Development of a fungal virulence assay using *Caenorhabditis elegans* as a model host to identify mechanisms of host pathogen interactions

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This thesis is dedicated to my mom,

Prabha Jain,

for all her struggles and efforts in raising me

in becoming the person I am.

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Abstract

Candida albicans is an opportunistic pathogen, which is responsible for causing systemic infection in immunocompromised patients in hospital settings (nosocomial infections). 90% of these nosocomial fungal infections are caused by *C. albicans*, and the estimated annual cost of treating them exceeds $1 billion per year. Despite this expenditure, there is a high mortality rate of 50%. There are two main routes of infections, first a mucosal infection that can spread and invades deeper into the tissues and gets disseminated into the bloodstream. Second, more frequently seen in hospital settings, is when *Candida* cells dislodge from a biofilm that has formed on intravenous devices or catheters. Treatment of these diseases is difficult due to a dearth of antifungal drugs and new strategies are required to explore mechanisms used by *Candida* in causing infection.

One way of approaching these significant scientific challenges is to identify virulence determinants and mechanisms, which apart from providing insightful knowledge of fungal pathogenesis, can also be used as targets for antifungal drug development. The innate immune responses in humans, which are important for defense against fungal infections, are conserved in *Caenorhabditis elegans*. In order to identify *Candida* virulence factors, I have developed a *C. elegans* based pathogenesis assay, where nematodes were infected with fungi (both *S. cerevisiae* and *C. albicans*) and observed for disease phenotypes including death. This assay can be used to study several aspects of disease progression in worms from swelling (inflammation a bio-marker of

infection) to colonization in the intestine, leading to intestinal distension and ultimately death of the host worm.

The assay offers a fast and simple way of identifying unknown genes, which when established as a virulence determinant in the worm model, can be further studied in mammalian models. I demonstrate the utility of this assay in multiple ways. First as proof of principle using this assay I have identified a fungal mutant *cap1*, which is susceptible to reactive oxygen species (ROS), and fails to cause disease, except in *bli-3* mutant worms that carry a mutation in an oxidase gene and is responsible for the oxidative stress. Second, we screened a library of ~1200 *C. albicans* mutants, and identified 7 genes, 3 known (*CMP1*, *IFF11* and *SAP 8*), validating the assay and 4 novel genes (*orf19.1219*, *orf19.6713*, *DOT4* and *ZCF15*) that play a role in fungal infection.

Third use of this assay is to test potential drugs in a high throughput fashion. Families of related compounds were identified through a screen of 30,000 compounds, for their ability as potential inhibitors of *C. albicans* adhesion to biological and inert surfaces. These compounds were further tested in this assay for their ability to reduce infection of *C. albicans* in worms. The assay provides us with a method to test efficacy of antifungals *in vivo*. Finally, using the survival assay, a test for mortality caused by infection, we can observe disparity in the different *C. albicans* fluconazole resistant strains isolated from AIDS patients. In addition this assay after small modification can be potentially employed to screen the *C. elegans* RNAi library to identify the modulators of innate immune responses during fungal infection.

Acknowledgements

It has been a long journey since the day I decided to come to the US for my Ph.D., and there are many people whom I would like to acknowledge for all their help. One thing that was reinforced during this time, is that you need to have a lot of patience to do research and for that I want to thank God for giving me the patience and the will to never give up.

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I cannot express my thanks to my mom for all her efforts and struggles in raising me to be the person I am and always encouraging me to do my best at everything in life. I want to thank her for all her support and belief in me and I hope I make her proud. I also want to thank my dad and my brother for all their help. I also want to thank my uncle Shishir Agrawal, who encouraged me to apply for my Ph.D. in the US and have been so supporting since the day I landed. I will never forget the happiness I heard in the voice of my aunt, Reeta Sharma, when I first told her I had joined the program at WPI. Thank you for all your help and encouragement through these years.

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Glossary of Abbreviations

ABC	ATP Binding Cassette
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
bp	base pairs
BSI	Bloodstream Infection
bZIP	Basic Leucine Zipper
cAMP	cyclic Adenosine Monophosphate
CFU	Colony Forming Units
CGC	Caenorhabditis Genetics Center
CRD	Cysteine Rich Domain
CWP	Cell Wall Proteins
^{o}C	degree Celsius
DIC	Differential interference contrast
DMEM	Dulbecoo's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
FBS	Fetal Bovine serum
5-FOA	5 Fluororotic acid
FGSC	Fungal Genetics Stock Center
g	grams

GI	Gastrointestinal
GPI	Glycophosphatidylinositol
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylase
HOG	High Osmolarity Glycerol
LB	Luria-Bertani broth
LOH	Loss of Heterozygosity
M	Molar
MAPK	Mitogen activated protein kinase
µl	microliter
µm	micrometer
min	minutes
mg	milligrams
ml	milliliter
mM	millimolar
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NGM	Nematode Growth Medium
NF-κB	Nuclear Factor *kappa*-light-chain-enhancer of activated *B* cells
OD	Optical Density
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
ROS	Reactive Oxygen Species

RNAi	RNA interference
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
TNF-α	Tumor Necrosis Factor-alpha
UTR	Untranslated Region
v/v	volume by volume
YPD	Yeast Extract Peptone Dextrose

Chapter 1

Introduction

1.1 Effects of fungal pathogens on humans

Fungal organisms have a huge impact on humans either from their beneficial uses in food and pharmaceutical industries or from the harmful effects of the pathogens. In the food industry different genera of fungus are used for different purposes, for example Baker's yeast or *Saccharomyces cerevisiae* has been used for making breads and to produce alcoholic beverages while others like *Agaricus bisporus* (button or Portobello mushroom) or truffles are eaten directly. In the pharmaceutical industry, fungi are used to produce antibiotics like Penicillin, Cyclosporin, Fusidic acid and others. Other than these favorable uses, fungi can also be pathogenic to humans. For the purpose of this thesis I am focused on role of fungus as a pathogen. The dire effects of fungal organisms can be felt by humans either directly, through infection or indirectly when their ecosystem is affected by fungal infections. There are many different genera of fungi that can cause infection in humans, plants and animals and these can have huge medical and economic6 impacts.

Fungal pathogens that impact agriculture are diverse. For example *Magnaporthe oryzae* causes rice blast in rice plants (COUCH *et al.* 2005) while *Fusarium oxysporum* targets multiple crops like tomatoes, sweet potatoes and was also responsible for causing Panama disease in banana plants (PLOETZ 2006). Apart from these, *Ustilago maydis* and *Cochliobolus heterostrophus* target maize (KÄMPER *et al.* 2006; LEV *et al.* 1999) while *Alternaria alternata* mainly targets citrus plants (PEEVER *et al.* 2000). As for

the direct effect of fungal organisms, people in their daily lives suffer from mild fungal infections such as thrush and athlete's foot. These infections are not life threatening and are relatively easy to manage, but difficult to cure. On the other hand, patients who are immunocompromised can get fungal infections as a consequence of AIDS, undergoing organ transplants, or using intravenous devices and catheters. These infections can range anywhere from mild to lethal. The frequency of nosocomial (hospital acquired) fungal infections have increased since the last decade. The fungal genera mainly involved in causing nosocomial diseases in humans are *Candida*, *Cryptococcus* and *Aspergillus*. For *Cryptococcus* and *Aspergillus*, *C. neoformans*, *C. gattii* and *A. fumigatus* are the medically relevant fungal organisms. Many different species of *Candida* are involved in causing infections including *C. albicans, C. tropicalis, C. krusei, C. dubliniensis, C. parapsilosis* and *C. glabrata*. Mortality rates of ~40% are seen in the patients suffering from infection due to these *Candida* species (HORN *et al.* 2009; WISPLINGHOFF *et al.* 2004). *Candida albicans* causes 46% of all nosocomial *Candida* infections (Figure 1) (HORN *et al.* 2009).

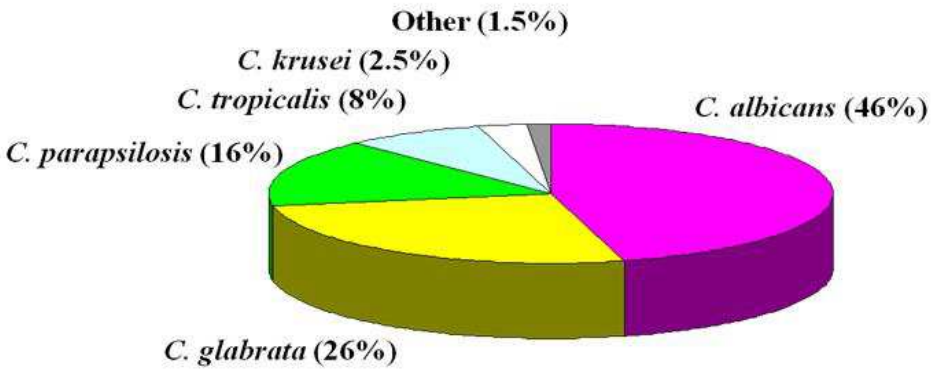

Figure 1. Distribution of *Candida* species isolated from 2019 patients with candidemia.

Some, but not all, of these different genera and species of fungi share common pathways in causing infection, such as Yap1, a transcription factor (Table 1). Yap1 belongs to a Yap family of 8 bZip proteins in *S. cerevisiae* (FERNANDES et al. 1997). This family is found only in fungi, as it binds to a specific DNA binding site containing TTA half site and central C-G pair (TTACTAA). This binding site is distinct in two positions when compared to the DNA binding site of AP-1 proteins which is a TGA half site with a central C-G pair (TGACTCA) (FERNANDES *et al.* 1997). In some cases the difference is only in 1 position. In order to maintain this specificity, the amino acid residue that are directly involved in the interaction with this DNA binding site are also different when compared to other AP-1 proteins but are mostly conserved within Yap family of proteins.

Yap1 binds to the above mentioned conserved sequence which is referred to as yAP-1 response element (YRE), and is present within the promoter of its targets and activates their transcription (WEMMIE *et al.* 1994). Yap1 has been implicated in resistance against variety of toxicants like cycloheximide (CYH), 4-nitroquinoline *N*-oxide (4-NQO), and cadmium, resistance against oxidative stress and multidrug resistance (MDR). Some of the targets of Yap1 include cadmium resistance ATP binding cassette (ABC) transporter gene *YCF1* (LI *et al.* 1997) as well as genes involved in oxidative stress response like *GSH1*, encoding gamma glutamylcysteine synthase (WU and MOYE-ROWLEY 1994), *TRX2*, encoding for thioredoxin (KUGE and JONES 1994), and *GLR1*, encoding for glutathione reductase (GRANT *et al.* 1996). Yap1 also controls the expression of FLZ1, encoding for a multidrug transporter belonging to the major facilitator (MF) superfamily (ALARCO *et al.* 1997a). Thus we can learn about these common mechanisms by

studying a few specific pathogens and since Yap1 and its homologs are fungal specific they can act as good targets for development of broad spectrum antifungals.

Table 1. Homologs of Yap1 and their role in pathogenicity

Organism	Orthologs	Requirement for virulence	Reference
Alternaria alternate	AaAP1	Y	(LIN *et al.* 2009)
Aspergillus fumigatus	Afyap1	N	(LESSING *et al.* 2007)
Candida glabrata	CgAP1	N	(CHEN *et al.* 2007)
Cochliobolus heterostophus	ChAP1	N	(LEV *et al.* 2005b)
Saccharomyces cerevisiae	Yap1, Yap2		(JAIN *et al.* 2009) This study
Candida albicans	Cap1		This study
Aspergillus parasiticus	ApyapA	Y	(REVERBERI *et al.* 2008)
Ustilago maydis	Yap1	Y	(MOLINA and KAHMANN 2007)
Magnaporthe grisea	Moap1	Y	(GUO *et al.* 2011)

1.2 *Candida albicans* Threat

In the last few decades, the incidence of nosocomial fungal infections especially candidemia has increased by 500% in large teaching hospitals and ~ 370% in small teaching and large nonteaching hospitals (PFALLER 1996). The rise in these cases of fungal infections is due to multiple reasons. These include disruption of normal balance

in the microbiota of the patient due to the use of broad spectrum antibiotics and, the increase in the number of immunocompromised patients who are either suffering from AIDS, are on immunosuppressants for transplants or undergoing chemotherapy and finally due to dearth of antifungal therapies. 90% of these infections are caused by *Candida* species (FRIDKIN and JARVIS 1996) and 50% of these infections are fatal (GUDLAUGSSON *et al.* 2003).

Two main types of infection are caused by these species, namely mucosal and systemic. Mucosal infection affects areas lined by epithelial cells including the mouth, upper gastrointestinal and uro-gential tract. At least 75% of women have at least one episode of vaginitis caused by *Candida* in their lifetime (SOBEL 1988), and there are approximately 13 million cases reported annually in the United States (KENT 1991). On the other hand, bloodstream infection (BSI), to some degree, is caused by the ability of this organism to form biofilms on intravenous devices and catheters (RICHARDS *et al.* 2000; VELASCO *et al.* 2000; WISPLINGHOFF *et al.* 2003). Among all BSI reported in US, *Candida albicans* is the fourth leading cause (EDMOND *et al.* 1999), resulting in approximately 5000 deaths per year (MORGAN *et al.* 2005), making it an important pathogen to study.

1.3 Antifungals available for treatment

Six main classes of drugs are currently available for treatment of fungal infections. Four classes of these drugs, target the outer structures of the fungal cell, while the fifth type of drug Flucytosine, targets DNA and RNA synthesis in the fungal cell (Figure 2). The

first class are the Azoles, for example fluconazole, which inhibits the synthesis of ergosterol by inhibiting the enzyme cytochrome P450 14α demethylase (HITCHCOCK *et al.* 1990). Ergosterol, a steroid alcohol, serves the same function in fungal membrane as cholesterol does in animal cell membrane. It is required to maintain membrane permeability and fluidity. Fluconazole is a fungistatic and is the drug most widely used against fungal infections. Due to its widespread use, drug resistance to the azoles has evolved in *Candida*.

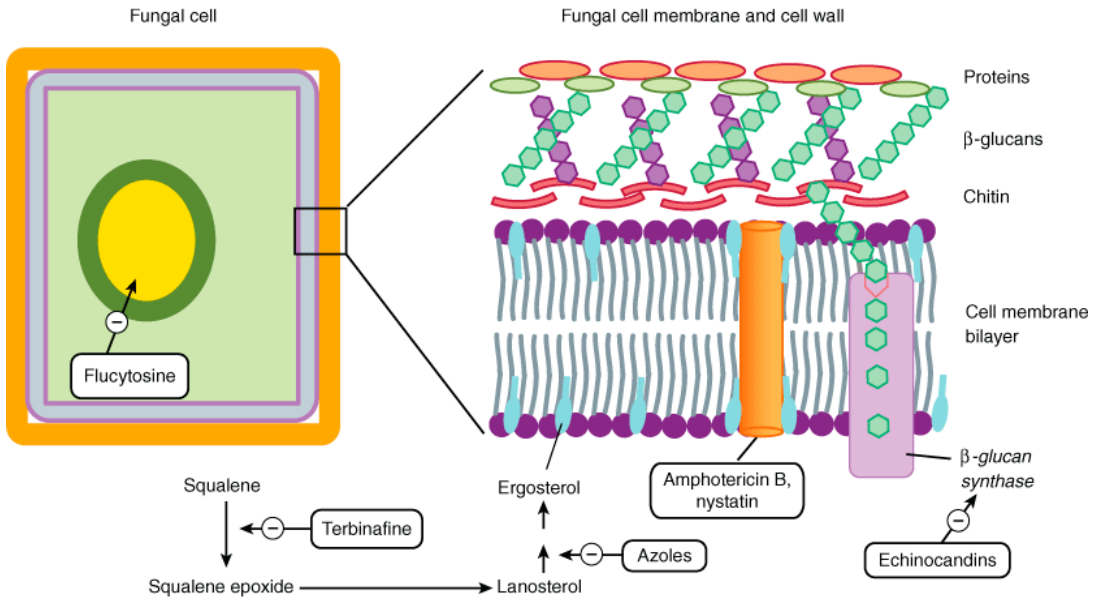

Figure 2. Fungal cell and the targets for five classes of antifungals (KATZUNG *et al.* 2009)

The second class of drugs less commonly used than the azoles are Allylamines like Naftifine or Terbinfine which also target ergosterol synthesis. They do this by inhibiting squalene monooxygenase (RYDER and DUPONT 1985). The third class of antimycotics, is the Polyenes such as Amphotericin B, which compromises the cell membrane by binding directly to ergosterol (BRAJTBURG *et al.* 1990). Polyenes do not have

widespread use but are the drug of choice for systemic infections. Another important class of drugs is the Echinocandins such as Caspofungin, which targets β 1, 3 glucan synthase, the enzyme that synthesizes β glucan, the main component of the fungal cell wall (MORRIS and VILLMANN 2006).

The final drug is Sordarin, which specifically target *C. albicans* elongation factor 2 (DOMÍNGUEZ *et al.* 1999), thus affecting protein synthesis. Despite the availability of these drugs for treatment there is an increasing demand for new drugs due to the toxicity caused by these drugs. For example, at high doses, fluconazole affects cholesterol synthesis – a component of human cell membranes (HITCHCOCK *et al.* 1990). Most of all, repeated use of these drugs has led to increased pathogen resistance to these drugs by different mechanisms [reviewed in (GHANNOUM and RICE 1999)]. Thus, there is an immediate need for identification of novel virulence factors that could be targeted for development of new drug therapies.

1.4 Methods to identify virulence factors

Virulence factors may be identified using a candidate gene approach. In this method, genes whose lose of function has been demonstrated to decrease virulence *in vitro* or in a model organism are specifically studied in a pathogen. This method requires previous knowledge of gene function and a testable hypothetical role in virulence. It allows for a thorough investigation of the suspected genes and a chance to learn more about them. The problem with this method is that it has limited potential for identification of novel factors. Another approach is the use of an unbiased mutant screen using a phenotypic

readout for virulence. Through such screens, genes that are responsible for that specific phenotype can be discovered, and thus their role as a virulence factor, can be identified. This may also help in identifying novel mechanisms or pathways that the pathogen uses during infection. A thorough investigation of the targets has to be done after an unbiased screen as loss of some nonessential genes can affect the organism in ways unrelated to virulence. Researchers need to devise a systemic approach to prioritize subsequent gene analysis. The disadvantage of this approach is that whole genome screens are not possible to conduct in most pathogens.

1.5 Steps involved in fungal pathogenesis

In order to cause disease, fungi need to adhere to specific surfaces, resist the host's innate immune responses and then proliferate and spread into the host body. Even though the order of these events is universal, there are other factors that can put pressure on pathogen survival *in vivo* (Figure 3).

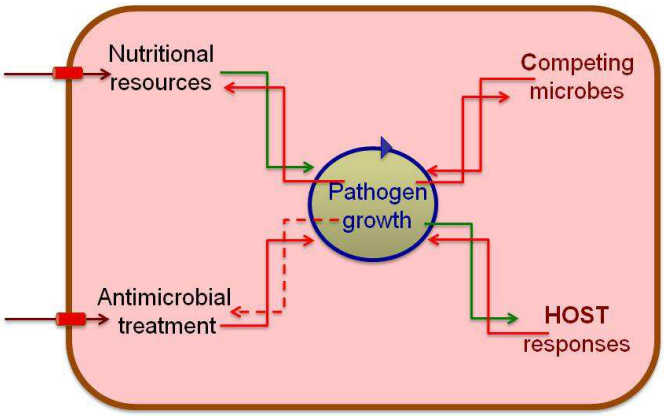

Figure 3. Factors affecting pathogen fitness *in vivo*.

Fungi employ diverse strategies to overcome these factors and cause disease. These strategies mainly involve sensing and responding to the environmental cues originating in the host niche. There are many different types of virulence determinants involved in these processes. The steps involved in initial stages of infection is adhesion, morphogenetic switching, invasion and in some cases phenotypic switching.

1.5.1 Adhesion

The initial step of adhesion is the recognition of host epithelial cells by the pathogen followed by attachment. The fungal cell is the major host determinant in this step. It is a dynamic structure whose architecture and composition is under the control of complex regulatory networks. β glucans are the main components of the cell wall with a chitin inside an outer mannan layer (Figure 4).

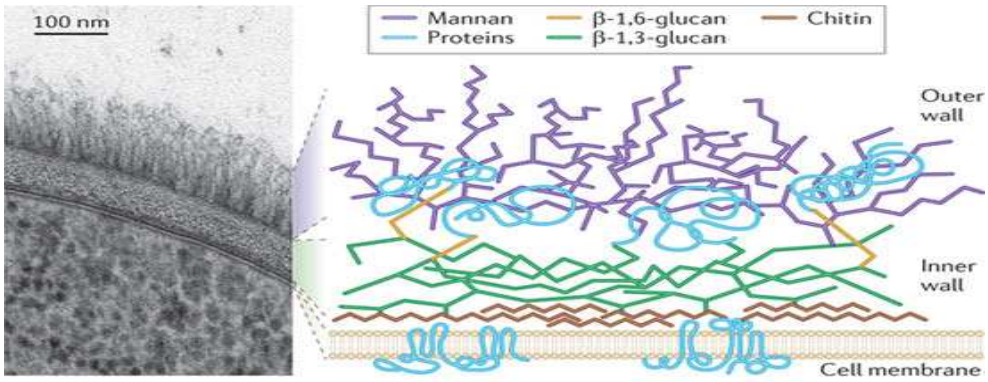

Figure 4. Cell wall architecture of *Candida albicans*. (GOW *et al.* 2012).

The mannan layer consists of heavily glycosylated mannoproteins secreted from the cell surface, and is mainly involved in cell-cell recognition. These mannoproteins are covalently linked to the β 1-3 glucans directly, or through β 1-6 glucans (CHAFFIN *et al.*

1998). The biomolecules that promote the adhesion of fungal cells to host cells or cell ligands are referred to as adhesins (CALDERONE and FONZI 2001). These adhesins are potent immnunogens in host organisms. Some of the genes that code for these host-recognition proteins are the cell wall proteins belonging to the ALS (Agglutinin Like Sequence) family (CHAFFIN 2008). Currently, a vaccine against ALS3 is under clinical trials (LIU and FILLER 2011). Proteins involved in maintaining cell wall composition are also equally important for the adherence of *Candida* to the host surface.

1.5.2 Morphogenetic Switching

Candida albicans is a dimorphic fungus as it can switch between the ovoid yeast forms and the elongated filamentous form. The filamentous form consists of extended tube like structures called hyphae or elongated cells which are considered pseudohyphae (Figure 5). These pseudohyphae coexist with the other two forms in cultures and are also found during infections (SUDBERY *et al.* 2004). For a long time it was believed that the filamentous form was the main requirement for virulence. Since then it has been shown that virulence is attenuated in mutants that are locked in either the yeast form or the filamentous form (MITCHELL 1998). This indicates that morphological switching is important during the process of pathogenesis. The ability of the organism to switch between the yeast and filamentous forms presumably allows the organism to invade a variety of body locations, adjust quickly to changes in host environment, evade immune responses and counteract drug therapies (CALDERONE 2002).

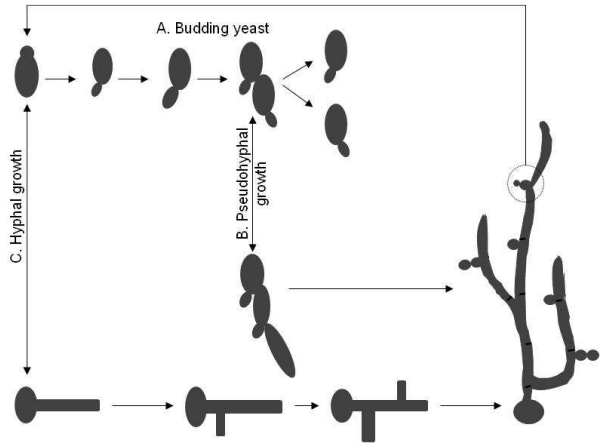

Figure 5. Different morphologies of *C. albicans*. (A) Budding yeast, (B) Psuedohyphal growth and (C) Hyphal growth.

Apart from many different proteins and cues that affect morphogenesis, 4 main pathways are involved in the process: Efg1, Cph1, Hog1 and PKC pathways. Efg1 and Cph1 are transcription factors, while Hog 1 and PKC are Map Kinase and Protein Kinase respectively. Morphogenetic switching is under the control of network of regulatory proteins which behave differently depending on the environmental niche and cues. For example in case of *PHR1* and *PHR2* which encode a glycosidase, the virulence phenotype of a *phr1* null mutant is attenuated in systemic infections but not in vaginal infections. In contrast *phr2* null mutants are virulent in systemic situation but avirulent in vaginal infection model (DE BERNARDIS *et al.* 1998; SAPORITO-IRWIN *et al.* 1995)

1.5.3 Invasion

Adherence of the fungal cell to the host surface is followed by invasion into the host tissue. Epithelial cell invasion is important for mucosal candidiasis since mutants

lacking this ability *in vitro* are not able to confer virulence in animal models. Invasion is usually carried out by two distinct mechanisms (Figure 6). Either the fungal cell induces endocytosis in host cell or it penetrates into or between the host cells (DALLE *et al.* 2010; VILLAR *et al.* 2007). (DALLE *et al.* 2010)(DALLE *et al.* 2010)(DALLE *et al.* 2010)(DALLE *et al.* 2010) Filamentation plays an important role in the process of invasion since null mutants of *efg1* (PARK *et al.* 2005) and *rim101* (NOBILE *et al.* 2008b) lack the capability of invading the host cell.

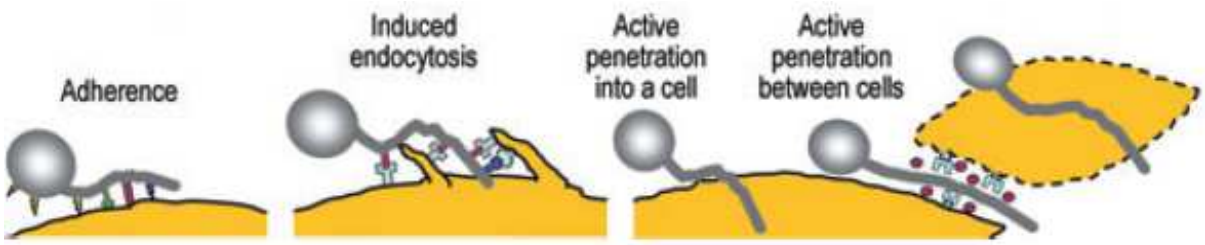

Figure 6. Mechanisms of fungal invasion into the host tissue. Adhesion is followed by either induced endocytosis or active penetration. Modified from (ZHU and FILLER 2010)

One of the proteins that plays a role in attachment, Als3, is also important during invasion as it can induce endocytosis by binding to E-cadherin, a glycoprotein on the epithelial surface (PHAN *et al.* 2007). Members of the Sap family and Plb1 have been shown to be important for the invasion of the host surface through penetration. All of these proteins will be discussed in detail later.

1.5.4 Phenotypic switching

Apart from the yeast-hyphae transitions, the other most extensively studied switching is white-opaque phenotypic switching. It was discovered that this phenotypic switching

occurred at a high frequency (10^{-2}) and white and opaque forms could be distinguished by cellular and colony morphologies, gene expression profiles and mating competence (SLUTSKY *et al.* 1987). The protein Wor1 was established as a master regulator of the opaque phase (HUANG *et al.* 2006) while Efg1 was main promoter of white phase (ZORDAN *et al.* 2007). It was also observed that expression of Sap1and Sap3 was specific for opaque cells while Sap2 was specific for white cells (MORROW *et al.* 1993). It has been observed that strains isolated from vaginitis and systemically infected patients show high frequency of switching. The ability to switch reversibly between these two cell types is important for the pathogenicity of *C. albicans*. Together, changes in the host environment and the virulence traits described above allow for the invasion and pathogenesis of *C. albicans.*

1.6 Known Virulence factors

The capability of *Candida* to switch from commensal to pathogen requires several virulence traits. Some of these include adhesion, dimorphic transition, antigenic variability, phenotypic switching, immunomodulation of host defense mechanisms, and the ability to sequester required nutrients from the host environment to survive and proliferate (Figure 3). Numerous proteins that contribute to these functions and are referred to as virulence factors or determinants have been identified in the last few decades. Most of these factors work in a complex network regulating the minute shifts in the intracellular and intercellular environment of the pathogen. Some of these factors can be broadly divided into specific groups while many have unique function.

1.6.1 Structural and Secreted Proteins

As mentioned above, the cell wall plays an important role in host recognition, attachment and invasion. There are many different Cell Wall Proteins (CWPs) that play crucial roles in pathogenesis. Most important, are the Adhesins including the Als family, Hwp1 (TSUCHIMORI *et al.* 2000) and Int1 (GALE *et al.* 1996). CWPs have become an important class of proteins to study because the absence of cell wall in the mammalian cells makes the fungal-specific protein components a good target for development of antifungal drugs. Apart from these, proteins like Mnt1 (BUURMAN *et al.* 1998) and Big1 (UMEYAMA *et al.* 2006) are not present on the cell wall but are nevertheless responsible for maintaining cell wall integrity and their loss makes the pathogen avirulent. There are also some secretory proteins like Saps and phospholipase, which are hydrolytic and play an important role in host tissue invasion. These are all discussed in more detail below.

Als family

The Agglutinin like sequence (Als) family consists of 9 genes which encode a shared three domain protein structure of which the 5' domain is the most conserved, while the 3' domain is highly variable. The central domain is comprised of variable numbers of tandemly repeated copies of 108bp motifs (Hoyer 2001). In *C. albicans*, the ALS proteins are Glycophosphatidylinositol (GPI) anchored and are linked to β 1-6 glucan (Kapteyn et al. 2000). Most of the Als proteins act as adhesins under different conditions and some of them may have overlapping functions. For example, it is known that Als2 and Als4 have compensatory functions (ZHAO *et al.* 2005) while Als1 and Als3

are redundant for biofilm formation (NOBILE *et al.* 2008a). Through expression in *S. cerevisiae*, Als5 was shown to cause adhesion to human epithelial, endothelial and extracellular matrix along with endothelial invasiveness by endocytosis (GAUR and KLOTZ 1997; GAUR *et al.* 1999).

Hwp1

Hyphal cell wall protein (Hwp1), as its name suggests, is only expressed in the hyphal cells. The protein is mannosylated and attached to β1-6 glucan through a remnant of GPI anchor. Hwp1 is responsible for the covalent attachment of germ tubes to epithelial cells, and this attachment is mediated by transglutaminases exposed on the host cell surface (STAAB *et al.* 1999). It has also been demonstrated that *HWP1* null mutants leads to reduced mortality in mice, less germination in infected mice kidneys and reduced endothelial damage, confirming its role in pathogenesis (TSUCHIMORI *et al.* 2000).

Sap family

The Secreted Aspartyl protease (Sap) family of proteins consists of 10 members and is the second largest family apart from ALS that has been well characterized. Saps are hydrolytic enzymes that are responsible for invasion of epithelial cells, which eventually leads to tissue damage. Different Saps function in different pH environments. Sap1-3 function at pH 3-5, while Sap 4-6 function at pH 5-7 (CHAFFIN 2008). This further shows that depending on niche pH, sap activation can be regulated. For example, Sap1 and

Sap2 are important for causing infection through vaginal epithelium (SCHALLER *et al.* 2003).

Int1

INT1 is an integrin like protein that is required for virulence in a murine systemic model of infection (GALE *et al.* 1996). Strains deleted in *INT1* are less virulent, show reduced adherence to epithelial cells and have defective filamentous growth on solid medium (GALE *et al.* 1998). This indicates that *INT1* plays an important role in adherence and filamentation.

Plb1

Plb1 is an extracellular phospholipase B1 which functions as a lipolytic enzyme. The gene is not required for host surface adherence but is important for host cell penetration (LEIDICH *et al.* 1998). The gene is also essential for wild type virulence in murine systemic model of infection.

1.6.2 Transcription factors in virulence

Transcription factors play an important role in activating or repressing gene functions in response to environmental or intracellular cues. They play a major role in pathogen response to host interaction by regulating the genes that either plays a role in causing infection or mounting an evasive response to host immune effectors. They might also help the pathogen in surviving the host environment by assimilating the available nutrients from the surroundings. The effect of transcription factors can be pleiotropic, so

only key transcription factors that have a clear role in pathogenesis are described here. These are Efg1 and Cph1, Tup1 and Stp1 and Stp2. The first three are involved in hyphal induction (Figure 7), while Stp1 and Stp2 are important for nutrient assimilation.

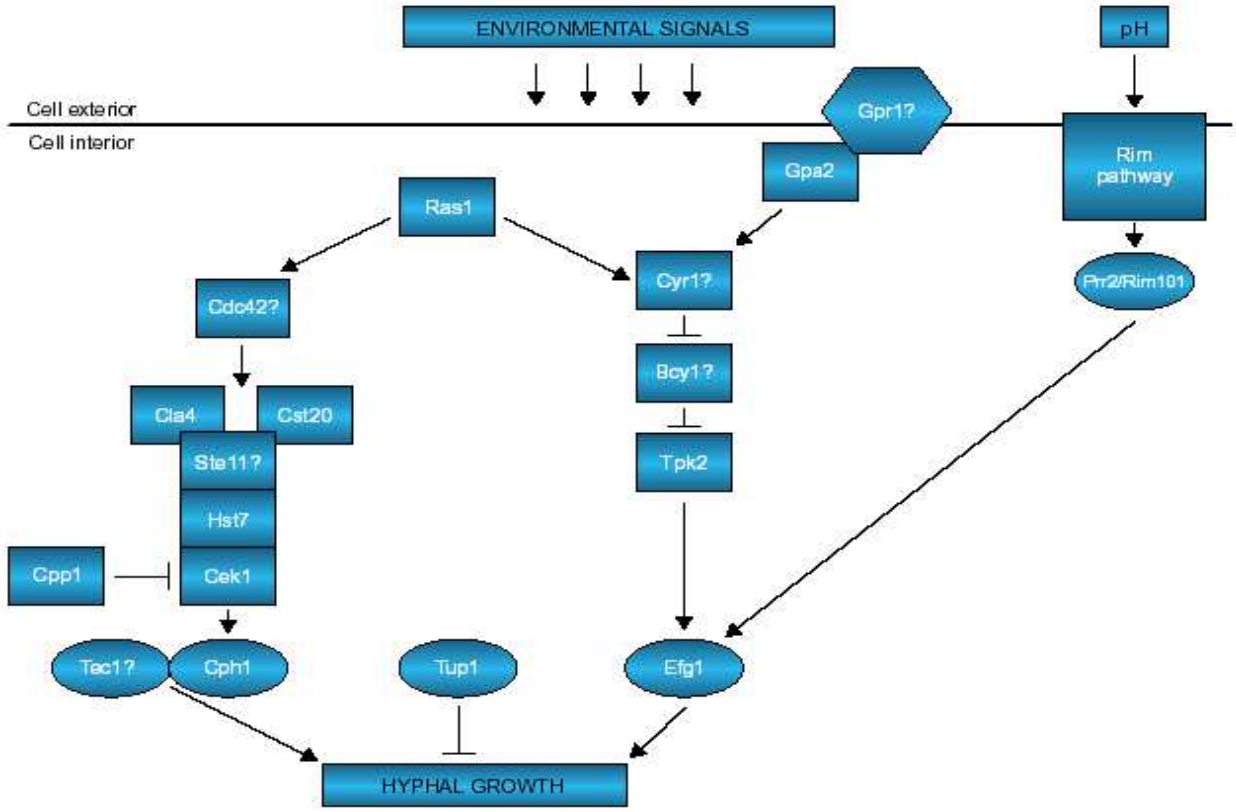

Figure 7. Variety of environmental cues can induce *C. albicans* hyphal formation through multiple pathways (SÁNCHEZ-MARTÍNEZ and PÉREZ-MARTÍN 2001)

Efg1 and Cph1

Filamentation in *C. albicans* is regulated by several pathways which converge and regulate known virulence genes (LANE *et al.* 2001). Efg1 stands for Enhanced

Filamentous Growth, while Cph1 represents Candida Pseudohyphal regulator. As their names suggest Efg1 and Cph1 are both important for the filamentous growth of *C. albicans*. Efg1 and Cph1 are transcription factors, which are responsible for regulating the expression of *SAP5* and *SAP6*. The pathway also controls the expression of the *HWP1* gene, which is important for adhesion. It was also shown that *efg1/efg1 cph1/cph1* double mutants are defective in filamentation and do not form pseudohyphae or hyphae in response to many stimuli including serum and macrophages (LO *et al.* 1997). Single mutants were also unable to induce hyphal formation, but a *cph1/cph1* mutation alone was not enough to make the strain avirulent in the *C. elegans* model, while an *efg1/efg1* mutation was (PUKKILA-WORLEY *et al.*). In the mouse model of candidemia, caused by translocation of *C. albicans* from the GI tract, the double mutant causes significantly less mortality compared to the wild type (KOH *et al.* 2008). The effect is much more pronounced when both of the genes were deleted.

We also know that Efg1 plays a major role in phenotypic switching, another important aspect of fungal infection. Efg1 is enriched in white phase of cells and is responsible for promoting the white phase by repressing *WOR1* which is a regulator of opaque phase. In turn another protein, Czf1, under the control of *WOR1* can repress *EFG1* and induce transition to opaque phase. So Efg1 not only controls genes important for filamentation, but also genes responsible for phenotypic switching in *C. albicans*. Thus it is responsible for controlling multiple aspects of fungal pathogenesis.

