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Mass spectrometric quantification of *Candida albicans* surface proteins to identify new diagnostic markers and targets for vaccine development

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Publication date
2013

[Link to publication](#)

Citation for published version (APA):

Heilmann, C. J. (2013). *Mass spectrometric quantification of Candida albicans surface proteins to identify new diagnostic markers and targets for vaccine development.*

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Chapter 1:

General Introduction

The role of fungi

Approximately 1.5 million species of fungi exist, ranging from unicellular fungi to species like *Fomitiporia ellipsoidea*, where a single fruiting body can weigh up to 500 kg [1]. The honey fungus *Armillaria ostoyae* forms the largest known fungal colony on the planet in eastern Oregon with an area of approx. 9 km², a mass of more than 600 tons and an estimated age of more than 2400 years [2]. Fungi also often form symbiotic relationships, especially with plants in the form of mycorrhiza, in which nutrients are shared among the partners [3]. Selected fungi are also appreciated for their industrial potential, especially baker's yeast, *Saccharomyces cerevisiae*, and some filamentous fungi such as *Aspergillus niger*. Fermentation carried out by *S. cerevisiae* is essential for the production of bread, cheese, beer, wine, and spirits among others [4]. It has been suggested that the empirical use of fermentation by fungi has played an important role in the transition from a hunter/gatherer society to a settled lifestyle by providing a staple of nutrition and entertainment [5].

Fungi form the backbone of decomposers in the earth's biosphere. They recycle dead plant and animal material and replenish nutrients in the soil [6]. Those saprophytic fungi secrete a variety of enzymes that break down complex carbohydrates and other compounds into their building blocks which can then be re-used by other organisms. While the large majority of fungi is not associated with disease, some species are able to damage and kill animals and plants. Fungal plant diseases like rice blast, caused by *Magnaporthe grisea*, or tomato wilt, caused by *Fusarium oxysporum*, result in significant economic damage in a multi-billion dollar range and crop losses which could feed millions [7,8]. Ash dieback, caused by *Chalara fraxinea*, has led to the death of ash trees all over Europe, leading to 60-90% losses in the ash population in some regions in Denmark [9]. The white nose fungus *Geomyces destructans* has already killed more than 6 million bats since 2006 [10]. The chytrid fungus *Batrachochytrium dendrobatidis* has led to a decline in population in more than a third of all amphibian species [11], possibly, the largest extinction event since the dinosaurs. A deeper understanding of fungi, their life cycle and their pathogenic potential will allow new industrial and clinical applications, help to preserve biodiversity and reduce human suffering and casualties.

In humans, fungal infections of the skin, for example by dermatophytes, are very common and often lead to mild infections, e.g. athlete's foot caused by fungi of the genus *Trichophyton*, or onychomycosis, fungal infection of the nails. Since the advent of modern medicine, the incidence of serious fungal in-

fections increases. This is mainly due to an increase in invasive surgery and the use of in-dwelling devices like catheters and stents, improved survival of immune-suppressed patients and an increase in infections acquired in a hospital setting. Human fungal pathogens can be divided into environmental fungi that enter the human body accidentally and opportunistic fungi that colonize and co-exist in the human host, but are kept in check by the immune system. Coccidioidomycosis, often referred to as valley fever, is caused by fungi of the *Coccidioides* genus found abundantly in dust and sand in Middle and North America, and leads to mild to severe life-threatening pulmonary infections. Especially fungi from the *Aspergillus* genus are often inhaled daily in their thousands as spores and often lead to asymptomatic infections in immune-competent humans. In immune-suppressed patients on the other hand, they lead to severe lung infections with possible secondary infections of the blood stream, organs and brain. In contrast, fungi, like the human fungal pathogen *Candida albicans*, that colonize humans are in constant interaction with host tissues and are recognized by the immune system even if no infection occurs. In immune-competent patients infections of the skin and mucosae can occur, but a reduction in immune status can lead to increased tissue invasion and damage and penetration of the fungus to the bloodstream. Upon dissemination, severe multi-organ infections often follow. This thesis focuses on virulence traits of *Candida albicans*.

Candida albicans history and epidemiology

Oral thrush has already been described by Hippocrates around 400 BC in “of the epidemics”. But only in 1847 a fungus was connected as the causal agent for thrush by the French mycologist Charles Phillippe Robin, naming the fungus he observed *Oidium albicans*. Later it was misclassified into the genus *Monilia*, fungi that mainly affect plants. In 1923 it was reclassified by the Dutch mycologist Christine Berkhout as *Candida albicans*, which was accepted as official nomenclature in 1954.

