LEBANESE AMERICAN UNIVERSITY

Tandem Mass Spectrometric Cell Wall Proteome Profiling of a *Candida albicans hwp2* Mutant Strain and Fluconazole Resistant Strains

By

Andy M. Awad

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology

School of Arts and Sciences

December 2017

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS APPROVAL FORM

Student Name: Andy Awad I.D. #: 200703400

Thesis Title Tandem Mass Spectrometric Cell Wall Proteome Profiling of a *Candida albicans hwp2* Mutant Strain and Fluconazole Resistant Strains

Program: MS Molecular Biology

Department: Natural Sciences

School: Arts and Sciences

The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment of the requirements for the degree of:

in the major of Molecular Biology

Thesis Advisor's Name	Roy Khalil	S[redacted]	DATE Dec 12 2017
Thesis Co-advisor	BRIGITTE WEX	S[redacted]	DATE Dec 12 2017
Committee Members Name	GEORGES KHAZEN	S[redacted]	DATE Dec 12 2017
Committee Member's Name	Rony Khnayzer	S[redacted]	DATE Dec 12 2017

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS COPYRIGHT RELEASE FORM

LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants the Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic formats and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: A[redacted]

Signature [redacted]

Date: De[redacted]

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that:

1. I have read and understood LAU's Plagiarism Policy.
2. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.
3. This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: [redacted]

Signatu

Date: December 6, 2017

ACKNOWLEDGMENT

The success of this thesis would have been impossible without the support of many people.

I would like to thank my advisors Dr. Roy Khalaf and Dr. Brigitte Wex who were always there to offer support and invaluable advice. I would also like to express immense gratitude to Dr. Roy Khalaf for his many contributions to this work.

Furthermore, I want to thank my committee members Dr. Rony Khnayzer and Dr. Georges Khazen who offered guidance and support.

Last but not least, the greatest thank you goes for Ms. Pamela El Khoury, my lab partner, with whom I have shared the ups and down experienced while working on this thesis project.

Tandem Mass Spectrometric Cell Wall Proteome Profiling of a *Candida albicans hwp2* Mutant Strain and Fluconazole Resistant Strains

Andy Awad

ABSTRACT

Candida albicans, often recognized as a harmless opportunistic yeast, is ordinarily present as part of the normal gut flora and detected in the oral cavities as well as the GI tracts of around forty to sixty percent of human adults. However benign *C. albicans* colonization can turn pathogenic and cause a variety of infections ranging from mild to severe in immune-compromised individuals. In addition and due to improper use of antifungal drug therapy, resistance rates to antifungals specifically fluconazole is on the rise. In a pathogen the cell wall and cell surface proteins are major antigenic determinants as they are the primary structure that contacts the host. Cell surface proteins perform a variety of functions that are necessary for virulence such as adhesion, host degradation, resistance to oxidative stress, and drug resistance amongst others. Our lab had previously characterized Hwp2, a *Candida albicans* cell wall adhesin that was shown to play a major role in the cell wall architecture and function since cells lacking Hwp2 were found to be deficient in chitin deposition, filamentation, adhesion and invasive growth, virulence, and resistance to oxidative stress. In this study, we utilized tandem mass spectrometry coupled with a bioinformatics approach to differentially profile the cell wall proteome of a wild type strain compared to an *hwp*2 null mutant to determine key differentially expressed proteins. Many such

proteins identified exclusively in the wild type go a long way in explaining the above mentioned phenotypes. These proteins include known virulence factors such as members of the SAP (secreted aspartyl proteases) family of proteins that include Sap4, Sap5, and Sap10 as well as several lipases involved in host degradation. We also identified members of the PGA Protein family (Pga28, Pga32, Pga41 and Pga50) that function in adhesion, Cht2 a chitinase involved in chitin remodeling, and several proteins that function in promoting filamentation (Phr1, Mts1, Rbr1). In parallel, we applied a similar approach to generate a differential profile of *C. albicans* fluconazole resistant strains and successfully identified proteins such as Sec4, a protein involved in resistance to drugs, as well as Pil1, a protein known to be involved in echinocandin resistance, suggesting a novel mechanism whereby resistance to fluconazole and caspofungin may be linked.

Keywords: *Candida albicans*, Fluconazole, Resistant, Sensitive, Hwp2, MALDI MS/MS, MASCOT.

Table of Contents

LIST OF FIGURES

List of Tables

Chapter 1

Introduction

1.1 Candida albicans: General Information and Statistics

Candida albicans, often recognized as a harmless opportunistic yeast in healthy organisms, was first described about one hundred and fifty years ago (Hoffmann et al. 2013). *C. albicans* is ordinarily present as part of the normal gut flora (Odds, 1988). As such, it is detected in the oral cavities as well as the gastrointestinal (GI) tracts of around forty to sixty percent of human adults if not more (Kerawala et al. 2014).

C. albicans cells share a commensal relation with their healthy hosts (Erdogan et al. 2015). The fungal cells typically colonize humans during infancy (Russell et al. 1973); furthermore, molecular typing indicates their clonal persistence over many years with minimal indication of any strain replacement (Odds et al. 2006). These studies as well as the inability to identify any *C. albicans* environmental reservoir, suggest that the fungus is restrictively adapted to proliferate in mammalian hosts (Perlroth et al. 2007).

However, the benign *C. albicans* colonization can turn pathogenic in patients with a compromised immunity, microbial dysbiosis, or epithelial damage (Perlroth et al. 2007). *C. albicans* can lead to a variety of infections (Pfaller et al. 2007). Such *Candida* infections in humans, which are the result of an overgrowth of the fungus, are

referred to as candidiasis (Martins et al. 2014) and range from superficial to deadly, systemic, disseminated infections (Warnock, 2007).

1.2 Cell Morphology

Organisms in the fungal kingdom exhibit wide morphological plasticity; their size can range anywhere between the micrometer in diameter microsporidia family of pathogens (Didier, 2005) to the honey fungus *Armillaria ostoyae* whose mycelium spreads over 9.6 km^2 (Ferguson et al. 2005). Since *C. albicans* is mainly restricted to mammalian hosts, it would be expected to have few morphological variations, but this is not the case. The fungus is often termed dimorphic because it grows as round yeast as well as filamentous cells. However, it can have many different morphologies (Hickman et al. 2016). So far, nine different cell morphologies have been identified and described (Noble, 2017).The four main types of *C. albicans* morphotypes are the yeast, pseudohyphae, hyphae, and chlamydospore. Round yeast and hyphae have been studied more extensively than chlamydospores and pseudohyphae (Sudbery, 2011).

Standard yeasts, commonly referred to as "white" cells, are morphologically similar to *Saccharomyces cerevisiae* i.e. they display a round or oval shape. Yeasts are considered unicellular organisms because they reproduce by budding; that is, a daughter cell grows as a bud and completely detaches from the mother cell by cytokinesis (Figure 1) (Noble, 2017).

During pseudohyphal growth, buds elongate but do not detach from the parent cell; this produces elongated protrusions that retain constrictions at the septal budding junction. The extent to which the buds may elongate varies greatly; pseudohyphal filaments can be so elongated that they are often mistaken for hyphae (Hornby et al.

2001). As such, cells with pseudohyphae showcase features of both round yeasts and true hyphae which has led to some controversy as to whether to consider them as a standalone cell type or a transitory intermediate between the other two (Carlisle et al. 2009). Having no record of any known *in vitro* culture conditions that induce and sustain stable and pure populations of pseudohyphal cells lends credibility to the latter theory (Martin et al. 2005).

True hyphae, on the other hand, consist of chains of cells with parallel walls and no constrictions at junctions and resemble a segmented hose (Hornby et al. 2001). After cytokinesis, hyphal cells stay attached to each other firmly from end to end, so that future cycles of cell division result in a sparingly branched, filamentous, and multicellular structure called mycelium (Carlisle et al. 2009).

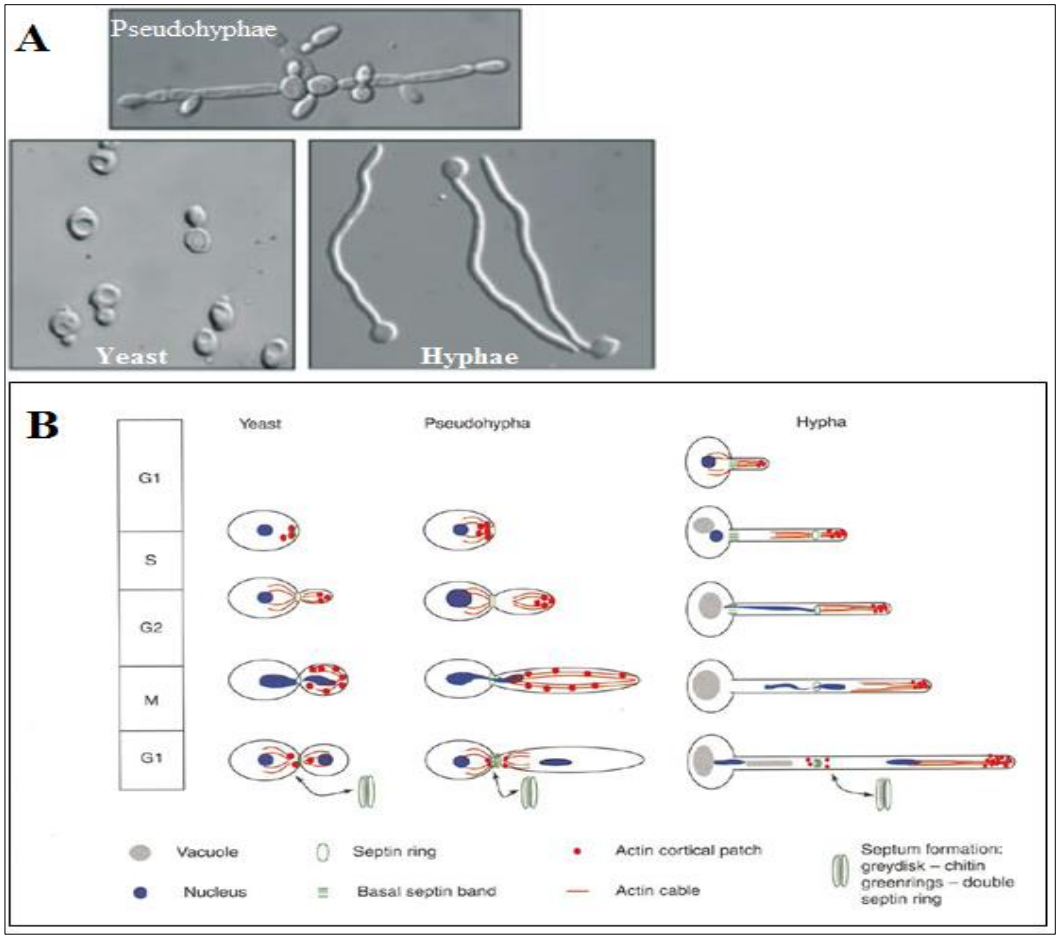


Figure 1 *C. albicans* morphologies: (A) DIC images of cells with Yeast, Hyphae, and Pseudohyphae growth morphologies of *C. albicans*. (B) Diagram detailing the cell cycle of yeast, hyphae, and pseudohyphae from unbudded cells and highlighting the structural differences between them (Sudbery et al. 2004).

The last of the classical cell shapes are the chlamydospores which are produced by suspensor cells at the outer ends of mycelia. Chlamydospores are large, globular, and thick-walled; they are typically observed under strenuous conditions *in vitro* namely starvation and hypoxia (Whiteway et al. 2007). More recently, other minor *C.*

albicans morphotypes have been observed and described: opaque, gastrointestinally induced transition (GUT), and grey.

When observed under a microscope, opaque cells are revealed to be more elongated than the white cells, to take up about three times more space, and to have pronounced vacuoles (Douglas, 2003). Additionally opaque cells include cell surface swellings that have no known biological function (Anderson et al. 1990). Furthermore, opaque cells are relatively resistant to phagocytosis by host phagocytes (Sasse et al. 2013); are more sensitive to specific filamentation causing cues (Si et al. 20013), and have changes in several gene expressions including genes involved in mating (Tuch et al. 2010).

Grey *C. albicans* are smaller than ordinary white or opaque yeasts, lack the cell surface pimples observed in opaque cells, and have a very low mating efficiency. The switch to the grey morphology is prompted by an exposure to nutrient-rich media (Tao et al. 2014).

Briefly, GUT cells are elongated compared to white cells and produce darker and flatter colonies. Intriguingly, experiments have shown that the cells switch to the GUT morphology during stages of high commensal fitness suggesting that this cell type could be specialized for commensalism specifically to the GI tract of mammals (Pande et al. 2013).

1.3 *C. albicans* Genotype and Mating

For a long time, scientists thought of *C. albicans* as a diploid organism with no haploid stage; however, today, it is known that alongside having a haploid stage, the yeast can also be tetraploid. The tetraploid cells form when two diploid *C. albicans* cells mate in their opaque form (Hickman et al. 2016). *C. albicans* cells were never observed to go through sporulation or meiosis and were long thought to be asexual. However, it was later clarified that mating at low frequencies does indeed occur between *C. albicans* "a" and "α" opaque cells (Magee et al. 2000). Most strains have a single copy of one of the two mating-type-like locus alleles (*MTL***a** or *MTL*α) on chromosome 5 (Hull et al. 1999). These cells have the ability to switch to opaque cells that can mate with each other: opaque "a" can mate with opaque "α" producing a minor number of tetraploid cells containing markers from both parent cells. Despite understanding the links between *MTL* genotype, white to opaque reversible switching, and mating, the sex cycle of *C. albicans* remains poorly understood (Hull et al. 2000).

