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Proteomic Analyses of the Cell Walls of *Candida albicans* *pga1* and *pir32* Null Mutants

By

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Proteomic Analyses of the Cell Walls of *Candida albicans pga1* and *pir32* Null Mutants

Pamela Milad El Khoury

Abstract

Though residing asymptomatically in mammalian tissues and mucosal surfaces, specific environmental cues can induce the opportunistic fungus *Candida albicans* to become pathogenic resulting in severe systemic and disseminated infection. The cell wall of this fungus is dynamic and its architecture is constantly modified and adapted as many virulence attributes are localized to the cell surface. Pga1 and Pir32 are two cell wall proteins that have been characterized by our lab following the generation of *pga1* and *pir32* null strains and comparing their respective phenotypes to the parental wild type strain. The *pga1* null strain was less adherent and virulent than the wild type strain and exhibited reductions in chitin deposition, biofilm formation, filamentation, and resistance to stress. The *pir32* null strain on the other hand showed more pronounced phenotypes such as hyperfilamentation ability, and increased resistance to oxidative stress in part due to a two fold increase in chitin deposition. In this study, cell wall proteomic profiles for both mutant strains were established and compared with the parental strain in order to explain the previously observed phenotypes. Major virulence proteins like Hsp70 and Mp65 were not detected in the *pga1* null strain. Lipases (Lip6, 8, and 10), Sap1 and Exg2 functioning in host degradation and nutrient

collection, were also undetected. Additionally, the mutant appears to lack Sod5, Erg1, Cdc11 and other proteins that have roles in cell wall integrity and resistance to environmental stress and antifungal treatment. On the other hand, key virulence factors required for proper dissemination were detected exclusively in the *pir32* null strain. Such factors include the adhesin Als3, in addition to lipases, superoxide dismutases and secreted aspartyl protease family members. The mutant also appeared to be differentially expressing proteins involved in filamentous growth such as Cdc42 and Ssu81, explaining the observed hyperfilamentous phenotype. As such, cell wall proteomic analysis is a powerful tool that allowed us to identify key virulence traits responsible for the observed phenotypes of the mutant strains.

Keywords: *Candida albicans*, proteomics, CWPs, Pga1, Pir32, MALDI MS/MS

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Chapter 1

Literature Review

1.1 Overview of *Candida albicans*

Candida albicans had first been isolated by B. Langenbeck in the year 1839 from white spots of oropharyngeal thrush from a patient having typhoid fever but he mistakenly hypothesized that it is the causing agent of the typhoid fever. In 1847, Charles-Phillipe Robin discovered that *C. albicans* is the causing agent of thrush and named it *Odium albicans.* Investigators then named it *Monilia albicans*, and it wasn't until 1923 that this yeast was correctly classified by the Dutch mycologist Christine Berkhout and given the name *Candida albicans* (Espinel-Ingroff, 2003).

As an ascomycete fungus belonging to the family *Saccharomycetaceae, C. albicans* is related to the model organism *Saccharomyces cerevisiae* (Kim & Sudbery, 2011). Regardless of the common features between these two organisms, they have important differences that highlight the mechanisms that permit *C. albicans* to survive within a mammalian host (Johnson, 2003). The codon CUG usually encodes serine; however, in members of the CUG clade, including *C. albicans* and other related species of pathogenic yeasts, this codon encodes leucine (Butler et al., 2009; Santos & Tuite, 1995). Additionally and until very recently, this yeast is mainly diploid with a 13.3-13.4 Mb genome that encodes almost 6,200 genes distributed over 8 chromosomes (Braun et al., 2005). These genes are grouped into 21 families such as transcription factors, oligopeptide transporters, lipases, ferric reductases, cell wall mannoproteins, and adhesions (Butler et al., 2009).

C. albicans is typically found in many homeotherms as a benign commensal organism residing asymptomatically on the skin and on mucosal surfaces (Sorgo et al., 2010). *C. albicans*, along with other *Candida* species, colonize the buccal cavity of almost 75% of the population without causing any harm (Mayer et al., 2013).

In the gastrointestinal tract of healthy individuals, fungal colonization and invasion are prevented by the bacterial microbiome, particularly by the lactic acid bacteria; thus, *C. albicans* remains benign (Kennedy et al., 1985). After antibiotic treatment, with the broad-spectrum cefoperazone for example, long term effects are seen regarding the indigenous microbiota with increased *C. albicans* growth and higher risk of candidiasis (Antonopoulos et al., 2009). Researchers have found that *C. albicans* recovers *Bacteroidetes* population that had been diminished in the cecum during the cefoperazone treatment, promotes *Enterococcus faecalis* population in the stomach, and reduces *Lactobacillus* spp. population (Mason et al., 2011, 2012). This shows that as a benign commensal organism of the GI tract, *C. albicans* plays a role in altering the bacterial microbiota.

However, *C. albicans* becomes an intermittent pathogen following a microbial imbalance or an ecological shift in the microbial flora. Factors triggering such disturbance include hormonal imbalances, uptake of broad-spectrum antibiotics, excessive carbohydrates intake, and malnutrition (Williams et al., 2013). Patients of radio- and chemo-therapies, organ transplantation, xerostomia, endocrine disorders, or HIV infection are prone to developing a *C. albicans* infection which can range from local to systemic candidiasis (Gyurko et al., 2000; Sorgo et al., 2010).

1.2 Prevalence of Candidiasis and Epidemiology

Of all the fungi living on Earth, only 600 species are human pathogens (Brown et al., 2012). *C. albicans* is an opportunistic pathogen capable of causing a variety of infections in humans ranging from mild and superficial to severe and systemic (Mayer et al., 2013). Candidiasis (plural form is candidosis) is an infection mainly caused by *C. albicans* and other *Candida* related species including *C. lusitaniae, C. parapsilosis, C. tropicalis, C. glabrata* and *C. krusei.* Yet, *C. albicans* is the predominant agent, responsible for around 50% of all reported cases (Quindós, 2014).

Statistically, *C. albicans* is one of the most often identified agents of nosocomial infections and the third most commonly isolated pathogen from the bloodstream according to the Centers for Disease Control (Tsui et al., 2016). This opportunistic pathogen has been the cause of 250 to 400 thousand deaths per year worldwide (Da Silva Dantas et al., 2016).

1.2.1 Mucosal Infections

A mucosal infection caused by *C. albicans* is termed pseudomembranous candidiasis, or thrush. It can affect the mucosae of the vagina, oropharynx, esophagus, and GI tract and is characterized by the existence of white spots on top of an inflamed membrane (Kim & Sudbery, 2011).

Almost 75% of all women suffer from vulvo vaginal candidiasis (VVC) at least once throughout their life (Sobel, 2007) 5 to 8% of those women face a minimum of four VVC recurrences per year (Foxman et al., 1998). Diabetes mellitus, pregnancy, oral contraception, and hormone therapy are the leading predisposing factors for vulvovaginal candidiasis (Fidel, 2004). VVC is usually treated with azole antifungals like albaconazole and fluconazole (Kim & Sudbery 2011).

Oral-pharyngeal candidiasis (OPC) is highly abundant among AIDS patients to the extent that it is used as an indicator of AIDS development in HIV-positive people (Klein et al., 1984; Thanyasrisung et al., 2014). Also, OPC is very common in patients with head and neck cancers and xerostomia, as well as those that are terminally ill and cannot produce enough saliva (Qui et al., 2017; Runkee, 2002). OPC and other oral and esophageal infections are treated using azoles but fungal resistance is developing against them (Mushi et al., 2017).

1.2.2 Disseminated Candidiasis

Regardless of their frequency, superficial infections caused by *C. albicans* are basically non-lethal. However, systemic candidiasis is lethal as reflected by the elevated mortality rate in spite of the treatment with antifungal therapy (Perlroth et al., 2007). Normally, neutrophils provide protection against blood stream infections. However, in patients with neutropenia following immunosuppressant therapy or blood cancers, *C. albicans* starts growing and colonizing in the blood stream leading to candidemia. In turn, candidemia might lead to disseminated candidiasis in which internal organs get colonized as well (Kim & Sudbery 2011). Almost all organs can be infected by *C. albicans* leading to Candida endophthalmia of the eyes, Candida osteomyelitis of the bone, Candida endocarditis of the heart, hepatosplenic candidiasis of the liver and spleen, and several others (Noble et al., 2016). Risk factors other than neutropenia include hospitalization, surgeries, catheters, and invasive procedures that permit disruption of mucosae, fungal overgrowth, and biofilm formation as well as prolonged treatment with antibiotics which diminishes bacterial competitors thus facilitating *C. albicans* to spread and infect (Pfaller & Diekema, 2007).

1.3 Pathogenicity Mechanisms

Numerous factors contribute to the switching of *C. albicans* from commensalism to pathogenicity, and in causing superficial and systemic infections. Virulence factors in addition to fitness attributes enable the opportunistic fungus to infect diverse niches in the mammalian host (Mayer et al., 2013).

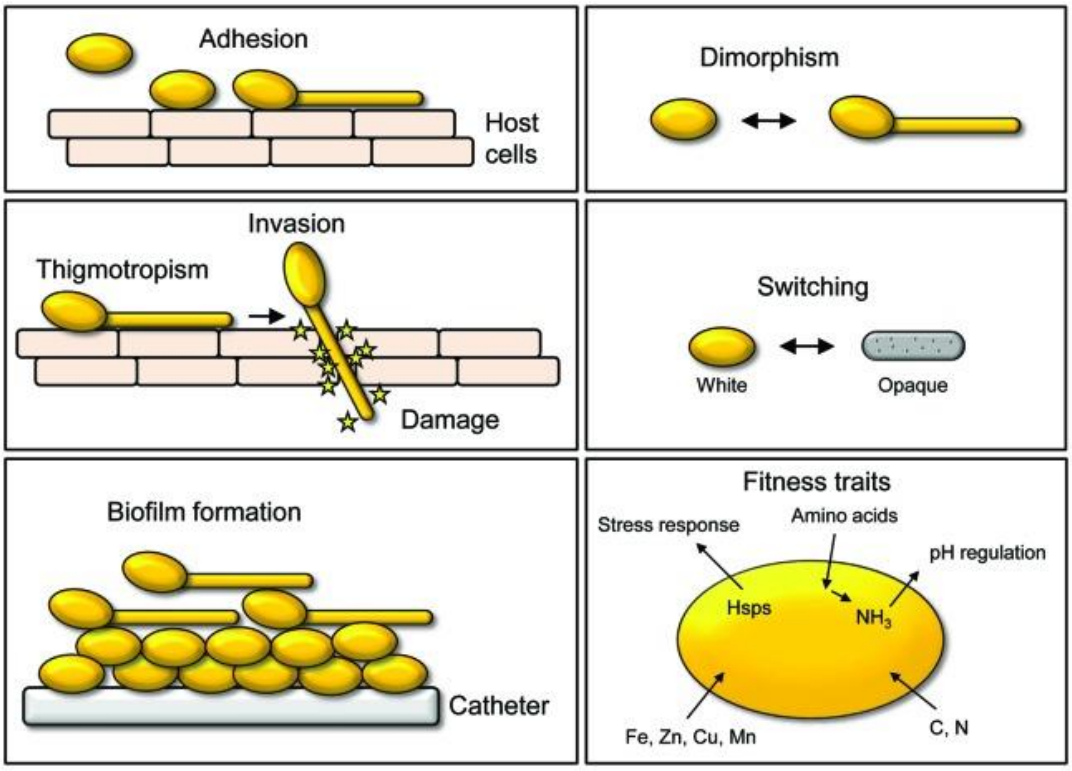

Figure 1. A representation of some *C. albicans* pathogenicity mechanisms. Adhesins are required for fungal attachment to the host cell surfaces. Upon contact, yeast cells transition into hyphae; then thigmotropism and invasins drive fungal-induced endocytosis into the underlying tissues and surfaces. When yeast cells attach to abiotic surfaces such as catheters or biotic surfaces, biofilms start forming consisting of yeast cells in the lower part and hyphae in the upper part. White-opaque switching is required for biofilm formation and antigenicity. *C. albicans* has developed many fitness traits that enable it to evade the host immune defenses. These include proteins involved in pH and stress responses, as well as nutrient and metal acquisition (Mayer et al., 2013).

1.3.1 Polymorphism

In response to varying environmental conditions, most fungal species undergo morphological changes such as the thermally dimorphic pathogens that can be found as multicellular and branched filaments in the soil and as unicellular buds in

mammalian hosts (Nemecek et al., 2006). Following this, and knowing that *C. albicans* does not proliferate outside a stable warm blooded host, the fact that this fungus has high morphological plasticity possessing at least 9 different cell shapes is surprising (Noble et al., 2016). Yeasts, pseudohyphae, hyphae, and chlamydospores are the first described, classical cell types and they differ in shape, occurrence, way of division, and virulence capability (Sudbery et al., 2004).