Tup1

Deoxy Thymidine monophosphate UPtake 1 (Tup1) is a transcriptional repressor of filamentation, as a *tup1* mutants grow exclusively as filaments in all common (YPD) and specialized media (Spider, Lee's medium) tested (BRAUN and JOHNSON 1997). Tup1 regulates the expression of HWP1 and other cell wall proteins (BRAUN *et al.* 2000). Tup1 also plays a role in phenotypic switching, as *TUP1* transcripts are 4 fold higher in the white phase cells compared to opaque phase cells (ZHAO *et al.* 2002).

Stp1 and Stp2

Stp1 and Stp2 are transcription factors are responsible for placing the virulence factors associated with nutrient acquisition under amino acid control (MARTÍNEZ and LJUNGDAHL 2005) . They show a divergent role in their ability to express genes required to assimilate the nitrogen source. The transcription factors are activated by induction through an amino acid sensor present in the plasma membrane. The activated form of Stp1 is then responsible for expressing genes that degrade the extracellular proteins through the expression of Sap2 and also helps in the uptake of peptides by expressing OPT1, a oligopeptide transporter. High concentrations of amino acids are known to prevent production of Saps (HUBE *et al.* 1994). Thus, amino acid induced processing of Stp1 is an important regulatory step that allows for the expression of Sap2. Stp2 is responsible for the uptake of amino acids through the activation of amino acid permeases like Gap1, Gap2 and Can1.

1.6.3 Signaling Pathways

Multiple signaling pathways are involved in different aspects of fungal pathogenesis. Some of these pathways cross-communicate, while others converge to give the same end results. Four main pathways have been identified to be important for fungal pathogenesis. One of these is the Efg1 pathway, which is under the control of cAMP-PKA, whose activation leads to hyphal development (BOCKMÜHL and ERNST 2001). The other parallel pathway is the Cek 1 MAP kinase pathway, which activates the transcription factor Cph1, which is important for hyphal development (CSANK *et al.* 1998) (Figure 6). The other two pathways of importance are the Mkc1 MAPK pathway and HOG pathway.

Mkc1 MAPK pathway

The Mkc1 pathway in *C. albicans* is important for maintaining cell wall integrity (NAVARRO-GARCÍA *et al.* 1995). Cell wall plays a very important role in adhesion to host cells (CHAFFIN *et al.* 1998). Glucan synthesis is also regulated by this pathway. *mkc1* mutants are sensitive to cell wall degrading enzymes and antifungals like Caspofungin, and are less virulent in a murine systemic model (DIEZ-OREJAS *et al.* 1997). Null mutants are also defective in producing biofilms and show reduced filamentation (KUMAMOTO 2005). The pathway also affects the immune response as it influences the exposure of glucan, which normally occurs in bud scars. This is important as the mammalian receptor Dectin-1 was shown to interact with the pathogen through the

exposed glucan layer (BROWN and GORDON 2001) and generate a receptor mediated response (BROWN *et al.* 2003).

Hog1 pathway

The Hog1 pathway is also important for the biogenesis of the *C. albicans* cell wall. This pathway is important for controlling responses against osmotic and oxidative stress. The null mutant of *hog1* shows reduced virulence in systemically infected mice (ALONSO-MONGE *et al.* 1999). The other components of the pathway include Pbs2 (MAPKK) and Ssk2 (MAPKKK) (CHEETHAM *et al.* 2007).

1.6.4 Histone Modifiers

Cellular genes are often controlled at the epigenetic levels by histone modifiers like Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs). These modifiers can also play a crucial role in fungal pathogenesis. Through the action of these modifiers, a number of genes can either be activated or repressed, which can affect their virulence of the organism. One example of such modifier is Rtt109, which is a HAT protein and is required for *C. albicans* pathogenesis. Rtt109 is responsible for acetylation of Histone 3 (H3K56), and is important for resistance against genotoxic agents that can cause DNA damage. During the process of infection, as a host immune response, *C. albicans* cells are engulfed into phagocytes and encounter high levels of reactive oxygen species (ROS). These ROS have the ability to cause DNA damage,

and also affect cell proteins and lipids. Rtt109 null mutants are also significantly attenuated in the murine systemic model (LOPES DA ROSA *et al.* 2010). Thus Rtt109 is important for pathogen's evasion of the host immune response.

On the other hand, HDACs are equally important. As we know white-opaque switching affects antigenicity and other virulent traits, so any impact on switching will also affect virulence of the pathogen. For example inhibitors against the HDAC HDA1, negatively affect the ability of that strain to adhere to human epithelia or serum induced filamentation, indicating that HDA1 could play an important role in fungal virulence (KLAR *et al.* 2001). Hst3 is a Histone H3K56 deacetylase which along with Rtt109 regulates white –opaque switching (STEVENSON and LIU 2011).

1.6.5 Other virulence factors

In addition to all the above mentioned specific type of virulence factors and pathways, many more determinants are equally important in virulence but might not fall in any of the specified categories. For example, Fas2, a component of fatty acid synthase is required for infection in a murine systemic model (ZHAO *et al.* 1997). Similarly, the FTR1 gene which encodes an iron permease important for growth in the bloodstream (RAMANAN and WANG 2000).

1.7 Model HOSTS used to study fungal virulence.

Despite all the information that has been gained through *in vitro* assays of filamentation and adhesion to inert and biological surfaces (human epithelium), it is imperative to eventually test these observations in a whole animal. *In vivo* experiments are important as they provide the conditions that the pathogen might actually be facing during the process of infection. These could be either the set of proteins that are expressed on the surface of the pathogen cell *in vivo* or the number of host immune responses that it has to overcome in order to establish itself. Whole animal models have to be used to explore the different aspects of fungal pathogenicity and host immune responses. The study of virulence factors and their specific roles in *in vivo* infection process requires the use of both a host organism and the pathogen. This gives a better understanding of the role of these factors in host pathogen interactions. It further helps in identifying general and specific host defense responses to the pathogen.

All these model hosts, share some general characteristics. They have short life cycles, are readily available and easy to maintain in the laboratory. They are genetically amenable and their genomes are sequenced. For most of these model hosts, there are genetic and molecular resources (availability of mutants and RNAi libraries) available for immediate use. A number of model hosts are available and have been used to identify virulence factors, such as *Caenorhabditis elegans, Drosophila melanogaster, Galleria mellonella*, *Danio rerio*, and *Mus musculus*. Of these *C. elegans* and *D. melanogaster* are the most widely used as host followed by *M. musculus*, while *G. mellonella* and *D. rerio* are more recent additions.

Use of invertebrate host models is beneficial as they are experimentally amenable, have conserved innate immune mechanisms between invertebrates and mammals, and several common virulence factors are involved in fungal pathogenesis (BEUTLER 2004). Comparative analysis has shown that a high proportion of human homologues that are involved in pathogen recognition, signal transduction and innate immune responses is conserved in *C. elegans* (55%) and *D. melanogaster* (60%).

1.7.1 *Caenorhabditis elegans*

C. elegans are transparent nematodes that are around 1mm in length. They are either self-fertilizing hermaphrodites or males. They are easy to maintain and grow on *E. coli* lawns, which is their food source. They have a life span of 2-3 weeks and a generation time of ~ 4 days at 20^{o}C. The larvae can be frozen and the thawed organisms are viable, allowing for long term storage of the different worm strains (BRENNER 1974). All the above mentioned attributes fulfill several of the main criteria for a useful model organism. Hermaphrodites are normally used for experiments. The adult hermaphrodites consists of 959 cells, and the developmental lineage of each somatic cell has been mapped out (SULSTON and HORVITZ 1977). The *C. elegans* genome was sequenced in 1998 (CONSORTIUM 1998), and a plethora of genetic and molecular tools have been developed for this organism. Some of these include availability of mutants, fluorescently labeled transgenic strains, microarrays and RNAi library (HARRIS *et al.* 2010).

C. elegans has a conserved innate immune system and thus has been used as a model host for studying the effects of many pathogenic bacteria like *P. aeruginosa* (TAN *et al.* 1999b), *E. faecalis* (SIFRI *et al.* 2002). Some fungal pathogens have been studied in *C. elegans* including *C. neoformans* (MYLONAKIS *et al.* 2002b), *Drechmeria coniospora* (JANSSON 1994) and very recently *C. albicans* (BREGER et al. 2007). *C. elegans* has 6 different signaling cascades, TGF β like, Insulin receptor like, programmed cell death (PCD) and 3 MAP kinase pathways (p38, JNK and ERK) that are preferentially activated depending on the type of pathogen encountered. RNAi knockdown of TIR1 (Toll and IL-1 receptor domain) protein, a component of the p38 MAP Kinase pathway, reduces the expression of neuropeptide like protein, NLP-31, which is necessary for immune response against *D. coniospora* (COUILLAULT et al. 2004). NLP-31 is a neuropeptide, which is a very potent antifungal peptide. TIR-1 is also responsible for regulating release of antimicrobial peptide NLP-29 against *S. marcescens* infection (COUILLAULT *et al.* 2004). All of these resources make *C. elegans* a useful model to study fungal pathogenesis.

1.7.2 *Drosophila melanogaster*

D. melanogaster is a fruit fly that has been developed as a model organism for many decades to study many different aspects of development. Its genome was sequenced in 2000 (ADAMS *et al.* 2000). Large number of *Drosophila* mutants and transgenic lines are available for use. Whole genome RNAi lines are also available (GELBART *et al.* 1997). *D. melanogaster* is also an established model organism in which innate immunity

to fungal and bacterial pathogens is studied (MYLONAKIS and ABALLAY 2005). Female flies are generally used for infection studies as they are larger in size.

D. melanogaster has no adaptive immune characteristics but the innate immune responses involve both cellular and humoral immunity. Plasmatocytes act as phagocytes and engulf pathogens, while innate immune pathways recognize the pathogen and induce the expression of antimicrobial compounds. These humoral pathways are well dissected and involve Toll and IMD signaling pathways. Interaction of the pathogen with specific pattern recognition receptors (PRR) leads to the activation of the Toll pathway which turns on a cascade of proteins that activate NF-κB-like transcription factors. This transcription factor then leads to the upregulation of antimicrobial peptide coding genes. IMD pathways turn on the Rel family transcription factor Relish (LECLERC and REICHHART 2004). Both transcription factors regulate the release of antimicrobial peptides to fight the invading pathogenic organisms. Of these, the Toll pathway is known to be more important in response to a fungal pathogen (LEMAITRE *et al.* 1996) by releasing the antifungal peptide drosomycin.

D. melanogaster has also been widely used to study fungal virulence factors of *C. albicans* using Toll receptor deficient flies (ALARCO *et al.* 2004). Aspects of phagocytosis against *C. albicans* have been studied using the S2 cell RNAi library of *D. melanogaster* (STROSCHEIN-STEVENSON *et al.* 2006). Thus, *D. melanogaster* can be used as a powerful model to study innate immunity response mechanisms to various microbial pathogens.

1.7.3 *Mus musculus*

The Mouse, *M. musculus* is one of the vertebrate model organisms that has been used to model multiple aspects of human biology. The mouse genome was sequenced in 2002 (WATERSTON *et al.* 2002) and is approximately same size as in humans, and many mouse genes have human homologues. Apart from many different aspects of human development and behavior, mice are also used to study fungal pathogenesis. Use of *in vitro* assays and invertebrate hosts has led to identification of a plethora of virulence factors in the pathogen. But, eventually all of these have to be proven in a vertebrate model to indicate that the genes are also required in vertebrate hosts. The relevance of these studies to human infections has been shown by the fact that, most of the genes that have been implicated as virulence determinants in human infection have been eventually proven to be important in either the murine oral candidiasis model, where colonization of mucosal surface leads to invasion of the underlying tissue, or the murine systemic model of infection, where *C. albicans* cells are present in the blood stream and are disseminated throughout the body. It is difficult to do a large scale screens in mice due to the expense and ethical issues concerning the killing of large number of mice. Recently, a systematic screen of a *C. albicans* homozygous deletion library in mice that overcomes these problems has been reported (NOBLE *et al.* 2010). Despite this, mice are currently used on a small scale to finally support either the role of virulence factors in fungal infection or the efficacy of antifungals in a vertebrate model.

1.7.4 *Galleria mellonella*

Use of invertebrate model organisms to study fungal pathogenesis has many advantages. But the biggest limitation is that it does not have an adaptive immune system and changes occurring in the pathogen due to the temperature of the human host (37^{o}C) cannot be studied. For these reasons the larvae of the wax moth *Galleria mellonella*, have been developed as a new model in the last decade for the study of pathogens, as they can mount both cellular and humoral immune response (TOJO *et al.* 2000) like *Drosophila*, and be infected at 37^{o}C. They possess phagocytic cells called haemocytes. These cells can perform the functions similar to phagocytes, by engulfing bacterial pathogens and producing ROS via homologous NADPH oxidase (BERGIN *et al.* 2005). These wax moth larvae have been proven for use in studying bacterial pathogens like *P. aeruginosa* (JANDER *et al.* 2000) and fungal pathogens like *C. albicans* (BRENNAN *et al.* 2002), *C. neoformans* (MYLONAKIS *et al.* 2005) and *A. fumigates* (JACKSON *et al.* 2009). Apart from the study of host-pathogen interactions, the larvae can also be used for testing antifungal or antibacterial agents (DESBOIS and COOTE 2011).

1.7.5 *Danio rerio*

D. rerio is a zebrafish that are minivertebrates which are ideal for use as model organisms for a number of reasons, among which is that the organisms have much more similarity to humans than any invertebrate system used. Zebrafish (*Danio rerio*) very recently had its genome sequenced and gene microarrays and insertional mutants are now available for studies. Gene specific knockdown even though transitory, can be

conducted through morpholino oligonucleotides. Zebrafish embryos are transparent and develop *ex utero* which allows for the real time visualization of developmental changes and other aspects under study. One limitation is that after 3 weeks postfertilization, this transparency is disrupted. To overcome this drawback, White and Zon in 2008 developed a transparent strain of zebrafish called Casper that enables visualization of the fish interior even in the adult stage (WHITE *et al.* 2008).

In the last few years, apart from studying other facets of biology, zebrafish have become a minivertebrate host model for not only innate immunity, but also adaptive immune responses (T cell and B cells, granulocytes present) to pathogens. Zebrafish have been used to study host-pathogen interactions especially through the use of strains that have fluorescent markers placed under tissue-specific promoters and use of fluorescently labeled pathogens. Many different bacterial pathogens for example *S. typhimurium* (VAN DER SAR *et al.* 2003) and *S. pyogens* (NEELY *et al.* 2002) have been very well studied in this host. Since then it was recently shown that zebrafish can also be used as a model host to study *C. albicans* infection (CHAO *et al.* 2010). The authors observed the effect of deletion of two known *C. albicans* genes, *EFG1* and *CPH1* on infection of zebrafish. They observed that lack of these genes resulted in delayed filamentation and influenced the expression of several virulence genes including *ALS3* and *HWP1*. Similar results have been reported for these strains in mouse model studies. This and other results from their work reveal that *C. albicans* infection can be studied in zebrafish. The Zebrafish Information Network (ZFIN) is now available online which contains almost all the known information about zebrafish in regards to genome sequence to commercially available strains.

All of the model organisms that have been used to study *C. albicans* infection have their advantages and disadvantages; the biggest one so far is that except for in *C. elegans*, in most cases *Candida* has to be injected into an individual host for infection to occur, which makes them undesirable for high throughput *in vivo* whole animal studies.

1.8 Summary

Treatment of systemic fungal infections is difficult because of the limited number of antimycotic drugs available. Thus, there is an immediate need for simple and innovative systems to assay the contribution of individual genes to fungal pathogenesis. Most of the pathogenesis studies are based on the ability of *Candida* to form hyphae and kill the host. The aim of this work is to develop a *Caenorhabditis elegans* based assay which will not only allow me to study the role of *Candida* in the yeast form during infection, but also help me monitor the entire infection process, not just death.

This can further help me in understanding how the disease progresses once the host is infected and also how the pathogen is able to stay fit and counteract all environmental pressure like host responses and presence of antimicrobials. Thus, I can identify novel virulence factors and pathways that are important in fungal pathogenesis which might further be used as potential targets for drug development. I can also study the general and specific *C. elegans* responses against this pathogen in order to identify host immune effectors. Apart from the candidate gene approach, the availability of mutant libraries for the pathogen and small molecule libraries for drug testing will provide me with great tools to not only identify novel virulence determinants and mechanisms responsible for causing infection, but also using a functional approach, to identify

inhibitors of *Candida* adhesion. Furthermore, I can also study the difference in pathogenicity of the progenitor and evolved strains isolated from patients suffering from *C. albicans* infection.

Chapter 2

Patho-assay using *S. cerevisiae* and *C. elegans* reveals novel roles for yeast AP-1, Yap1 and host Dual Oxidase, BLI-3 in fungal pathogenesis.

In order to study the process of fungal pathogenesis in the model host *C. elegans*, I chose to use *S. cerevisiae* as a benchmark. *C. elegans* has been used as a model host to identify both bacterial and fungal virulence factors. The assay that was developed for understanding fungal pathogenesis relies on the ability of the fungi to undergo hyphal transition and uses death as the disease indicator. In order to study other intermediate effects of fungal infection and the entire infection process, not just death as disease marker, I wanted to develop a virulence assay which could mimic normal host pathogen interaction conditions.

Once the assay was developed, in order to further prove that I can use this assay to identify virulence factors, I decided to identify the role of a transcription factor, Yap1 (Yeast Activator Protein1).

Except for figure 3B and 3C, I performed and analyzed all the work in this chapter. I was also involved in designing the experiments. This work was published as JAIN, C., M. YUN, S. M. POLITZ and R. P. RAO, 2009 A pathogenesis assay using *Saccharomyces cerevisiae* and *Caenorhabditis elegans* reveals novel roles for yeast AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis. Eukaryot Cell **8:** 1218-1227.

A Patho-assay using *S. cerevisiae* and *C. elegans* Reveals Novel Roles for Yeast AP-1, Yap1 and Host Dual Oxidase, BLI-3 in Fungal Pathogenesis.

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Running Head: Novel patho-assay for infection

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2.1 Abstract

Treatment of systemic fungal infections is difficult because of the limited number of antimycotic drugs available. Thus, there is an immediate need for simple and innovative systems to assay the contribution of individual genes to fungal pathogenesis. We have developed a patho-assay using *Caenorhabditis elegans,* an established model host and *Saccharomyces cerevisiae* as the invading fungus. We have found that yeast infects nematodes causing disease and death. Our data indicate that the host produces reactive oxygen species (ROS) in response to fungal infection. Yeast mutants *sod1*Δ and *yap1*Δ, which cannot withstand ROS, fail to cause disease, except in *bli-3* worms that carry a mutation in a dual oxidase gene. Chemical inhibition of the NADPH oxidase activity abolishes ROS production in worms exposed to yeast. This patho-assay is useful for conducting systematic, whole-genome screens to identify fungal virulence factors as alternative targets for drug development and exploration of host responses to fungal infections.

2.2 Introduction

Nosocomial microbial infections are a growing health problem. Among these, fungal infections are especially threatening with an estimated mortality rate of 40% (WISPLINGHOFF *et al.* 2003). The key reason for this alarming mortality rate is the limited range of antifungal agents. Identification of new drug targets requires high throughput infection assays that are complicated by the very fact that they involve two organisms: a host and a pathogen.

We have taken a reductionist approach to studying host-pathogen interactions and have developed a *Saccharomyces cerevisiae*-based assay to understand the genetic and molecular mechanisms of fungal pathogenesis. Using *Caenorhabditis elegans* as a model host, we have found that *S. cerevisiae* infects the worm, producing visible disease phenotypes. The two organisms used in our study are specifically suited for host-pathogen infection studies because both genomic sequences have been completely determined and mutants are readily available. A complete genome knockout collection is available in *S. cerevisiae*, a resource that does not exist for any fungal pathogen. Likewise an RNAi mediated knockdown genomic library is available in *C. elegans.* These unique tools are key in the context of a genetic screen and allow us to systematically scan the entire genomes to identify fungal virulence factors and modulators of host immunity that combat a fungal pathogen.

The budding yeast *S. cerevisiae* has recently been described as an emerging pathogen and has been isolated from human patients (McCusker 2006; McCusker *et al.* 1994). It is routinely used as a model for pathogenic fungi because a large proportion of its genes are conserved in pathogenic fungi (for review see (Liu 2001)). Homologs of genes and pathways identified in *S. cerevisiae* have been shown to be important in bona fide pathogens. It has also been used for the identification of gene products important for fungal survival in the mammalian host environment (Goldstein and McCusker 2001; Wheeler *et al.* 2003). For example the *SSD1* allele type affects pathogenicity of yeasts, indicating that allelic variation at the *SSD1* locus may be important for survival under various conditions (Wheeler *et al.* 2003). This has allowed

investigators to use reverse genetic approaches to study contributions of genes whose importance has been established in *S. cerevisiae.*

Caenorhabditis elegans has emerged as a valuable model host in which to study pathogenesis and innate immunity [for review, see (22)]. Microbial genes essential for virulence in mammalian models have been shown to be required for pathogenicity in nematodes (SIFRI *et al.* 2005). These studies have primarily explored bacterial species and have tested only a few fungi, such as *Cryptococcus neoformans* and *Candida albicans,* to explore virulence strategies. These studies focus on a killing assay using *C. elegans* and have identified several virulence factors with homologs in *S. cerevisiae* (APIDIANAKIS *et al.* 2004; MYLONAKIS *et al.* 2002a) suggesting that genes and pathways we identify in *S. cerevisiae* are likely to be found in pathogens. Moreover other pathogenic fungi tested are limited in the repertoire of laboratory tools available for their study, making them recalcitrant to genetic manipulation and inappropriate for whole genome high throughput approaches to studying fungal virulence. Recently, Breger, *et al.* described the application of a *C. elegans*-based infection assay as a tool to screen a chemical library for candidate antifungal compounds (BREGER *et al.* 2007). Our investigation complements these studies in two significant ways. First, it allows us to identify genes that exacerbate as well as attenuate the pathogenic process because we use an intermediate disease phenotype, while most other studies have used death as an endpoint phenotype. This aspect, taken together with the fact that *S. cerevisiae* share significant genetic identity with pathogenic fungi, suggests that our study will yield a basic understanding of fungal pathogenesis. Second, it allows us to conduct a systematic, unbiased, whole genome screen, which is currently not available in

pathogenic fungi. Furthermore, genes and pathways identified may be targeted for antimycotic drug development.

Facets of innate immunity are evolutionarily conserved from nematodes to mammals. For example, a common defense strategy of mammals (phagocytes), (CROSS and SEGAL 2004), plants (APEL and HIRT 2004), and insects (HA *et al.* 2005a), is to produce Reactive Oxygen Species (ROS), which directly damage pathogens. In human phagocytes, a NADPH-oxidase enzyme complex produces ROS in host defense (GAUSS *et al.* 2007a; QUINN and GAUSS 2004). In *Drosophila melanogaster*, ROS are generated in the intestine by a NADPH-oxidase to combat ingested bacteria (HA *et al.* 2005a). Loss of NADPH-oxidase activity makes the fly susceptible to the bacterial infection (HA *et al.* 2005a; HA *et al.* 2005b). Likewise *C. elegans* has also been shown to produce ROS such as superoxide and/or hydrogen peroxide when it ingests bacterial pathogens (CHAVEZ *et al.* 2007). In each case, pathogen death can be abrogated by the addition of enzymes such as catalase that break down ROS (BOLM *et al.* 2004; JANSEN *et al.* 2002; MOY *et al.* 2004) suggesting that ROS productions plays a key role in a variety of pathogenic interactions.

We have found that *S. cerevisiae* can cause infection and death in *C. elegans.* Our data indicate that the nematode host produces ROS in response to fungal infection. We demonstrate that mutant yeast carrying deletions of genes that mediate oxidative stress responses fail to induce the Dar disease phenotype except in mutant worms with an altered dual oxidase gene, suggesting that the generation of ROS is a part of the defense strategy for the host and the neutralization of ROS is needed for persistent fungal infection.

2.3 Materials and Methods

Strains, Media and Growth Conditions

Strains used for the study, for both *S. cerevisiae* and *C. elegans* are listed in Table 1. Deletions in the yeast-deletion set strains were confirmed by PCR or recreated using a PCR mediated gene disruption cassette (WACH *et al.* 1994).

Table 1. Strains used in this study

Strains	**Description**	**Source**
S. cerevisiae		
BY4741	MATa;*his3*Δ1;*leu2*Δ0;*met15*Δ0;*ura3*Δ0	IDL*
Pdc1::mcherry	*PDC1*::mcherry in BY4741	G. Fink, MIT
*yap1*Δ	*yap1*Δ in BY4741	IDL
*yap2*Δ	*yap2*Δ in BY4741	IDL
*yap4*Δ	*yap4*Δ in BY4741	IDL
*sod1*Δ	*sod1*Δ in BY4741	IDL
*pdr1*Δ	*pdr1*Δ in BY4741	IDL
*pdr15*Δ	*pdr15*Δ in BY4741	IDL
C. elegans		
N2 Bristol	Wild type	CGC†
bli-3(e767)I	Dual oxidase	CGC
jnk-1(gk7)IV	MAPK of JUN MAP kinase pathway	CGC
mek-2(n1989)I	MAPKK of ERK MAP kinase pathway	CGC

IDL* – Invitrogen Deletion Library

CGC† – Caenorhabditis Genetics Center

The *C. elegans* wild-type strain was var. Bristol, strain N2. Mutant and wild type *C. elegans* strains were obtained from the Caenorhabditis Genetics Center (CGC, Minnesota). *C. elegans* stocks used for the study were grown on Nematode Growth agar medium (NGM) on *E. coli* OP50 and maintained as described previously (BRENNER 1974). *E. coli* OP50 was grown overnight in Luria Broth at 37^{o}C. Yeast growth media were prepared as described by Sherman *et al.* (SHERMAN *et al.* 1986) and strains were grown overnight at 30^{o}C.

Egg Preparation

Worms were grown for 4-5 days on NGM agar containing *E. coli* OP50 at 20^{o}C. Eggs and worms were washed off four plates with M9 buffer (BRENNER 1974) and centrifuged at 900 xg for 2 min. The pellet was resuspended and washed twice with M9. This was then resuspended in a 1:4 dilution of commercial bleach (5.25%) containing 0.25 M Sodium Hydroxide solution, mixed gently by inversion for 3 minutes, and centrifuged for 2 min at 2000 xg. The pellet was washed and centrifuged twice with M9 buffer at 2000 xg for 2 min each and then finally resuspended in M9 buffer. The egg suspension was diluted or concentrated with M9 as required to obtain approximately 5-6 eggs/µl.

Pathogenesis Assay

E. coli and yeast strains were grown overnight at 37^{o}C and 30^{o}C respectively. Culture aliquots were centrifuged at full speed in a micro-centrifuge, washed twice in sterile

water and finally resuspended to a final concentration of 200mg/ml and 20mg/ml respectively. A 3:1 ratio of *E. coli*: yeast by weight (an estimated 30:1 ratio by number of organisms) mixture was prepared. A mixture of 10 μl of 50 mg/ml streptomycin sulfate stock and 10 μl of *E. coli*: yeast mix (2.5 μl:7.5 μl) was spotted on each NGM plates and 5 μl of *C. elegans* egg suspension was transferred to each plate. Plates were observed for 4-5 days unless specified otherwise. ANOVA was used to check the statistical significance of the differences observed between mutants and wild type yeast strains.

Test of Koch's postulates and measurement of colony-forming units

Twenty worms each from *S. cerevisiae* test-plates and *E. coli* control-plates were picked and washed four times with sterile water. Worms were then crushed using a freeze fracture technique in individual microfuge tubes and resuspended in 100 μl of sterile water. Appropriate dilutions of the mixture were transferred to YPD plates and incubated overnight. Colonies were counted to estimate colony-forming units (CFUs). Resulting colonies were replica plated to selective media to test for auxotrophic markers. Two of these colonies were retested using the patho-assay described above.

Microscopy of *C. elegans*

A 2 % agarose pad containing 0.01 M sodium azide, as anesthetic was prepared on a slide. A 3 μl drop of M9 buffer was added to the pad. Worms were picked and

transferred to the drop on the slide. Mounted worms were then covered with a cover slip and observed at 40 X and 20 X magnifications using an Axiovision Zeiss Microscope under DIC (Nomarski) and epifluorescent optics. An ApoTome attachment was used to enhance fluorescence images.

Amplex Red Hydrogen Peroxide Assay

NGM plates were spotted with 20 µl of 1:1 (v:v) mixture of streptomycin (50 mg/ml) and overnight yeast culture. These plates were then kept overnight at 30 ^{o}C. Approximately L3-L4 stage worms were washed off stock plates with M9 and then transferred to these plates and kept at 20 ^{o}C for 10 hr. Amplex Red Assay Kit (Molecular Probes) was used to detect hydrogen peroxide. Post incubation the worms were washed four times with 1ml of reaction buffer and resuspended to a final volume of 100 worms/ 50 µl. 50 µl of this suspension was added to the wells of a 96 well polystyrene plate. Diphenyleneiodonium (DPI) was added to some samples to make a final concentration of 100 µM and allowed to stand for 10 min. Then 50 µl of Amplex Red reaction buffer was added to each well and color change was observed over 3-5 hours.

***C. elegans* survival analysis**

For survival analysis, test plates and *C. elegans* eggs were prepared as described in the Pathogenesis Assay methods section. Each plate was started with 30 ± 5 eggs; each experiment included three yeast and three *E. coli* plates per strain. Beginning on

the second day after plating eggs, the number of dead and live worms on each plate was recorded daily. Live worms were transferred to new plates as necessary to avoid confusing the original worms with their offspring. All plates were of the same composition as the original test plates.

SigmaStat 3.5 (Systat Software, Inc.) was used to analyze the survival curve data. Significance, defined as $p<0.05$, was assessed using the Gehan-Breslow test. In our experiments, worms that left the plates in the first several days were "censored", i.e., removed from the counts of subsequent days. The Gehan-Breslow test assumes that the data from early survival times are more accurate than later times and weights the data accordingly. The number of censored worms was taken as the total number of worms (dead plus live worms) on that day minus total worms on the previous day. All of the data were collected from two independent experiments.

2.4 Results

Development of an assay for fungal pathogenesis

Study of host-pathogen relationships would be greatly simplified by the use of two well characterized model systems with readily available mutants. This would allow simultaneous study of the genetics of both host and pathogen. To this end, we developed a patho-assay using *C. elegans* as the model host and *S. cerevisiae* as the invading fungus. This system will allow us to use systematic whole genome approaches to study fungal pathogenesis as well as host responses to fungal infections.

In order to study the interaction of *S. cerevisiae* with *C. elegans*, the organisms were co-cultured on NGM plates. L3 -L4 stage larval or adult hermaphrodites that were offered only *S. cerevisiae* as food successfully laid eggs but their progeny arrested at an early (L1-L2 larval) stage of development. One possible explanation for the larval-stage growth arrest phenotype is that the yeast cells were too large for young larvae to ingest. To test this we co-cultured hermaphrodite worms on lawns of RFP labeled yeast. Within a few hours, labeled yeast (or yeast particles) was visible in the intestinal lumen of adult and late stage larvae (data not shown) but not in the early-larval stages. Therefore in subsequent assays *E. coli* was added as a nutritional supply along with small amounts of *S. cerevisiae*. In this way small larvae could overcome the growth arrest while being exposed to yeast.

C. elegans grown on *E. coli* as control (Figure 1A) and mixed lawns of *E. coli*, strain OP50 and small amounts of *S. cerevisiae*, strain S288c, (BY4741) exhibited the **d**eformed **a**nal **r**egion (Dar) phenotype (Figure 1B). This phenotype was never seen in worms grown on *E. coli* OP50 alone (Fig. 1A). The Dar phenotype, which has been established as a disease symptom of *C. elegans* infected with bacteria *Microbacterium nematophilum* (GRAVATO-NOBRE *et al.* 2005; HODGKIN *et al.* 2000; NICHOLAS and HODGKIN 2004) is characterized as a distinctive swelling in the post- anal region. It has been suggested that the anal region of the worm is enlarged in response to infection, as worm mutants that do not show this phenotype are much more adversely affected by *M. nematophilum* infection. This deformity has been genetically characterized as a part of a defense reaction of the worm and can be used as a marker for infection (GRAVATO-NOBRE *et al.* 2005). An intermediate phenotype allows us to identify factors that

exacerbate (for example Dar visible earlier or death) as well as attenuate the pathogenic response. Late stage larvae or adult worms grown on yeast alone also display the Dar response. In this study we used Dar as a phenotypic marker and previously characterized host genes, involved in the Dar response, as genotypic markers of the disease condition.

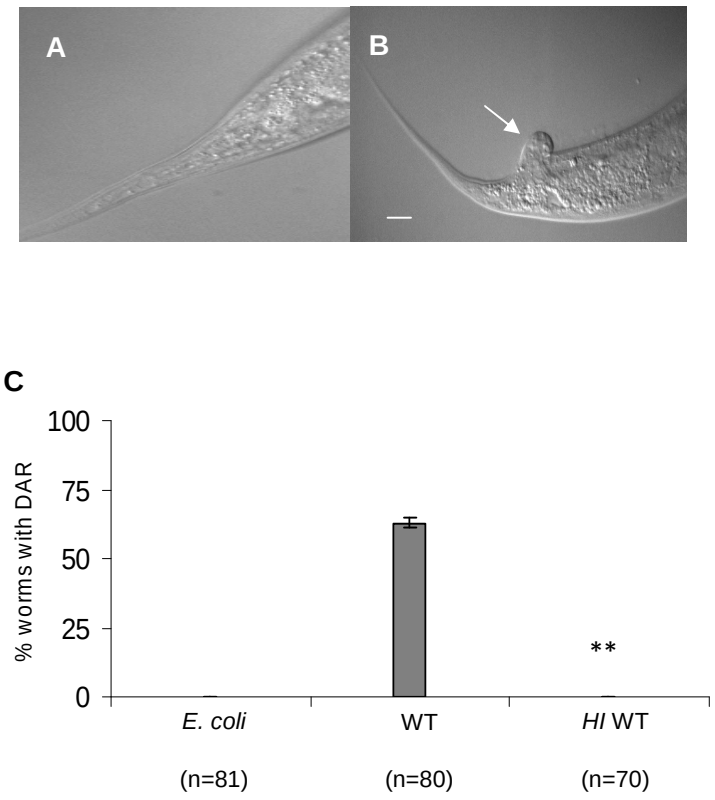


Figure 1. *S. cerevisiae* causes a deformity in the post anal region (Dar) in *C. elegans*. (A) *C. elegans* exposed to *E. coli* as control. (B) Worms exposed to *S. cerevisiae* show the Dar phenotype (arrow). Scale – 20µm. (C) The Dar phenotype was scored on Day 4 for nematodes exposed to *E. coli*, *S. cerevisiae* (WT) and heat inactivated (HI) wild-type *S. cerevisiae*. The difference between WT and HI is statistically significant ($p<0.01$, ANOVA).

Worms were monitored twice daily from the time the eggs were added to the microbial cultures up to day four, when the Dar disease was clearly visible. At day four the Dar phenotype was scored for worms exposed to yeast or heat-inactivated yeast and compared to worms reared on *E. coli* (Figure 1C). These results clearly indicated that the Dar phenotype affected worms exposed to yeast and that only metabolically active yeast cells were capable of eliciting Dar. To test whether the Dar phenotype is reversible, we transferred the affected worms to plates containing *E. coli*. The worms were 'cured' of the Dar phenotype within two days of transfer. This supports the notion that yeast causes the deformity in the anal region and that worms can recover by clearing the yeast when they are no longer exposed to it.

The nematode's response to yeast is unlikely to be due to starvation because microscopic observation of the patho-assay plates indicated that at the time when the Dar phenotype was visible, ample food was present. Furthermore the inability of heat-inactivated yeast and mutant yeast (described in later section) to evoke the Dar phenotype strongly supports the notion that this manifestation of infection is a response to the pathogen rather than a response to starvation. We also ruled out the possibility that the worms are unable to digest the yeast because L3-L4 stage larvae that are reared on yeast develop into fertile adults suggesting that they are able to meet their nutritional requirements from yeast.

As a test of Koch's postulates, we reisolated and cloned microbes from an infected worm after extensive washing to remove externally-associated yeast cells (see Materials and Methods). Genotypic characterization of the reisolated yeast demonstrated that they harbored the same mutant markers as the original yeast strain.

Furthermore, yeast cells from two independent colonies isolated from the worm were used to re-infect wild type worms. These worms showed the same extent of Dar disease progression observed with the original yeast strain. These results indicate that the etiological agent for the Dar disease was the one that we introduced, not a spurious contaminant.

Progressive distension and accumulation of yeast in the intestinal lumen.

To visualize yeast present in the intestine and clearly differentiate them from yeast sticking to the outside of the worm, we used a fluorescent-tagged housekeeping protein to follow progression of disease and specifically test the hypothesis that the intestinal distension is due to the accumulation of yeast. We chose an in-frame fusion of the yeast protein pyruvate decarboxylase (Pdc1) with the Red Fluorescent Protein (RFP) because its RFP fluorescence was clearly visible and maintained over the five-day period of our assay. Moreover, it was indistinguishable from a yeast strain containing the native untagged version of *PDC1* in all phenotypic tests (personal communication Dr. G. Fink, MIT).

