG_05381,CNAG_05413,CNAG_05430,CNAG_05533,CNAG_05616,CNAG_05633,CNAG_05654,CNAG_05698,CNAG_05702,CNAG_05732,CNAG_05760,CNAG_05784,CNAG_05817,CNAG_05866,CNAG_05888,CNAG_05915,CNAG_06024,CNAG_06031,CNAG_06323,CNAG_06381,CNAG_06548,CNAG_06561,CNAG_06654,CNAG_06714,CNAG_06776,CNAG_06875,CNAG_06911,CNAG_06925,CNAG_07317,CNAG_07387,CNAG_07538,CNAG_07647,CNAG_07685,CNAG_07693,CNAG_07695,CNAG_07751,CNAG_07810,	9.7	1.13	1.22	0.03662460708	0.4166956765	1
GO:0031224	intrinsic component of membrane	1358	132	CNAG_00064,CNAG_00096,CNAG_00331,CNAG_00433,CNAG_00453,CNAG_00476,CNAG_00517,CNAG_00529,CNAG_00540,CNAG_00554,CNAG_00574,CNAG_00664,CNAG_00668,CNAG_00691,CNAG_00744,CNAG_00749,CNAG_01043,CNAG_01073,CNAG_01175,CNAG_01180,CNAG_01208,CNAG_01228,CNAG_01283,CNAG_01298,CNAG_01318,CNAG_01378,CNAG_01384,CNAG_01452,CNAG_01472,CNAG_01473,CNAG_01475,CNAG_01575,CNAG_01588,CNAG_01592,CNAG_01611,CNAG_01647,CNAG_01691,CNAG_01702,CNAG_01704,CNAG_01707,CNAG_01713,CNAG_01761,CNAG_01850,CNAG_01864,CNAG_01930,CNAG_01960,CNAG_02010,CNAG_02026,CNAG_02081,CNAG_02083,CNAG_02223,CNAG_02254,CNAG_02297,CNAG_02487,CNAG_02715,CNAG_02728,CNAG_02733,CNAG_02747,CNAG_02753,CNAG_027	9.7	1.14	1.22	0.04283962002	0.4166956765	1

				61,CNAG_02775,CNAG_02806,CNAG_02848,CNAG_02955,CNAG_02975,CNAG_03013,CNAG_03079,CNAG_03391,CNAG_03426,CNAG_03488,CNAG_03565,CNAG_03605,CNAG_03651,CNAG_03735,CNAG_03824,CNAG_03858,CNAG_03909,CNAG_04126,CNAG_04363,CNAG_04386,CNAG_04488,CNAG_04534,CNAG_04556,CNAG_04651,CNAG_04681,CNAG_04752,CNAG_04758,CNAG_04911,CNAG_04943,CNAG_04949,CNAG_04956,CNAG_04982,CNAG_05130,CNAG_05227,CNAG_05300,CNAG_05340,CNAG_05381,CNAG_05413,CNAG_05430,CNAG_05533,CNAG_05616,CNAG_05633,CNAG_05654,CNAG_05698,CNAG_05702,CNAG_05732,CNAG_05760,CNAG_05784,CNAG_05817,CNAG_05866,CNAG_05888,CNAG_05915,CNAG_06031,CNAG_06323,CNAG_06381,CNAG_06548,CNAG_06561,CNAG_06654,CNAG_06714,CNAG_06776,CNAG_06875,CNAG_06911,CNAG_06925,CNAG_07317,CNAG_07387,CNAG_07538,CNAG_07647,CNAG_07685,CNAG_07693,CNAG_07695,CNAG_07751,CNAG_07810,						
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Appendix 3. List of GO terms generated from FungiDB based on the molecular function ontology.

ID	Name	Bgd count	Result count	Result gene list	Pct of bgd	Fold enrichment	Odds ratio	P-value	Benjamini	Bonferroni
GO:0015927	trehalase activity	2	2	CNAG_03525,CNAG_03785,	100	11.69	inf	0.007305839656	0.233786869	1
GO:0004555	alpha,alpha-trehalase activity	2	2	CNAG_03525,CNAG_03785,	100	11.69	inf	0.007305839656	0.233786869	1
GO:0000987	cis-regulatory region sequence-specific DNA binding	4	3	CNAG_00156,CNAG_01173,CNAG_07924,	75	8.77	32.28	0.002331221342	0.08816255256	0.9697880781
GO:0009987	cellular process	3	2	CNAG_01208,CNAG_03605,	66.7	7.79	21.47	0.02067251366	0.3999743411	1
GO:0001216	DNA-binding transcription activator activity	3	2	CNAG_00156,CNAG_01173,	66.7	7.79	21.47	0.02067251366	0.3999743411	1
GO:0000978	RNA polymerase II cis-regulatory region sequence-specific DNA binding	3	2	CNAG_00156,CNAG_01173,	66.7	7.79	21.47	0.02067251366	0.3999743411	1

