Infection related stress adaptations in the secretome and wall proteome of Candida albicans

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Chapter 1

General introduction

Fungal diversity

Fungal biodiversity is hotly debated: while the widely accepted number up till now is about 1.5 million, estimates range from about 500,000 up to 9.9 million fungal species [reviewed in (79)]. However, only a minor fraction has been described so far. Some of them have great ecological value, due to their contribution to the decomposition of organic matter, while others are appreciated as food source or exploited for the production of industrial enzymes and antibiotics (9). Although some fungi are facilitating and improving human life, others are causing severe problems. Fungi can be a serious concern in the food industry as the causative agent of food spoilage (84), infect crops, but they can also cause infections in the human population. Nevertheless, of the many fungi known, only very few are capable of causing disease in humans.

Mammalian endothermy is clearly contributing to this low number since most fungi cannot survive the higher temperatures encountered in a mammalian host (14). It is hypothesized that fungi might even have contributed to the replacement of reptiles by mammals as the dominating animals on land (13). Analysis of the thermotolerance of 4802 fungal strains by Robert *et al.* showed that most fungi grew well up to 30°C, but above that temperature every increase by 1°C inhibited the growth of about 6% of the strains (70). Intriguingly, when calculating the optimal temperature in terms of protection against fungal infections and the energy costs to maintain it, the result was 36.7°C, which is very close to the mammalian body temperature (14). Accordingly, primitive mammals like the platypus, with a body core temperature of about 32°C, are more susceptible to fungal infections (60). In addition, superficial skin infections are much more common compared to systemic infections, because the lower surface temperature allows growth of certain temperature-sensitive fungi.

An effective immune system is crucial for the protection against fungal infections as well. Since severe fungal diseases are generally of opportunistic nature they can only arise when the body defense is weakened (56). Indeed,

human mycoses are rarely contagious, unlike bacterial or viral infections. Additionally, the limitation of certain nutrients, such as iron, is strongly limiting the survival of infectious microorganisms, a phenomenon known as nutritional immunity, as will be discussed in a later section.

The numbers of immunocompromised patients, like aids patients, neonates, or people undergoing immunosuppressive therapies are constantly increasing. Due to the high medical standards, severely ill patients can survive longer but that makes them in turn susceptible for microbial pathogens. As a consequence fungal infections have been steadily rising over the last decades (46, 53), as shown by the increase of fungal systemic infections in the US from 1979 to 2001 (Figure 1), with a trend that is still ongoing (66).

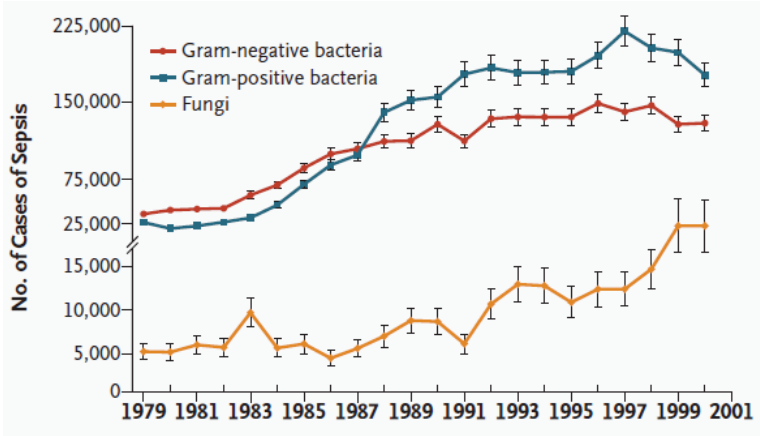

Figure 1. Causative agents of sepsis in the US from 1979-2000. Reproduced with permission from (53), Copyright Massachusetts Medical Society.

Among the fungi causing the majority of severe infections in the human population are *Aspergillus* spp., *Cryptococcus* spp., and *Coccidioides* spp., but *Candida* species are the dominating causative agent (65, 69).

Candida albicans – the major fungal pathogen

The majority of the approximately 150 *Candida* species are environmental saprophytes and only a few are actually capable of thriving in a human host and

causing diseases. Nonetheless, *Candida* species are causing about 8-10% of all the hospital-acquired blood stream infections in the US, with a mortality rate of about 40% [reviewed in (65)]. While exact infection numbers vary between studies and countries, *C. albicans* is the dominating pathogenic *Candida* species (Table 1). Nevertheless, over the recent years also *C. glabrata, C. tropicalis*, *C. parapsilosis*, *C. krusei* and other non - *C. albicans* species are more and more frequently isolated from ill patients (65, 82), partially due to the improvement of identification methods.

Table 1. Species distribution of invasive candidiasis.