C. albicans is present on the skin and mucosae of approx. 80% of the population. In immune-competent humans, the pathogen is kept in check by the immune system and can occasionally result in topical, acute infections. Oral and vaginal thrush is a result of overgrowth of the fungus and 4 out of 5 women have at least one episode of vaginal candidiasis in their lifetime. The abundant presence of *C. albicans* on the surfaces of the human body is especially problematic when the barrier function of skin and mucosae is broken and *C. albicans* gains

access to the blood stream. This can occur through injury, major surgery or invasive growth of the fungus itself.

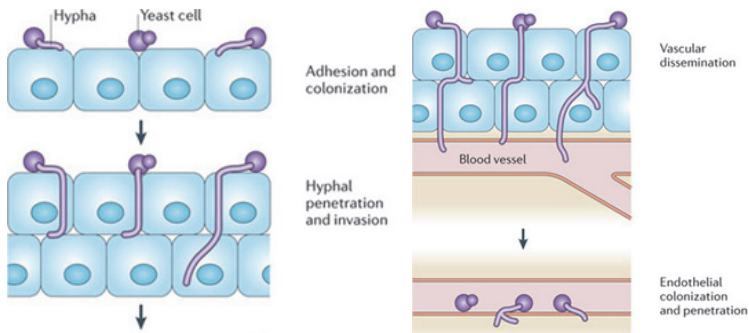


Figure 1.1. Schematic representation of distinct steps during *Candida albicans* infections (from [12]).

Candida cells adhere to the mucosal surface and harmlessly colonize it, being kept in check by the immune system. When the balance is perturbed, e.g. through immune-suppression, hyphal cells start piercing the physical barrier of the epithelium and invade. When the cells reach a blood vessel, fungal cells start disseminating throughout the body.

Furthermore, the increased use of in-dwelling devices like catheters and intravenous lines can serve as ports of entry. Up to 10% of all blood stream infections are of fungal origin, which are difficult to diagnose early and if not treated in time are often fatal [13]. The mortality of *C. albicans* blood stream infections is ~40% [14]. Especially neonates and immune-compromised patients, who for example suffer from neutropenia, are prone to fungal infections and the prevalence of *C. albicans* infections has soared during the AIDS epidemic in the 1980s and 1990s. Also, patients undergoing chemotherapy are vulnerable to infections. Additionally, more severely immune-suppressed patients survive longer due to improved therapies and care. This combined with the increase in hospitalization of patients with polytrauma, extensive burns or after major surgical intervention results in a significant increase in the number of nosocomial infections.

C. albicans has been the leading cause of fungal infections in humans, although recently, infections caused by other *Candida* species are emerging. This is in part due to better diagnostics but is also most likely related to the fact that *C. albicans* has a relatively low base resistance to antifungals like fluconazole which are regularly used in the clinics. Nonetheless, *C. albicans* is also able to acquire a plethora of mutations that result in increased resistance through differ-

ent mechanisms [15]. In contrast to antibiotics, the number of targets for antifungals is more limited, due to the fact that both mammalian and fungal cells are eukaryotic cells and many potential drugs would affect both host and pathogen, often with severe side effects. Currently, there are three main classes of antifungals: all three target the cell surface. Polyenes and azoles act on the fungal-specific membrane sterol ergosterol, either directly or through its biosynthesis. Echinocandins target the biosynthesis of β -glucan, an important carbohydrate constituent of the cell wall. The cell wall is a distinct feature compared to mammalian cells and is therefore a promising target for antifungal intervention.