GO: 000 122 8	DNA-binding transcription activator activity, RNA polymerase II-specific	3	2	CNAG_00156,CNAG_01173,	66.7	7.79	21.47	0.020 67251 366	0.3999743 411	1
GO: 000 097 7	RNA polymerase II transcription regulatory region sequence-specific DNA binding	4	2	CNAG_00156,CNAG_01173,	50	5.84	10.73	0.039 01380 597	0.3999743 411	1
GO: 001 802 4	histone-lysine N-methyltransferase activity	4	2	CNAG_01882,CNAG_05404,	50	5.84	10.73	0.039 01380 597	0.3999743 411	1
GO: 004 239 3	histone binding	4	2	CNAG_01882,CNAG_05977,	50	5.84	10.73	0.039 01380 597	0.3999743 411	1
GO: 001 715 0	tRNA dihydrouridine synthase activity	4	2	CNAG_00234,CNAG_06997,	50	5.84	10.73	0.039 01380 597	0.3999743 411	1
GO: 004 205 4	histone methyltransferase activity	4	2	CNAG_01882,CNAG_05404,	50	5.84	10.73	0.039 01380 597	0.3999743 411	1
GO: 000 097 6	transcription regulatory region sequence-specific DNA binding	7	3	CNAG_00156,CNAG_01173,CNAG_07924,	42.9	5.01	8.06	0.016 76969 964	0.3999743 411	1
GO: 000 106 7	regulatory region nucleic acid binding	7	3	CNAG_00156,CNAG_01173,CNAG_07924,	42.9	5.01	8.06	0.016 76969 964	0.3999743 411	1
GO: 000 098 1	DNA-binding transcription factor activity, RNA polymerase II-specific	125	40	CNAG_00156,CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01487,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_03018,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04534,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07867,CNAG_07901,CNAG_07924,	32	3.74	5.43	3.31E-14	6.89E-12	1.38E-11
GO: 000	3'-5' exonuclease activity	9	3	CNAG_02490,CNAG_02773,CNAG_03345,	33.3	3.9	5.37	0.035 37540 906	0.3999743 411	1

8408											
GO:1990837	sequence-specific double-stranded DNA binding	9	3	CNAG_00156,CNAG_01173,CNAG_07924,	33.3	3.9	5.37	0.03537540906	0.3999743411	1	
GO:0015103	inorganic anion transmembrane transporter activity	9	3	CNAG_01472,CNAG_06925,CNAG_07647,	33.3	3.9	5.37	0.03537540906	0.3999743411	1	
GO:0003700	DNA-binding transcription factor activity	162	47	CNAG_00156,CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07435,CNAG_07593,CNAG_07867,CNAG_07901,CNAG_07924,	29	3.39	4.77	1.13E-14	4.70E-12	4.70E-12	
GO:0140110	transcription regulator activity	183	48	CNAG_00156,CNAG_00192,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_05687,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07435,CNAG_07593,CNAG_07867,CNAG_07901,CNAG_07924,	26.2	3.07	4.14	4.00E-13	5.55E-11	1.66E-10	
GO:0043565	sequence-specific DNA binding	41	11	CNAG_00156,CNAG_00514,CNAG_01173,CNAG_01883,CNAG_02241,CNAG_03116,CNAG_03401,CNAG_03637,CNAG_06762,CNAG_07405,CNAG_07924,	26.8	3.14	3.99	0.0004711439509	0.01959958836	0.1959958836	
GO:0035091	phosphatidylinositol binding	16	4	CNAG_01707,CNAG_02676,CNAG_02730,CNAG_02989,	25	2.92	3.59	0.04205120552	0.3999743411	1	
GO:0098772	molecular function regulator	271	55	CNAG_00156,CNAG_00192,CNAG_00818,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01004,CNAG_01069,CNAG_01173,CNAG_01454,CNAG_01487,CNAG_01557,CNAG_01977,CNAG_02066,CNAG_02310,CNAG_02322,CNAG_02364,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03115,CNAG_03116,CNAG_03132,CNAG_03183,CNAG_03316,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04090,CNAG_04184,CNAG_04534,CNAG_04630,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_05687,CNAG_05977,CNAG_06339,CNAG_06483,CNAG_06871,CNAG_07011,CNAG_07435,CNAG_07566,CNAG_07593,CNAG_07867,CNAG_07901,CNAG_07924,	20.3	2.37	2.97	4.40E-10	4.57E-08	1.83E-07	
GO:000	zinc ion binding	268	53	CNAG_00192,CNAG_00299,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01883,CNAG_01972,CNAG_01977,CNAG_02066,CNAG_02322,CN	19.8	2.31	2.86	2.52E-09	2.10E-07	1.05E-06	