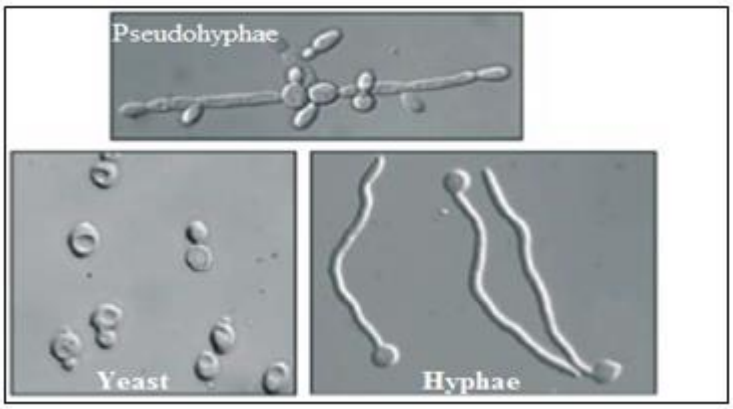

Figure 2. DIC images of *C. albicans* cells with pseudohyphal, yeast, and hyphal growth forms. Yeasts are round cells, hyphae are elongated cells arranged in filaments, while pseudohyphae are thought to be a transition phase between both morphologies (Sudbery et al. 2004).

Yeasts are round, white cells that reproduce by budding where nuclear division and cytokinesis completely separate the daughter cell from its mother cell (Warenda & Konopka, 2002). Yeasts are unicellular and their favored growth conditions include a temperature of 30℃ and an acidic pH of 4 (Kim & Sudbery 2011).

Hyphae are chains of tubular cells that remain attached after cytokinesis, and following several cycles of cell division they generate mycelia; i.e. multicellular, branched filaments (Sudbery et al., 2004). Hyphal growth is favored at 37℃, neutral pH, availability of serum, high concentration of carbon dioxide, and presence of N-acetylglucosamine (Sudbery, 2011).

Pseudohyphal cells have an ellipsoid shape and are intermediates in morphology as well as in growth conditions between the already described cell types;

thus they are commonly viewed as a transitional stage. These cells are more related to yeasts than they are to hyphae when compared on the basis of polarized growth, septum hydrolysis following cytokinesis, and cell cycle organization (Crampin et al., 2005). Pseudohyphal growth is favored at 35℃ and a pH of 5.5 (Carlisle et al., 2009).

Chlamydospores are thick-walled spherical cells with a relatively large size when compared to the previously mentioned cell types (Jansons & Nickerson, 1970). They are produced by the suspensor cells of the mycelia only under extremely harsh conditions like hypoxia and starvation (Whiteway & Bachewich, 2007).

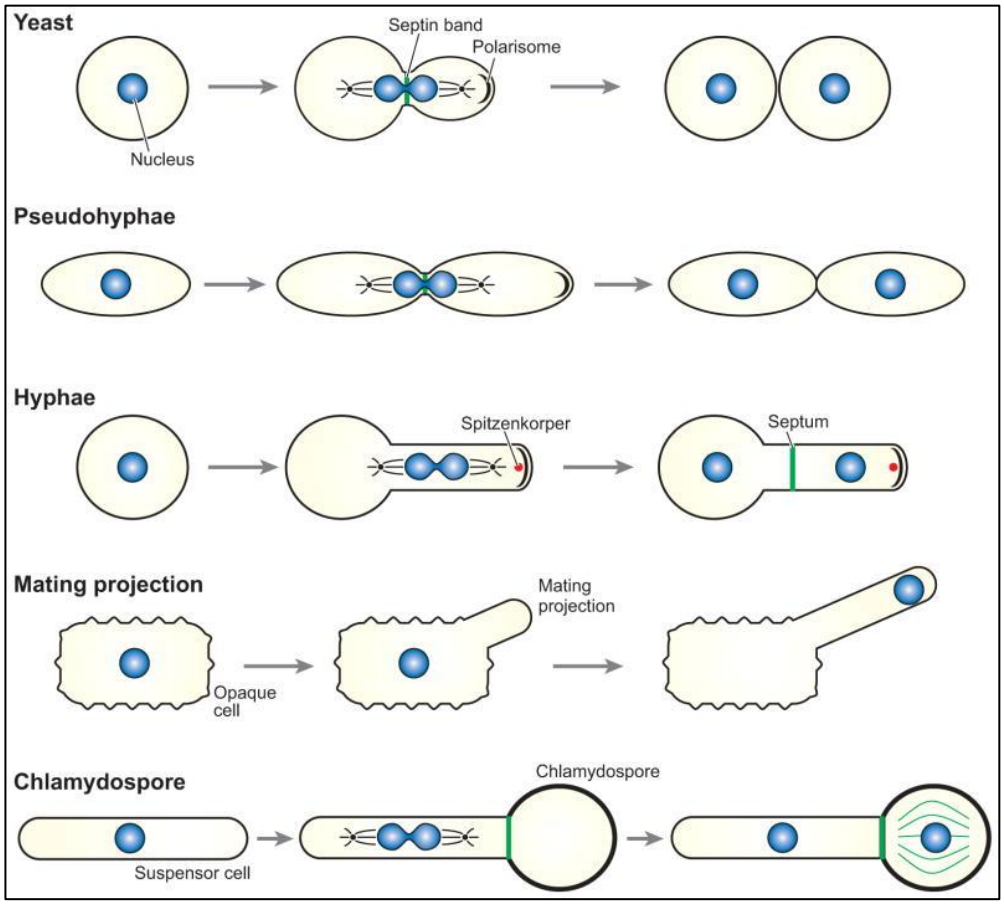

Figure 3. Distinct structural characteristics of the main *C. albicans* morphological forms. Yeasts reproduce by budding that gives completely separated mother and daughter cells at the septin band. The polarisome at the end of the growing bud defines polarized growth of these cells. Pseudohuphae grow in the same manner however, the mother and daughter cells are not completely separated after cytokinesis and the cells are much more elongated. Hyphae are chains of tubular cells that remain attached after cytokinesis, and are characterized by a Spitzenkorper at the end of the growing hyphae. The mating opaque cells produce a shmoo, an elongated mating projection, so that the nucleus can

migrate across this structure and fuse with the nucleus of the other mating competent cell. Chlamydospores form at the ends of suspensor cells. The nucleus divides inside the suspensor cell, then it migrates across the septin band into the chlamydospore (Whiteway & Bachewhich, 2007).

The purpose behind pseudohyphae and chlamydospores formation is not very well understood, but yeasts and hyphae are well characterized (Sudbery et al., 2004). The morphological switching between yeast and hyphae is named dimorphism where it has been suggested that the two growth types are essential for developing and maintaining pathogenicity by *C. albicans* (Jacobsen et al., 2012). On one hand, the hyphal form has been proven to be needed for invasion more than the yeast type, and on the other hand, the yeast type has been proven to be required for dissemination (Saville et al., 2003). Initially, it was believed that only the hyphal form is required for the *C. albicans* to divert to the pathogenic state since when mutants being locked into the yeast form were found to be avirulent (Lo et al., 1997). However, following the construction of mutants that are locked in the hyphal form, these mutants were surprisingly avirulent as well (Braun et al., 2000). Thus, it is now commonly hypothesized that dimorphism, rather than any specific morphology, is the key factor for virulence and subsequent development of disseminated candidiasis though molecular data clearly depicting this relation is still lacking (Gow et al., 2002). Also, pseudohyphae are believed to play an important role in biofilm formation and virulence mainly during candidemia (Ramage et al., 2009); but chlamydospores are induced in vitro and hardly ever isolated from specimens indicating that their role is still unclear (Alicia et al., 2006).

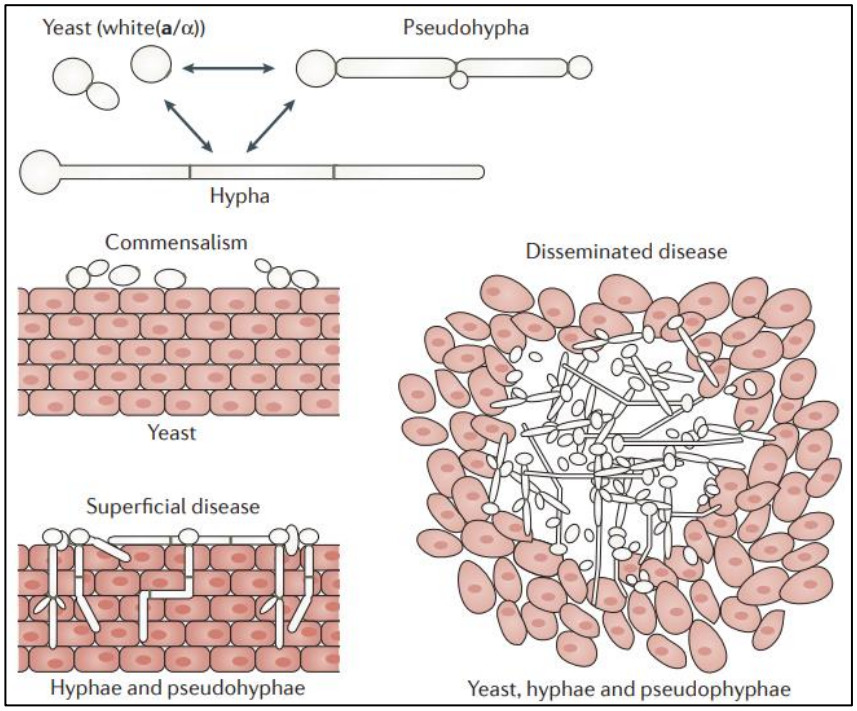

Figure 4. The reversible transition between the different morphological forms is essential for the virulence of *C. albicans*. This reversible transition is under the effect of different environmental cues. As a commensal colonizer of mammalian surfaces, *C. albicans* exists mainly in its yeast form. Invasion and damage are associated with hyphal and pseudohyphal forms especially in mucocutaneous infections. In disseminated infections, all three morphologies are present as they all contribute for disease development (Noble et al., 2016).

In addition to the already described round white yeast cells, *C. albicans* can transition into other yeast-like types with distinct characteristics and roles in host interactions; mainly opaque, grey, and GUT cells (Noble et al., 2016).

In comparison to the white cells, the opaque cells are more elongated as they are rather ellipsoidal in appearance, larger in volume since they occupy triple the space, and have surface pimples (Douglas, 2003). In addition to these structural differences, opaque cells are more resistant to the actions of the host phagocytes and as such can evade the immune system while being more sensitive to filamentation favoring cues (Sasse et al., 2013; Si et al., 2013). They also have differences in gene expressions mainly of the genes functioning in respiration and mating (Tuch et al. 2010). The transition from white to opaque yeast-like form is essential for mating and

is favored in the presence of N-acetylglucosamine, high levels of carbon dioxide, and an acidic medium (Huang et al., 2009; Huang et al., 2010; Sun et al., 2015). It had been believed that *C. albicans* is a diploid asexual organism with no signs of meiosis and sporulation. But in the year 2000, researchers have reported few tetraploid cells thus hinting for infrequent-mating between a and α opaque cells (Magee et al., 2000). Most *C. albicans* are heterozygous for the mating type loci (MTL a/α) and thus are unable to mate and locked in the white phase. However, isolates that are homozygous (MTL a/a or MTL α /α) can switch to the opaque phase and become mating competent. Mating cells form a shmoo through which they can exchange genetic material (Whiteway & Bachewhich, 2007; Kim & Sudbery 2011).

Grey *C. albicans* have an ellipsoidal shape and are the smallest morphotype. They lack any pimples and have a reduced mating efficiency. Upon high nutrient availability, transition to the grey type is induced. These grey cells possess high fitness on host epithelial surfaces especially in tongue infections where they have the fastest doubling time (Tao et al., 2014).

As for the GUT cells, they are also ellipsoidal in shape where the colonies are much darker and flatter than those of the white cells. They lack surface pimples and cannot mate. This cell type has been hypothesized to be particularly essential for commensalism in the mammalian GI tract where the host induces the expression of *WOR1* in *C. albicans* yeasts thus switching from the white to the GUT type. This hypothesis has been supported by the switching of the GUT cells back to the white form when present outside the mammalian host and by the lack of virulence of the GUT cells in the bloodstream (Pande et al., 2013).