A time course of microscopic evaluation of infection using RFP-labeled yeast revealed that by day three, RFP-labeled yeast cells had started to accumulate in the pharynx and the intestine (Figure 2A and 2D). By day four (Figure 2B and 2E) and day five (Figure 2C and 2F), progressively more yeast had accumulated in the pharynx and intestine, causing the lumen to be severely distended compared to uninfected worms in which the intestinal lumen is a narrow tube. Intestinal distension is also observed in *M.*

nematophilum infection, but bacteria accumulate at the anus and in the rectum near the Dar tail swelling and do not extend into the intestine (NICHOLAS and HODGKIN 2004). This is in contrast to what we observed - four days post exposure, the intestinal lumen appeared to be packed with yeast, and the yeast were not observed to accumulate in the rectum. This striking difference may be indicative of the primary route of entry for the fungal pathogen, which appears to be oral ingestion. Distension of the intestinal lumen was also visible in DIC (Nomarski) micrographs of infected worms (Figure 1B).

Consistent with these microscopic observations, numbers of intact yeast cells released from infected worms increased during the course of infection. CFUs were determined for yeast recovered from the worm intestine on days 2, 3 and 5. A 4-fold increase in the number of yeast CFUs was observed between day 2 and day 3 and a10-fold increase between day 2 and day 5.

Between days five and seven, a 5–17 % decrease in viability was observed on yeast test-plates. The progeny of some dead worms appeared to have hatched inside the animal. Upon closer inspection on days four and five, we noted a pronounced swelling in the vulval region of these worms (Figure 2H and 2I). It is possible that this swelling impeded eggs from being laid and caused them to hatch within the worm resulting in matricidal death. These qualitative assessments suggest that viable *S. cerevisiae* causes visible lesions near two of the three body openings of *C. elegans*. Coincidentally, such body openings are common sites of fungal infection in mammals. Having established phenotypic characteristics of the diseased condition in the worm host, we wanted to assay the contribution of known molecular markers of bacterial disease in our *S. cerevisiae – C. elegans* based patho-assay.

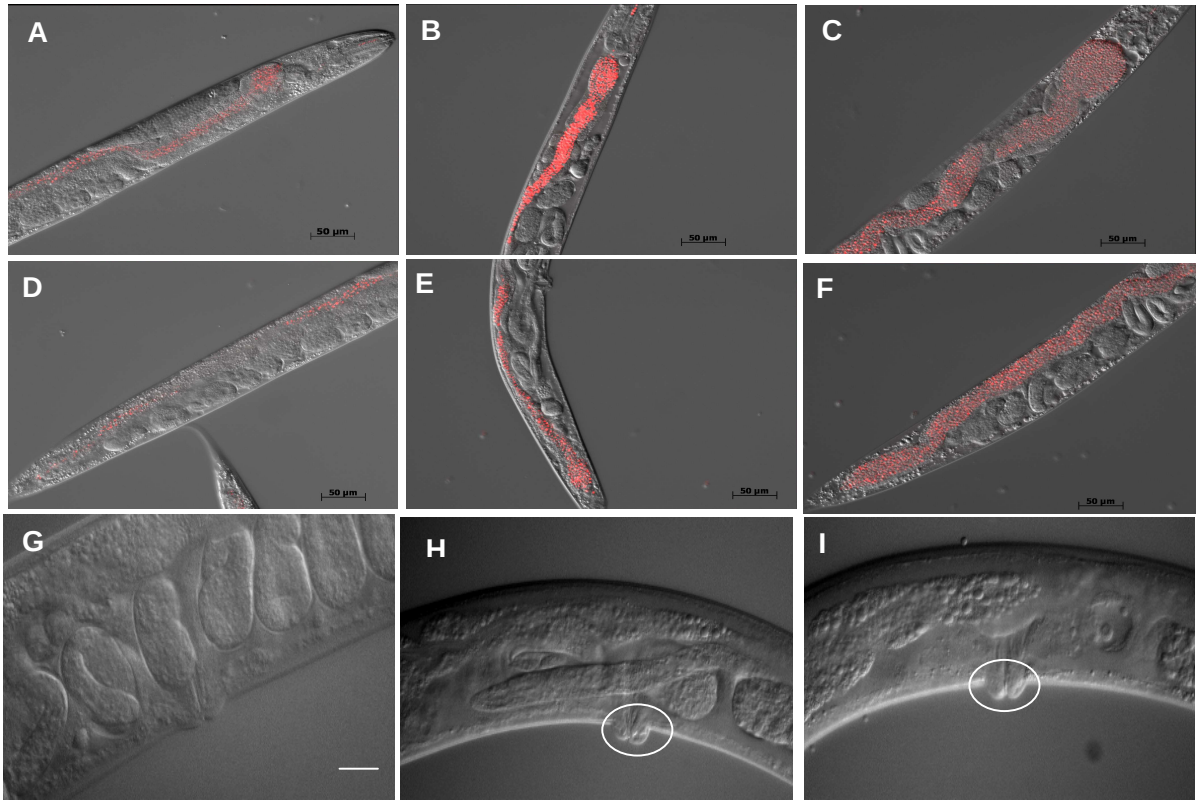
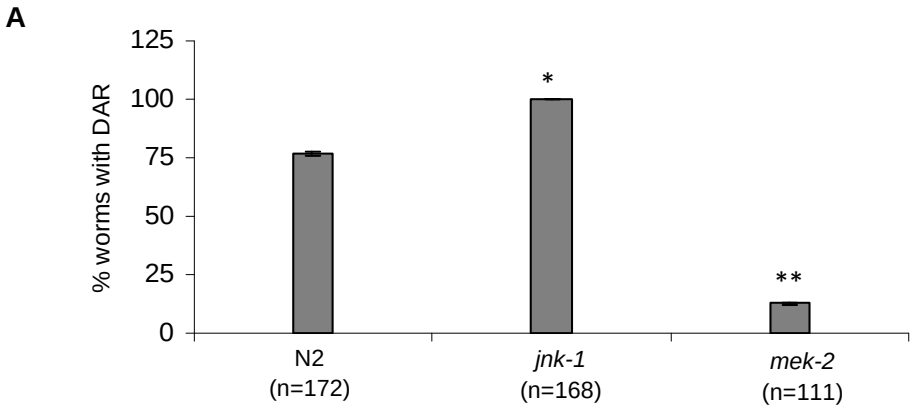
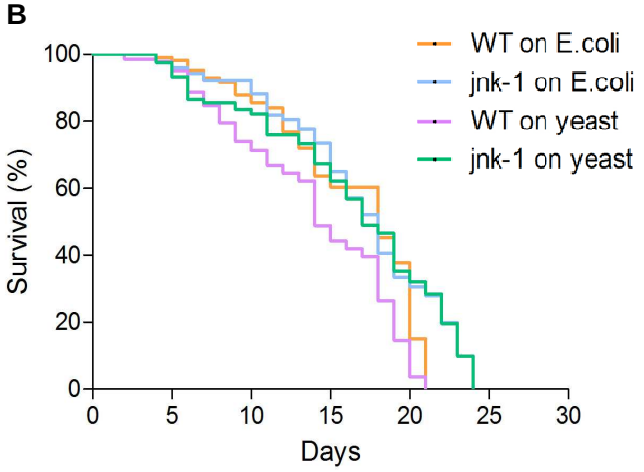
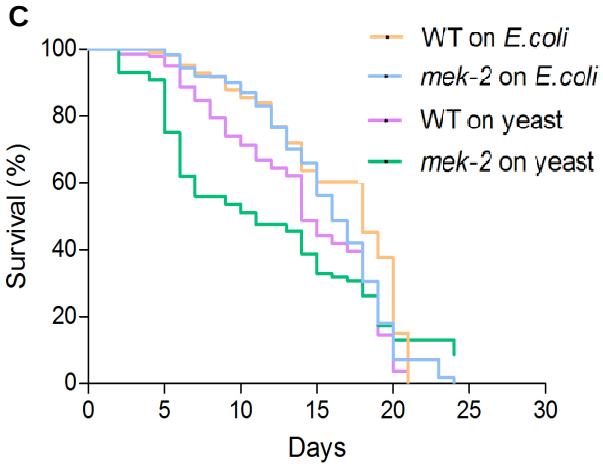


Figure 2. Time course of intestinal distention in *C. elegans* exposed to *S. cerevisiae*. Worms were exposed to RFP marked, wild type yeast from hatching and photographed on day 3 (A and D), day 4 (B and E) and day 5 (C and F). The experiment was done 3 times and 60-75 worms were observed over a 3-day period. A – C shows the anterior region of the worm and D – F shows the posterior region of the worm. Accumulation of yeast began in the pharynx region (compare A and D) and proceeded to the posterior. *S. cerevisiae* also induces vulval swelling in the worms. Worms exposed to *S. cerevisiae* (H – I) show abnormal vulval swelling (white circles) as compared to the control sample grown on *E. coli* (G). Scale: A – G = 50µm, H – I = 20µm.


A
% worms with DAR
125
100
75
50
25
0
*
**
N2
(n=172)
jnk-1
(n=168)
mek-2
(n=111)


B
Survival (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
WT on E.coli
jnk-1 on E.coli
WT on yeast
jnk-1 on yeast


C
Survival (%)
100
80
60
40
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5
10
15
20
25
30
Days
WT on E.coli
mek-2 on E.coli
WT on yeast
mek-2 on yeast

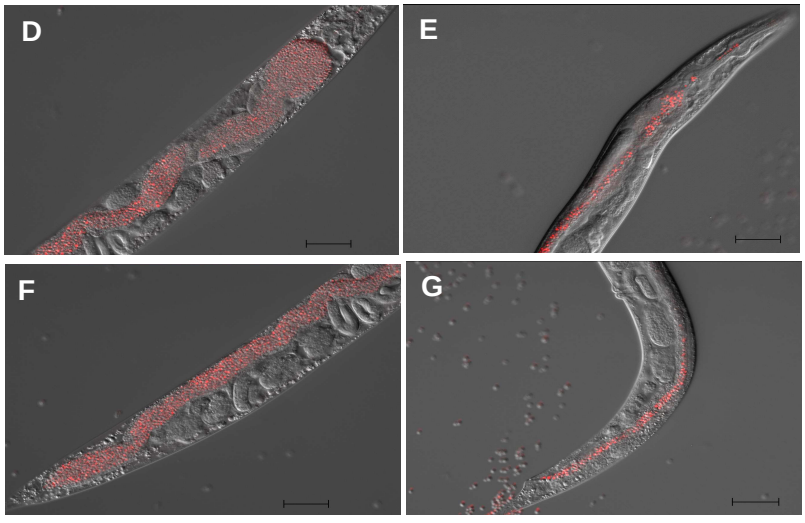
D
E
F
G

Figure 3. An active ERK MAPK pathway is required for the DAR phenotype and survival of worms upon yeast infection. (A) Worm mutants *jnk–1(gk7)* of JUN MAPK pathway and *mek–2(n1989)* of ERK MAPK pathway were exposed to wild-type yeast and DAR phenotype was scored on Day 4. The *mek-2(n1989)* mutants, but not *jnk-1(gk7)* showed decreased Dar compared to the wild-type N2 worms. Differences between *mek-2(n1989)* or *jnk-1(gk7)* and wild-type are statistically significant compared to the wild type. Values for *mek-2(n1989)* and *jnk-1(gk7)* are significantly different from wild-type values (*p<0.01, **p<0.001, ANOVA). (B-C) Survival curves for the *mek-2(n1989)* and *jnk-1(gk7)* mutants indicate that *mek-2(n1989)* mutant worms were more susceptible to yeast than wild-type worms ($p < 0.001$, Gehan-Breslow test). The *jnk-1(gk7)* mutants survived just as long as their wild type counterparts. (D-G) Worms showing Dar accumulated less yeast, at both, the anterior and posterior regions as compared to worms without Dar. Wild-type nematodes were exposed to RFP marked, wild type yeast and photographed on Day 5. The experiment was done 3 times and 20-25 worms were observed in each experiment. D – E shows the anterior region of the worm and F – G the posterior end of the worm. E and G are the worms with Dar.

Requirements for *C. elegans* ERK MAPK pathway in yeast infection - a verification of molecular markers of host response

C. elegans response to pathogens involves the MAPK pathways. An active ERK pathway has been shown to be required for the Dar response of worms exposed to *M. nematophilum* (NICHOLAS and HODGKIN 2004) while the *jnk-1* gene, encoding a JNK-like MAP kinase, is not required. The JNK pathway has been implicated in immunity in other organisms (DONG *et al.* 2002). We tested representative loss-of-function mutants in the ERK and JNK MAPK pathways for effects on *S. cerevisiae* pathogenesis in *C. elegans* (Table 1). The *mek-2* gene encodes a MAPKK in the *C. elegans* ERK pathway

(Wu *et al.* 1995). The loss of function mutant *mek-2(n1989)* showed a very low percentage of tail deformity when exposed to yeast compared to N2 wild-type worms (Figure 3A). However, a loss-of-function mutation in the JNK-like MAP kinase *jnk-1(gk7)* exhibited the Dar phenotype in response to *S. cerevisiae*. These effects are analogous to the Dar response of nematodes with *M. nematophilum* infections suggesting that the ERK MAPK pathway is required for the tail deformities while the JNK MAPK pathway is not.

To study the contribution of these MAPK pathways to the survival of the host worm upon exposure to yeast we conducted survival assays. This assay measured the survival of mutant worms as compared to wild-type worms when exposed to *S. cerevisiae*. Survival of wild-type worms exposed to yeast versus *E. coli* was also monitored. Worm mutants were grown on *E. coli* to evaluate their general health. Our data indicated that the *mek-2* mutant that was unable to exhibit the Dar response also showed enhanced susceptibility to yeast killing compared to wild-type *C. elegans* on yeast ($p<0.001$, Gehan-Breslow test, Figure 3B). By contrast, the *jnk-1* mutant that showed no defect in its Dar phenotype also showed no difference in survival compared to wild-type *C. elegans* on yeast, and in fact appeared to be less susceptible than wild type ($p=0.055$, Gehan-Breslow test, Figure 3C). Wild-type *C. elegans* showed a slight yet significant reduction in survival on *S. cerevisiae* compared to *E. coli* ($p < 0.008$, Gehan-Breslow test). The direct correlation between survival of the worm mutants and the severity of their Dar response (Figure 3A correlated to Figure 3B and 3C) suggests that the Dar response might be a protective phenotype. To address this we observed the extent of intestinal distention (or constipation) in worms exhibiting the Dar

phenotype as compared to worms that did not show the Dar response. We used the RFP-marked yeast strain for visualization (Table 1). We found that wild-type worms exhibiting the Dar phenotype accumulated less yeast in their intestine thus enhancing survival (Figure 3D- 3G). Together these results suggest that the ERK MAPK pathway is important for the Dar response and protecting the worms from *S. cerevisiae* infection. Albeit the exact defensive mechanism is not clearly understood, a similar observation was noted for *M. nematophilum* infection of *C. elegans* (GRAVATO-NOBRE *et al.* 2005; NICHOLAS and HODGKIN 2004).

Fungal resistance to oxidative stress is required to establish disease.

In order to use this patho-assay to identify fungal virulence factors, we used a candidate gene approach in which yeast gene-specific deletion mutants were tested for their ability to induce the Dar phenotype in *C. elegans*.

We chose Yap1 and its paralog Yap2 for this study because these genes have been shown to be important in host–pathogen interactions of plant and human fungal pathogens (ALARCO and RAYMOND 1999b; LEV *et al.* 2005a; PRUSTY *et al.* 2004). It has been shown that Yap1 is activated in conidial germ tubes of *Cochliobolus heterostrophus*, a fungal pathogen of maize, at the earliest stage of plant infection and persists during infection (LEV *et al.* 2005a). Furthermore, overexpression of *CAP1* (the *C. albicans* ortholog of *YAP1*) confers resistance to the popular clinical antifungal fluconazole, an azole derivative (ALARCO *et al.* 1997a). However, until this study no one had demonstrated that Yap1, or its orthologs, are required for

pathogenesis. Yap1 has been recognized as a likely candidate for antimycotic drug development because it is a fungal-specific transcription factor of the AP-1 family that is involved in multidrug resistance (ALARCO and RAYMOND 1999b; LEV *et al.* 2005a; PRUSTY *et al.* 2004). It also regulates oxidative stress responses in fungi as the mutants show lack in growth in the presence of reactive oxygen species (COLEMAN *et al.* 1999; ZHANG *et al.* 2000b). Otherwise there are no apparent growth defects in *yap1*Δ and *yap2*Δ mutants. Therefore we chose to test *yap1*Δ and its paralog *yap2*Δ in our patho-assay.

C. elegans exposed to either *yap1*Δ or *yap2*Δ mutants failed to exhibit the Dar phenotype (Figure 4A). As a control we showed that a mutant in a related transcription factor, Yap4, did exhibit Dar phenotype (Figure 4A). These results strongly support previous observations in bona fide fungal pathogens that Yap1 plays a key role in the infection process. These studies suggest that Yap1 and its paralog, Yap2, may be important virulence factors.

In addition to the regulation of oxidative stress responses, Yap1 plays a role in regulating multidrug resistance genes (ALARCO *et al.* 1997a; ALARCO and RAYMOND 1999b). To define which aspect of Yap1 is required to evoke the Dar response we tested mutants that were defective in one or the other responses but not both. The specific mutants tested were Superoxide dismutase (*sod1*Δ) and Pleiotropic Drug resistance (*pdr1*Δ, *pdr15*Δ) genes using the same patho-assay. The physiological role for Sod1 is to guard cells against oxidative damage by neutralizing reactive oxygen species and the mutants show normal growth except when in the presence of reactive oxygen species (BERMINGHAM-MCDONOGH *et al.* 1988; CHANG *et al.* 1991; LONGO *et al.*

1999). Pdr1, like Yap1, is a transcription factor, and regulates resistance to a variety of drugs while *PDR15* encodes an ATP binding cassette (ABC) transporter of the plasma membrane implicated in general cellular detoxification (DECOTTIGNIES *et al.* 1998; HIKKEL *et al.* 2003; WOLFGER *et al.* 1997; WOLFGER *et al.* 2001; WOLFGER *et al.* 2004). Like *yap1*Δ and *yap2*Δ mutants, s*od1*Δ mutants failed to elicit the Dar response while *pdr1*Δ and *pdr15*Δ mutants were indistinguishable from their wild-type counterparts (Figure 4B).

These experiments demonstrate that the fungal-specific transcription factors, Yap1 and Yap2, are required to elicit the Dar response. These proteins mediate oxidative stress responses and drug resistance. Using mutants defective in only one of these two pathways made it unlikely that multi-drug resistance is required for virulence; we believe that the inability of Yap mutants to tolerate oxidative stress compromises their ability to elicit Dar. Furthermore, these results suggest that fungi must be able to neutralize ROS in order to cause disease. This presents a testable hypothesis that this requirement stems from the fact that *C. elegans* produce ROS when they are infected with *S. cerevisiae.*

Worms produce reactive oxygen species in response to a fungal infection

ROS have been shown to play an important role in pathogenesis and defense. Human phagocytes are known to produce ROS when fighting infections (BABIOR *et al.* 1976). It has recently been shown that *C. elegans* produce ROS when infected with bacteria (CHAVEZ *et al.* 2007; MOY *et al.* 2004), presumably as a part of their defense

mechanisms. Worms also produce lipofuscin, a marker of oxidative stress, in intestinal cells as they age (GERSTBREIN *et al.* 2005) and lipofuscin is produced earlier in development when young worms are infected with a pathogen that elicits ROS production (12). We tested the hypothesis that young worms exposed to yeast produce more ROS by measuring relative amounts of ROS produced in worms exposed to yeast as compared to worms reared on bacteria. We used a commercially available biochemical assay (Amplex Red peroxidase kit, Molecular Probes) to test ROS produced by the worms in response to pathogenic attack (CHAVEZ *et al.* 2007). Hydrogen peroxide produced by the host oxidizes the substrate, Amplex Red to form a red product. Here we show that worms exposed to yeast produced more ROS than the control population grown on *E. coli* (Figure 4C). Furthermore, we showed that ROS production can be chemically compromised using Diphenyleneiodonium sulfate (DPI), a specific inhibitor of NADPH-oxidase activity that does not inhibit peroxidase (BINDSCHEDLER *et al.* 2006). ROS production in phagocytes is catalyzed by an NADPH-oxidase (BABIOR *et al.* 1976) and DPI has recently been shown to inhibit production of ROS in nematodes in response to pathogenic attack (CHAVEZ *et al.* 2007). Worms exposed to yeast that are treated with DPI oxidize Amplex Red only as well as unexposed worms (Figure 4C) suggesting that DPI mediated inhibition of the NADPH-oxidase activity, impedes the ability of the worm to produce ROS. These results directly implicate NADPH-oxidase activity in the host response to fungal infection and suggest that such an activity might constitute a protective response against yeast infection. These observations prompted us to look for *C. elegans* proteins that contain a domain similar to the mammalian NADPH-oxidase domain, *gp91phox.*

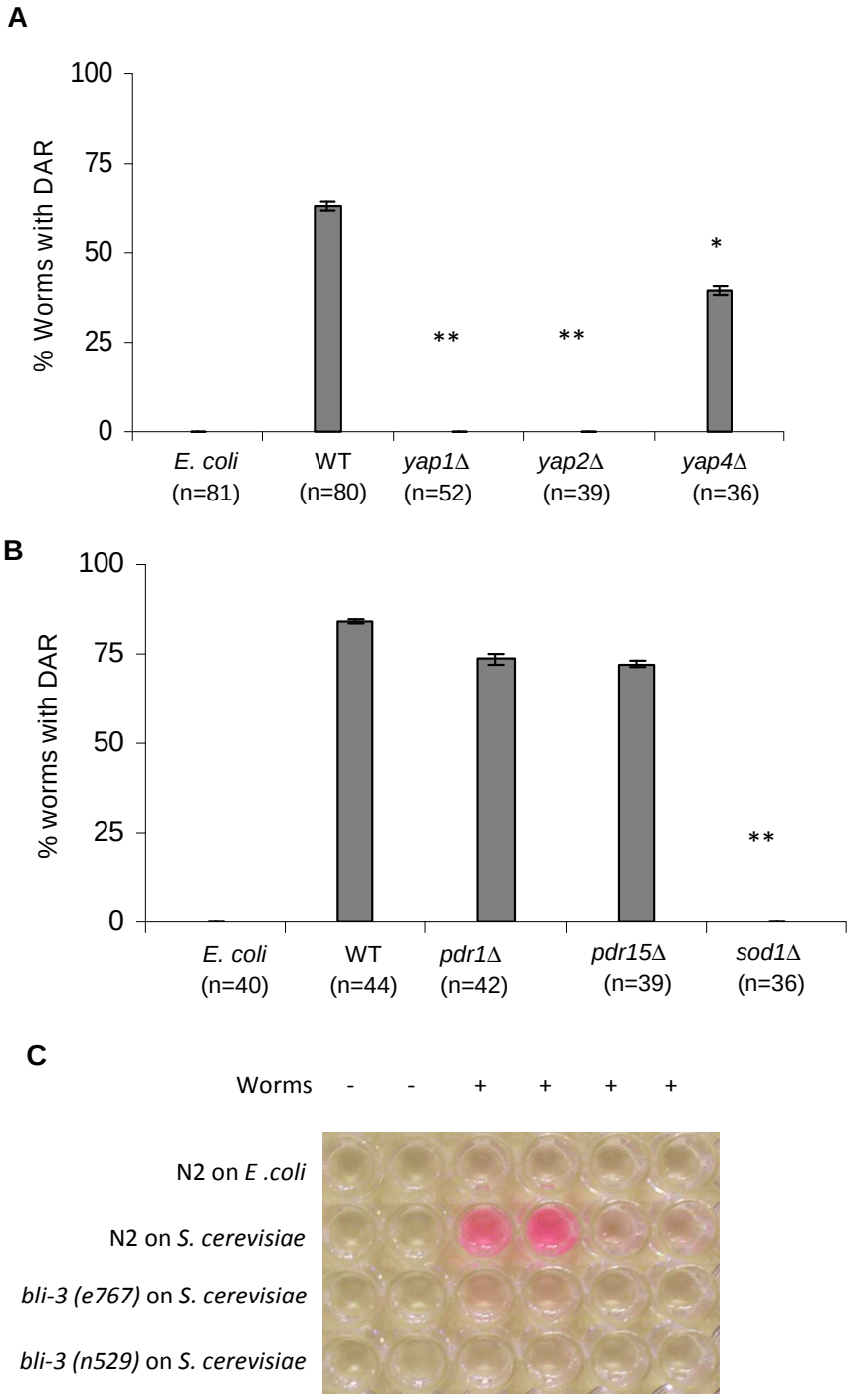

Figure 4. Yap1, Yap2 and Sod1 are required for fungal virulence. The Dar phenotype was scored on Day 4 for nematodes exposed to: (A) *yap1*Δ, *yap2*Δ and *yap4*Δ mutants, (B) *sod1*Δ, *pdr1*Δ or *pdr15*Δ mutants and compared to *E. coli* or the isogenic wild-type yeast strains. Values for *yap1*Δ, *yap2*Δ, *yap4*Δ or *sod1*Δ are significantly different from

wild-type values (*p<0.01, **p<0.001, ANOVA). The *pdr1*Δ and *pdr15*Δ mutants are not significantly different from wild type (p>0.01, ANOVA). (C) Production of ROS was monitored using Amplex Red reagent in wild type and*bli-3* (alleles e767 and n529) mutant worms exposed to *S. cerevisiae* in the presence or absence of DPI. Each experiment was performed in duplicate and repeated 3 times. Worms treated similarly with *E. coli* was used as a control.

Homology searches revealed that an NADPH-oxidase motif is present in the coding sequence of the *bli-3* (or *Blister-3*) locus. In the encoded protein, the *gp91phox* motif is juxtaposed with a peroxidase domain in a single polypeptide, hence it is referred to as a Dual oxidase (CeDuox1) (EDENS *et al.* 2001; LAMBETH *et al.* 2000; SIMMER *et al.* 2003). Therefore we tested the hypothesis that CeDuox1 is responsible for generating an environment of elevated ROS.

Mutant worms that are unable to produce ROS are susceptible to *S. cerevisiae.*

Two mutations, *bli-3*(e767) and *bli-3*(n529) have been identified in the gene encoding CeDuox1 (SIMMER *et al.* 2003) The mutant phenotypes of these strains recapitulate those produced by RNAi of CeDuox1 (EDENS *et al.* 2001). Using the Amplex Red assay described above we showed that both mutant alleles of *bli-3* produced less ROS when exposed to yeast (Figure 4C). We hypothesized that mutant yeast that are sensitive to ROS, hence unable to induce Dar in a wild-type nematode host, would be able to do so in a *bli-3* mutant worm. To address this we tested the *bli-3*(e767) mutant allele in our patho-assay. The *bli-3* gene encodes the dual oxidase (CeDuox1) that we believe is

involved in creating an environment of elevated ROS. Yeast *yap1*∆ and s*od1*∆ mutants that are unable to evoke the Dar response in wild type worms are competent to induce Dar in *bli-3* mutant worms (Figure 5). Furthermore, the *bli-3* mutant elicits the Dar disease earlier, after three days, in contrast to the wild type, which typically shows Dar only after four days. No Dar phenotype was observed when wild-type N2 and *bli-3* mutant were grown on *E. coli* as control (data not shown). These results strongly support the interpretation that worms exposed to yeast generate a burst of ROS via the action of the *bli-3* gene product CeDuox1 that inhibits the ability of yeast to induce Dar. In the absence of this defensive response, even the *yap1*∆ and s*od1*∆yeast mutants can induce Dar.

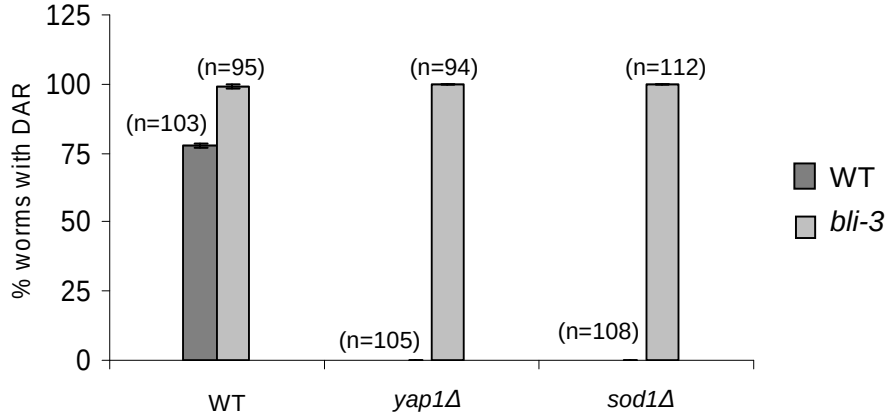

Figure 5. The yeast *yap1*∆ and *sod1*∆ mutants are able to cause Dar disease in *C. elegans bli-3(e767)* mutant. The *bli-3(e767)* gene encodes a Dual oxidase (CeDuox1), which contains a NADPH oxidase motif that has been implicated in ROS production in phagocytes. *C. elegans bli-3(e767)* mutants were exposed to *yap1*∆ and *sod1*∆ mutants and the isogenic wild type as control. Their Dar response was scored on Day 4 and compared to wild type worms that were exposed to the yeast *yap1*∆ and *sod1*∆ mutants and wild-type counter parts.

We have used genetic and biochemical approaches on both sides of the host-pathogen equation in a patho-assay to demonstrate a role for ROS in the host defense against fungal pathogenesis. We have identified Yap1 and its homolog Yap2 as factors, which may be used as targets for antifungal drug development. We have also identified CeDuox1 as a modulator of host responses to yeast infections. Studies such as the current one have the potential of conducting unbiased, whole genome screens to identify novel fungal virulence factors and will greatly enhance our knowledge of host defense mechanisms in response to fungi.

2.5 Discussion

Our studies have demonstrated that ROS play a central role in mediating host-pathogen interactions in our model. Using our novel patho-assay with *S. cerevisiae* as a model pathogen and *C. elegans* as the model host we find that yeast can infect worms resulting in intestinal distension, a deformity in the post anal region (DAR) and death of the host nematode. In this study we show that ROS play an important role in fungal pathogenesis and host defense. The *C. elegans bli-3* gene encodes a dual oxidase known to have mammalian homologs capable of producing ROS. Our results show that two *bli-3* mutants with point mutations in the peroxidase domain (SIMMER *et al.* 2003) do not produce hydrogen peroxide in the Amplex Red assay. This may seem surprising, because classically, superoxide produced by NADPH oxidase is converted to hydrogen peroxide by dismutation, then to antimicrobial oxidants by peroxidase. According to this model, a mutation in the peroxidase domain would not be expected to alter production

of the hydrogen peroxide. However, it has been suggested that the peroxidase domain of the dual oxidases catalyzes dismutation as well as production of antimicrobial oxidants (LETO and GEISZT 2006) In that case, a mutation in the peroxidase domain might well cause a decrease in hydrogen peroxide production. Alternatively, a mutation in the peroxidase domain may alter the topology of the oxidase domain and decrease its activity. Further studies of the dual oxidases will be required to understand the details of their catalytic mechanisms.

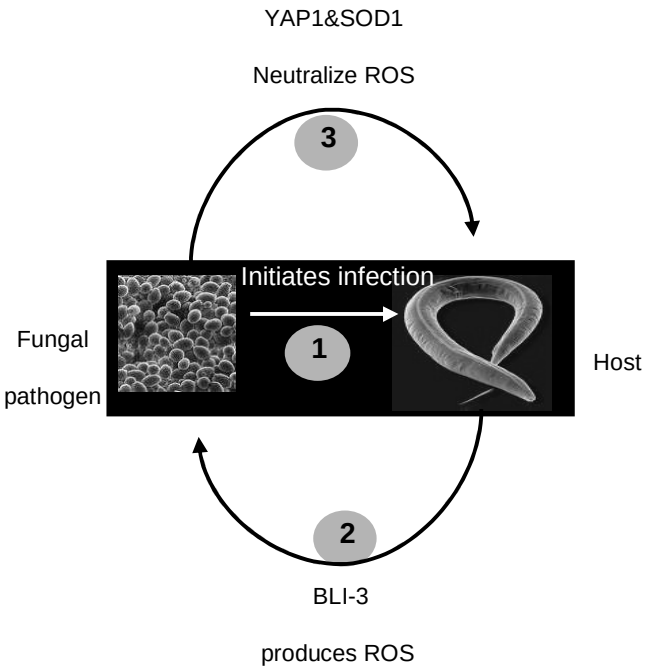

Figure 6. Working model for the role of ROS in early events in host defense and fungal pathogenesis.

Our working model (Figure 6) is based on the following results. In *C. elegans* the Dual oxidase, CeDuox1, encoded by *bli-3* is responsible for creating an environment of

elevated ROS in response to the presence of yeast. Yeast mutants such as *yap1*Δ and *sod1*Δ that are sensitive to oxidizing environments are unable to evoke the Dar response in wild-type worms but are able to induce it in *bli-3* mutants. Based on our data we predict a mechanism in which the nematode produces reactive oxygen species (ROS) in response to yeast. The Yap1 and Sod1 gene products of the invading fungus must neutralize these ROS before it can cause Dar. CeDuox1 is expressed in the cuticle (EDENS *et al.* 2001) and our data predicts that it should also be active in the intestinal lumen of the worms. In the future, *in situ* localization of the CeDuox1 can be used to further test our hypothesis. Mammals also express the Nox family oxidases, which include the Duox enzymes, in epithelial tissues including the intestine (EL HASSANI *et al.* 2005; KAWAHARA *et al.* 2004) and evidence is mounting that these enzymes are involved in host defense (LETO and GEISZT 2006).

C. elegans has successfully been used as a model host for several microbial pathogens. Likewise *S. cerevisiae* has proved its worth as a prototype for fungal pathogens. This is the first study that demonstrates a pathogenic interaction between these two powerful genetic model organisms. The advantages of being able to study both the host and pathogen genetically have been noted previously(PERSSON and VANCE 2007). The present study demonstrates how insights from one side of the host-pathogen interaction can be used to suggest experiments that can inform what is going on in the other side of the interaction.

This is exciting because in principle, such a study can be extended to the whole genome using existing methodologies. *S. cerevisiae* is the only fungal species with a systematic single gene deletion library. This assay will enable us to conduct a high

throughput, unbiased, whole genome screen to identify fungal virulence factors. Promising genes and pathways identified may be targeted for antifungal drug development. On the host side, there are plasmid libraries available containing RNAi constructs that down-regulate single transcripts in *C. elegans*. Our patho-assay will allow us to screen such a library and identify host factors that may be different from those identified in bacterial interactions.

Fungal infections are hard to treat because chemicals that are toxic to fungi are also often harmful to human patients. Thus genes that are unique to fungi are potential targets for antifungal agents. It has previously been suggested that Yap1, a fungal specific transcription factor, is involved in pathogenesis (ALARCO and RAYMOND 1999b; LEV *et al.* 2005a; PRUSTY *et al.* 2004), however it has not been shown to be required for pathogenesis. We use this patho-assay to demonstrate that Yap1 and Yap2 are required to elicit a potentially protective host response, the Dar phenotype. This key observation, along with the finding that Yap1 is a fungal-specific transcription factor, makes it a likely target for broad-spectrum antimycotic drug development.

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were designed by CJ, SMP, and RPR. MY performed experiments and analyzed data for Figures 3B and 3C. CJ performed all other experiments. RPR wrote the paper. This work was done at and supported by Worcester Polytechnic Institute.

Chapter 3

The role of reactive oxygen species in the virulence of *Candida albicans.*

We have previously developed a virulence assay with *S. cerevisiae* to identify potential virulence factors. Despite all the advantages in using *S. cerevisiae*, especially plethora of tools available for its manipulation, it is not a true pathogen. *C. albicans* is the main cause of fungal infections, though recently due to fluconazole drug resistance observed by *Candida,* other *Candida* species such as *C. dubliniensis* and *C. glabrata* are emerging as pathogens. My work demonstrates that pathogenesis assay I developed works for testing virulence of several diverse *Candida spp*.

Using the Dar disease phenotype, I was able to identify a potential role of the *S. cerevisiae* gene, Yap1, in fungal virulence. I also wanted to test whether the Yap1 homologue in *C. albicans*, Cap1, is a virulence factor, not only in this invertebrate model, but also in our mammalian models. I found that Cap1 is required for pathogenesis in a nematode host as well as in an *in vitro* macrophage model. However, in the murine model, deletion of *cap1* only partially rescued mouse survival. This indicates that though Cap1 is playing a role in pathogenesis, other factors are involved.

Cap1 is a basic leucine zipper (bZIP) transcription factor and along with Yap1, Yap2, *Schizosaccharomyces pombe* Pap1 and others constitute a subgroup in Yap family of transcription activators. These proteins in the subgroup have a highly conserved

cysteine rich domain (CRD) in their C-terminal. This domain contains 3 cysteine residues which have been shown in Yap1 to be important in regulating nuclear localization of the protein in response to oxidative stress (KUGE *et al.* 1997). Cap1 along with regulating the oxidative stress response genes like *Ca.GLR1*, *Ca.TRX2*, also regulates the expression of *MDR1*, encoding a multidrug efflux pump and a homologue of *FLR1* (MOGAVERO et al. 2011). Treatment with the antifungal Caspofungin also induces the nuclear localization of Cap1 (KELLY *et al.* 2009).

The experiments shown in table 2 and figures 1, 2 and 4 were performed by me and I was involved in designing and analysis of the data collected from the experiments. The work shown in this chapter will be submitted for publication.