8270				AG_02364,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_02998,CNAG_03018,CNAG_03055,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_04983,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05404,CNAG_05538,CNAG_05642,CNAG_05784,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07679,CNAG_07867,CNAG_07901,						
GO:0046983	protein dimerization activity	49	9	CNAG_00460,CNAG_00791,CNAG_01648,CNAG_02516,CNAG_04321,CNAG_05375,CNAG_06751,CNAG_07420,CNAG_07924,	18.4	2.15	2.43	0.02127462269	0.3999743411	1
GO:0046914	transition metal ion binding	311	54	CNAG_00192,CNAG_00299,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01069,CNAG_01487,CNAG_01883,CNAG_01972,CNAG_01977,CNAG_02066,CNAG_02322,CNAG_02364,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_02998,CNAG_03018,CNAG_03055,CNAG_03115,CNAG_03132,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03710,CNAG_03902,CNAG_03914,CNAG_04184,CNAG_04518,CNAG_04534,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_04983,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05333,CNAG_05380,CNAG_05404,CNAG_05538,CNAG_05642,CNAG_05784,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07679,CNAG_07867,CNAG_07901,	17.4	2.03	2.42	1.94E-07	1.35E-05	8.08E-05
GO:0003677	DNA binding	286	47	CNAG_00156,CNAG_00240,CNAG_00514,CNAG_00566,CNAG_00828,CNAG_00830,CNAG_01069,CNAG_01173,CNAG_01648,CNAG_01883,CNAG_01972,CNAG_02241,CNAG_02525,CNAG_02555,CNAG_02603,CNAG_03018,CNAG_03116,CNAG_03183,CNAG_03401,CNAG_03431,CNAG_03449,CNAG_03637,CNAG_04184,CNAG_04450,CNAG_04518,CNAG_04534,CNAG_04637,CNAG_04841,CNAG_05049,CNAG_05177,CNAG_05333,CNAG_05380,CNAG_05642,CNAG_06203,CNAG_06327,CNAG_06339,CNAG_06762,CNAG_06793,CNAG_06814,CNAG_06871,CNAG_07011,CNAG_07405,CNAG_07435,CNAG_07464,CNAG_07867,CNAG_07901,CNAG_07924,	16.4	1.92	2.23	6.34E-06	0.0003770125661	0.002639087963
GO:0046872	metal ion binding	686	87	CNAG_00192,CNAG_00290,CNAG_00299,CNAG_00394,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01019,CNAG_01069,CNAG_01209,CNAG_01378,CNAG_01487,CNAG_01557,CNAG_01610,CNAG_01704,CNAG_01718,CNAG_01746,CNAG_01883,CNAG_01972,CNAG_01977,CNAG_02066,CNAG_02081,CNAG_02292,CNAG_02322,CNAG_02364,CNAG_02408,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02602,CNAG_02603,CNAG_02604,CNAG_02741,CNAG_02775,CNAG_02998,CNAG_03018,CNAG_03055,CNAG_03115,CNAG_03132,CNAG_03174,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03406,CNAG_03431,CNAG_03449,CNAG_03607,CNAG_03710,CNAG_03785,CNAG_03902,CNAG_03914,CNAG_04043,CNAG_04184,CNAG_04268,CNAG_04518,CNAG_04534,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_04983,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05252,CNAG_05264,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05404,CNAG_05538,CNAG_05642,CNAG_05784,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06793,CNAG_06871,CNAG_06997,CNAG_07011,CNAG_07405,CNAG_07591,CNAG_07679,CNAG_07848,CNAG_07862,CNAG_07867,CNAG_07901,	12.7	1.48	1.69	5.07E-05	0.002636902444	0.02109521955
GO:0043169	cation binding	689	87	CNAG_00192,CNAG_00290,CNAG_00299,CNAG_00394,CNAG_00514,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01019,CNAG_01069,CNAG_01209,CNAG_01378,CNAG_01487,CNAG_01557,CNAG_01610,CNAG_01704,CNAG_01718,CNAG_01746,CNAG_01883,CNAG_01972,CNAG_01977,CNAG_02066,CNAG_02081,CNAG_02292,CNAG_02322,CNAG_02364,CNAG_02408,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02602,CNAG_02603,CNAG_02604,CNAG_02741,CNAG_02775,CNAG_02998,CNAG_03018,CNAG_03055,CNAG_03115,CNAG_031	12.6	1.48	1.68	5.98E-05	0.002765763028	0.02489186725