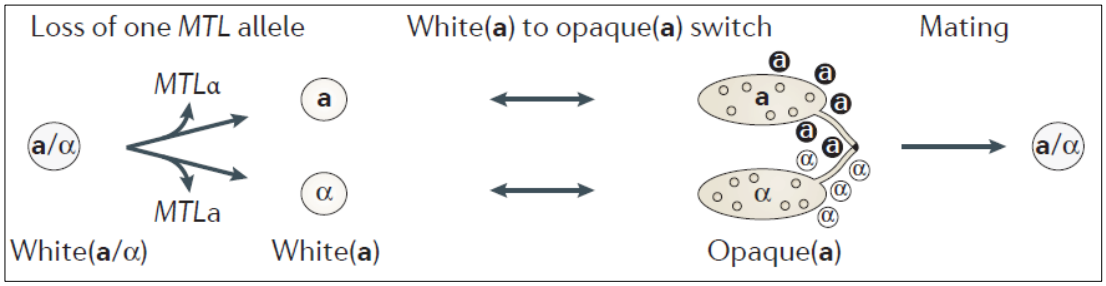


Figure 2. Mating in *C. albicans*. The loss of one MTL allele confers the ability to switch to the opaque mating competent form.

1.4 *C. albicans* pathogenicity

1.4.1 Overview of *C. albicans* infections

Surprisingly and despite advancements in modern day medical treatments, the numbers of patients susceptible to *Candida* infections have increased, and the *C. albicans* fungi are ranked as the third or fourth cause of invasive bloodstream nosocomial infections in the United States of America (Pfaller et al. 2007).

The most common type of candidiasis is referred to as thrush or pseudomembranous candidiasis and is characterized by the white spots covering an area of inflammation. The thrush usually affects the mucosal layers of the vagina, oropharynx, esophagus, and GI tract (Sobel, 1997). Vulvo Vaginal Candidiasis affects up to 75% of all women and may recur more than once in a lifetime (Fidel, 2007). Candidiasis of the oropharynx is specific to patients suffering from AIDS; its mere appearance signals the advent of the disease in HIV^+ individuals (Klein et al., 1984). It also affects patients suffering from oral cancers, using dentures, and failing to produce enough saliva (Runke, 2002).

C. albicans infections that disseminate into the blood stream are referred to as candidemia. In healthy hosts, neutrophils protect against such infections; however, patients suffering from neutropenia caused by cancers or immunosuppressive medication can develop candidemia. Surgeries that may breach the GI tract and spread *C. albicans* cells into the blood as well ICU catheters that result in the formation of fungal biofilm are both common causes of candidemia (Kim et al. 2011). *C. albicans* blood stream infections may spread and infect internal organs leading to disseminated

candidiasis. Both infection types are very serious medical conditions with a mortality rate between 30% and 50% depending on the cause (Kibbler *et al.*, 2003).

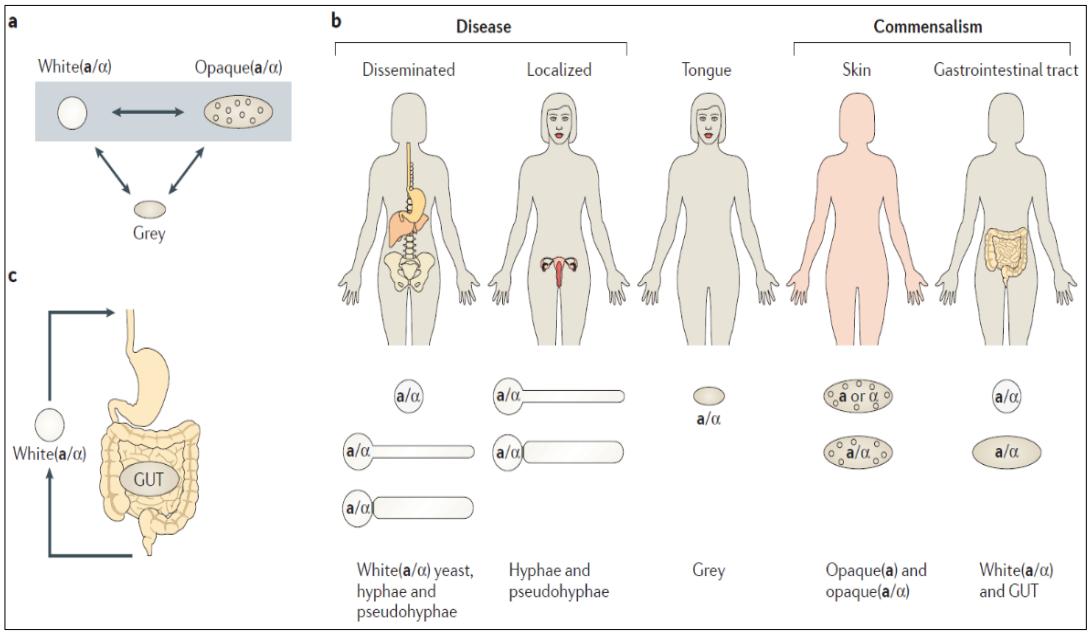


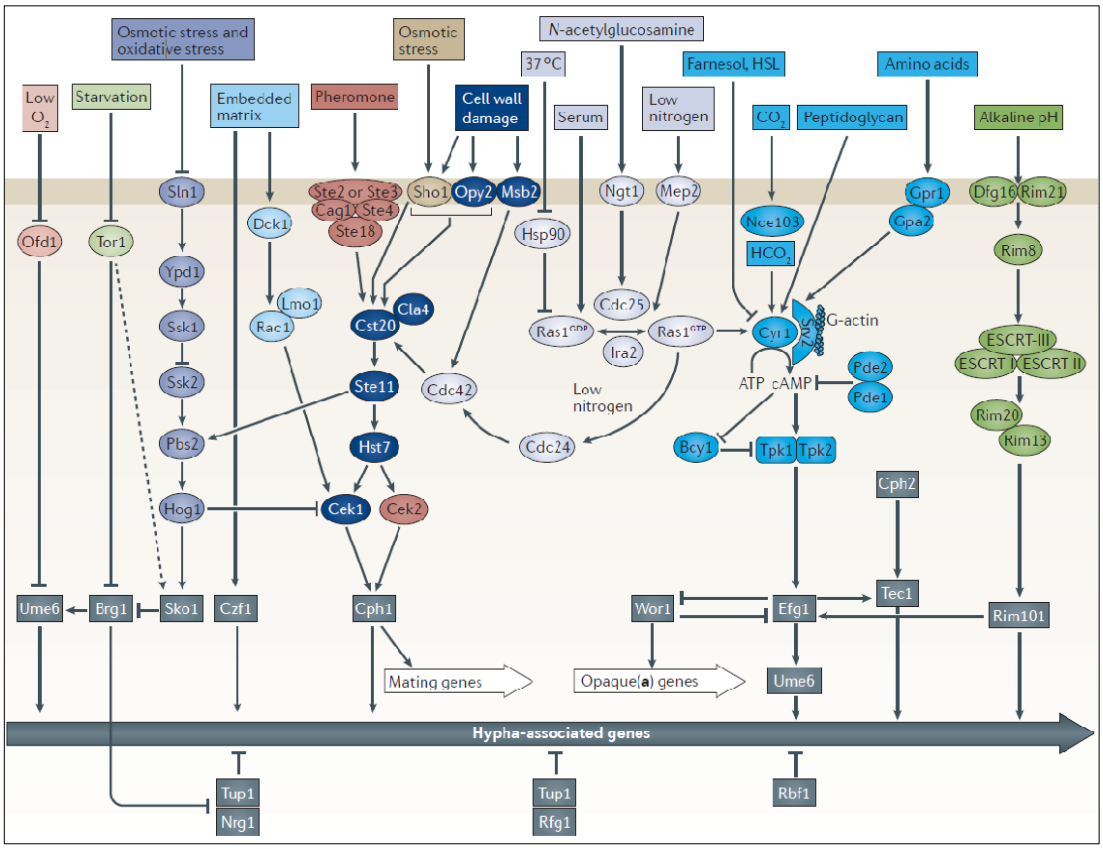
Figure 3. Diagram showing the "fitness" of every cell morphology with candidiasis type and localization. **(a)** Cells with an a/α (MTL) locus can switch between the round to oval white morphology and the elongated opaque morphology that exhibit cell surface pimples. **(b)** Filamentous cell types are typically associated with infection; the disseminated infections require all three cell types. Round yeast forms are usually harmless. **(c)** GUT cells are specifically fit to exist in the GUT, but change back to white the moment they leave the GI environment (Noble et al. 2016)

1.4.2 *C. albicans* virulence factors

The most important virulence factor of *C. albicans* is its ability to grow hyphae and interchange between the three main morphologies: yeast, pseudohyphae, and hyphae (Kim et al. 2011). The round yeast form is needed for rapid clonal expansion while the filamentous hyphal form is necessary for tissue invasion and penetration both of which are essential for successful dissemination (Karkowska-Kuleta, 2009). The transition to hyphae depends on several filamentation inducing environmental cues such as low nitrogen, normal body temperature, exposure to serum, CO_2, and the availability of peptidoglycan and amino acids (Bockmühl et al. 2001).

Furthermore, the hyphal cell wall expresses an arsenal of specific virulence factors that are involved in altering or digesting the host's cell membranes thus facilitating tissue invasion. They also express factors engaged in resisting the reactive oxygen species (Dantas, 2015). These cell wall virulence factors include adhesins such as the hyphal wall protein family of proteins and the agglutinin-like (Als) protein family; several GPI-anchored proteins; dismutases to resist oxidative stress such as Sod5; and tissue-degrading enzymes such as the secreted aspartyl protease (Sap) protein family (Noble et al. 2016). Hyphae display a higher virulence potential compared to the other cell types in models of superficial candidiasis, such as oropharyngeal and vulvovaginal infection (Peters, 2014). For instance, hyphal adhesins like Als3 interact with the host's epithelial E-cadherin inducing hyphal endocytic uptake (Phan et al. 2007). The hyphae then damage the hosts' cells and activate numerous inflammatory pathways. In contrast, round yeast cells that simply

form colonies at the surface of the host tissues without damaging them, trigger a more subdued inflammatory response (Peters, 2014).



Interestingly, in cases of disseminated candidiasis, both the yeast and the virulent hyphae cell types contribute to the disease. Samples recovered from patients with disseminated candidiasis show that all three main cell types, i.e. yeasts, hyphae and pseudohyphae, exist in infected tissues (Chin et al. 2014). Furthermore in some studies, *C. albicans* mutants were genetically altered to be constitutively filamentous or trapped as yeasts, and both proved to be defective in candidemia. This suggests that

switching between the different cell morphologies is necessary for virulence (Brown et al. 2007). Originally, it was hypothesized that yeasts, being round and small, spread through the bloodstream; while, filamentous hyphae were believed to escape the blood vessels and disseminate and damage internal organs. Surprisingly, a recent study that used a strain that can be indefinitely trapped in the yeast or hyphal forms showed that *C. albicans* locked in the yeast form were able to find a way out of blood vessels and penetrate the internal organs just like hyphae. Nevertheless, this strain failed to kill the host organism thus supporting the idea that the yeast-to-hypha shift is vital for virulence in models of disseminated infections (Saville et al. 2003).

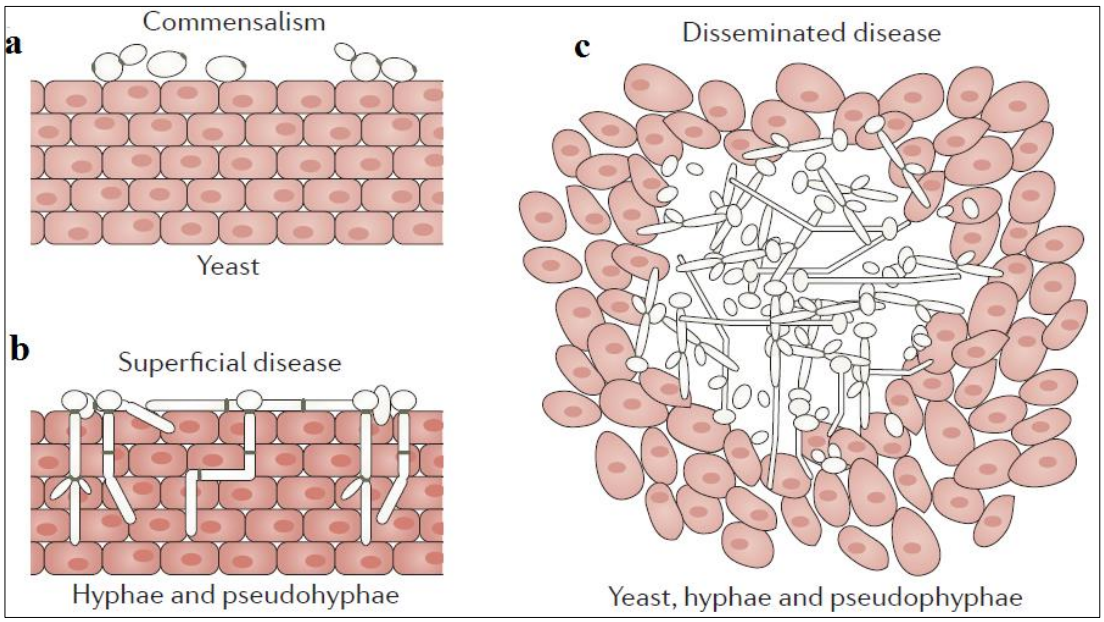


Figure 5. *C. albicans* morphotypes to disease association. **(a)** Yeast cell types are commensal; **(b)** filamentous forms are associated with penetration and tissue damage; and **(c)** all three cell types are engaged in disseminated infections (Noble et al. 2016)

1.4.3 Biofilm formation

Biofilms refer to communities of microbes that form on surfaces such as plastics or inside a host organism. Biofilms associated with certain medical devices are very important clinically due to their high incidence and inherent resistance to drugs and to the human immune system (Soll et al. 2016). *C. albicans* form biofilms readily; first, the "white" yeast cells adhere to a solid surface. Then, they flourish to form micro-colonies. This is followed by the emergence of pseudohyphae and hyphae. An extracellular matrix of proteins, nucleic acids, and polysaccharides ends up constituting the majority of the biofilm alongside the hyphae and the pseudohyphae. Another danger of biofilms is their ability to disperse when a white cell separates from the biofilm and reattaches to the surface at a new location (Daniels et al. 2013). Furthermore, these *C. albicans* cells haploid for the mating type locus have the ability to form sexual biofilms that are different from normal biofilms in several ways: increased permeability and decreased resistance to antifungals as well as to the immune response. It is hypothesized that the prime function of such cell biofilms is to make mating between sexually competent cells easier (Park et al. 2013).