	% of total per year							
Species	**1999**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
C. albicans	69.8	65.4	61.4	62.3	62.8	65.9	65.1	64.0
C. glabrata	9.7	11.1	10.7	12.1	11.7	11.2	11.7	12.0
C. tropicalis	5.3	7.5	7.4	7.6	7.5	7.6	8.0	8.3
C. parapsilosis	4.9	6.9	6.6	7.3	6.7	5.6	5.9	5.4
C. krusei	2.2	2.5	2.6	2.7	2.3	2.4	2.5	2.6
C. guilliermondii	0.8	0.7	1.0	0.8	0.7	0.7	0.5	0.5
C. lusitaniae	0.5	0.6	0.5	0.6	0.6	0.6	0.7	0.7
C. kefyr	0.4	0.3	0.4	0.5	0.5	0.6	0.5	0.6
C. inconspicua		0.1	0.2	0.3	0.3	0.3	0.3	0.4
C. famata	0.2	0.2	0.4	0.3	0.4	0.3	0.2	0.3
C. rugosa	0.03	0.7	0.6	0.4	0.2	0.1	0.1	0.2
C. dubliniensis		<0.1	0.1	<0.1	0.1	0.2	0.2	0.2
C. norvegensis		0.1	<0.1	0.1	0.1	0.1	0.2	0.1
C. lipolytica		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. sake		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. pelliculosa		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. apicola							0.2	<0.1
C. zeylanoides		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. valida				<0.1	<0.1	<0.1	<0.1	<0.1
C. indermedia			<0.1		<0.1	<0.1	<0.1	<0.1
C. pulcherrima			<0.1	<0.1		<0.1	<0.1	<0.1
C. haemuloide			<0.1	<0.1	<0.1	<0.1	<0.1	
C. stellatoidae								<0.1
C. utilis							<0.1	<0.1
C. humicola					<0.1		<0.1	<0.1
C. ciferrii							<0.1	<0.1
C. colliculosa							<0.1	<0.1
C. holmii								<0.1
C. marina								<0.1
C. sphaerica								<0.1
NOS	6.0	3.3	7.8	4.8	5.8	4.2	3.5	4.3
Total/year	22998	21804	24680	33106	33406	28412	29167	31078

The data are combined from Pfaller and Diekema 2007 and 2010 (65, 66) and are obtained from the ARTEMIS Global surveillance program from 142 institutions and 41 countries. NOS, *Candida* species not otherwise identified.

C. albicans is living in the majority of the human population as harmless commensal without causing any noticeable problems. Whether this colonization has any beneficial effects for the host, like the stimulation of the immune system, is still under debate. Under some circumstances it can cause diseases, which range from superficial infections of the skin and mucosal layers in healthy individuals, to deep-seated invasive infections that can lead to dissemination of fungal cells, colonization of various organs and ultimately to the death of the patient. Systemic infections are generally the result of a defective immune defense, as in HIV^+ and cancer patients or in organ transplant patients that receive immunosuppressive therapy (65). Infections may originate from the own flora, facilitated by the disruption of the gastrointestinal barrier during surgery, or fungal cells might enter the body via medical devices, like catheters, stents, etc.

C. albicans is a polymorphic fungus. It can grow as single-celled budding yeast, or form elongated pseudohyphae, like most of the pathogenic *Candida* species. Additionally, *C. albicans* can also form true hyphae, a unique feature that it only shares with *C. dubliniensis* (31). Importantly, the switch between yeast and hyphae is considered to be a relevant virulence attribute, since *C. albicans* mutants that are locked in either morphology display strongly attenuated virulence (32). Under certain conditions *C. albicans* can also form spore-like chlamydospores (54), with a yet unknown function, and it can switch between the white and the mating-competent opaque state (55). Various factors are influencing the morphology of *C. albicans*, like temperature, pH, oxygen, and carbon dioxide levels, culture density, the carbon source and availability of certain nutrients, antifungals, and the contact with immune cells (10). The presence of serum, the amino sugar *N*-acetylglucosamine, or certain amino acids and temperatures close to 37°C can trigger the reversible switch from yeast-to-hypha while elevated glucose concentrations inhibit hyphal growth (51). Also the pH is an important determinant – while pH 4 favors yeast growth, near neutral pH leads to hyphal formation (Figure 2), as reviewed in (85). The

transition from yeast to hyphae is strictly controlled by a regulatory network integrating the transcription factors Cph1, Efg1 and the negative regulator Tup1 (6). Hyphae are generally more adhesive than yeast cells and it has been suggested that hyphae are more important for the invasion of tissues, both by induced endocytosis as well as active penetration (18). Invasion by yeast cells occurs only rarely, but as the switch from yeast to hyphal growth is reversible, yeast cells are more relevant for dissemination in the body. Hyphae of *C. albicans*, but not yeast cells, have been shown to bind the iron-storage protein ferritin via the GPI-protein Als3 and subsequently use it as an iron source (1). In addition, iron starvation was also shown to induce hyphal formation (37). Yeast cells and short hyphae can be taken up very efficiently by phagocytes. While neutrophils effectively kill the fungus, some *C. albicans* cells can survive in macrophages, form hyphae, breach the engulfing cell and finally escape (27, 49). Understandably, the interactions with the immune system are highly complex and depend on fungal morphology and site of infection (39).