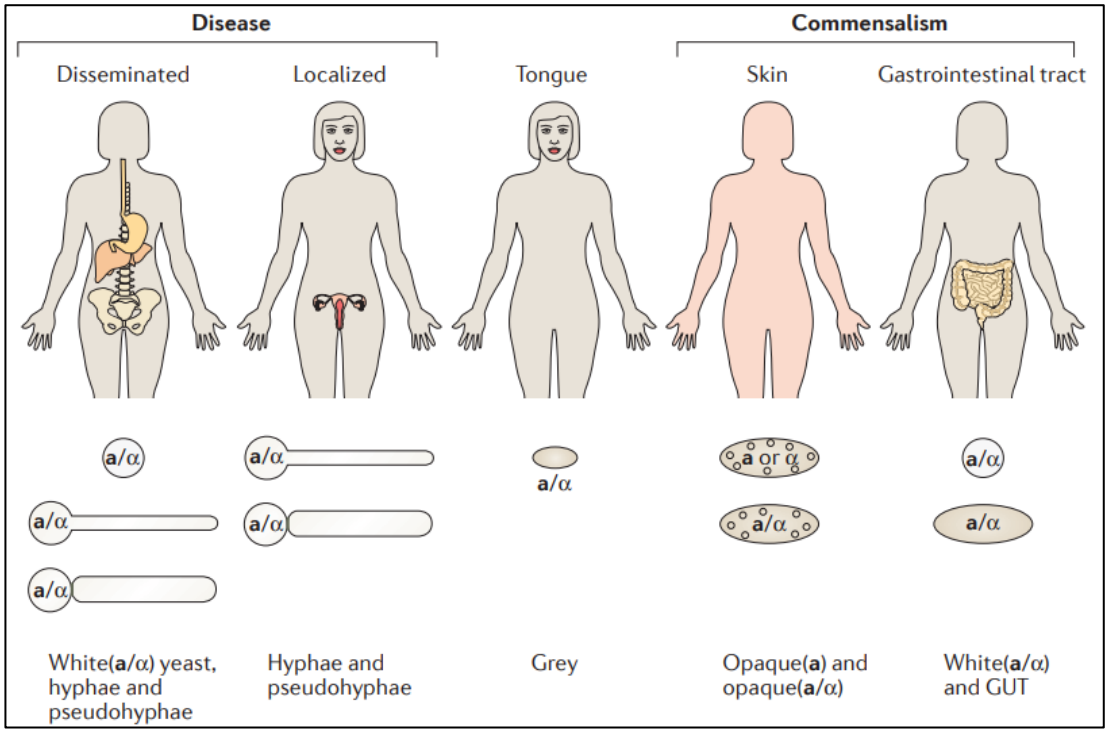

Figure 5. Diagram showing the niche preferred by each cell morphology in relation to disease and commensalism states. In disseminated diseases, white yeast, hyphae, and pseudohyphae are all needed while in localized infections such as oral candidiasis, only hyphae and pseudohyphae are required. In tongue infections, grey cells are the fastest proliferating form. Opaque cells are outcompete the other forms in skin colonization, while white cells and gastrointestinally induced transition cells have higher fitness in the gastrointestinal tract than the other forms (Noble et al. 2016).

1.3.2 Adhesins and invasins

Adherence of *C. albicans* cells to other fungal cells, microorganisms, host cells, and abiotic surfaces is mediated by a specialized group of proteins called adhesins (Verstrepen & Klis, 2006). These adhesins vary with respect to the morphological type of the fungus (Chaffin, 2008). Initially, yeast cells contact a surface and adhere to it, then hyphae start growing from them to invade across the surface. It is at this stage that the hyphal-expressed adhesins become major determinants of pathogenesis and infection extent (Moyes et al., 2015).

The most studied adhesins are the 8 proteins of the ALS family. One of these proteins is Als3, a hypha-associated protein that is overexpressed during vaginal and oral infections (Cheng et al., 2005). Another hyphal adhesin is Hwp1 that is required

for acting on the mammalian transglutaminases during adhesion to epithelial surfaces (Sundstrom et al., 2002). Various other adhesins are morphology-independent such as Iff4, Mp65, Int1 and much more (Naglik et al., 2011).

Many proteins function as invasins. Ssa1 is a major heat shock protein involved in interacting with the host cadherin thus promoting endocytosis of the fungus (Dalle et al., 2010). Als3 is also an invasin aiding Ssa1 during endocytosis (Cheng et al., 2005). Following proper adhesion of *C. albicans* to host cell surfaces hyphae start secreting hydrolases to actively penetrate into these cells and to acquire enough extracellular nutrient supply. Such proteins belong to the three families: secreted aspartic proteases (Sap1-10), lipases (Lip1-10), or phospholipases (5 classes: A, B, C, and D) all of which are required for pathogenicity (Mayer et al., 2013).

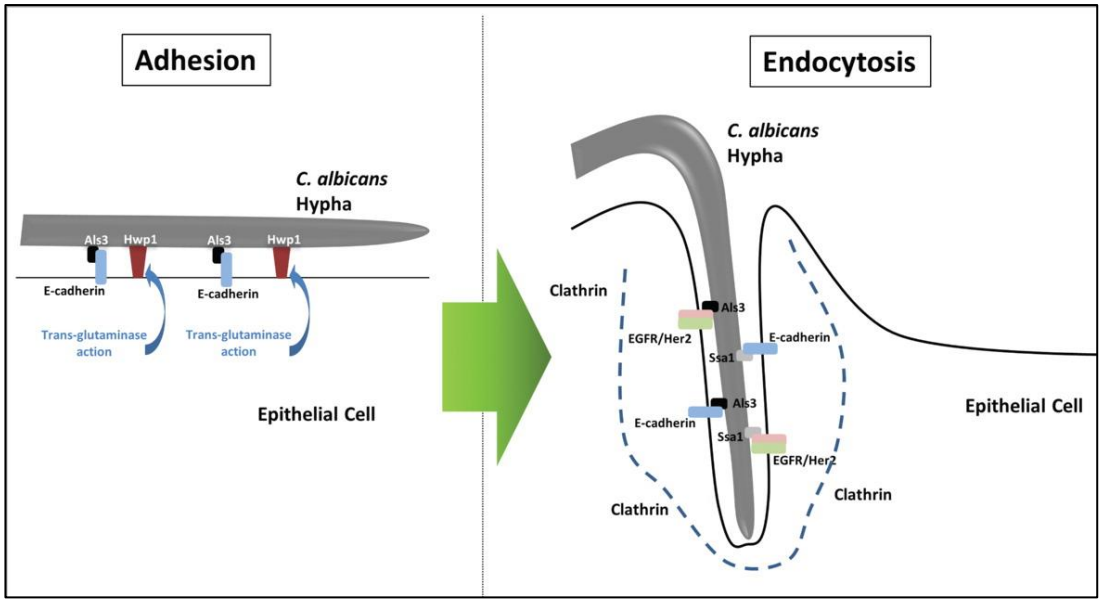

Figure 6. Some proteins required for proper adhesion and endocytosis of hyphal *C. albicans* by epithelial cells. Als3 is an important adhesin that allows *C. albicans* to attach to the host by binding its E-cadherin receptor. Hwp1 is another protein that allows adhesion after it gets processed by host trans-glutaminases. Following this contact, the fungus drives its own endocytosis and uptake into the host cell by the action of its various invasins. Als3 also acts in this process along with Ssa1 that interact with EGFR/Her2 heterodimer and E-cadherin of the host respectively. As such, clathrin is recruited to form the invasion pocket (Moyes et al., 2015).

1.3.3 Biofilm formation

A major virulence factor of *C. albicans* is its ability to form biofilms on abiotic (catheters and dentures) and biotic (skin and mucosae) surfaces (Fanning & Mitchell 2012). Biofilms start forming sequentially starting with the adherence of the yeast cells to their substrate, followed by their proliferation, formation of hyphae, accumulation of extracellular matrix, and ending in the dispersal of the more virulent yeast cells from this complex architecture (Finkel & Mitchell, 2011). This complex structure provides resistance to antifungal drugs and host defense stresses thereby contributing to disseminated infections (Uppuluri et al., 2010).

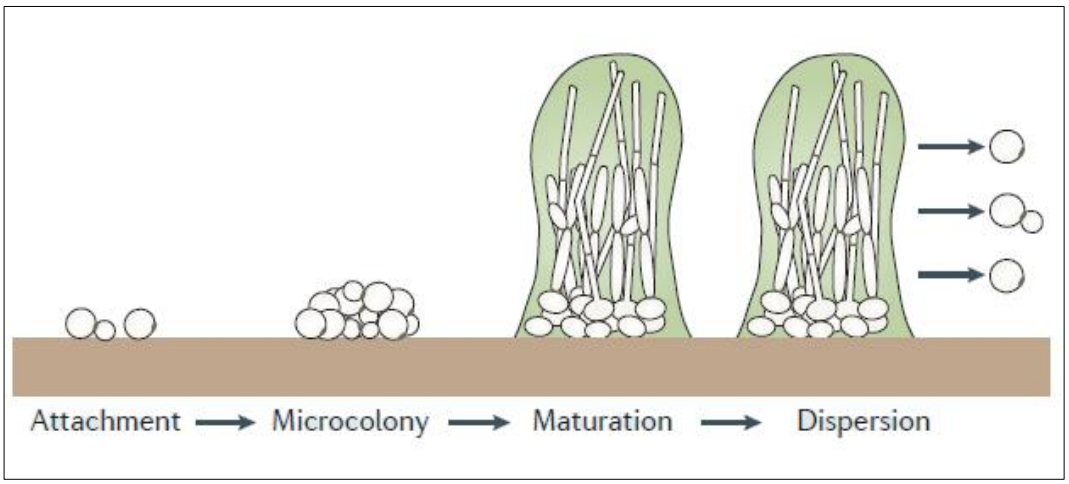

Figure 7. Life cycle of *C. albicans* biofilm. Biofilm formation is induced upon yeast attachment to a solid surface. They start proliferating and arranging into a microcolony. Hyphal and pseudohyphal forms start developing on the apical part to form the mature biofilm that contains proteins, nucleic acids, and polysaccharides as extracellular matrix components. White yeasts start detaching from the formed biofilm in order to spread and reattach to other surfaces (Noble et al., 2016).

It has also been supported that *C. albicans* can form sexual biofilms that facilitate mating process between competent cells by putting them in close proximity (Noble et al., 2016).

Various transcriptional factors contribute to biofilm formation like Tec1, Bcr1, Efg1, Rob1, Ndt80 and Brg1. The major regulator of dispersion and antifungal resistance of biofilms is Hsp90 (Nobile et al., 2012).

1.3.4 Contact sensing and thigmotropism

Hyphal growth and biofilm formation are triggered by contact sensing. When *C. albicans* contacts a biotic surface it starts forming hyphal filaments that invade underlying strata. If it contacts an abiotic surface it rather forms a biofilm (Kumamoto, 2008). As described previously in the polymorphism section and in Figure 8, several signals induce hyphal growth by activating various signaling pathways. In addition to these environmental cues, dimorphism is regulated by quorum sensing such that upon high cell densities yeast growth is favored while upon low cell densities the hyphal growth is promoted (Mayer et al., 2013). If the surface contacted by *C. albicans* has ridges, hyphae will start developing in a directional pattern; a process called thigmotropism essential for full epithelial damage and virulence (Brand et al., 2008).

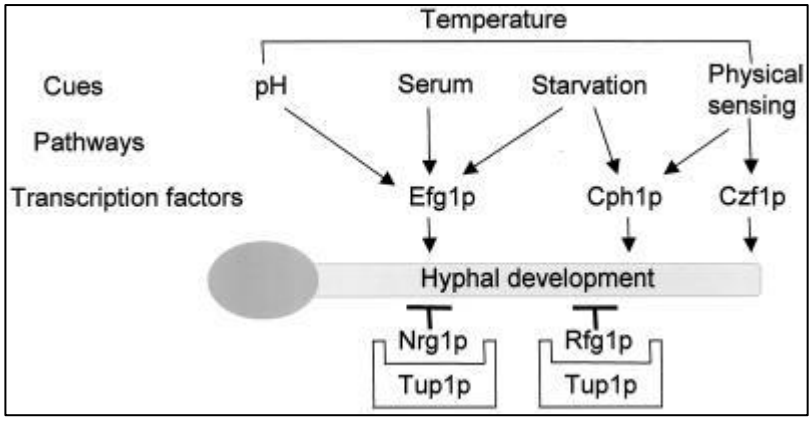

Figure 8. Schematic representation of the regulators of hyphal growth in *C. albicans.* Aside from the transcriptional activators (Efg1, Cph1, and Czf1) and the transcriptional repressors (Tup1, Nrg1 and Rfr1), there are various environmental cues that can affect hyphal development (Saville et al., 2003).

1.3.5 Fitness attributes

Since *C. albicans* can colonize a wide variety of organs and niches in the mammalian host, it is subjected to different environmental conditions. To begin with, this fungus is subjected to varying pH values ranging from slightly alkaline pH of the blood to the high acidity of the vagina and the varying pH of the GI tract (Davis, 2009). Phr1 and Phr2 are cell wall glycosidases required for adapting to changing pH (Fonzi,

1999). In addition to sensing the environmental pH, *C. albicans* can control and modify this pH by alkalizing it under nutrient scarcity and by inducing hyphal development (Vylkova et al., 2011).

Nutrient availability varies with respect to the niche as well. Gluconeogenesis, glycolysis, and starvation responses add up to the fungal ability to colonize the host cells and cause infections. During disseminated candidiasis, *C. albicans* accesses the bloodstream which is very rich in glucose (Mayer et al., 2013). However, once phagocytized by a macrophage or neutrophil, the environment becomes completely altered for the fungus since the phagocyte produces highly reactive intermediates and antimicrobial peptides while restricting the availability of nutrients. The fungus adapts to this hostile environment by switching to the hyphal form and utilizing lipids and amino acids as energy sources (Lorenz et al., 2004). *C. albicans* detoxifies the produced reactive oxygen species (ROS) by the actions of superoxide dismutases and catalases such as Sod1 and Cta1 (Mayer et al., 2013). Following those adaptations, the fungus can drive its own expulsion from the phagocyte, or escape it after hyphal induced lysis or pyroptosis (Da Silva Dantas et al., 2016).