				32,CNAG_03174,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03406,CNAG_03431,CNAG_03449,CNAG_03607,CNAG_03710,CNAG_03785,CNAG_03902,CNAG_03914,CNAG_04043,CNAG_04184,CNAG_04268,CNAG_04518,CNAG_04534,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_04983,CNAG_05019,CNAG_05049,CNAG_05170,CNAG_05252,CNAG_05264,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05404,CNAG_05538,CNAG_05642,CNAG_05784,CNAG_06339,CNAG_06483,CNAG_06762,CNAG_06793,CNAG_06871,CNAG_06997,CNAG_07011,CNAG_07405,CNAG_07591,CNAG_07679,CNAG_07848,CNAG_07862,CNAG_07867,CNAG_07901,						
GO: 004 316 7	ion binding	1328	133	CNAG_00154,CNAG_00192,CNAG_00234,CNAG_00290,CNAG_00299,CNAG_00394,CNAG_00396,CNAG_00514,CNAG_00565,CNAG_00566,CNAG_00654,CNAG_00682,CNAG_00828,CNAG_00830,CNAG_00896,CNAG_01019,CNAG_01069,CNAG_01209,CNAG_01373,CNAG_01378,CNAG_01464,CNAG_01487,CNAG_01557,CNAG_01575,CNAG_01610,CNAG_01704,CNAG_01707,CNAG_01718,CNAG_01746,CNAG_01865,CNAG_01883,CNAG_01972,CNAG_01977,CNAG_02059,CNAG_02066,CNAG_02081,CNAG_02116,CNAG_02148,CNAG_02285,CNAG_02292,CNAG_02307,CNAG_02322,CNAG_02364,CNAG_02373,CNAG_02408,CNAG_02490,CNAG_02525,CNAG_02555,CNAG_02575,CNAG_02602,CNAG_02603,CNAG_02604,CNAG_02676,CNAG_02730,CNAG_02741,CNAG_02745,CNAG_02775,CNAG_02989,CNAG_02998,CNAG_03018,CNAG_03055,CNAG_03115,CNAG_03132,CNAG_03134,CNAG_03174,CNAG_03183,CNAG_03345,CNAG_03401,CNAG_03406,CNAG_03431,CNAG_03449,CNAG_03565,CNAG_03607,CNAG_03637,CNAG_03710,CNAG_03769,CNAG_03785,CNAG_03902,CNAG_03914,CNAG_04043,CNAG_04108,CNAG_04118,CNAG_04174,CNAG_04184,CNAG_04268,CNAG_04450,CNAG_04505,CNAG_04518,CNAG_04534,CNAG_04678,CNAG_04800,CNAG_04807,CNAG_04841,CNAG_04878,CNAG_04952,CNAG_04983,CNAG_05004,CNAG_05019,CNAG_05049,CNAG_05134,CNAG_05170,CNAG_05216,CNAG_05252,CNAG_05264,CNAG_05301,CNAG_05333,CNAG_05380,CNAG_05404,CNAG_05444,CNAG_05538,CNAG_05616,CNAG_05642,CNAG_05784,CNAG_06140,CNAG_06339,CNAG_06440,CNAG_06483,CNAG_06552,CNAG_06568,CNAG_06632,CNAG_06762,CNAG_06793,CNAG_06871,CNAG_06997,CNAG_07011,CNAG_07347,CNAG_07405,CNAG_07591,CNAG_07679,CNAG_07848,CNAG_07862,CNAG_07867,CNAG_07901,	10	1.17	1.27	0.016 73887 241	0.3999743 411	1