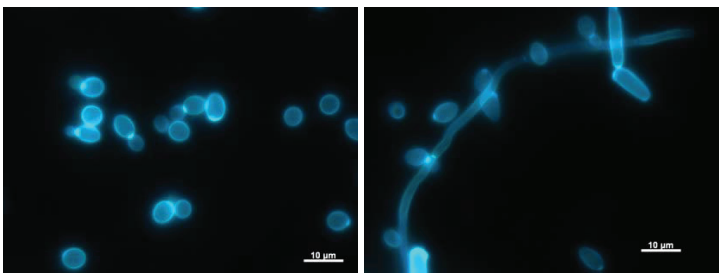

Figure 2. Calcofluor white staining of *C. albicans* SC5314 grown for 18 hours at 37°C. On the left, yeast cells grown in YNBS at pH 4, on the right a mix of yeast and hyphal cells at pH 7.4.

Diagnostic challenges of fungal infections

Life-threatening systemic fungal infections need to be treated as quickly as possible, but before treatment can start, a fast and correct diagnosis is indispensable [reviewed in (45)]. Blood cultures are most frequently employed, but they are time-consuming and yield very frequently false-negative results

(64). Other routinely used techniques are microscopy and histopathology, but these lack specificity and sensitivity and might require invasive procedures. Serological diagnoses are either based on the detection of antigens from the invading pathogen, or on detection of antibodies produced in response to the pathogen. Antibody responses are generally very specific (72). On the other hand, also healthy individuals can host *C. albicans* as commensal, and might therefore also have produced antibodies directed against *C. albic*ans. Distinguishing between antibody levels due to commensalism or infection can be very difficult, and might lead to false-positive test results. On the other hand also false-negative results can be easily obtained, *e.g.* when the immune status of the patient is very weak and only low levels of antibodies are produced. The detection of the antigens can be complicated as well. They may be rapidly cleared from the bloodstream, requiring a very sensitive detection method (24, 45). In the last decade also newer DNA-based detection methods were developed, using PCR or sequencing for pathogen identification. Although very promising due to the high sensitivity and rapid results, the lack of standardization for these methods until now and the rather high false positive rate due to contamination or commensalism are drawbacks as well (64). Despite the high instrument costs, mass spectrometry is already widely used in a clinical setting for toxicology and endocrinology since it is a very fast and sensitive new identification tool (33). Additionally it has been shown that it could become a suitable tool for the identification of proteins and diagnostic of microorganisms as well (52).

Antifungal intervention

The emergence of resistances and the increase in fungal infections has added to the need to find new antifungals and discover novel targets. Ideally, an antifungal drug should specifically target processes or enzymes that do not have a mammalian counterpart, thus minimizing its side-effects. Additionally, it should be effective against a broad spectrum of microorganisms, easily

bioavailable and allow oral administration. The majority of the currently used drugs, in one way or another, targets ergosterol, the major sterol of fungal membranes and the analog of mammalian cholesterol (Table 2). Ergosterol is contributing to membrane fluidity and integrity and is therefore crucial for the function of membrane-bound enzymes and growth. The azoles, which target ergosterol biosynthesis, form the largest group of antifungals. Currently, the most prominent member is fluconazole, since it is very well tolerated and can be orally administered, but in the last few years some very promising, new azoles with even a broader antifungal spectrum, like voriconazole and posaconazole, became approved as well. Azoles inhibit the synthesis of ergosterol by binding to Erg11, a cytochrome P-450 enzyme, thus preventing the 14α-demethylation of lanosterol (86). In some fungi and depending on the concentration used, fluconazole can also inhibit Δ^{22}-desaturase activity (42). As a consequence of the azole treatment, ergosterol is depleted and replaced by toxic sterols, which affects membrane fluidity and subsequently inhibits growth. Azoles are generally fungicidal for molds, such as *Aspergillus* species, but they are only fungistatic against *Candida* species and other yeasts, favoring the emergence of resistant strains.

Also targeting the biosynthesis of ergosterol are the allylamines and the morpholines. While allylamines, like terbinafine, inhibit the synthesis at a very early step by inhibiting the squalene epoxidase Erg1, morpholines affect Δ^{14}-reductase Erg24 and the Δ^{8}- Δ^{7}-isomerase Erg2, which are required at a late stage during ergosterol synthesis (35). Polyenes, the most prominent being Amphothericin B, bind directly to ergosterol, causing leakage of the plasma membrane. It is fungicidal for many fungi, including *Aspergillus* spp., *Candida* spp., and *Cryptococcus* spp., but due to its limited specificity it is also toxic to mammalian cells (61).

Another target, that is absent in mammalian cells, besides ergosterol, is the fungal cell wall. Among the newer drug classes which only became approved in the last decade are the echinocandins. Echinocandins, like caspofungin, inhibit

the synthesis of β-1,3-glucans, an essential constituent of the fungal wall (20), resulting in the loss of cell wall integrity. Due to their unique fungal target echinocandins are very well tolerated, but unfortunately they cannot be applied orally. They generally act fungistatic against molds, while they are fungicidal against yeasts and are also effective against some azole-resistant *Candida* strains, but they do not show antifungal activity against *C. neoformans* (20, 80).
Besides cell wall and ergosterol inhibitors also other fungal drug classes exist, like flucytosines. This drug is converted to 5-fluorouracil, which is then incorporated into nascent RNA causing premature chain termination. Additionally flucytosines inhibit DNA synthesis by affecting thymidylate synthase. However, due to its limited antifungal spectrum and the high frequency of resistance development, flucytosines are rarely used alone against fungal infections (61).