C. albicans morphology

C. albicans is able to form five distinct morphological states. Chlamydo spores are thick-walled cells whose function is not entirely understood yet [16]. *C. albicans* is the most successful *Candida* pathogen and one of the few *Candida* species that is able to form true hyphae. The yeast-to-hypha transition is the most visually striking change in morphology and comprises three distinct morphologies (Figure 1.4) [17,18]. The yeast form is almost identical in appearance to baker's yeast with ovoid cells that propagate by budding. Pseudohyphae are branched chains of elongated cells that are restricted at their septation sites. A true hypha is characterized by parallel walls that are not restricted at their septation sites. Intriguingly, these three distinct morphologies have been shown to be linked by the expression of Ume6, a transcriptional regulator that governs hyphal extension [19]. Ume6 functions downstream of the master regulators Efg1, Tup1 and Cph1 [20]. A gradient of Ume6 determines the morphological appearance of *C. albicans*, with low levels corresponding to yeast cells, median levels to pseudohyphae, and high levels to true hyphae [21]. Yeast cells most likely play a role in dissemination through the blood stream and the seeding of biofilms. Hyphal growth plays an important role in tissue invasion and destruction, through the increased secretion of degrading enzymes, e.g. Sap4-6 [22]. Hyphae are also important for adhesion and biofilm maturation. Hyphal formation is also induced inside macrophages and enables *C. albicans* to escape from phagosomes by bursting the immune cell [23]. The production of a superoxide dismutase, Sod5, that detoxifies reactive oxygen species is also induced in true hyphae [24]. It is important to note that locking *C. albicans* genetically in one morphology results in avirulence [25]. This underlines the importance of the

yeast-to-hypha transition and vice versa in the life cycle and virulence of *C. albicans*. The effect of the yeast-to-hypha transition on the secretome and wall proteome is described in chapters 3 and 5.

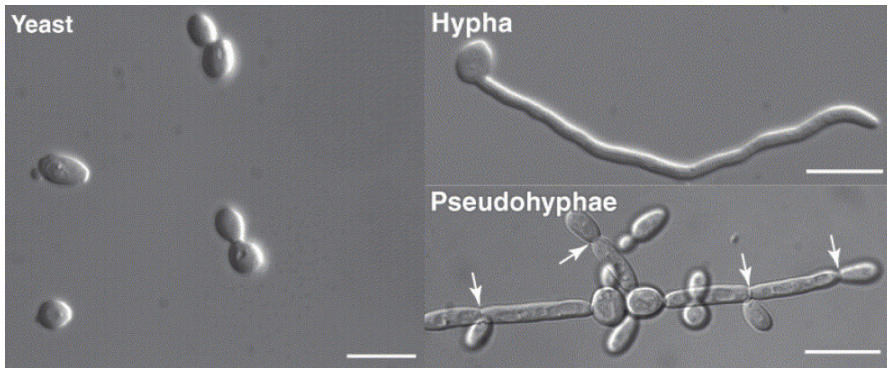


Figure 1.4. Distinct morphological states during the yeast-to-hypha transition from [17].

Left: Yeast cells are oval and form buds that are subsequently separated. Lower right: Pseudohyphae are clusters of cells that are elongated but are still clearly restricted at their septation sites (white arrows). Upper right: Hyphae emerge from the mother cell as germ tubes and form parallel walls and show no septation.

The white-opaque transition plays an important role in the sexual reproduction of *C. albicans*. Opaque cells are mating-competent and are elongated in appearance compared to the typical yeast form, which in this context is referred to as the white state (reviewed in [26]). The terms white and opaque are derived from their colony appearance on a plate. White and opaque colonies are also easily distinguishable because opaque colonies can be specifically stained pink with the dye phloxine B. To transition from white to opaque, a cell has to be homozygous in the mating type locus (MTL). White-opaque-switching is determined by two allelic variants in the Mating Type Locus, *MTLa* and *MTL α* , as well as the master regulator *WOR1*. *Wor1* regulates itself as well as the transcription factors *WOR2*, *CZF1*, and *EFG1* and the expression of opaque specific genes [27]. The large majority of cells are MTL heterozygous and gene expression leads to the production of the dimeric $\alpha1$ - $\alpha2$ repressor, which prevents the expression of *WOR1*. Since loss of heterozygosity regularly occurs in *C. albicans* [28], *MTL*-homozygous cells lose the activity of the repressor and begin expression of *WOR1*. If a threshold level of *Wor1* is reached, the cells convert to the opaque state, in which *Wor1* is continuously produced and activates opaque-

associated proteins. Opaque **a** and α cells can mate and produce tetraploid, recombinant cells that switch back to the white state due to the renewed production of the $\alpha 1$ - $\alpha 2$ repressor. White-opaque-switching plays a role in introducing additional genetic variation, but additionally many opaque-associated genes have functions beyond mating. Opaque cells show metabolic changes and do not readily undergo yeast-to-hypha transition. Interestingly, while opaque cells are able to avoid host defenses, especially phagocytic cells and leukocytes, more effectively than white cells [29], they are more sensitive to reactive oxygen species and killing by leukocytes [30]. This suggests that opaque cells might be more suited to some host niches (*e.g.* infections of the skin) than others and supports the notion of specific morphologies being geared towards selected sites of infection. The morphologies described in this section also affect the cell wall.