Appendix 4. Hypotitanising screen gene list from Venn diagram

area <2	k-means <2	percent <2	AI<2	circularity <2
CNAG_05074	CNAG_00744	CNAG_03582	CNAG_03384	CNAG_02373
CNAG_04637	CNAG_05074	CNAG_02373	CNAG_05010	CNAG_02989
CNAG_05010	CNAG_04353	CNAG_01249	CNAG_00396	CNAG_03387
CNAG_03384	CNAG_05728	CNAG_00558	CNAG_04911	CNAG_03391
CNAG_03605	CNAG_04637	CNAG_06753	CNAG_07435	CNAG_02975
CNAG_03387	CNAG_06762	CNAG_00919	CNAG_03582	CNAG_02020
CNAG_01173	CNAG_01173	CNAG_03488	CNAG_01947	CNAG_03651
CNAG_00744	CNAG_01557	CNAG_04630	CNAG_02874	CNAG_02190
CNAG_04495	CNAG_02922	CNAG_06871	CNAG_01281	CNAG_00903
CNAG_07924	CNAG_07464	CNAG_00460	CNAG_06871	CNAG_01216
CNAG_07464	CNAG_03320	CNAG_04462	CNAG_01069	CNAG_00029
CNAG_02190	CNAG_01281	CNAG_00654	CNAG_02753	CNAG_02148
CNAG_04630	CNAG_03605	CNAG_03003	CNAG_04558	CNAG_05264
CNAG_02922	CNAG_03003	CNAG_03605	CNAG_02281	CNAG_02575
CNAG_03352	CNAG_00654	CNAG_03320	CNAG_04507	CNAG_01272
CNAG_00554	CNAG_01638	CNAG_07464	CNAG_02724	CNAG_02310
CNAG_05686	CNAG_05010	CNAG_01557	CNAG_04636	CNAG_07780
CNAG_05781	CNAG_04462	CNAG_01173	CNAG_01541	CNAG_05413
CNAG_04462	CNAG_00554	CNAG_06762	CNAG_05538	CNAG_00827
CNAG_00514	CNAG_05642	CNAG_04637	CNAG_00411	CNAG_04151
CNAG_04807	CNAG_00927	CNAG_05728	CNAG_02307	CNAG_02728
CNAG_03003	CNAG_00460	CNAG_04353	CNAG_05533	CNAG_06978
CNAG_03115	CNAG_05781	CNAG_00744	CNAG_04106	CNAG_05373
CNAG_06130	CNAG_03387	CNAG_05074	CNAG_00144	CNAG_03075
CNAG_06140	CNAG_06871	CNAG_02922	CNAG_02955	CNAG_00396
CNAG_00674	CNAG_02447	CNAG_02604	CNAG_06561	CNAG_03055
CNAG_00460	CNAG_07924	CNAG_06498	CNAG_01713	CNAG_05451
CNAG_01281	CNAG_04630	CNAG_00927	CNAG_01089	CNAG_01934
CNAG_00331	CNAG_03006	CNAG_04450	CNAG_02026	CNAG_04505
CNAG_00654	CNAG_03488	CNAG_00411	CNAG_01864	CNAG_00414
CNAG_04353	CNAG_04495	CNAG_03387	CNAG_02555	CNAG_01154
CNAG_02122	CNAG_00919	CNAG_05781	CNAG_02660	CNAG_04674
CNAG_04268	CNAG_02998	CNAG_04678	CNAG_00461	CNAG_03320
CNAG_06762	CNAG_00674	CNAG_01638	CNAG_02528	CNAG_05010
CNAG_02998	CNAG_06245	CNAG_04985	CNAG_01373	CNAG_05838
CNAG_00919	CNAG_04620	CNAG_02998	CNAG_04160	CNAG_03754
CNAG_01454	BWC2- white collar 2 protein	CNAG_01550	CNAG_04311	CNAG_06577