Table 2. Summary table of the three major antifungal drug classes, their targeted pathways, advantages and disadvantages.

Antifungal	Target	Advantages	Disadvantages
Azoles	ergosterol	few side effects, oral administration	not effective against *C. glabrata* and *C. krusei*
Polyenes	ergosterol	broad spectrum	high host toxicity
Echinocandins	β-glucans	few side effects, highly effective	not orally applicable, not active against *Cryptococcus* spp.,

The fungal wall as barrier and support

C. albicans possesses a robust cell wall that serves as a protective barrier against manifold environmental factors. It opposes the intracellular turgor pressure, is crucial for budding and cell division, influences cell shape and is indispensable for maintaining the integrity of the cell. Because a cell wall is absent in mammals it provides a unique antifungal target. As depicted in Figure 3, the fungal wall is composed of a basal layer of polysaccharides, which is

covered by a dense layer of heavily glycosylated and phosphorylated wall proteins that are mainly linked to the wall via the truncated version of their glycosylphosphadidylinositol [GPI] –anchor, as reviewed previously (44).

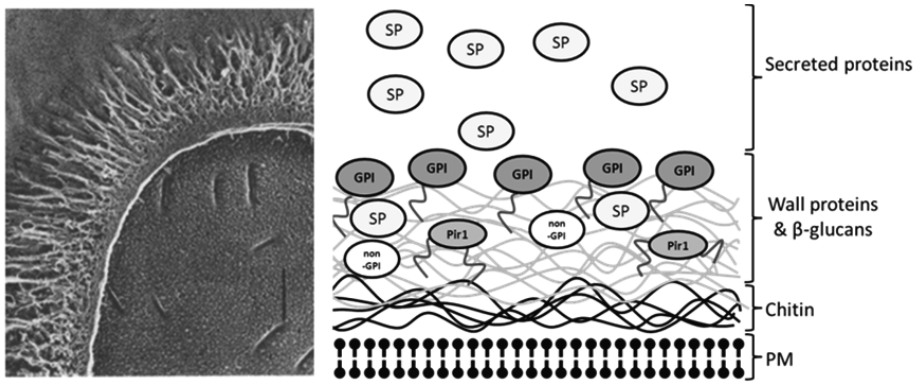

Figure 3. Left side: electron microscopy of a *C. albicans* cell (38). The bar represents 0.2 µm. Right side: schematic representation of a *C. albicans* wall, with its covalently attached wall proteins that can be linked to the wall via a GPI-anchor, interconnecting Pir proteins and secreted proteins SP. PM, plasma membrane.

The basal carbohydrate layer comprises a network of β-1,3-glucans, which account for the major part of this layer and are responsible for its mechanical strength, as well as β-1,6-glucans and small amounts of chitin. The carbohydrate network becomes further strengthened by Pir (Protein with Internal Repeats) proteins which are interconnecting β-1,3-glucans (41). β-1,6-glucans are highly branched and flexible and serve to connect the outer layer of GPI-proteins with the wall, mainly to β-1,3-glucans, but also to a minor extent to chitin. Likely dityrosine residues are contributing to the cell wall strength as well (44). During growth and cell separation the wall is locally loosened and therefore subjected to severe stress. Also external factors like oxidative stress, stretch of the plasma membrane, cell wall-degrading enzymes and wall-perturbing compounds are reported to significantly weaken the fungal wall (47). Serving manifold crucial functions, a healthy cell wall is essential for fungal fitness and virulence. Consequently, the cell invests a lot of energy into sensing and maintaining the integrity of the wall. Three mitogen-activated protein

[MAP] kinase pathways - the Mkc1, Hog1 and Cek1 pathway - and the calcineurin pathway are amongst the key pathways involved in regulating varying aspects of cell wall integrity [CWI] in *C. albicans*, as shown in Figure 4. One of the strategies to counteract cell wall stress is the increased incorporation of chitin into the wall to provide strength and support. Intriguingly, the Mkc1, Hog1 and calcineurin pathway all have been shown to contribute to the increased expression of chitin synthases (58) and therefore to the elevated chitin levels observed upon cell wall stress.