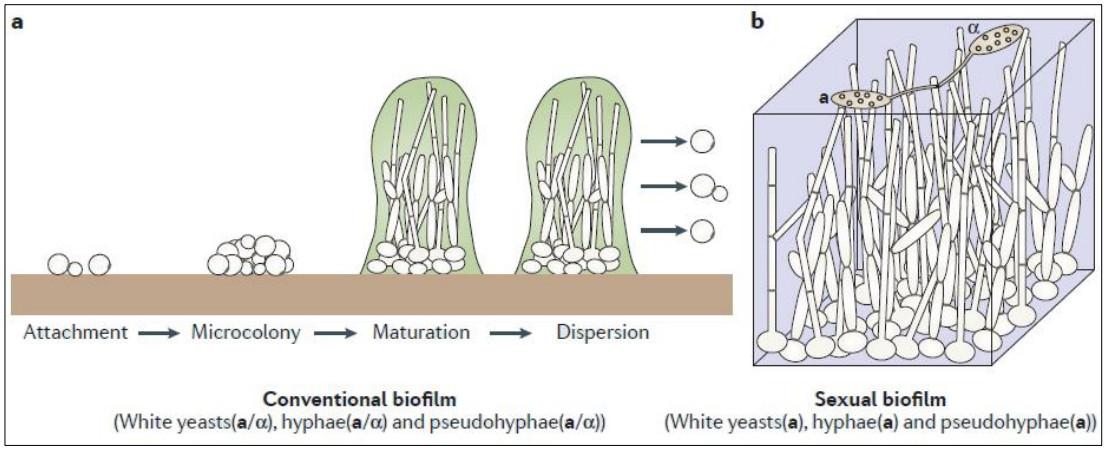


Figure 6. *C. albicans* biofilms. **(a)** Diagrammatic representation of the four stages of biofilm formation: attachment, colony formation, maturation and the appearance of hyphae and pseudohyphae, and lastly detachment of yeast cells and dispersion. **(b)** Sexual biofilm that facilitates mating between two competent opaque cells (Noble et al. 2016).

1.4.4 *C. albicans* cell wall structure

The fungal cell wall is an active and versatile structure that is integral for cell survival, morphogenesis, and virulence. It is a dynamic cell component that influences the biology of fungi and whose composition actively changes in response to stresses from a shifting environment. About twenty percent of the total yeast genes function in the cell wall synthesis, structure, and function (Gow et al. 2017).

Many of the virulence factors discussed in the previous subsection are directly related to the *C. albicans* cell wall. The different components of the cell wall establish adhesion, the principal step of any infection. It also includes cell wall proteins (CWPs) responsible for resisting stress, switching between the different morphotypes, and antigenicity (Masuoka, 2004). The dynamic nature the cell wall makes it well adapted

for infection. For instance, hyphal cell walls are thicker due to increased chitin deposition and the addition of several hypha specific cell wall proteins (Kapteyn et al. 2000). Carbohydrates, namely, mannan, chitin, and β- glucan make up close nine tenths of the *Candida* cell wall (Chaffin et al. 1998). The inner structure of the *Candida albicans* cell wall is made up of β-(1,3) glucan covalently attached to chitin. This structural component resists the considerable hydrostatic pressure applied by the cell's cytoplasm and membrane (Latgé, 2007). The outer cell wall is composed of a layer of mannosylated glycoproteins that are attached to β-(1,6) glucan that links CWPs to the β-(1,3) glucan-chitin inner matrix through glycosylphosphatidyl inositol (GPI) anchors. (Rappleye et al. 2007). Some proteins with internal repeats (Pir) can directly attach to the β-(1,3) glucan via an alkali-sensitive linkage. CWPs make up around 40% of the dry mass of the *C. albicans* cell wall (Klis et al. 2001).

Most characterized adhesins, needed to adhere to the cell surface of host cells, attach to the cell wall by GPI anchors added post transnationally to the C-terminus. There are over one hundred GPI anchored proteins in *C. albicans* (de Groot et al. 2003) many of which are regulated at the transcriptional level during the switch to hyphae or during infection (MacCallum et al. 2009). Besides adhesins, there are many CWPs that play an important role in virulence and cell wall synthesis such as transglycosidases, yapsins, hydrolases, deacetylase, and hydrophobins (Gow et al. 2017). Being a pathogen, the wall of *C. albicans* induces the human immune response, and the flexible nature of cell wall often incorporates immune shields (Erwig et al. 2016). It is a valuable source of diagnostic antigens used to detect fungal infections, and it is also a source of potential targets for chemotherapeutic treatment of pathogenic fungi (Gow et al. 2017).

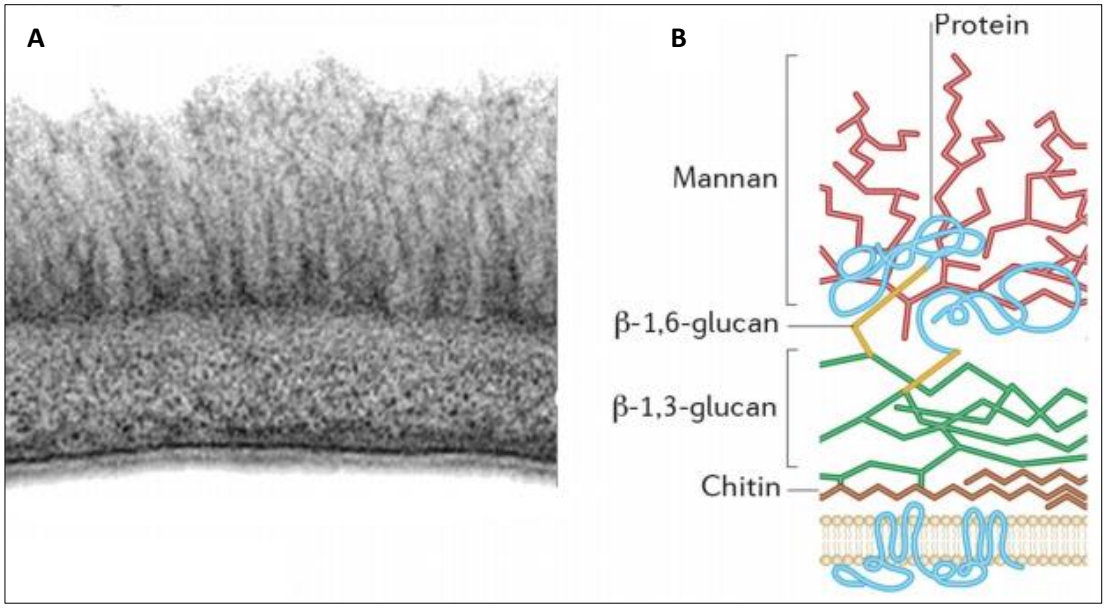


Figure 7. *C. albicans* cell wall structure. **(A)** Transmission Electron Microscopy of a section of the *C. albicans* cells wall. **(B)** Schematic representation of the cell wall (Gow et al. 2017).

1.5 The development of antifungal resistance

Typically, the treatment of *C. albicans* infections is limited to only four major classes of antifungal drugs: azoles, polyenes, fluoropyrimidines, and echinocandins. Echinocandins are used as the last resort for strains resistant to all the other classes of antifungal drugs (Bitar et al. 2014). The azole Fluconazole is the most commonly used method for treating Candidiasis. It inhibits the synthesis of ergosterol which is a main component of the cell membrane. The onset of resistance to antifungals is clinically important and is controlled by several mechanism. For instance, mutations in *ERG11*, the gene encoding the drug's target enzyme result in the reduced ability of the enzyme to bind fluconazole. (Morio et al. 2010).

In a similar fashion, mutations in *FKS1* that codes for the target of echinocandins result in a reduced affinity to bind the drug (Perlin, 2015). Furthermore, gain-of-function gene mutations in some transcription factors lead to constitutive up regulation of numerous target genes and increased fluconazole resistance. For example, mutations in Mrr1 and Tac1 constitutively overexpress the multidrug efflux pumps *MDR1* and *CDR1*/*CDR2* which considerably enhances fluconazole resistance in *C. albicans* (Hampe et al. 2017).

Table 1. Summary of Fluconazole resistance methods (Casalinuovo, 2004)

Causes of Fluconazole resistance	Effect on Resistance Mechanisms
Mutation or overexpression of *ERG11* gene	Decreased drug affinity between fluconazole and the target protein
Mutations to enzymes in the ergosterol synthetic pathway	Production of sterols with cross resistance to azoles
Overexpression of efflux pump genes	Reduced fluconazole concentrations in the cell
Other Mechanisms yet to be identified	Alterations to CWPs

1.6 Hyphal Wall Protein 2 (Hwp2)

The Hwp family comprises two proteins, Hwp1 and Hwp2. Hwp2 is a 909 amino acid long GPI anchored cell wall protein that has been detected in filamentous *C. albicans* cells only in the presence of Efg1, the hyphal transcriptional activator (Sohn et al. 2003). *HWP2* is repressed under non filamentous conditions by the general yeast

repressor Tup1 (de Groot et al. 2003). Our lab has previously characterized the function of *HWP2* by creating a null strain through marker cassette recombination. The null strain was found to be defective in adhesion, invasive growth, and filamentation on solid media. However, it showed no significant loss of filamentation ability in liquid media. Furthermore the mutant strain was shown to be less resistant to oxidative stress, and less capable of forming biofilms on plastic surfaces, resulting in a slight reduction in virulence compared to the wild strain (Hayek et al. 2010, Younes et al. 2011).

1.7 MALDI TOF/TOF MS/MS

Matrix-assisted laser desorption/ionization (MALDI) uses laser energy absorbing matrices to generate ions from larger molecules with marginal fragmentation (Hillenkamp et al. 1991). It is amongst the chief ionization methods used for analyzing biological molecules. Mass spectrometry is a vital tool used for characterizing proteins; measuring the molecular weight of polypeptides; and identifying peptides. The main application of mass spectrometry in the field of proteomics is the identification of isolated proteins represented in protein databases (Yergey et al. 2002). MALDI is applied in three steps. First, samples are mixed with an ionization matrix and spotted on a metal plate. Then, a pulsed laser treats the sample forcing the sample and matrix material to desorp. Finally, the sample molecules are typically protonated or deprotonated and can thus be accelerated into whichever mass spectrometer is being used (Karas et al. 2003).

1.8 Purpose of the Study

The *hwp2* null strain previously generated by our lab had been found to be severely filamentation deficient on solid media, displayed reduced chitin content, decreased adhesiveness, increased susceptibility to oxidative stress, decreased biofilm formation, and decreased virulence when compared to the wild type *RM1000* strain (Younes et al. 2009). These findings suggest that Hwp2 plays an important role in the cell wall organization, plasticity, and biosynthesis. Therefore, our aim is to analyze the entire cell wall proteome of the *hwp2* null mutant as well as well as the wild type RM1000 strain and to generate a comparative analysis profile of both cell wall proteomes using MALDI TOF/TOF MS/MS. We believe that proteins exclusively identified in the wild type strain, and deficient in the null strain will help explain the observed mutant phenotypes listed above.

In parallel, our lab previously obtained numerous *C. albicans* isolates from local Beirut medical centers. Many isolates were found to be either resistant or sensitive to fluconazole. With emerging evidence that fluconazole resistance is the result of an up regulation of efflux pumps that are localized at the cell surface, we applied a similar proteomic approach as above to the cell surface proteins to try to obtain a comparative protein profile that may help in further understanding the mechanism of fluconazole resistance.

Chapter 2

Materials and Methods

2.1 Strain utilized

In this study, we used two *C. albicans* strains: the wild type strain, RM1000 (*ura3*Δ*::_imm434/ura3*Δ*::_imm434his1::hisG/his1::hisG*) (Negredo et al. 1997) histidine and uridine auxotroph, and the *HWP2:URA3/HWP2::HIS1* mutant null strain (Hayek et al. 2010).

For the fluconazole resistance study, five sensitive and five resistant *C. albicans* strains were used. The strains were previously donated by two major medical centers in Beirut (Bitar et al. 2014).

2.2 Media Preparation, Culture Conditions, and Epsilometer Test

For invasive and non-invasive growth on solid media, strains were grown on Sabouraud agar with dextrose (Oxoid, UK) supplemented with Uridine and Histidine for up to two weeks at 37 ºC. The media was prepared based on the manufacturer's instructions.