The cell wall of *C. albicans*

The cell wall is a layered structure consisting of carbohydrates and proteins. The carbohydrate layer is composed of chitin, β -1,3- and β -1,6-glucan [31,32]. While β -1,3-glucan forms more ordered structures, β -1,6-glucan is mainly a linker molecule. Associated with these carbohydrates are covalently and non-covalently anchored proteins. These proteins are highly glycosylated, mainly with mannose residues (Figure 1.2). The cell wall confers structural integrity, maintains the shape of the cell, and counteracts the turgor pressure of the protoplast. Despite its name, the cell wall is a dynamic structure and its carbohydrate components are affected by enzymes associated with the wall or secreted into the environment [33]. Wall remodeling is required during isotropic growth, cell separation, and cell wall loosening as a preparatory step to bud formation, and hyphal branching [34,35]. In response to stress, the carbohydrate composition can change, *e.g.* the relative amount of chitin increases, mainly through increased chitin synthesis [36]. Although the thickness of the wall is tightly regulated, it can change in response to a change in carbon source [37]. The elasticity, the porosity and the hydrophobicity of the cell wall can also change in response to outside stimuli [37].

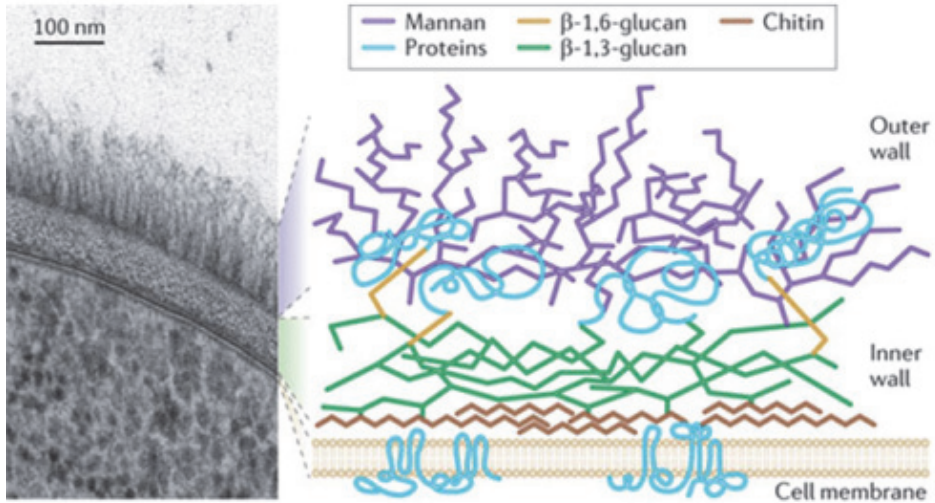


Figure 1.2. Overview of the cell wall composition (from [12]).

Left: Transmission electron micrograph of the cell wall showing the fibrillar layer of mannoproteins attached to a carbohydrate matrix on top of the cell membrane. Right: Schematic of the cell wall and its constituents. The inner wall is mainly composed of β -glucans with a small amount of chitin. The outer wall is dominated by proteins attached to the inner wall that are highly mannoseylated.

Properties of *C. albicans* wall proteins

The majority of covalently anchored wall proteins in *C. albicans* is attached to highly branched β -1,6-glucan which acts as a flexible linker to the more structured β -1,3-glucan lattice. This attachment is mediated by a truncated GPI-anchor. GPI-proteins follow the secretory pathway while anchored to the luminal leaflet of the secretory pathway membranes. While all GPI-proteins are initially inserted into the plasma membrane, the GPI-proteins that are to become resident proteins of the wall get cleaved off and the GPI-remnant is attached to β -1,6-glucan [38]. This is supported by the detection of free GPI-proteins in the wall which have a processed GPI-anchor but are not attached to β -1,6-glucan yet [39]. It is important to note that the distinction between the final destination of wall proteins is not absolute and that GPI-proteins can be found at both locations [40,41].