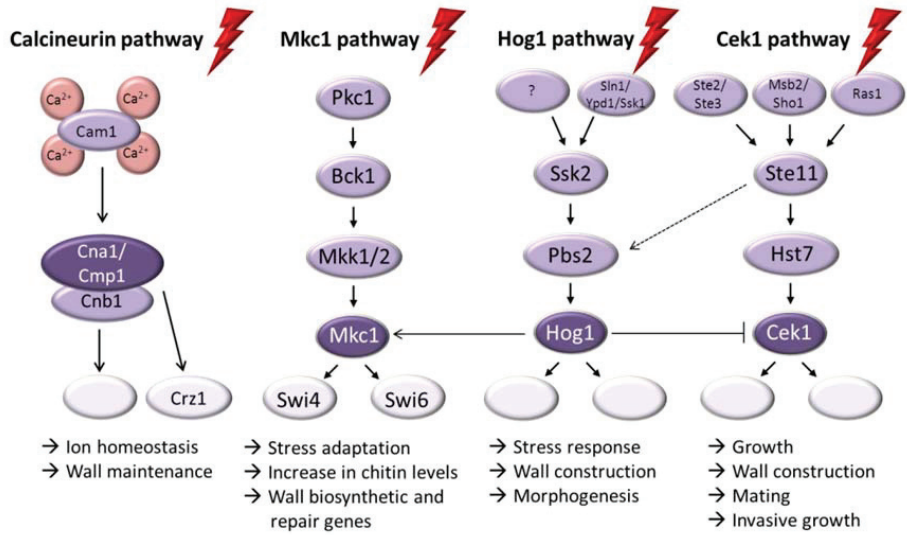

Figure 4. Signaling pathways involved in the response to surface stresses.

The Mkc1 pathway occupies the central role in CWI maintenance and regulation. Additionally to increased sensitivity to cell wall stress and an altered cell wall composition, *mkc1* mutants show attenuated virulence during systemic infections of mice (21). It also participates in the response to oxidative stress, both via Pkc1 and also Hog1 (59). As a consequence of Mkc1 phosphorylation, genes involved in cell wall biosynthesis become activated to strengthen the weakened wall (59). As in *S. cerevisiae,* the Hog1 pathway in *C. albicans* is crucial for osmotic adaptations and its activation leads to the intracellular

accumulation of glycerol to counteract hyperosmotic challenges (75). In addition, the pathway is involved in morphogenesis, since *hog1* and *pbs2* mutants show enhanced filamentation even under non-inducing conditions (2, 3). It is important for the response to oxidative stress (59) and is involved in cell wall biosynthesis. As depicted in Figure 4, activation of the Hog1 pathway occurs either via the three proteins Sln1, Ypd1 and Ssk1, a route via Ste11 or possibly by an additional yet unknown signaling branch. The Cek1 pathway can be triggered by different stimuli, while it is repressed by Hog1 (23). It is required for efficient mating and is activated by the binding of mating pheromones to the corresponding pheromone receptors Ste2 or Ste3. In addition, it is involved in CWI maintenance since it senses defective *N*- and *O*-glycosylation of the wall by the membrane proteins Msb2 and Sho1 (12, 71). Besides, the Cek1 pathway is involved in hyphal growth, mainly via Ras1, and mutants in this pathway show reduced hyphal development and also reduced virulence (17). Calcineurin, a heterodimeric Ca^{2+}-calmodulin-regulated serine/ threonine protein phosphatase, is conserved from fungi to humans. Upon diverse environmental stresses the influx of Ca^{2+} into the cytoplasm is triggered. Ca^{2+} associates with calmodulin, which subsequently binds to calcineurin and triggers a conformational change in the catalytic subunit of calcineurin. In *C. albicans* calcineurin is important for the response to surface stresses, since mutants display hypersensitivity to membrane perturbants, like SDS and azole drugs (16), as well as to perturbants of the cell wall (76). Although hyphal development is unaffected, mutants also show attenuated virulence, presumably due to their reduced tolerance to calcium stress encountered in serum (5, 7). In *S. cerevisiae* calcineurin is also involved in regulating wall biosynthesis, since it positively regulates β-1,3-glucan synthesis and it is crucial in the absence of the CWI pathway (29).

Wall proteome and secretome: indispensable for survival and stress resistance

As already discussed, the fungal wall is essential for the integrity of the cell and

survival. Wall proteins and secreted proteins are needed to maintain and strengthen this barrier, which is subjected to constant adaptations. Surface proteins are not only essential for the integrity of the wall but they are also necessary for many other crucial processes, like adherence, biofilm formation, tissue destruction and invasion, nutrient acquisition and immune evasion, which makes them major virulence factors.

As all eukaryotic surface proteins, wall proteins and secreted proteins of *C. albicans* generally follow the classical secretory pathway [reviewed in (26)]. An N-terminal signal sequence triggers the entry of the newly synthesized secretory protein, either already during or after its translation, into the endoplasmic reticulum [ER]. Transmembrane proteins get directly inserted into the ER membrane. In the ER, proteins become properly folded, N- and O-glycosylated and to certain proteins with a GPI-attachment signal a GPI-anchor is connected at their C-terminus. After being further modified in the Golgi, the proteins are transported to the cell membrane. While transmembrane proteins and a subset of GPI-proteins remain there, other GPI-proteins are cleaved off the membrane and become covalently associated to the cell wall via a truncated version of their GPI-anchor. Some secretory proteins are retained in the wall by forces other than a GPI-anchor, while others are simply released and will most likely end up in the environment (Figure 3).