Sensitive and resistant strains were grown on RPMI 1640 Media with L-Glutamine and without Sodium Bicarbonate. *C. albicans* colonies, previously grown on PDA plates as described above, were suspended in 0.154 M sterile saline solution up to a turbidity of 0.5 McFarland. *C. albicans* samples were spread over the RPMI1640

plates with a sterile cotton swab; a Fluconazole strip was then placed at the center of each. The plates were incubated for 48 hours at 28 ºC.

2.3 Cell Growth and Harvesting

For the *HWP2* strains, cells grown under non-invasive conditions were harvested from the surface of the agar at day 5; whereas, cells grown under invasive conditions were collected from the surface of the agar for the hwp2 null non-invasive strain, as well as from within the filamentation and invasively grown cells for the wild type strain present at day 14.

Fluconazole sensitive cells were harvested from the edges of the growth inhibition zones to ensure that the acquired cells had some level of exposure to the antifungal. Fluconazole resistant cells were harvested from the edges of the strip outwards to ensure the acquired cells were properly exposed to the antifungal.

2.4 Cell Wall Isolation and Protein Extraction

Twelve cell wall extractions were performed for each strain under each condition (invasive, noninvasive). Cells were spun at 4,000 rpm for 5 min, and were re-suspended in 5 mL Tris (5 mM, pH=7.8). Protease Inhibitor Cocktail (6 µL, abcam ab65621) and cold glass beads were added. Vortexing (30 cycles) was performed for breakage as follows: 30 sec on vortex then 30 sec on ice. Samples turned orange due to a reaction of acidic cytosol with the Protease inhibitor. The efficiency of breakage was monitored under the microscope. The supernatant was poured off and pellet re-suspended in NaCl (40 mL, 1 M) and spun. The wash step was repeated 3-4X. SDS extraction buffer (50 mM Tris, 2% SDS, 100 mM Na-EDTA, 150 mM NaCl, pH 7.8)

with β-ME (8 µL per 1 mL SDS extraction buffer) was added (0.5 mL buffer per 100 mg wet weight walls) and the pellet re-suspended. Tubes were boiled for 10 min and spun for 5 min at 3,000 rpm. The supernatant was collected and analyzed for SDS extractable proteins. SDS extraction buffer and β-ME were added as before to re-suspend pellet. Samples were boiled, cooled, and spun for 5 min at 3,000 rpm and suspended in water. Wash steps with water (Type 2+) were performed to remove excess SDS. The final pellet was frozen in liquid N_2 and freeze-dried. Lyophilized cell walls were stored at -20^{0}C until use.

2.5 Extraction of Alkali Labile Cell Wall Proteins

The cell wall pellets were incubated with NaOH (30 mM) at 4°C overnight. The mixture was then neutralized with aqueous acetic acid (30 mM) (Mrsă et al. 1997). Samples were spun, supernatant was collected and subjected to tryptic digestion followed by MALDI-TOF-TOF analysis.

2.6 Glucanase Treatment of Cell Wall Pellets

To maximize the extraction of cell wall proteins, 10^8 cells were treated with 1 mg of Glucanase in sodium acetate buffer (1 mL, 150 mM, pH 5) at 37 °C overnight (Klippel et al. 2010). Cell numbers were estimated using spectrophotometric analysis at 600 nm. Supernatants were collected and subjected to tryptic digestion.

2.7 Tryptic Digestion

The cell wall extracts were placed at 55 °C for 1 h in reducing buffer (10 mM DTT, 100 mM NH_4HCO_3). Samples were cooled to room temperature and spun. Pellets were incubated for 45 min at room temperature in the dark with an alkylating buffer (65 mM iodoacetamide, 100mM NH_4HCO_3). A quenching solution (55 mM DTT, 100 mM NH_4HCO_3) was subsequently added to the samples for 5 min at room temperature. Samples were washed 5 times with ammonium bicarbonate buffer (50 mM). Pellets were re-suspended in ammonium bicarbonate (50 mM) and a trypsin solution was added (1µg/µL). Samples were incubated at 37 °C for 16 h. Then, the samples were spun, and the supernatants were collected and prepped for Zip Tipping by adding TFA (0.1% V/V).

2.8 Peptide Concentration

ZipTip C18 clean up tips were wetted in acetonitrile wetting solution and subsequently equilibrated in a 0.1% TFA HPLC water solution. Sample binding was performed by full pressing the pipette at least 10 times in the digest tube. The membrane was then washed in a 0.1% TFA HPLC water solution. The sample was eluted using 10 µL of elution buffer (0.1% TFA (v/v) in HPLC water/acetonitrile (1:1).

2.9 MALDI TOF/TOF MS/MS

Digested proteins were spotted on stainless steel target plate and overlaid with CHCA matrix solution and dried. MALDI-TOF-TOF MS spectra were acquired using the

4800 MALDI-TOF-TOF analyzer (operated by the 4000 Series Explorer software version 3.7). The instrument was externally calibrated using TOF/TOF Calibration Mixture (Mass Standards Kit for Calibration of AB SCIEX TOF/TOF™ Instruments). The chosen mass range was 499 Da-2500 Da and the focus mass was 1500 Da. The resulting mass lists were manually scanned for known contaminant mass peaks: keratin, matrix, BSA, and trypsin autolysis. The identified contaminant mass peaks were used to create an exclusion list that was applied in the acquisition method used for the all MS/MS data acquisitions performed throughout this thesis.

2.10 Protein identification

Protein identification was done using MASCOT Server, MS/MS Ion Search was carried out using a custom database. The database contained protein sequences of all curated proteins in the Swissprot database (retrieved on September 18, 2016) filtered for proteins with GO cell wall, membrane, transmembrane localization tags. It consisted of protein sequences of all curated *C. albicans* proteins (taxon id: 237561) present in the Swissprot database with gene ontologies: cellwall (GO: 0005618), plasma membrane (GO: 0005886), transmembrane (GO: 00 16021), extra cellular region (GO:0005576), cell surface (GP:0009986), hyphal cell wall (GO:0030446), and fungal type cell wall (GO:0009277) localization tags. The peptide and fragment tolerance values were specified at ±2 Da. This may seem too tolerant; however, the resolution per mass peak as displayed by the machine is on average 4000 under our operating conditions. In this experiment, the default machine settings were used. As such we had to choose a slightly more lenient tolerance level. Carbamidomethyl C was assigned as a fixed modification because iodoacetamide was

used as the alkylating agent, while Oxidation at M was chosen as a variable modification. Up to two missed cleavages were allowed for trypsin. Proteins with a minimum of 2% sequence coverage were included. The nature of the work (MS/MS) ion search results in greater peptide confidence than PMF which makes the issue of sequence coverage less important. 2% coverage equates to an average of 12 amino acids identified in a specific order which is enough for unmistakable protein identification (Barrett et. al, 2004). We added the 2 % cut off as an extra step to reduce false positives. Peptide sequences identified by MASCOT but not linked to proteins were blasted on Candidagenome.org. The cut off e-value was < 0.05 in both MASCOT searches as well as BLAST searches. In BLAST we used the default BLASTP options but we did not allow gaps.

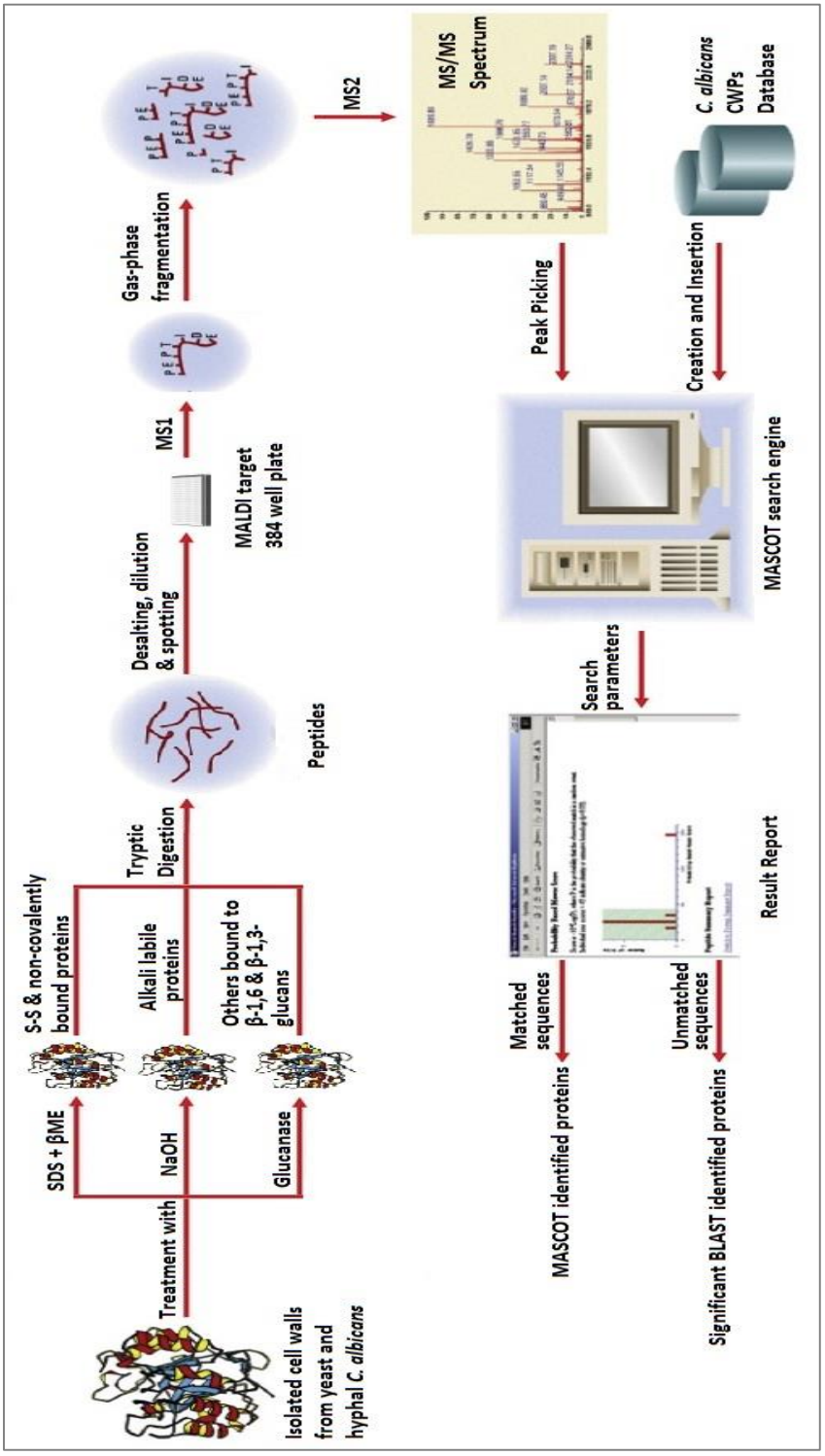


Figure 8. Graphical abstract summarizing the experimental work flow. Dotted lines represent the unsuccessful approaches that were discontinued.

Chapter 3

Results

3.1 Proteomic Analysis of *hwp2* mutant versus wild type strains

Table 2 lists the proteins consistently identified regardless of the growth conditions. In total, we identified 36 proteins with 10 being essential (E) and another 8 are putative essential proteins (P). The remaining 18 proteins were proteins consistently identified.

Table 3 displays a total of 14 proteins identified exclusively in the wild type strain by MASCOT. It is important to note the secreted aspartyl proteases (SAPs) that are key virulence factors. Furthermore, as seen in Table 4, BLAST identified 12 more proteins exclusively present in the wild type bringing the total up to 26. Many of these proteins are potent virulence factors and proteins required for invasive growth and filamentation and will be expanded upon in the discussion section.

Table 5 shows 16 proteins exclusively identified in the wild type strain grown under non-invasive conditions. It is remarkable how the proteins involved in virulence and penetration have decreases as opposed to their presence in cells harvested after the onset of invasive growth. For instance, EXG2 is involved in the metabolism of β-Glucan but is not needed for Glucanase functions involved in adhesion (Tsai et al. 2011). SSU1 functions in the response to starvation, and its presence in the wild type signals that cells were getting ready to transition into the invasive growth phase (Uhl et al. 2003). In addition, Table 7 shows 17 proteins have been further identified in the

wild type strain. It is worth noting that in our lab's previous assessment of the mutant, the main differences manifested during virulence and conditions requiring filamentation on solid media. Therefore, it is not surprising that under non-invasive conditions both the wild type and the mutant display many PGA family proteins. On the other hand, the detection of MTS1 is significant because it encodes a protein required for forming hyphae on solid media (Oura, et al. 2010) which is consistent with our lab's previous observations.

Table 2. Common proteins identified in both strains under both growth conditions.