The role of reactive oxygen species in the virulence of *Candida albicans*

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3.1 ABSTRACT

Candida albicans is a major fungal pathogen of humans, causing mucosal infections that are difficult to eliminate and systemic infections that are often lethal, primarily due to defects in the host's innate status. Here we demonstrate the utility of *Caenorhabditis elegans*, a model host to study innate immunity, by exploring the role of reactive oxygen species (ROS) as a critical innate response against *C. albicans* infections. Much like a human host, the nematode's innate immune response is activated to produce ROS in response to fungal infection. We use the *C. albicans cap1*Δ mutant, which is susceptible to ROS, as a tool to dissect this physiological innate immune response and show that *cap1*Δ mutants fail to cause disease and death, except in *bli-3* mutant worms that are unable to produce ROS because of a defective NADPH oxidase. We further validate the ROS-mediated host defense mechanism in mammalian phagocytes by demonstrating that chemical inhibition of the NADPH oxidase in cultured macrophages enables the otherwise susceptible *cap1*Δ mutant to resists ROS-mediated phagolysis. Loss of *CAP1* confers minimal attenuation of virulence in a disseminated mouse model, suggesting that *CAP1*-independent mechanisms contribute to pathogen survival *in vivo*. Our findings underscore a central theme in the process of infection — the intricate balance between the virulence strategies employed by *C. albicans* and the host's innate immune system and validates *C. elegans* as a simple model host to dissect this balance at the molecular level.

3.2 INTRODUCTION

The incidence of invasive fungal infections has escalated in recent years, primarily in hospital settings (PFALLER 1996); 90% of these infections are caused by various *Candida* species (FRIDKIN and JARVIS 1996), 50% of which are fatal (GUDLAUGSSON *et al.* 2003). The estimated annual cost of treating nosocomial *Candida* infections exceeds $1 billion per year and has an attributable mortality of about 5000 deaths per year in the United States (MILLER *et al.* 2001; MORGAN *et al.* 2005; PAPPAS *et al.* 2003). *Candida* species are the fourth leading cause of bloodstream infections (BSI), with *C. albicans* responsible for about half the cases. *C. albicans* form robust biofilms on medical implants, such as intravenous catheters, prosthetic joints, or artificial heart valves, which can seed potentially lethal disseminated infections (RICHARDS *et al.* 2000; VELASCO *et al.* 2000; WISPLINGHOFF *et al.* 2003). Approximately 75% of women have at least one episode of vaginitis caused by *Candida* in their lifetime (SOBEL 1988) and oropharyngeal thrush and esophagitis are common in both infants and in patients with AIDS (DAROUICHE 1998). *Candida* species also cause superficial infections on mucosal surfaces in the body, including the mouth, upper gastrointestinal (GI) and uro-gential tract. The frequency of these superficial infections combined with the treatment challenges posed by disseminated infections make *C. albicans* an important pathogen for further study.

A variety of *in vitro*, *ex vivo*, and *in vivo* models have been employed to study the interaction between the host and this fungal pathogen. Fully *in vitro* studies of hyphal morphogenesis and biofilm formation, among many others, have yielded important insights into virulence (FINKEL and MITCHELL 2011; NOBILE and MITCHELL 2005). *Ex vivo*

models, such as co-culturing *C. albicans* with isolated macrophages, neutrophils, epithelial or endothelial cells, and even intact, perfused organs, have demonstrated that *C. albicans* has very complex responses to host cell contact, which can differ dramatically between cell types (FRADIN *et al.* 2005; LORENZ *et al.* 2004; PARK *et al.* 2009; RUBIN-BEJERANO *et al.* 2003; THEWES *et al.* 2007; ZAKIKHANY *et al.* 2007). A murine model of disseminated candidiasis has been frequently used to validate the role of specific genes on overall virulence. There is a general appreciation that each of these models has provided important insights into fungal pathogenesis. Recently, invertebrate models have become additional tools to dissect the roles of components of the antifungal host defense system, including flies, wax moth larvae (*Galleria mellonella*), and the nematode *Caenorhabditis elegans* (BRENNAN *et al.* 2002; CHAMILOS *et al.* 2006; PUKKILA-WORLEY *et al.* 2009).

C. elegans has emerged as a useful model to study infectious disease for several reasons. First, facets of its innate immune system are conserved in humans (KIM *et al.* 2002; MALLO *et al.* 2002) and the nematode reacts to pathogens in a manner similar to mammals, such as activation of specific signal transduction pathways (JAIN *et al.* 2009; KIM *et al.* 2002; MALLO *et al.* 2002; PUKKILA-WORLEY *et al.*). A rich body of literature demonstrates that human pathogens, both bacteria and fungi, also infect *C. elegans* in ways that are mechanistically similar to humans. For example the opportunistic human pathogens *Pseudomonas aeruginosa* (DARBY *et al.* 1999; KIM *et al.* 2002; MAHAJAN-MIKLOS *et al.* 1999; TAN *et al.* 1999a; TAN *et al.* 1999b) and *Serratia marcescens* (KURZ *et al.* 2003; MALLO *et al.* 2002), produce toxins that are required for pathogenesis in disparate eukaryotic hosts. Mutant studies in *Salmonella typhimurium*, typically thought

to have a narrow host range, shows a direct correlation in virulence between humans and *C. elegans* (ABALLAY *et al.* 2000; LABROUSSE *et al.* 2000). More recently a comparative study in *C. elegans* using fungal pathogens of the genus *Cryptococcus* (MYLONAKIS *et al.* 2002b; MYLONAKIS *et al.* 2004) showed that only the human pathogen *C. neoformans*, but not other related yeasts *(C. kurtzingii or C. laurentii),* killed the nematode. These studies further demonstrated that a virulence factor such as Kin1, first identified in nematodes, was also important in mammals (49). More recent whole genome analyses of *C. elegans* infected with *C. albicans* reveal that the nematode induces immune defenses with known antifungal properties (PUKKILA-WORLEY *et al.*).

Studies of C. elegans infected with bacterial pathogens reveal that the generation of reactive oxygen species (ROS) is an important part of the nematode's defense response (CHAVEZ et al. 2009; JAIN et al. 2009), a hallmark shared with mammalian innate immune responses. We previously reported a *C. elegans*-based assay to study several aspects of disease progression, namely Dar (deformity in the anal region), an early marker of infection; intestinal distension, resulting from colonization of the intestine; swelling in the vulva, representing infection of other epithelial layers; and ultimately death of the host worm (JAIN *et al.* 2009). Developed initially using *Saccharomyces cerevisiae*, here we have adapted the assay for *C. albicans* and, using genetic tools available in both the fungus and nematode, are able to dissect the role of ROS in antifungal innate immunity. We employ genetic and pharmacological tools to alter the intricate balance between the host and the pathogen and demonstrate that ablating either the ability of the worm to produce ROS or *C. albicans* to detoxify it (via mutation of the Cap1 transcription factor) has dramatic effects on the outcome of this

infection. Results in the worm were recapitulated in macrophage co-cultures, validating this model. Surprisingly, the *C. albicans cap1*Δ mutant retained virulence in the disseminated murine bloodstream model, suggesting that additional layers of regulation of antioxidant defense exist in the context of a mammal. This work thus provides an avenue to investigate fungal pathogenesis and has allowed us to identify further complexity in the pathogenic *C. albicans*–host interaction.

3.3 MATERIALS AND METHODS

Strains, media and growth conditions

The *C. albicans* strains used are listed in Table 1 and are based on SC5314 and its auxotrophic derivative CAI4-F2. *C. albicans* transformations were performed via electroporation (REUSS *et al.* 2004). Fungal growth medium was prepared as described previously (SHERMAN *et al.* 1986) and strains were grown overnight in yeast extract-peptone-dextrose (YPD) at 37°C. The *C. elegans* strains were grown at 20°C on nematode growth agar medium (NGM), spotted with *Escherichia coli* OP50 and maintained as described previously (BRENNER 1974). *E. coli* OP50 was grown overnight in Luria broth at 37°C.

Table 1. Strains used in this study

Strains	Relevant genotype	Complete genotype	Source
C. elegans strains			
N2 Bristol	Wild type	Wild type	(BRENNER 1974)
CB767	*bli-3*	*bli-3(e767)I*	(BRENNER 1974)
S. cerevisiae strains			
BY4741	Wild type	*MATa; his3Δ1; leu2Δ0; met15Δ0; ra3Δ0*	(BRACHMANN *et al.* 1998)
C. albicans strains			
SC 5314	Wild type	Wild type	(FONZI and IRWIN 1993)
Caf 2-1	*URA3/ura3Δ*	*URA3/ura3Δ::imm434*	(FONZI and IRWIN 1993)
CJD21	*cap1Δ/Δ*	*cap1Δ::hisG/cap1Δ::hisG-URA3-hisG ura3Δ/ura3Δ*	(ALARCO and RAYMOND 1999a)
AGC2	*cap1Δ/Δ*	*cap1Δ::hisG/cap1Δ::hisG ura3Δ/ura3Δ RPS10/rps1::URA3-CIP10*	This study
AGC4	*cap1Δ/Δ* complement	*cap1Δ::hisG/cap1Δ::hisG ura3Δ/ura3Δ RPS10/rps1::CAP1-URA3-CIP10*	This study
	efg1Δ/Δ	*efg1Δ::hisG/efg1Δ::hisG-URA3-hisG ura3Δ/ura3Δ*	(LO *et al.* 1997)
	cph1Δ/Δ	*cph1Δ::hisG/cph1Δ::hisG-URA3-hisG ura3Δ/ura3Δ*	(LO *et al.* 1997)
	efg1Δ/Δ cph1Δ/Δ	*efg1Δ::hisG/efg1Δ::hisG-URA3-hisG cph1Δ::hisG/ cph1Δ::hisG ura3Δ/ura3Δ*	(LO *et al.* 1997)
Other *Candida* strains			
C. albicans	Wild type	Clinical isolates	(WHITE 1997a)
C. dubliniensis	Wild type	Clinical isolates	(MORAN *et al.* 1997)
C. krusei	Wild type	Clinical isolates	A.B. Onderdonk
C. tropicalis	Wild type	Clinical isolates	A.B. Onderdonk
C. parapsilosis	Wild type	Clinical isolates	(KUHN *et al.* 2004; LAFFEY and BUTLER 2005)
C. glabrata	Wild type	Clinical isolates	(MUNDY and CORMACK 2009)

Generation of *cap1*Δ mutant strains

The existing *cap1*Δ/Δ strain CJD21 (ALARCO and RAYMOND 1999a) expresses *URA3* from the disrupted *cap1* locus, a strategy that has been subsequently demonstrated to potentially affect virulence through mis-expression of the *URA3* marker (LAY *et al.* 1998; SUNDSTROM *et al.* 2002); this can be overcome through ectopic integration of *URA3* at the *RPS10* locus using plasmid CIp10 (BRAND *et al.* 2004; MURAD *et al.* 2000). To generate the *cap1*Δ/Δ mutant and complemented strains with *URA3* at *RPS10*, we grew CJD21 on YPD for two overnight passages in YPD, then plated to media containing 5-fluororotic acid (5-FOA) to select for *ura3* auxotrophs (BOEKE *et al.* 1987). PCR amplified the *CAP1* open reading frame from genomic DNA of strain SC5314, plus ~1000 bp of 5' UTR and ~350 bp of 3' UTR. This fragment was ligated between the *Xho*I and *Hin*DIII sites in CIp10. This plasmid was digested with *Stu*I and used to transform the 5-FOA-selected *cap1*Δ/Δ strain. In parallel, the empty CIp10 plasmid was digested with *Stu*I and used to transform the same strain. After selection on SD-Ura, correct integration at *RPS10* was confirmed by PCR to generate *cap1*Δ/Δ strains with either *URA3* or *CAP1–URA3* expressed from the same genomic site as the mutant (AGC2) and complemented (AGC4) strains, respectively.

Microscopic analysis of *C. elegans*

A 2% agarose pad containing 0.01 M sodium azide as anesthetic was prepared on a slide. A 5 µl drop of M9 buffer was added to the pad. Worms exposed to wild-type *Candida* or RFP-labeled *Candida* were picked and transferred to the drop on the slide.

Mounted worms were then covered with a coverslip and observed at 200X and 400X magnifications using an Axiovision Zeiss microscope under differential interference contrast (Nomarski) and epifluorescence optics. An ApoTome attachment was used to enhance the fluorescence images.

Egg preparation

Three worms each in the L3/L4 stage were transferred to two NGM agar plates containing *E. coli* OP50 and grown at 20°C for 4 days. On the day of the experiment, worms were washed off the plates with M9 buffer and centrifuged at 900X *g* for 2 min. The supernatant was removed and the worms were then re-suspended in a bleach solution (1:4 dilution of commercial bleach (5.25%) containing 0.25 M sodium hydroxide). The worm suspension was mixed gently by inversion for 3 min and centrifuged for 2 min at 2000X *g*. The pellet was washed and centrifuged with M9 buffer at 2000X *g* for 2 min and then finally re-suspended in 500 µl M9 buffer. The egg suspension was diluted or concentrated with M9 buffer as required to obtain approximately 30–40 eggs/5 µl.

Pathogenesis assay

E. coli and *Candida* strains were grown overnight at 37°C. Culture aliquots were centrifuged at full speed for 1 min in a table top microcentrifuge and the supernatant removed. Pellets were washed twice in sterile deionized water, and re-suspended to a

final concentration of 200 mg/ml and 10 mg/ml, respectively. Next, a mixture of 10 µl of a 50-mg/ml streptomycin sulfate stock to inhibit *E. coli* growth, 7 µl of distilled water, 2.5 µl of *E. coli* and 0.5 µl of *Candida* was spotted on to each NGM plate. *E. coli* spotted plates were used as control. Finally, 5 µl of *C. elegans* egg suspension was transferred to each plate. Plates were then kept in a 20°C incubator and were observed for 5 days. All the experiments were done in triplicate. Student *t*-test was used to check the statistical significance of the differences observed between wild type and other *Candida* strains.

C. elegans **survival analysis**

Survival analysis was done on the *CAP1/CAP1, cap1*Δ/Δ + *CAP1*, and *cap1*Δ/Δ strains and plates were prepared as described under egg preparation and pathogenesis assay. Starting on Day 3 following the initial assay, the number of worms alive, dead, and dead on the rim was recorded each day. In order to differentiate between the first-generation worms and their offspring, worms were transferred every 2 days to new plates that were prepared the day before as previously discussed and incubated overnight at 20°C.

For data analysis, SPSS (IBM, Inc.) was used to generate Kaplan–Meier survival curves. In this experiment, each worm that died on the plate was entered as a "1", indicating the event of death due to fungal disease took place. Worms that were found dead on the rim of the plate were censored and entered as a "0", since death occurred for a non-related reason. Significance, as defined as a p value < 0.05, was assessed using the Gehan–Breslow test. This test assumes that data from earlier survival times

are more accurate than later times and weights these data accordingly. Data were combined from three plates and another independent experiment gave the same results.

Macrophage growth inhibition assay

Macrophage cell line RAW 264.7 (ATCC) was used in the assay. The cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The protocol was slightly modified from the original (LOPES DA ROSA *et al.* 2010). Briefly, macrophages, on reaching 80–90% confluence, were scraped and brought up in DMEM supplemented with 10% FBS, 100 U/ml penicillin or 100 µg/ml streptomycin, then 2×10^6 macrophage cells were plated on 35 mm^2 plates and allowed to adhere for 5 h. *Candida* strains were grown overnight at 37°C and diluted 1:10 and allowed to grow for another 5 h. *Candida* cells were then washed with supplemented DMEM and were added to the plates containing macrophages in a ratio 1:15 macrophage and to a final volume of 2 ml. Yeast strains were grown in parallel without macrophages under identical conditions to calculate percent survival. Plates were incubated overnight at 37°C and 5% CO_2. Cells were then brought up to 24 ml in a tube using 0.05% Triton X-100 (v/v) in water to osmotically lyse the macrophage cells. Dilutions were prepared and plated on YPD plates and grown overnight at 37°C. Colony forming units (CFUs) were counted and percent survival was calculated by taking the ratio of CFU from co-culture of *Candida* and macrophage to the CFU obtained for *Candida* alone.

Experiments involving diphenyleneiodonium chloride (DPI) were performed as

mentioned above but with the addition of 0.05 µM DPI from a 31.8 mM stock solution in DMSO. Controls without DPI had the same concentration of DMSO. Statistical analysis was done using Student's *t*-test.

Mouse virulence assay

Mouse virulence assays were carried out as described previously (RAMIREZ and LORENZ 2007). Female, adult (21–25 g) ICR mice (Harlan) were maintained on a normal laboratory diet. *C. albicans* strains were passaged twice in overnight cultures in YPD, then diluted into fresh YPD and grown for 3 hours at 30°C. Cell were collected by centrifugation, washed with water and re-suspended in PBS, then diluted and counted with a haemocytometer. Cells were diluted to 1×10^8 cells/ml in PBS. Mice were injected with 100 µl of this suspension via the tail vein, with groups of 10 mice/strain. Animals were monitored 2–3 times daily for signs of infection and were euthanized when moribund. Survival data were analyzed with Prism5 (Graphpad Software) using the log rank test. All animal experiments were conducted in accordance with protocols approved by the University of Texas Health Science Center Animal Welfare Committee.

3.4 RESULTS

Generation of *CAP1* homozygous and complemented strains

The original *cap1*Δ/Δ mutant strain CJD21 was generated by Raymond and colleagues (ALARCO and RAYMOND 1999a) using an approach that was subsequently shown to be

inappropriate for animal experiments due to the variability of expression of the *URA3* selectable marker. We selected a *ura3*- derivative of the *cap1*Δ/Δ strain CJD21 on 5-FOA, then integrated either *URA3* or *URA3–CAP1* at the *RPS10* locus using plasmid CIp10, a strategy shown to stably express *URA3* during infection (MURAD *et al.* 2000). This generated a *cap1*Δ/Δ mutant strain (AGC2) and a complemented strain (AGC4); the mutant strain is phenotypically identical to the original CJD21 during *in vitro* challenge with ROS (data not shown).

In vivo virulence assay for *Candida* infection

We previously described a pathogenesis assay where a small quantity of *S. cerevisiae*, a model pathogen, was introduced with the nematode's standard diet of *E. coli* OP50. The *E. coli* was attenuated to avoid interactions with the fungal species under investigation (JAIN *et al.* 2009). We uncovered molecular mechanisms of fungal virulence and the reciprocal innate immune response of nematodes, which is also conserved in mammals (GAUSS *et al.* 2007b; ZU *et al.* 1998). Here we have adapted this assay to test various fungal pathogens of the *Candida* genus, including *C. albicans, C. dubliniensis, C. krusei, C. tropicalis*, and *C. glabrata*. We decreased the concentration of *Candida* in the feeding mixture by 30-fold because of the increased pathogenicity of *Candida* species, and even at this lower fungal burden, several species induced Dar in 100% worms, compared with *S. cerevisiae,* which induced 0% Dar at the same concentration of inoculums (Table 2).

Table 2: Percentage Dar observed for different clinical isolates

Strains	% Dar	*n* =
C. albicans (SC 5314)	100	114
C. albicans	100	111
C. dubliniensis	100	122
C. glabrata	59.7 ± 1.1	119
C. parapsilosis	4.9 ± 0.3	133
C. krusei	100	115
C. tropicalis	100	134
S. cerevisiae (BY4741)	0	76

(± standard error)

Disease phenotypes of *C. elegans* upon *C. albicans* infection

Subsequent studies focused on *C. albicans* because it is the most prevalent infectious species in this genus (GUDLAUGSSON *et al.* 2003) and adequate genomic and molecular tools have been developed. To visualize infection, disease progression and death, we exposed nematodes to *C. albicans* and observed them daily over a 6-day period. The Dar phenotype was clearly visible in every worm on Day 4 (Fig. 1 and Table 2). Swelling in the vulva region was also noted in the worms infected with *C. albicans* (Fig. 2B).

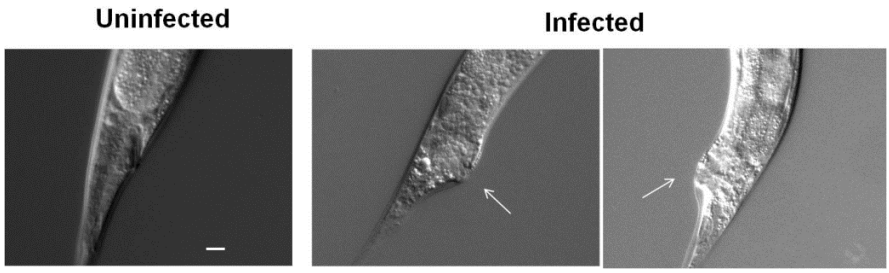


Figure 1: *C. albicans* induces deformed anal region (Dar) in wild-type worms. Worms were exposed to *E. coli* as control (uninfected) and *C. albicans* (shown are two examples of infected worms) and pictures were taken on Day 4 (arrow indicates the Dar region). Scale = 20 µm.

The worm succumbed to the infection following these phenotypic observations. To observe colonization of the intestinal lumen, worms were exposed to mCherry-labeled *C. albicans* (mCherry labeled SC5314, gift from Dr. Robert Wheeler, Univ. of Maine). Time-series micrographs of worms infected with *C. albicans* indicated that the intestinal lumen was colonized 2 days post-exposure compared with uninfected worms. Considerable intestinal distension was observed in infected worms on subsequent days (Fig. 2A) compared with the uninfected control population. In general, the disease phenotypes were more robust with *C. albicans* compared with *S. cerevisiae*, even with lower inoculum for infection.

To further validate our assay we tested known mutants *efg1Δ and cph1Δ* — previously documented virulence factors that regulate hyphal transition of *C. albicans* (LO *et al.* 1997) and have been shown to be important in *in vivo* infections of mice and nematodes (KOH *et al.* 2008; PUKKILA-WORLEY *et al.*). The *efg1Δ and cph1Δ* single mutants showed decreased Dar ~10% and ~50%, respectively, compared with the cognate wild type, while the *efg1Δ cph1Δ* double mutant failed to induce any Dar (0% Dar).

These results recapitulate the pattern of virulence in mice, where the *cph1*Δ mutant is slightly attenuated, the *efg1*Δ mutant is significantly attenuated, and the double mutant is completely avirulent (LO *et al.* 1997). Accordingly, our assay is suitable for exploring virulence strategies of *C. albicans* and the reciprocal host defenses that may be correlated to aspects of innate immunity that is conserved in mammals (KIM *et al.* 2002; MALLO *et al.* 2002).

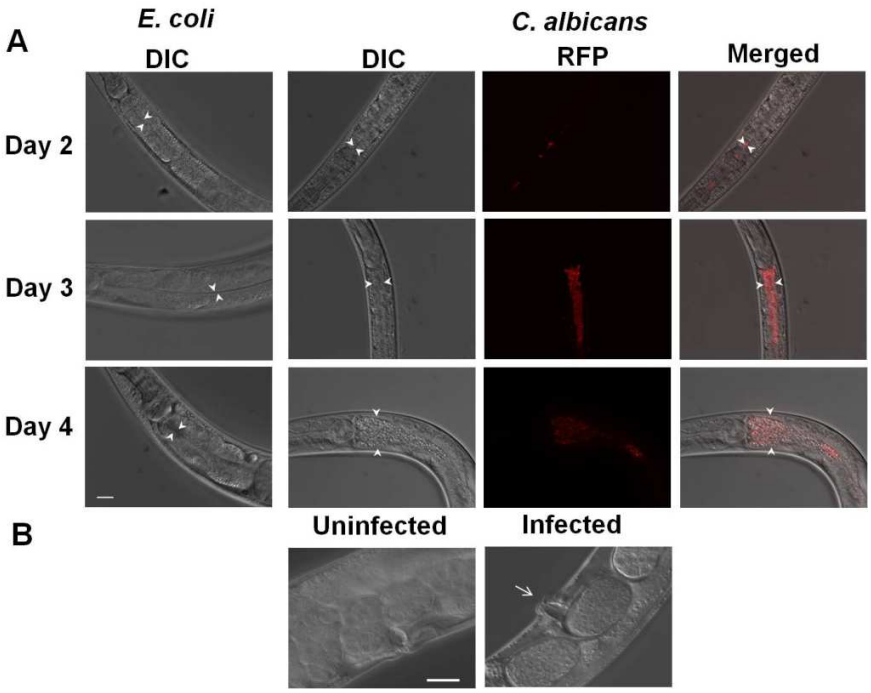


Figure 2: Different phenotypes are observed due to *Candida* infection. (A) Exposure to *C. albicans* causes intestinal distention in the worms over time (Days 2, 3 and 4). DIC (Nomarski) pictures of worms feeding on *E. coli* was taken as control and the DIC, RF and merged pictures of worms infected with *C. albicans* are shown over 3 days. (B) Vulva swelling is observed (arrow points to the vulva region) by Day 4 when worms are exposed to *Candida* compared with worms exposed *E. coli* as control (uninfected). Scale = 20 µm.

CAP1 is required to establish infection in nematodes

In vitro, Cap1 regulates the response to oxidative stress (ALARCO and RAYMOND 1999a; ENJALBERT *et al.* 2006; WANG *et al.* 2006; ZHANG *et al.* 2000a), which is a key component of the human innate immune response to fungal infections and is induced in the presence of neutrophils (FRADIN *et al.* 2005). To correlate these studies, performed at 30°C and 37°C, respectively, with our *in vivo* infection, performed at 20°C, we compared the ROS sensitivity profiles of *cap1*Δ/Δ, *CAP1*-complemented, and the cognate wild-type strain at the following temperatures: 20°C, 30°C and 37°C. Our reconstructed *cap1*Δ/Δ mutant failed to grow in the presence of hydrogen peroxide (at a final concentration of 2 mM) while the *CAP1*-complemented strain showed the same growth as the wild type at all temperatures tested (data not shown). These results indicate that ROS sensitivity of the *cap1*Δ/Δ strain is independent of temperature and that all *C. albicans* strains used in this study show the same phenotype at all temperatures used in this study.

To test whether the mechanism by which Cap1 promotes virulence is related to the production of ROS by the host, we compared the Dar response of worms exposed to null mutant (*cap1*Δ/Δ) and wild-type strain (*CAP1/CAP1*) as well as a *CAP1*-complemented strain (*cap1*Δ/Δ + *CAP1*), where a single wild-type copy *CAP1* is replaced at the native locus. The wild-type strain was able to induce Dar in 100% of the worms while the *cap1*Δ/Δ homozygous mutant showed a significant reduction in Dar induction (Fig. 3A). The *CAP1*-complemented strain showed an intermediate Dar response that is commonly observed for complemented strains because a diploid

deletion is complemented with a single copy. This result indicates that Cap1 function is required for full virulence in the nematode model.

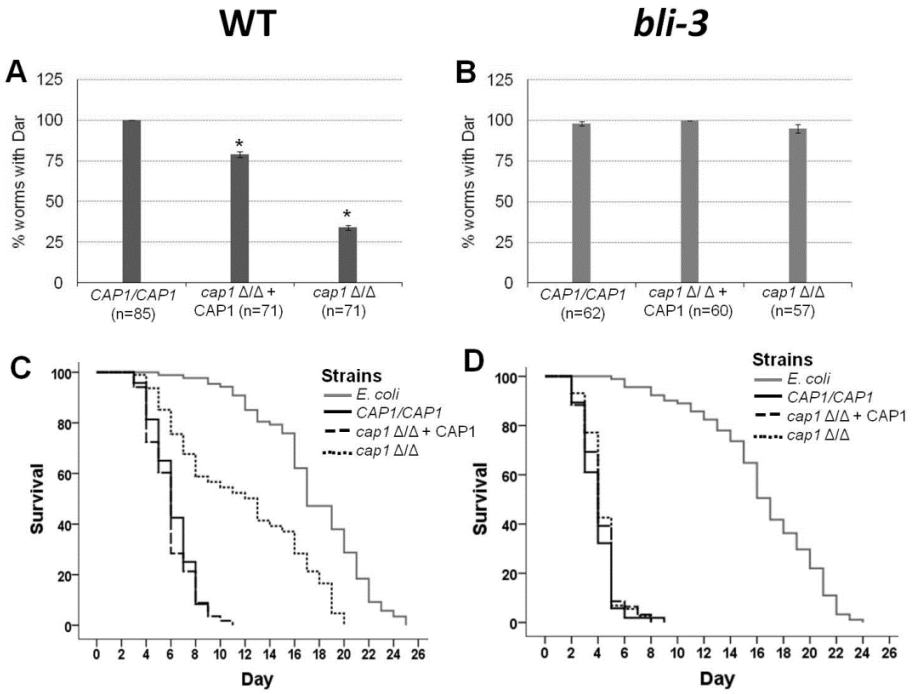


Figure 3: Cap1 is a virulence factor that is required for counteracting oxidative stress created by the host during fungal pathogenesis. The Dar phenotype is observed for both (A) wild type and (B) *bli-3* mutant worms (which lack the ability to produce ROS) exposed to *CAP1/CAP1, cap1*Δ/Δ + *CAP1*, and *cap1*Δ/Δ strains (* denotes $p < 0.001$; *n* denotes the number of worms exposed to a particular strain). Experiment was done in triplicate. (C) Survival curves for wild-type worms when exposed to *CAP1/CAP1, cap1*Δ/Δ + *CAP1*, and *cap1* Δ/Δ with *E. coli* OP50 as control ($p < 0.01$ for *CAP1/CAP1* and the *cap1Δ/ Δ* mutant) show that worms exposed to *cap1Δ/Δ* survive longer than worms exposed to wild type, while (D) *bli-3* mutant worms exposed to *Candida* strains show no difference in survival between exposure to wild-type *CAP1/CAP1* and the *cap1*Δ/Δ mutant.

The *bli-3* gene contains the only NADPH oxidase moiety in *C. elegans* genome, where it is found fused the peroxidase domain (hence called Dual oxidase, or Duox) (EDENS *et*

al. 2001). We and others previously reported that Bli-3 produces ROS in response to pathogenic insult (CHAVEZ *et al.* 2009; JAIN *et al.* 2009). To test the hypothesis that Cap1 is responsible for counteracting this oxidative environment upon *C. albicans* infection, we exposed *bli-3* mutant worms to *cap1*Δ/Δ, *CAP1*-complemented, and wild-type *CAP1/CAP1* strains. As shown in Figure 3B, the Dar phenotype of *bli-3* mutant worms infected with the *cap1*Δ/Δ null mutant is indistinguishable from that of the wild type and complemented counterparts, suggesting that Cap1 function is not required for infection if the host cannot produce ROS. Thus, Cap1 is dispensable when the ROS production part of the host's defense repertoire has been compromised, as in the *bli-3* mutant.

CAP1 is required to sustain infection and ultimately kill the nematode host

We and others previously showed that Dar is an early indicator (HODGKIN *et al.* 2000; JAIN *et al.* 2009) of an eventually lethal infection, which is reduced in worms exposed to the *cap1*Δ/Δ null mutant. To test the hypothesis that these mutants are avirulent, we measured the life span of worms infected with the c*ap1*Δ/Δ null mutant, complemented, and wild-type strains relative to uninfected worms. The average lifespan of *C. elegans* infected with wild-type *C. albicans (CAP1/CAP1*) or *a CAP1*-complemented strain was significantly shorter (median survival is 6 days) than those infected with the *cap1*Δ/Δ null mutant (median survival is 13 days) (Fig. 3C). These data indicate Cap1 is required for the infection to persist and ultimately kill the hosting worms. To test the hypothesis that Cap1 is required to neutralize host ROS we challenged an ROS-deficient *bli-3*

mutant host with the *C. albicans cap1*Δ/Δ mutant. Lifespan plots reveal that the kinetics of killing are indistinguishable between the *cap1*Δ/Δ mutant and the wild type or complemented strains (Fig. 3D), suggesting that ROS is the primary defense of nematodes against *C. albicans* but that *C. albicans* requires Cap1 for a lethal infection to persist. The lifespan of uninfected *bli-3* mutants is the same as their wild-type counterparts (Fig. 3), eliminating confounding factors other than ROS production as a likely cause of death of infected worms.

Cap1 is responsible for neutralizing ROS produced by phagocytes

Part of the mammalian host defense against *Candida* infection is production of ROS within phagosomes that have engulfed the fungal pathogen (VAZQUEZ-TORRES and BALISH 1997). Wild-type *C. albicans* can effectively neutralize ROS to survive (ARANA *et al.* 2007; FROHNER *et al.* 2009; LORENZ *et al.* 2004; WELLINGTON *et al.* 2009; WYSONG *et al.* 1998) and eventually cause the macrophages to lyse. To test whether Cap1 mediates this key innate immune response that allows *C. albicans* to withstand the oxidative environment, we co-cultured macrophages with either wild type or *cap1*Δ/Δ strains. Our data indicate that the CFUs of *C. albicans* recovered from macrophages were significantly lower for *cap1*Δ/Δ null mutant compared to the cognate wild type (Fig. 4); the complemented strain survived macrophage phagocytosis to an extent similar to the wild type.

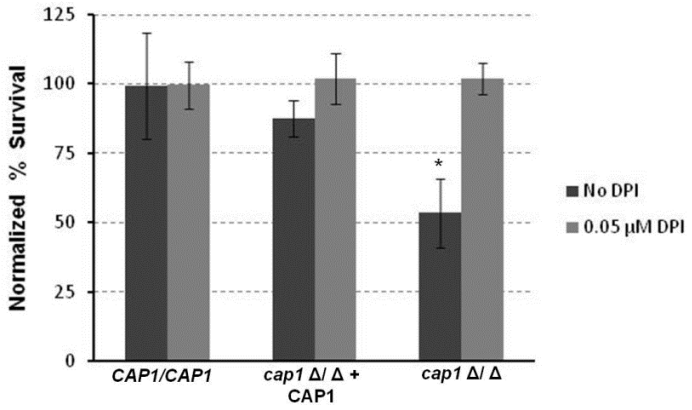


Figure 4: Cap1 is required for survival in macrophages. Macrophages were exposed to different strains of *Candida* in a ratio of 1:15 macrophages with and without DPI. Percentage survival was calculated by dividing the CFUs obtained for *Candida* grown with macrophages to *Candida* alone. Data were then normalized to the wild type. Percent survival of *cap1*Δ/Δ mutant is significantly less (**$p < 0.05$) than the wild type and the complemented Cap1 strain.

To further correlate the survival of phagocytized *C. albicans* with ROS production within the phagocytes, we chemically inhibited ROS production in the phagosomes with Diphenyleneiodonium chloride (DPI) (HANCOCK and JONES 1987). DPI specifically inhibits NADPH oxidase (BABIOR 2004), a homolog of Bli-3 responsible for ROS production in macrophages. In the presence of DPI, there was no difference in CFUs recovered from macrophages between the three strains, suggesting that pharmacological inhibition of the host ROS-production machinery allows *cap1*Δ/Δ, which is otherwise attenuated, to survive within the phagosome. These results mimic our genetic studies in nematodes and suggest that *C. albicans* relies on Cap1 to neutralize the oxidative environment within the phagosome, and that eliminating the source of ROS in phagosomes eliminates the need for Cap1.

CAP1 has a limited effect during mammalian infection

The *cap1*Δ/Δ mutant is clearly attenuated in the nematode model and during contact with macrophage, and has previously been reported to be hypersensitive to several sources of oxidative stress (ALARCO and RAYMOND 1999a). We tested it in the standard mouse model of disseminated candidiasis, in which *C. albicans* is introduced directly into the circulatory system via the tail vein. We injected outbred ICR mice with 10^6 *C. albicans* cells and monitored for signs of infection (Fig. 5; see Materials and Methods).

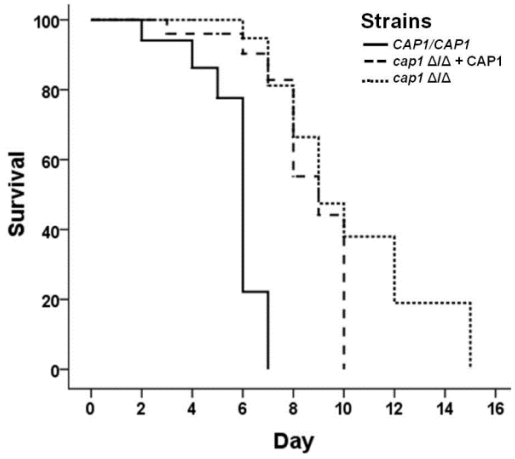


Figure 5: *Candida* virulence is reduced when Cap1 is deleted. Ten mice/strain were injected in the tail vein with 10^6 cells of the respective *Candida* strain. Survival curves of mice when infected with *CAP1/CAP1, cap1*Δ/Δ + *CAP1*, and *cap1*Δ/Δ strains show that mice infected with the *cap1*Δ/Δ mutant were able to survive significantly longer than those injected with the wild type ($p < 0.01$ for *CAP1/CAP1* and the *cap1*Δ/Δ mutant).

Mice inoculated with the *cap1*Δ/Δ strain had a statistically significant increase in median survival times relative to wild type (from 6.0 to 8.5 days, $p = 0.0003$). However, the *CAP1*-complemented strain showed roughly the same virulence (median survival 8.0

days). Thus, despite the worm and macrophage data, *in vitro cap1*Δ/Δ phenotypes, and the general assumption that the oxidative burst is a key part of the anti-fungal innate immune response, we show that loss of *CAP1* alters virulence only slightly.