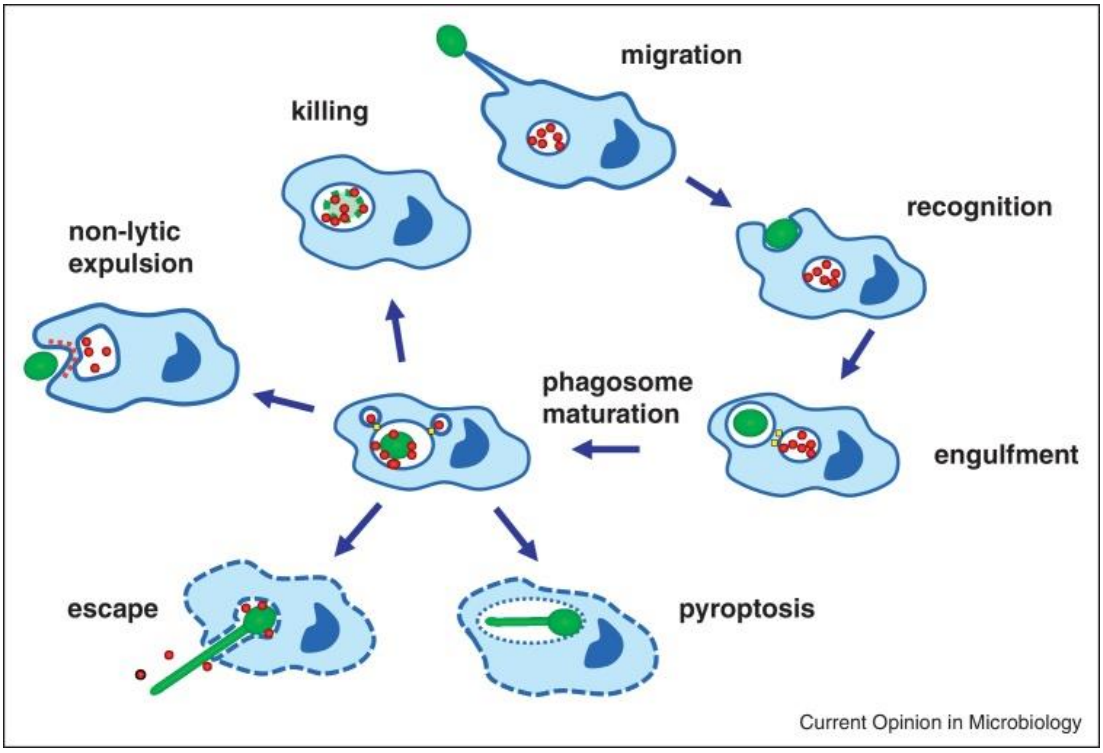

Figure 9. Ways by which *C. albicans* can escape the phagosome. A macrophage targets the fungus by chemotaxis, and after fungal recognition it starts engulfing it. The phagosomal vesicle containing the engulfed fungus fuses with lysosomes and puts the fungus under oxidative and nitrosative stresses. *C. albicans* has the ability to resist such harsh conditions as it detoxifies oxidative and nitrosative elements and drives its own expulsion from the phagolysosome. It can also lyse the phagocyte by pyroptosis and forming hyphae (Da Silva Dantas et al., 2016).

Metal acquisition is another pathogenicity mechanism whereby the fungus tries to utilize the various metals from its environment in order to grow and disseminate. Those metals include iron, zinc, copper and manganese that are needed for the correct functioning of various enzymes (Hood & Skaar., 2012). *C. albicans* can acquire iron from hemoglobin; a process mediated by the hemoglobin receptor Rbt5 and the heme oxygenase Hmx1 (Almeida et al., 2008; Okamoto-Shibayama et al., 2014). It can also utilize free iron present in its surrounding environment and iron in the form of transferrin through the reductive uptake system consisting of ferric reductases, multicopper oxidases, and iron permeases. The adhesin and invasin Als3 was also found to mediate the iron acquisition process from ferritin (Almeida et al., 2008).

Also, *C. albicans* has lots of heat shock proteins (Hsps) that help it adapt to stressful conditions by acting as chaperones that bind to their substrates and subsequently prevent protein unfolding and aggregation. Heat shock proteins in this fungus are distributed into 5 major families: small Hsps, Hsp60s, Hsp70s, Hsp90, and Hsp100s (Ritcher et al., 2010).

1.4 Cell Wall Organization

Whether hyphal or yeasts, *C. albicans* cells are enveloped by a dynamic cell wall that is continuously amended upon growth and morphogenesis. In addition to providing shape, this cell wall also protects the fungus against osmotic pressure and other chemical and physical aggressions and contains many proteins acting as adhesins and immunomodulators (Castillo et al., 2008). The cell wall is integral for fungal survival and virulence as it contains many proteins involved in virulence pathways and several pumps required for drug resistance. In a yeast, more than 20% of its genes regulate cell wall structure and function (Gow et al., 2017).

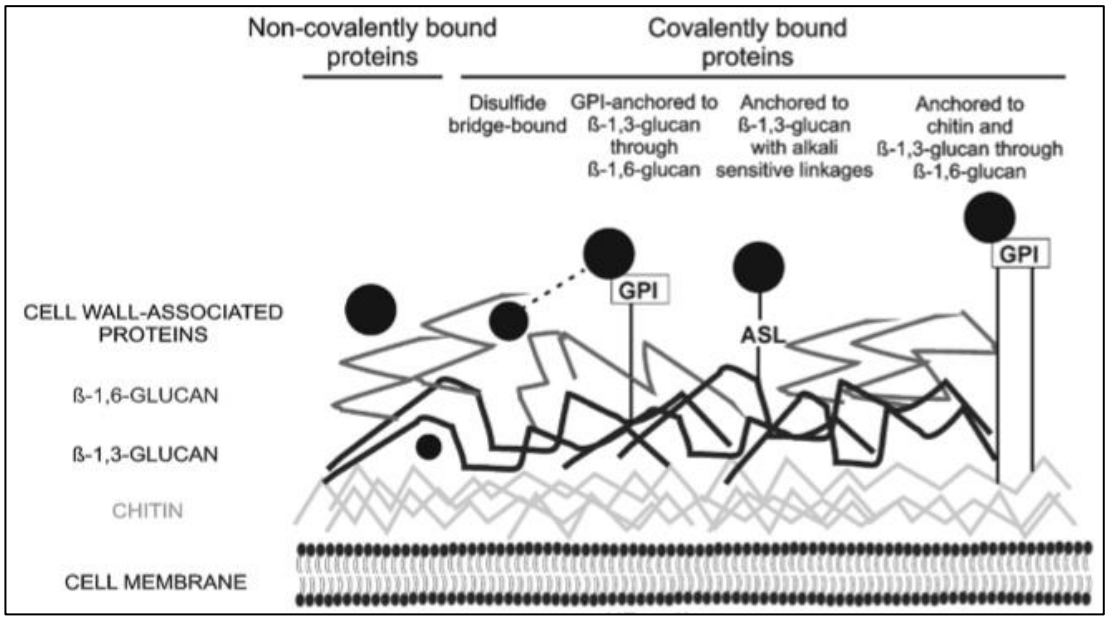

Figure 10. *C. albicans* cell wall structure and major types of CWPs. The inner layer is composed of chitin, the two consecutive layers consist of β-1,3-glucans and β-1,6-glucans. The outer layer mannan

layer contains proteins with diverse linkages to the underlying chitin and β-1,3-glucan layers (Karkowska-Kuleta & Kozik, 2015).

As shown in Figure 10, the cell wall is composed of 4 layers where the inner one is formed of chitin also known as N-acetylglucosamine, followed by a layer of β-1,3-glucan, then a layer of β-1,6-glucan, and ending in an outer layer of mannan (Kapteyn et al., 2000).

Thirty percent of the total cell dry weight is represented by the cell wall where glucans are the main constituents and chitin is the minor constituent. These two constituents are absent from the host; therefore, they are potential targets, along with their biosynthetic pathways, for antifungal drugs (Shepherd, 1987).

Proteins represent up to 40% of the cell wall and govern the cell surface properties required for adhesion, fighting the host defenses, and overall virulence (Chaffin et al., 1998). β-1,3-glucan molecules are branched with many non-reducing ends thus forming a three-dimensional matrix around the cell. Accordingly, these ends act as attachment sites to which cell wall proteins (CWPs) bind as seen in Figure 11. Major CWPs are glycosylphosphatidylinositol-dependent proteins (GPI-CWPs) and proteins with internal repeats (Pir-CWPs) (Kapteyn et al., 1999). GPI-CWPs attach indirectly to β-1,3-glucan branches by first attaching to the β-1,6-glucan via a GPI structure. On the other side, Pir-CWPs attach directly to β-1,3-glucan branches by alkali-labile linkages (Kollár et al., 1997).

Most CWPs function in host adhesion including lectins and adhesins (Gow et al., 2002). Not all CWPs are covalently linked to the cell wall. Some proteins are non-covalently linked by disulfide bridges or secreted. Lipases and proteases are secreted proteins that aid in fungal invasion and endocytosis by degrading the external structures of the host membranes (Chaffin, 2008). Mannosidases and chitinases act during cell wall synthesis and remodeling processes thus influencing cell wall rigidity

and fungal resistance to environmental stresses. A wide variety of proteins acting as virulence markers are associated with this dynamic cell wall (López-Ribot et al., 2004).

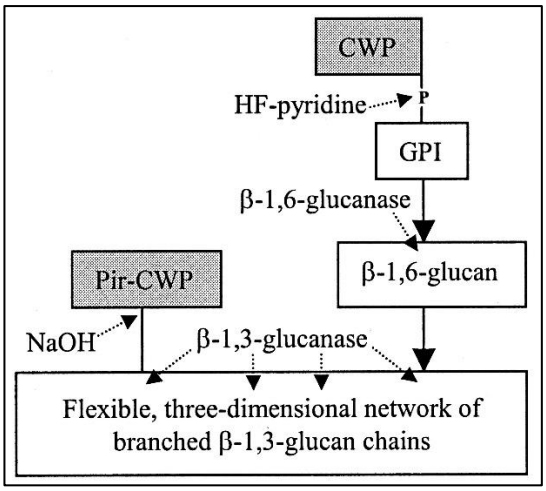

Figure 11. *C. albicans* contains two classes of mannoproteins that are covalently linked to the β-1,3-glucan branches of the cell wall. Some CWPs have a GPI anchor at their C-termini that attaches them to the these branches via a β-1,6-glucan chain. Other CWPs are called Pir-CWPs because they have internal repeats and are covalently linked to the β-1,3-glucan branches via alkali-labile bonds. Dotted arrows indicate the linkages that can be broken upon the respective chemical or enzymatic treatments (De Groot et al., 2004).

As such, the cell wall is a rigid structure that provides *C. albicans* with mechanical stability and shape maintenance. The spatial and temporal arrangement of the cell wall components determines the final morphological type of the fungus (Shepherd, 1987). Hyphae show tripled level of chitin within the cell wall and increased amounts of glucans when compared to yeast cells (Chattaway et al., 1968).

1.5 Pga1: a GPI-CWP

Pga1 belongs to a family of putative GPI anchored (PGA) proteins consisting of 65 members where the majority are still uncharacterized so far (Hashash et al., 2011). These proteins have a hydrophobic domain at their C-termini that is cleaved off post-translationally and replaced with a GPI anchor, a lipid preformed in the

endoplasmic reticular membrane by a transamidase enzyme complex (De Groot et al., 2003; Plaine et al., 2008). Then, this GPI anchor either directly links the protein to the chitin layer or links it indirectly to the cell wall by first binding to the β-1,6-glucan that is in turn attached to the β-1,3-glucan branches (Kollár et al., 1997). As such, PGA proteins are exposed on the external surface of the fungus thus contributing to fungal adhesion and invasion of host surfaces as well as cell wall assembly and virulence (Plaine et al., 2008).

Pga1, a 133 amino acid long CWP, has an amidated asparagine as a GPI anchor. Similar to ideal CWPs, Pga1 has a signal peptide of 19 amino acid residues at the N-terminus to drive its secretion (Ruiz-Herrera et al., 2006). Our lab had previously characterized Pga1 by means of homologous marker cassette recombination. Pga1 was found to play major roles in virulence, adhesion, chitin deposition, biofilm development, and resistance to stresses (Hashash et al., 2011).

1.6 Pir32: a Pir-CWP

Pir1 and Pir32 are the only members of the Pir family of proteins in *C. albicans* (Bahnan et al., 2012). These proteins are highly O-glycosylated and attached to the β-1,3-glucan branches by base-labile bonds, possibly by O-glycosidic linkages. They include a signal peptide at the N-terminus, a variable number of 2 till 11 internal repeats, a Kex2 site, and 4 cysteine residues (C) at the C-terminus located as follows: repeats-C–66aa–C–16aa–C–12aa–C–COOH (Ruiz-Herrera et al., 2006). The signal peptide leads this protein to the cell wall for secretion under proper conditions, and the Kex2 site is a proregion that is believed to keep the protein in an inactive state until it gets cleaved by a proprotein convertase (Newport & Agabian, 1997).