The majority of wall proteins possess a truncated GPI-anchor that attaches them covalently to the cell wall. As already mentioned some GPI-proteins are predominantly localized to the cell membrane, some to the wall, while some can be found in both. GPI-proteins generally possess a modular structure: the functional domain is located at the N-terminus which protrudes into the environment, the middle comprises a serine- and threonine-rich spacer domain which is heavily glycosylated, and at the C-terminus the GPI-anchor is attached [reviewed in (44)]. Apart from GPI-modified proteins also proteins without a GPI-anchor can be covalently attached to the wall, like the glucan-crosslinking Pir1. Soluble secreted proteins, which are not covalently linked to the wall,

might nevertheless be retained for a while and only slowly diffuse out of the dense wall (Chapter 2 will focus on secreted proteins).

C. albicans can survive in many different host niches, like the skin, mucosal surfaces, the blood stream and inner organs. Each niche can differ in pH, temperature, oxygen, and nutrient availability as well as immune defenses, and requires therefore special adaptations (Figure 5). That might explain the evolution of many families among wall and secreted proteins with a similar function but different optima. The GPI-proteins Phr1 and Phr2, both transglucosylases, are differently expressed depending on pH. While *PHR1* is expressed and required at near neutral pH, the expression of *PHR2* is induced at acidic pH (57, 77).

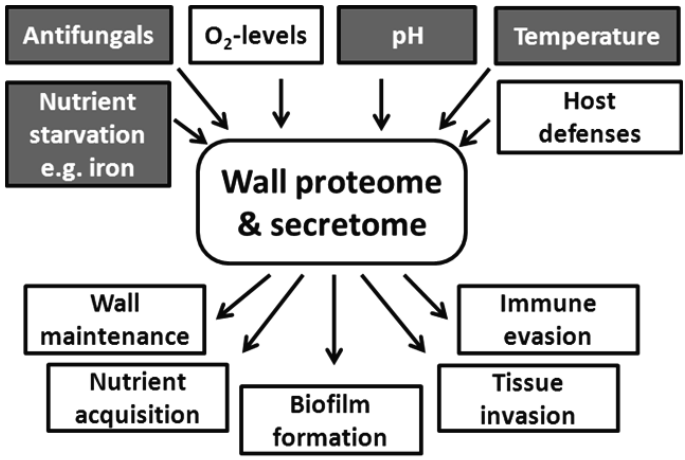

Figure 5. Environmental stress conditions that influence wall proteins and secreted proteins of *C. albicans* and some of their affected functions.

The Als (agglutinin-like sequence) protein family consists of eight members – all of them GPI-proteins with similar domain structure that can act as adhesins with broad, but also distinct, substrate specificity (74, 81). Besides this general role, some Als proteins contain amyloid-forming sequences, which could be involved in the formation of biofilms (48). In addition, some family members serve also a more specific role, like in the case of Als3, which acts as ferritin

receptor (1), facilitating iron uptake, and can bind to N- and E-cadherins on host cells, inducing endocytosis of *C. albicans* (67).

Adhesion is a prerequisite of establishing an infection, followed by invasion of the tissue, which is promoted by the secretion of hydrolytic enzymes, like lipases and proteases. The large Sap (secreted aspartyl proteinases) family is consisting of ten members. Eight of them are secreted into the environment, namely Sap1 to Sap8, while the yapsin-like Sap9 and Sap10 GPI-proteins that become at least partially covalently linked to the wall. Sap1 to Sap3 have their optimal activity at pH 3-5 and are rather yeast-associated, while pH 5-7 is optimal for Sap4 to Sap6 [reviewed in (78)], correlating with their hyphal expression at near neutral pH (25). Saps are important for manifold processes, like tissue degradation, invasion and nutrient uptake. In addition, they are also involved in the defense against the host immune system, *e.g.* by degrading certain complement proteins (34). The family of superoxide dismutases is also an important asset against the immune defenses of the host. In addition to intracellular Sods, that are required to remove noxious reactive oxygen species [ROS] arising from various intracellular processes, *C. albicans* also employs the extracellular GPI-linked proteins Sod4, Sod5 and Sod6. While little is known about Sod6, Sod4 and Sod5 are needed to cope with ROS produced during the oxidative burst (28), which is a common defense mechanism of certain immune cells against invading pathogens.

Nutrient acquisition is without a doubt crucial for survival as well. The already mentioned hydrolytic enzymes of *C. albicans* are important for tissue destruction and unclosing new food sources. Iron is essential for the pathogen, but also the host in which it is living. Iron homeostasis is tightly regulated in a mammalian host, both because of the reactive nature of iron, which can easily lead to the formation of toxic radicals, and to restrict growth of invading microorganisms. Iron is needed as a prosthetic group for manifold reactions, like in the respiratory chain, or for oxygen transport in hemoglobin. Excessive iron is safely stored being bound to ferritin. Defects in iron homeostasis are

leading to certain diseases, either by iron deficiency if there is too little, or hemochromatosis by too much iron. The latter is frequently associated with certain types of infections that can only arise if iron is present in excessive amounts (11). *C. albicans* is not reliant on an excess of iron. Adapted to a life in a host it developed special uptake mechanisms that ensure its survival in an iron-deprived world, as will be discussed in more depth in chapter 5. Some wall proteins belonging to the CFEM-family, named after the conserved eight-cysteine containing motive, have been shown to be involved in hemoglobin iron acquisition (87, 88) and one of the various functions of Als3 is the binding of ferritin (1) in order to subsequently exploit the stored iron.