Protein	Description
Alo1	Has a protective role against oxidative damage
Als7	ALS family protein; hypermutable contingency gene; growth-regulated
Ape2^{E}	Neutral arginine, alanine, leucine specific metallo-aminopeptidase
Bmh1^{E}	14-3-3 protein in C. albicans; role in hyphal growth; possibly regulated by host interaction
Bna4^{E}	Putative kynurenine 3-monooxygenase; oral infection upregulated. No homozygous null strain has yet been successfully generated.
Bud2	GTPase activating protein for Rsr1
Bud4	Bud site selection protein B
Cdc11	Septin; cell and hyphal morphology, agar-invasive growth
Cdr1	Multidrug transporter of ABC superfamily

Cdr2	Multidrug transporter
Eft2^{E}	Elongation Factor 2 (eEF2); GTPase; essential; highly expressed; target of sordarin antifungals
Eno1^{E}	Enolase; glycolysis and gluconeogenesis; major cell-surface antigen
Erg6	Delta(24)-sterol C-methyltransferase, converts zymosterol to fecosterol, ergosterol biosynthesis
Gmo1	Phosphoglycerate mutase; surface protein that binds host complement Factor H and FHL-1; antigenic
Hsp21	Small heat shock protein; role in stress response and virulence
Hsp90^{E}	Essential chaperone, regulates several signal transduction pathways and temperature-induced morphogenesis
Met6^{E}	Essential 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
Mlt1	Vacuolar membrane transporter
Pan1^{E}	Essential protein involved in endocytosis and polarized growth
Pdc1^{P}	Pyruvate decarboxylase; antigenic; on hyphal not yeast cell surface
Pgk1^{P}	Phosphoglycerate kinase; localizes to cell wall and cytoplasm
Phr2	Glycosidase; role in vaginal not systemic infection
Pmt2^{E}	Protein mannosyltransferase (PMT) with roles in hyphal growth and drug sensitivity
Prt1^{P}	Putative translation initiation factor eIF3
Rps1^{P}	Putative ribosomal protein

Sa6	Biofilm-specific aspartyl protease; expressed during hyphal growth
Sap9	Secreted aspartyl protease; roles in adhesion, cell surface integrity; induced by antifungal drugs
Sec4^{E}	Small GTPase of Rab family; role in post-Golgi secretion
Sln1	Histidine kinase involved in a two-component signaling pathway that regulates cell wall biosynthesis
Ssa1	HSP70 family chaperone; cell wall fractions; antigenic
Ssa2	HSP70 family chaperone; cell wall fractions; antigenic
Ssc1^{P}	Heat shock protein
Stp3	Transcription factor; regulates SAP2, OPT1 expression and thereby protein catabolism
Tef1^{P}	Translation elongation factor 1-alpha; at cell surface; binds human plasminogen
Tim50^{P}	Predicted component of the Translocase of the Inner Mitochondrial membrane
Ugp1^{P}	UTP-glucose-1-phosphaturidyl transferase

Table 3. Proteins exclusive to the wild type RM1000 strain as detected by MASCOT. The strains were grown under invasive conditions.

Protein	Description	Protein Coverage (%)	Peptide Sequence	Missed Cleavages
Pga5	1,3-beta-glucanosyltransferase PGA5	7.6	EVQEEEPGVPGLPGSNK	0
			EPHYIDPLANPFTCLR	0
			ELCFKVDCEDINANGR	1
Sec62	Translocation protein SEC62	9.9	TGLLDNTNDIEFFR	0
			ALLSDDYKQKQQNPK	2
Egd2	Nascent polypeptide-associated complex subunit alpha	3.9	QIKGISR	1
Sap5	Candidapepsin-5	4.5	SAGSYSPASSRTSQNLNTR	1
Sap10	Candidapepsin-10	10.4	NTTSSSSGNCQTR	0
			ILYTTELEIGSNK	0
			ILYTTELEIGSNKDK	1

			MDLVIMNFVFLLYLTSVVK	0
Sho1	High osmolarity signaling protein SHO1	4.7	DEILEVDDIDGKWWQARR	2
Sap4	Candidapepsin-4	6.2	TLSVGLR	0
			KISMAQVKYTSQSNIVAIN	2
Cht2	Chitinase 2	3.3	LFVGVPATSNIAGYVDTSK	0
Erg1	Squalene monooxygenase	2.4	GFILLGDSLNMR	0
Iff11	Cell wall protein IFF11	8.2	SFVNTGEIILAFK	0
Sla1	Actin cytoskeleton-regulatory complex	3.7	LTDGPLRR	1

	protein SLA1			
Pga53	GPI-anchored protein 53	9.4	SNSTSSSSSSSKK	1
Mp65	Cell surface mannoprotein MP65	3.7	LYGVDCDQVSAVLK	0
Pns1	Protein PNS1	4.6	SAKDTFDLIRFK	2
			DPEIFQMTNRNR	1

Table 4. Proteins exclusive to the wild type RM1000 strain as detected by BLAST. The strains were grown under invasive conditions.

Protein	Description	Sequence	E-value
Cht4	Chitinase	QLITFDNLQCAR	2.00E-07
Erg11	Lanosterol 14-alpha-demethylase	HLTTPVFGKGVIYDCPNSR	2.00E-15
Fmp13	Mitochondrial inner membrane protein	DIESVIGR	4.10E-02
		SEIPPPPPPPPPPKAKR	8E-14
Hyr4	Putative GPI-anchored adhesin-like protein	MFFSASGK	1.00E-02
Lip8	Secreted lipase	SSKITNIK	5.30E-01
Pga50	Adhesin-like protein	GSITSGLASKTETSK	9.00E-09
Qcr2	Ubiquinol-cytochrome-c reductase	FTFLNNGAKSALR	1.00E-07
Qdr1	Putative antibiotic resistance transporter	MPGNREEFDIEK	3.00E-07
Qdr2	Predicted MFS membrane transporter	SLDWYYR	2.00E-02

Rbr1	Glycosylphosphatidylinositol (GPI)-anchored cell wall protein	SGASSVASAAK	2.00E-04
Sap7	Pepstatin A-insensitive secreted aspartyl protease	YTNLPFAMKEQNLIAK	2.00E-11
Sec62	Putative endoplasmic reticulum (ER) protein-translocation complex subunit	RLQRALLSDDYK	1.00E-06

Table 5. Proteins exclusive to the wild type RM1000 as detected by MASCOT. The strains were grown under non-invasive conditions.

Protein	Description	Protein Coverage	Peptide Sequence	Missed Cleavages
Pan1	Actin cytoskeleton-regulatory complex protein	4.5	KAEVFKLELLK	2
			EIMAVQTKLDER	1
			KFEHLFRTAVPK	2
			GYVDSNVALNVFTK	0
			NQFAVAMHLIYRR	1
			KGYVDSNVALNVFTK	1
Tim21	Mitochondrial import inner membrane translocase subunit	6	MHMIQRVSSIGLRTLR	2
			MHMIQRVSSIGLRTLR	2
Qdr2	MFS antiporter QDR2	2.8	RMNNDITTIVPATR	1
Pga32	Probable GPI-anchored adhesin-like protein	6	TTLGQVTTPSR	0
			LSSSKSIYSNSTTSR	1
Ssu1		7.5	DTMDHLSYLSTIDSKR	1

	Sulfite efflux pump SSU1		IRPFAKNPNPSHTNRFGLLK	2
Cbr1	NADH-cytochrome b5 reductase	7.1	ASNDTNLLLCGPPPMVSAMKK	1
Cht2	Chitinase 2	3.3	LFVGVPATSNIAGYVDTSK	0
Pga41	Probable cell wall protein	2.7	SSYYNNLR	0
Pga31	Cell wall protein	2.4	DSYAVVK	0
Prm1	Plasma membrane fusion protein	6.1	EISVSLNSTIVEFLDK	0
			MGIDLNERAIWMLSFMFSKISR	2
			MGIDLNERAIWMLSFMFSKISR	2
Qdr1	MFS antiporter QDR1	2.2	RIVGNGSIRPK	1
Fba1	Fructose-bisphosphate aldolase	6.4	GFAIPAINVTSSSTVVAALEAAR	0
Eft2	Elongation factor 2	3.2	DLEGKALLKVVMR	2
			NCDPNADLMLYVSK	0
Pns1	Protein PNS1	2.3	SAKDTFDLIRFK	2

Lip6	Lipase 6	5.8	QSGKATLDSIK	1
			LGSGVTSDDVSVNGLR	0
Exg2	Glucan 1,3-beta-glucosidase	3.8	EAGLNMVRIPIGYWSFEK	1

Table 6. Proteins exclusive to the wild-type RM1000 strain as detected by BLAST. The strains were grown under non-invasive conditions.

Protein	Description	Sequence	E-Value
Fba1	Fructose-bisphosphate aldolase	APPAVLSKSGVIYGKDVK	4.00E-09
Fmp13	Mitochondrial inner membrane protein	DIESVIGR	4.10E-02
		QQLEAKLNQK	2.00E-04
		LNEERNGRLANLEK	3.00E-08
Lip6	Secreted lipase	SNGHTTETVVGAPAALTWIDAR	3.00E-17
Mts1	Sphingolipid C9-methyltransferase	ITSVEMAEHVGIR	6.00E-08
Pga28	Putative GPI-anchored adhesin-like protein	TSETGGVSSTANSEAKSGSVTTSK	1.00E-17
Pga30	GPI-anchored protein of cell wall	FIGGGKSSSVTK	2.00E-06
Pga45	Putative GPI-anchored cell wall protein	LVLSQKNVLLYIFAGVLSK	2.00E-13
Pga5	Putative GPI-anchored beta-1,3-glucanosyltransferase	EEEKEVQEEEPGVPGLPGSNK	4.00E-14
Pga53	GPI-anchored cell surface protein of unknown function	LINDDELMVQDAQFDYPAIVNLK	2.00E-18

Phr1	Cell surface glycosidase	MTDVWSGGIVYMYFEEANK	1.00E-15
Plb1	Phospholipase B	QGMISNGFEIATR	4.00E-08
		SSGSNTAISQSK	4.00E-06
Qcr2	Ubiquinol-cytochrome-c reductase	FTFLNNGAKSALR	1.00E-07
Qdr1	Putative antibiotic resistance transporter	RIVGNGSIRPK	9.00E-06
Rim9	pH-response regulator protein	FFESEYRYANDDMRIMR	1.00E-13
Ssu1	Protein similar to S. cerevisiae Ssu1 sulfite transport protein	DTMDHLSYLSTIDSKR	3.00E-11
Tim21	Component of the Translocase of the Inner Mitochondrial membrane	LIEKNEQAQK	2.00E-04
Tsa1B	Putative peroxidase	MAPVVQQPAPSFKK	3.00E-07

3.2 Proteomic profile of resistant *C. albicans* strains

In total, 8 proteins were uniquely detected in the resistant *C. albicans* cells. Their functions and potential ties to drug resistance will be elaborated in the discussion

Table 7. Proteins exclusively detected in the *C. albicans* fluconazole resistant strain.

Protein	Protein description	Sequence coverage %	missed cleavages	Peptide sequence	Identified by
Sec4	Ras-related protein	5.2	0	LQVWDTAGQER	MASCOT
Pns1	Protein PNS1	2.3	2	SAKDTFDLIRFK	MASCOT
Pam17	Presequence translocated-associated motor subunit PAM17, mitochondrial	5.9	1	LTWVDYFQLKK	MASCOT
Tsa1	Peroxiredoxin	7.7	1	GVLRQITINDLPVGR	MASCOT

Ipp1	Inorganic pyrophosphatase	5.6	0	VLGVMALLDEGETDWK	MASCOT
Protein	**Protein description**	**E-value**	**Sequence**		**Identified by**
C1_13530W_Ap	pseudogene	3.00E-10	SGIDTTKVYGGLANYGR		BLAST
Ywp1	Yeast-form wall protein	2.00E-12	NLYGAGAVPFFQVHLEK		BLAST
Pil1	Eisosome component; predicted role in endocytosis; echinocandin-binding protein	2.0e-06	LSQFIKMEKNFM		BLAST

Chapter 4

Discussion

4.1 Wild Type cell wall proteome profile

The Hwp2 protein was previously characterized as a cell wall protein with important roles in host cell adhesion, resistance to oxidative stress and cell wall disrupting agents, cell wall chitin deposition, and biofilm formation. As such, it was found that Hwp2 is necessary for proper invasion and virulence in *C. albicans* (Hayek et al. 2010). Earlier studies done by our lab came up with this characterization by comparing an *hwp2* mutant strain with a wild-type strain and observing that the former displayed reduced adherence to human endothelial and epithelial cells with drop rates of 70% and 75% respectively, lowered resistance to hydrogen peroxide and SDS, decreased chitin content in the cell wall, and lower biofilm mass by almost 30% (Younes et al., 2011). This same mutant strain was found to have deficiency in filamentation on solid agar media as well (Hayek et al., 2010). Since Hwp2 is a cell wall protein, we hypothesized that knocking out Hwp2 would result in changes at the level of the cell surface architecture in general and changes at the level of cell surface proteome in particular. The purpose here was to detect the differentially expressed proteins in the cell wall of the wild-type strain that are unique to this strain and are responsible for this variation in the observed phenotypes.

To achieve that purpose, proteins were extracted from the cell walls of cells harvested from both strains under invasive and non-invasive growth conditions

followed by identification through MALDI mass spectrometry coupled with MASCOT and BLAST database searches. Comparative analysis of the obtained hits was done to find the proteins present in the cell walls of the wild-type cells and not detected in the mutant cells thus reflecting the above mentioned phenotypes. It is worth noting that many proteins were found in both mutant and wild type pools,

It is important to note that if a protein was not detected in one strain, this does not firmly imply its complete absence from the sample. It might be present at trace amounts that could not be detected by the machine while in the other strain it is found at much higher concentration (Castillo et al., 2008); resulting in the respective phenotypic variation. This is supported in our study by the detection of the essential protein Exg2 only in the non-invasive wild-type cells. Exg2 is a cell wall protein with GPI anchorage and functions as an essential exo-β-1,3-glucanase (Tsai et al., 2011) that cleaves glucose from the ends of glucans during cell wall regeneration (Castillo et al., 2006). Pan1 is also another essential protein yet only detected in the non-invasive wild-type cells. This protein is required for polarized growth and actin-mediated endocytosis as well as for regulation of actin cytoskeleton (Martin et al., 2007). As such, both proteins cannot be totally absent from the other samples; they are present at very low concentrations. Since they play roles in cell wall polarization, rigidity, and regeneration, their low concentration in the mutant explains the inability of this mutant to form invasive filaments on agar and its increased susceptibility to disrupting agents.