Most GPI-wall proteins are organized in distinct domains (Figure 1.3). The N-terminus contains a signal peptide which is cleaved off when exiting the secretory pathway. The remaining N-terminal domain constitutes the active part of the protein and often involves the enzymatic activity of carbohydrate-active en-

zymes of the cell wall. The majority of peptides used for wall protein identification with mass spectrometry originate from this domain. The C-terminal part of the protein contains the truncated GPI-anchor at the very end. Between the GPI-anchor and the active domain, the S/T-rich domain is located. This domain is sometimes referred to as a “stem domain” and has mainly a structural function and houses the majority of glycosylation sites, which makes it difficult to digest using proteases. It also allows the extension of the active domain further into the surrounding of the wall. Recently, micro-domains that are able to form amyloid-like interactions have been described [42]. These amyloids are involved in the recruiting of an array of adhesins that facilitate adherence to tissues and abiotic surfaces [43].



Figure 1.3. Domain structure of a typical GPI-wall protein.

From left to right: N-terminal signal peptide (SP), active domain (*e.g.* active site of enzymes), structural domain containing the majority of glycosylation sites, truncated GPI-anchor attached to β -1,6-glucan.

Covalently anchored wall proteins serve many functions including adhesion, biofilm formation, and acquisition of micro-nutrients (*e.g.* iron) and interaction with the immune system. Furthermore, GPI-proteins also detoxify superoxides produced by immune cells and facilitate the invasion and damaging of tissues [44]. Most wall proteins form families with the same function, but slightly different specificities with regard to pH, temperature or morphological state. For example, Phr1 and Phr2 are both transglucosylases acting on the wall carbohydrates, but are expressed at either near neutral or acidic pH, respectively [45]. The carbohydrate-active enzymes and other GPI-wall proteins will be discussed throughout this thesis in more detail.

Properties of secretome proteins of *C. albicans*

Similar functions are fulfilled by proteins released into the environment, but their structure is more diverse. Although there are more than 400 proteins predicted to contain signal peptides, not all are released into the environment. Proteins can be retained in the ER-lumen or become inserted into the cell mem-

brane. Of the 225 proteins that are secreted and not retained approx. 60 are GPI-wall proteins, while about 165 proteins are predicted to be soluble [46] (see also chapter 4). Only approximately a quarter of these proteins are expressed at any given time, underlining the high degree of regulation involved. Like for the wall proteins, protein families allow the selection of the optimal protein with a specific function for the actual conditions. The secretome contains more proteins directed towards breaking down carbohydrates, proteins and lipids for uptake by *C. albicans* than the wall proteome. The families of the secreted aspartic proteases (Sap), Phospholipases (Plb) and lipases (Lip) are among the most prominent [47]. Furthermore, members of the secretome also provide carbohydrate material for the generation of the extracellular matrix and assist in the formation and subsistence of a biofilm [48,49]. Although not by definition belonging to the secretome, GPI-wall proteins are consistently found in the environment, most likely due to the extensive wall remodeling discussed before and the temporal gap between cleavage off the plasma membrane and attachment to β -glucan. The composition of the secretome and the functions of its members will be discussed in detail in chapters 3, 4 and 6.

Immune recognition of the cell wall

The cell wall carbohydrates and proteins as well as the secretome present also important pathogen-associated molecular patterns (PAMPS). These patterns are recognized by many receptors on the surface of immune cells. The interplay between the immune system and the fungus is essential for a state of co-existence and if the system is disturbed, e.g. by a reduction in immune status, infections occur (reviewed in [50]). With regard to the carbohydrates, the recognition of chitin and its components is not fully understood, while β -1,3-glucan is mainly recognized by Dectin-1 and leads to a strong inflammatory response [51] and β -1,6-glucan is often opsonized and targeted by the complement system [52]. The strongly proinflammatory carbohydrates are at least in part shielded by the wall proteins and their mannosyl residues and damage to this “glycoshield” leads to an increase in the strength of inflammation [53].