3.5 DISCUSSION

Mammalian biology has been effectively modeled using a variety of species that present substantial advantages in complexity, genetic tractability, ethical considerations and cost. The last decade has seen the acceptance of invertebrates, such as *C. elegans*, as relevant hosts that can contribute to the molecular understanding of microbial pathogenesis. The *C. elegans* model, which has been generally used to study systemic pathogens such as *Cryptococcus, Pseudomonas* and *Enterococcus*, clearly differs from mammalian infection in several respects, including the site of infection (gut vs. bloodstream), growth temperature (25–30°C vs. 37°C) and the absence of an adaptive immune response. Yet, equally clearly, *C. elegans* and other invertebrates have provided important insights into bacterial and fungal pathogenesis and many mutants have been uncovered that attenuate virulence both in the worm gut and in the mouse bloodstream (KURZ and EWBANK 2000; MACCALLUM 2012; PRADEL and EWBANK 2004). We show here a case in which the worm offers opportunities to dissect the important components of the innate immune response to fungal pathogens. More importantly, we are able to tease out the differences between mammalian and nematode models using the *C. albicans cap1*Δ/Δ strain.

We previously demonstrated that the model yeast *S. cerevisiae* can cause pathology in the nematode (JAIN *et al.* 2009). In this work we show that this assay is far more robust when using pathogenic *Candida* species and that the virulence of these species in the worm roughly correlates with virulence in humans. The worm has emerged as a valuable model to investigate host responses because aspects of its innate immune system are conserved in humans. In addition, our nematode infection assay faithfully recapitulates the ROS-mediated innate response observed in humans.

Generation of ROS has been shown to be an important component of the worm's defense against bacterial infections (CHAVEZ *et al.* 2009) and we demonstrate that here for fungal infections as well. Data presented here indicate that Cap1, a fungal-specific transcription factor known to regulate oxidative stress responses (ALARCO and RAYMOND 1999a; WANG *et al.* 2006) and drug resistance pumps (ALARCO *et al.* 1997b), is required for virulence of *C. albicans* in nematodes and cultured macrophages. Specifically, strains lacking *CAP1* induce the Dar phenotype less frequently and attenuate virulence in a worm-killing assay relative to the wild-type strain. This is directly due to the increased sensitivity of this mutant to ROS because mutant worms that cannot produce ROS due to a mutation in the host oxidase do not show early signs of infection, nor do they succumb to an infection with the *cap1*Δ/Δ null mutant. Furthermore when the NADPH oxidase is chemically inactivated in cultured macrophages, *cap1*Δ/Δ null mutants are able to survive just as well as wild-type *Candida*.

Given the data above and the known *in vitro* functions of *CAP1*, the results of the murine model are surprising, as it is difficult to conclude that loss of *CAP1* alters virulence. Since some other antioxidant proteins, such as the secreted superoxide

dismutase *SOD5* and catalase *CAT1*, are attenuated in this same model (MARTCHENKO *et al.* 2004; NAKAGAWA *et al.* 2003; WYSONG *et al.* 1998), it seems clear that anti-oxidant defenses are important *in vivo*. There is a small difference in the mutant and wild-type strain, but the difference is not eliminated in the complemented strain. It is possible that the reintroduction of a single copy of *CAP1* is haploinsufficient in a mouse, though this strain fully complements all *in vitro* phenotypes. A more intriguing possibility is that there may be Cap1-independent mechanisms for their induction in the context of the mammalian host. Indeed, *SOD4* and *SOD5* are both more highly expressed in hyphal cells, which can be induced in response to several host-associated cues (FROHNER *et al.* 2009; MARTCHENKO *et al.* 2004). Thus, our *C. elegans* model may allow analysis of critical fungal responses to the innate immune system that could be masked by phenotypic redundancy in the context of the intact mammal.

Genetic tractability is a key advantage of the *C. elegans* assay, even over other invertebrates, and our model is intended to maximize the potential of the nematode. By feeding *C. albicans* to *C. elegans* we avoid manipulation of individual animals, a time-consuming aspect of screens in both vertebrate and other invertebrate species such as mice, zebrafish, *Drosophila* or *Galleria* larvae. Further, our virulence assay can be differentiated from other *C. elegans*-based assays because we include attenuated *E. coli* as the primary nutritional source and spike in *C. albicans* as the infectious agent. This avoids any complicating factors that may come from altered nutritional states, since we have found that *C. elegans* does not feed well on a lawn of fungal cells alone (M.L. and R.P.R., unpublished observations). In addition, this system can be easily adapted to conduct systematic mutant screens for modulators of the innate immune response using

the existing RNAi knockdown collection, available as *E. coli* strains expressing double-stranded RNA that is specific to any *C. elegans* gene and that knock down the gene's protein concentration to a negligible amount. In conclusion, we have shown that this model system offers a robust means to probe both innate immunity and fungal pathogenesis.

ACKNOWLEDGMENTS

We would like to thank Dr. Ryder and L. Gaffney for their critical review of the manuscript.

Chapter 4

Mutant screen to identify novel potential virulence factors.

Prevalence of *C. albicans* infection in hospital settings has been increasing in the last few decades. In order to understand the repertoire of virulence strategies used by *C. albicans,* we used the *C. elegans – C. albicans* pathogenesis assay that I have described earlier to conduct a mutant screen. We screened a transposon-mutagenized insertion library of ~1200 *Candida* clones to identify novel factors required for *Candida* infection. Using the assay, we identified some known and some novel genes that showed altered virulence in *C. elegans*. Some of these genes are uncharacterized and pose future possibilities for investigation.

The screening of the mutant library was done by 4 undergraduates, under my guidance. I was involved in designing and analyzing the data. I was also responsible for troubleshooting any problems that occurred during the screen.

4.1 INTRODUCTION

C. albicans is an opportunistic pathogen that is normally found in the mouth, gastrointestinal and uro-genital tract of humans. Under conditions such as prolonged use of broad-spectrum antibiotics or a compromised immune system due to AIDS or transplants, this commensal can proliferate and cause infection. The use of invasive devices like catheters and other implants facilitates systemic candida infection (FINKEL and MITCHELL 2011; VELASCO *et al.* 2000). *C. albicans* is the fourth leading cause of hospital-acquired blood stream infection with a mortality rate of 50% (GUDLAUGSSON *et al.* 2003). Drug resistance exhibited by *Candida* exacerbates the problem of treatment, and limited availability of different drugs adds to the problem. Thus, there is a need to understand the full repertoire of virulence strategies used by *Candida*. Studies are been conducted to better understand the triggers that facilitate the commensal to pathogenic switch and other processes required for pathogenesis and host adaptation during the process of fungal infection (ZHU and FILLER 2010).

I*n vivo* screens have been developed using both invertebrate and mammalian models to study different aspects of fungal pathogenesis. The mammalian study is a fitness assay in which a mouse is injected with a mixed population of barcoded mutants of *C. albicans*. Usually mutants with a virulence defect have reduced competitive fitness and are selectively depleted in the host. Abundance of *C. albicans* strains is determined with real time PCR and mutants missing when compared to the inoculum are considered avirulent (NOBLE *et al.* 2010). However, using mice for high throughput screening can be cost prohibitive. Use of invertebrates for screens is not only inexpensive but helps in avoiding ethical issues associated with using mammalian

systems. They are also beneficial for screening both the aspects of host-pathogen interaction; virulence factors and host immune effectors. The invertebrates that are normally used for such screens are either *D. melanogaster* or *C. elegans*. One such screen for host immune effectors that has been reported is the RNAi based *D. melanogaster* S2 cells screen to explore the role of 7,216 genes in phagocytosis of *Candida* (STROSCHEIN-STEVENSON *et al.* 2006). Both of these models have a conserved innate immune system which is responsible for fighting fungal infections; thus these model organisms are widely used to study virulence factors (MYLONAKIS and ABALLAY 2005). *C. elegans* has been shown to be useful for performing *in vivo*, whole animal high throughput screens for identifying antimicrobials (BREGER *et al.* 2007).

We have previously reported a pathogenesis assay in which we infect *C. elegans* with *C. albicans* and observe the deformed anal region (Dar) phenotype in the worms, which we use as biomarker for altered virulence (JAIN *et al.* 2009). This is a simple, straightforward assay that is also amenable to high throughput screening for fungal virulence factors. Using this assay, we have screened ~1200 mutants from a knockout library of *Candida* clones submitted to FGSC by Dr. Aaron Mitchell. We identified 7 virulence factors. Three of these genes (*CMP1*, *IFF11* and *SAP8*) are known virulence factors. In addition we identified 4 novel genes (*orf19.1219, orf19.6713, DOT4* and *ZCF15*). Not much is known about these genes and provides a foundation for future work in order to decipher their role in fungal pathogenesis.

4.2 MATERIALS AND METHODS

Strains, media and growth conditions

The *C. albicans* strains used are listed in Table 1. *S*trains were grown overnight in yeast extract-peptone-dextrose (YPD) at 37°C. The *C. elegans* strains were grown at 20°C on nematode growth agar medium (NGM) spotted with *Escherichia coli* OP50 and maintained as described previously (BRENNER 1974). *E. coli* OP50 was grown overnight in Luria broth at 37°C. The *C. albicans* knockout library was obtained from the fungal genetics stock center (MCCLUSKEY *et al.* 2010).

Table 1: Strains used in this study

Strains	Relevant Genotype	Complete Genotype
C. elegans strains		
N2 Bristol	Wild type	Wild type
***C. albicans* strains**		
SC 5314	Wild type	Wild type
SN250	Reference strain *Arg4* Δ/Δ	*his1Δ/his1 Δ, leu2 Δ::C. dubliniensis HIS1 /leu2 Δ::C. maltosa LEU2, arg4 Δ /arg4 Δ, URA3/ura3 Δ::imm434, IRO1/iro1 Δ::imm*^{434}
MS1- 335	*orf19.1219*Δ/Δ	*his1Δ/his1Δ, leu2Δ/leu2Δ, arg4Δ/arg4Δ, URA3/ura3 Δ::imm434, IRO1/iro1Δ::imm434, orf19.1219Δ::C.dubliniensisHIS1/orf19.1219Δ::C.maltosaLEU2*
MS1- 596	*dot4*Δ/Δ	*his1Δ/his1Δ, leu2Δ/leu2Δ, arg4Δ/arg4Δ, URA3/ura3 Δ::imm434, IRO1/iro1Δ::imm434, dot4Δ::C.dubliniensisHIS1/dot4Δ::C.maltosaLEU2*
MS1- 221	*sap8*Δ/Δ	*his1Δ/his1Δ, leu2Δ/leu2Δ, arg4Δ/arg4Δ, URA3/ura3 Δ::imm434, IRO1/iro1Δ::imm434, sap8Δ::C.dubliniensisHIS1/sap8Δ::C.maltosaLEU2*
MS1- 529	*zcf15*Δ/Δ	*his1Δ/his1Δ, leu2Δ/leu2Δ, arg4Δ/arg4Δ, URA3/ura3 Δ::imm434, IRO1/iro1Δ::imm434, zcf15Δ::C.dubliniensisHIS1/zcf15::C.maltosaLEU2*

Egg Preparation

Three wild type worms in the L3/L4 stage were transferred to each of two NGM agar plate containing *E. coli* OP50 and grown at 20°C for four days. On the day of the experiment worms were washed off the plates with M9 buffer and centrifuged at 900X *g* for 2 min. The supernatant was removed, and the worms were then resuspended in a bleach solution (1:4 dilution of commercial bleach (5.25%) containing 0.25 M sodium hydroxide). The worm suspension was mixed gently by inversion for 3 min, and centrifuged for 2 min at 2,000X *g*. The pellet was washed and centrifuged with M9 buffer at 2,000X *g* for 2 min and then finally resuspended in 500 µl M9 buffer. The egg suspension was diluted or concentrated with M9 buffer as required to obtain approximately 30-40 eggs/5µl.

Pathogenesis Assay

E. coli and *Candida* strains were grown overnight at 37°C. Culture aliquots were centrifuged at full speed for 1 minute in a table top microcentrifuge and the supernatant was removed. Pellets were washed twice in sterile deionized water, and finally resuspended to a final concentration of 200 mg/ml and 10 mg/ml, respectively. A mixture of 10 µl of a 50-mg/ml streptomycin sulfate stock, 7 µl of distilled water, 2.5 µl of *E. coli* and 0.5µl of *Candida* was spotted on to each NGM plate. *E. coli* spotted plates was used as control. Finally, 5 µl of *C. elegans* egg suspension was transferred to each plate. Plates were then kept in a 20°C incubator and were observed over next 5 days. Screen was done on a single plate/mutant. Experiments other then the screen were

done in triplicate. Student t-test was used to check the statistical significance of the differences observed between wild type and other *Candida* strains.

4.3 RESULTS

Screening the *Candida* transposon mutagenized library

We have previously reported a pathogenesis assay in which *C. elegans* is infected by the pathogen *C. albicans*. One consequence of this infection in *C. elegans* is an induction of the deformed anal region (Dar) phenotype. Using Dar as a biomarker for infection, number of worms showing this phenotype in response to infection can be quantitated. In order to identify novel virulence factors and thus new pathways that may be important for virulence, we screened ~1200 mutants in the Mitchell *Candida* transposon library (NOBILE and MITCHELL 2009). The strategy that was used to do the screen is shown in the schematic (Figure 1).

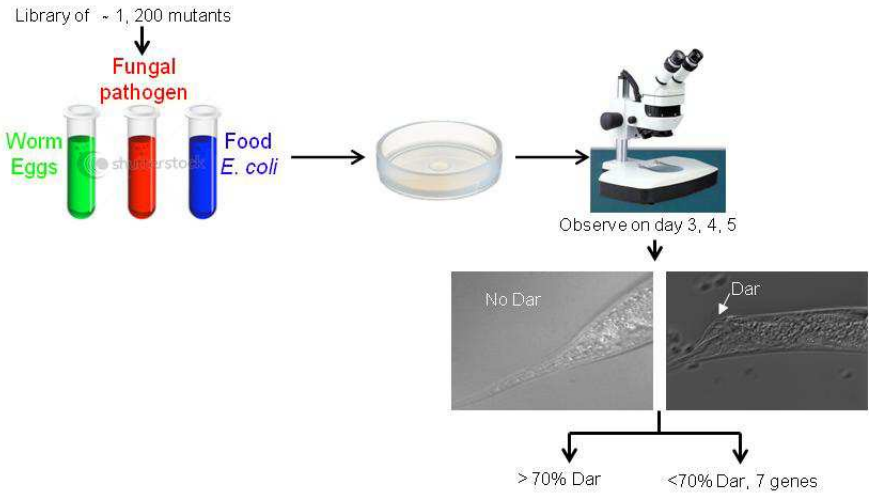


Figure 1. Schematic of the strategy used to screen the mutant library

The library was created by insertion of the *Tn7-UAU1* cassette into *C. albicans* genomic library by *in vitro* transposition. The *UAU1* cassette consists of a functional *ARG4* gene flanked by nonfunctional *URA3* deletion derivatives. Insertion of the cassette into the first allele gives the Arg^+, Ura^- phenotype. Due to mitotic gene conversion, heterozygous insertion of *UAU1* becomes homozygous and has to be selected for *URA3* transformants. In this second step *UAU1* cassette can recombine and excise *ARG4* and yield functional *URA3* and the transformants screened for Arg^+, Ura^+ phenotype (Figure 2). Thus, use of this cassette results in the formation of homozygous mutants from single *C. albicans* transformation. The library consists of a collection of homozygous mutants representing genes coding for transcription factors, cell wall proteins and kinases.

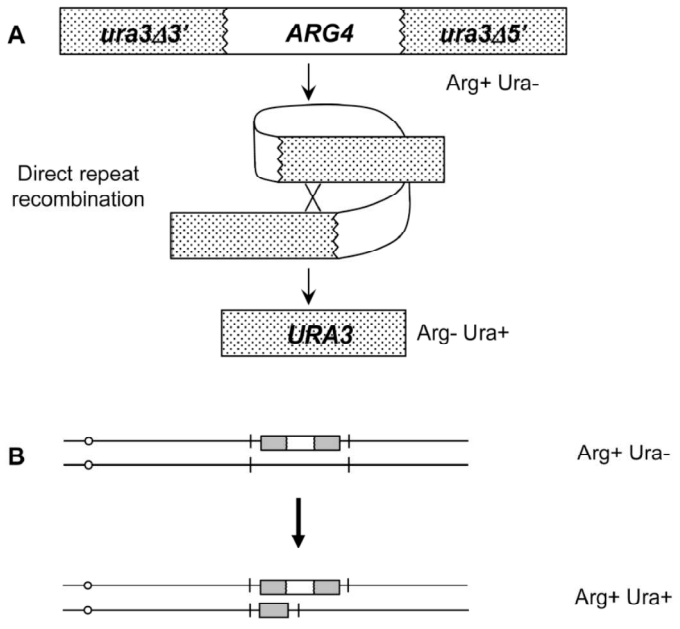


Figure 2. Genetic properties of *UAU1* cassette. (A) *UAU1* conversion to *URA3* due to recombination excision of *ARG4*. (B) Selection for double disruption results in formation of homozygous insertion mutants. Modified from (NOBILE and MITCHELL 2009)

From the screen we identified 7 mutants that induced a reduced percentage of worms with Dar, and thus showed altered virulence. These are listed in Table 2. Of these, 3 (*CMP1*, *IFF11* and *SAP8*) are known to be involved in virulence. The novel genes (orf19.6713, orf19.1219, *DOT4* and *ZCF15*) that we have identified through our screen have never been implicated before for their role in virulence. This is the first time where we are suggesting their potential role as virulence factors.

Table 2: List of mutants identified in the screen for their role in pathogenesis

ORF #	**% Dar**	**Description**
Wild type	90.6 ± 2.5	
Known genes		
orf19.6033 (*CMP1*)	44	Catalytic subunit of calcineurin
orf19.12854 (*IFF11*)	46	Secreted protein required for cell wall structure
orf19.242 (*SAP8*)	68	Secreted Aspartyl Proteinase highly expressed in opaque cells
Novel genes		
orf19.6713	30	Uncharacterized
orf19.1219	36	Uncharacterized
orf19.3370 (*DOT4*)	48	Cell surface protein in hyphal cells similar to Ubiquitin hydrolase
orf19.2753 (*ZCF15*)	47	Predicted Zinc finger protein

From previous work we know that Iff11, a secreted protein is important for organization of cell wall and the null mutant is attenuated in virulence in the mouse model (BATES *et al.* 2007). Similarly, from the work previously done on Cmp1, which codes for the catalytic subunit of calcineurin, is essential for virulence in a mouse model for systemic candidiasis (BADER *et al.* 2003). Sap 8, a secreted aspartyl proteinase, on the other hand, has shown to be preferentially expressed in vaginal *Candida* infection rather than oral infections (NAGLIK *et al.* 2003). Of the novel genes, orf19.1219 and orf19.6713 are uncharacterized, with probable nucleic acid and zinc ion binding motifs identified through computational analysis for the latter. Dot 4 codes for a protein which might be a homologue of Ubiquitin hydrolase and the protein specifically localizes to surface of hyphal cells (URBAN *et al.* 2003). Zcf15 is also uncharacterized, with a predicted zinc finger protein with a probable sequence specific transcription factor activity.

In order to confirm the phenotypes that we were observing were caused by loss of function; we used gene deletion mutants for some of the genes and repeated the Dar assay. We tested *sap8Δ, orf191219Δ, dot4Δ* and *zcf15Δ* mutants and compared them to the respective wild type SN250 strain in the Dar assay. We observed that all the above mentioned mutants showed significant reduction in Dar (Fig.3, $p<0.05$ for all strains) compared to the wild type, indicating these mutants indeed show altered virulence.

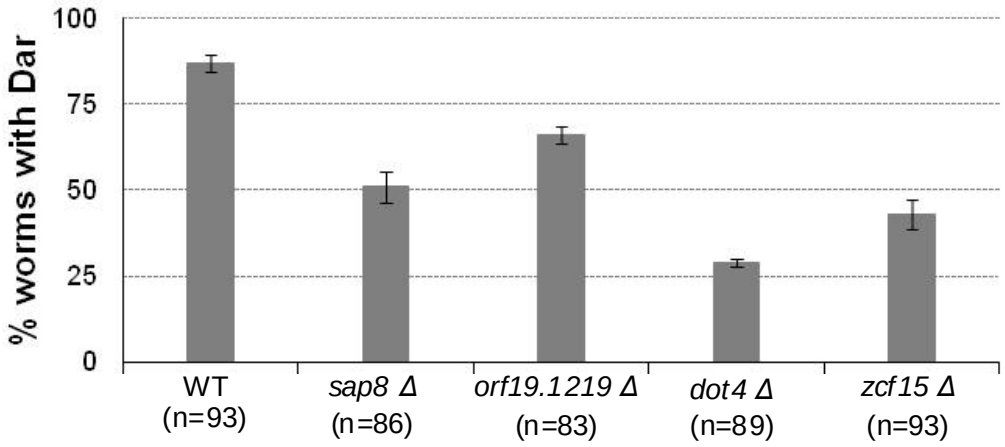


Figure 3. Wild type worms were exposed to *sap8 Δ, orf19.1219 Δ, dot4 Δ, zcf15 Δ* deletion mutants and their corresponding wild type *Candida* strains in order to confirm the results from the screen. % worms that exhibited Dar were scored on Day 3 and the data plotted. All mutant strains show significant reduction ($p<0.05$) compared to the wild type.

4.4 DISCUSSION

Large scale screens for identification of virulence factors can lead to identification of novel genes with role in pathogenesis as well as identification of pathways and new interconnectivity between known pathways. *C. elegans* provides us with an excellent tool to do an *in vivo* whole animal screen to look for the virulence determinants of. Using the pathogenesis assay described in this work, we have screened a *C. albicans* mutant library just by feeding the worms with the specific mutant strains. Though not part of this work, it is feasible to screen for host immune effectors in *C. elegans* using a slightly modified version of the assay and feeding them the *C. elegans* RNAi library. Despite these advantages, we have to remember that the gut of the worm provides a specific niche for *Candida* to colonize in. We also know from previous work, that in some cases that is a differential expression of genes depending on their surroundings. For example in case of *PHR1* and *PHR2* which encode a glycosidase, the virulence phenotype of a *phr1* null mutant is attenuated in systemic infections but not in vaginal infections. In contrast *phr2* null mutants are virulent in systemic situation but avirulent in vaginal infection model (DE BERNARDIS *et al.* 1998; SAPORITO-IRWIN *et al.* 1995). This indicates that in our screen along with novel potential virulence factors, we might not be able to pick all known virulence factors present in the library. Instead we will pick those that are known to be required for fungal pathogenesis in an environment similar to the one present in *C. elegans* gut.

Through this screen we have identified 3 genes (*CMP1*, *IFF11* and *SAP8*) known for their role in fungal virulence, and 4 genes (orf19.1219, orf19.6713, *DOT4* and *ZCF15*) that were not known to play a part in fungal pathogenesis. Mutation in these 4 genes

leads to altered virulence in the nematode. Most of these genes are not characterized and the information available on the others is very limited. Despite this paucity of information of their role in *C. albicans*, homologues of these genes in *S. cerevisiae* are known and have been well studied. Extrapolation from their function in *S. cerevisiae* can help us speculate about their gene function in *Candida*, and focus our study.

The homolog of orf19.1219, an uncharacterized gene in *C. albicans*, is the *S. cerevisiae* *RKR1* gene. In *S. cerevisiae*, the product of *RKR1* is a conserved nuclear RING domain protein that modulates chromatin structure, function, and transcription. This complex is required for several histone modifications and transcription, and cells lacking this gene show impaired sub-telomeric gene silencing (HOYER 2001).

In regards to *DOT4* we have very little information available. For *C. albicans* we do know that the gene codes for a protein that is similar to a Ubiquitin C-terminal hydrolase which normally localizes to the surface of hyphal cells (URBAN *et al.* 2003). This suggests that the gene plays a role during pathogenesis, as hyphal formation is one of the main traits of pathogenesis. Its homologue in *S. cerevisiae* is a ubiquitin protease known to be important for sub-telomeric gene silencing and chromatin modification (KAHANA and GOTTSCHLING 1999). Loss of this gene in *S. cerevisiae* leads to expression of sub-telomeric genes. Thus it seems that its function is contrary to that of *RKR1* in *S. cerevisiae*.

ZCF15 was identified in an *In silico* screen of the *C. albicans* genome, as an ORF possessing a Zn(II)(2)C(6)-type binuclear cluster that might be involved in DNA recognition (MAICAS *et al.* 2005). Thus, it possesses a DNA binding domain and may behave as a transcription factor. There is currently some debate as to its homologue in

S. cerevisiae. According to the Candida Genome Database, its homologue in *S. cerevisiae* is *PDR1*, which is a transcription factor, but according to the orthology website of Broad Institute (http://www.broadinstitute.org/regev/orthogroups/), the nearest homologue is PUF2, which is a RNA binding protein. This indicates that detailed studies need to be done in order to characterize this gene. Some preliminary work does indicate that deletion of this gene in *C. albicans* leads to more filamentation compared to the wild type.

The final gene identified in the screen was orf19.6713 which is uncharacterized. Computational analysis indicates that it might have a nucleic acid binding domain, while the ortholog search on the Broad website identifies MSB1 in *S. cerevisiae* as its ortholog. Msb1 is a protein involved in positive regulation of β1-3 glucan and Pkc1-MAPK pathways.

The above information clearly reflects the need to characterize these genes and study their role in pathogenesis.

Chapter 5

Small molecule screen to identify inhibitors of *C. albicans* adhesion.

Use of catheters and intravenous devices is one of the main causes of disseminated *Candida* infection. Use of these devices compromises the physical barriers to function, and patients who undergo implantation are already immunocompromised. Simple use of a urinary catheter by immunocompromised patients can lead to fatal systemic infections.

These devices can act as an inert substrate for adhesion of microbes, including *C. albicans*, and thus provide a platform on which the microbes can grow and spread. The process of biofilm formation is generally divided in multiple steps. Once the *Candida* cells bind to the inert surface (Adhesion) they start dividing and undergoing morphological changes to hyphal and pseudohyphal forms (Initiation). This is followed by the production of extracellular material (shown in green). This results in the formation of a biofilm (Figure 1). *Candida* from these biofilms can then disperse and either attach to host surfaces or get released in blood from where they can infect multiple organs.

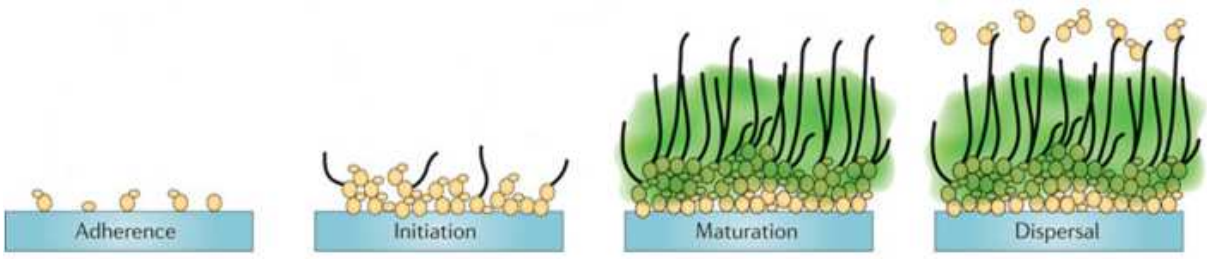


Figure 1. Steps involved in biofilm formation in *C. albicans.* Modified from (FINKEL and MITCHELL 2011)

Biofilms are relatively more resistant to antifungal drugs or host immune responses. Currently, research has been focused on developing new strategies to inhibit biofilm formation and one of these strategies is coating the devices with a compound that can prevent adhesion and thus, biofilm formation. Many such compounds have been suggested but most of them have not been successful in fulfilling their roles. The following chapter describes identification of one such putative compound.

I was involved in doing the screen, part of which is shown in figure 3. I was responsible for designing, collecting the data and its analysis in figure 5. I was also involved in designing rest of the experiments and analysis of data.

5.1 Introduction

Candida albicans is a fungus that is part of normal human microbial flora found on the skin, mouth, gastrointestinal tract and vagina. Under certain conditions of a compromised immune system e.g., patients undergoing organ transplant, chemotherapy treatment or suffering from AIDS, this commensal can become pathogenic and cause disease (DUPONT 1995; WEIG *et al.* 1998). It can cause infections ranging from mild mucosal infection to lethal systemic infections. *C. albicans* is the 4th leading cause of blood stream or systemic infection (EDMOND *et al.* 1999) and has a high mortality rate of 50% in the cases of nosocomial infections (GUDLAUGSSON *et al.* 2003). Currently ~ $ 1 billion per year is spent on treatment of nosocomial infection (MILLER *et al.* 2001). Systemic or disseminated candidiasis in most cases is observed in immunocompromised patients, and one of the main causes of this is the presence of contaminated intravascular devices and catheters (VELASCO *et al.* 2000). *Candida* from these devices is disseminated into the bloodstream and spreads by infecting multiple organs. The first course of action for these fungal organisms in the blood is to adhere to the endothelial surface and then to penetrate the endothelial lining of the blood vessel and the extracellular matrix; in this way they can infect multiple organs.

Adhesion to the host cell surface, either epithelial or endothelial, is a first and critical step in establishing a fungal infection. From the multiple studies that have been conducted in relation to the adhesion of *Candida* to the host surface, it is believed that the yeast form cells of *Candida* are most likely responsible for adhesion either to epithelial/endothelial cells (BENDEL *et al.* 2003; SAVILLE *et al.* 2003) or to the inert surface. This is then followed by morphogenesis of cells from the yeast form into the

hyphal form which then further helps in the invasion of the host tissue or forming biofilms. Biofilm associated infections normally do not respond to conventional treatment and it sometimes becomes necessary to remove the devices in order to completely treat the infection (BAUTERS *et al.* 2002). There are multiple strategies that are being used to prevent biofilm-associated infections (HETRICK and SCHOENFISCH 2006); one of these consists of coating the devices with chemicals (WU and GRAINGER 2006). Some antifungal compounds have been used as a coating on these catheters (DAROUICHE *et al.* 2006; PIGNO *et al.* 1994). Other studies have used nystatin, or zinc pyrithione as coating material (DE PRIJCK *et al.* 2010). Nystatin is an antifungal that binds to ergosterol, a fungal specific component of cell membrane and forms pores while zinc pyrithione blocks the proton pump and disrupt membrane transport.

Thus there is a need to identify better coating materials or compounds that can prevent adhesion and thus biofilm formation. This work is focused on screening a library of small molecule compounds and identifying some compounds that could be potentially used as coating material to prevent biofilm formation.

5.2 Material and Methods

Strains and Growth conditions

The *C. albicans* strains used were the wild type, SC5314, and the mutant *edt1-/-* which is defective in adhesion. The strains were grown overnight at 30^{o}C in YPD or Synthetic complete (SC) medium with 0.15% glucose depending on the experiment. The *C.*

elegans wild type strain N2 was grown at 20°C on nematode growth agar medium (NGM) spotted with *Escherichia coli* OP50 and maintained as described previously (BRENNER 1974). *E. coli* OP50 was grown overnight in Luria broth at 37°C.

Adhesion Assay for the Screen

A 5 ml starter culture of SC5314 and two, 5ml starter cultures of *edt1-/-* in SC+0.15% glucose media at 30^{o}C were started 2 days before the experiment. A day before the experiment, from these starter cultures a 100ml culture was started for each strain, in SC+0.15% glucose media at 30^{o}C. On the day of the experiment OD600nm was measured for both of the cultures. Appropriate number of cells for each strain were spun down and resuspended in fresh SC+0.15% glucose media to a final concentration of 0.5 OD/ml.

200µl/well of 0.5 OD/ml *edt1-/-* culture was added to the first column of a 96 well Immulon ELISA plate. Then 200µl/well of 0.5 OD/ml SC5314 culture was added to columns 2 through 12. This was followed by robotic addition of 2µl of compound each, which were dissolved in DMSO, to each well from columns 2 through 11 and of DMSO for the negative control in column 1 and positive control in column 12. The cells and the compounds were mixed robotically 3 times by pipetting the mixture up and down. The plates were then covered with Al foil and allowed to incubate at 37^{o}C for 4 hours. The content of the wells were then decanted and 50 µl of 0.5% crystal violet was added to each well of the plate. The plates were covered again and allowed to sit at room temperature for 45 minutes. This was followed by decanting the contents of the well.

Plates were then gently rinsed by submerging them in an ice bucket filled with distilled water and then decanting the water to wash off the excess dye. This was repeated 4 more times and then wells were rinsed with water. The rinsing was done 5 more times. The plates were then gently tapped on a paper towel to remove any excess water and the sides and the bottom were wiped down. 200 µl of 75% Methanol was then added to each well of the plate. The plates were allowed to sit for 30 minutes at room temperature and then OD was taken at 590nm. Plate with wells that showed significant less color and OD values were photographed.

Egg Preparation

Three worms in the L3/L4 stage each were transferred to two NGM agar plate containing *E. coli* OP50 and grown at 20°C for four days. On the day of the experiment worms were washed off the plates with M9 buffer and centrifuged at 900X *g* for 2 min. The supernatant was removed, and the worms were then resuspended in a bleach solution (1:4 dilution of commercial bleach (5.25%) containing 0.25 M sodium hydroxide). The worm suspension was mixed gently by inversion for 3 min, and centrifuged for 2 min at 2,000X *g*. The pellet was washed and centrifuged with M9 buffer at 2,000X *g* for 2 min and then finally resuspended in 500 µl M9 buffer. The egg suspension was diluted or concentrated with M9 buffer as required to obtain approximately 30-40 eggs/5µl.

Pathogenesis Assay

E. coli and *Candida* strains were grown overnight at 37°C. Culture aliquots were centrifuged at full speed for 1 minute in a table top microcentrifuge and the supernatant was removed. Pellets were washed twice in sterile deionized water, and finally resuspended to a final concentration of 200 mg/ml and 10 mg/ml, respectively. A mixture of 10 µl of a 50-mg/ml streptomycin sulfate stock, 7 µl of distilled water, 2.5 µl of *E. coli* and 0.5µl of *Candida* was spotted on to each NGM plate. Drugs in a final concentration of 12.5 µM were added to the mix. *E. coli* spotted plates were used as control. Finally, 5 µl of *C. elegans* egg suspension was transferred to each plate. Plates were then kept in a 20°C incubator and were observed over next 5 days. All the experiments were done in triplicate. Student t-test was used to check the statistical significance of the differences observed between wild type and other *Candida* strains.

Macrophage Growth Inhibition Assay with the Compounds

Macrophage cell line RAW 264.7 (ATCC) was used in the assay. The cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The protocol was slightly modified from the original (LOPES DA ROSA *et al.* 2010). Briefly, macrophages on reaching 80-90% confluence were scraped and brought up in DMEM supplemented with 10% FBS, 100 U/ml Penicillin, and 100µg/ml Streptomycin. 2 X 10^6 macrophage cells were placed in a 35mm^2 plate and allowed to adhere for 5 hours. *Candida* strain SC5314 was grown overnight at 37˚C and diluted 1:10 and allowed to grow for another 5 hours. *Candida* cells were then washed with supplemented DMEM

and were added to the plates containing macrophages in a ratio of 1*Candida*:15 macrophage cells to a final volume of 2 ml. SC5314 was also grown in parallel without macrophages to calculate % survival. Compounds of interest in specific concentrations were added to each plate so that each sample concentration was tested in duplicate or triplicate. DMSO alone at a volume similar to that of compounds was also added as control to SC5314 and SC5314 – macrophage co- cultures in the absence of compounds. The plates were incubated overnight (15-16hours) at 37˚C and 5% CO_2. Cells were then brought up to 24 ml in a tube using 0.05% Triton X-100 (v/v) in water to osmotically lyse the macrophage cells. Dilutions were prepared and plated on YPD plates and grown overnight at 37˚C. Colony forming units (CFU) were counted and percentage survival was calculated by taking the ratio of CFU from co culture of *Candida* and macrophage to the CFU obtained for *Candida* alone.

5.3 Results

Screening a Small molecule library to identify inhibitors of *C. albicans* adhesion to an inert surface.

Traditionally drug discovery begins with target identification - a protein that plays a key role in the disease. The next step is focused on developing assays used to identify small molecules that will inhibit the target protein. This is further followed by in vitro and in vivo toxicity and efficacy studies as part of pre-clinical trials. This target-based approach can sometimes become very lengthy and is one of the reasons for the slow adoption of new drugs. We took an alternative approach that is based on function,

rather than a single protein. We screened a small molecule library (UMass Medical School, Worcester) of 30,000 compounds (50 µM) together with 0.1 OD of log phase cells of *C. albicans* wild type strain SC5314 in 96 well plates. The positive hits were the wells that showed >50% inhibition measured as final OD at 590nm (Figure 3, shown in red). We used a modified crystal violet adhesion assay that measures the extent of adhesion of *C. albicans* cells to an inert polystyrene surface (REYNOLDS and FINK 2001). *C. albicans edt1-/-* mutants lacking a protein that facilitates adhesion to surfaces was used as positive control, while wild type *C. albicans* SC5314 with DMSO was used as negative control.