It is important to note that the results of Table 2 add credibility to our methods. The constant detection of 18 essential proteins regardless of the growth conditions is very important in adding credibility to all the other results obtained throughout the

study. There are 40 essential proteins in the cell wall in total which means that were able to detect 45% of the essential proteins consistently. The protein that were not detected are perhaps expressed in very low concentrations under our experimental conditions.

All of the extracted proteins should theoretically be cell wall proteins since the extraction protocol targeted the cell wall and extracts its different proteins while discarding the cytosolic and intracellular ones. Surprisingly, some of the identified proteins were cytosolic, mitochondrial, or nuclear proteins. Such detected proteins can be contaminants from the cell wall isolation process. However, recently many proteins detected among such cell wall fractions in yeasts are being referred to as “atypical” proteins involved in atypical secretory pathways (Nombela et al., 2006). Those proteins lack the usual signal peptides that normally direct their translocation to the cell surface so that they could be secreted following the classical secretory pathway involving the endoplasmic reticulum and Golgi apparatus (Nickel, 2003). Still, those atypical proteins - mainly cytoplasmic enzymes involved in metabolic activities, ribosomal proteins, and elongation factors - are able to reach the cell surface and get secreted in a yet unresolved mode (Nickel and Rabouille, 2009). Probably, atypical proteins with already known functions in cytoplasmic reactions and processes can also be retained in the *C. albicans* cell wall by non-covalent bonds (Castillo et al., 2008).

Of the proteins solely detected in the wild-type strain that are either artifacts or atypical proteins, we identified Tsa1B, Eft2, Egd2, Fmp13, Fmp17, and Qcr2. Fmp13, also known as Mic60, is a component of the mitochondrial inner membrane (Nobile et al., 2012) while Fmp17, also known as Tim21, functions in transporting proteins across

the mitochondrial inner membrane (Hewitt et al., 2012); both proteins are most probably artifacts.

On the other hand, Tsa1B is atypical and has been previously described as a moonlighting protein for it could be found in the nucleus, cytoplasm, and cell wall with distinct functions (Urban et al., 2005). In the cell wall, Tsa1B is a peroxidase that plays a major role in resisting heat shock and oxidative stress (Jang et al., 2004); its lack of detection in the mutant cells supports the observed increased sensitivity to hydrogen peroxide. Eft2 is antigenic and was assigned as an atypical protein in a previous study (Castillo et al., 2008). Additionally, Qcr2, being antigenic (Pitarch et al., 2004), and Egd2 are both atypical proteins found only in hyphae and not in yeast cells (Hernández et al., 2004) which is supported by this study since they were both found in the wild-type strain under invasive growth conditions and not detected in the mutant which is filamentation-deficient.

The rest of the detected proteins are typical cell wall proteins. Out of these, nine proteins, detected only in the wild-type strain, belong to the putative GPI-anchored (PGA) family that includes around 65 proteins where the majority are yet to be characterized (Hashash et al., 2011). Identified PGA proteins with unknown functions include Pga30, Pga45, and Pga53. On the other side, many of the identified proteins are adhesin-like such as Pga28, Pga32, Pga41 and Pga50 that play roles in adhesion of the fungus to the host cell surface and subsequently in virulence (Chaudhuri et al., 2011); thus clarifying the reduced adhesion to epithelial and endothelial host cells and the decreased virulence levels noticed in the mutant strain. Another protein only detected in the wild-type is Pga31, which is involved in the regulation and assembly

of chitin within the cell wall. This reflects the drop in chitin content and reduction cell wall integrity which in turn affect resistance to perturbing agents (Plaine et al., 2008). Also, Pga5 is a β-1,3-glucanosyltransferase that cleaves β-1,3-glucan chains and forms new glycosidic linkages leading to the elongation and branching of these chains in the cell wall (Kováčová et al., 2015). This shows the important role of Pga5 in cell wall strength and assembly on which cell wall integrity and morphogenesis rely, (Latgé, 2007) all of which are reduced in the mutant strain.

The mutant was found to exhibit reduction in biofilm mass when compared to the wild-type and to be unable to form invasive filaments on solid agar. This could be attributed to the lack of detection of certain proteins with invasion related functions in the mutant samples. Phr1 is a glycosidase that is crucial for morphogenesis as a *phr1* null mutant is locked in the round morphology and cannot filament (Saporito-Irwin et al., 1995). Mts1 is a sphingolipid C9-methyltransferase that functions in glucosylceramide biosynthesis necessary for proper elongation on solid media, virulence, and the development of hyphae (Oura and Kajiwara, 2009). Rbr1 and Rim9 have roles in filamentous growth (Lotz et al., 2004) as well as Ssu1 which induces filamentous growth by acting as a sulfite efflux pump to grow within keratinized host structures (Hennicke et al., 2013). Collectively, these proteins explain the variation in the ability to filament and invade between the mutant and the wild-type strains.

Several proteins involved in cell wall biosynthesis and organization were differentially detected as well. Cht2 and Cht4 are chitinases functioning in chitin remodeling with additional roles in cell separation and sporulation respectively (McCreath et al., 1995; Dünkler et al., 2008). Hyr4 is an adhesin-like GPI-anchored

protein that helps the fungus adhere to host tissues and functions in the proper organization of the cell wall and in the subsequent hyphal growth (De Groot et al., 2003). Sla1 is involved in actin cytoskeleton assembly and actin-mediated endocytosis; thereby promoting cell polarization and hyphal growth (Zeng et al., 2011). Proteins involved in cell wall organization and remodeling are key factors for maintaining cell wall integrity necessary for resisting environmental stresses and for regulating morphogenesis (Chaffin, 2008). As such, the lack of detection of these proteins in the mutant strain clarify its higher sensitivity to SDS and osmotic stress than the wild-type.

In healthy hosts, the innate immune cells, including macrophages and neutrophils which are active phagocytes, generate toxic reactive oxygen species (ROS) like superoxide and hydrogen peroxide (Kaloriti et al., 2014). However, the pathogenic *C. albicans* has developed mechanisms to detoxify ROS that involve various proteins (Dantas et al., 2015). One important protein that mediates resistance to oxidative stress is Ssu81, an osmosensor present at the plasma membrane. This protein mediates such resistance by affecting the assembly of the cell wall mannan, increasing invasive filamentation, and activating morphogenesis (Roman et al., 2005). Another protein involved in the activation of the oxidative stress response pathway is Iff11, but this protein is a secreted protein that lacks a GPI-anchor and acts as a hydroperoxide scavenger (Bates et al., 2007). Ssu1 and Iff11 were not detected in any of the *HWP2* mutant fractions; thus explaining the increased sensitivity of the mutant to oxidative stress when compared to the wild-type.

Ergosterol is a major component of the fungal plasma membrane that affects cell permeability and rigidity (Sorgo et al., 2011). Two proteins necessary for ergosterol biosynthesis were detected in fractions of the wild-type strain. These proteins are Erg1 and Erg11 which are major targets of allylamine and azole antifungal drugs respectively; thus being crucial for antifungal drug resistance (Liu et al., 2005). Fungal membrane permeability is also affected by transporters localized in the plasma membrane such as Pns1 and Qdr family of proteins. Though Qdr1 and Qdr2 do not contribute to drug transport, they are required for nutrient uptake, biofilm thickness, and virulence attenuation (Shah et al., 2014). As such, the *hwp2* mutant strain lacking these proteins is less virulent than the wild-type strain.

Mp65, a cell wall mannoprotein, is a major virulent factor with multiple roles in pathogenesis (Calderone et al., 2001). It is a β-glucanase adhesin that aids the fungus in adhering to the host tissues and catheter surfaces, then in the following invasion and biofilm development (Sandini et al., 2007). Additionally, it contributes to the carbohydrate content of the cell wall and glucan metabolism during cell wall regeneration (Sandini et al., 2011). Consequently, Mp65 affects cell wall rigidity and response to environmental factors. This protein was not detected in the mutant strain which was found to be less virulent than the wild-type with higher sensitivity to stresses and perturbing agents and decreased adherence to surfaces.

Many of the detected proteins are secreted proteins that act as hydrolytic enzymes with important roles in the pathogenicity of the fungus. In the wild-type, we detected 2 secreted lipases (Lip6 and 8), 1 phospholipase (Plb1), and 4 aspartyl proteases (Sap4, 5, 7 and 10) that supply *C. albicans* with its nutritional requirements by degrading the

host's membrane and macromolecules; thus aiding the pathogen in overcoming the host defenses and enhancing invasion (Schaller et al., 2005). Accordingly, the mutant, in which those hydrolytic enzymes were not identified, turned out to be invasion-deficient.

4.2 Cell wall proteome profile of fluconazole resistant strains

A fairly decent amount of work has been generated recently about the onset of resistance to azoles, chemical antifungals grouped into triazoles, such as Fluconazole, and imidazoles. Fluconazole is an antifungal drug first discovered around 25 years ago. It has been shown to be a powerful antifungal capable of treating yeast infections. Recently, resistant *C. albicans* strains have been identified in immune-incompetent patients. Fluconazole, compared to other antifungals, seems free of serious adverse effects and is thus administered in both intravenous and oral formulations. Its low toxicity and ease of distribution within the body have made it the treatment of choice for both superficial candidiasis, candidemia, and disseminated candidiasis (Casalinuovo, 2004).

Interestingly, we were not able to detect any efflux pumps in the resistant strains used in our experiments; however, we have been able to detect some interesting proteins that have a role to play in resistance. For instance, Sec4 was detected in the resistant strain; it is known to have a role in the cellular response to drugs. Also, Sec4 heterozygous cells are resistant to ergosterol analogues which shows that this protein in involved in the ergosterol biosynthetic pathway and is thus linked to the mode of action of fluconazole (Roemer, 2003). Furthermore, mutation in Sec4 have been shown to cause an increase in the accumulation of post Golgi secretory vesicles which directly

led to an increased accumulation of fluconazole in the cells (Basso et al. 2010). Pns1 has an unknown function but is thought to be involved in transport through the plasma membrane suggesting a potential role in fluconazole resistance. Pam17 is a mitochondrial protein and is probably an artifact of the extraction process. Tsa1 is another interesting protein that has been detected. It has been shown to be important for the proper formation of the hyphal cell wall and for proper resistance to oxidative stress. Its absence increases the mutant cells' susceptibility to cell wall perturbing agents (Urban et al. 2005). Its effect on fluconazole resistance hasn't been investigated, but its role in the proper assembly of hyphal cell walls coupled with its detection in the resistant strain only suggest it may have a role in antifungal resistance as well. IPP1 is a protein involved in virulence and is antigenic during *C. albicans* infections. It is found in soluble form inside the fungal hyphae. There is no evidence so far to support its role in fluconazole resistance. Ywp1 encodes a cell wall glycoprotein that is thought to function in yeast cell dispersal during infections because it has anti-adhesive properties. It is ample in the yeast cell walls but not in hyphae. It is expressed mainly by budding *C. albicans* yeast (Granger, 2012). It would interesting to study its relation with fluconazole resistance. The last protein to be identified is Pil1 which is an echinocandin binding protein. Echinocandins are anti-fungals that inhibit 1, 3-β glucan synthase, an enzyme required for glucan biosynthesis within the cell wall. By binding echinocandins, Pil1 allows the standard cell wall biosynthesis to proceed thus making the cells resistant to echinocandin action. It is known that fluconazole down regulates Pil1 (Rogers et.al 2006). Differential regulation of Pil1 in the resistant strain is very interesting here, implying that Pil 1 might also play a role in azole resistance by maybe also binding fluconazole and preventing it from destabilizing the cell membrane,

resulting in increased resistance. In sensitive strains, Pil1 is not present to bind either drug resulting in sensitivity to both.

4.3 Conclusions and Future Work

The lack of detection of many proteins in the *hwp2* mutant strain can explain the previously observed decreased levels of chitin content, adhesion, virulence, and resistance to oxidative stress and SDS. This can also clarify the inability of the mutant to form hyphae on solid media. The detected proteins are virulence factors each affecting a certain pathogenesis pathway in *C. albicans* and were all affected by the deletion of *HWP2* from the genome of this fungus. Thus, we conclude that *HWP2* has a fundamental effect on the *C. albicans* cell wall architecture and secretome.

Furthermore, our results support the notion that fluconazole resistance is not merely a matter of overexpressing efflux pumps but can rather involve different proteins that are still poorly described. Interestingly, we may have stumbled upon evidence that echinocandins and fluconazole resistance are positively correlated, and identified a novel mechanism of action of azole resistance.

It would be very interesting to conduct further experiments to characterize the different pathways in which Hwp2 is involved as well as conduct similar studies that quantify the amounts of all detected proteins instead of relying simply on the presence or absence of a protein. Moreover, it would be of extreme interest to further explore the potential link between echinocandin and fluconazole resistance which may result in a novel treatment approach.

Bibliography

Anderson, J., Mihalik, R., & Soll, D. R. (1990). Ultrastructure and antigenicity of the unique cell wall pimple of the candida opaque phenotype. *Journal of Bacteriology, 172*(1), 224-235.

Barrett, J., Brophy, P. M., & Hamilton, J. V. (2005). Analysing proteomic data. *International Journal for Parasitology, 35*(5), 543-553.

Basso, L. R., Gast, C. E., Mao, Y., & Wong, B. (2010). Fluconazole transport into candida albicans secretory vesicles by the membrane proteins Cdr1p, Cdr2p, and Mdr1p. *Eukaryotic Cell, 9*(6), 960-970.

Bates, S., Jos, M., MacCallum, D. M., Brown, A. J., Gow, N. A., & Odds, F. C. (2007). Candida albicans Iff11, a secreted protein required for cell wall structure and virulence. *Infection and Immunity, 75*(6), 2922-2928.