	(response to external stimulus, interspecies interaction), senses light			
CNAG_03006	CNAG_05431	CNAG_01825	CNAG_01557	CNAG_01073
CNAG_02919	CNAG_04575	CNAG_01281	CNAG_04956	CNAG_02081
CNAG_04518	CNAG_03710	CNAG_00674	CNAG_00682	CNAG_02263
CNAG_04575	CNAG_03352	CNAG_03710	CNAG_02673	CNAG_06813
CNAG_00830	CNAG_02122	CNAG_05642	CNAG_02241	CNAG_01707
CNAG_06814	CNAG_01454	CNAG_02508	CNAG_03041	CNAG_07696
CNAG_00927	CNAG_02604	BWC2- white collar 2 protein (response to external stimulus, interspecies interaction), senses light	CNAG_01972	CNAG_02737
CNAG_05642	CNAG_06130	CNAG_02834	CNAG_02759	CNAG_02724
CNAG_02447	CNAG_02401	CNAG_06245	CNAG_03044	CNAG_06896
CNAG_07415	CNAG_05524	CNAG_04620	CNAG_00558	CNAG_00565
CNAG_04620	CNAG_03282	CNAG_02761	CNAG_06762	CNAG_01648
BWC2- white collar 2 protein (response to external stimulus, interspecies interaction), senses light	CNAG_00331	CNAG_02113	CNAG_00322	CNAG_01192
CNAG_00453	CNAG_02487	CNAG_04126	CNAG_04363	CNAG_02223
CNAG_06327	CNAG_07415	CNAG_03006	CNAG_04185	CNAG_05134
CNAG_02113	CNAG_05686	CNAG_01758	CNAG_04863	CNAG_00079
CNAG_07425	CNAG_00514	CNAG_01132	CNAG_04344	CNAG_05415
CNAG_02989	CNAG_04450	CNAG_00554	CNAG_03525	CNAG_04293
CNAG_00828	CNAG_05663	CNAG_05010	CNAG_01943	CNAG_03587
CNAG_05663	CNAG_01825	CNAG_01078	CNAG_05560	CNAG_00710
CNAG_03320	CNAG_03115	CNAG_00154	CNAG_01865	CNAG_00664
CNAG_03637	CNAG_04678	CNAG_05817	CNAG_04064	CNAG_02730
CNAG_03710	CNAG_06140	CNAG_01720	unknown	CNAG_06145
CNAG_00240	CNAG_01132	CNAG_05019	CNAG_05633	CNAG_02088
CNAG_02775	CNAG_02285	CNAG_01487	CNAG_00079	CNAG_04238
CNAG_04488	CNAG_04268	CNAG_07924	CNAG_05312	CNAG_02745
CNAG_04752	CNAG_04985	CNAG_05791	CNAG_02678	CNAG_04785

CNAG_06245	CNAG_00830	CNAG_04043	CNAG_04104	CNAG_02548
CNAG_06404	CNAG_05538	CNAG_00834	CNAG_01180	CNAG_02741
CNAG_05874	CNAG_06753	CNAG_02447	CNAG_04585	CNAG_00995
CNAG_00039	CNAG_02773	CNAG_03391	CNAG_05728	CNAG_00394
CNAG_05331	CNAG_04146	CNAG_05431	CNAG_01047	CNAG_02526
CNAG_01960	CNAG_00453	CNAG_04717	CNAG_00657	CNAG_01611
CNAG_06339	CNAG_06232	CNAG_04085	CNAG_02747	CNAG_01883
CNAG_05431	CNAG_04752	CNAG_02926	CNAG_04068	CNAG_01473
CNAG_03391	CNAG_03391	CNAG_01576	CNAG_04506	CNAG_03902
CNAG_05177	CNAG_04518	CNAG_07642	CNAG_04943	CNAG_00096
CNAG_00349	CNAG_00240	CNAG_05663	CNAG_00903	CNAG_00971
CNAG_02395	CNAG_02190	CNAG_03282	CNAG_02059	CNAG_05227
CNAG_07752	CNAG_06654	CNAG_02760	CNAG_07322	CNAG_07538
CNAG_04678	CNAG_02775	CNAG_00830	CNAG_02737	CNAG_00192
CNAG_04544	CNAG_06327	CNAG_05767	CNAG_02408	CNAG_04878
CNAG_04985	CNAG_05869	CNAG_05772	CNAG_01691	CNAG_05482
CNAG_02285	CNAG_00517	CNAG_05004	CNAG_01109	CNAG_01041
CNAG_02490	CNAG_04807	CNAG_00566	CNAG_05333	CNAG_02292
CNAG_02373	CNAG_06814	CNAG_07338	CNAG_04556	CNAG_03044
CNAG_06675	CNAG_03637	CNAG_06381	CNAG_07591	CNAG_01209
CNAG_02254	CNAG_06404	CNAG_02401	CNAG_01921	CNAG_00299
CNAG_00896	CNAG_00558	CNAG_06483	CNAG_05749	CNAG_00416
CNAG_07027	CNAG_02395	CNAG_02432	CNAG_00156	CNAG_02482
CNAG_06232	CNAG_04085	CNAG_01746	CNAG_05616	CNAG_00411
CNAG_04174	CNAG_02997	CNAG_00691	CNAG_02715	CNAG_05965
CNAG_02020	CNAG_04344	CNAG_02997	CNAG_05415	CNAG_05301
CNAG_01825	CNAG_06837	CNAG_03183	CNAG_07387	CNAG_02688
CNAG_07420	CNAG_01249	CNAG_01454	CNAG_00540	CNAG_05866
CNAG_03858	CNAG_02927	CNAG_02722	CNAG_05225	CNAG_06651
CNAG_07558	CNAG_00834	CNAG_03909	CNAG_02081	CNAG_06312
CNAG_05728		CNAG_02359	CNAG_04118	CNAG_00399
CNAG_02487		CNAG_07468	CNAG_02116	CNAG_03007
CNAG_00517		CNAG_05538	CNAG_01702	CNAG_02893
CNAG_02096		CNAG_01109	CNAG_02602	CNAG_02955
CNAG_03282		CNAG_02468		CNAG_05200
CNAG_02401		CNAG_06323		CNAG_01865
CNAG_07517		CNAG_06130		CNAG_04982
CNAG_03769		CNAG_01228		CNAG_06776
		CNAG_04990		CNAG_03746
		CNAG_04208		CNAG_06568