The differences between yeast and hyphal growth, but also between white and opaque cells, result in changes in recognition and attack by the immune system. As already mentioned before, opaque cells seem to be able to evade some types of immune cells, but are more susceptible to specific killing mechanisms. Interestingly, Hyr1, a strongly hypha-associated protein [24] (see also chapter 5), is able to confer resistance to neutrophil killing, as was for example shown by

heterologous expression in *C. glabrata* [54]. Hyr1 is a member of the Hyr/Iff family with 11 members that are expressed upon specific conditions and it is conceivable that other members of the family lead to similar protection in another morphology. Also the absence of bud-scars and therefore surface-exposed β -1,3-glucan in hyphae leads to reduced Dectin-1 activation. Conversely, the soluble protein Pra1, important for zinc uptake and secreted by hyphae, is detected by human neutrophils and leads to increased killing [55]. Strikingly, while Pra1 is a prime target for immune recognition of hyphae [56], it is also released into the environment and can act in a decoy function for complement [57]. Similarly, the signaling mucin Msb2 is located in the membrane, but upon processing by proteases, a large extracellular domain is released into the environment, which has recently been shown to be able to bind antimicrobial peptides [58]. These few examples underline the constant tug of war between the host immune system and the fungus. It also reveals potential targets for aiding the immune system in its constant fight against the fungal invaders.

Current diagnostic methods for *C. albicans* detection

The detection of a bloodstream infection of *C. albicans* is difficult and has to be achieved as early as possible to mitigate the high mortality rate. Usually, mortality rates increase towards the end of the first week of infection, but most diagnostic methods take multiple days from sampling to identification and starting antifungal treatment. When a fungal infection is suspected, blood cultures are ordered immediately, but growth of the fungus takes at least 2 days and differentiation between fungal species, which is important for the correct choice of treatment options, requires a battery of biochemical assays and microscopic staining assays. While methods based on the detection of nucleic acids are very sensitive, their specificity is often lacking. Especially for a fungus co-existing in the human body, the differentiation of a pathogenic infection and normal interaction with the host is difficult. Assays based on antibodies against the fungus are affected by the same drawback and are unreliable in immune-suppressed patients where the B-cell mediated immune response is virtually non-existent. Nonetheless, approaches using either duplex PCRs with multiple target sequences or fluorescence *in situ* hybridization (FISH) are useful in a clinical setting [59,60]. Therefore it is critical to develop new methods for rapid detection of a fungal infection, in the best case using blood serum. Distinguishing an infection from normal colonization could be improved by targeting proteins

known to be associated with virulence, *e.g.* the secreted Saps 4-6, strongly associated with tissue damage, or the hypha-associated Hyr1. Additionally, defining acceptable levels as well as a pathogenic threshold for these indicators would lead to better diagnostic methods.

The state of *C. albicans* vaccine development

In general, preventative measures in health care are preferred and the success of vaccinations is best exemplified by the small pox eradication campaign. A vaccine against *C. albicans* has a number of issues to contend with. First, differentiation between invasive disease and normal co-existence is complicated in the early stages of disease. Here, a vaccination could be specifically targeted to prevent penetration of the fungus into the blood stream, *e.g.* by stimulating patrolling immune cells in the blood to detect *C. albicans*. Secondly, especially immune-suppressed patients are at risk and vaccination after immune suppression would most likely not lead to an effective immunization. This could be mitigated by identifying at risk groups, *e.g.* patients with planned major surgery like organ transplants and extended hospitalization. Lastly, the identification of targets is still on-going. The cell wall and secreted proteins, with their front position in the host-pathogen interactions are promising targets for the development of a vaccine. Indeed, the GPI-anchored adhesin Als3 is already in clinical trials and shows protection against both *C. albicans* and the bacterial pathogen *Staphylococcus aureus* [61]. Similarly, Hyr1 has also shown promise in mice immunization studies and has entered the clinical testing phase as well [54]. It has also been proposed to combine the antigenicity of the carbohydrate portion of the wall with the accessibility of the wall proteins, by interconnecting β -1,2-linked mannotrioses to immunogenic peptides [62]. This approach has been used for hyphal wall protein 1 (Hwp1), a cell wall protein, as well as for three enzymes involved in glycolysis. Immunization of mice with these constructs led to protection. Although glycolytic enzymes are probably not actively directed to the cell surface by healthy cells [63], they might be released or leaked by apoptotic or stressed cells. Glycolytic enzymes could be important due to their high abundance and immunogenicity for recruiting additional immune cells after lysis of some fungal cells to the site of infection. In any case, although first steps have been taken towards the development have been taken, some hurdles still remain. The cell wall and secreted proteins of *C. albicans* most likely still contain addi-

tional effective targets for vaccine development and the development of innovative strategies to combat fungal infections.