The first member in this family is Pir1 that has been found to be encoded by an essential gene in *C. albicans* since homozygous null mutants were inviable. This protein is required for maintaining the stability and rigidity of the cell wall since heterozygous mutant cells were elongated and formed clumps with deteriorated growth rate and hypersensitivity to disrupting agents (Martínez et al., 2004).

Pir32, a 422 amino acid CWP, follows this consensus sequence for the 4 cysteines, and its signal peptide is located at position 1-21 amino acids as indicated in Uniprot (retrieved on November 20, 2017). Our lab had previously characterized Pir32 by creating a *pir32* null mutant *C. albicans* strain in a way similar to the one used for characterizing Pga1. This protein was found to be involved in various pathogenicity mechanisms and cell wall proteome pathways. The null mutant was found to have increased virulence, doubled chitin deposition, elevated resistance to stress responses, and enhanced filamentation ability (Bahnan et al., 2012).

1.7 MALDI-TOF/TOF-MS/MS

Protein identification through mass spectrometry (MS) is crucial in proteomic profiling studies and is achieved by peptide mass fingerprinting (PMF) or by peptide sequence analysis. In the former method, the mass to charge ratios of peptides in a sample are determined by a matrix assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF) and then compared to theoretical ones in a database in order to identify the unknown protein. In the second method, peptides are subjected to tandem mass spectrometry (MS/MS) after which they become fragmented into daughter ions. The mass to charge ratios of these provide the amino acid sequences of

the unknown peptides. Then these sequences along with their masses are compared to databases for protein identification (Damodaran et al., 2007).

MS has been recently incorporated into routine laboratory techniques for the fast identification of infection causing microorganisms (Putignani et al., 2011). Researchers are using this method for correctly identifying *Candida* at the species level from clinical isolates (Pulcrano et al., 2013). In particular, MS/MS has been applied to test the effect of different surface stresses on *C. albicans* cell wall proteome (Heilmann et al., 2012). Also, it has been used to characterize *C. albicans* Dse1 protein, and other secreted proteins (Sorgo et al., 2010; Zohbi et al., 2014).

1.8 Aim of the Study

Previously in our lab, *pga1* and *pir32* null strains were generated and each showed distinctive phenotypes when compared to the wild type strain. The phenotypes exhibited by the *pga1* null strain are decreased cell wall chitin content, reduced virulence, lessened adhesion to host surfaces, and increased sensitivity to stresses (Hashash et al., 2011). On the other hand, the *pir32* null strain showed improved filamentation ability, doubled chitin deposition within the cell wall, enhanced virulence, and decreased sensitivity to stresses (Bahnan et al., 2012). An explanation to this rather unexpected phenotype is that the cell compensated for the deletion by thickening its surface through an increased chitin deposition.

Since both proteins are cell surface proteins, we hypothesize that changes in the cell surface proteome architecture occurred in response to the deletions. As such, we decided to use tandem-MS to identify differentially expressed proteins that might explain the previously observed phenotypes. In order to achieve this goal, cells from

both null strains and the wild type strain were grown under filamentous and non-filamentous conditions, and their cell walls were isolated by mechanical disruption. Multiple chemicals and enzymes were used to ensure linkage-dependent extraction and fractionation of the CWPs. Tandem-MS, following tryptic protein digestion, was applied with subsequent data analysis so as to point out the CWPs that are exclusive to each strain.

Chapter 2

Materials and Methods

2.1 Strains utilized

The *C. albicans* utilized in this project are the wild type RM1000 strain with pAbSK (*ura3*Δ*::_imm434/ura3*Δ*::_imm434his1::hisG/his1::hisG*) which is a histidine and uridine auxotroph, and the null strains *pir32:URA3/pir32::HIS1* and *pga1:URA3/pga1::HIS1* (Bahnan et al., 2012; Hashash et al., 2011).

2.2 Media preparation and culture conditions

The strains were full-grown in a liquid media of rich potato dextrose broth (PDB) (Hi Media, India) supplemented with histidine and uracil until exponential phase. For non-filamentous growth, strains were incubated at 28 ℃ under aerobic conditions. For filamentous growth, PDB media was supplemented with 10% fetal bovine serum (FBS) and incubated at 37℃ under aerobic conditions.

2.3 Cell wall isolation and protein extraction

Twelve cell wall extractions from each strain and for each of the growth conditions were performed independently. Cells were first centrifuged at 4,000 rpm for 5 min, then re-suspended in 5 mL Tris (5 mM, pH=7.8). Protease Inhibitor Cocktail (6 µL, abcam ab65621) along with cold glass beads were added. 30 cycles of vortexing were applied to ensure breakage as follows: 30 sec on vortex followed by 30 sec on ice. Samples turned orange reflecting a reaction between the acidic cytosol and the Protease inhibitor. Beads were then removed and the efficiency of breakage was

examined under the microscope. The samples were spun, **t**he supernatants containing intracellular proteins were poured off, while pellets were re-suspended in NaCl (40 mL, 1 M) and spun. This NaCl washing step was repeated 3-4X. Protein 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 pellets re-suspended. Tubes were boiled for 10 min and spun for 5 min at 3,000 rpm. The supernatants containing SDS extractable proteins were collected for later analysis. Protein extraction buffer and β-ME were added again to re-suspend pellet. Samples were boiled, cooled, centrifuged for 5 min at 3,000 rpm, and re-suspended in water. Wash steps with Type 2+ water were done to remove excess SDS. The final pellets were frozen in liquid N_2 and freeze-dried. Lyophilized cell walls were finally stored at -20 ℃ until use.

2.4 Extraction of alkali labile CWPs

The cell wall pellets were subjected to overnight incubation with NaOH (30 mM) at 4 ℃. They were then neutralized with aqueous acetic acid (30 mM) (Sorgo et al., 2010). Samples were spun, and supernatants were collected and subjected to tryptic digestion.

2.5 Glucanase treatment of cell wall pellets

10^8 cells were incubated at 37 ℃ with 1 mg of Glucanase in sodium acetate buffer (1 mL, 150 mM, pH=5) overnight (Cabezon et al., 2009). Spectrophotometric analysis was used to estimate cell numbers. Supernatants were collected and subjected to tryptic digestion.

2.6 Tryptic digestion

The cell wall extracts were incubated in a reducing buffer (10 mM DTT, 100 mM NH_4HCO_3) at 55 ℃ for 1 h. Samples were cooled to room temperature and spun. An alkylating buffer (65 mM iodoacetamide, 100mM NH_4HCO_3) was added to the pellets that were kept for 45 min at room temperature in the dark. Subsequently, a quenching solution (55 mM DTT, 100 mM NH_4HCO_3) was added to the samples for 5 min at room temperature. Ammonium bicarbonate buffer (50 mM) was used to wash the samples 5 times. Pellets were re-suspended in solution containing ammonium bicarbonate (50 mM) and trypsin (1 µg/µL). Samples were left at 37 ℃ for 16 h. Then, they were spun, and the supernatants were collected and prepared for Zip Tipping by adding TFA (0.1% V/V).

2.7 Peptide concentration

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

2.8 Mass spectrometry

Digested CWPs were spotted on a stainless steel target plate (Opti-TOF TM 384 Well Insert, 128x81 mm RevA, Applied Biosystems). BSA digests were also spotted and used as a standard solution. The sample and BSA digest spots were overlaid with α-cyano-4-hydroxy-cinnamic acid matrix solution (10 mg CHCA matrix in 50% acetonitrile with 0.1% TFA) and air-dried. MALDI-TOF-TOF MS spectra were

acquired on 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). MS reflector positive mode at a laser intensity of 2500 was used as an acquisition method. The selected mass range was 499 Da-2500 Da with a focus mass of 1500 Da. Reflector positive default was used as a processing method with a minimum signal-to-noise ratio of 5. The resulting mass lists were manually scanned for known contaminant mass peaks including keratin, matrix, and trypsin autolysis. The identified contaminant mass peaks were used to create an exclusion list that applied in the interpretation method for the MS/MS data acquisition. The minimum signal-to-noise filter for the monoisotopic precursor selection for MS/MS was also assigned 5. “Strongest precursors first” option was selected for precursor sorting order per spot and “weakest precursors first” option was selected for MS/MS acquisition order per spot with a maximum of 30 precursors per spot for each. MS/MS 1kV positive was used as an MS/MS acquisition method with a fixed laser intensity of 3500 and a precursor mass of 1570.677 Da. CID was turned on with specifying medium gas pressure and air gas type. Metastable suppressor was also turned on. MS/MS positive default was used as an MS/MS processing method with a signal-to-noise threshold of 5 for monoisotopic peaks.

2.9 Protein identification

MS/MS Ion Search was performed first using the contaminants and cRAP databases, in order to eliminate any additional contaminants whose peaks were not included within the exclusion list, then using a custom database on the MASCOT Server in order to identify the proteins within the samples. The database consisted of

protein sequences of all curated *C. albicans* proteins (taxon id: 237561) present in the Swissprot database (2016_07, retrieved on September 18, 2016) with gene ontology: cell wall (GO: 0005618), plasma membrane (GO: 0005886), and transmembrane (GO: 0016021) localization tags. The peptide and fragment tolerance values were specified at ±2 Da. This may seem too tolerant; however, the default settings of the 4800 MALDI-TOF-TOF analyzer were used where the resolution per mass peak as displayed by the machine is on average 4000 which is lower than the preferred acceptable values. This is another limitation of the machine. As such we had to choose a slightly more lenient tolerance level. Carbamidomethyl C was chosen as a fixed modification, whereas Oxidation at M was selected as a variable modification. Up to two missed cleavages were permitted for trypsin. A peptide charge of 1+ was assigned and MALDI-TOF-TOF was picked in the instrument type option. After these parameters were assigned, the data file was chosen and searched.

In the exported peptide summary report generated by MASCOT, proteins with less than 2% sequence coverage were disregarded. 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, 2005). We added the 2% cut off as an extra step to reduce false positives.

Peptide sequences identified by MASCOT but not assigned to any protein were blasted on Candidagenome.org where the selected target genome and target sequence dataset were "*Candida albicans* SC5314 Assembly 22" and "Proteins – translation of coding sequence (PROTEIN)" respectively. The BLASTP program was selected and

no gapped alignments were allowed. The cut off e-value was < 0.05 for MASCOT searches as well as BLAST searches.

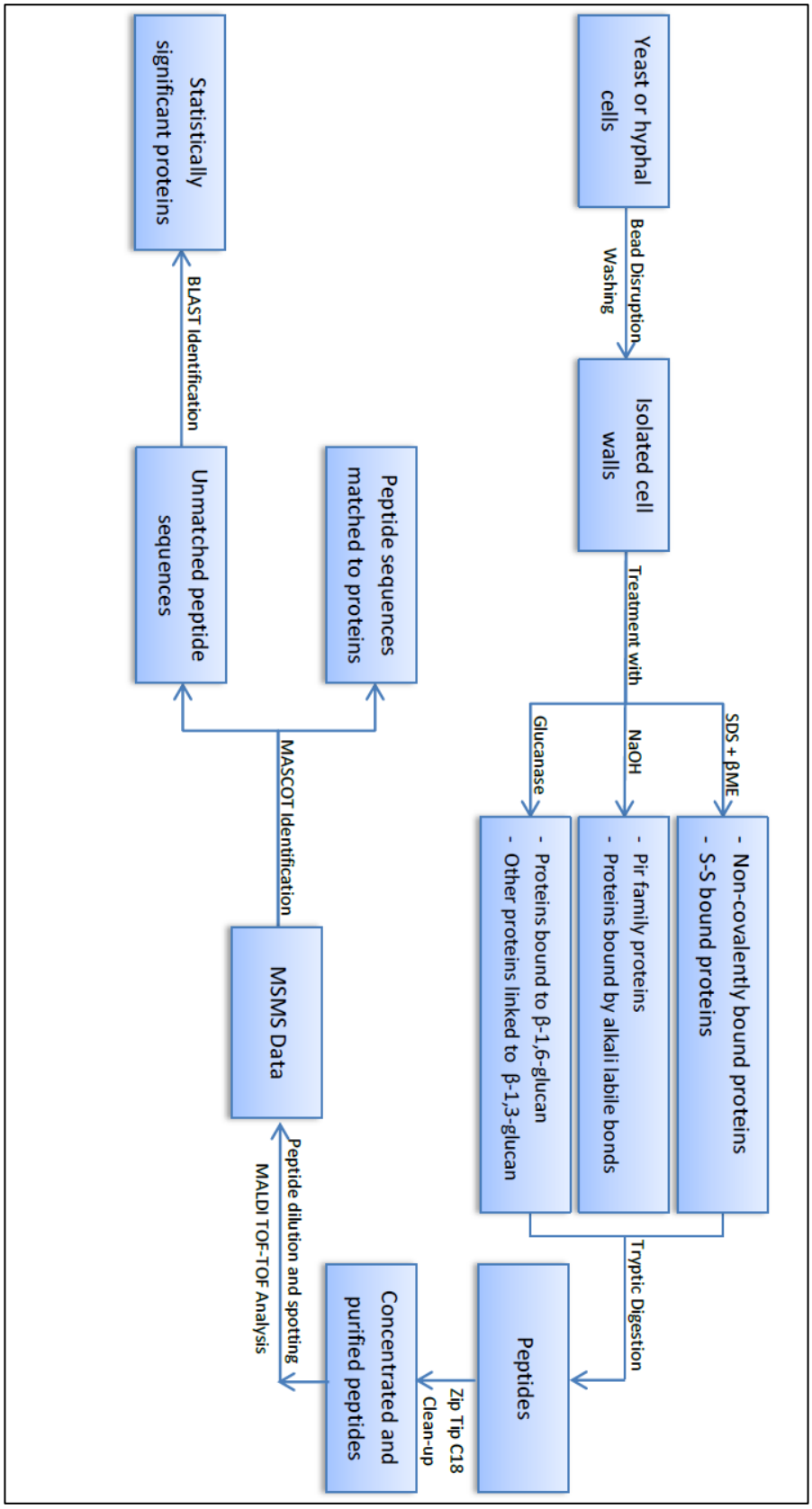

Figure 12. Graphical representation of the experimental procedure: this figure shows the steps followed in this study to extract the cell wall proteins and successively identify them in the wild type strain as well as in both *pga1* and *pir32* null mutant strains.