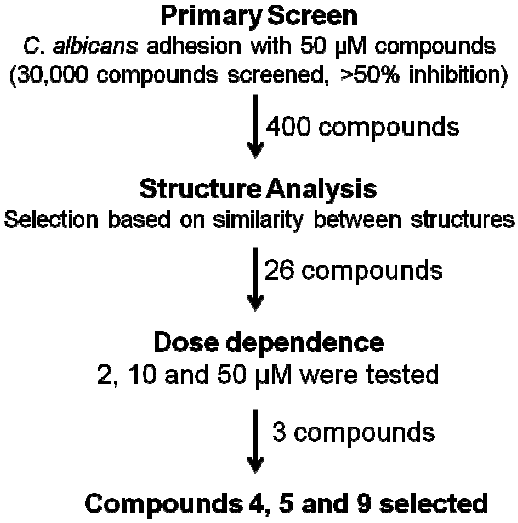
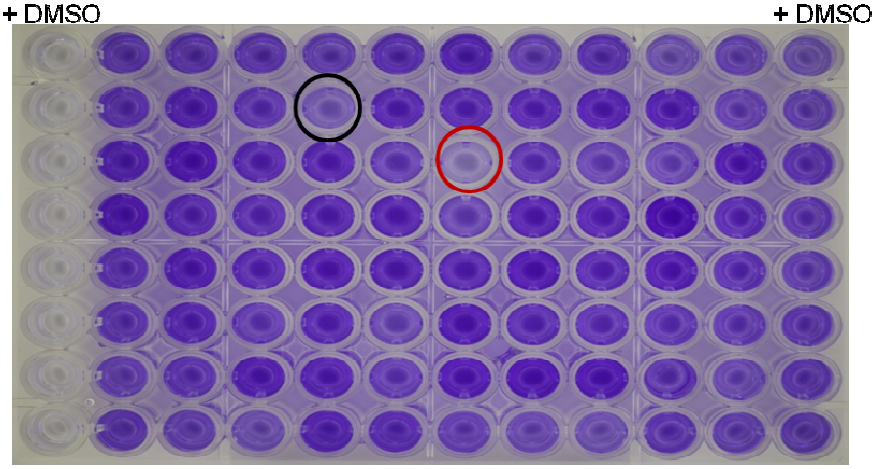


Figure 2. Flow chart of the steps taken to identify an inhibitor of adhesion.

Compounds that were able to inhibit *Candida* adhesion by more than 50% were considered positive hits. 1.3% of the library gave a positive result. These compounds were then analyzed based on their commonality and differences in the structure (Figure 2). Through this structural analysis, we picked a family of 26 related compounds (Table 1) for follow up studies. Structural analysis was later on followed up by a study of dose dependence of these 26 compounds in the crystal violet assay. This enabled us to

focus on 3 compounds, whose ids in Chembridge are 6714151 (compound 4), 7021797 (compound 5) and 7140676 (compound 9).

A



B

0.10	0.87	0.89	0.91	0.83	1.07	1.37	0.81	1.03	0.61	0.79	0.62
0.10	0.97	1.30	0.86	0.47	1.12	1.12	1.11	1.28	1.36	0.74	0.76
0.12	1.39	1.69	1.02	1.13	0.73	0.29	0.84	0.65	0.62	1.43	0.95
0.11	1.72	1.04	0.95	1.06	1.11	0.62	1.19	1.14	2.12	1.02	0.83
0.12	0.99	1.41	1.01	1.26	1.13	1.05	1.44	1.02	1.36	0.90	0.77
0.10	0.94	1.18	0.74	1.05	0.58	1.37	0.94	0.88	0.83	0.79	0.87
0.11	0.81	0.94	1.39	1.19	0.76	1.50	1.67	1.60	0.63	0.64	0.89
0.11	1.00	0.81	0.62	1.37	1.15	0.81	0.69	0.63	1.02	0.81	0.90

Figure 3. Drugs causing more than 50% inhibition in adhesion of SC5314 were selected as positive hits. SC5314 without the drug was used as a positive control while *edt1-/-* without the drug was the negative control. Since the drug added was dissolved in DMSO, the same concentration of the solvent was also added in control wells. (A) A photograph of a representative plate that was observed during the screen. (B) Corresponding OD590nm values for the plate shown above. The difference in the color intensity correlates with the range of inhibition. As we can see the well with red circle was considered a positive hit but the one with the black circle was not.

Compound 4 and 5 decrease the extent of *C. albicans* infections in *C. elegans*.

We have previously reported a pathogenesis assay in which we infected the nematode *Caenorhabditis elegans* with the pathogen *C. albicans* and quantitated the number of worms showing the disease phenotype Dar (deformed anal region). Using this assay we were able to screen for novel virulence factors in *C. albicans* which are important for virulence. Using the same assay we wanted to test the ability of these drugs in inhibiting the infection process in *C. elegans*. We exposed the eggs to *Candida* alone or with drug in 12.5 µM concentration and quantitated for Dar on Day 4. We observe that compound 4 and 5 were able to significantly reduce the number of worms exhibiting Dar (Figure 4). This shows that compounds 4 and 5 do play some role in inhibiting fungal infection in *C. elegans*.

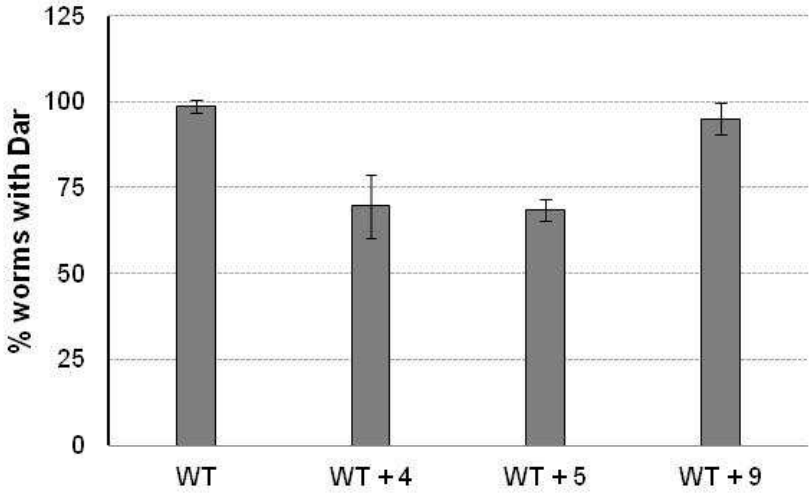


Figure 4. Compound 4 and 5 inhibit *Candida* infection in *C. elegans*. Worm eggs were exposed to mixed lawns of *E. coli* and *Candida* without and with the compounds 4, 5 and 9 at 12.5 µM concentrations and observed for Dar on day 4. Compounds 4 and 5 significantly ($p<0.05$) reduced the % of worms with Dar compared to the worms exposed to WT strain without the compound.

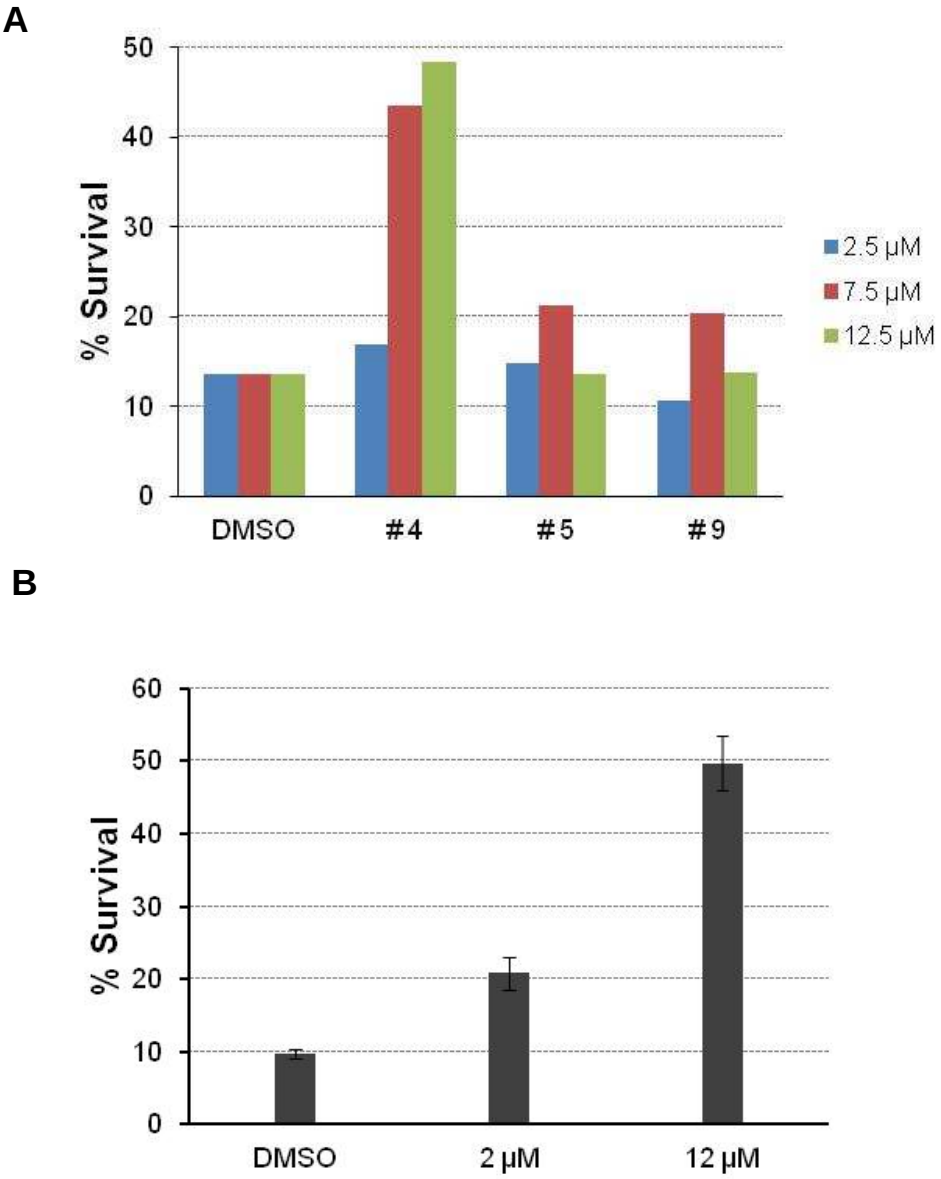


Figure 5. Compound 4 shows a dose dependence increase in the % survival of *C. albicans* when exposed to RAW264.7 macrophages. SC5314 was exposed to macrophages in the presence of the solvent DMSO as control and (A) dose dependence of compounds 4, 5 and 9. Since the experiment was performed in duplicates and an observable change was see with compound 4, the experiment was repeated in triplicate with (B) dose dependence of compound 4. There was a significant increase in the *Candida* survival.

Compound 4 inhibits *Candida* attachment to mammalian phagocytes.

In order to assess compounds 4, 5 and 9 as potential inhibitors of adhesion in mammalian cells, we exposed murine macrophage cell line RAW264.7 to *C. albicans* SC5314 in 15:1 ratio with and without the drugs at different concentrations and % *Candida* survival was calculated. We hypothesized that *Candida* will not attach to phagocytes in the presence of the drug and will escape phagocytosis. Compounds 5 and 9 were not significantly different compared to DMSO control. In presence of compound 4, we observed a ~ 5 times increase in the % survival of the *Candida* cells (Figure 5A). Compound 4 was retested at concentrations 2 and 12 µM and exhibited dose dependent inhibition of *Candida* adhesion (Figure 5B). Thus, compound 4 affects the adhesion of *Candida* to macrophages and prevents phagocytosis.

5.4 Discussion

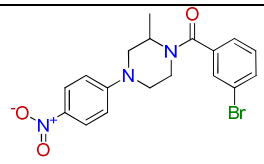
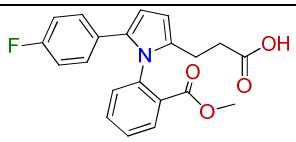
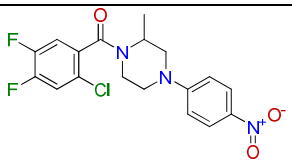
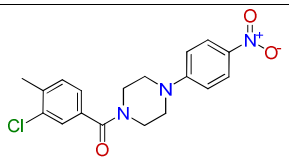
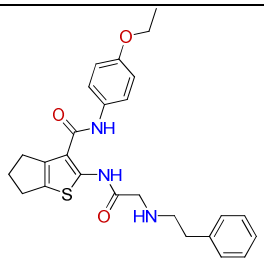
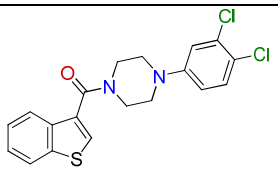
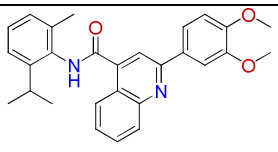
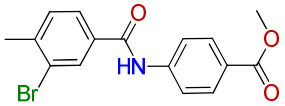
Adhesion to the host mucosal epithelium is required for the fungal organism to establish itself, colonize and cause infection. If not treated, in some very few cases these mucosal infections can become systemic. Chances of a systemic infection are higher with the use of intravascular devices and catheters. *Candida* forms biofilms on these devices and thus obtains access to the bloodstream of the patients. Any compound that can inhibit the adhesion of *Candida* to these inert surfaces can potentially be used as one of the preventives for systemic infections.

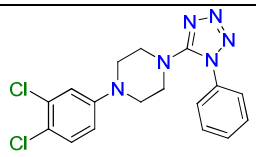
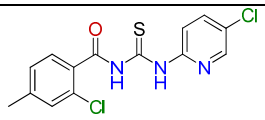
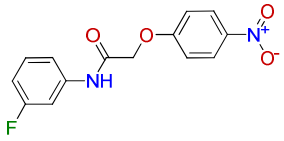
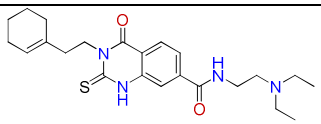
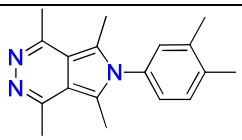
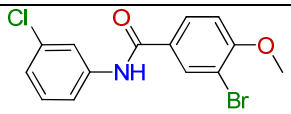
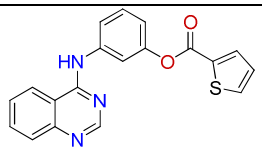
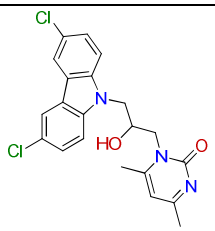
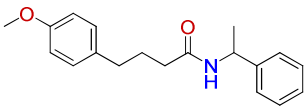
To identify such compound we screened a library of 30,000 compounds and identified one compound which has the potential of being an inhibitor of *Candida* adhesion.

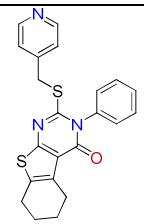
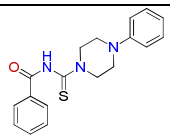
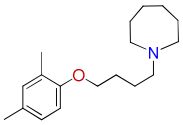
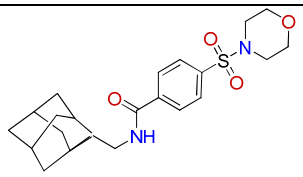
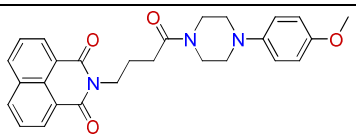
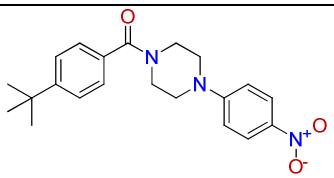
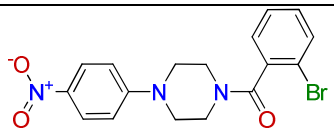
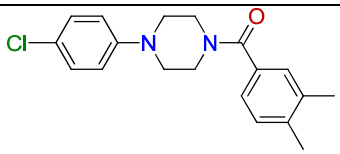
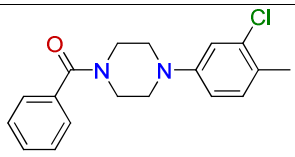
Following the steps in the scheme (Figure 2), we eventually tested 3 compounds in an *in vitro* mammalian model of phagocytic cells and identified a potential role of compound 4. Further testing confirms a dose dependent effect of compound 4 on adhesion of *Candida* to macrophages. This means that the drug might be altering the fungal cell surface in such a way, that it can no longer adhere to macrophages.

These exciting results show the potential of compound 4 as an inhibitor of adhesion. The next step would be to test the adhesion of *C. albicans* on human epithelial cells, silicon catheter material and other implant materials in the presence of compound 4.

Table 1. List of 26 compounds

Compound #	Compound IDs	Structure	Molecular Formula	Mol. Weight
1	6177408		C18H18BrN3O3	404.258
2	6870699		C21H18FNO4	367.37
3	6139415		C18H16ClF2N3O3	395.788
4	**6714151**		**C18H18ClN3O3**	**359.807**
5	7021797		C26H29N3O3S	463.592
6	7288338		C19H16Cl2N2OS	391.314
7	7291583		C28H28N2O3	440.534
8	7052098		C16H14BrNO3	348.191

9	7140676		C17H16Cl2N6	375.255
10	6942569		C14H11Cl2N3OS	340.228
11	6988606		C14H11FN2O4	290.247
12	7115759		C23H32N4O2S	428.591
13	7024394		C18H21N3	279.379
14	6777751		C14H11BrClNO2	340.6
15	6886186		C19H13N3O2S	347.39
16	5605598		C21H19Cl2N3O2	416.3
17	6873245		C19H23NO2	297.391

18	7111893		C22H19N3OS2	405.536
19	7146132		C18H19N3OS	325.428
20	7319890		C18H29NO	275.429
21	6937280		C23H32N2O4S	432.576
22	7113600		C27H27N3O4	457.521
23	5742793		C21H25N3O3	367.442
24	5221077		C17H16BrN3O3	390.231
25	7239106		C19H21ClN2O	328.836
26	7276058		C18H19ClN2O	314.809

Chapter 6

Significance and Future Directions

6.1 Significance

In this work, I have demonstrated the utility of the pathogenesis assay in multiple ways (Figure 1). First I have shown that the assay is useful in identification of virulence determinants using a candidate gene approach. Thus, I identified Cap1 to be important in fungal response against stress caused by ROS (Chapter 3).

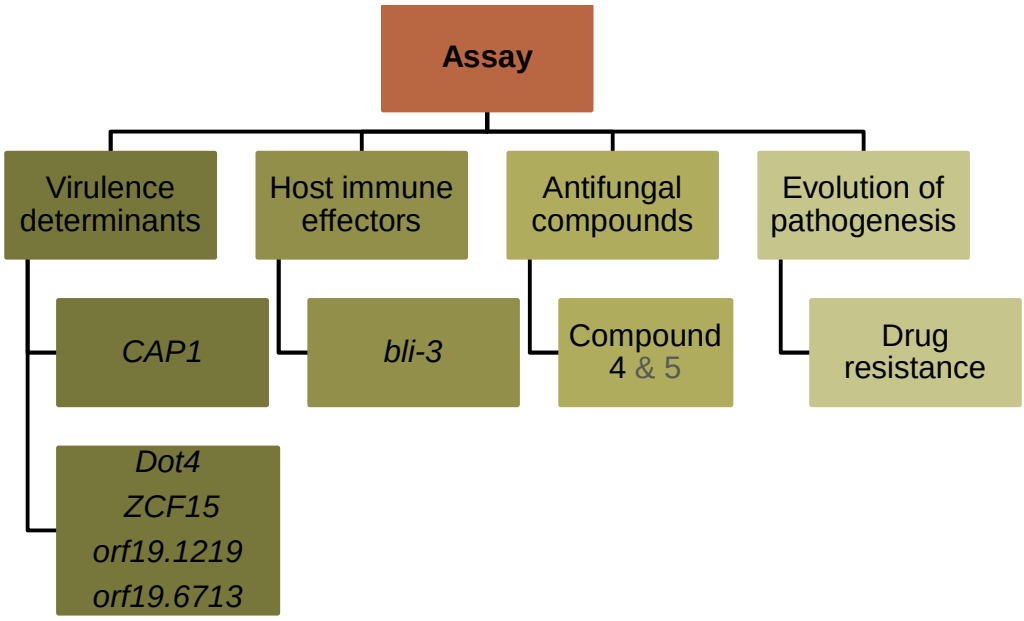


Figure 1. An overview of various ways in which pathogenesis assay was used.

I have also shown that using the assay it is possible to screen a library of *Candida* mutants. Through such screen we can look for novel virulence factors and pathways that add to the repertoire of *Candida*, that it uses to overcome the selective pressure in

the host environment. We screened a library of ~1200 *C. albicans* insertion mutants, and identified 4 novel (*orf19.1219*, *orf19.6713*, *DOT4* and *ZCF15*) genes that potentially play a role in fungal infection (Chapter 4).

Second, I can use the assay to identify host immune effectors that are involved in resistance against *Candida* infection. I have shown that BLI-3 is an important part of host innate immune response, as it produces ROS to counteract *Candida* infection (Chapter 3). Although not part of this work, this assay after some modifications can also be employed to screen the *C. elegans* RNAi library to order to identify the modulators of innate immune responses during fungal infection.

Third use of this assay is for drug screens or to test for potential drugs in a whole animal model. Families of related (same scaffold) compounds were identified through an adhesion screen of 30,000 compounds, for their ability as potential inhibitors of *C. albicans* adhesion to biological and inert surfaces. These compounds were further tested in the pathogenesis assay for their ability to reduce infection of *C. albicans* in *C. elegans* (Chapter 5).

Finally, using the survival assay to test for the mortality caused by infection, I could observe disparity in the different *C. albicans* fluconazole resistant strains isolated from AIDS patients (Appendix). This shows that we can study the differences in the *Candida* strains that have been exposed to the host environment and evolved to counter the select pressure of the environment.

Candida albicans has 8 chromosomes and belongs to the pre duplication clade of fungi (Figure 2) but its most important characteristic is that the genome exhibits a high degree

of plasticity. This plasticity includes gross chromosomal rearrangements, aneuploidy and loss of heterozygosity (LOH) (RUSTCHENKO 2007). *C. albicans* reproduces through asexual clonal division (IWAGUCHI *et al.* 1992) and even though most of its mating and meiosis machinery is retained (TZUNG *et al.* 2001), meiosis is never observed (MAGEE and MAGEE 2004).

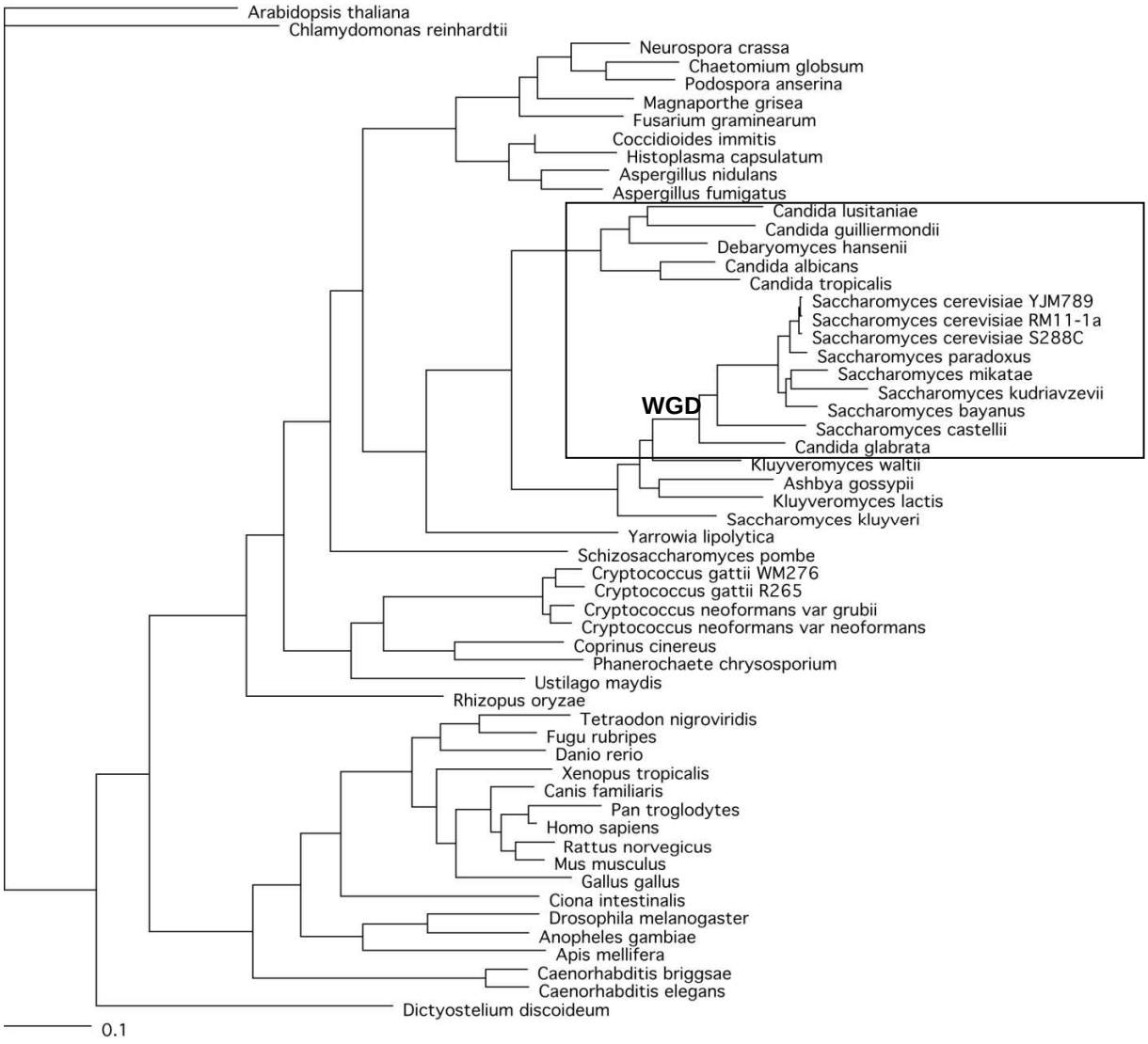


Figure 2. *C. albicans* belongs to the pre duplication clade. Boxed region is the clades of interest. WGD – Whole genome duplication. Adapted from (FITZPATRICK *et al.* 2006)

C. albicans have mating type like alleles (MTL) a and α. In some cases strains that are homozygous for MTL, which only occurs in 3 to 10% of clinical isolates are mating competent (LEGRAND *et al.* 2004). The strains that mate become tetraploid and undergo random loss of multiple chromosomes until they reach a near diploid state (ALBY *et al.* 2009). This parasexual cycle provides an alternative to meiosis and gives rise to genomic diversity. Along with these diversity arising in clinical isolates through parasexuality, current models support the occurrence of adaptive mutations that can occur, e.g., during host pathogen interaction or with the acquisition of drug resistance (BENNETT and JOHNSON 2005).

Maintenance of heterozygosity is one of the ways that *C. albicans* maintains genetic variability. Despite this, LOH is very common and is considered to be an important factor in acquiring azole resistance. Homozygosity of hyperactive alleles like *ERG11* (encodes target of azole drug) is associated with increased azole resistance (WHITE 1997c). Furthermore some of the evolved clinical isolates that showed azole resistance and were confirmed for LOH were tested in our assay and compared to their progenitors which were drug sensitive. Our assay clearly shows an increase in mortality of *C. elegans* exposed to LOH strains compared to the progenitors (Appendix, Figure 3).

Thus it will be interesting to study the different aspects of *Candida* genome diversity which can potentially lead to increased virulence in our pathogenesis assay.

6.2 Future Directions

The work presented in this thesis has helped us gain insightful knowledge about fungal pathogenesis. Use of pathogenesis assay and other corroborating experiments has answered a few of the questions in the field but has raised more queries that need to be addressed.

The 4 potential virulence factors, *orf19.1219, orf19.6713, Dot 4* and *Zcf15* that were identified in the screen, are uncharacterized in *C. albicans*. Their roles can only be speculated from their homologs in *S. cerevisiae*. Future work would entail the characterization of these genes and assessment of their role in fungal pathogenesis. Apart from the normal tests that are done to characterize a mutant such as study shape of the mutant cells and colonies, and compare growth in different media, we need to do genome level studies to identify their protein targets for interaction. Extrapolating their role in *S. cerevisiae*, either these proteins are transcription factors or enzymes important in the ubiquitination pathway. Either way they could target a number of proteins and thus be responsible for their co-regulation. Identifying these targets will further help us tease out the specific steps for which these factors might be important. Microarrays are one of the ways through which we can identify their direct or indirect targets, as expression of these targets will either be substantially lowered or raised in the mutants compared to the wild type.

The one limiting factor of our assay is that quantification of the biomarker, Dar can be time consuming, making the use of this assay in high throughput screenings a little difficult if not impossible. There are now automated approaches available to image

thousands of samples per day. Using these approaches combined with cell imaging software's like CellProfiler (CARPENTER *et al.* 2006) could allow us to do functional genomics or chemical screens in a high throughput manner. Using the software we can quantitatively address complex morphological changes such as cell/organelle shape or potentially Dar phenotype. Optimizing this software to "read" Dar will truly make the use of patho-assay a high throughput assay for screening fungal virulence factors, host immune effectors or chemicals that could potentially have a role as an antifungal.

High-throughput antifungal screens are normally performed *in vitro* and then followed up by a much smaller scale testing in *in vivo* models. Use of this assay will provide us with the means to skip the first step and directly screen the potential drugs in an *in vivo* manner. Furthermore, use of *E. coli* in the assay also provides us with the potential of utilizing the *C. elegans* RNAi collection library to identify probable, universal or specific, host immune effectors and pathways that are activated during fungal infections in a high throughput fashion.

We also observed the role of evolved fluconazole resistant strains in fungal infection through our assay. The normal *Candida* strains in the human body are under selective pressure from host immune responses, and any favorable genetic changes they experience in the host environment may make it possible for them to spread and infect. Attempts to control this process in humans through activation of host immune responses or from administration of antifungals, has led to the evolution of these organisms. Our assay would be a great advantage in studying the evolution of these strains in whole animal model.

Appendix

Resistance of *C. albicans* to antifungal drugs is a growing problem in the medical field. A study was done by *Perea et. al.* in 2001 to identify the molecular mechanism for azole resistance. They isolated the pathogen from AIDS patients suffering from oropharyngeal candidiasis. An initial sample (Progenitor) was collected, patients were administered the drug, and a second sample (Evolved) was then collected from the patients that were not cured by the drugs. In some cases, more samples (Evolved) were collected in a time course (Figure 1). They observed that there was an increase in the expression of efflux pumps in the resistant strains (PEREA *et al.* 2001).

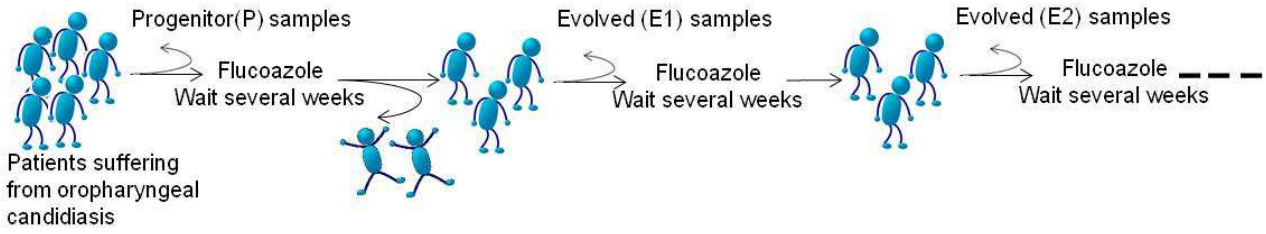


Figure 1. Schematic of sample collection done to study changes in the drug resistant strains.

Recently, all of the strains reported in the paper were subjected to genomic sequencing at the Broad Institute in order to identify genes showing recurrent single nucleotide polymorphism (SNPs) in the evolved strain compared to the progenitor. Some of the genes showing polymorphism in the evolved strains but not the progenitors are the genes that we identified in the screen (Chapter 4) except for orf19.6713. The SNPs in genes of interest (described in Chapter 4) were also observed in recurrent cases.

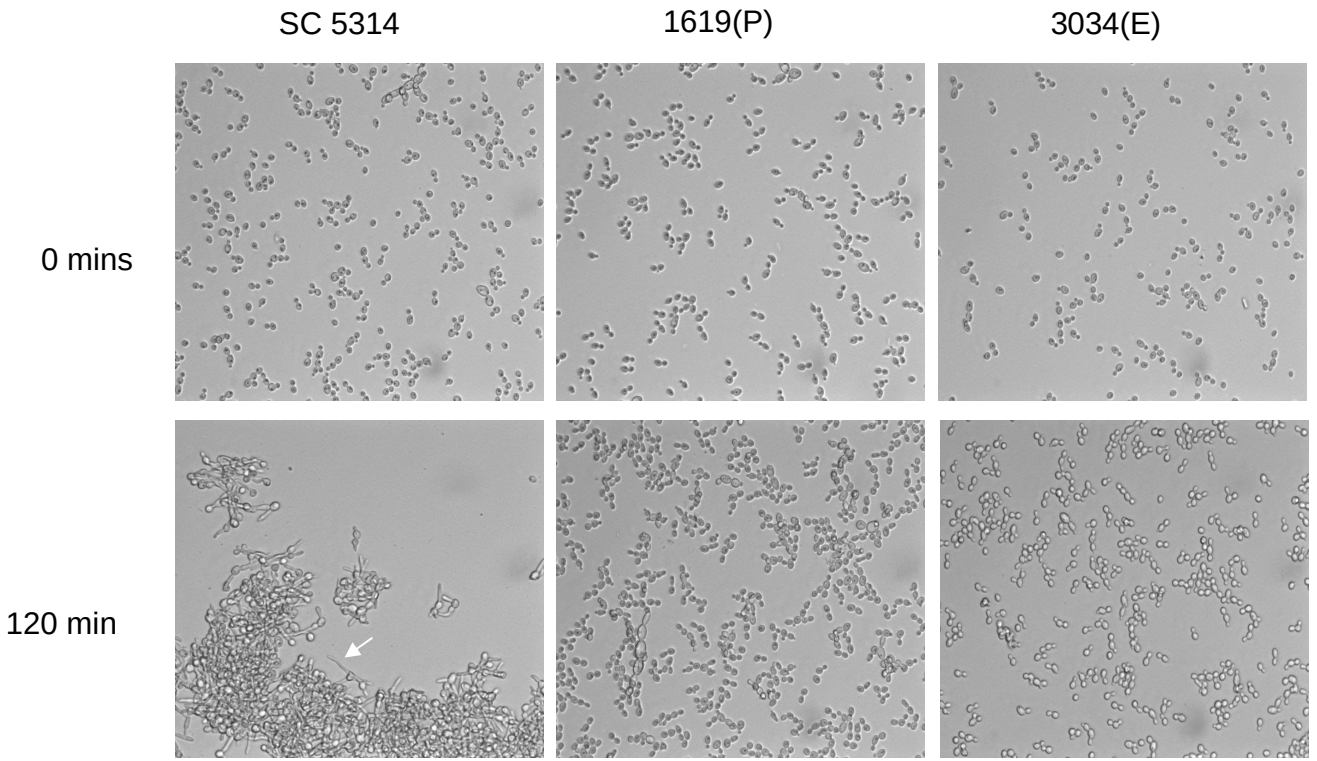
In order to characterize these clinical isolates, especially in a tractable host, we performed a *C. elegans* survival assay in which we followed up 3 sets of samples (showing SNPs in our gene of interests) that are listed in Table 1(modified from Perea et. al. 2001) below. In table 1, first isolate in each set of sample (e.g. 1649 in patient no. 43) is the progenitor strain followed by the evolved strain in the time course and MIC stands for Minimum Inhibitory Concentration of the drug. Clinical isolates 1, 12 and 13 were from a time course used in a separate study (WHITE 1997b). These clinical isolates were also characterized using an *in vitro* assay.

TABLE 1. Antifungal susceptibilities of *C. albicans* isolates

Patient no.	Isolate no.	MIC (μg/ml) at 24 h/48 h				
		Fluconazole	Itraconazole	Voriconazole	Posaconazole	AmB
43	1649	0.25/0.5	0.015/0.015	0.03/0.03	0.015/0.015	0.06/0.125
	3034	>64/>64	0.5/0.5	0.5/2	0.25/0.5	0.25/0.5
59	3917	2/4	0.06/0.125	0.125/0.125	0.03/0.06	0.125/0.5
	4617	32/64	0.125/0.25	0.25/1	0.125/0.125	0.125/0.25
	4639	64/>64	0.25/0.25	0.5/1	0.125/0.125	0.125/0.25
30	5106	4/8	0.03/0.06	0.06/0.06	0.015/0.25	0.06/0.125
	5108	32/64	0.06/0.25	0.5/0.5	0.06/0.125	0.125/0.5

The adhesion and survival assays were performed as mentioned before. Germ tube formation assay was performed as follows: Overnight cultures of the strains were grown in YPD at 30^{o}C. Next day, 0.05 OD_{600}nm were collected and resuspended in 3ml of fresh RPMI media + 10% FBS. A 10 μl sample was immediately placed on a slide, observed and photographed for (t=0min). Micrographs were then taken at every half hour interval. For convenience purposes only 0 and 2 hour pictures are shown.