	CNAG_05979	CNAG_01378
	CNAG_04807	CNAG_01930
	CNAG_05444	CNAG_07685
	CNAG_00517	CNAG_01575
	CNAG_07848	CNAG_03449
	CNAG_03096	CNAG_07941
	CNAG_04575	CNAG_05130
	CNAG_04344	CNAG_05760
	CNAG_02096	CNAG_02676
	CNAG_06814	CNAG_01972
	CNAG_03352	CNAG_01097
	CNAG_04146	CNAG_01704
	CNAG_01464	CNAG_03041
	CNAG_05874	CNAG_05687
	CNAG_02898	CNAG_01298
	CNAG_07593	CNAG_07601
	CNAG_03236	CNAG_04737
	CNAG_02427	CNAG_06302
	CNAG_01019	CNAG_01920
	CNAG_03115	CNAG_02311
	CNAG_04518	CNAG_06973
	CNAG_04248	CNAG_04454
	CNAG_00574	CNAG_04651
	CNAG_06357	CNAG_01208
		CNAG_05429
		CNAG_03983
		CNAG_00290
		CNAG_03884
		CNAG_02297
		CNAG_06917
		CNAG_05733
		CNAG_04901
		CNAG_05040
		CNAG_07631
		CNAG_00690
		CNAG_07862
		CNAG_02141
		CNAG_02874
		CNAG_03426
		CNAG_07677

				CNAG_04090
				CNAG_05533
				CNAG_01004
				CNAG_06024
				CNAG_06912
				CNAG_05397
				CNAG_04961

Appendix 5. Hypertitanising screen gene list from Venn diagram

area >2	k-means >2	percent>2	AI>2	circularity >2
CNAG_01476	CNAG_01476	CNAG_01476	CNAG_06793	CNAG_05252
CNAG_03401	CNAG_00234	CNAG_06925	CNAG_06706	
CNAG_06925	CNAG_06925	CNAG_02766	CNAG_06873	
CNAG_02766	CNAG_04758	CNAG_06924	CNAG_06553	
CNAG_06924	CNAG_06924	CNAG_00234	CNAG_03914	
CNAG_06912		CNAG_04759	CNAG_03013	
CNAG_00029		CNAG_06912	CNAG_00150	
CNAG_01092		CNAG_04758	CNAG_06405	
CNAG_00234		CNAG_03401	CNAG_02926	
CNAG_05702		CNAG_03079	CNAG_05373	
CNAG_04759		CNAG_05264	CNAG_03401	
CNAG_05264		CNAG_01384	CNAG_03387	
CNAG_03079		CNAG_00144	CNAG_03345	
CNAG_03914		CNAG_03494	CNAG_05157	
CNAG_04758		CNAG_03565	CNAG_00039	
CNAG_07693		CNAG_07396	CNAG_06911	
CNAG_07396		CNAG_07317	CNAG_03259	
CNAG_03316		CNAG_07867	CNAG_04952	
CNAG_04841		CNAG_03316	CNAG_03174	
CNAG_01154		CNAG_07693	CNAG_02575	
CNAG_03494		CNAG_00791	CNAG_04909	
CNAG_02944		CNAG_00688	CNAG_06632	
CNAG_01384		CNAG_07616	CNAG_06675	
CNAG_00791		CNAG_00710	CNAG_07733	
CNAG_03587		CNAG_02701	CNAG_02989	
CNAG_07616		CNAG_02944	CNAG_07027	
CNAG_04663		CNAG_05702	CNAG_05130	
CNAG_00688		CNAG_04663	CNAG_07558	
CNAG_07867		CNAG_06145	CNAG_06738	