Chapter 3

Results

Cell walls of *C. albicans* null and wild type strains grown under different conditions were isolated. CWPs were fractionated according to their type of linkage to the cell wall molecules. These proteins were then prepared for mass spectrometry. The acquired data was analyzed using MASCOT engine to achieve significant protein identification. Aside the protein hits, MASCOT also provides unassigned sequences which we successfully identified by blasting on the *Candida* genome database (candidagenome.org). The proteome profiles of each of the null strains were compared to that of the wild type strain in order to find common and differential proteins that explain the varying phenotypes. These differentially identified proteins are responsible for the observed phenotypes in *pga1* and *pir32* null strains. The count of these differentially identified proteins by either MASCOT or BLAST engines under each growth condition is shown in Table. 1. It is noteworthy that at least 20 extractions for each condition were performed per strain and that the listed proteins were found in a minimum of 5 extractions. A schematic representation of the methods performed leading to protein identification is represented in Figure 12.

Table 1. Number of proteins exclusively found in each of the wild type and *pir32* null strains under each growth condition using MASCOT and BLAST search engines.

Search Engine	Growth condition	Wild type strain	*pir32* null strain
MASCOT	Non-filamentous	11	6
	Filamentous	6	12
BLAST	Non-filamentous	20	11
	Filamentous	7	16

3.1 Pga1 characterization by comparing the cell wall proteomes of *pga1* null strain with the parental wild type strain:

In total, 44 proteins were common between the wild type and the *pga1* null strains irrespective of the growth conditions, Table 2. Six of these proteins are known to be essential or are probably essential proteins in *C. albicans* and these essential proteins were expected to be common among all strains and growth conditions. The total count of essential proteins within the customized database is 40. This means that only 15% of these essential proteins are detected and found to be common between both strains. This low percentage might be due to the presence of the other undetected proteins at low concentrations in one of the strains or in both under the studied growth condition.

Table 2. Common proteins between the wild type and the *pga1* null strains regardless of the growth conditions. The superscript "a" indicates essential and probably essential proteins.

Protein	Description
Alo1	D-Arabinono-1,4-lactone oxidase
Bmh1	14-3-3 protein homolog
Bna4	Putative kynurenine 3-monooxygenase
Bud2	GTPase activating protein for Rsr1
Bud4	Bud site selection protein
Cbr1	NADH-cytochrome b5 reductase
Cdc19	Pyruvate kinase at yeast cell surface
Cdr1	Multidrug resistance protein
Cdr2	Multidrug resistance protein
Cht2	Chitinase
Eft2[a]	Elongation Factor 2
Eno1[a]	Enolase
Erg11	Lanosterol 14-alpha-demethylase
Erg6	Delta(24)-sterol C-methyltransferase
Fba1	Fructose-bisphosphate aldolase
Gpm1	Phosphoglycerate mutase
Hsp21	Small heat shock protein
Hsp90[a]	Small heat shock protein
Hwp2	GPI-anchored, glycosylated cell wall protein
Met6[a]	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
Mic60	MICOS complex subunit

Mlt1	Vacuolar membrane transporter
Mpg1	Mannose-1-phosphate guanyltransferase
Pan1[a]	Actin cytoskeleton-regulatory complex protein
Pdc11	Pyruvate decarboxylase
Pga41	Putative GPI-anchored adhesin-like protein
Pgk1	Phosphoglycerate kinase
Phr2	pH-responsive protein 2, Glycosidase
Prt1	Putative translation initiation factor eIF3
Qcr2	Cytochrome b-c1 complex subunit 2, mitochondrial
Sap4	Secreted aspartyl proteinase
Sap5	Secreted aspartyl proteinase
Sap6	Biofilm-specific aspartyl protease
Sap9	Secreted aspartyl protease
Sec4	Small GTPase of Rab family
Sec62	Putative endoplasmic reticulum protein-translocation complex subunit
Sln1	Histidine protein kinase
Ssa1	HSP70 family chaperone
Ssa2	HSP70 family chaperone
Ssu1	Sulfite efflux pump
Stp3	sulfite transport protein
Tef1[a]	Transcription factor
Tim50	Component of the translocase of the inner mitochondrial membrane
Ugp1	UTP-glucose-1-phosphaturidyl transferase

Since the null strain showed reduced phenotypes when compared to the wild type strain, we focused in this study on the proteins that were exclusively detected in the wild type and missing from the mutant that explain the varying phenotypes. These proteins are divided into 2 categories depending on their identification engine.

3.1.1 Proteins identified through MASCOT

Under non filamentous growth conditions, this category consists of 11 proteins with relatively high sequence coverage reaching up to 10.5%. This value adds to the confidence of correct protein identification. The presence of key virulence factors like Lip9, Exg2, Mp65, Sod5 and many Pga family proteins is noteworthy.

Table 3. MASCOT identified proteins that are exclusive to the wild type strain grown under non filamentous conditions.

Protein	Protein Accession	Protein Description	Sequence Coverage (%)	Peptide Sequence	# Missed Cleavages
Cdc11	G1UB61	Septin	10.5	EEQIKLEEERLR	2
				KFEERVHQDLINK	2
				KSINFSIMIIGESGSGR	1
Exg2	Q5AIA1	Glucan 1,3-beta-glucosidase 2	3.8	EAGLNMVRIPIGYWSFEK	1
				DTTMSSYMSSKK	1
				AVEKSPFLDALK	1
Lip9	Q9P4E6	Lipase	3.3	QSGQAVLNSIRASLK	1
Mp65	Q59XX2	Cell surface mannoprotein	2.9	SNQQAAISSIK	0
Pga31	Q5A5U9	Cell wall protein	2.4	DSYAVVK	0
Pga32	Q5ADQ7	Probable GPI-anchored adhesin-like protein	6	TTLGQVTTPSR	0
				LSSSKSIYSNSTTSR	1
Pga52	Q59L72	GPI-anchored protein	6.3	QAGADVATTLATVTGQSATTTSKK	1
Pir1	Q59SF7	Cell wall mannoprotein	6.6	ACSSANNLEMTLHDSVLKDTHER	1
Pns1	Q5AB93	Protein Pns1	2.3	SAKDTFDLIRFK	2
Sla1	Q5ALV2	Actin cytoskeleton-regulatory complex protein	4.4	DWWMVENIATRR	1
				SKDWWMVENIATR	1
				LTDGPLR	0
				LSVEDLEYVER	0
				DWWMVENIATR	0
				EGDEVYIIDQKK	1
Sod5	Q5AD07	Cell surface Cu-only superoxide dismutase	7	FTPANNGTVSVSVDLK	0

Table 4 lists the 6 proteins identified only in the wild type strain that was grown under filament-inducing conditions. Proteins required for resisting antifungals and stresses such as Qdr1 and Sho1 along with a secreted aspartyl protease Sap10 have been detected.

Table 4. MASCOT identified proteins that are exclusive to the wild type strain grown under filamentous conditions.

Protein	Protein Accession	Protein Description	Sequence Coverage (%)	Peptide Sequence	# Missed Cleavages

Egd2	Q5ANP2	Nascent polypeptide-associated complex subunit alpha	3.9	QIKGISR	1
Erg1	Q92206	Squalene monooxygenase	2.4	GFILLGDSLNMR	0
Pga5	Q59VW6	1,3-beta-glucanosyltransferase	7.6	EVQEEEPGVPGLPGSNK	0
				EPHYIDPLANPFTCLR	0
				ELCFKVDCEDINANGR	1
Qdr1	Q5A6P6	MFS antiporter	3.1	RIVGNGSIRPK	1
				RIVGNGSIRPKNVLNK	2
Sap10	Q5A651	Candidapepsin	4.2	MDLVIMNFVFLLYLTSVVK	0
Sho1	Q5AQ36	High osmolarity signaling protein	4.7	DEILEVDDIDGKWWQARR	2

3.1.2 Proteins identified through Blast

Twenty proteins were identified exclusively in the cell walls of the wild type strain under non filamentous conditions. This subset contains a large number of proteins and most of them are enrolled in pathways related to chitin deposition, nutrient supply, antifungal resistance, and cell wall structure. Noteworthy proteins are the lipases, Qdr1, Phr1, Rim9, and Tsa1B.

Table 5. BLAST identified proteins that are exclusive to the wild type strain grown under non filamentous conditions. The asterisk symbol "*" marks the proteins that were already identified by MASCOT.

Protein	Description	Sequence	E-value
Ape2	Neutral arginine, alanine, leucine specific metallo-aminopeptidase	YMATTQMEPTDCRR	2.00E-10
		TSEGVDESSVLETRSK	9.00E-10
Cfl1	Oxidoreductase	ATGKYNTR	1.30E-02
Egd2*	Nascent polypeptide associated complex protein alpha subunit	GISRVTFKQR	7.00E-05
Fmp13	Mitochondrial inner membrane protein	DIESVIGR	4.10E-02
		QQLEAKLNQK	2.00E-04
		LNEERNGRLANLEK	3.00E-08
Lip10	Secreted lipase	DSFYSPPVGFATAKPGDILK	3.00E-15
Lip6	Secreted lipase	SNGHTTETVVGAPAALTWIDAR	3.00E-17
Lip8	Secreted lipase	SSKITNIK	5.30E-01
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
Pga53	GPI-anchored cell surface protein of unknown function	LINDDELMVQDAQFDYPAIVNLK	2.00E-18
Phr1	Cell surface glycosidase	MTDVWSGGIVYMYFEEANK	1.00E-15
Qdr1*	Putative antibiotic resistance transporter	RIVGNGSIRPK	9.00E-06
Rim9	pH-response regulator protein	FFESEYRYANDDMRIMR	1.00E-13
Rps1	Putative ribosomal protein 10	KKMIEIMQR	2.00E-04
Stp1	Transcription factor	ALHFIYPAGVKASQR	3.00E-08
Tim21	Component of the Translocase of the Inner Mitochondrial membrane	LIEKNEQAQK	2.00E-04
Tsa1b	Putative peroxidase	MAPVVQQPAPSFKK	3.00E-07
Vma2	Vacuolar H(+)-ATPase	LSLEFLEKFEKNFISQGAYENR	3.00E-17

The number of identified proteins is reduced to 7 under filamentous growth conditions as seen in Table 6. These proteins include a variety of proteins involved in different pathogenicity pathways and cell wall maintenance and assembly mechanisms.

Table 6. BLAST identified proteins that are exclusive to the wild type strain grown under filamentous conditions. The asterisk symbol "*" marks the proteins that were already identified by MASCOT.

Protein	Description	Sequence	E-value
Cdc11*	Septin	KFEERVHQDLINK	3.00E-08
Fmp13	Mitochondrial inner membrane protein	DIESVIGR	4.10E-02
		SEIPPPPPPPPPPKAKR	8.00E-14
Hsp70	Putative chaperone	IDKSQVEEIVLVGGSTR	5.00E-04
Int1	Bud site selection protein, also known as Bud4	DLNFANYSNNTNRPR	2.00E-10
Pga4	ß-1,3-glucanosyltransferase	LPSGLYFNCGDDDMAR	8.00E-12
Qdr2	Predicted MFS membrane transporter	SLDWYYR	2.00E-02
Rbr1	GPI-anchored cell wall protein; Repressed By RIM101 protein	SGASSVASAAK	2.00E-04

3.2 Pir32 characterization by comparing the cell wall proteomes of *pir32* null strain with the parental wild type strain:

Table 7 lists the common proteins between the *pir32* null strain and the wild type strain irrespective of the growth condition amounting to 56 proteins. Ten of these

proteins are known to be essential or are probably essential proteins in *C. albicans*. This means that only 25% of the reviewed essential proteins with cell wall, plasma membrane and transmembrane localization tags are detected and found to be common between both strains. This low percentage might be due to the presence of the other undetected proteins at low concentrations in one of the strains or in both under the studied growth conditions.