A



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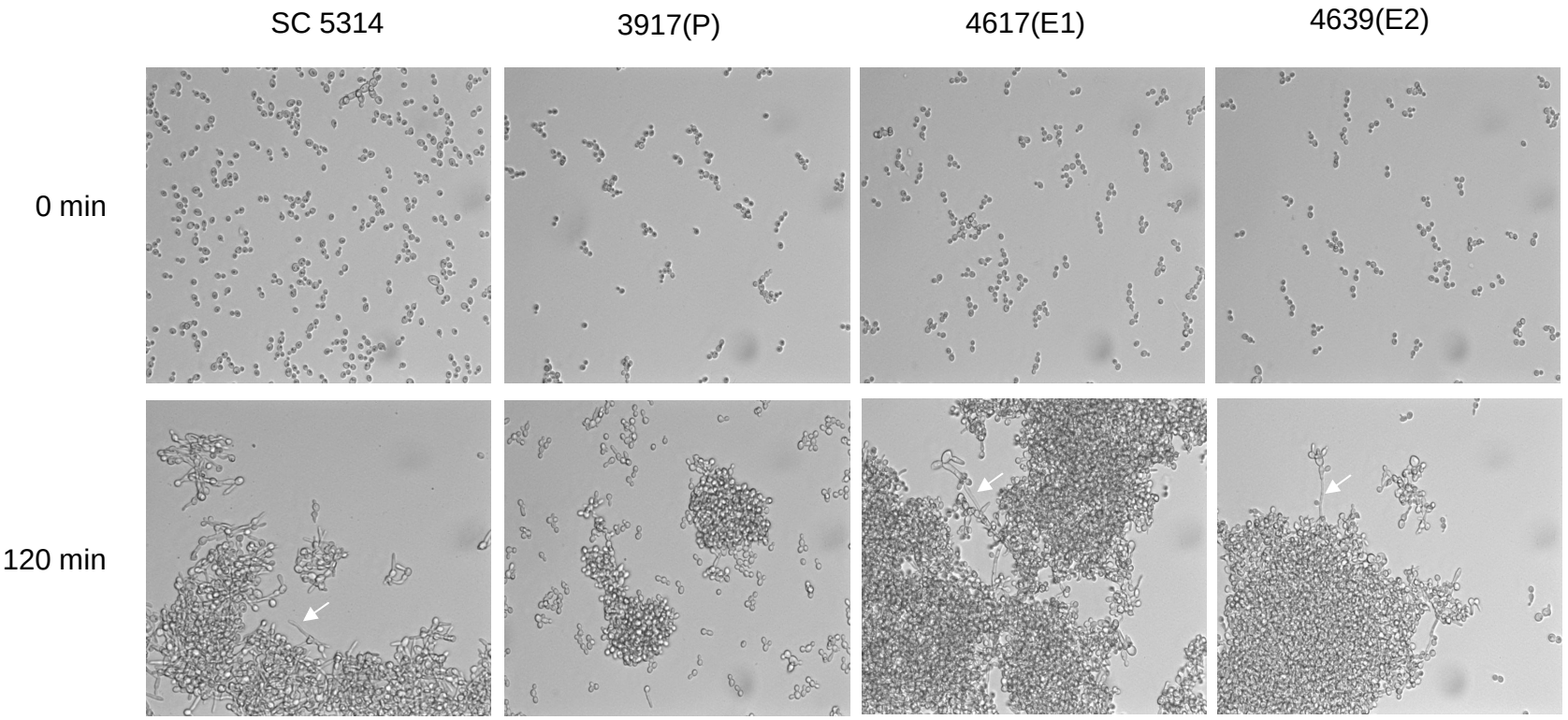

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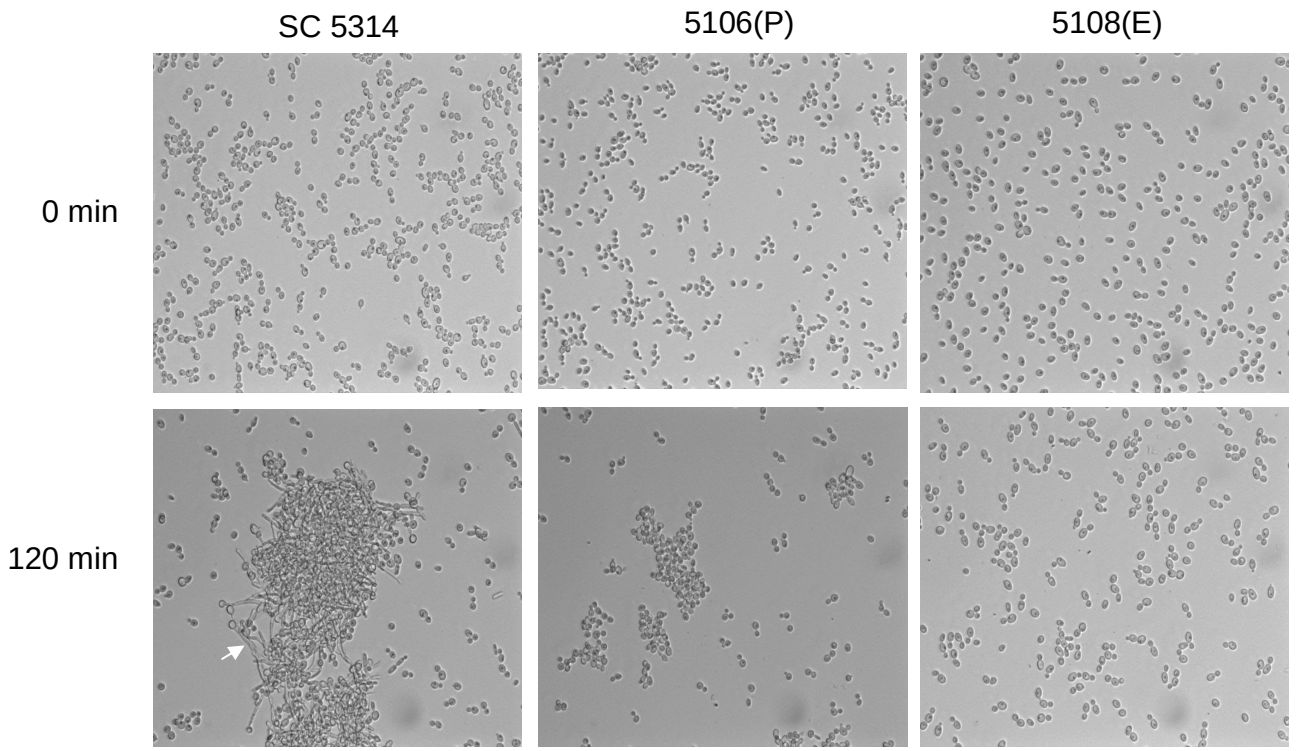

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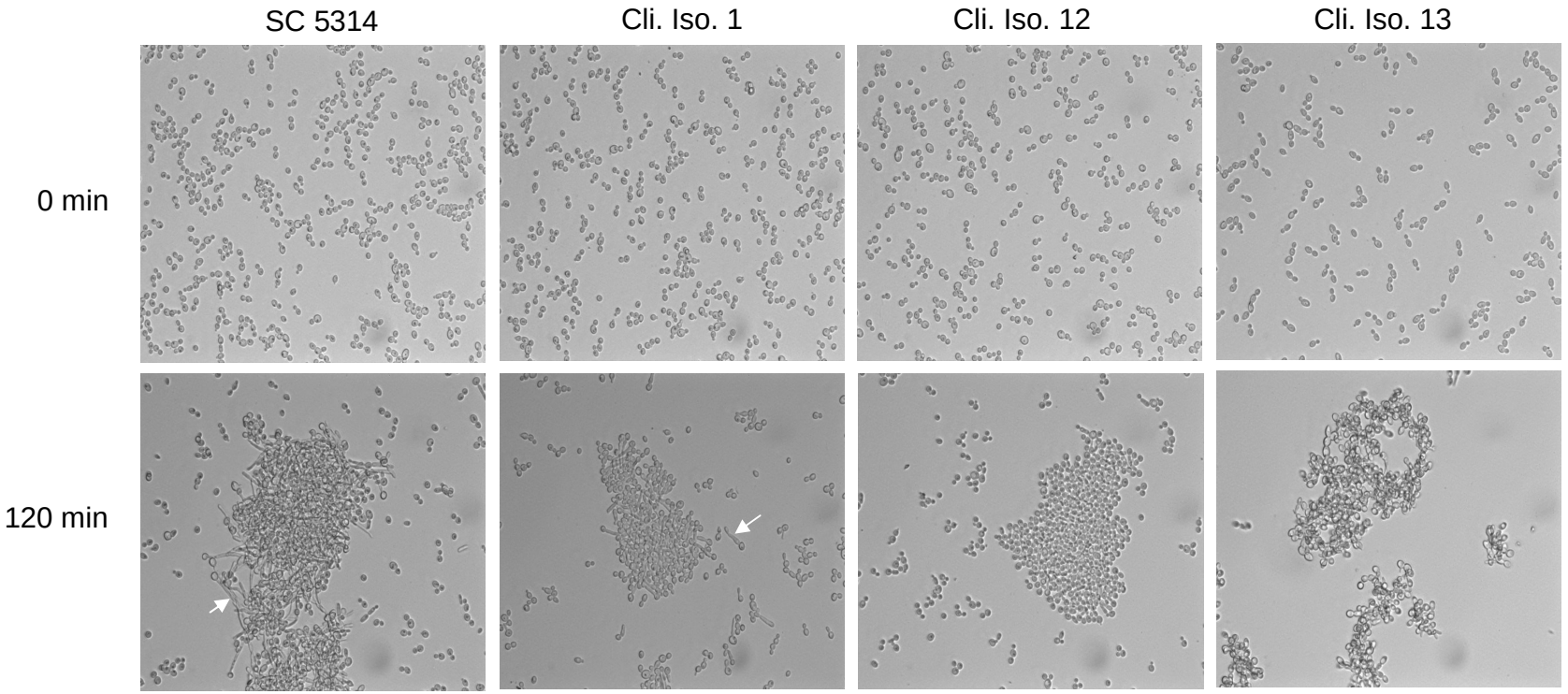
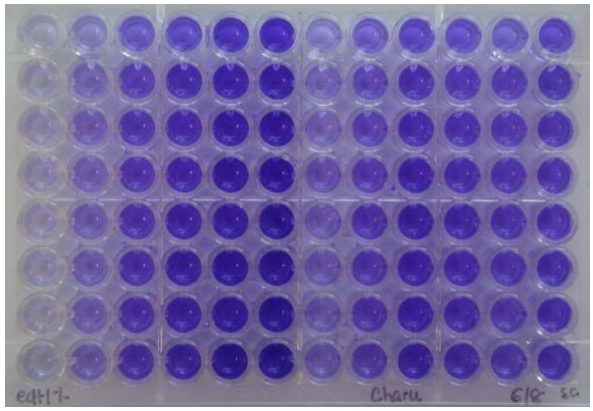


Figure 2. Germ tube formation (shown by white arrows) in some cases is affected in the evolved strain compared to progenitor. SC5314 was used as a control. Cultures were grown in filament inducing media containing 10% FBS and pictures were taken at t=0 min and t=120 min. Panels **(A)** 1649(P) and 3034(E), (**B)** 3917(P) and 4617(E1) and 4639(E2), **(C)** 5106(P) and 5108(E) and **(D)** Clinical Isolates 1, 12 and 13, are samples from different patients. Altered in germ tube formation is observed between in the evolved strains when compared to the progenitors. Though strains 4617(E1) and 4639(E2) are hyper filamentous.

A



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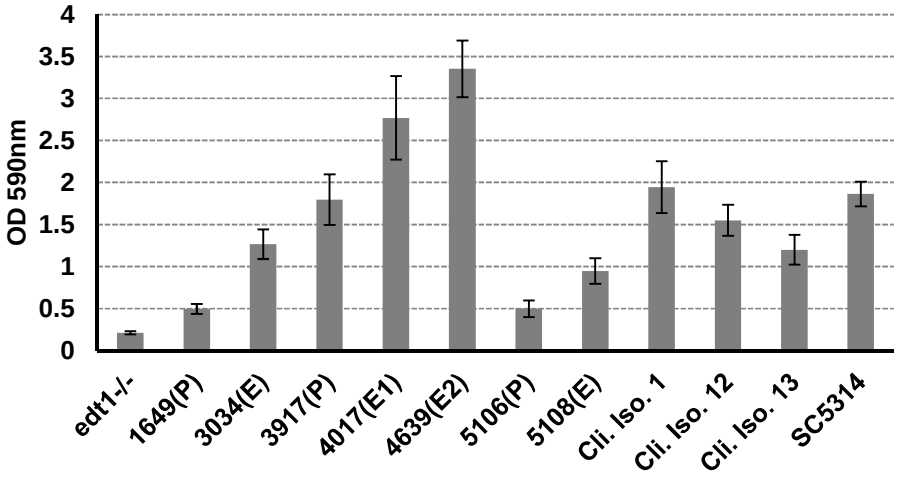

Figure 3. Evolved strains are more adhesive on a polystyrene surface than their respective progenitor. *edt1-/-* is the positive control and SC5314 is the negative control for the Adhesion Assay. P is progenitor and E is evolved strain. The evolved strains are shown immediately after their respective progenitor strains. (A) Photograph taken of the crystal violet adhesion plate. Strains are in the same order as shown in the graph. (B) Graph depicting the average of OD 590nm values taken from 8 wells for each strain. Except for, comparison between SC5314-3917 and SC5315-Clinical Isolate 1, all other comparisons (SC5314 with other strains and Progenitors with the Evolved) by Student's t-test had $p<0.01$.

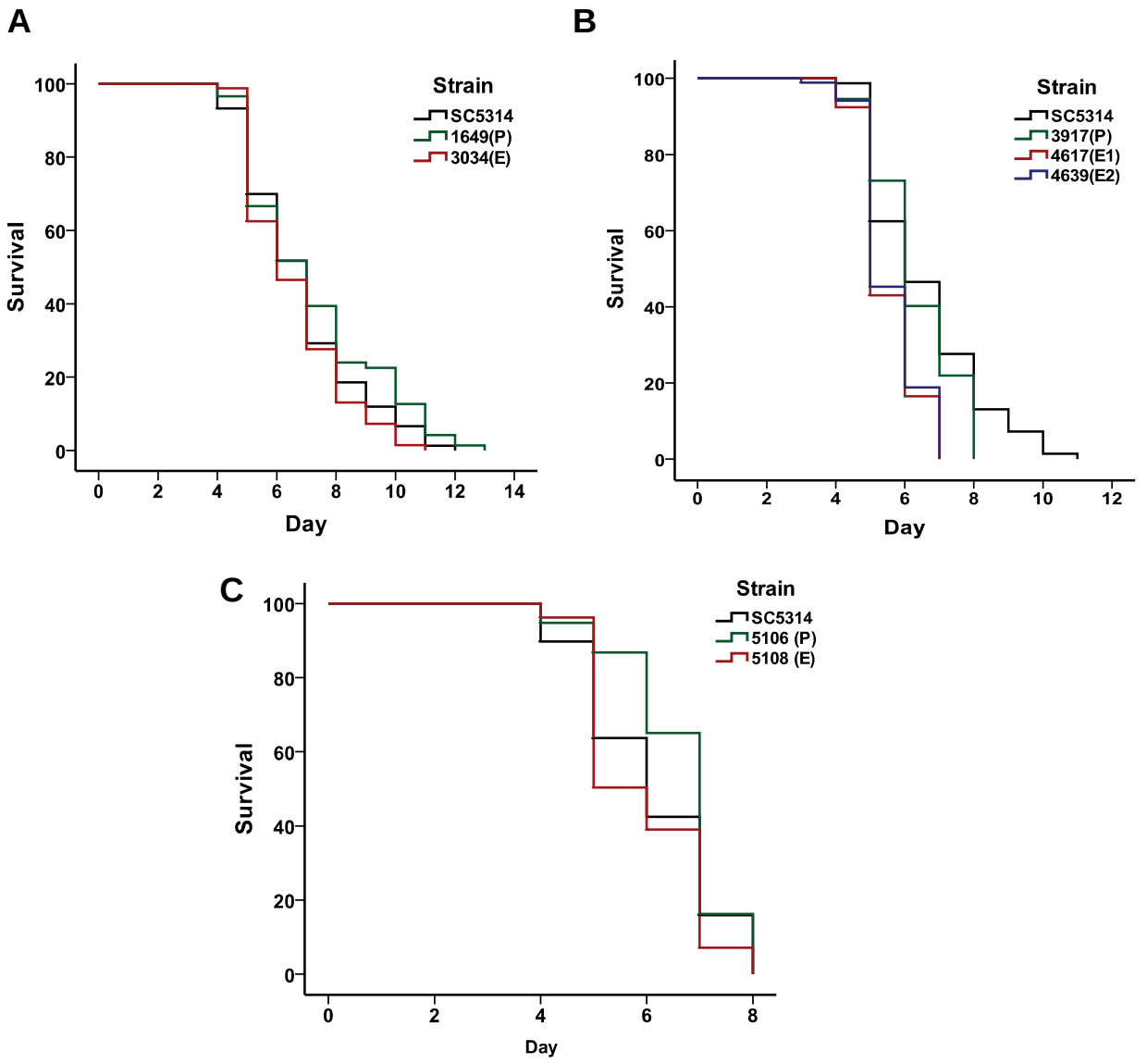

Figure 3. Evolved strains show increased virulence in the worms compared to their respective progenitors. Survival assays were performed along with SC5314, a laboratory wild type strain as control, for each group of strains **(A)** 1649(P) and 3034(E) (**B)** 3917(P) and 4617(E1) and 4639(E2) and **(C)** 5106(P) and 5108(E). All the evolved strains killed the worms significantly faster than their respective progenitors. Log rank statistical analysis for the comparison between all progenitor and respective evolved strains had $p<0.05$. Kelly Pastor performed the experiment in panels A and B. I am responsible for performing experiment in panel C, designing all the experiments and analyzing the data.

From the germ tube formation (Figure 2), adhesion (Figure 3) and survival criteria (Figure 4) it is quite clear that the evolved strains do show an enhanced phenotype than their respective progenitors. Thus the changes taking place in these strains through their evolution in the human host due to exposure to antifungal drugs does not seem to be restricted to the genes involved in antifungal resistance but seem to make the strain overall even more virulent. Additional experiments needs to be performed in order to corroborate this. Especially for 4617(E1) and 4639(E2), the results are interesting as they are also causing more adhesion and kill the worms faster than even SC5314. Also note that survival experiments have been performed only once and need to be repeated again to reconfirm the data.

References

1. ABALLAY, A., P. YORGEY and F. M. AUSUBEL, 2000 Salmonella typhimurium proliferates and establishes a persistent infection in the intestine of Caenorhabditis elegans. Curr Biol **10:** 1539-1542.
2. ADAMS, M. D., S. E. CELNIKER, R. A. HOLT, C. A. EVANS, J. D. GOCAYNE *et al.*, 2000 The genome sequence of Drosophila melanogaster. Science **287:** 2185-2195.
3. ALARCO, A. M., I. BALAN, D. TALIBI, N. MAINVILLE and M. RAYMOND, 1997a AP1-mediated multidrug resistance in Saccharomyces cerevisiae requires FLR1 encoding a transporter of the major facilitator superfamily. J Biol Chem **272:** 19304-19313.
4. ALARCO, A. M., I. BALAN, D. TALIBI, N. MAINVILLE and M. RAYMOND, 1997b AP1-mediated multidrug resistance in Saccharomyces cerevisiae requires FLR1 encoding a transporter of the major facilitator superfamily. J Biol Chem **272:** 19304-19313.
5. ALARCO, A. M., A. MARCIL, J. CHEN, B. SUTER, D. THOMAS *et al.*, 2004 Immune-deficient Drosophila melanogaster: a model for the innate immune response to human fungal pathogens. J Immunol **172:** 5622-5628.
6. ALARCO, A. M., and M. RAYMOND, 1999a The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in Candida albicans. J Bacteriol **181:** 700-708.
7. ALARCO, A. M., and M. RAYMOND, 1999b The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in Candida albicans. J Bacteriol **181:** 700-708.

8. ALBY, K., D. SCHAEFER and R. J. BENNETT, 2009 Homothallic and heterothallic mating in the opportunistic pathogen Candida albicans. Nature **460:** 890-893.
9. ALONSO-MONGE, R., F. NAVARRO-GARCÍA, G. MOLERO, R. DIEZ-OREJAS, M. GUSTIN *et al.*, 1999 Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of Candida albicans. J Bacteriol **181:** 3058-3068.
10. APEL, K., and H. HIRT, 2004 Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373-399.
11. APIDIANAKIS, Y., L. G. RAHME, J. HEITMAN, F. M. AUSUBEL, S. B. CALDERWOOD *et al.*, 2004 Challenge of Drosophila melanogaster with Cryptococcus neoformans and role of the innate immune response. Eukaryot Cell **3:** 413-419.
12. ARANA, D. M., R. ALONSO-MONGE, C. DU, R. CALDERONE and J. PLA, 2007 Differential susceptibility of mitogen-activated protein kinase pathway mutants to oxidative-mediated killing by phagocytes in the fungal pathogen Candida albicans. Cell Microbiol **9:** 1647-1659.
13. BABIOR, B. M., 2004 NADPH oxidase. Curr Opin Immunol **16:** 42-47.
14. BABIOR, B. M., J. T. CURNUTTE and B. J. MCMURRICH, 1976 The particulate superoxide-forming system from human neutrophils. Properties of the system and further evidence supporting its participation in the respiratory burst. J Clin Invest **58:** 989-996.
15. BADER, T., B. BODENDORFER, K. SCHRÖPPEL and J. MORSCHHÄUSER, 2003 Calcineurin is essential for virulence in Candida albicans. Infect Immun **71:** 5344-5354.

16. BATES, S., J. M. DE LA ROSA, D. M. MACCALLUM, A. J. BROWN, N. A. GOW *et al.*, 2007 Candida albicans Iff11, a secreted protein required for cell wall structure and virulence. Infect Immun **75:** 2922-2928.
17. BAUTERS, T. G., M. MOERMAN, H. VERMEERSCH and H. J. NELIS, 2002 Colonization of voice prostheses by albicans and non-albicans Candida species. Laryngoscope **112:** 708-712.
18. BENDEL, C. M., D. J. HESS, R. M. GARNI, M. HENRY-STANLEY and C. L. WELLS, 2003 Comparative virulence of Candida albicans yeast and filamentous forms in orally and intravenously inoculated mice. Crit Care Med **31:** 501-507.
19. BENNETT, R. J., and A. D. JOHNSON, 2005 Mating in Candida albicans and the search for a sexual cycle. Annu Rev Microbiol **59:** 233-255.
20. BERGIN, D., E. P. REEVES, J. RENWICK, F. B. WIENTJES and K. KAVANAGH, 2005 Superoxide production in Galleria mellonella hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils. Infect Immun **73:** 4161-4170.
21. BERMINGHAM-MCDONOGH, O., E. B. GRALLA and J. S. VALENTINE, 1988 The copper, zinc-superoxide dismutase gene of Saccharomyces cerevisiae: cloning, sequencing, and biological activity. Proc Natl Acad Sci U S A **85:** 4789-4793.
22. BEUTLER, B., 2004 Innate immunity: an overview. Mol Immunol **40:** 845-859.
23. BINDSCHEDLER, L. V., J. DEWDNEY, K. A. BLEE, J. M. STONE, T. ASAI *et al.*, 2006 Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. Plant J **47:** 851-863.

24. BOCKMÜHL, D. P., and J. F. ERNST, 2001 A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of Candida albicans. Genetics **157:** 1523-1530.

25. BOEKE, J. D., J. TRUEHEART, G. NATSOULIS and G. R. FINK, 1987 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol **154:** 164-175.

26. BOLM, M., W. T. JANSEN, R. SCHNABEL and G. S. CHHATWAL, 2004 Hydrogen peroxide-mediated killing of Caenorhabditis elegans: a common feature of different streptococcal species. Infect Immun **72:** 1192-1194.

27. BRACHMANN, C. B., A. DAVIES, G. J. COST, E. CAPUTO, J. LI *et al.*, 1998 Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115-132.

28. BRAJTBURG, J., W. G. POWDERLY, G. S. KOBAYASHI and G. MEDOFF, 1990 Amphotericin B: current understanding of mechanisms of action. Antimicrob Agents Chemother **34:** 183-188.

29. BRAND, A., D. M. MACCALLUM, A. J. BROWN, N. A. GOW and F. C. ODDS, 2004 Ectopic expression of URA3 can influence the virulence phenotypes and proteome of Candida albicans but can be overcome by targeted reintegration of URA3 at the RPS10 locus. Eukaryot Cell **3:** 900-909.

30. BRAUN, B. R., W. S. HEAD, M. X. WANG and A. D. JOHNSON, 2000 Identification and characterization of TUP1-regulated genes in Candida albicans. Genetics **156:** 31-44.

31. BRAUN, B. R., and A. D. JOHNSON, 1997 Control of filament formation in Candida albicans by the transcriptional repressor TUP1. Science **277:** 105-109.

32. BREGER, J., B. B. FUCHS, G. APERIS, T. I. MOY, F. M. AUSUBEL *et al.*, 2007 Antifungal chemical compounds identified using a C. elegans pathogenicity assay. PLoS Pathog **3:** e18.

33. BRENNAN, M., D. Y. THOMAS, M. WHITEWAY and K. KAVANAGH, 2002 Correlation between virulence of Candida albicans mutants in mice and Galleria mellonella larvae. FEMS Immunol Med Microbiol **34:** 153-157.

34. BRENNER, S., 1974 The genetics of Caenorhabditis elegans. Genetics **77:** 71-94.

35. BROWN, G. D., and S. GORDON, 2001 Immune recognition. A new receptor for beta-glucans. Nature **413:** 36-37.

36. BROWN, G. D., J. HERRE, D. L. WILLIAMS, J. A. WILLMENT, A. S. MARSHALL *et al.*, 2003 Dectin-1 mediates the biological effects of beta-glucans. J Exp Med **197:** 1119-1124.

37. BUURMAN, E. T., C. WESTWATER, B. HUBE, A. J. BROWN, F. C. ODDS *et al.*, 1998 Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of Candida albicans. Proc Natl Acad Sci U S A **95:** 7670-7675.

38. **CALDERONE, R. A.**, 2002 *Candida* and candidiasis., pp. 3-13. ASM Press, Washington, D. C.

39. CALDERONE, R. A., and W. A. FONZI, 2001 Virulence factors of Candida albicans. Trends Microbiol **9:** 327-335.

40. CARPENTER, A. E., T. R. JONES, M. R. LAMPRECHT, C. CLARKE, I. H. KANG *et al.*, 2006 CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol **7:** R100.

41. CHAFFIN, W. L., 2008 Candida albicans cell wall proteins. Microbiol Mol Biol Rev **72:** 495-544.

42. CHAFFIN, W. L., J. L. LÓPEZ-RIBOT, M. CASANOVA, D. GOZALBO and J. P. MARTÍNEZ, 1998 Cell wall and secreted proteins of Candida albicans: identification, function, and expression. Microbiol Mol Biol Rev **62:** 130-180.

43. CHAMILOS, G., M. S. LIONAKIS, R. E. LEWIS, J. L. LOPEZ-RIBOT, S. P. SAVILLE *et al.*, 2006 Drosophila melanogaster as a facile model for large-scale studies of virulence mechanisms and antifungal drug efficacy in Candida species. J Infect Dis **193:** 1014-1022.

44. CHANG, E. C., B. F. CRAWFORD, Z. HONG, T. BILINSKI and D. J. KOSMAN, 1991 Genetic and biochemical characterization of Cu,Zn superoxide dismutase mutants in Saccharomyces cerevisiae. J Biol Chem **266:** 4417-4424.

45. CHAO, C. C., P. C. HSU, C. F. JEN, I. H. CHEN, C. H. WANG *et al.*, 2010 Zebrafish as a model host for Candida albicans infection. Infect Immun **78:** 2512-2521.

46. CHAVEZ, V., A. MOHRI-SHIOMI and D. A. GARSIN, 2009 Ce-Duox1/BLI-3 generates reactive oxygen species as a protective innate immune mechanism in Caenorhabditis elegans. Infect Immun **77:** 4983-4989.

47. CHAVEZ, V., A. MOHRI-SHIOMI, A. MAADANI, L. A. VEGA and D. A. GARSIN, 2007 Oxidative Stress Enzymes are Required for DAF-16 Mediated Immunity due to Generation of Reactive Oxygen Species by C. elegans. Genetics.

48. CHEETHAM, J., D. A. SMITH, A. DA SILVA DANTAS, K. S. DORIS, M. J. PATTERSON *et al.*, 2007 A single MAPKKK regulates the Hog1 MAPK pathway in the pathogenic fungus Candida albicans. Mol Biol Cell **18:** 4603-4614.

49. CHEN, K. H., T. MIYAZAKI, H. F. TSAI and J. E. BENNETT, 2007 The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in Candida glabrata. Gene **386:** 63-72.

50. COLEMAN, S. T., E. A. EPPING, S. M. STEGGERDA and W. S. MOYE-ROWLEY, 1999 Yap1p activates gene transcription in an oxidant-specific fashion. Mol Cell Biol **19:** 8302-8313.

51. CONSORTIUM, C. E. S., 1998 Genome sequence of the nematode C. elegans: a platform for investigating biology. Science **282:** 2012-2018.

52. COUCH, B. C., I. FUDAL, M. H. LEBRUN, D. THARREAU, B. VALENT *et al.*, 2005 Origins of host-specific populations of the blast pathogen Magnaporthe oryzae in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. Genetics **170:** 613-630.

53. COUILLAULT, C., N. PUJOL, J. REBOUL, L. SABATIER, J. F. GUICHOU *et al.*, 2004 TLR-independent control of innate immunity in Caenorhabditis elegans by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. Nat Immunol **5:** 488-494.

54. CROSS, A. R., and A. W. SEGAL, 2004 The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems. Biochim Biophys Acta **1657:** 1-22.

55. CSANK, C., K. SCHRÖPPEL, E. LEBERER, D. HARCUS, O. MOHAMED *et al.*, 1998 Roles of the Candida albicans mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect Immun **66:** 2713-2721.

56. DALLE, F., B. WÄCHTLER, C. L'OLLIVIER, G. HOLLAND, N. BANNERT *et al.*, 2010 Cellular interactions of Candida albicans with human oral epithelial cells and enterocytes. Cell Microbiol **12:** 248-271.

57. DARBY, C., C. L. COSMA, J. H. THOMAS and C. MANOIL, 1999 Lethal paralysis of Caenorhabditis elegans by Pseudomonas aeruginosa. Proc Natl Acad Sci U S A **96:** 15202-15207.

58. DAROUICHE, R. O., 1998 Oropharyngeal and esophageal candidiasis in immunocompromised patients: treatment issues. Clin Infect Dis **26:** 259-272; quiz 273-254.

59. DAROUICHE, R. O., M. D. MANSOURI and E. M. KOJIC, 2006 Antifungal activity of antimicrobial-impregnated devices. Clin Microbiol Infect **12:** 397-399.

60. DE BERNARDIS, F., F. A. MÜHLSCHLEGEL, A. CASSONE and W. A. FONZI, 1998 The pH of the host niche controls gene expression in and virulence of Candida albicans. Infect Immun **66:** 3317-3325.

61. DE PRIJCK, K., N. DE SMET, K. HONRAET, S. CHRISTIAEN, T. COENYE *et al.*, 2010 Inhibition of Candida albicans biofilm formation by antimycotics released from modified polydimethyl siloxane. Mycopathologia **169:** 167-174.

62. DECOTTIGNIES, A., A. M. GRANT, J. W. NICHOLS, H. DE WET, D. B. MCINTOSH *et al.*, 1998 ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem **273:** 12612-12622.

63. DESBOIS, A. P., and P. J. COOTE, 2011 Wax moth larva (Galleria mellonella): an in vivo model for assessing the efficacy of antistaphylococcal agents. J Antimicrob Chemother **66:** 1785-1790.

64. DIEZ-OREJAS, R., G. MOLERO, F. NAVARRO-GARCÍA, J. PLA, C. NOMBELA *et al.*, 1997 Reduced virulence of Candida albicans MKC1 mutants: a role for mitogen-activated protein kinase in pathogenesis. Infect Immun **65:** 833-837.

65. DOMÍNGUEZ, J. M., M. G. GÓMEZ-LORENZO and J. J. MARTÍN, 1999 Sordarin inhibits fungal protein synthesis by blocking translocation differently to fusidic acid. J Biol Chem **274:** 22423-22427.

66. DONG, C., R. J. DAVIS and R. A. FLAVELL, 2002 MAP kinases in the immune response. Annu Rev Immunol **20:** 55-72.

67. DUPONT, P. F., 1995 Candida albicans, the opportunist. A cellular and molecular perspective. J Am Podiatr Med Assoc **85:** 104-115.

68. EDENS, W. A., L. SHARLING, G. CHENG, R. SHAPIRA, J. M. KINKADE *et al.*, 2001 Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. J Cell Biol **154:** 879-891.

69. EDMOND, M. B., S. E. WALLACE, D. K. MCCLISH, M. A. PFALLER, R. N. JONES *et al.*, 1999 Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis **29:** 239-244.

70. EL HASSANI, R. A., N. BENFARES, B. CAILLOU, M. TALBOT, J. C. SABOURIN *et al.*, 2005 Dual oxidase2 is expressed all along the digestive tract. Am J Physiol Gastrointest Liver Physiol **288:** G933-942.

71. ENJALBERT, B., D. A. SMITH, M. J. CORNELL, I. ALAM, S. NICHOLLS *et al.*, 2006 Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen Candida albicans. Mol Biol Cell **17:** 1018-1032.

72. FERNANDES, L., C. RODRIGUES-POUSADA and K. STRUHL, 1997 Yap, a novel family of eight bZIP proteins in Saccharomyces cerevisiae with distinct biological functions. Mol Cell Biol **17:** 6982-6993.

73. FINKEL, J. S., and A. P. MITCHELL, 2011 Genetic control of Candida albicans biofilm development. Nat Rev Microbiol **9:** 109-118.

74. FITZPATRICK, D. A., M. E. LOGUE, J. E. STAJICH and G. BUTLER, 2006 A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. BMC Evol Biol **6:** 99.

75. FONZI, W. A., and M. Y. IRWIN, 1993 Isogenic strain construction and gene mapping in Candida albicans. Genetics **134:** 717-728.

76. FRADIN, C., P. DE GROOT, D. MACCALLUM, M. SCHALLER, F. KLIS *et al.*, 2005 Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. Mol Microbiol **56:** 397-415.

77. FRIDKIN, S. K., and W. R. JARVIS, 1996 Epidemiology of nosocomial fungal infections. Clin Microbiol Rev **9:** 499-511.

78. FROHNER, I. E., C. BOURGEOIS, K. YATSYK, O. MAJER and K. KUCHLER, 2009 Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. Mol Microbiol **71:** 240-252.

79. GALE, C., D. FINKEL, N. TAO, M. MEINKE, M. MCCLELLAN *et al.*, 1996 Cloning and expression of a gene encoding an integrin-like protein in Candida albicans. Proc Natl Acad Sci U S A **93:** 357-361.

80. GALE, C. A., C. M. BENDEL, M. MCCLELLAN, M. HAUSER, J. M. BECKER *et al.*, 1998 Linkage of adhesion, filamentous growth, and virulence in Candida albicans to a single gene, INT1. Science **279:** 1355-1358.

81. GAUR, N. K., and S. A. KLOTZ, 1997 Expression, cloning, and characterization of a Candida albicans gene, ALA1, that confers adherence properties upon Saccharomyces cerevisiae for extracellular matrix proteins. Infect Immun **65:** 5289-5294.

82. GAUR, N. K., S. A. KLOTZ and R. L. HENDERSON, 1999 Overexpression of the Candida albicans ALA1 gene in Saccharomyces cerevisiae results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of Candida albicans. Infect Immun **67:** 6040-6047.

83. GAUSS, K. A., L. K. NELSON-OVERTON, D. W. SIEMSEN, Y. GAO, F. R. DELEO *et al.*, 2007a Role of NF-kappaB in transcriptional regulation of the phagocyte NADPH oxidase by tumor necrosis factor-alpha. J Leukoc Biol **82:** 729-741.

84. GAUSS, K. A., L. K. NELSON-OVERTON, D. W. SIEMSEN, Y. GAO, F. R. DELEO *et al.*, 2007b Role of NF-kappaB in transcriptional regulation of the phagocyte NADPH oxidase by tumor necrosis factor-alpha. J Leukoc Biol **82:** 729-741.

85. GELBART, W. M., M. CROSBY, B. MATTHEWS, W. P. RINDONE, J. CHILLEMI *et al.*, 1997 FlyBase: a Drosophila database. The FlyBase consortium. Nucleic Acids Res **25:** 63-66.

86. GERSTBREIN, B., G. STAMATAS, N. KOLLIAS and M. DRISCOLL, 2005 In vivo spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in Caenorhabditis elegans. Aging Cell **4:** 127-137.

87. GHANNOUM, M. A., and L. B. RICE, 1999 Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev **12:** 501-517.

88. GOLDSTEIN, A. L., and J. H. MCCUSKER, 2001 Development of Saccharomyces cerevisiae as a model pathogen. A system for the genetic identification of gene products required for survival in the mammalian host environment. Genetics **159:** 499-513.

89. GOW, N. A., F. L. VAN DE VEERDONK, A. J. BROWN and M. G. NETEA, 2012 Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol **10:** 112-122.

90. GRANT, C. M., L. P. COLLINSON, J. H. ROE and I. W. DAWES, 1996 Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. Mol Microbiol **21:** 171-179.