Besides pH and nutrients also the temperature can vary depending on the host and body niche. While the human body core temperature is around 37°C it can decrease in the extremities or on the skin, while during fever it can even be higher. Apart from humans, *C. albicans* also frequently colonizes other warm-blooded animals. These may have a significantly lower, or, like in the case of birds, even a higher body temperature compared to humans (40, 68). Temperature has a known strong impact on the physiology and growth of all microorganisms. As in *Saccharomyces cerevisiae* also in *C. albicans* prolonged thermal stress leads to trehalose accumulation in order to facilitate proper protein folding (4). The elevated temperature influences the fluidity of inner membranes but also of the plasma membrane, resulting in surface stress. In *S. cerevisiae* thermal stress results in wall stress and leads to the activation of the CWI pathway and consequently affects the wall proteome and secretome as well. The effects on *C. albicans* are not so clearly understood and will be the focus of chapter 4.

Apart from all these manifold natural challenges, *C. albicans* has to face additional, rather recent forms of stress. Only in the 1960s, the first orally available antifungals were introduced (83). Since then several drug classes have been approved, as discussed above. This certainly poses an additional challenge on fungal pathogens and requires novel adaptation strategies. Some of the

adaptations are reflected in the wall composition, the wall proteome and secretome and will be further discussed in chapter 3.

Mass spectrometry for protein identification

As already discussed, wall proteins are glycoproteins, due to their heavy N- and O-glycosylation. This will generally constrain identification on a proteomic level. In a classical proteomic approach using 2D gel electrophoresis, proteins are released from the wall (Figure 6), separated based on their isoelectric point and their mass, and are subsequently identified by peptide mass fingerprinting.

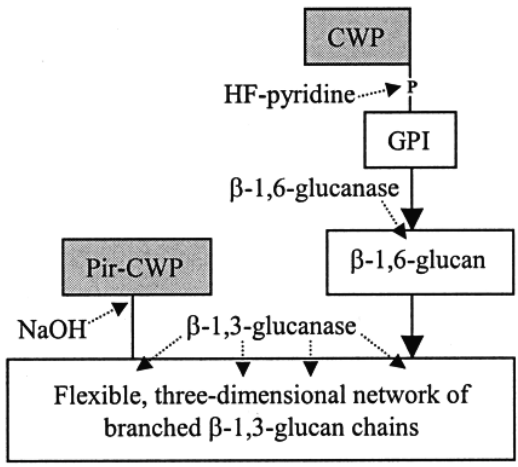

Figure 6. Chemical and enzymatic release of GPI-linked proteins and proteins with alkali-sensitive linkage, like Pir proteins, from the fungal wall (19).

Both, isoelectric point and protein mass, will be affected by glycosylation (89). Carbohydrate side-chains often contain various numbers of negative charges *e.g.* due to phosphodiester bridges, therefore influencing the isoelectric point (30). Besides, the side-chains also differ in length within a protein species, which will change the protein mass (90). Therefore fungal wall proteins frequently appear in the gel as multiple and blurry spots, complicating their identification. Avoiding this error-prone and time-consuming gel-based separation step, wall proteins can be directly identified by mass spectrometry

(*e.g.* in our case by liquid chromatography coupled with tandem mass spectrometry: LC-MS/MS or Fourier Transform mass spectrometry: FTMS). Protein glycosylation is of course a problem for direct mass spectrometric identification as well. Heavily glycosylated regions might restrict the access of trypsin, impeding tryptic digestion, or – if digestion was successful – make identification highly problematic. But heavy glycosylation of wall proteins is generally limited to serine/threonine-rich regions, which still leaves the functional domain at the N-terminal region of the protein for analysis. This domain generally yields unique tryptic peptides for accurate protein identification by mass spectrometry. There exist several deglycosylation strategies [reviewed in (22)], like treatment with trifluoromethanesulfonic acid or the enzymatic removal of carbohydrate chains. Although in other studies deglycosylation strategies resulted in the identification of some additional *C. albicans* wall proteins (15, 50), we found that identification and quantification became much less reliable, and we therefore omitted deglycosylation prior to protein digestion.