Bitar, I., Khalaf, R. A., Harastani, H., & Tokajian, S. (2014). Identification, typing, antifungal resistance profile, and biofilm formation of candida albicans isolates from lebanese hospital patients. *BioMed Research International, 2014*

Bockmhl, D. P., Krishnamurthy, S., Gerads, M., Sonneborn, A., & Ernst, J. F. (2001). Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of candida albicans. *Molecular Microbiology, 42*(5), 1243-1257.

Brown, A. J., Odds, F. C., & Gow, N. A. (2007). Infection-related gene expression in candida albicans. *Current Opinion in Microbiology, 10*(4), 307-313.

Calderone, R. A., & Fonzi, W. A. (2001). Virulence factors of candida albicans. *Trends in Microbiology, 9*(7), 327-335.

Casalinuovo, I. A., Di Francesco, P., & Garaci, E. (2004). Fluconazole resistance in candida albicans: A review of mechanisms. *European Review for Medical and Pharmacological Sciences., 8*, 69-78.

Castillo, L., Calvo, E., Martínez, A. I., Ruiz-Herrera, J., Valentín, E., Lopez, J. A., & Sentandreu, R. (2008). A study of the candida albicans cell wall proteome. *Proteomics, 8*(18), 3871-3881.

Castillo, L., Martnez, A. I., Garcer, A., Garca-Martnez, J., Ruiz-Herrera, J., Valentn, E., & Sentandreu, R. (2006). Genomic response programs of candida albicans following protoplasting and regeneration. *Fungal Genetics and Biology, 43*(2), 124-134.

Chaffin, W. L. (2008). Candida albicans cell wall proteins. *Microbiology and Molecular Biology Reviews, 72*(3), 495-544.

Chaffin, W. L., Lpez-Ribot, J. L., Casanova, M., Gozalbo, D., & Martnez, J. P. (1998). Cell wall and secreted proteins ofCandida albicans: Identification, function, and expression.*Microbiology and Molecular Biology Reviews, 62*(1), 130-180.

Chaudhuri, R., Ansari, F. A., Raghunandanan, M. V., & Ramachandran, S. (2011). FungalRV: Adhesin prediction and immunoinformatics portal for human fungal pathogens. *BMC Genomics, 12*(1), 192.

Chin, V., Foong, K., Maha, A., Rusliza, B., Norhafizah, M., & Chong, P. P. (2014). Multi-step pathogenesis and induction of local immune response by systemic candida albicans infection in an intravenous challenge mouse model. *International Journal of Molecular Sciences, 15*(8), 14848-14867.

Daniels, K. J., Park, Y., Srikantha, T., Pujol, C., & Soll, D. R. (2013). Impact of environmental conditions on the form and function of candida albicans biofilms. *Eukaryotic Cell, 12*(10), 1389-1402.

Dantas, A. d. S., Day, A., Ikeh, M., Kos, I., Achan, B., & Quinn, J. (2015). Oxidative stress responses in the human fungal pathogen, candida albicans. *Biomolecules, 5*(1), 142-165.

de Groot, P. W., Hellingwerf, K. J., & Klis, F. M. (2003). Genome-wide identification of fungal GPI proteins. *Yeast, 20*(9), 781-796.

Didier, E. S. (2005). Microsporidiosis: An emerging and opportunistic infection in humans and animals. *Acta Tropica, 94*(1), 61-76. doi:10.1016/j.actatropica.2005.01.010

Dnkler, A., Jorde, S., & Wendland, J. (2008). An ashbya gossypii cts2 mutant deficient in a sporulation-specific chitinase can be complemented by candida albicans CHT4.*Microbiological Research, 163*(6), 701-710.

Douglas, L. J. (2003). Candida biofilms and their role in infection. *Trends in Microbiology, 11*(1), 30-36.

Erdogan, A., & Rao, S. S. C. (2015). Small intestinal fungal overgrowth. *Current Gastroenterology Reports, 17*(4), 16. doi:10.1007/s11894-015-0436-2

Erwig, L. P., & Gow, N. A. (2016). Interactions of fungal pathogens with phagocytes. *Nature Reviews Microbiology, 14*(3), 163-176.

Esteban, P. F., Ríos, I., García, R., Dueñas, E., Plá, J., Sánchez, M., . . . del Rey, F. (2005). Characterization of the CaENG1 gene encoding an endo-1, 3-β-glucanase involved in cell separation in candida albicans. *Current Microbiology, 51*(6), 385-392.

F. C. Odds, A. D. Davidson, M. D. Jacobsen, A. Tavanti, J. A. Whyte, C. C. Kibbler, . . . N. A. R. Gow. (2006). Candida albicans strain maintenance, replacement, and

microvariation demonstrated by multilocus sequence typing. *Journal of Clinical Microbiology, 44*(10), 3647-3658. doi:10.1128/JCM.00934-06

Ferguson, B. A., Dreisbach, T. A., Parks, C. G., Filip, G. M., & Schmitt, C. L. (2003). Coarse-scale population structure of pathogenic armillaria species in a mixed-conifer forest in the blue mountains of northeast oregon. *Canadian Journal of Forest Research, 33*(4), 612-623. doi:10.1139/x03-065

Fidel, P. L. (2007). History and update on host defense against vaginal candidiasis. *American Journal of Reproductive Immunology, 57*(1), 2-12.

Gaur, M., Puri, N., Manoharlal, R., Rai, V., Mukhopadhayay, G., Choudhury, D., & Prasad, R. (2008). MFS transportome of the human pathogenic yeast candida albicans. *BMC Genomics, 9*(1), 579.

Gow, N. A., Latge, J., & Munro, C. A. (2017). The fungal cell wall: Structure, biosynthesis, and function. *Microbiology Spectrum,*

Granger, B. L. (2012). Insight into the antiadhesive effect of yeast wall protein 1 of candida albicans. *Eukaryotic Cell, 11*(6), 795-805.

Hager, C. L., & Ghannoum, M. A. (2017). The mycobiome: Role in health and disease, and as a potential probiotic target in gastrointestinal disease. *Digestive and Liver Disease,*

Hampe, I. A., Friedman, J., Edgerton, M., & Morschhuser, J. (2017). An acquired mechanism of antifungal drug resistance simultaneously enables candida albicans to escape from intrinsic host defenses. *PLoS Pathogens, 13*(9), e1006655.

Hashash, R., Younes, S., Bahnan, W., El Koussa, J., Maalouf, K., Dimassi, H. I., & Khalaf, R. A. (2011). Characterisation of Pga1, a putative candida albicans cell wall protein necessary for proper adhesion and biofilm formation. *Mycoses, 54*(6), 491-500.

Hayek, P., Dib, L., Yazbeck, P., Beyrouthy, B., & Khalaf, R. A. (2010). Characterization of Hwp2, a candida albicans putative GPI-anchored cell wall protein necessary for invasive growth.*Microbiological Research, 165*(3), 250-258.

Hennicke, F., Grumbt, M., Lermann, U., Ueberschaar, N., Palige, K., Bttcher, B., . . . Monod, M. (2013). Factors supporting cysteine tolerance and sulfite production in candida albicans.*Eukaryotic Cell, 12*(4), 604-613.

Hernández, R., Nombela, C., Diez-Orejas, R., & Gil, C. (2004). Two-dimensional reference map of candida albicans hyphal forms. *Proteomics, 4*(2), 374-382.

Hewitt, V. L., Heinz, E., Shingu-Vazquez, M., Qu, Y., Jelicic, B., Lo, T. L., . . . Traven, A. (2012). A model system for mitochondrial biogenesis reveals evolutionary

rewiring of protein import and membrane assembly pathways. *Proceedings of the National Academy of Sciences, 109*(49), E3366.

Hickman, M. A., Zeng, G. S., Forche, A., Hirakawa, M. P., Abbey, D., Harrison. (2016). Corrigendum: The 'obligate diploid' candida albicans forms mating-competent haploids. *Nature, 530*(7589), 242. doi:10.1038/nature16134

Hillenkamp, F., Karas, M., Beavis, R. C., & Chait, B. T. (1991). Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Analytical Chemistry, 63*(24), 1203A.

Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G. D., . . . Bushman, F. D. (2013). Archaea and fungi of the human gut microbiome: Correlations with diet and bacterial residents. *PloS One, 8*(6), e66019. doi:10.1371/journal.pone.0066019

Hull, C. M., & Johnson, A. D. (1999). Identification of a mating type-like locus in the asexual pathogenic yeast candida albicans. *Science, 285*(5431), 1271-1275.

Hull, C. M., Raisner, R. M., & Johnson, A. D. (2000). Evidence for mating of the" asexual" yeast candida albicans in a mammalian host. *Science, 289*(5477), 307-310.

Jacob M. Hornby, Ellen C. Jensen, Amber D. Lisec, Joseph J. Tasto, Brandon Jahnke, Richard Shoemaker, . . . Kenneth W. Nickerson. (2001). Quorum sensing in the dimorphic FungusCandida albicans is mediated by farnesol. *Applied and Environmental Microbiology, 67*(7), 2982. doi:10.1128/AEM.67.7.2982-2992.2001

Jang, H. H., Lee, K. O., Chi, Y. H., Jung, B. G., Park, S. K., Park, J. H., . . . Yun, J. W. (2004). Two enzymes in one: Two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell, 117*(5), 625-635.

Kaloriti, D., Jacobsen, M., Yin, Z., Patterson, M., Tillmann, A., Smith, D. A., . . . Bohovych, I. (2014). Mechanisms underlying the exquisite sensitivity of candida albicans to combinatorial cationic and oxidative stress that enhances the potent fungicidal activity of phagocytes. *MBio, 5*(4), 1334.

Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Müller, W. H., Andel, A., Verkleij, A. J., . . . Klis, F. M. (2000). The cell wall architecture of candida albicans wild-type cells and cell wall-defective mutants. *Molecular Microbiology, 35*(3), 601-611.

Karas, M., & Krger, R. (2003). Ion formation in MALDI: The cluster ionization mechanism. *Chemical Reviews, 103*(2), 427-440.

Karkowska-Kuleta, J., & Kozik, A. (2015). Cell wall proteome of pathogenic fungi. *Acta Biochim Pol, 62*(3), 339-351.

Kerawala, C., & Newlands, C. (2014). *Oral and maxillofacial surgery* (Second edition. ed.). GB: Oxford University Press.

Kibbler, C. C., Seaton, S., Barnes, R. A., Gransden, W. R., Holliman, R. E., Johnson, E. M., . . . Wilson, J. A. (2003). Management and outcome of bloodstream infections due to candida species in england and wales. *Journal of Hospital Infection, 54*(1), 18-24.

Kim, J., & Sudbery, P. (2011). Candida albicans, a major human fungal pathogen. *The Journal of Microbiology, 49*(2), 171.

Klein, R. S., Harris, C. A., Small, C. B., Moll, B., Lesser, M., & Friedland, G. H. (1984). Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *New England Journal of Medicine, 311*(6), 354-358.

Klippel, N., Cui, S., Groebe, L., & Bilitewski, U. (2010a). Deletion of the candida albicans histidine kinase gene CHK1 improves recognition by phagocytes through an increased exposure of cell wall beta-1,3-glucans. *Microbiology (Reading, England), 156*(Pt 11), 3432. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20688824

Klippel, N., Cui, S., Groebe, L., & Bilitewski, U. (2010b). Deletion of the candida albicans histidine kinase gene CHK1 improves recognition by phagocytes through an increased exposure of cell wall β-1, 3-glucans. *Microbiology, 156*(11), 3432-3444.

Klis, F. M., Groot, P. D., & Hellingwerf, K. (2001). Molecular organization of the cell wall of candida albicans. *Medical Mycology, 39*(1), 1-8.

Kováčová, K., Degani, G., Stratilová, E., Farkaš, V., & Popolo, L. (2015). Catalytic properties of phr family members of cell wall glucan remodeling enzymes: Implications for the adaptation of candida albicans to ambient pH. *FEMS Yeast Research, 15*(2)

Latgé, J. (2007). The cell wall: A carbohydrate armour for the fungal cell. *Molecular Microbiology, 66*(2), 279-290.

Liu, T. T., Lee, R. E., Barker, K. S., Lee, R. E., Wei, L., Homayouni, R., & Rogers, P. D. (2005). Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in candida albicans. *Antimicrobial Agents and Chemotherapy, 49*(6), 2226-2236.

Lotz, H., Sohn, K., Brunner, H., Mhlschlegel, F. A., & Rupp, S. (2004). RBR1, a novel pH-regulated cell wall gene of candida albicans, is repressed by RIM101 and activated by NRG1.*Eukaryotic Cell, 3*(3), 776-784.

MacCallum, D. M., Castillo, L., Nather, K., Munro, C. A., Brown, A. J., Gow, N. A., & Odds, F. C. (2009). Property differences among the four major candida albicans strain clades. *Eukaryotic Cell, 8*(3), 373-387.

Magee, B. B., & Magee, P. T. (2000). Induction of mating in candida albicans by construction of MTLa and MTLα strains. *Science, 289*(5477), 310-313.

Martin, R., Hellwig, D., Schaub, Y., Bauer, J., Walther, A., & Wendland, J. (2007). Functional analysis of candida albicans genes whose saccharomyces cerevisiae homologues are involved in endocytosis. *Yeast, 24*(6), 511-522.

Martins, N., Ferreira, I., Barros, L., Silva, S., & Henriques, M. (2014). Candidiasis: Predisposing factors, prevention, diagnosis and alternative treatment. *Mycopathologia, 177*(5), 223-240. doi:10.1007/s11046-014-9749-1

Masuoka, J. (2004). Surface glycans of candida albicans and other pathogenic fungi: Physiological roles, clinical uses, and experimental challenges. *Clinical Microbiology Reviews, 17*(2), 281-310.