Outline of this thesis

Throughout this thesis I discuss the importance of the cell surface for *C. albicans* life cycle, morphology and virulence. Especially the changes in protein level of covalently anchored wall proteins are examined during morphological changes and thermal stress. Additionally, the proteins secreted into the environment and their role in both maintaining cell wall integrity and affecting host cells are discussed. To study these two important groups of proteins, we tailored mass spectrometric methods to their analysis and quantification. We also identified candidates for diagnostic use in the secretome and suggest strategies and potential targets for the development of vaccines.

In **chapter 1**, fungi are introduced as both important in the global ecosystem but also as pathogens that are an increasing risk in hospital settings, especially for immune-compromised patients. The characteristics of the human fungal pathogen *Candida albicans* are introduced and current treatment options are briefly summarized. Functionally, the cell wall plays an important role in host-pathogen interaction as well as fungal growth in general. The cell wall is also distinct from mammalian cells and is therefore a preferred target for antifungals. The role of the cell wall as a target for the host immune system is introduced and the current status of vaccine development is discussed.

Chapter 2 introduces mass spectrometry as a powerful analytical tool for protein identification. Strategies for protein quantification are examined and the importance of protein quantification is explained. Then, the application and tailoring of these strategies to our specific biological question are outlined. Workflows for identification and quantification of wall anchored and secreted proteins are presented. Finally, future steps for the improvement and expansion of these strategies are proposed.

Chapter 3 shows the application of the methods described in chapter 2 to the secreted proteome, or secretome, of *C. albicans* during different environmental conditions. Temperature, pH and morphological changes impact the secretome significantly and a method for spotting trends in proteomic data is presented.

Although the protein content only represents 0.1-0.2% of dry biomass, a total of 44 proteins was identified with functions ranging from adhesion to wall maintenance. A core set of proteins that are mainly involved in wall maintenance has been detected in all measured conditions. Additionally, a list of 29 highly immunogenic peptides is presented which originate from 18 proteins, underlining the potential of the secreted proteins in virulence and as diagnostic candidates.

Chapter 4 puts the findings of chapter 3 in context by analyzing existing literature for secreted proteins in fungi. Furthermore, the results of additional mass spectrometric studies performed by our groups in *C. albicans* and related fungi are presented and help refine the message that secreted proteins are essential for wall maintenance and virulence. Not all potentially secreted proteins have been identified due to the conditionality of protein secretion. This discrepancy between the predicted secretome size and the actual observed secretomes is discussed as well as secretome size differences between pathogenic and saprophytic fungi. The presence of wall-anchored GPI-proteins in the medium is also addressed.

Chapter 5 focuses on the changes in the wall proteome upon the morphological switch from yeast to hypha, an essential virulence trait. Three methods to induce the formation and maintenance of hyphae *in vitro* over 18h are compared. Three categories of wall proteins were identified after quantification of 21 wall proteins. The response to the three induction methods used was highly similar. The first category contains five hypha-associated proteins, while the second contains three proteins associated with yeast growth. In the remaining category proteins only showed a very limited response to the morphological change. This category is enriched for enzymes involved in wall remodeling and maintenance.

Chapter 6 explores the importance of the wall proteome and the secretome in dealing with thermal stress. Cells grown in the presence of surface stress, *e.g.* fluconazole treatment or growth at 42°C, were shown to contain more chitin and exhibit a cell separation defect. The sets of proteins involved in wall maintenance and remodeling identified in chapter 3 and chapter 5 are important for coping with surface stress. Relative quantification of the wall proteome revealed the same set of proteins also described previously for fluconazole stress. Also, a relative decrease of chitinases in the secretome at high temperatures is a probable link between the increased chitin content and the decrease in cell separation.

The phosphorylation patterns of the MAP kinase Mkc1, important for the cell wall integrity (CWI) pathway, are consistent for all surface stressors tested. This suggest that any kind of surface stress leads to a conserved response, resulting in increased chitin content to reinforce the cell wall structurally and an increase in the level of cell wall remodeling enzymes.

Chapter 7 aims at summarizing my findings by distilling them into 15 main concepts I discovered and learned from my research. Furthermore, new potential targets for vaccine and diagnostic development are proposed. Lastly, future research directions based on this work are suggested.

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