91. GRAVATO-NOBRE, M. J., H. R. NICHOLAS, R. NIJLAND, D. O'ROURKE, D. E. WHITTINGTON *et al.*, 2005 Multiple genes affect sensitivity of Caenorhabditis elegans to the bacterial pathogen Microbacterium nematophilum. Genetics **171:** 1033-1045.

92. GUDLAUGSSON, O., S. GILLESPIE, K. LEE, J. VANDE BERG, J. HU *et al.*, 2003 Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis **37:** 1172-1177.

93. GUO, M., Y. CHEN, Y. DU, Y. DONG, W. GUO *et al.*, 2011 The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus Magnaporthe oryzae. PLoS Pathog **7:** e1001302.

94. HA, E. M., C. T. OH, Y. S. BAE and W. J. LEE, 2005a A direct role for dual oxidase in Drosophila gut immunity. Science **310:** 847-850.

95. HA, E. M., C. T. OH, J. H. RYU, Y. S. BAE, S. W. KANG *et al.*, 2005b An antioxidant system required for host protection against gut infection in Drosophila. Dev Cell **8:** 125-132.

96. HANCOCK, J. T., and O. T. JONES, 1987 The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. Biochem J **242:** 103-107.

97. HARRIS, T. W., I. ANTOSHECHKIN, T. BIERI, D. BLASIAR, J. CHAN *et al.*, 2010 WormBase: a comprehensive resource for nematode research. Nucleic Acids Res **38:** D463-467.

98. HETRICK, E. M., and M. H. SCHOENFISCH, 2006 Reducing implant-related infections: active release strategies. Chem Soc Rev **35:** 780-789.

99. HIKKEL, I., A. LUCAU-DANILA, T. DELAVEAU, P. MARC, F. DEVAUX *et al.*, 2003 A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. J Biol Chem **278:** 11427-11432.

100. HITCHCOCK, C. A., K. DICKINSON, S. B. BROWN, E. G. EVANS and D. J. ADAMS, 1990 Interaction of azole antifungal antibiotics with cytochrome P-450-

dependent 14 alpha-sterol demethylase purified from Candida albicans. Biochem J **266:** 475-480.

101. HODGKIN, J., P. E. KUWABARA and B. CORNELIUSSEN, 2000 A novel bacterial pathogen, Microbacterium nematophilum, induces morphological change in the nematode C. elegans. Curr Biol **10:** 1615-1618.

102. HORN, D. L., D. NEOFYTOS, E. J. ANAISSIE, J. A. FISHMAN, W. J. STEINBACH *et al.*, 2009 Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin Infect Dis **48:** 1695-1703.

103. HOYER, L. L., 2001 The ALS gene family of Candida albicans. Trends Microbiol **9:** 176-180.

104. HUANG, G., H. WANG, S. CHOU, X. NIE, J. CHEN *et al.*, 2006 Bistable expression of WOR1, a master regulator of white-opaque switching in Candida albicans. Proc Natl Acad Sci U S A **103:** 12813-12818.

105. HUBE, B., M. MONOD, D. A. SCHOFIELD, A. J. BROWN and N. A. GOW, 1994 Expression of seven members of the gene family encoding secretory aspartyl proteinases in Candida albicans. Mol Microbiol **14:** 87-99.

106. IWAGUCHI, S., M. HOMMA and K. TANAKA, 1992 Clonal variation of chromosome size derived from the rDNA cluster region in Candida albicans. J Gen Microbiol **138:** 1177-1184.

107. JACKSON, J. C., L. A. HIGGINS and X. LIN, 2009 Conidiation color mutants of Aspergillus fumigatus are highly pathogenic to the heterologous insect host Galleria mellonella. PLoS One **4:** e4224.

108. JAIN, C., M. YUN, S. M. POLITZ and R. P. RAO, 2009 A pathogenesis assay using Saccharomyces cerevisiae and Caenorhabditis elegans reveals novel roles for yeast AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis. Eukaryot Cell **8:** 1218-1227.

109. JANDER, G., L. G. RAHME and F. M. AUSUBEL, 2000 Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. J Bacteriol **182:** 3843-3845.

110. JANSEN, W. T., M. BOLM, R. BALLING, G. S. CHHATWAL and R. SCHNABEL, 2002 Hydrogen peroxide-mediated killing of Caenorhabditis elegans by Streptococcus pyogenes. Infect Immun **70:** 5202-5207.

111. JANSSON, H. B., 1994 Adhesion of Conidia of Drechmeria coniospora to Caenorhabditis elegans Wild Type and Mutants. J Nematol **26:** 430-435.

112. KAHANA, A., and D. E. GOTTSCHLING, 1999 DOT4 links silencing and cell growth in Saccharomyces cerevisiae. Mol Cell Biol **19:** 6608-6620.

113. KAPTEYN, J. C., L. L. HOYER, J. E. HECHT, W. H. MÜLLER, A. ANDEL *et al.*, 2000 The cell wall architecture of Candida albicans wild-type cells and cell wall-defective mutants. Mol Microbiol **35:** 601-611.

114. KATZUNG, B. G., S. B. MASTERS and A. J. TREVOR, 2009 *Basic and Clinical Pharmacology*. McGraw-Hill Medical.

115. KAWAHARA, T., Y. KUWANO, S. TESHIMA-KONDO, R. TAKEYA, H. SUMIMOTO *et al.*, 2004 Role of nicotinamide adenine dinucleotide phosphate oxidase 1 in oxidative burst response to Toll-like receptor 5 signaling in large intestinal epithelial cells. J Immunol **172:** 3051-3058.

116. KELLY, J., R. ROWAN, M. MCCANN and K. KAVANAGH, 2009 Exposure to caspofungin activates Cap and Hog pathways in Candida albicans. Med Mycol **47:** 697-706.

117. KENT, H. L., 1991 Epidemiology of vaginitis. Am J Obstet Gynecol **165:** 1168-1176.

118. KIM, D. H., R. FEINBAUM, G. ALLOING, F. E. EMERSON, D. A. GARSIN *et al.*, 2002 A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity. Science **297:** 623-626.

119. KLAR, A. J., T. SRIKANTHA and D. R. SOLL, 2001 A histone deacetylation inhibitor and mutant promote colony-type switching of the human pathogen Candida albicans. Genetics **158:** 919-924.

120. KOH, A. Y., J. R. KÖHLER, K. T. COGGSHALL, N. VAN ROOIJEN and G. B. PIER, 2008 Mucosal damage and neutropenia are required for Candida albicans dissemination. PLoS Pathog **4:** e35.

121. KUGE, S., and N. JONES, 1994 YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. EMBO J **13:** 655-664.

122. KUGE, S., N. JONES and A. NOMOTO, 1997 Regulation of yAP-1 nuclear localization in response to oxidative stress. EMBO J **16:** 1710-1720.

123. KUHN, D. M., P. K. MIKHERJEE, T. A. CLARK, C. PUJOL, J. CHANDRA *et al.*, 2004 Candida parapsilosis characterization in an outbreak setting. Emerg Infect Dis **10:** 1074-1081.

124. KUMAMOTO, C. A., 2005 A contact-activated kinase signals Candida albicans invasive growth and biofilm development. Proc Natl Acad Sci U S A **102:** 5576-5581.

125. KURZ, C. L., S. CHAUVET, E. ANDRÈS, M. AUROUZE, I. VALLET *et al.*, 2003 Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. EMBO J **22:** 1451-1460.

126. KURZ, C. L., and J. J. EWBANK, 2000 Caenorhabditis elegans for the study of host-pathogen interactions. Trends Microbiol **8:** 142-144.

127. KÄMPER, J., R. KAHMANN, M. BÖLKER, L. J. MA, T. BREFORT *et al.*, 2006 Insights from the genome of the biotrophic fungal plant pathogen Ustilago maydis. Nature **444:** 97-101.

128. LABROUSSE, A., S. CHAUVET, C. COUILLAULT, C. L. KURZ and J. J. EWBANK, 2000 Caenorhabditis elegans is a model host for Salmonella typhimurium. Curr Biol **10:** 1543-1545.

129. LAFFEY, S. F., and G. BUTLER, 2005 Phenotype switching affects biofilm formation by Candida parapsilosis. Microbiology **151:** 1073-1081.

130. LAMBETH, J. D., G. CHENG, R. S. ARNOLD and W. A. EDENS, 2000 Novel homologs of gp91phox. Trends Biochem Sci **25:** 459-461.

131. LANE, S., C. BIRSE, S. ZHOU, R. MATSON and H. LIU, 2001 DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in Candida albicans. J Biol Chem **276:** 48988-48996.

132. LAY, J., L. K. HENRY, J. CLIFFORD, Y. KOLTIN, C. E. BULAWA *et al.*, 1998 Altered expression of selectable marker URA3 in gene-disrupted Candida

albicans strains complicates interpretation of virulence studies. Infect Immun **66:** 5301-5306.

133. LECLERC, V., and J. M. REICHHART, 2004 The immune response of Drosophila melanogaster. Immunol Rev **198:** 59-71.

134. LEGRAND, M., P. LEPHART, A. FORCHE, F. M. MUELLER, T. WALSH *et al.*, 2004 Homozygosity at the MTL locus in clinical strains of Candida albicans: karyotypic rearrangements and tetraploid formation. Mol Microbiol **52:** 1451-1462.

135. LEIDICH, S. D., A. S. IBRAHIM, Y. FU, A. KOUL, C. JESSUP *et al.*, 1998 Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of Candida albicans. J Biol Chem **273:** 26078-26086.

136. LEMAITRE, B., E. NICOLAS, L. MICHAUT, J. M. REICHHART and J. A. HOFFMANN, 1996 The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86:** 973-983.

137. LESSING, F., O. KNIEMEYER, I. WOZNIOK, J. LOEFFLER, O. KURZAI *et al.*, 2007 The Aspergillus fumigatus transcriptional regulator AfYap1 represents the major regulator for defense against reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. Eukaryot Cell **6:** 2290-2302.

138. LETO, T. L., and M. GEISZT, 2006 Role of Nox family NADPH oxidases in host defense. Antioxid Redox Signal **8:** 1549-1561.

139. LEV, S., R. HADAR, P. AMEDEO, S. E. BAKER, O. C. YODER *et al.*, 2005a Activation of an AP1-Like Transcription Factor of the Maize Pathogen

Cochliobolus heterostrophus in Response to Oxidative Stress and Plant Signals. Eukaryot Cell **4:** 443-454.

140. LEV, S., R. HADAR, P. AMEDEO, S. E. BAKER, O. C. YODER *et al.*, 2005b Activation of an AP1-like transcription factor of the maize pathogen Cochliobolus heterostrophus in response to oxidative stress and plant signals. Eukaryot Cell **4:** 443-454.

141. LEV, S., A. SHARON, R. HADAR, H. MA and B. A. HORWITZ, 1999 A mitogen-activated protein kinase of the corn leaf pathogen Cochliobolus heterostrophus is involved in conidiation, appressorium formation, and pathogenicity: diverse roles for mitogen-activated protein kinase homologs in foliar pathogens. Proc Natl Acad Sci U S A **96:** 13542-13547.

142. LI, Z. S., Y. P. LU, R. G. ZHEN, M. SZCZYPKA, D. J. THIELE *et al.*, 1997 A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci U S A **94:** 42-47.

143. LIN, C. H., S. L. YANG and K. R. CHUNG, 2009 The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus Alternaria alternata in citrus. Mol Plant Microbe Interact **22:** 942-952.

144. LIU, H., 2001 Transcriptional control of dimorphism in Candida albicans. Curr Opin Microbiol **4:** 728-735.

145. LIU, Y., and S. G. FILLER, 2011 Candida albicans Als3, a multifunctional adhesin and invasin. Eukaryot Cell **10:** 168-173.

146. LO, H. J., J. R. KÖHLER, B. DIDOMENICO, D. LOEBENBERG, A. CACCIAPUOTI *et al.*, 1997 Nonfilamentous C. albicans mutants are avirulent. Cell **90:** 939-949.

147. LONGO, V. D., L. L. LIOU, J. S. VALENTINE and E. B. GRALLA, 1999 Mitochondrial superoxide decreases yeast survival in stationary phase. Arch Biochem Biophys **365:** 131-142.

148. LOPES DA ROSA, J., V. L. BOYARTCHUK, L. J. ZHU and P. D. KAUFMAN, 2010 Histone acetyltransferase Rtt109 is required for Candida albicans pathogenesis. Proc Natl Acad Sci U S A **107:** 1594-1599.

149. LORENZ, M. C., J. A. BENDER and G. R. FINK, 2004 Transcriptional response of Candida albicans upon internalization by macrophages. Eukaryot Cell **3:** 1076-1087.

150. MACCALLUM, D. M., 2012 Hosting infection: experimental models to assay Candida virulence. Int J Microbiol **2012:** 363764.

151. MAGEE, P. T., and B. B. MAGEE, 2004 Through a glass opaquely: the biological significance of mating in Candida albicans. Curr Opin Microbiol **7:** 661-665.

152. MAHAJAN-MIKLOS, S., M. W. TAN, L. G. RAHME and F. M. AUSUBEL, 1999 Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model. Cell **96:** 47-56.

153. MAICAS, S., I. MORENO, A. NIETO, M. GÓMEZ, R. SENTANDREU *et al.*, 2005 In silico analysis for transcription factors with Zn(II)(2)C(6) binuclear cluster DNA-binding domains in Candida albicans. Comp Funct Genomics **6:** 345-356.

154. MALLO, G. V., C. L. KURZ, C. COUILLAULT, N. PUJOL, S. GRANJEAUD *et al.*, 2002 Inducible antibacterial defense system in C. elegans. Curr Biol **12:** 1209-1214.

155. MARTCHENKO, M., A. M. ALARCO, D. HARCUS and M. WHITEWAY, 2004 Superoxide dismutases in Candida albicans: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. Mol Biol Cell **15:** 456-467.

156. MARTÍNEZ, P., and P. O. LJUNGDAHL, 2005 Divergence of Stp1 and Stp2 transcription factors in Candida albicans places virulence factors required for proper nutrient acquisition under amino acid control. Mol Cell Biol **25:** 9435-9446.

157. MCCLUSKEY, K., A. WIEST and M. PLAMANN, 2010 The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. J Biosci **35:** 119-126.

158. MCCUSKER, J. H., 2006 *Saccharomyces cerevisiae: an emerging and model pathogenic fungus.* ASM Press, Washington, DC.

159. MCCUSKER, J. H., K. V. CLEMONS, D. A. STEVENS and R. W. DAVIS, 1994 Genetic characterization of pathogenic Saccharomyces cerevisiae isolates. Genetics **136:** 1261-1269.

160. MILLER, L. G., R. A. HAJJEH and J. E. EDWARDS, 2001 Estimating the cost of nosocomial candidemia in the united states. Clin Infect Dis **32:** 1110.

161. MITCHELL, A. P., 1998 Dimorphism and virulence in Candida albicans. Curr Opin Microbiol **1:** 687-692.

162. MOGAVERO, S., A. TAVANTI, S. SENESI, P. D. ROGERS and J. MORSCHHÄUSER, 2011 Differential requirement of the transcription factor Mcm1 for activation of the Candida albicans multidrug efflux pump MDR1 by its regulators Mrr1 and Cap1. Antimicrob Agents Chemother **55:** 2061-2066.

163. MOLINA, L., and R. KAHMANN, 2007 An Ustilago maydis gene involved in H2O2 detoxification is required for virulence. Plant Cell **19:** 2293-2309.

164. MORAN, G. P., D. J. SULLIVAN, M. C. HENMAN, C. E. MCCREARY, B. J. HARRINGTON *et al.*, 1997 Antifungal drug susceptibilities of oral Candida dubliniensis isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob Agents Chemother **41:** 617-623.

165. MORGAN, J., M. I. MELTZER, B. D. PLIKAYTIS, A. N. SOFAIR, S. HUIE-WHITE *et al.*, 2005 Excess mortality, hospital stay, and cost due to candidemia: a case-control study using data from population-based candidemia surveillance. Infect Control Hosp Epidemiol **26:** 540-547.

166. MORRIS, M. I., and M. VILLMANN, 2006 Echinocandins in the management of invasive fungal infections, Part 2. Am J Health Syst Pharm **63:** 1813-1820.

167. MORROW, B., T. SRIKANTHA, J. ANDERSON and D. R. SOLL, 1993 Coordinate regulation of two opaque-phase-specific genes during white-opaque switching in Candida albicans. Infect Immun **61:** 1823-1828.

168. MOY, T. I., E. MYLONAKIS, S. B. CALDERWOOD and F. M. AUSUBEL, 2004 Cytotoxicity of hydrogen peroxide produced by Enterococcus faecium. Infect Immun **72:** 4512-4520.

169. MUNDY, R. D., and B. CORMACK, 2009 Expression of Candida glabrata adhesins after exposure to chemical preservatives. J Infect Dis **199:** 1891-1898.

170. MURAD, A. M., P. R. LEE, I. D. BROADBENT, C. J. BARELLE and A. J. BROWN, 2000 CIp10, an efficient and convenient integrating vector for Candida albicans. Yeast **16:** 325-327.

171. MYLONAKIS, E., and A. ABALLAY, 2005 Worms and flies as genetically tractable animal models to study host-pathogen interactions. Infect Immun **73:** 3833-3841.

172. MYLONAKIS, E., F. M. AUSUBEL, J. R. PERFECT, J. HEITMAN and S. B. CALDERWOOD, 2002a Killing of Caenorhabditis elegans by Cryptococcus neoformans as a model of yeast pathogenesis. Proc Natl Acad Sci U S A **99:** 15675-15680.

173. MYLONAKIS, E., F. M. AUSUBEL, J. R. PERFECT, J. HEITMAN and S. B. CALDERWOOD, 2002b Killing of Caenorhabditis elegans by Cryptococcus neoformans as a model of yeast pathogenesis. Proc Natl Acad Sci U S A **99:** 15675-15680.

174. MYLONAKIS, E., A. IDNURM, R. MORENO, J. EL KHOURY, J. B. ROTTMAN *et al.*, 2004 Cryptococcus neoformans Kin1 protein kinase homologue, identified through a Caenorhabditis elegans screen, promotes virulence in mammals. Mol Microbiol **54:** 407-419.

175. MYLONAKIS, E., R. MORENO, J. B. EL KHOURY, A. IDNURM, J. HEITMAN *et al.*, 2005 Galleria mellonella as a model system to study Cryptococcus neoformans pathogenesis. Infect Immun **73:** 3842-3850.

176. NAGLIK, J. R., C. A. RODGERS, P. J. SHIRLAW, J. L. DOBBIE, L. L. FERNANDES-NAGLIK *et al.*, 2003 Differential expression of Candida albicans secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. J Infect Dis **188:** 469-479.

177. NAKAGAWA, Y., T. KANBE and I. MIZUGUCHI, 2003 Disruption of the human pathogenic yeast Candida albicans catalase gene decreases survival in mouse-model infection and elevates susceptibility to higher temperature and to detergents. Microbiol Immunol **47:** 395-403.

178. NAVARRO-GARCÍA, F., M. SÁNCHEZ, J. PLA and C. NOMBELA, 1995 Functional characterization of the MKC1 gene of Candida albicans, which encodes a mitogen-activated protein kinase homolog related to cell integrity. Mol Cell Biol **15:** 2197-2206.

179. NEELY, M. N., J. D. PFEIFER and M. CAPARON, 2002 Streptococcus-zebrafish model of bacterial pathogenesis. Infect Immun **70:** 3904-3914.

180. NETEA, M. G., G. D. BROWN, B. J. KULLBERG and N. A. GOW, 2008 An integrated model of the recognition of Candida albicans by the innate immune system. Nat Rev Microbiol **6:** 67-78.

181. NICHOLAS, H. R., and J. HODGKIN, 2004 The ERK MAP kinase cascade mediates tail swelling and a protective response to rectal infection in C. elegans. Curr Biol **14:** 1256-1261.

182. NOBILE, C. J., and A. P. MITCHELL, 2005 Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr Biol **15:** 1150-1155.

183. NOBILE, C. J., and A. P. MITCHELL, 2009 Large-scale gene disruption using the UAU1 cassette. Methods Mol Biol **499:** 175-194.

184. NOBILE, C. J., H. A. SCHNEIDER, J. E. NETT, D. C. SHEPPARD, S. G. FILLER *et al.*, 2008a Complementary adhesin function in C. albicans biofilm formation. Curr Biol **18:** 1017-1024.

185. NOBILE, C. J., N. SOLIS, C. L. MYERS, A. J. FAY, J. S. DENEAULT *et al.*, 2008b Candida albicans transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell Microbiol **10:** 2180-2196.

186. NOBLE, S. M., S. FRENCH, L. A. KOHN, V. CHEN and A. D. JOHNSON, 2010 Systematic screens of a Candida albicans homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet **42:** 590-598.

187. PAPPAS, P. G., J. H. REX, J. LEE, R. J. HAMILL, R. A. LARSEN *et al.*, 2003 A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. Clin Infect Dis **37:** 634-643.

188. PARK, H., Y. LIU, N. SOLIS, J. SPOTKOV, J. HAMAKER *et al.*, 2009 Transcriptional responses of candida albicans to epithelial and endothelial cells. Eukaryot Cell **8:** 1498-1510.

189. PARK, H., C. L. MYERS, D. C. SHEPPARD, Q. T. PHAN, A. A. SANCHEZ *et al.*, 2005 Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell Microbiol **7:** 499-510.

190. PEEVER, T. L., L. OLSEN, A. IBAÑEZ and L. W. TIMMER, 2000 Genetic Differentiation and Host Specificity Among Populations of Alternaria spp. Causing

Brown Spot of Grapefruit and Tangerine x Grapefruit Hybrids in Florida. Phytopathology **90:** 407-414.

191. PEREA, S., J. L. LÓPEZ-RIBOT, W. R. KIRKPATRICK, R. K. MCATEE, R. A. SANTILLÁN *et al.*, 2001 Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother **45:** 2676-2684.

192. PERSSON, J., and R. E. VANCE, 2007 Genetics-squared: combining host and pathogen genetics in the analysis of innate immunity and bacterial virulence. Immunogenetics **59:** 761-778.

193. PFALLER, M. A., 1996 Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. Clin Infect Dis **22 Suppl 2:** S89-94.

194. PHAN, Q. T., C. L. MYERS, Y. FU, D. C. SHEPPARD, M. R. YEAMAN *et al.*, 2007 Als3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. PLoS Biol **5:** e64.

195. PIGNO, M. A., M. C. GOLDSCHMIDT and J. C. LEMON, 1994 The efficacy of antifungal agents incorporated into a facial prosthetic silicone elastomer. J Prosthet Dent **71:** 295-300.

196. PLOETZ, R. C., 2006 Fusarium Wilt of Banana Is Caused by Several Pathogens Referred to as Fusarium oxysporum f. sp. cubense. Phytopathology **96:** 653-656.

197. PRADEL, E., and J. J. EWBANK, 2004 Genetic models in pathogenesis. Annu Rev Genet **38:** 347-363.

198. PRUSTY, R., P. GRISAFI and G. R. FINK, 2004 The plant hormone indoleacetic acid induces invasive growth in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **101:** 4153-4157.

199. PUKKILA-WORLEY, R., F. M. AUSUBEL and E. MYLONAKIS, Candida albicans Infection of Caenorhabditis elegans Induces Antifungal Immune Defenses. PLoS Pathog **7:** e1002074.

200. PUKKILA-WORLEY, R., A. Y. PELEG, E. TAMPAKAKIS and E. MYLONAKIS, 2009 Candida albicans hyphal formation and virulence assessed using a Caenorhabditis elegans infection model. Eukaryot Cell **8:** 1750-1758.

201. QUINN, M. T., and K. A. GAUSS, 2004 Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. J Leukoc Biol **76:** 760-781.

202. RAMANAN, N., and Y. WANG, 2000 A high-affinity iron permease essential for Candida albicans virulence. Science **288:** 1062-1064.

203. RAMIREZ, M. A., and M. C. LORENZ, 2007 Mutations in alternative carbon utilization pathways in Candida albicans attenuate virulence and confer pleiotropic phenotypes. Eukaryot Cell **6:** 280-290.

204. REUSS, O., A. VIK, R. KOLTER and J. MORSCHHAUSER, 2004 The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene **341:** 119-127.

205. REVERBERI, M., S. ZJALIC, A. RICELLI, F. PUNELLI, E. CAMERA *et al.*, 2008 Modulation of antioxidant defense in Aspergillus parasiticus is involved in aflatoxin biosynthesis: a role for the ApyapA gene. Eukaryot Cell **7:** 988-1000.

206. REYNOLDS, T. B., and G. R. FINK, 2001 Bakers' yeast, a model for fungal biofilm formation. Science **291:** 878-881.

207. RICHARDS, M. J., J. R. EDWARDS, D. H. CULVER and R. P. GAYNES, 2000 Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol **21:** 510-515.

208. RUBIN-BEJERANO, I., I. FRASER, P. GRISAFI and G. R. FINK, 2003 Phagocytosis by neutrophils induces an amino acid deprivation response in Saccharomyces cerevisiae and Candida albicans. Proc Natl Acad Sci U S A **100:** 11007-11012.

209. RUSTCHENKO, E., 2007 Chromosome instability in Candida albicans. FEMS Yeast Res **7:** 2-11.

210. RYDER, N. S., and M. C. DUPONT, 1985 Inhibition of squalene epoxidase by allylamine antimycotic compounds. A comparative study of the fungal and mammalian enzymes. Biochem J **230:** 765-770.

211. SAPORITO-IRWIN, S. M., C. E. BIRSE, P. S. SYPHERD and W. A. FONZI, 1995 PHR1, a pH-regulated gene of Candida albicans, is required for morphogenesis. Mol Cell Biol **15:** 601-613.

212. SAVILLE, S. P., A. L. LAZZELL, C. MONTEAGUDO and J. L. LOPEZ-RIBOT, 2003 Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. Eukaryot Cell **2:** 1053-1060.

213. SCHALLER, M., M. BEIN, H. C. KORTING, S. BAUR, G. HAMM *et al.*, 2003 The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an in vitro

model of vaginal candidiasis based on reconstituted human vaginal epithelium. Infect Immun **71:** 3227-3234.

214. SHERMAN, F., G. R. FINK and J. B. HICKS, 1986 *Laboratory course manual for methods in yeast genetics*. Cold Spring Harbor, NY.

215. SIFRI, C. D., J. BEGUN and F. M. AUSUBEL, 2005 The worm has turned--microbial virulence modeled in Caenorhabditis elegans. Trends Microbiol **13:** 119-127.

216. SIFRI, C. D., E. MYLONAKIS, K. V. SINGH, X. QIN, D. A. GARSIN *et al.*, 2002 Virulence effect of Enterococcus faecalis protease genes and the quorum-sensing locus fsr in Caenorhabditis elegans and mice. Infect Immun **70:** 5647-5650.

217. SIMMER, F., C. MOORMAN, A. M. VAN DER LINDEN, E. KUIJK, P. V. VAN DEN BERGHE *et al.*, 2003 Genome-wide RNAi of C. elegans using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol **1:** E12.

218. SLUTSKY, B., M. STAEBELL, J. ANDERSON, L. RISEN, M. PFALLER *et al.*, 1987 "White-opaque transition": a second high-frequency switching system in Candida albicans. J Bacteriol **169:** 189-197.

219. SOBEL, J. D., 1988 Pathogenesis and epidemiology of vulvovaginal candidiasis. Ann N Y Acad Sci **544:** 547-557.

220. STAAB, J. F., S. D. BRADWAY, P. L. FIDEL and P. SUNDSTROM, 1999 Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. Science **283:** 1535-1538.

221. STEVENSON, J. S., and H. LIU, 2011 Regulation of white and opaque cell-type formation in Candida albicans by Rtt109 and Hst3. Mol Microbiol **81:** 1078-1091.

222. STROSCHEIN-STEVENSON, S. L., E. FOLEY, P. H. O'FARRELL and A. D. JOHNSON, 2006 Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS Biol **4:** e4.

223. SUDBERY, P., N. GOW and J. BERMAN, 2004 The distinct morphogenic states of Candida albicans. Trends Microbiol **12:** 317-324.

224. SULSTON, J. E., and H. R. HORVITZ, 1977 Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol **56:** 110-156.

225. SUNDSTROM, P., J. E. CUTLER and J. F. STAAB, 2002 Reevaluation of the role of HWP1 in systemic candidiasis by use of Candida albicans strains with selectable marker URA3 targeted to the ENO1 locus. Infect Immun **70:** 3281-3283.

226. SÁNCHEZ-MARTÍNEZ, C., and J. PÉREZ-MARTÍN, 2001 Dimorphism in fungal pathogens: Candida albicans and Ustilago maydis--similar inputs, different outputs. Curr Opin Microbiol **4:** 214-221.

227. TAN, M. W., S. MAHAJAN-MIKLOS and F. M. AUSUBEL, 1999a Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci U S A **96:** 715-720.

228. TAN, M. W., L. G. RAHME, J. A. STERNBERG, R. G. TOMPKINS and F. M. AUSUBEL, 1999b Pseudomonas aeruginosa killing of Caenorhabditis elegans

used to identify P. aeruginosa virulence factors. Proc Natl Acad Sci U S A **96:** 2408-2413.

229. THEWES, S., M. KRETSCHMAR, H. PARK, M. SCHALLER, S. G. FILLER *et al.*, 2007 In vivo and ex vivo comparative transcriptional profiling of invasive and non-invasive Candida albicans isolates identifies genes associated with tissue invasion. Mol Microbiol **63:** 1606-1628.

230. TOJO, S., F. NAGANUMA, K. ARAKAWA1 and S. YOKOO1, 2000 Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, Galleria mellonella. J Insect Physiol **46:** 1129-1135.

231. TSUCHIMORI, N., L. L. SHARKEY, W. A. FONZI, S. W. FRENCH, J. E. EDWARDS *et al.*, 2000 Reduced virulence of HWP1-deficient mutants of Candida albicans and their interactions with host cells. Infect Immun **68:** 1997-2002.

232. TZUNG, K. W., R. M. WILLIAMS, S. SCHERER, N. FEDERSPIEL, T. JONES *et al.*, 2001 Genomic evidence for a complete sexual cycle in Candida albicans. Proc Natl Acad Sci U S A **98:** 3249-3253.

233. UMEYAMA, T., A. KANEKO, H. WATANABE, A. HIRAI, Y. UEHARA *et al.*, 2006 Deletion of the CaBIG1 gene reduces beta-1,6-glucan synthesis, filamentation, adhesion, and virulence in Candida albicans. Infect Immun **74:** 2373-2381.

234. URBAN, C., K. SOHN, F. LOTTSPEICH, H. BRUNNER and S. RUPP, 2003 Identification of cell surface determinants in Candida albicans reveals Tsa1p, a protein differentially localized in the cell. FEBS Lett **544:** 228-235.

235. VAN DER SAR, A. M., R. J. MUSTERS, F. J. VAN EEDEN, B. J. APPELMELK, C. M. VANDENBROUCKE-GRAULS *et al.*, 2003 Zebrafish embryos as a model host for the

real time analysis of Salmonella typhimurium infections. Cell Microbiol **5:** 601-611.

236. VAZQUEZ-TORRES, A., and E. BALISH, 1997 Macrophages in resistance to candidiasis. Microbiol Mol Biol Rev **61:** 170-192.

237. VELASCO, E., L. C. THULER, C. A. MARTINS, M. NUCCI, L. M. DIAS *et al.*, 2000 Epidemiology of bloodstream infections at a cancer center. Sao Paulo Med J **118:** 131-138.

238. VILLAR, C. C., H. KASHLEVA, C. J. NOBILE, A. P. MITCHELL and A. DONGARI-BAGTZOGLOU, 2007 Mucosal tissue invasion by Candida albicans is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. Infect Immun **75:** 2126-2135.

239. WACH, A., A. BRACHAT, R. POHLMANN and P. PHILIPPSEN, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:** 1793-1808.

240. WANG, Y., Y. Y. CAO, X. M. JIA, Y. B. CAO, P. H. GAO *et al.*, 2006 Cap1p is involved in multiple pathways of oxidative stress response in Candida albicans. Free Radic Biol Med **40:** 1201-1209.

241. WATERSTON, R. H., K. LINDBLAD-TOH, E. BIRNEY, J. ROGERS, J. F. ABRIL *et al.*, 2002 Initial sequencing and comparative analysis of the mouse genome. Nature **420:** 520-562.

242. WEIG, M., U. GROSS and F. MÜHLSCHLEGEL, 1998 Clinical aspects and pathogenesis of Candida infection. Trends Microbiol **6:** 468-470.

243. WELLINGTON, M., K. DOLAN and D. J. KRYSAN, 2009 Live Candida albicans suppresses production of reactive oxygen species in phagocytes. Infect Immun **77:** 405-413.

244. WEMMIE, J. A., M. S. SZCZYPKA, D. J. THIELE and W. S. MOYE-ROWLEY, 1994 Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. J Biol Chem **269:** 32592-32597.

245. WHEELER, R. T., M. KUPIEC, P. MAGNELLI, C. ABEIJON and G. R. FINK, 2003 A Saccharomyces cerevisiae mutant with increased virulence. Proc Natl Acad Sci U S A **100:** 2766-2770.

246. WHITE, R. M., A. SESSA, C. BURKE, T. BOWMAN, J. LEBLANC *et al.*, 2008 Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell **2:** 183-189.

247. WHITE, T. C., 1997a Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother **41:** 1482-1487.

248. WHITE, T. C., 1997b Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother **41:** 1482-1487.

249. WHITE, T. C., 1997c The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14alpha demethylase in Candida albicans. Antimicrob Agents Chemother **41:** 1488-1494.

250. WISPLINGHOFF, H., T. BISCHOFF, S. M. TALLENT, H. SEIFERT, R. P. WENZEL *et al.*, 2004 Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis **39:** 309-317.

251. WISPLINGHOFF, H., H. SEIFERT, S. M. TALLENT, T. BISCHOFF, R. P. WENZEL *et al.*, 2003 Nosocomial bloodstream infections in pediatric patients in United States hospitals: epidemiology, clinical features and susceptibilities. Pediatr Infect Dis J **22:** 686-691.

252. WOLFGER, H., Y. MAHE, A. PARLE-MCDERMOTT, A. DELAHODDE and K. KUCHLER, 1997 The yeast ATP binding cassette (ABC) protein genes PDR10 and PDR15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators. FEBS Lett **418:** 269-274.

253. WOLFGER, H., Y. M. MAMNUN and K. KUCHLER, 2001 Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Res Microbiol **152:** 375-389.

254. WOLFGER, H., Y. M. MAMNUN and K. KUCHLER, 2004 The yeast Pdr15p ATP-binding cassette (ABC) protein is a general stress response factor implicated in cellular detoxification. J Biol Chem **279:** 11593-11599.

255. WU, A. L., and W. S. MOYE-ROWLEY, 1994 GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. Mol Cell Biol **14:** 5832-5839.

256. WU, P., and D. W. GRAINGER, 2006 Drug/device combinations for local drug therapies and infection prophylaxis. Biomaterials **27:** 2450-2467.

257. WU, Y., M. HAN and K. L. GUAN, 1995 MEK-2, a Caenorhabditis elegans MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. Genes Dev **9:** 742-755.

258. WYSONG, D. R., L. CHRISTIN, A. M. SUGAR, P. W. ROBBINS and R. D. DIAMOND, 1998 Cloning and sequencing of a Candida albicans catalase gene and effects of disruption of this gene. Infect Immun **66:** 1953-1961.

259. ZAKIKHANY, K., J. R. NAGLIK, A. SCHMIDT-WESTHAUSEN, G. HOLLAND, M. SCHALLER *et al.*, 2007 In vivo transcript profiling of Candida albicans identifies a gene essential for interepithelial dissemination. Cell Microbiol **9:** 2938-2954.

260. ZHANG, X., M. DE MICHELI, S. T. COLEMAN, D. SANGLARD and W. S. MOYE-ROWLEY, 2000a Analysis of the oxidative stress regulation of the Candida albicans transcription factor, Cap1p. Mol Microbiol **36:** 618-629.

261. ZHANG, X., M. DE MICHELI, S. T. COLEMAN, D. SANGLARD and W. S. MOYE-ROWLEY, 2000b Analysis of the oxidative stress regulation of the Candida albicans transcription factor, Cap1p. Mol Microbiol **36:** 618-629.

262. ZHAO, R., S. R. LOCKHART, K. DANIELS and D. R. SOLL, 2002 Roles of TUP1 in switching, phase maintenance, and phase-specific gene expression in Candida albicans. Eukaryot Cell **1:** 353-365.

263. ZHAO, X., S. H. OH, K. M. YEATER and L. L. HOYER, 2005 Analysis of the Candida albicans Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. Microbiology **151:** 1619-1630.

264. ZHAO, X. J., G. E. MCELHANEY-FESER, M. J. SHERIDAN, S. E. BROEDEL and R. L. CIHLAR, 1997 Avirulence of Candida albicans FAS2 mutants in a mouse model of systemic candidiasis. Infect Immun **65:** 829-832.

265. ZHU, W., and S. G. FILLER, 2010 Interactions of Candida albicans with epithelial cells. Cell Microbiol **12:** 273-282.

266. ZORDAN, R. E., M. G. MILLER, D. J. GALGOCZY, B. B. TUCH and A. D. JOHNSON, 2007 Interlocking transcriptional feedback loops control white-opaque switching in Candida albicans. PLoS Biol **5:** e256.

267. ZU, Y. L., J. QI, A. GILCHRIST, G. A. FERNANDEZ, D. VAZQUEZ-ABAD *et al.*, 1998 p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. J Immunol **160:** 1982-1989.