Table 7. Common proteins between the wild type and *pir32* null strains irrespective of the growth condition. The superscript "a" indicates essential and probably essential proteins.

Protein	Description
Aim36	Altered inheritance of mitochondria protein 36
Alo1	D-Arabinono-1,4-lactone oxidase
Ape2[a]	Neutral arginine, alanine, leucine specific metallo-aminopeptidase
Bmh1	14-3-3 protein homolog
Bna4	Putative kynurenine 3-monooxygenase
Bud2	GTPase activating protein for Rsr1
Bud4	Bud site selection protein
Cdc11	Septin
Cdc19	Pyruvate kinase at yeast cell surface
Cdr1	Multidrug transporter of ABC superfamily
Eft2[a]	Elongation Factor 2
End3	Actin cytoskeleton-regulatory complex protein
Eng1	Endo-1,3-beta-glucanase
Eno1[a]	Enolase
Erg11	Lanosterol 14-alpha-demethylase
Erg6	Delta(24)-sterol C-methyltransferase
Gpm1	Phosphoglycerate mutase
Hsp21	Small heat shock protein
Hsp90[a]	Heat shock protein 90
Hwp2	GPI-anchored, glycosylated cell wall protein
Ino1	Inositol-1-phosphate synthase
Ipp1[a]	Putative inorganic pyrophosphatase
Lip10	Secreted lipase
Lip8	Secreted lipase
Mdr1	Plasma membrane MDR/MFS multidrug efflux pump
Met6[a]	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
Mic60	MICOS complex subunit
Mlt1	Vacuolar membrane transporter
Mnt1	Alpha-1,2-mannosyl transferase
Pan1[a]	Actin cytoskeleton-regulatory complex protein

Pdc11	Pyruvate decarboxylase
Pga41	Putative GPI-anchored adhesin-like protein
Pgk1	Phosphoglycerate kinase
Phr1	pH-responsive protein 1, Glycosidase
Phr2	pH-responsive protein 2, Glycosidase
Pmt2[a]	Protein mannosyltransferase
Prm1	Putative membrane protein
Prt1	Putative translation initiation factor eIF3
Sap4	Secreted aspartyl proteinase
Sap6	Biofilm-specific aspartyl protease
Sap7	Pepstatin A-insensitive secreted aspartyl protease
Sap8	Secreted aspartyl protease
Sap9	Secreted aspartyl protease
Sec4	Small GTPase of Rab family
Sec62	Putative endoplasmic reticulum protein-translocation complex subunit
Sln1	Histidine protein kinase
Ssa1	HSP70 family chaperone
Ssa2	HSP70 family chaperone
Ssc1[a]	Heat shock protein
Ssu1	Sulfite transport protein
Stp3	sulfite transport protein
Tef1[a]	Transcription factor
Tim21	Component of the translocase of the inner mitochondrial membrane
Tim50	Component of the translocase of the inner mitochondrial membrane
Ugp1	UTP-glucose-1-phosphaturidyl transferase
Vma2	Vacuolar H(+)-ATPase

Virulence characteristics and pathogenicity mechanisms were more pronounced in the *pir32* null strain than they were in the wild type. Therefore, in this study we focused on the proteins that were exclusively detected in this mutant that can explain the varying phenotypes. These proteins are divided into 2 categories depending on their identification engine.

3.2.1 Proteins identified through MASCOT

Table 8 shows the 6 proteins that were extracted under non-filamentous growth conditions and that were identified only in the mutant. Remarkable virulence proteins are Dfg5, Dse1, Nce102, and Sap2.

Table 8. MASCOT identified proteins that are exclusive to the *pir32* null strain grown under non filamentous conditions.

Protein	Protein accession	Protein description	Sequence coverage (%)	Peptide sequence(s)	# Missed cleavages
Dfg5	Q5ACZ2	Mannan endo-1,6-alpha-mannosidase	3.8	NSISNGCLFHLAARLAR	1
				LSANIVK	0
Dse1	Q59Y20	Protein DSE1	3.5	KPTTTIK	0
				DSQWEENDNTK	0
Lip7	Q9P4E7	Lipase 7	3.3	DLETSQLGDVLKWR	1
Nce102	Q5ANE3	Non-classical export protein 102	7.6	GGLFGHSSRPAPR	0
Pga27	Q5AD23	Predicted GPI-anchored protein 27	2.9	TDIPVSQRIDTISK	1
Sap2	P0DJ06	Candidapepsin-2	3.3	SAGFVALDFSVVK	0

A total of 12 proteins listed in Table 9 were identified exclusively in *pir32* null strain under filamentous growth conditions. A key virulence factor in *C. albicans* is the adhesin Als3 was detected in this strain. Other significant proteins are Csa2, Dfi1, Ssr1 and multiple members of the PGA family.

Table 9. MASCOT identified proteins that are exclusive to the *pir32* null strain grown under filamentous conditions.

Protein	Protein accession	Protein description	Sequence coverage (%)	Peptide sequence	# Missed cleavages
Als3	Q59L12	Agglutinin-like protein 3	3.7	AGTNTVTFNDGDKK	1
				CFKAGTNTVTFNDGDK	1
				ISINVDFER	0
				NSDAGSNGIVIVATTR	0
Csa2	Q5A0X8	Surface antigen protein 2	7.5	CRGADVTNFRK	2
Dfi1	Q5AFI4	Cell-surface associated glycoprotein	8.3	LGSDEFFNGELGVR	0

				RNNRDYEGGWTFWR	2
Erv25	Q5A302	Endoplasmic reticulum vesicle protein 25	9.3	AREERMR	2
				VTDSLGNEYRNKK	2
Lip9	Q9P4E6	Lipase 9	2.6	NATVGDILQFRK	1
Pga17	Q5AHA4	Predicted GPI-anchored protein 17	2.7	NIVGEVVIEALPLIK	0
Pga31	Q5A5U9	Cell wall protein	2.4	DSYAVVK	0
Pga49	Q59QA5	Predicted GPI-anchored protein 49	4.7	QPVSLSMPTK	0
				NRSKFYNHFLR	2
				LHSPTSTDTKSSK	1
Rbt5	Q59UT4	Repressed by TUP1 protein 5	9.1	IYDQLPECAK	0
				AEETSKAAETTK	1
Rpl13	O59931	60S ribosomal protein L13	11.9	TIGISVDHRRQNK	2
				QNKSQETFDANVAR	1
Ssr1	Q5AFN8	Covalently-linked cell wall protein 14	3.8	NSDVEKCLK	1
Stp2	Q5AL16	Transcriptional regulator STP2	2.1	MIDPELVPFASK	0

3.2.2 Proteins identified through BLAST

Table 10 holds 11 BLAST identified proteins exclusive to *pir32* null strain under non-filamentous conditions. Notable proteins are Int1, C1_13530wp_a, Sod4, Ssu81, and Xog1. Several PGA family proteins affecting cell wall integrity were also identified.

Table 10. BLAST identified proteins that are exclusive to the *pir32* null strain grown under non filamentous conditions. The asterisk symbol "*" marks the proteins that were already identified by MASCOT.

Protein	Description	Sequence	E-value
C1_00310w_a	Putative protein of unknown function	DPEIFQMTNR	2.00E-05
Int1	Integrin-like protein 1	KESISSKPAKLSSASPR	7.00E-11
		DLNFANYSNNTNRPR	2.00E-10
Lip4	Secreted lipase	IFKSGWNILKNPTISK	1.00E-11
C1_13530wp_a	Pseudogene	SGIDTTKVYGGLANYGR	3.00E-10
Pga14	Putative GPI-anchored protein	KFTTVATVFAISSLAAAKGGEK	3.00E-16
Pga27*	Predicted GPI-anchored protein of unknown function	DGVPINFHRAPAIIMK	8.00E-12
Pga4	GPI-anchored cell surface protein; beta-1,3-glucanosyltransferase	YFQELGINTIRVYSIDNTK	1.00E-14
Sod4	Cu-containing superoxide dismutase	NSTNGSSGSSTSASQGSGAGR	7.00E-15

Ssu81	High osmolarity signaling protein	NTIYTDSETGTGITFR	4.00E-11
Stp2*	Amino-acid-regulated transcription factor	HFNYAKPIKSAERSK	2.00E-10
Xog1	Exo-1,3-beta-glucanase; 5 glycosyl hydrolase family member	QLSFILTSSVFILLLEFVK	2.00E-13

Similarly, identified proteins under filamentous conditions are listed in Table 11. The total count is 16. The most prominent of these are Cdc42, Dcw1, Lip5, Pga26, Sap3, and Sod6.

Table 11. BLAST identified proteins that are exclusive to the *pir32* null strain grown under filamentous conditions. The asterisk symbol "*" marks the proteins that were already identified by MASCOT.

Protein	Description	Sequence	E-value
Cdc42	Rho-type GTPase	LSPITQEQGEKLAKELR	3.00E-11
Dcw1	Mannan endo-1,6-alpha-mannosidase	NSVSNGALFHLAARLAR	3.00E-11
Dfi1*	Cell-surface associated glycoprotein	LSINNNNNNR	2.00E-04
Exg2	GPI-anchored cell wall protein, Glucan 1,3-beta-glucosidase 2	VGSCAEFNKSPDK	4.00E-08
		NGIMPQPLDNYK	1.00E-07
Kar2	Hsp70 family ATPase	DAGTIAGLNVLR	5.00E-06
Lip5	Cold-activated secreted lipase	QTVSGCQHIQR	6.00E-07
Pga26	GPI-anchored adhesin-like protein of the cell wall	GGSSSGSSSGSSSGSRGGSSSGSSSSGSR	3.00E-22
		NVAGALVGVVAIAAAMM	5.00E-10
Pga32	Putative GPI-anchored adhesin-like protein	ITPIASASASSGSSTK	1.00E-09
Pga37	Putative GPI-anchored protein	GGSSSGSSSGSSSGSRGGSSSGSSSSGSR	3.00E-22
Pga49*	Predicted GPI-anchored protein 49 of unknown function	LDKPKFPDIFTIIR	2.00E-09
Pmt5	Protein mannosyltransferase	FSFWSKLIETHK	7.00E-08
Rbt5*	Repressed by TUP1 protein 5	AEETSKAAETTK	7.00E-06
Rpl13*	60S ribosomal protein L13	AISKNLPLLNNHFRK	5.00E-10
Sap3	Secreted aspartyl proteinase	NVTGPQGEINTNVNVK	8.00E-11
Sod6	Copper-containing superoxide dismutase	HGCINTTCFELK	2.00E-08
		EGKHVNVHIDMTGLPK	1.00E-11
Tpi1	Triose-phosphate isomerase	VILCIGETLEER	9.00E-07
Ucf1	Upregulated by cAMP in filamentous growth	LELDVSCTNESAMVDVEYKSIPM	1.00E-18
		FRRDLQHHIQK	5.00E-07

Chapter 4

Discussion

This thesis aimed at further characterizing the *C. albicans* GPI-anchored CWP Pga1 and mannoprotein Pir32. Tandem mass spectrometry is a useful technique for identification of extracted CWPs from *pga1* and *pir32* null strains and the wild type RM1000 strain. Unassigned peptide sequences generated by MASCOT were also aligned to sequences and matched to proteins in *Candida* genome database. This enabled the identification of extra proteins. As such, cell wall proteome profile of each of the null strains was unraveled and compared to that of the wild type. Proteins exclusive to each strain were of interest as they explain the phenotypic variations between the tested strains.

As stated in earlier chapters of this thesis, our lab has characterized the *pga1* null strain. This strain showed diminished virulence highlighted by its defect in adhesion, incapability of proper biofilm formation, reduced level of cell wall chitin deposition, and declined resistance to stresses when compared to the wild type strain (Hashash et al., 2011). Since *pga1* null strain exhibited reduced overall virulence phenotypes than the wild type strain, proteins exclusive to the wild type and not detected in the *pga1* null strain explain these variations and are discussed below. Surprisingly, these virulence phenotypes were more pronounced in the *pir32* null strain which was hyper-filamentous showing enhanced resistance to oxidative and osmotic stresses, doubled cell wall chitin content, and improved biofilm forming ability (Bahnan et al., 2012). In this case, proteins exclusive to the *pir32* null strain underlying these variations are discussed below.

4.1 Essential proteins

In each of our 2 comparison analyses, proteins detected in both analyzed strains regardless of the growth conditions were highlighted. These common proteins, with many being essential, add more credibility to the results and assertion that *C. albicans* cell wall is a dynamic structure whereby several proteins are involved in its assimilation and rigidity (Kapteyn et al., 2000).

It is important to note that the lack of detection of a certain protein from one strain does not necessarily imply that this protein is completely absent from the analyzed sample. This is confirmed by the detection of essential proteins in only one of the strains rather than being equally detected. One reason behind this failed protein detection could be its presence at minimal amounts in one strain thus falling below the detection range of the machine (Castillo et al., 2008). So, if a protein was detected in only one of the compared strains, this could also signify that it is present at a higher concentration in this strain than it is in the other one; resulting in phenotypic variations.