CNAG_00144		CNAG_06973	CNAG_06875	
CNAG_06973		CNAG_01092	CNAG_00674	
CNAG_03565		CNAG_01154	CNAG_06552	
CNAG_06145		CNAG_06328	CNAG_05607	
CNAG_03116		CNAG_04974	CNAG_00422	
CNAG_07317		CNAG_04184	CNAG_05049	
CNAG_04184		CNAG_00029	CNAG_02464	
CNAG_04974		CNAG_01375	CNAG_06548	
CNAG_01318		CNAG_03914	CNAG_06667	
CNAG_00710		CNAG_03587	CNAG_03735	
CNAG_01375		CNAG_02516	CNAG_06339	
CNAG_02224		CNAG_05350	CNAG_06551	
CNAG_01475		CNAG_05429	CNAG_05702	
CNAG_06772		CNAG_02603	CNAG_07647	
CNAG_01611		CNAG_07901	CNAG_06714	
CNAG_05350		CNAG_06561	CNAG_06910	
CNAG_02701		CNAG_06568	CNAG_02364	
CNAG_06568		CNAG_01475	CNAG_04841	
CNAG_05429		CNAG_00064	CNAG_02806	
CNAG_00064		CNAG_01318	CNAG_00029	
CNAG_02223		CNAG_04841	CNAG_00749	
CNAG_01704		CNAG_02223	CNAG_06707	
CNAG_07901		CNAG_01704	CNAG_05170	
CNAG_05340		CNAG_06772	CNAG_05654	
CNAG_06328		CNAG_03234	CNAG_03824	
CNAG_02516		CNAG_04321		
CNAG_06203		CNAG_07347		
CNAG_07347		CNAG_02224		
CNAG_04321		CNAG_06267		
CNAG_02603		CNAG_02225		
CNAG_05973		CNAG_06203		
CNAG_02225		CNAG_07405		
CNAG_05533		CNAG_04064		
CNAG_04064		CNAG_05533		
CNAG_06561		CNAG_01934		
CNAG_00126		CNAG_07679		
CNAG_05991		CNAG_05081		
CNAG_03018		CNAG_07567		
CNAG_07567		CNAG_00476		
CNAG_07541		CNAG_05451		

CNAG_06896		CNAG_05340		
CNAG_07405		CNAG_05991		
CNAG_02088		CNAG_00126		
CNAG_05760		CNAG_06896		
CNAG_03234		CNAG_06235		
CNAG_01718		CNAG_03134		
CNAG_01934		CNAG_01796		
CNAG_06235		CNAG_07541		
CNAG_01610		CNAG_03018		
CNAG_06267		CNAG_01611		
CNAG_05081		CNAG_00608		
CNAG_01796		CNAG_04238		
CNAG_05451		CNAG_02088		
CNAG_00476		CNAG_01929		
CNAG_02848		CNAG_01850		
CNAG_05977		CNAG_05039		
CNAG_04238		CNAG_05760		
CNAG_01010		CNAG_02955		
CNAG_01417		CNAG_01010		
CNAG_06144		CNAG_01718		
CNAG_06194		CNAG_02733		
CNAG_02733		CNAG_03145		
CNAG_02728		CNAG_05300		
CNAG_01929		CNAG_05973		
CNAG_07679		CNAG_06997		
CNAG_03134		CNAG_02848		
CNAG_02955		CNAG_02728		
CNAG_01272		CNAG_07434		
CNAG_00608		CNAG_02525		
CNAG_04956		CNAG_05977		
CNAG_05265		CNAG_01761		
CNAG_02525		CNAG_05265		
CNAG_05039		CNAG_02083		
CNAG_04068		CNAG_04956		
CNAG_05915		CNAG_06144		
CNAG_02010		CNAG_05915		
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		CNAG_05846		