McCreath, K. J., Specht, C. A., & Robbins, P. W. (1995). Molecular cloning and characterization of chitinase genes from candida albicans. *Proceedings of the National Academy of Sciences, 92*(7), 2544-2548.

Mohan, d. V., & Ballal, M. (2008). Proteinase and phospholipase activity as virulence factors in candida species isolated from blood. *Revista Iberoamericana De Micologia, 25*(4), 208-210.

Morio, F., Loge, C., Besse, B., Hennequin, C., & Le Pape, P. (2010). Screening for amino acid substitutions in the candida albicans Erg11 protein of azole-susceptible and azole-resistant clinical isolates: New substitutions and a review of the literature. *Diagnostic Microbiology and Infectious Disease, 66*(4), 373-384.

Mrsă, V., Seidl, T., Gentzsch, M., & Tanner, W. (1997). Specific labelling of cell wall proteins by biotinylation. identification of four covalently linked O-mannosylated proteins of saccharomyces cerevisiae. *Yeast (Chichester, England), 13*(12), 1145-1154. doi:AID-YEA163>3.3.CO;2-P

Murillo, L. A., Newport, G., Lan, C., Habelitz, S., Dungan, J., & Agabian, N. M. (2005). Genome-wide transcription profiling of the early phase of biofilm formation by candida albicans.*Eukaryotic Cell, 4*(9), 1562-1573.

Nickel, W. (2003). The mystery of nonclassical protein secretion. *The FEBS Journal, 270*(10), 2109-2119.

Nickel, W., & Rabouille, C. (2008). Mechanisms of regulated unconventional protein secretion. *Nature Reviews Molecular Cell Biology, 10*(2), nrm2617.

Nobile, C. J., Fox, E. P., Nett, J. E., Sorrells, T. R., Mitrovich, Q. M., Hernday, A. D., . . . Johnson, A. D. (2012). A recently evolved transcriptional network controls biofilm development in candida albicans. *Cell, 148*(1), 126-138.

Noble, S., Suzanne M Noble, Brittany A Gianetti, & Jessica N Witchley. (1). Candida albicans cell-type switching and functional plasticity in the mammalian host. *Nature Reviews.Microbiology, 15*(2), 96; 96.

Nombela, C., Gil, C., & Chaffin, W. L. (2006). Non-conventional protein secretionin yeast. *Trends in Microbiology, 14*(1), 15-21.

Odds, F. C. (1988). *Candida and candidosis*. England: Retrieved from http://catalog.hathitrust.org/Record/000915985

Oura, T., & Kajiwara, S. (2010). Candida albicans sphingolipid C9-methyltransferase is involved in hyphal elongation. *Microbiology, 156*(4), 1234-1243.

Pande, K., Chen, C., & Noble, S. M. (2013). Passage through the mammalian gut triggers a phenotypic switch that promotes candida albicans commensalism. *Nature Genetics, 45*(9), 1088-1091.

Park, Y., Daniels, K. J., Pujol, C., Srikantha, T., & Soll, D. R. (2013). Candida albicans forms a specialized "sexual" as well as "pathogenic" biofilm. *Eukaryotic Cell, 12*(8), 1120-1131.

Patricia L. Carlisle, Mohua Banerjee, Anna Lazzell, Carlos Monteagudo, José L. López-Ribot, David Kadosh, & Aaron Mitchell. (2009). Expression levels of a filament-specific transcriptional regulator are sufficient to determine candida albicans morphology and virulence. *Proceedings of the National Academy of Sciences of the United States of America, 106*(2), 599-604. doi:10.1073/pnas.0804061106

Perlin, D. S. (2015). Echinocandin resistance in candida. *Clinical Infectious Diseases, 61*(suppl_6), S617.

Perlroth, J., Choi, B., & Spellberg, B. (2007). Nosocomial fungal infections: Epidemiology, diagnosis, and treatment. *Medical Mycology, 45*(4), 321-346.

Peters, B. M., Palmer, G. E., Nash, A. K., Lilly, E. A., Fidel, P. L., & Noverr, M. C. (2014). Fungal morphogenetic pathways are required for the hallmark inflammatory response during candida albicans vaginitis. *Infection and Immunity, 82*(2), 532-543.

Pfaller, M. A., & Diekema, D. J. (2007). Epidemiology of invasive candidiasis: A persistent public health problem. *Clinical Microbiology Reviews, 20*(1), 133-163.

Phan, Q. T., Myers, C. L., Fu, Y., Sheppard, D. C., Yeaman, M. R., Welch, W. H., . . . Filler, S. G. (2007). Als3 is a candida albicans invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biology, 5*(3), e64.

Pitarch, A., Abian, J., Carrascal, M., Sánchez, M., Nombela, C., & Gil, C. (2004). Proteomics-based identification of novel candida albicans antigens for diagnosis of systemic candidiasis in patients with underlying hematological malignancies. *Proteomics, 4*(10), 3084-3106.

Plaine, A., Walker, L., Da Costa, G., Mora-Montes, H. M., McKinnon, A., Gow, N. A., . . . Richard, M. L. (2008). Functional analysis of candida albicans GPI-anchored proteins: Roles in cell wall integrity and caspofungin sensitivity. *Fungal Genetics and Biology, 45*(10), 1404-1414.

Rappleye, C. A., Eissenberg, L. G., & Goldman, W. E. (2007). Histoplasma capsulatum α-(1, 3)-glucan blocks innate immune recognition by the β-glucan receptor. *Proceedings of the National Academy of Sciences, 104*(4), 1366-1370.

Roemer, T., Jiang, B., Davison, J., Ketela, T., Veillette, K., Breton, A., . . . Marta, C. (2003). Large-scale essential gene identification in candida albicans and applications to antifungal drug discovery. *Molecular Microbiology, 50*(1), 167-181.

Rogers, P. D., & Barker, K. S. (2003). Genome-wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in Candida albicans clinical isolates.*Antimicrobial agents and chemotherapy*, *47*(4), 1220-1227

Romn, E., Nombela, C., & Pla, J. (2005). The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen candida albicans. *Molecular and Cellular Biology, 25*(23), 10611-10627.

Russell, C., & Lay, K. M. (1973). Natural history of candida species and yeasts in the oral cavities of infants. *Archives of Oral Biology, 18*(8), 957-962. doi:10.1016/0003-9969(73)90176-3

Sandini, S., La Valle, R., De Bernardis, F., Macrì, C., & Cassone, A. (2007). The 65 kDa mannoprotein gene of candida albicans encodes a putative β-glucanase adhesin required for hyphal morphogenesis and experimental pathogenicity. *Cellular Microbiology, 9*(5), 1223-1238.

Sandini, S., Stringaro, A., Arancia, S., Colone, M., Mondello, F., Murtas, S., . . . De Bernardis, F. (2011). The MP65 gene is required for cell wall integrity, adherence to epithelial cells and biofilm formation in candida albicans. *BMC Microbiology, 11*(1), 106.

Saporito-Irwin, S. M., Birse, C. E., Sypherd, P. S., & Fonzi, W. A. (1995). PHR1, a pH-regulated gene of candida albicans, is required for morphogenesis. *Molecular and Cellular Biology, 15*(2), 601-613.

Sasse, C., Hasenberg, M., Weyler, M., Gunzer, M., & Morschhuser, J. (2013). White-opaque switching of candida albicans allows immune evasion in an environment-dependent fashion.*Eukaryotic Cell, 12*(1), 50-58.

Saville, S. P., Lazzell, A. L., Monteagudo, C., & Lopez-Ribot, J. L. (2003). Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of candida albicans during infection. *Eukaryotic Cell, 2*(5), 1053-1060.

Schaller, M., Borelli, C., Korting, H. C., & Hube, B. (2005). Hydrolytic enzymes as virulence factors of candida albicans. *Mycoses, 48*(6), 365-377.

Shah, A. H., Singh, A., Dhamgaye, S., Chauhan, N., Vandeputte, P., Suneetha, K. J., . . . Ghannoum, M. A. (2014). Novel role of a family of major facilitator transporters in biofilm development and virulence of candida albicans. *Biochemical Journal, 460*(2), 223-235.

Si, H., Hernday, A. D., Hirakawa, M. P., Johnson, A. D., & Bennett, R. J. (2013). Candida albicans white and opaque cells undergo distinct programs of filamentous growth. *PLoS Pathogens, 9*(3), e1003210.

Sobel, J. D. (1997). Vaginitis. *New England Journal of Medicine, 337*(26), 1896-1903.

Sohn, K., Urban, C., Brunner, H., & Rupp, S. (2003). EFG1 is a major regulator of cell wall dynamics in candida albicans as revealed by DNA microarrays. *Molecular Microbiology, 47*(1), 89-102.

Soll, D. R., & Daniels, K. J. (2016). Plasticity of candida albicans biofilms. *Microbiology and Molecular Biology Reviews, 80*(3), 565-595.

Sorgo, A. G., Heilmann, C. J., Dekker, H. L., Bekker, M., Brul, S., de Koster, C. G., . . . Klis, F. M. (2011). Effects of fluconazole on the secretome, the wall proteome, and wall integrity of the clinical fungus candida albicans. *Eukaryotic Cell, 10*(8), 1071-1081.

Stelzner, A. (1990). F. C. odds,candida and candidosis, A review and bibliography (second edition). X + 468 S., 97 abb., 92 tab. u. 22 farbtafeln. London—Philadelphia—Toronto—Sydney—Tokyo 1988. baillière tindall (W. B. saunders). £ 35.00. ISBN: 0–7020–1265–3. *Journal of Basic Microbiology, 30*(5), 382-383. doi:10.1002/jobm.3620300522

Stephen W. Martin, Lois M. Douglas, & James B. Konopka. (2005). Cell cycle dynamics and quorum sensing in candida albicans chlamydospores are distinct from budding and hyphal growth. *Eukaryotic Cell, 4*(7), 1191-1202. doi:10.1128/EC.4.7.1191-1202.2005

Sudbery, P. E. (2011). Growth of candida albicans hyphae. *Nature Reviews Microbiology, 9*(10), 737-748. doi:10.1038/nrmicro2636

Sudbery, P., Gow, N., & Berman, J. (2004). The distinct morphogenic states of candida albicans. *Trends in Microbiology, 12*(7), 317-324. doi:10.1016/j.tim.2004.05.008

Suzanne M Noble, Brittany A Gianetti, & Jessica N Witchley. (2017). Candida albicans cell-type switching and functional plasticity in the mammalian

host. *Nature Reviews. Microbiology, 15*(2), 96-108. doi:10.1038/nrmicro.2016.157

Tao, L., Du, H., Guan, G., Dai, Y., Nobile, C. J., Liang, W., . . . Huang, G. (2014). Discovery of a "white-gray-opaque" tristable phenotypic switching system in candida albicans: Roles of non-genetic diversity in host adaptation. *PLoS Biology, 12*(4), e1001830.

Tsai, P., Yang, C., Chang, H., & Lan, C. (2011). Characterizing the role of cell-wall β-1, 3-exoglucanase Xog1p in candida albicans adhesion by the human antimicrobial peptide LL-37.*PLoS One, 6*(6), e21394.

Tuch, B. B., Mitrovich, Q. M., Homann, O. R., Hernday, A. D., Monighetti, C. K., Francisco, M., & Johnson, A. D. (2010). The transcriptomes of two heritable cell types illuminate the circuit governing their differentiation. *PLoS Genetics, 6*(8), e1001070.

Uhl, M. A., Biery, M., Craig, N., & Johnson, A. D. (2003). Haploinsufficiency-based large-scale forward genetic analysis of filamentous growth in the diploid human fungal pathogen *C. albicans*. *The EMBO Journal, 22*(11), 2668-2678.

Urban, C., Xiong, X., Sohn, K., Schrppel, K., Brunner, H., & Rupp, S. (2005). The moonlighting protein Tsa1p is implicated in oxidative stress response and in cell wall biogenesis in candida albicans. *Molecular Microbiology, 57*(5), 1318-1341.

Vylkova, S., Li, X. S., Berner, J. C., & Edgerton, M. (2006). Distinct antifungal mechanisms: B-defensins require candida albicans Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. *Antimicrobial Agents and Chemotherapy, 50*(1), 324-331.

Warenda, A. J., & Konopka, J. B. (2002). Septin function in candida albicansMorphogenesis. *Molecular Biology of the Cell, 13*(8), 2732-2746.

Whiteway, M., & Bachewich, C. (2007). Morphogenesis in candida albicans. *Annu.Rev.Microbiol., 61*, 529-553.

Yergey, A. L., Coorssen, J. R., Backlund, P. S., Blank, P. S., Humphrey, G. A., Zimmerberg, J., . . . Vestal, M. L. (2002). De novo sequencing of peptides using MALDI/TOF-TOF. *Journal of the American Society for Mass Spectrometry, 13*(7), 784-791.

Younes, S., Bahnan, W., Dimassi, H. I., & Khalaf, R. A. (2011). The candida albicans Hwp2 is necessary for proper adhesion, biofilm formation and oxidative stress tolerance.*Microbiological Research, 166*(5), 430-436.

Zeng, G., Wang, Y., & Wang, Y. (2012). Cdc28–Cln3 phosphorylation of Sla1 regulates actin patch dynamics in different modes of fungal growth. *Molecular Biology of the Cell, 23*(17), 3485-3497.

Zohbi, R., Wex, B., & Khalaf, R. A. (2014). Comparative proteomic analysis of a candida albicans DSE1 mutant under filamentous and non-filamentous conditions. *Yeast*, *31*(11), 441-448.