Upon comparing proteome profiles of *pga1* null and RM1000, 3 essential proteins were only detected in RM1000 cells grown under non-filamentous conditions: Ape2, Exg2, and Pga52. When the profile of RM1000 strain was compared to that of *pir32* null strain, essential protein Dse1 was exclusive to the null cells grown under non-filamentous conditions and essential proteins Exg2 and Tpi1 were exclusive under filamentous growth conditions. These proteins are essential for guarding cell wall regeneration, cell wall rigidity, and filamentation (Daher et al., 2011; Seweryn et al., 2015; Tsai et al., 2011). We assume that these differentially detected essential proteins have been downregulated in the *pga1* null strain and overexpressed in the *pir32* null strain.

4.2 Artifacts and atypical CWPs

In reference to the cell wall isolation and protein extraction methods performed in this study, all detected proteins are supposed to be CWPs. Hypothetically, intracellular and cytosolic proteins were discarded at the early steps of the cell wall extraction procedure. Nevertheless, many detected proteins in both comparison analyses were nuclear, cytosolic, or mitochondrial. It could be possible that these proteins are artifacts or contaminants during the early isolation process. Also, they could be atypical proteins that lack a signal peptide needed to translocate them towards the cell surface via the classical secretory pathway (Nickel, 2003). Rather they are secreted in a yet undetermined manner (Nombela et al., 2006). Studies are suggesting that these atypical proteins, with previously assigned cytosolic functions, are retained by non-covalent linkages in the cell walls of *C. albicans* (Castillo et al., 2008; Nickel & Rabouille, 2009).

Artifacts detected exclusively in RM1000 strain are Fmp13, Tim21, and Rps1. Fmp13 is an inner membrane component of the mitochondria and Tim21 is a protein transporter also located in the mitochondrial inner membrane, whereas Rps1 is a ribosomal subunit (Hewitt et al., 2012; Nobile et al., 2012). Exclusive to *pir32* null strain are Rpl13 and Kar2 which are a ribosomal subunit and a component of the endoplasmic reticulum respectively (Nobile et al., 2012). One notable atypical protein is Tsa1B that is called a moonlighting protein as it has varying location-dependent functions (Urban et al., 2005). It acts as a peroxidase in the cell wall. The lack of detection of this peroxidase from the *pga1* null strain explain the increased sensitivity to cell wall perturbing agents like hydrogen peroxide. Another identified atypical protein is Egd2 which has a role in hyphal growth; a phenotype reduced in the *pga1* null strain (Hernández et al., 2004).

4.3 Detected PGA family members

The remaining identified proteins are all CWPs each being a virulence factor involved in a specific signaling pathway. Many of the proteins solely detected in the wild type strain or the *pir32* null strain belong to the PGA family.

Nine members of this family were only detected in the wild type. Pga30, 45 and 53 have unknown functions. Pga28, 32 and 55 are adhesin-like proteins required for adequate adhesion to host surfaces which is the initial step for any infection (Chaudhuri et al., 2011). Pga31 regulates the assembly of chitin within the cell wall during cell wall regeneration process and upon morphogenesis (Plaine et al., 2008). Pga4 is a key virulence factor required for the elongation of the ß-1,3-glucan branches during cell wall biosynthesis and filamentation (Ene et al., 2012). The lack of detection of these proteins in the mutant cell wall is behind this strain's reduction in virulence, chitin deposition, rigidity, and adhesion.

On the other side, 9 other members of this family were solely detected in *pir32* null strain. Pga27, 37, and 49 have undetermined functions. Pga14 and 17 have roles in cell wall regeneration, Pga32 is an adhesin and Pga31 functions in chitin binding and deposition (Plaine et al., 2008). Pga26 acts as a glycoprotein that maintains cell wall integrity thus affecting resistance to antifungal drugs and other stresses (Laforet et al., 2011). Being detected only in this strain clarify the doubling of chitin content and the enhanced rigidity, integrity, and resistance of the cell wall to perturbing agents. Pga4 was also present in this strain however under non-filamentous growth conditions. This sheds light on its observed hyperfilamentous phenotype as it was able to grow filaments and hyphae even under non-inducing conditions.

4.4 Proteins involved in filamentation and biofilm formation

Reduced filamentation ability observed in *pga1* null strain is attributed to many hypha-related proteins being undetected; namely Sla1, Phr1, Mts1, and Rbr1. The first two are involved in actin-mediated endocytosis that promotes dimorphism, polarized growth and thigmotropism (Saporito-Irwin et al., 1995; Zeng et al., 2011). The last two ensure proper hyphal growth and elongation thus permitting the formation of biofilms (Lotz et al., 2004; Oura & Kajiwara, 2009). Also, Sho1 belongs to this group as it senses osmotic changes in the fungal environment and impose switching to hyphal shape (Román et al., 2005). As such, it is not surprising that the *pga1* null strain missing these proteins has defected filamentation and biofilm forming abilities.

Proteomic analysis of the *pir32* null strain revealed that it encompasses many proteins related to these abilities: Cdc42, Dfi1, Int1, Ssu81 and Ucf1 (Nobile et al., 2012). Int1 is required for the axial budding of yeast cells and for allocating the septation sites in hyphal cells. This protein induces hyphal growth and promotes morphogenesis (Gale et al., 1998). Ssu81, also known as Sho1 is a remarkable osmosensor mediating *C. albicans* resistance to oxidative stress through activating the switch to hyphal type, manipulating the cell wall mannan layer, and promoting host invasion (Biswas et al., 2007). The detection of these two proteins in particular in this strain under non-filamentous conditions highlight its distinctive hyperfilamentation ability. The other proteins were found exclusively in the *pir32* null strain under filamentous growth conditions. Cdc42 is required for budding and hyphal growth (Mirbod et al., 1997). Dfi1 is a glycoprotein that binds calmodulin upon matrix sensing which initiates a signaling cascade that in turn promotes the formation of invasive filaments (Davis et al., 2013).

4.5 Proteins involved in cell wall assembly and rigidity

Many differentially detected proteins have roles in cell wall rearrangement and rigidity. Pir1 and Cdc11 were solely found within the cell wall of the wild type. The mannoprotein Pir1 is a cell wall constituent whose expression is elevated under acidic and hypoxic conditions and it also varies upon dimorphism (Martínez et al., 2004). Cdc11 is a septin affecting chitin deposition within the fungal cell wall (Warenda & Konopka, 2002). As such, these proteins are key proteins for cell wall rigidity. They were not detected in *pga1* null strain cell wall that was reduced in chitin content and rigidity as it was more permeable to antifungal drugs and stresses.

It has been previously hypothesized that sometimes a cell compensates for the deletion of a certain CWP by increasing the thickness and rigidity of its cell wall (Plaine et al., 2008). This is the case in the studied *pir32* null strain that showed two-fold increase in chitin content and improved cell wall rigidity as it showed higher resistance to osmotic stress and to SDS. Proteins behind this observation are Ssr1, Xog1, Dfg5, and Dcw1 (Garcerá et al., 2005; Spreghini et al., 2003; Tsai et al., 2011). Ssr1 has a GPI anchorage that is directly attached to the ß-1,3-glucan layer and might form disulfide bridges with other CWPs (Garcerá et al., 2005). It was found that when *SSR1* gene was either deleted or overexpressed, amplified sensitivity to Congo red and calcofluor white were observed; thus indicating the crucial role of ssr1 in cell wall structure and rigidity (Garcerá et al., 2003). Xog1 is an exoglucanase that is involved in chitin biosynthesis and ß-1,3-glucan synthesis that are major components of the cell wall; hence this protein is needed for chitin deposition and overall cell wall assembly (Tsai et al., 2011). Additionally, Dfg5 and Dcw1 promote growth under harsh pH conditions by inflicting changes in the assembly of cell wall components and making it more rigid (Spreghini et al., 2003).

4.6 Proteins involved in metabolic processes

Upon nutrient deprivation, *C. albicans* cells start breaking down amino acids to use them as carbon sources. This results in elevated levels of ammonia which in turn raises the pH in the fungal environment; a signal for morphology switching (Vylkova et al., 2011).

In the wild type, Rim9 was exclusively detected which is a factor in the pH-dependent signaling pathway (Cornet et al., 2009). Also, lipases (Lip6, 8 and 10), Sap1, and Stp1 were found. These proteins provide the fungus with its nutrient requirements by degrading host cell membrane and associated proteins (Hube et al., 2000; Martínez & Ljungdahl, 2005; Schaller et al., 2001). Being undetected in the cell wall of the *pga1* null strain, the reduced fitness and virulence phenotype of this strain were observed.

The detection of several lipases (Lip4, 5, 7 and 9), aspartyl proteases (Sap2 and 3) and Stp2 in the *pir32* null strain contributed to its more pronounced phenotypes related to virulence and filamentation.

4.7 Proteins involved in iron utilization and evading immune defenses

Fitness and virulence of *C. albicans* are governed by its pool of proteins functioning in iron scavenging and detoxification of ROS, as indicated in chapter 1 of this thesis.

The *pga1* null strain was less virulent than the wild type since the oxidoreductase Cfl1 and the superoxide dismutase Sod5 involved in these functions respectively were not detected in it.

On the other hand, *pir32* had a more pronounced virulence due to the exclusive detection of Rbt5, Csa2, Sod4 and Sod6 in the protein fractions of this strain. Rbt5 is a heme receptor and Csa2 is a secreted protein activated during iron acquisition process (Okamoto-Shibayama et al., 2014).

4.8 Proteins involved in drug resistance

Proteins required for drug resistance like Erg1, Qdr1 and Qdr2 were not detected in *pga1* null mutant (Liu et al., 2005). Erg1 is a protein required for ergosterol biosynthesis, a component of the plasma membrane that affects cell permeability (Sorgo et al., 2011). This protein is targeted by allylamine antifungal drugs (Liu et al., 2005). Also, Qdr1 and Qdr2 are transporters needed for biofilm thickness and nutrient uptake (Shah et al., 2014). Accordingly, the *pga1* null mutant lacking these proteins showed reduced efficiency to resist antifungal drugs.

When comparing the proteome profiles of the wild type and the *pga32* null strains, no differential proteins functioning in drug resistance were found. This is expected since no significant alterations in sensitivity to fluconazole, caspofungin, or amphotericin B were observed between the parental wild type strain and the *pir32* null strain (Bahnan et al., 2012).

4.9 Major virulence proteins

The *pir32* null strain is the most virulent among the studied strains followed by the parental wild type strain. The more pronounced virulence phenotype could be attributed to Als3, a key virulence factor only detected in *pir32* null strain. Als3 is an adhesin and an invasion specific to the hyphal *C. albicans* (Almeida et al., 2008). It

promotes endocytosis and aggregation that are essential steps in pathogenesis (Cheng et al., 2005).

Other major virulence proteins are MP65 and Hsp70 which is also known as Ssa1. MP65 is an adhesin that is also enrolled in various pathogenicity mechanisms. It has roles in biofilm formation, glucan metabolism, and cell wall regeneration following morphogenesis (Sandini et al., 2011). Hsp70 is a heat shock protein that drives endocytosis following the action of Als3 (Dalle et al., 2010). These proteins were not detected in *pga1* null strain thus reflecting its reduced adhesion to host surfaces and diminished virulence.

Moreover, many proteins common between the *pir32* null and wild type strains regardless of the growth conditions and listed in Table 6 explain the enhanced virulence phenotype in these strains. These virulence proteins include members of the lipase family (Lip 8 and 10), Sap family (Sap4, Sap6-9), and Hsp family (Ssa1 and 2, Ssc1, Hsp21, and Hsp90) in addition to pH-responsive proteins (Phr1 and 2), pumps and drug transporters (Cdr1, Mdr1, Ssu1, and Stp3) and other factors involved in the various pathogenesis mechanisms discussed above.

Chapter 5
Conclusion and Insights

In this study, we identified differentially expressed proteins that might explain the observed phenotypes in *pga1* and *pir32* null strains by obtaining the cell wall proteome profile of each of the null strains and comparing them to that of the wild type.

The lack of detection of the above mentioned virulence proteins in the *pga1* null strain when comparing its cell wall proteome profile to that of the wild type explains the previously observed phenotypes: decreased chitin content, defect in filament and biofilm formations, reduced resistance to stresses, and overall diminished virulence.

Following the same logic, the phenotypes expressed by the *pir32* null strain were due to the exclusive detection of multiple virulence proteins in this strain upon comparing its cell wall proteome profile to that of the wild type.

We conclude from this that *C. albicans* modulates responses to CWP deletions differently. Upon the deletion of *pga1*, the fungus lost many of its virulence attributes making it an unsuccessful pathogen. However, following *pir32* deletion, *C. albicans* compensated by incorporating crucial virulence factors into the cell wall and thickening the cell wall thus making it a life-threatening pathogen.

Future work would require conducting additional protein quantification studies as they would give supporting information for the overexpression or downregulation of the differentially detected proteins for each strain. In addition, elucidating the pathways and cues involved in expressing Pga1 and Pir32 would establish full characterization of these proteins.

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