Wall proteins can be released from the fungal wall before identification by various methods (Figure 6). They can be released enzymatically by glucanases or chemically by HF-pryridine, that will specifically release GPI-proteins, or by NaOH treatment to release proteins that are associated with wall polysaccharides through an alkali-sensitive linkage (19). While the release of wall proteins is indispensable for gel-based applications, it is not necessary for direct trypsination of cell walls in combination with mass spectrometry. During the so called cell wall shaving step, proteins covalently linked to the wall are directly digested by trypsin, releasing tryptic peptides for subsequent mass spectrometric identification (89). To ensure that only covalently attached proteins are analyzed, for both cell wall shaving and prior protein release, the cell walls need to be purified by extensive washing with hot SDS. The purified walls where only covalently anchored proteins remain attached can then be further processed. In addition to identification, protein levels can also be

quantified. By comparing the intensities of the corresponding protein spots, relative protein levels can be estimated from protein gels. For relative quantification by mass spectrometry corresponding peptide peak areas are compared (8, 63).

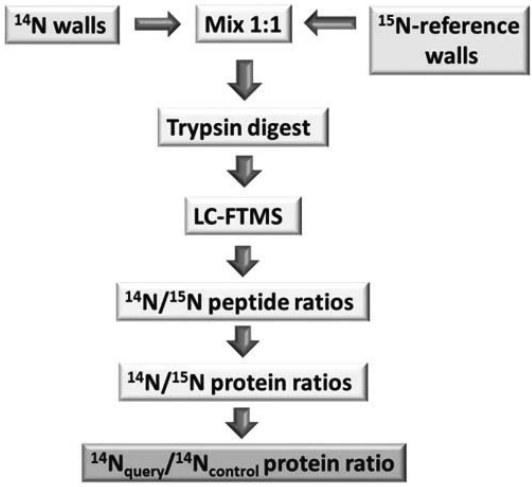

Figure 7. Relative quantification workflow. Isolated walls from a ^{14}N-query culture are mixed with walls from a reference culture that was grown in the presence of ^{15}N ammonium sulfate as sole nitrogen source. The mixed walls are digested with trypsin and peptides are analyzed by Fourier Transform mass spectrometry. From the ^{14}N/^{15}N peptide ratios the protein ratios can be calculated. Subsequently all the query cultures can be compared with each other.

In order to distinguish two different samples, proteins can already be labeled in the growing culture by stable isotope-labeled amino acids (SILAC), or by another compound that will be integrated into the newly synthesized protein like ^{15}N-labeled ammonium sulfate, or they can be labeled at a later time point *e.g.* by isotope-coded affinity tags (ICAT) (36, 43, 62, 73). The first method certainly has the advantage that the labeling is introduced at the beginning of the experiment, avoiding the influence of labeling on the quantification outcome. Nevertheless it is important that the growth conditions allow for maximal enrichment of the introduced isotope and avoid remnants of the undesirable isotopes. For the relative quantification of *C. albicans* wall proteins we applied metabolic labeling of a reference culture (Figure 7), which will be further discussed in chapter 3-5 and the general discussion.

Thesis outline

The wall proteins and secreted proteins of *C. albicans* are highly adaptable and required to master the numerous challenges in the life of an opportunistic fungal pathogen. Having discussed the importance of these two subproteomes for the survival and virulence of *C. albicans* a better understanding of their regulation could provide valuable insights for preventing, detecting or combating occurring infections. **Chapter 1** gives a general overview on the problem of fungal infections, their detection and antifungal therapy nowadays. It focuses on the opportunistic fungal pathogen *Candida albicans* and highlights the importance of the fungal wall as a protective barrier and the role of secreted proteins and wall proteins in survival and stress adaptation. Furthermore, it mentions mass spectrometry as a powerful tool for the identification and quantification of fungal wall proteins and secreted proteins. **Chapter 2** serves to establish the method for the analysis of the *C. albicans* secretome. In addition, the versatility of the secretome under different growth conditions, like pH, temperature, and the hyphal inducer GlcNAc, will be analyzed. **Chapter 3** will provide new insides on the action of azoles with their impact on the cell wall of *C. albicans* itself, the wall proteome and secretome. Fluconazole is very often the weapon of choice against fungal infections. It is known to affect the cell membrane by inhibiting ergosterol biosynthesis, nevertheless its effect on the wall proteome and secretome are hardly understood. Like fluconazole also thermal stress affects membrane fluidity. In **chapter 4** similarities between these two conditions and additional surface stresses will be disclosed. Effects on morphology and growth will be tested. By applying relative quantification effects on the wall proteome will be analyzed. Furthermore the composition of secreted proteins and the involvement of different regulatory pathways will be described. Another stress regularly encountered in a mammalian host is iron starvation, which will be the focus of **chapter 5**. The fungus requires iron for the function of many enzymes, amongst others also for enzymes involved in ergosterol biosynthesis. *C. albicans* has developed special means to ensure a

sufficient supply with iron. The contribution of wall proteins during iron starvation in different growth media will be analyzed and insights into possible new ways of iron acquisition will be given. **Chapter 6** will summarize and discuss the results obtained from the previous studies. Protein identification and quantification strategies used throughout this thesis will be briefly discussed. Secretomes of different fungi will be compared and the presence of different protein classes in the medium will be explained. Furthermore similarities and differences in adaptations in the wall proteome as a consequence of different stresses will be compared. Secreted proteins and wall proteins will be also discussed as clinical markers and vaccine candidates.

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